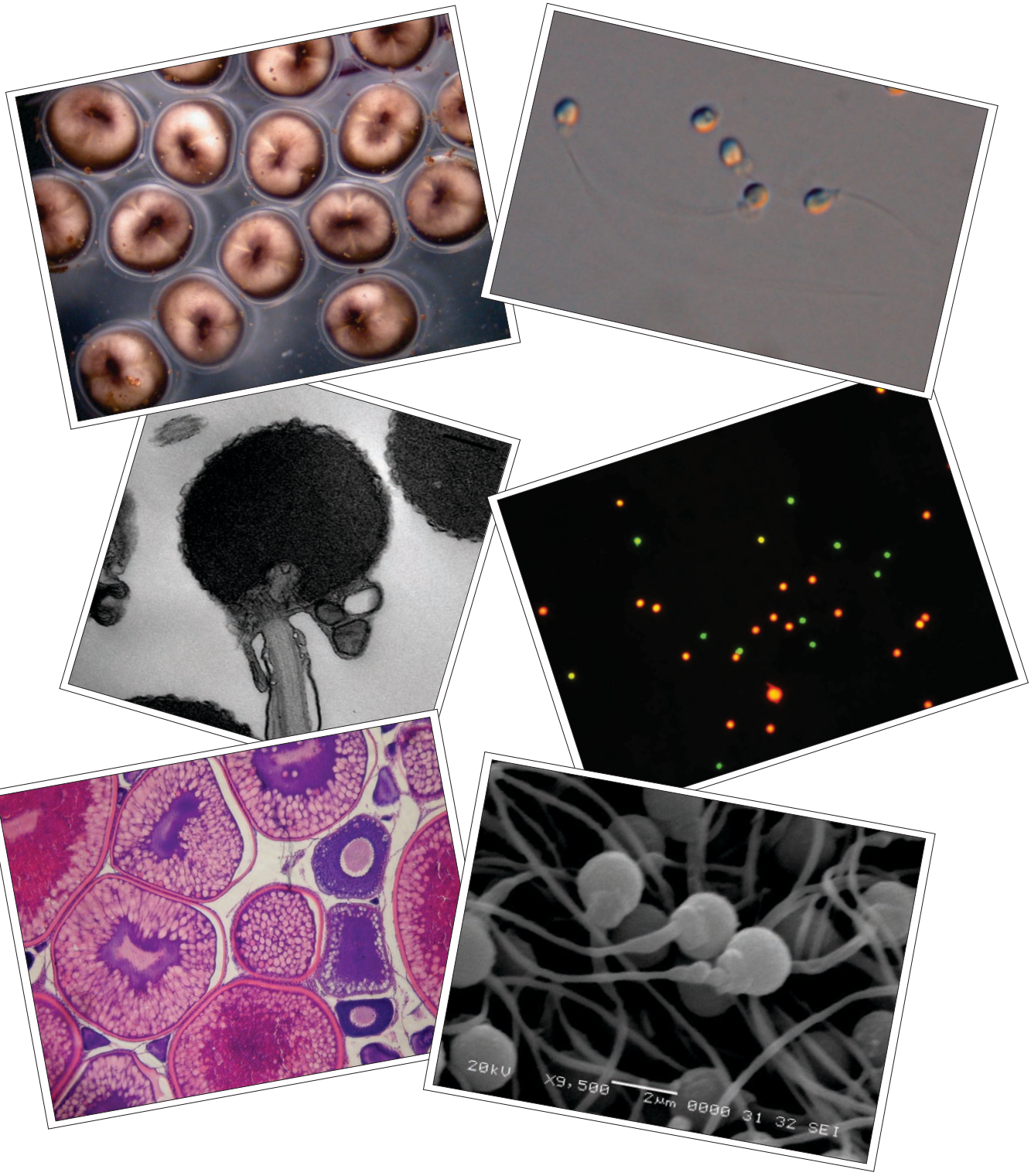
3RD International Workshop on the Biology of Fish Gametes

September 7-9, 2011

Budapest, Hungary



Final Programme

Book of Abstracts

3rd International Workshop on the Biology of Fish Gametes

Final Programme & Book of Abstracts

Budapest, Hungary, September 7-9, 2011

Edited by:

Robert Hohol
Diamond Congress Ltd.
H-1255, Budapest, POB 48.

ISBN 978-963-88019-7-5

TABLE OF CONTENTS

FOREWORD

It is our pleasure to present you this Book of Abstracts of the 3rd International Workshop on the Biology of Fish Gametes held in Budapest and Gödöllő, Hungary on September 7-9, 2011. This series of scientific workshops was initiated by our colleagues of the Research Institute of Fish Culture and Hydrobiology in Vodňany (currently part of the Faculty of Fisheries and Water Protection at the University of South Bohemia) with the first event being held in Vodňany, Czech Republic on August 29-31, 2007. This was followed two years later by the second workshop organized by the Institute of Animal Science and Technology at the Polytechnic University of Valencia on September 9-11, 2009 in Valencia, Spain.

Over the years, this series of workshops attracted an increasing attention of the scientific community working on problems of gametogenesis, gamete quality and gamete interaction in fish. While the first workshop concentrated on fish sperm, the second already incorporated topics on eggs, as well, and it was decided to extend the scientific scope of the meeting to the gametes of other aquatic animals, i.e. aquatic invertebrates. From the very beginning, authors of abstracts were encouraged to submit their materials in the form of complete manuscripts which were later published in the Journal of Applied Ichthyology following peer review.

Abstracts submitted to this current workshop went through a process of review by members of the Scientific Committee. We have received 83 abstracts from authors representing 24 countries. Of these 79 were accommodated in the workshop with 31 oral presentations (including 3 by invited speakers) and 48 posters. The scientific program of the workshop is divided into 3 oral sessions (Gametogenesis, Gamete Preservation and Gamete Quality), 2 poster sessions and a day of practical presentations and roundtable discussions. In addition, several socializing events are organized for the workshop that allow participants to engage in personal discussions which can be just as important for establishing scientific relationships as attendance of the sessions.

We would also like to use this opportunity to thank all people who helped us with the organization of this workshop. First and foremost we would like to express our gratitude to all participants of the workshop who expressed their interest in this event and honored us with their attendance. Our sincere gratitude goes to Dr. Otomar Linhart, Dr. Juan Asturiano and Dr. Harald Rosenthal for organizing the first and second workshops and for allowing publication of materials submitted to the workshops in the Journal of Applied Icthyology. We are grateful to the members of the Scientific Committee who have committed their time and energy to review the abstracts submitted to the workshop and provided useful suggestions to matters of scientific organization. Last but definitely not least, we would like to personally thank the team of Diamond Congress Kft. whose professional organizing work was the key to the success of this meeting.

Ákos Horváth PhD and Béla Urbányi PhD
Department of Aquaculture
Szent István University

COMMITTEES

Scientific Committee

- Asturiano, Juan F., Polytechnic University of Valencia, Spain
- Arai, Katsutoshi, Hokkaido University, Japan
- Cabrita, Elsa, ICMAN (CSIC), Spain/Portugal
- Carnevali, Oliana, Polytechnic University of Marche, Italy
- Cosson, Jacky, CNRS, France
- Ciereszko, Andrzej, Polish Academy of Sciences, Poland
- Fauvel, Christian, IFREMER, France
- Horváth, Ákos, Szent István University, Hungary
- Krasznai, Zoltán, University of Debrecen. Hungary
- Linhart, Otomar, University of South Bohemia in České Budějovice, Czech Rep.
- Morisawa, Masaaki, Tokyo Kasei Gakuin University, Japan
- Pérez, Luz, Polytechnic University of Valencia, Spain
- Rosenthal, Harald, Editor of the Journal of Applied Icthyology, Germany
- Tiersch, Terrence R., Louisiana State University, USA
- Trippel, Edward A., Centre for Marine Biodiversity, Canada

Organizing Committee

- Urbányi, Béla
- Horváth, Ákos
- Csenki, Zsolt
- Bokor, Zoltán
- Kotrik, László
- Kovács, Balázs
- Varga, Attila

Conference Secretariat

Diamond Congress Ltd.
1015 Budapest, Csalogány u. 28., Hungary
Phone: +36 1 225 0210
Fax: +36 1 201 2680
E-mail: diamond@diamond-congress.hu

SCIENTIFIC PROGRAMME

TUESDAY, SEPTEMBER 6TH

15:00-18:00 Registration

18:00-20:00 Welcome party at Danubius Hotel Helia

WEDNESDAY, SEPTEMBER 7TH

8:00-9:00 **Registration**

9:00-9:30 **Opening and general information**

9:30-12:30	**Session 1: Gametogenesis** ***Chairs: Edward A. Trippel, Christian Fauvel***	
9:30-10:00	**Invited speaker:** **O-1/01 ZEBRAFISH GONAD TRANSFORMATION: A MODEL FOR SEX REVERSAL IN SEQUENTIALLY HERMAPHRODITIC TELEOSTS** Laszlo Orban, Junhui Jiang, Preethi Ravi, Woei Chang Liew, Jolly M. Saju, Mohammad S. Hossain, Hsiao Yuen Kwan, Natascha May, Rajini Sreenivasan	17
10:00-10:20	**O-1/02 FERTILITY AND PLOIDY OF GAMETES OF DIPLOID, TRIPLOID AND TETRAPLOID LOACHES, *MISGURNUS ANGUILLICAUDATUS*, IN CHINA** Ya-Juan Li, Ming-Zhao Zhang, Cong Qian, Min Gao, Katsutoshi Arai	19
10:20-10:40	**O-1/03 EVALUATION OF SPERMIATION INDEXES DURING MULTIPLE SPERM COLLECTIONS IN STERLET (ACIPENSER RUTHENUS)** A. Shaliutina, B. Dzyuba, M. Hulak, S. Boryshpolets, P. Li, O.Linhart	21
10:40-11:00	**Coffee break**	
11:00-11:20	**O-1/04 AQUAPORINS AND TELEOST SPERMATOGENESIS: INSIGHTS INTO THEIR PHYSIOLOGICAL ROLES AND POTENTIAL USE FOR SPERM CRYOPRESERVATION** François Chauvigné, Loredana Zilli, Sebastiano Vilella, Joan Cerdà	23
11:20-11:40	**O-1/05 PROBIOTICS INDUCE OOCYTE MATURATIONAL COMPETENCE IN DANIO RERIO** Giorgia Gioacchini, Elisabetta Giorgini, Daniel L. Merrifield, Gary Hardiman, Andrea Borini, Lisa Vaccari, Oliana Carnevali	25
11:40-12:00	**O-1/06 MELATONIN EFFECTS ON KILLIFISH GAMETES** Francesco Lombardo, Adele Fabbrocini, Elisabetta Giorgini, Paolo Ferraris, Raffaele D'Adamo, Giorgia Gioacchini, Oliana Carnevali	27
12:00-12:20	**O-1/07 ORIGIN AND MIGRATION OF PRIMORDIAL GERM CELLS IN STURGEON** Taiju Saito, Rie Goto, Martin Psenicka, Yutaka Kawakami, Shinji Adachi, Katsutoshi Arai, Etsuro Yamaha	29
12:30-14:00	**Lunch**	
14:00-17:00	**Session 5: Gamete Preservation** ***Chairs: Elsa Cabrita, Béla Urbányi***	
14:00-14:30	**Invited speaker:** **O-5/01 HIGH-THROUGHPUT CRYOPRESERVATION FOR AQUATIC SPECIES** Terrence R. Tiersch	31

14:30-14:50	**O-5/02 FERTILITY, VELOCITIES AND MOTILITY OF SURUBIM-DO-PARAÍBA STEINDACHNERIDION PARAHYBAE SPERM CRYOPRESERVED IN LACTOSE AND LACTOSE-FREE MEDIA** Rafael V. Araujo, Isabel C. Carvalho, Eduardo A. Sanches, Danilo Caneppele, Marcelo C. Leal, Ana T. M. Viveiros	34
14:50-15:10	**O-5/03 OXIDATIVE STRESS INDUCED BY CRYOPRESERVATION ON COMMON CARP SPERM** Ping Li, Zhi-Hua Li, Boris Dzyuba, Martin Hulak, Marek Rodina, Otomar Linhart	36
15:10-15:30	**O-5/04 TREATMENT BY HYPOTONIC SOLUTIONS OF COMMON CARP (CYPRINUS CARPIO) SPERMATOZOA PRIOR TO FREEZING IMPROVES SURVIVAL TO CRYOINJURIES** Boris Dzyuba, Gunes Yamaner, Olga Bondarenko and Otomar Linhart	38
15:30-15:50	**Coffee break**	
15:50-16:10	**O-5/05 NEW TOOLS FOR GENOME PRESERVATION: GRAFTING SPERMATOGONIA IN BROWN TROUT (SALMO TRUTTA)** C. Fernández-Díez, R. Pérez-Sanchiz, C. Sarasquete, E. Cabrita, M.P. Herráez, José Beirao	40
16:10-16:30	**O-5/06 QUANTITATIVE DNA DAMAGE EVALUATION IN CRYOPRESERVED ZEBRAFISH PRIMORDIAL GERM CELLS** M.F. Riesco, V. Robles	42
16:30-16:50	**O-5/07 VITRIFICATION OF PRIMORDIAL GERM CELLS USING WHOLE EMBRYOS FOR GENE-BANKING IN LOACH, MISGURNUS ANGUILLICAUDATUS** Daisuke Inoue, Takafumi Fujimoto, Yutaka Kawakami, George Shigueki Yasui, Etsuro Yamaha and Katsutoshi Arai	44
16:50-17:10	**O-5/08 EFFECT OF OXYGENE ATMOSPHERE AND ANTIOXIDANTS ON THE COMMON CARP (CYPRINUS CARPIO L.) MILT SHORT TERM STORAGE** R. K. Kowalski, B. I. Cejko1, B. Sarosiek, K. Targońska, D. Żarski, D. Kucharczyk, J. Glogowski	47
17:10-18:30	**Session 3: Poster session 1**	

THURSDAY, SEPTEMBER 8TH

9:00-12:00	**Session 2: Gamete Quality** ***Chairs: Oliana Carnevali, Jacky Cosson***	
9:00-9:30	**O-2/01 IDENTIFICATION OF SEMINAL PROTEINS IN FISH: FROM TRADITIONAL APPROACH TO PROTEOMICS** Andrzej Ciereszko, Mariola A. Dietrich, Joanna Nynca	49
9:30-9:50	**O-2/02 MARATHON VS SPRINT RACERS : AN ADAPTATION OF SPERM CHARACTERISTICS TO THE REPRODUCTIVE STRATEGY OF PACIFIC OYSTER, TURBOT AND SEABASS** M. Suquet, J. Cosson, A. Donval, C. Labbé, P. Haffray, I. Bernard, C. Fauvel	51
9:50-10:10	**O-2/03 SPERM MOTILITY MULTIPLE ACTIVATIONS: PHYSIOLOGICAL BACKGROUND AND PRACTICAL USE IN AQUACULTURE** S. Boryshpolets, B. Dzyuba, M. Rodina and O. Linhart	53
10:10-10:30	**O-2/04 STERLET SPERMOTOZOA BEAHAVIOUR AT DIFFERENT LEVELS INSIDE THE DROP** S. Boryshpolets, V. Bondorenko, M. Rodina, B. Dzyuba, J. Cosson and O. Linhart	55
10:30-10:50	**Coffee break**	

17:10-18:30	**Session 6: Poster session 2**	

FRIDAY, SEPTEMBER 9TH

8:00-9:30	**Bus trip to Szent István University, Gödöllő**	
9:30-12:00	**Session 7: Standardization or Inter-calibration in Gamete Research and Publication** (Open discussion, moderated by Harald Rosenthal and Ákos Horváth)	
10:30-10:50	**Coffee break**	
10:50-12:00	**Session 8: Discussion of the proposed COST Action** (Moderated by Juan Asturiano and Ákos Horváth)	
12:00-12:30	**Closing remarks and vote on the organizer of the following workshop**	
12:30-14:00	**Lunch at Erzsébet Királyné Hotel, Gödöllő**	
14:00-15:30	**Demonstration of the new MiBio sperm analysis system and Tecniplast aquatic systems**	
15:30-15:45	**Coffee break**	
15:45-17:15	**Demonstration of the laboratories of the Department of Aquaculture of Szent István University**	
17:15-17:45	**Travel to Lázár Equestrian Park, Domonyvölgy**	
17:45-21:00	**Dinner at Lázár Equestrian Park**	
21:00-22:30	**Bus trip to Budapest**	

LIST OF POSTERS – SESSION 1

LIST OF POSTERS – SESSION 2

Abstracts of oral presentations

O-1/01
ZEBRAFISH GONAD TRANSFORMATION: A MODEL FOR SEX REVERSAL IN SEQUENTIALLY HERMAPHRODITIC TELEOSTS

Laszlo Orban[1,2,3], Junhui Jiang[1,2,4], Preethi Ravi[1], Woei Chang Liew[1,5], Jolly M. Saju[1], Mohammad S. Hossain[1], Hsiao Yuen Kwan[1], Natascha May[6], Rajini Sreenivasan[1]

[1] *Reproductive Genomics Group, Temasek Life Sciences Laboratory, Singapore*
[2] *Department of Biological Sciences, National University of Singapore, Singapore*
[3] *Department of Animal Sciences and Animal Husbandry, Georgikon Faculty, University of Pannonia, Keszthely, Hungary*
[4] *Agri-Food and Veterinary Authority, Singapore*
[5] *School of Biological Sciences, Nanyang Technological University, Singapore*
[6] *Bioinformatics Support Team, Temasek Life Sciences Laboratory, Singapore*

E-mail: laszlo@tll.org.sg

Background
Teleosts form the biggest vertebrate group: their 34 thousand species outnumber all the other vertebrates combined. In addition to their stunning phenotypic diversity, teleosts also exhibit a wide range of reproductive systems from gonochorism to parallel hermaphroditism [review: (*1*)].

We have been studying the sex determination and gonad differentiation of zebrafish for the past 12 years by using the tools of molecular genetics and genomics [review: (*2*)]. Lately, our attention has turned towards the process of gonad transformation that involves the conversion of 'juvenile ovary' to testis in developing zebrafish males. In this presentation, I will describe our current knowledge on this complicated process and propose that it can serve as a model not only for protogynous, but even for protandrous natural sex reversal.

Testis differentiation in zebrafish
Zebrafish sex appears to be unusual for two reasons. The first is the apparent lack of heteromorphic sex chromosomes [review: (*3*)] and wide-ranging familial sex ratios across stocks (*4*). Based on these features we proposed the presence of a polygenic system (*2*), as opposed to a sex chromosomal one suggested by (*5*). Experimental evidence for polygenic sex determination in zebrafish has also been provided recently by a QTL search (*6*). The second reason is the necessity for future males to undergo a 'juvenile ovary' stage during their early gonadal development (*7*) that forces them through a drastic transformation process prior to the beginning of testis differentiation. We have analyzed this transformation using a transgenic zebrafish line (*8*) and a home-made cDNA microarray (*9*). Later, the same array was used to analyze the effect of chemical and biological treatments on the gonad differentiation process. Our data show the involvement at least two major signal transduction pathways in the gonadal transformation process in zebrafish.

Can the zebrafish be a model for protandrous hermaphrodites?
During natural (and artificial) sex reversal [review: (*10*)], a large set of genes and pathways cooperate during the conversion of a male gonad into a female one or vice versa. Based on published data and our observations [e.g. (*11, 12*)], we propose that a largely overlapping set of genes participate in both female-to-male (protogynous) and male-to-female (protandrous) sex reversal. In addition, we also suggest that the transformation of an immature ovary to immature testis involves processes similar to the conversion of a mature gonad type to another one. Consequently, we suggest that the zebrafish could be a potential model for all kinds of natural sex reversal processes, independently from their direction and timing.

Molecular analysis of sex change in asian seabass
We have tested the validity of our hypothesis on the Asian seabass *(Lates calcarifer)*, a euryhaline protandrous hermaphrodite. In absence of detailed information on the transcriptome and genome of the species, first we had to generate the tools for our study. We have had contract sequencing performed on the Asian seabass transcriptome by SOLiD sequencing technology. The resulting transcripts were used to design a PCR array

and later, an expression microarray. These tools were used to analyze the gonadal transcriptome of immature males, mature males, individuals undergoing transformation and mature females. Comparison of the resulting gene expression profiles with histological data yielded preliminary results that seem to support the working hypothesis described above.

References

1. R. H. Devlin, Y. Nagahama, Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208, 191 (2002).
2. L. Orban, R. Sreenivasan, P. E. Olsson, Long and winding roads: testis differentiation in zebrafish. *Mol Cell Endocrinol* 312, 35 (2009).
3. L. Sola, E. Gornung, Classical and molecular cytogenetics of the zebrafish, *Danio rerio* (Cyprinidae, Cypriniformes): an overview. *Genetica* 111, 397 (2001).
4. W. C. Liew, R. Bartfai, Z. Lim, R. Sreenivasan, L. Orban, Zebrafish sex is determined by a polygenic system, in preparation. (2011).
5. S. K. Tong, H. J. Hsu, B. C. Chung, Zebrafish monosex population reveals female dominance in sex determination and earliest events of gonad differentiation. *Dev Biol* 344, 849 (2010).
6. K. M. Bradley *et al.*, A SNP-based linkage map for zebrafish reveals sex determination loci. *G3: Genes, Genomes, Genetics* 1, 3 (2011).
7. H. Takahashi, Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. *Bull Fac Fish Hokkaido Univ* 28, 57 (1977).
8. A. V. Krovel, L. C. Olsen, Expression of a vas :: EGFP transgene in primordial germ cells of the zebrafish. *Mech Dev* 116, 141 (2002).
9. R. Sreenivasan *et al.*, Transcriptomic analyses reveal novel genes with sexually dimorphic expression in the zebrafish gonad and brain. *PLoS ONE* 3, e1791 (2008).
10. J. C. Avise, J. E. Mank, Evolutionary perspectives on hermaphroditism in fishes. *Sex Dev* 3, 152 (2009).
11. X. G. Wang, R. Bartfai, I. Sleptsova-Freidrich, L. Orban, The timing and extent of 'juvenile ovary' phase are highly variable during zebrafish testis differentiation. *Journal of Fish Biology* 70, 1 (2007).
12. X. G. Wang, L. Orban, Anti-Mullerian hormone and 11 beta-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. *Dev Dyn* 236, 1329 (2007).

O-1/02
FERTILITY AND PLOIDY OF GAMETES OF DIPLOID, TRIPLOID AND TETRAPLOID LOACHES, *MISGURNUS ANGUILLICAUDATUS*, IN CHINA

Ya-Juan Li[1], Ming-Zhao Zhang[1], Cong Qian[1], Min Gao[1], Katsutoshi Arai[2]

[1] *Dalian Ocean University, Life Science and Technology Institute, Dalian, China*
[2] *Hokkaido University, Faculty and Graduate School of Fisheries Sciences, Hakodate, Japan*

E-mail: araikt@fish.hokudai.ac.jp

Introduction

Among wild loach (*Misgurnus anguillicaudatus*) specimens, mainly collected from the Chang Jiang River and its adjacent area, natural tetraploid individuals with 100 chromosomes appear with sympatric diploid individuals with 50 chromosomes (Li et al. 2008). Low frequencies of natural triploid individuals have also been recorded in Chinese wild populations from the same area (Li et al. 2008). Although reproductive capacity and cytogenetics of gametes have been investigated in polyploid specimens collected from Japanese fish market and wild populations (Arai 2001, 2003), Chinese tetraploid and triploid loach specimens have not been examined from the viewpoints of fertility and ploidy of gametes, yet. In the present study, we analyzed concentration per unit volume (cells / ml), duration of active progressive motility (s) and rate of motile spermatozoa after activation (%). Then, we also examined fertility of gametes from diploid and polyploid specimens by observing developmental capacity of the resultant progeny which occurred from crosses using eggs and sperm of diploid, triploid and tetraploid loaches. After fertilization, sizes were measured in eggs from diploid, triploid and tetraploid females. Finally, chromosomes of the resultant progeny were analyzed to estimate ploidy level of the gametes.

Materials and methods

All the specimens were collected in Hubei province, China in 2009-2010. Sperm was taken to hematocrit capillary by gentle squeezing abdomen. Then, concentration per unit volume (cells per ml sperm), duration (s) of active progressive motility and approximate rate (%) of motile spermatozoa after activation were measured according to Yoshikawa et al. (2007). Artificial ovulation and fertilization were conducted according to Fujimoto et al. (2004). Using eggs of three diploid females and three tetraploid females and sperm of three diploid males and three tetraploid males, all possible crosses were made (2n female x 2n male, 2n x 4n, 4n x 2n, and 4n x 4n). Using eggs of two triploid females and sperm of one triploid male, 3n x 2n, 3n x 4n and 2n x 3n crosses were made. Diameter was measured in fertilized eggs from 2n x 2n, 3n x 2n, 4n x 2n and 4n x 4n crosses 30 min after fertilization. Fertilization rate was calculated by counting cleaved eggs relative to total egg number used for each cross within 8 h after insemination. Hatching rate was estimated by counting number of hatched larvae relative to initial number of fertilized eggs. Normal hatching rate was calculated by counting hatched larvae with normal appearance relative to total number of hatched larvae. After 18 to 20h after fertilization, about 30 embryos were taken from each cross to prepare cell-suspension and then chromosome slides were made by air-drying after colchicine treatment, hypotonic treatment and fixation by Carnoy fixative, basically according to Fujimoto et al. (2007).

Results

More than 80% of spermatozoa exhibited progressive motility after activation with ambient water under a view of microscope. Duration of motility was mean 154.58 ± s.d. 8.98 s in diploid and 160.65 ± 6.07 s in tetraploid specimens. There was no significant difference in concentration of spermatozoa between diploid (5.20 ± 0.99 x 10^9 cells/ml) and tetraploid (6.21 ± 0.21 x 10^9 cells/ml).

Progeny produced by using eggs and sperm of diploid and tetraploid loaches exhibited high rates of normal larvae (86 to 98%) after hatching. Progeny from 3n x 2n and 3n x 4n also showed similar rates of normal larvae (94 % and 83 %, respectively). Although above mentioned progeny showed relatively higher survival rates (66 to 92 %) at 7 days after fertilization, progeny from 2n x 3n gave 43% normal hatching rate and only 8% survived at 7 days after fertilization.

Average egg diameter was not significantly different between diploid (0.84 ± 0.03 mm) and triploid (0.82 ± 0.04 mm), but triploid showed a very small number of exceptional large-size eggs (about 1 mm). Significantly larger egg diameter was observed in tetraploid (1.02 ± 0.04 mm).

Chromosome analyses showed diploid-range chromosome numbers (CN) in 2n x 2n (mean CN 47.6 ± s.d. 2.3) and 3n x 2n embryos (47.3 ± 3.2), triploid-range CN in 3n x 4n (71.8 ± 5.1), 2n x 4n (73.8 ± 5.6) and 4n x 2n embryos (73.0 ± 3.5), and tetraploid-range CN in 4n x 4n progeny (93.8 ± 6.6). On the other hand, 2n x 3n embryos demonstrated hyper-diploid or hypo-triploid CN (67.4 ± 5.5).

Discussion and conclusion

Concentration and motility of spermatozoa of tetraploid males are similar to those of normal diploid males. Thus, spermatozoa of tetraploid males are able to fertilize eggs from diploid and tetraploid females. In fact, viable progeny were produced in 2n x 2n, 2n x 4n, 4n x 2n and 4n x 4n crosses. Triploid females are concluded to spawn fertile eggs, because the progeny from 3n x 2n and 3n x 4n were viable and normal. However, fertility of spermatozoa of triploid male is low, because the 2n x 3n progeny showed low survival rate and normal appearance.

Eggs of triploid females are similar to those of diploid in size, but smaller than those of tetraploid females, suggesting difference in genome size of egg nucleus between diploid or triploid and tetraploid.

Cytogenetic results suggest that diploid and tetraploid loaches should generate haploid and diploid gametes (eggs and spermatozoa), respectively. However, triploid females generate fertile haploid eggs, while triploid male produces aneuploid spermatozoa with 1.7n chromosomes in average. Thus, resultant progeny from eggs of triploid develop normally, but those from fertilization with sperm of triploid exhibit lower survival capacity, probably due to aneuploidy.

Thus, diploid and tetraploid loaches are concluded to reproduce bisexually. Triploid specimens are fertile in female, but males generate aneuploid spermatozoa which reduce viability of the progeny.

Acknowledgments

This study is supported in part by Grants-in-Aid for Scientific Research (B) (No.21380114) from the Japan Society for the Promotion of Science (JSPS) to KA and for Ronpaku (Dissertation Ph.D.) Program (CSC-10610) from JAPS to YJL.

REFERENCES

Arai K., 2001: Genetic improvement of aquaculture finfish species by chromosome manipulation techniques in Japan. Aquaculture 197: 205-228.

Arai K., 2003: Genetics of the loach, Misgurnus anguillicaudatus: recent progress and perspective. Folia Biologica (Krakow) 51 suppl:107-117.

Fujimoto T.; Kataoka T.; Otani S.; Saito T.; Aita T.; Yamaha E.; Arai K. ,2004: Embryonic stages from cleavage to gastrula in the loach Misgurnus anguillicaudatus. Zool. Sci. 21: 747-755.

Fujimoto T.; Sakao S.; Yamaha E.; Arai K., 2007: Evaluation of different doses of UV irradiation to loach eggs for genetic inactivation of the maternal genome. J. exp. Zool. 307A: 449-462.

Li Y.-J. ; Yin J.; Wang J.-B.; Yuan X.; Wei J.; Sun X.-W.; Arai K., 2008: A study on the distribution of polyploidy loaches in China. Nippon Suisan Gakkaishi 74: 177-182.

Yoshikawa H.; Morishima K.; Kusuda S.; Yamaha E.; Arai K., 2007: Diploid sperm produced by artificially sex-reversed clone loaches. J. exp. Zool. 307A: 75-83.

O-1/03
EVALUATION OF SPERMIATION INDEXES DURING MULTIPLE SPERM COLLECTIONS IN STERLET (*ASIPENCER RUTHENUS*)

A. Shaliutina[1], B. Dzyuba[1,2], M. Hulak[1], S. Boryshpolets[1], P. Li[1], O.Linhart[1]

[1] *University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Vodňany, Czech Republic*

[2] *Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of the Ukraine, Kharkov, Ukraine*

E-mail: shalia00@frov.jcu.cz

Introduction

The sterlet (*Acipenser ruthenus*) is a common Eurasian species of sturgeon. The species has undergone a large population decline, but local populations are still surviving in most parts of its distribution range. Nowadays, the major problem faced by development of sturgeon culture is the lack of domestic brood-stocks and dependence of fish farmers on wild brood-stocks capture, which is not only unreliable, but also severely restricted by governments regulations (Chebanov and Billard, 2001). Moreover, it should be noted that, in contrast to almost always limited number of sexually mature females, another important problem during artificial reproduction is the availability of sexually mature males capable to produce sufficient amount of sperm of desired quality at the needed time. This important aspect is one among several crucial factors that ultimately determine the success of artificial reproduction in sturgeon farms (Billard, 2000). However, due to limited access of mature males that are capable to produce sperm of desired quality, a very common practice consists in striping mature sturgeon males more than once during hatchery operation. So far, the lack of information exists about physiological consequences of such sequential or multiple sperm collection on sturgeon sperm physiology.

Therefore, the present work was designed to better understand the effects of multiple sperm collection on selected quantitative parameters (e.g. sperm volume, spermatozoa concentration) as well as functional parameters including spermatozoa motility and velocity by using sterlet males as model.

Materials and methods

Three different experimental groups were used (A, B and C). Each group consists of six sexually mature males. Spermiation was stimulated by intramuscular injection of carp pituitary powder at a dosage of 4 mg /kg of body weight. In each experimental group sperm was collected three times per each male and the time intervals between subsequent collections was 5 hours: in group A the initial sperm collection started at 12 h after hormonal injection followed by two subsequent stripping that were realized at 17 h and 22 h after hormonal treatment, in group B the first sperm collection started at 24 h after hormonal injection followed by two subsequent collections at 29 h and 34 h after hormonal treatment and in group C the initial sperm collection started at 36 h after hormonal injection followed by two subsequent collections at 41 h and 46 h after hormonal treatment, respectively.

During sperm sequential stripping, volume was measured and expressed as ml. The spermatozoa concentration was calculated by Burker haemocytometer using X400 magnification. Percent motile spermatozoa (%) and spermatozoa velocity (μms^{-1}) were determined after triggering motility under dark-field microscopy (Olympus BX 50, Japan) (×20 objective magnification). Motility was observed and recorded immediately after dilution until 2 min post-activation using a CCD video camera (SONY, SSC-DC50AP). The positions of the spermatozoa heads were measured from 5 consecutive video frames at 15, 30, 45, 60 and 90 s post-activation and then analyzed using Micro-image software (version 4.0.1. for Windows with a special macro created by Olympus, Czech Republic).

The osmolality of seminal plasma was measured using a Vapour Pressure Osmometer and expressed in mOsmol kg^{-1}. The protein concentration in seminal plasma was determined by the bicinchoninic acid assay, using a photometer Infinite M200 (Tecan, Switzerland).

Statistical comparison was obtained by analysis of variance (ANOVA) followed by Tukey's HSD test. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

Results

The average volume of sperm collected during experiment varied from 12.74 ± 6.9 to 4.73 ± 4.67 ml in all experimental groups used. Furthermore, the sperm volume was significantly ($p<0.05$) altered by multiple collection in experimental groups B and C. In terms of spermatozoa concentration, significant differences ($p<0.05$) were observed between collections in all experimental groups but insignificant differences were found between experimental groups.

The average protein concentration in seminal fluid ranged from 0.50 ± 0.18 to 0.57 ± 0.11 mg/ml. However, insignificant differences were found in protein concentrations between and within experimental groups. The osmolality of seminal fluid was significantly ($p<0.05$) altered by multiple collections in experimental group B. In the other two experimental groups (A and C), significant changes in osmolality of seminal plasma between subsequent collections were not detected.

Investigation of spermatozoa percentage shows that 100% of spermatozoa were motile at 15 s after activation with a velocity of 181.12 ± 19.10 μms^{-1}; after 90 s, the velocity decreased to 130 ± 26 μms^{-1}; and motility was maintained for up to 4 min. In general, both the highest percentage of motile spermatozoa and spermatozoa velocity were observed after third stripping in all experimental groups.

Discussion and conclusions

Our results show that within small time intervals (5 hours) between collections, sterlet males are able to produce an additional new portion of sperm.

The spermatozoa concentration increased during the second stripping as compared to the first and third collection in all experimental groups. Also, the lowest spermatozoa concentration was detected after third sperm collection: this could result from an increased production of seminal fluid. However, no significant differences in protein concentration between sequential stripping in all experimental groups were presented. Thus, based on our results, we hypothesize that dilution of seminal plasma with urine during each subsequent stripping is similar and consequently, the protein concentration in seminal plasma is not affected.

The increase of spermatozoa velocity and motility, that we observed, could be explained by a so-called "effect of sperm aging". This aging phenomenon could arise from the fact that, during spermatogenesis, spermatozoa would appear and be stored in testis several months before their release at spawning: during this period of ageing process, the population of mature sperm could be partly degraded in testes, which would affect spermatozoa motility and other sperm indexes.

Acknowledgements

Special thanks are expressed to the projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

References

Alavi, S.M.H., Cosson, J., Kazemi, R., 2006. Semen characteristics in Acipenser persicus in relation to sequential stripping. Appl. Ichthyol. 22, 400-405.

Billard, R., 2000. Biology and control of reproduction of sturgeons in fish farms. J. Fisheries Sci. 2(2), 1-20.

Chebanov, M., Billard R., 2001. The culture of sturgeons in Russia: production of juveniles for stocking and meat for human consumption. Aquatic Living Resource 14, 375-381.

Podushka, S., 2003. Sustainable scheme of males sterlet exploitation. INEHKO, 2003, №6, 29-31 (Russian).

O-1/04
AQUAPORINS AND TELEOST SPERMATOGENESIS: INSIGHTS INTO THEIR PHYSIOLOGICAL ROLES AND POTENTIAL USE FOR SPERM CRYOPRESERVATION

François Chauvigné[1], Loredana Zilli[2], Sebastiano Vilella[2], Joan Cerdà[1]

[1] *Laboratory of Institut de Recerca i Tecnologia Agroalimentàries (IRTA)-Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas (CSIC), 08003 Barcelona, Spain*

[2] *Laboratory of Comparative Physiology, Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy*

E-mail: joan.cerda@irta.cat

Introduction

Despite that fluid homeostasis is essential during vertebrate spermatogenesis to produce viable sperm, limited information is available on the molecular mechanisms involved. In many tissues, water transport across cell membranes is mediated by molecular water channels or aquaporins which are permeable to water and small non-charged solutes and conserved in all organisms. In lower vertebrates, such as fish, the diversity, structure and substrate specificity of aquaporins are yet largely unknown. However, recent studies have shown that teleosts harbor a large repertoire of functional aquaporins which retain the substrate specificity characteristics of the tetrapod counterparts (reviewed by Cerdà and Finn 2010).

In teleosts, the composition of the seminal fluid is highly regulated to maintain spermatozoa immotile until the time of release into the aqueous environment. In marine fish, the hyperosmotic shock encountered by spermatozoa when facing saline water induces a rapid cytoplasmic water efflux and membrane hyperpolarization, which results in the activation of cell motility. In the gilthead sea bream (*Sparus aurata*), we recently demonstrated the presence of two aquaporin isoforms, Aqp1a and -10b, in the head and flagellum of spermatozoa, and based on functional expression and sperm activation assays in vitro it was suggested that Aqp1a may be involved in sperm activation (Zilli et al. 2009). However, the role of Aqp10b in spermatozoa is yet unknown. In addition, considering that fluid homeostasis is most likely associated with maturation, activation, and viability of male germ cells, more information is necessary to understand the physiological roles that aquaporins may play during fish spermatogenesis. Therefore, the present work was aimed at identifying the specific aquaporin isoforms expressed during gilthead sea bream spermatogenesis and establish their cellular localization patterns.

Materials and methods

Partial cDNAs encoding additional aquaporins from the gilthead sea bream (Aqp0a, -3a, -4, -7, -9b) were cloned by RT-PCR using degenerate oligonucleotide primers. Using these sequences, and the previous cloned Aqp1a, -1b, -8b, and -10b cDNAs, we determined which isoforms were expressed in testis, sperm, or in both, by RT-PCR. The 5' and 3' ends of the positive cDNAs were amplified, and the full-length cDNAs functionally expressed in *Xenopus laevis* oocytes to determine their substrate preferences. In situ hybridization (ISH) and immunocytochemistry using specific antisera were carried out to localize the cellular sites of expression in testis.

Results and discussion

In the zebrafish (*Danio rerio*), eight aquaporin isoforms have been found to be expressed in testis, including water-selective aquaporins (Aqp1a, -1b, -4, and -12) and water and solute transporting aquaporins (aquaglyceroporins, Aqp-3a, -3b, -7, -8aa, and -10b) (Tingaud-Sequeira et al. 2010). In the testis of the gilthead sea bream, we found transcripts encoding Aqp0a, -1a, -7, -8b, -9b, and -10b, whereas only Aqp1a and -10b mRNAs were found in spermatozoa. Interestingly, *X. laevis* oocytes expressing sea bream Aqp8b were permeable to water and urea, but not to glycerol, as it occurs for the zebrafish Aqp8aa and -8ab, but not for zebrafish Aqp8b (Tingaud-Sequeira et al. 2010). However, the permeability of sea bream Aqp8b to urea was unusually high, i.e. as high as that of human aquaporin-3 (AQP3).

The specific cellular localization of aquaporins in the sea bream testis was studied by ISH and immunohistochemistry. The results indicated that Aqp1a was localized in spermatocytes and spermatids, in addition to spermatozoa, whereas Aqp8b was found only in spermatids. These observations suggest that these aquaporins may be involved in spermatid formation/differentiation and maturation of spermatozoa. The presence of other aquaglyceroporins in testis, such as Aqp7 and -9b, and of Aqp10b in spermatozoa, suggest the role of neutral solute transport via aquaporins both during gametogenesis and spermatozoa viability. However, the specific physiological functions of these aquaporins remain unknown.

The presence of aquaglyceroporins in spermatozoa is also of interest for sperm cryopreservation because these channels are permeated by cryoprotectant agents such as propylene glycol or ethylene glycol (Chauvigné et al. 2011). Optimization of protocols for sperm cryopreservation are of importance for fishes of commercial interest showing low production of sperm or sperm of low quality (Cabrita et al. 2008). Artificial expression of aquaglyceroporins in gametes, such as mammalian AQP3, enhances cell permeability to water and cryoprotectants, thus potentially reducing the formation of intracellular ice during freezing as well as cryoprotectant toxicity and osmotic stress (reviewed by Chauvigné and Cerdà, 2009). Recently, we engineered two zebrafish Aqp3b mutants that exhibit enhanced ethylene glycol permeability or were insensitive to changes in pH (Chauvigné et al. 2011), and methods to deliver these aquaporins into fish spermatozoa are being developed.

Acknowledgements
This work is supported by the Spanish Ministry of Science and Innovation (AGL2010-15597, AQUAZOA) to J.C.

References

Cabrita, E.; Robles, V.; Herraez, P., 2008: Methods in reproductive aquaculture: marine and freshwater species. CRC Press, Taylor & Francis Group, 568 pp.

Cerdà, J.; Finn, R.N., 2010: Piscine aquaporins: an overview of recent advances. J. Exp. Zool. A Ecol. Genet. Physiol. 313, 623-650.

Chauvigné, F.; Cerdà, J., 2009: Expression of functional aquaporins in oocytes and embryos and the impact on cryopreservation. In: Preservation of Human Oocytes, Coticchio, G., Borini, A., eds. London: Informa Healthcare. pp. 104-115.

Chauvigné, F.; Lubzens, E.; Cerdà, J., 2011: Design and characterization of genetically engineered zebrafish aquaporin-3 mutants highly permeable to the cryoprotectant ethylene glycol. BMC Biotechnol. 11, 34.

Tingaud-Sequeira, A.; Calusinska, M.; Finn, R.N.; Chauvigné, F.; Lozano, J.; Cerdà, J., 2010: The zebrafish genome encodes the largest vertebrate repertoire of functional aquaporins with dual paralogy and substrate specificities similar to mammals. BMC Evol. Biol. 10, 38.

Zilli, L.; Schiavone, R.; Chauvigné, F.; Cerdà, J.; Storelli, C.; Vilella, S., 2009: Evidence for the involvement of aquaporins in sperm motility activation of the teleost gilthead sea bream (Sparus aurata). Biol. Reprod. 81, 880-888.

O-1/05
PROBIOTICS INDUCE OOCYTE MATURATIONAL COMPETENCE IN *DANIO RERIO*

Giorgia Gioacchini[1], Elisabetta Giorgini[2], Daniel L. Merrifield[3], Gary Hardiman[4, 5], Andrea Borini[6], Lisa Vaccari[7], Oliana Carnevali[1]

[1] *Dipartimento di Scienze del Mare, Università Politecnica delle Marche, 60131 Ancona, Italy*
[2] *Dipartimento di Idraulica, Strade, Ambiente e Chimica ISAC, sezione Chimica, Università Politecnica delle Marche, 60131 Ancona, Italy*
[3] *Aquaculture and Fish Nutrition Research Group, School of Biomedical and Biological Sciences, The University of Plymouth, PL4 8AA, UK*
[4] *Department of Medicine, University of California San Diego, La Jolla, CA, USA*
[5] *BIOGEM, School of Medicine, University of California San Diego, La Jolla, CA, USA*
[6] *Tecnobios Procreazione,Centre for Reproductive Health, 40125 Bologna, Italy*
[7] *SISSI beamline, ELETTRA Synchrotron Light Laboratory, S.S. 14, Km 163.5, 34149, Basovizza, Trieste, Italy*

E-mail: o.carnevali@univpm.it

Introduction
Recent research on the molecular biology and genomics of probiotics has focused on the interaction of gut microbiota with the immune system (Verdenelli et al. 2009), brain development (Diaz Heijtz et al. 2001), anti-cancer potential (Hirayama & Rafter 2000) and potential as a biotherapeutic agent against many diseases (Iannitti & Palmieri 2010). Very recently, our group observed a positive role of a probiotic, *Lactobacillus rhamnosus* IMC 501®, on fecundity and on endocrine and paracrine control of follicle development in zebrafish (*Danio rerio*) females (Gioacchini et al. 2010; Giorgini et al. 2010).

The current study examined, for the first time, the effects of the probiotic *L. rhamnosus* IMC 501®, administered as functional food, on oocyte maturational competence acquiring in zebrafish.

Materials and methods
Zebrafish (*D. rerio*) adults were divided into two experimental groups: a control group (CTRL) fed only on a commercial diet and a probiotic fed group (PROBIO) fed on the commercial diet supplemented with the probiotic *Lactobacillus rhamnosus* IMC 501® at 10^6 CFU ml^{-1} for 10 days. PCR-DGGE was utilised to detect *L. rhamnosus* IMC 501® in the GI tract.

Follicle *in vitro* maturation assays were performed and the expression of relevant genes of interest, were carried out using Real Time PCR (Sybr green method) on follicle stage IIIa and IIIb mechanically isolated from both treated and control females. Finally, a DNA microarray and FPA (Focal Plane Array) FT-IR Imaging were conducted from the same follicles.

Results
Ten days administration of *L. rhamnosus* significantly enhanced responsiveness of incompetent follicles (IIIa) to 17,20-dihydroxy-4-pregnen-3-one (MIH) and their *in vitro* maturation rate. Real Time PCR results revealed changes of expression of genes recently identified as key regulators of oocyte maturational competence acquisition; in particular, the probiotic treatment induced *lhr*, *activinβA1* and *mprβ* levels and concomitantly reduced *tgfβ1* and *gdf9* mRNA levels in stage IIIa follicles in comparison with the control group. In addition, from the DNA microarray experiment and the relative gene ontology (GO) data analysis it emerged that several gene family members involved in signal transduction and transmission were modulated by the probiotic treatment in class IIIa follicles. In class IIIb follicles several gene families involved in transcription were modulated by the probiotic treatment. Molecular analysis using PCR-DGGE revealed that the probiotic was able to populate the GI tract and modulate the microbial communities causing a clear shift in microbial communities,

Finally, by FPA (Focal Plane Array) FT-IR Imaging, modifications in proteins secondary structures, as well as in hydration and in bands related to phosphate moieties were observed in IIIa follicles isolated from probiotic fed females.

Discussion and conclusion

These findings highlight the capability of the probiotic *L. rhamnosus* IMC 501® to populate and modulate the microbiota of the GI tract and revealed an enhancing role of *L. rhamnosus* on acquisition of maturational competence in zebrafish oocytes. The significance of the results obtained herein underline the importance of diet and gut microbes in the reproductive process, supporting the hypothesis that feed additives could improve fecundity. Considering that the zebrafish has been clearly established as an excellent vertebrate model for reproduction and development research, these results support the potentiality of feed additives such as probiotics, frequently used in aquaculture practice, as a new technology to improve reproduction.

Acknowledgements

Special thanks to Francesca Maradonna, Chiara Piccinetti and Silvia Falcinelli at Università Politecnica delle Marche for their help at various stages of this study; special thank also to James Sprague at the University of California San Diego Biomedical Genomics laboratory (BIOGEM) for Agilent microarray processing and Dr. Roman Sasik for help with the data analysis

References

Diaz Heijtz, R.; Wang, S.; Anuar, F.; Qian, Y.; Björkholm, B.; Samuelsson, A.; Hibberd, M.L.; Forssberg, H.; Pettersson, S., 2011: Normal gut microbiota modulates brain development and behavior. PNAS 108, 3047–3052.

Gioacchini, G.; Maradonna, F.; Lombardo, F.; Bizzaro, D.; Olivotto, I.; Carnevali, O., 2010: Increase of fecundity by Probiotic administration in zebrafish (Danio rerio). Reproduction 140, 953-959.

Giorgini, E.; Conti, C.; Ferraris, P.; Sabbatini, S.; Tosi, G.; Rubini, C.; Vaccari, L.; Gioacchini, G.; Carnevali, O., 2010: Effects of Lactobacillus rhamnosus on zebrafish oocyte maturation: an FTIR imaging and biochemical analysis. Analytical and Bioanalytical Chemistry 398, 3063-3072.

Hirayama, K.; Rafter, J., 2000: The role of probiotic bacteria in cancer prevention. Microbes and Infection 2, 681-686.

Iannitti, T.; Palmieri, B., 2010: Therapeutical use of probiotic formulations in clinical practice Clinical Nutrition 29, 701-725.

Verdenelli, M. C.; Ghelfi, F.; Silvi, S.; Orpianesi, C.; Cecchini, C.; Cresci, A. 2009: Probiotic properties of Lactobacillus rhamnosus and Lactobacillus paracasei isolated from human faeces. European Journal of Nutrition 48, 355-363.

O-1/06
MELATONIN EFFECTS ON KILLIFISH GAMETES

Francesco Lombardo[1], Adele Fabbrocini[2], Elisabetta Giorgini[3], Paolo Ferraris[3], Raffaele D'Adamo[2], Giorgia Gioacchini[1], Oliana Carnevali[1]

[1] *Dipartimento di Scienze del Mare, Università Politecnica delle Marche, via Brecce Bianche, Ancona, Italy*
[2] *Consiglio Nazionale delle Ricerche, Istituto di Scienze Marine, UOS Lesina (FG), Italy*
[3] *Dipartimento ISAC, Università Politecnica delle Marche, via Brecce Bianche, Ancona, Italy*

E-mail: f.lombardo@univpm.it

Introduction
The surrounding environment, with its cyclic changes plays a crucial role in the regulation of reproduction in many animals, including fish (Chaudhuri 1997), influencing different aspects of their physiology and behaviour (Leatherland et al. 1992; Zimecki 2006). Processing of environmental message is one of the most essential events in the regulation of reproduction and so in the life cycle (Maitra & Chattoraj 2007).

This study aimed at investigating the effects of exogenous melatonin administration on *Fundulus heteroclitus* egg and sperm by fecundity, embryo survival, hatching rate and sperm motility analyses.

An *in vitro* oocyte maturation assay was also carried out to test the ability of melatonin (50 pg/ml and 100 pg/ml) to affect maturation of *F. heteroclitu*s postvitellogenic-prematurational follicles.

Moreover, the effects of melatonin on follicles and sperm macromolecular composition were highlighted by FT-IR analysis.

Materials and methods
Mature killifish, *Fundulus heteroclitus* L., under controlled condition (24°C; 14L/10D, 30‰) were used to set up two experimental groups: one control (CTRL) and one group (MEL) exposed via water, for 8 days, to 1 μM melatonin, following Zhdanova et al. 2008. The experiment was repeated three times.

Thus, to evaluate the effects of melatonin exposure on *F. heteroclitus* gametes, daily egg collection was performed at 09 am by siphoning out the spawning tank bottom. The fertilized eggs were then incubated at room temperature (24°C) for 8 days and embryo survival monitored till hatching. Sperm was collected by stripping at the end of melatonin treatment to evaluate the effects on sperm motility and to prepare sperm samples for FT-IR analysis.

Sperm motility parameters [curvilinear velocity (VCL); % of total motile sperm (VCL>35 μm/sec); % of rapid sperm (VCL>200 μm/sec)] were analyzed by Sperm Class Analyzer® after 1:50 dilution in sea water (15‰) up to the decay of motility (15 min). FT-IR microspectroscopic analyses were carried out in reflectance mode, on centrifuged sperm samples (400 g for 10 min) while the ovaries were isolated and cryosectioned at a predefined thickness of 5 μm. All the samples were deposed on steel supports (spatial resolution 100x100 μm, spectral resolution 4 cm^{-1}). Follicles containing postvitellogenic-prematurational oocytes (1,25-1,45 mm in diameter) were surgically removed from the ovary and incubated for 24h in a humidified, temperature-controlled incubator, with: L15 medium alone (CTRL), DHP (17α,20β-dihydroxy-4-pregnen-3-one, the maturation-inducing hormone (MIH) 10 ng/ml alone (DHP), DHP 10ng/ml+melatonin 50pg/ml (DHP+MEL50), DHP 10ng/ml+melatonin 100pg/ml (DHP+MEL100), 50 pg/ml melatonin alone (MEL50) and 100pg/ml melatonin alone (MEL100). Thus, oocytes maturation was evaluated after 24h of incubation at 24°C by the incidence of resumption of meiosis (oocyte maturation) scored as the percentage of germinal vesicle breakdown (GVBD).

Results
In this experiment, 8 days-melatonin exposure significantly ($P<0,05$) increased *F. heteroclitus* fecundity and embryo survival with respect to control group, while the hatching rate was not affected by this treatment.

The *in vitro* maturation assay clearly demonstrated, for the first time in this specie, the melatonin stimulatory effects on follicles maturation both administrated alone or in combination with DHP, resulting in the greatest GVBD rate in the DHP+MEL100 group.

On activation, the percentage of total motile spermatozoa and the VCL were significantly ($P<0,05$) higher in the treated group with respect to the control one;

these differences were more evident in rapid spermatozoa. In the following minutes no differences in the analyzed parameters were recorded.

FT-IR microspectroscopy analysis evidenced changes in Amide I and II bands, which increased their absorbances in treated samples, respect to the control, and in the ratio between the bands at 1080 and 1240 cm^{-1}, attributable to the symmetric and asymmetric stretching modes of phosphates, respectively. Representative spectra for I to IV oocyte stages were investigated in controls to find specific vibrational patterns corresponding to different maturation degrees. On going from I to IV oocyte stages, relevant spectral differences were found, with stage III exhibiting an intermediate spectroscopic behaviour. Analyses on the effects of melatonin on macromolecular composition in follicles at different stages are in progress.

Discussion and conclusions

The present study demonstrated that via water melatonin administration significantly enhanced fecundity and *in vitro* oocyte GVBD rate in *F. heteroclitus,* showing that this hormone may act on the last step of oogenesis, thus, on the GVBD and subsequent ovulation, increasing the amount of spawned eggs as recently demonstrated also in zebrafish (Carnevali et al. 2010; 2011). In addition, melatonin enhanced sperm motility, both in terms of percentage of motile spermatozoa and velocity. These results are consistent with the increase of fecundity and embryo survival observed in melatonin treated group (Kime et al. 1999). The FTIR analysis showed that melatonin treatment causes an increase in the proteic component of sperm. As a matter of fact, the observed decrease of the band ratio 1080/1240 cm-1 could be attributable to a higher consumption of phosphates, enhanced by the increasing in sperm motility (Perchec et al. 1995). In conclusion, these results support the beneficial role of melatonin on *F. heteroclitus* reproduction.

References

Carnevali, O.; Gioacchini, G.; Piccinetti, C.C.; Maradonna, F.; Lombardo, F.; Giorgini, E.; Tosi, G., 2010: Melatonin control of oogenesis and metabolic resources in Zebrafish. J. Appl. Ichthyol. 26, 826-830.

Carnevali, O.; Gioacchini, G.; Maradonna, F.; Olivotto, I.; Migliarini, B., 2011: Melatonin induces follicle maturation in Danio rerio. Plus One, In Press.

Chaudhuri, H., 1997: Environmental regulation of gonadal maturation and spawning in Fishes. In: Frontiers in environmental and metabolic endocrinology, Maitra S.K., ed. University of Burdwan Publications, Burdwan, India, pp 91-100.

Leatherland, J.F.; Farbridge, K.J.; Boujard, T., 1992: Lunar and semi-lunar rhythms in fishes. In: Rhythms Fish, Ali, M.A., ed. Plenum Press, NYC, pp. 83-107.

Kime, D.E.; Van Look, K.J.W.; McAlllister, B.G.; Huyskens, G.; Rurangwa, E.; Ollevier, F., 1999: Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fishes. Comp. Biochem. Physiol. C130, 425-433.

Maitra, S.K.; Chattoraj, A., 2007: Role of photoperiod and melatonin in the regulation of ovarian functions in Indian carp Catla catla: basic information for future application. Fish Physiol. Biochem. 33, 367-382.

Perchec, G.; Jeulin, C.; Cosson, J.; André, F.; Billard, R.,1995: Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747-753.

Zhdanova, I.V.; Yu, L.; Lopez-Patino, M.; Shang, E.; Kishi, S.; Guelin, E., 2008: Aging of the circadian system in zebrafish and the effects of melatonin on sleep and cognitive performance. Brain Res. Bull. 75, 433-441.

Zimecki, M., 2006: The lunar cycle: effects on human and animal behaviour and physiology. Postepy Hig. Med. Dosw. 60, 1-7.

O-1/07
ORIGIN AND MIGRATION OF PRIMORDIAL GERM CELLS IN STURGEON

Taiju Saito[1,4], Rie Goto[1], Martin Psenicka[2], Yutaka Kawakami[1], Shinji Adachi[3], Katsutoshi Arai[4], Etsuro Yamaha[1]

[1] *Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Nanae 041-1105, Japan*
[2] *Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in Ceske Budejovice, Zatisi 728/II, 389 25 Vodnany, Czech Republic.*
[3] *Laboratory of Aquaculture Biology, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan*
[4] *Laboratory of Aquaculture Genetics & Genomics, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan*

E-mail: taiju@hokudai-nanae.jp

Introduction

Acipenseridae is one of the oldest families of bony fish. Gonadal development of sturgeon species has already been investigated by histological and ultrastructural procedures from late embryonic stage (Grandi and Chicca, 2008). However, migration of primordial germ cells (PGCs) in embryo has not been studied in sturgeons because of the difficulty to distinguish these cells from somatic cells by the morphological features during embryonic stages. Eggs of sturgeons have holoblastic cleavage as seen in amphibian, whereas many bony fish utilize meroblastic cleavage. It has been suggested that the sturgeon embryo share many developmental mechanisms with the amphibian *Xenopus laevis* (Ballard and Ginsburg, 1980). In this study, we tried to visualize PGCs in living embryos of the F2 bester, a hybrid between a beluga (*Huso huso*) and a sterlet (*A. ruthenus*), by injecting GFP-*nos*1 3'UTR mRNA into fertilized eggs. The *nos*1 3'UTR was of zebrafish, *Danio rerio*, origin (Köprunner et al., 2001). Moreover, to investigate whether the migratory mechanisms of PGCs is conserved among bony fish, the visualized sturgeon PGCs were transplanted into goldfish, *Carassius auratus*, embryos (each of them belong to the Class Actinopterygii and teleostei: time of divergence based on Inoue et al., 2005: 342-379 million years ago).

Materials and methods

To determine what region of the fertilized eggs generates PGCs, GFP-*nos*1 3'UTR mRNA (MW=446,000) was injected into the animal pole or vegetal pole region of fertilized eggs. Injection was performed in Ringer's solution after removing the outer layer of the chorion (L2 and L3). Embryos were cultured in tap water containing penicillin and streptomycin at 15 °C, and the culture water was replaced every day. mRNA injected embryos were continuously observed under a fluorescence stereomicroscope (Leica MZ16F) equipped with a digital camera (Leica DFC300FX). Embryos with GFP-positive PGCs-like cells were fixed with Bouin's fixative after the eggs hatched and prepared for histology. GFP-positive cells on the sections were stained immunohistochemically and identified as PGCs with the localization patterns and the morphological characteristics. In order to determine the specific blastomeres that generate PGCs, FITC-biotin-dextrans (MW=10,000) was injected into a single vegetal blastomere at the 64- to 128-cell stage. Visualized PGC and somatic cell mass was removed from the embryo with fine forceps at the neural stage and dissociated into single cell in 1% citric acid trisodium in Ringer's solution by gentle pipetting. Single PGCs were transplanted into goldfish blastula embryo as according to Saito et al., 2008. Endogenous PGCs in goldfish embryos were labeled with RFP by injecting RFP-*nos*1 3UTR mRNA at 1- to 4-cell stage.

Results

PGC-like cells were visualized when mRNA was injected into the vegetal pole region of sturgeon egg. In total, 47 embryos were injected with mRNA at vegetal pole region and 35 developed normally, of which 33 embryos contained PGC-like cells that showed strong GFP expression. In these embryos, the number of PGC-like cells was 23.5 on average (SD: 17.1; Range: 0-59). On the other hand, when mRNA was injected into the animal pole region (total number was 44 and 33 developed normally), PGC-like cells were not appeared in any embryos. At first, PGC-like cells were observed at the surrounding region of the tailbud after the closed neural tube stage. During somitogenesis stage, PGC-like cells migrated dorsal-ward on close to the surface of embryo, and finally, these cells aligned along the newly formed alimentary canal at least until 6-day post fertilization (dpf). Immunohistochemical analysis revealed that the labeled cells were PGCs. A FITC-biotin labeled blastomere also gave rise to PGCs during development. FITC-labeled PGCs detached from the main cluster of FITC-labeled yolky vegetal cells and migrated toward the gonadal region. In total, 68 embryos were injected and 33 developed normally, of which 32 embryos contained FITC-labeled PGCs. In these embryos, the number of FITC-labeled PGCs was 17.0 (SD: 13.0; Range: 0-52) on average. We transplanted a GFP-labeled sturgeon PGC into goldfish blastula embryo in which endogenous PGCs were labeled with RFP by injecting RFP-nos1 3'UTR mRNA. 36 chimeras were produced and of which 2 embryos contained a transplanted donor PGCs at the gonadal region at 3 dpf. Each donor-derived PGC was localized side by side with host PGCs, indicating sturgeon PGCs could recognize the guidance signals for goldfish PGCs during migration correctly. However, in all chimeras, transplanted PGCs disappeared until 4 dpf.

Disscussion and conclusions

In this study, we visualized sturgeon PGCs by injecting GFP-*nos*1 3'UTR mRNA into vegetal pole region of 1- to 2-cell egg. Visualized PGCs were observed in almost all embryos. This result clearly showed that mechanism of *nos*1 3'UTR, subject to degradation in somatic cells and stabilization in PGCs by interaction with the microRNA, miR-430, is conserved between these two phylogenetically distantly related fish species, as previously shown among other species. Sturgeon PGCs was also labeled by injecting FITC-biotin into a single vegetal blastomere at 64- to 128-cell stage. These results strongly suggest that PGCs originate from vegetal hemisphere of eggs. It is reasonable to think that PGCs formation of sturgeon is similar to the equivalent processes in amphibian embryos rather than teleost species. When sturgeon PGCs were transplanted into goldfish embryos, however, these PGCs were able to migrate toward the gonadal region of host embryo, although donor PGCs disappeared until 4 dpf. This result gives strong evidence that the migration mechanisms of PGC are highly conserved between these two fishes irrespective of superficial differences between them.

Acknowledgements

This study was supported in part by Grant-in-Aid for PROBRAIN from the Bio-oriented Technology Research Advancement Institution and PD fellowships for foreign researchers (P09325) from JSPS.

References

Ballard, W.W. and Ginsburg, A.S., 1980: Morphogenetic movements in acipenserid embryos. J. Exp. Zool. 213, 69-103.

Grandi, G. and Chicca, M., 2008: Histological and ultrastructural investigation of early gonad development and sex differentiation in Adriatic sturgeon (Acipenser naccarii, Acipenseriformes, Chondrostei). J. Morphol. 269, 1238-1262.

Inoue, J.G., Miya, M., Venkatesh, B. and Nishida, M., 2005: The mitochondrial genome of Indonesian coelacanth Latimeria menadoensis (Sarcopterygii: Coelacanthiformes) and divergence time estimation between the two coelacanths. Gene. 349, 227-235.

Köprunner, M., Thisse, C., Thisse, B. and Raz, E., 2001: A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes. Dev. 15, 2877-2885.

Saito, T., Goto-Kazeto, R., Arai, K. and Yamaha, E., 2008: Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol. Reprod. 78, 159-166.

O-5/01
HIGH-THROUGHPUT CRYOPRESERVATION FOR AQUATIC SPECIES

Terrence R. Tiersch

Aquaculture Research Station, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana, USA

E-mail: ttiersch@agctr.lsu.edu

Introduction

High-throughput processing has been widely applied for decades for sperm cryopreservation in livestock. The technology was based on advances made in the laboratory and was scaled up for increased speed, mass production, and quality assurance. Cryopreserved germplasm combined with high-throughput processing constitutes an independent industry encompassing animal breeding, preservation of genetic diversity, and medical research. High-throughput includes efficient large-scale production, uniformity and quality assurance, and standardized and streamlined procedures. Fish sperm cryopreservation began in the 1950's, about the same time as for humans and livestock, but it has remained at a research scale for fish despite study of hundreds of species (Tiersch and Green 2011). In the past decade, equipment and processes in place for livestock have been tested for feasibility with fish (e.g., Haffray et al. 2008) or directly by using the protocols developed for dairy bulls for blue catfish *Ictalurus furctatus* (Lang et al. 2003), and shellfish such as Pacific oysters *Crassostrea gigas* (Dong et al. 2005, 2007a).

Overview

Large-scale sperm cryopreservation and germplasm banking of aquatic species can allow easy and safe maintenance and transport of genetic resources, and is a readily transferable form of bankable wealth that can be accumulated, archived, and cataloged. Genetically relevant germplasm banking must deal with large numbers of individuals, populations, and species while providing quality assurance to guarantee recovery of specific gene pools. Thus, high-throughput approaches need to be developed encompassing biological and cryobiological principles, equipment and facility development, process control for sample handling, inventory and database maintenance, quality control and assessment, standardization and establishment of industrial practices, and institution of biosecurity systems. High-throughput processing should be scalable to the needs of individual users and a central pathway should be established to accommodate current and future levels and methods of application. These activities should be funneled into a standardized approach that can utilize industrial methods supported by commercial vendors of specialized equipment, supplies, and reagents, and industrial-level service providers for cryopreservation, storage, and quality control.

Automation

One of the most efficient methods to achieve high-throughput is by adoption of automated systems developed for mammals that provide loading, sealing, labeling, and reading of straws. Such systems have been evaluated for aquatic species for commercial-scale cryopreservation (e.g., Hu et al. 2011). After addition of the cryoprotectant, sperm samples are drawn into plastic straws (0.25 - 0.5 mL), transferred to a sealing platform, labeled and verified with alphanumeric information and bar-coding and undergo quality control evaluation. These systems can routinely process as many as 15,000 straws per hour before standardized controlled-rate freezing (Hu and Tiersch 2011) and storage in liquid nitrogen.

Pathway development

In addition to the use of automated equipment, high-throughput systems require arrangement of the sequence of procedures, and balancing of the inputs and outputs between connected steps. For any sequence developed, the production utility and processing costs can be evaluated. Based on previous studies, a minimum of five major steps can be identified: sample collection (including shipping of broodstock or gametes), sample processing, freezing and sorting, storage, and utilization. The time and cost can be reduced at each step to increase the efficiency of production. Therefore, high-throughput cryopreservation would include: 1) optimization of each step individually and in relation to other steps, 2) assembly and streamlining of the steps into a pathway, and 3) standardization of the pathway for application to improve overall efficiency, sample usage (e.g., quantifying steps on a per-sperm basis), post-thaw quality, and fertilization success (Tiersch et al. 2004, Tiersch 2011).

Need for standardization

A considerable and potentially troublesome lack of standardization characterizes the methods and reporting for cryopreservation studies. Comparisons among different studies are difficult to perform and could well be invalid in most cases due to the procedural and reporting variations. Studies utilizing the sperm agglutination phenomenon (Dong et al. 2007b) clearly demonstrated the requirement to standardize sperm concentration and methods for oysters (and by extension, all aquatic species) during cryopreservation. Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. Controversy and inconsistency would be reduced if more congruent approaches were utilized and results among various studies could be directly compared. Suggestions for improvement include the creation and widespread acceptance of standard reference works to assist in harmonizing terminology, and the development and utilization of congruent educational programs. Standardization of research practices and reporting could be facilitated through establishment of guidelines for publication of results. These guidelines could be made available to journal editors and reviewers to assist in evaluation of research reports.

Conclusions

High-throughput technology has wide potential application. In biomedical science, it can preserve valuable strains for research purposes; in fisheries management, it can assist hatcheries in recovery efforts, and in aquaculture, it can enable and accelerate selective breeding and genetic improvement. High-throughput processing will serve as a fundamental technology for aquatic germplasm banking. These efforts will proceed more easily with large-bodied fishes or species that can provide copious volumes of sperm such as salmon, but can also proceed with small biomedical fishes such as zebrafish (Yang et al. 2007a) or *Xiphophorus* (Yang et al. 2007b) by pooling of samples to represent lines and increase the volume available for automated processing. Eventual development of processing equipment and containers that can rapidly handle small volumes (e.g., 10-50 uL) in a standard format or by use of microfluidic technologies would allow high-throughput of individual small fishes or samples.

Acknowledgements

This work supported in part by funding from the US National Institutes of Health, US Department of Agriculture, and the Louisiana Sea Grant Program.

References

Dong, Q.; Eudeline, B.; Huang, C.; Allen, S.K.; Tiersch, T.R., 2005: Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, Crassostrea gigas. Cryobiology 50, 1-16.

Dong, Q.; Huang, C.; Eudeline, B.; Tiersch, T.R., 2007a: Cryoprotectant optimization for sperm of diploid Pacific oysters by use of commercial dairy sperm freezing facilities. Aquacult. 271, 537-545.

Dong, Q; Huang, C.; Tiersch, T.R., 2007b: Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. Cryobiology 54, 87–98.

Haffray, P.; Labbe, C.; IMV Technologies; Maisse. G., 2008: Fish sperm cryopreservation in France: from laboratory studies to application in selective breeding programs. Cybium 32 supplement, 127-129.

Hu, E.; Tiersch, T.R., 2011: Development of high-throughput cryopreservation for aquatic species. In: Cryopreservation in Aquatic Species, 2nd edition, Tiersch T.R. and Green C.C., eds. World Aquaculture Society: Baton Rouge, Louisiana. pp. 995-1003.

Hu, E.; Yang, H.; Tiersch, T.R., 2011: High-throughput cryopreservation of spermatozoa of blue catfish: Establishment of an approach for commercial-scale processing. Cryobiology 64, 74-82.

Lang, R.P.; Riley, K.L.; Chandler, J.E.; Tiersch, T.R., 2003: The use of dairy protocols for sperm cryopreservation of blue catfish Ictalurus furcatus. J. World Aquacult. Soc. 34, 66-75.

Tiersch, T.R., 2011: Process pathways for cryopreservation research, application and commercialization. In: Cryopreservation in Aquatic Species, 2nd edition, Tiersch T.R. and Green C.C., eds. World Aquaculture Society: Baton Rouge, Louisiana. pp. 646-671.

Tiersch, T.R.; Green, C.C. (editors), 2011: Cryopreservation in Aquatic Species, second edition, World Aquaculture Society, Advances in World Aquaculture, Baton Rouge, Louisiana, 1005 pages.

Tiersch, T.R.; Wayman, W.R.; Skapura, D.P.; Grier, H.J.; Neidig C.L., 2004: Transport and cryopreservation of sperm of the common snook, Centropomus undecimalis (Bloch). Aquacult. Research 35, 278-288.

Yang, H.; Jones, C.; Varga, Z.; Tiersch, T.R., 2007a: Development of a simplified and standardized protocol for sperm cryopreservation with potential for high throughput in zebrafish Danio rerio. Theriogenology 68, 128-136.

Yang, H.; Hazlewood L.; Heater, S.J.; Guerrero P.A.; Walter R.B.; T.R. Tiersch, 2007b: Production of F1 interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the swordtail Xiphophorus helleri. Biol. Reprod. 76, 401-406.

O-5/02
FERTILITY, VELOCITIES AND MOTILITY OF SURUBIM-DO-PARAÍBA *STEINDACHNERIDION PARAHYBAE* SPERM CRYOPRESERVED IN LACTOSE AND LACTOSE-FREE MEDIA

Rafael V. Araujo[1], Isabel C. Carvalho[1], Eduardo A. Sanches[2], Danilo Caneppele[3], Marcelo C. Leal[4], Ana T. M. Viveiros[1]

[1] *Animal Science Dept, Federal University of Lavras, MG, Brazil*
[2] *Aquaculture Centre, State University of São Paulo, Jaboticabal, SP, Brazil*
[3] *Hydrobiology and Aquaculture Unit of the Energetic Company of São Paulo State (CESP), Paraibuna, SP, Brazil*
[4] *R. Topázio 524, Condomínio Stone Village, Lavras, MG, Brazil*

E-mail: ana.viveiros@dzo.ufla.br; anatmviveiros@hotmail.com.

Introduction
The surubim-do-paraíba *Steindachneridion parahybae* is a migratory Siluriformes Brazilian fish species, native to the Paraíba do Sul River Basin, located in the States of São Paulo, Minas Gerais and Rio de Janeiro (Oliveira & Moraes Jr. 1997). Until 1950, *S. parahybae* was a very important species for fisheries (Machado & Abreu 1952), however, due to overfishing, pollution and hydroelectric dams, the status of this species is currently set as endangered (Rosa & Lima 2008). The use of sperm cryopreservation as a technique to store genetic material of endangered species has long been used. However, this technique has not yet been tested for *S. parahybae*. The aim of this study was to develop a method for sperm cryopreservation of S. parahybae. Post-thaw sperm quality was evaluated for motility and velocities using a computer-assisted sperm analyzer (CASA) and for fertility.

Materials and methods
S. parahybae broodfish were selected from tanks at the Hydrobiology and Aquaculture Station of the Hydroelectric Company of São Paulo (CESP) in Paraibuna city, São Paulo state, Brazil, during the spawning season (January and February). Sperm was collected without hormone induction and diluted in one of the six freezing media comprising the combination of 3 extenders (5% glucose, 10% glucose and BTS™) and 2 concentration of lactose (0 and 10%). Beltsville Thawing Solution™ (BTS™) is a commercial extender for boar sperm (Minitub™, Tiefenbach/Landshut, Germany) and is composed of 0.02% gentamycin sulfate, 4.0% glucose, 0.63% sodium citrate, 0.13% EDTA, 0.13% $NaHCO_3$, and 0.08% KCl. Dimethyl sulfoxide (DMSO) was used as cryoprotectant. Diluted sperm was then loaded into 0.5-mL straws (n = 6 replicate straws x 6 media x 3 males), frozen in nitrogen vapor (Cryoporter™ LN_2 dry vapor shipper) and stored in liquid nitrogen. Three replicate straws were used for fertilization within a week, while the other three were transported to the Laboratory of Semen Technology at the Federal University of Lavras (UFLA) for motility and velocities analysis. All straws were thawed in a water bath at 25 °C for 20 seconds. A pool of oocytes stripped from three females after carp pituitary treatment (0.5 and 5 mg Kg^{-1}) was divided in aliquots of 0.5 g (~140 oocytes). Each aliquot was fertilized at an approximate ratio of ~1 x 10^6 spermatozoa per oocyte. As control, fresh sperm (n = 5 males) was used to fertilize three aliquots of oocytes from the same females. Fertilization rate was calculated 10 hours after fertilization at ~24 °C. Motility and velocities were evaluated using CASA. Post-thaw sperm was activated in a Leja™ counting chamber placed on a phase contrast microscope (Nikon™ E200, Japan), 100 x magnification, green filter, and pH1 position. The microscope was connected to a video camera (Basler Vision Technologies™ 602FC, Ahrensburg, Germany) which generated 100 frames per second. Video recording was started at ~10 seconds post-activation in NaCl 50 mM. Each image was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™, Microptics, S.L. Version 5.1, Barcelona, Spain). Sperm was considered immotile when velocity was <20 μm/s. Progressive motility, curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity were calculated.

Results

In general, the presence of lactose was benefic when combined with 5% glucose, malefic when combined with 10% glucose and indifferent when combined with BTS™, regarding velocities and fertilization rate (Table 1). Post-thaw motility varied between 58 and 66% in all samples, except for lactose-10% glucose medium (36%). In general, the best results were achieved when sperm was frozen in lactose-5% glucose medium: 64% motility, 69 μm/s of VCL, 48 μm/s of VSL, 58 μm/s of VAP and 45% fertilization rate.

Table 1: Progressive motility, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) and fertilization rate of Steindachneridion parahybae sperm cryopreserved in lactose and lactose-free media

Extender	Lactose (%)	Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	Fertilization (%)
5% Glucose	0	66 ± 10[a]	55 ± 5[c]	35 ± 7[b]	44 ± 7[c]	34 ± 9[b]
	10	64 ± 6[a]	69 ± 11[a]	48 ± 12[a]	58 ± 11[a]	45 ± 7[a]
10% Glucose	0	65 ± 3[a]	63 ± 8[b]	41 ± 7[b]	50 ± 7[b]	48 ± 5[a]
	10	36 ± 5[b]	39 ± 3[d]	10 ± 3[c]	17 ± 4[d]	35 ± 11[b]
BTS™	0	59 ± 11[a]	52 ±7[d]	34 ± 10[b]	42 ± 10[c]	30 ± 10[c]
	10	62 ± 7[a]	60 ± 7[b]	35 ± 7[b]	44 ± 7[c]	22 ± 8[d]
Fresh sperm	--	80 ± 6	--	--	--	74 ± 6

[a-e] *Mean ± SD followed by different superscript differ (Scott-Knott; P<0.05).*

Discussion and conclusions

The Siluriformes order contains 33 families, but only 9 species of 6 families have been subjected to sperm cryopreservation studies during the last decade (Viveiros 2011). This is the first report on the sperm cryopreservation of *S. parahybae*. The use of commercial extenders, such as 5% glucose and BTS™ facilitates the procedures for cryopreservation, as no laboratory equipments such as scale, chemicals and pH meter, are necessary. The use of 5% glucose as sperm extender was improved when combined with lactose, possibly because of the increase on the global osmolality. However, when lactose was combined with 10% glucose, a negative effect was observed in all parameters evaluated, probably due to a hyperosmotic shock. The use of CASA to evaluate sperm quality is a great tool to predict fertilization rate as many aspects of motility and velocities can be estimated (Peña & Linde-Forsberg 2000; Rurangwa et al. 2001).

Acknowledgements

CAPES, ANEEL P&D FURNAS (017965), ANEEL P&D CESP (0061-017/2006).

References

Machado C.E.; Abreu H.C.F., 1952: Notas preliminares sobre a caça e a pesca no Estado de São Paulo. A pesca no Vale do Paraíba. Bol. Ind. Animal 13 145-160.

Oliveira J.C.; Moraes Jr D.F, 1997: Dados adicionais à descrição de *Steindachneridion parahybae* (Steindachner, 1876) (Teleostei, Siluroidei, Pimelodidae). Bol. Museu Nac. 384, 1-11.

Peña A.I.; Linde-Forsberg C., 2000: Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. Theriogenology 54, 703- 718.

Rosa R.S.; Lima F.C., 2008: Os peixes brasileiros ameaçados de extinção. In: Livro vermelho da fauna brasileira ameaçada de extinção. Machado A.B.M.; Drummond A.M; Paglia, A.P., eds. Brasília: Fundação Biodiversitas. vol. 2.

Rurangwa E.; Kime D.E.; Ollevier F.; Nash J.P., 2004: The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1-28.

Viveiros A.T.M., 2011: Current status of sperm cryopreservation in Siluriform catfishes. In: Cryopreservation in Aquatic Species. 2nd edition. Tiersch T.R.; Green C.C., eds. Baton Rouge: World Aquaculture Society. pp. 387-397.

O-5/03
OXIDATIVE STRESS INDUCED BY CRYOPRESERVATION ON COMMON CARP SPERM

Ping Li[1], Zhi-Hua Li[1], Boris Dzyuba[1,2], Martin Hulak[1], Marek Rodina[1], Otomar Linhart[1]

[1] *Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in Ceske Budejovice, Vodnany, Czech Republic*

[2] *Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Kharkov, Ukraine*

E-mail: pingli06@yahoo.com

Introduction

Cryopreservation leads to the generation of reactive oxygen species (ROS), which impair postthaw motility, viability, intracellular enzymatic activity, fertility, and other sperm parameters. Oxidative stress can be assessed through the direct quantification of ROS and antioxidants, or by measurement of oxidative stress end products (Li et al. 2010). ROS associated with cryopreservation damage have been characterized in mammal spermatozoa. However, in fish there is a lack of information regarding the effects of commonly used extenders such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG) on sperm function, lipid peroxidation (LPO), and antioxidant activity. The aim of this study was to examine cryopreservation procedures with respect to oxidative stress in common carp spermatozoa. Spermatozoa motility, membrane integrity, and oxidative stress indicators were measured in unfrozen sperm and after freeze/thaw processes.

Materials and methods

Common carps were reared at the experimental station of Faculty of Fisheries and Protection of Waters, Vodnany, Czech Republic. Functional parameters of cryopreserved sperm, including oxidative stress indicators (thiobarbituric-acid-reactive substance [TBARS] and carbonyl groups [CP]) and antioxidant indices (superoxide dismutase [SOD], glutathione reductase [GR], and glutathione peroxidase [GPx]) were investigated. The experimental groups were: 1) SB (control): Sperm was diluted 1:1 (v/v) with saline buffer (SB, ~430 mOsmol/kg) containing 200 mM KCl, 30 mM Tris (pH 8.0); 2) DMSO: Sperm was diluted 1:1 (v/v) with DMSO extender (~2050 mOsmol/kg) composed of 360 mg of NaCl, 1000 mg of KCl, 22 mg of $CaCl_2$, 8 mg of $MgCl_2$, and 20 mg of $NaHCO_3$ in 89 mL distilled water and 11 mL DMSO to a final volume of 100 ml (Kurokura et al. 1984). Samples were placed on ice and subjected to one of three treatment periods: 0, 15, or 30 min; 3) EG: Sperm was diluted 1:1 (v/v) with EG extender (~2810 mOsmol/kg): 59 mM NaCl, 6.3 mM KCl, 0.68 mM $CaCl_2$, 1.2 mM $MgCl_2$, 27 mM $NaHCO_3$, 3.4 mM sucrose, 69 mM D-mannitol, 118 mM Tris-HCl (pH 8.1), and 16% (v/v) EG (Kopeika 1986). Samples were placed on ice and subjected to one of three treatment periods: 0, 15, and 30 min. 4) DMSO-CRYO and EG-CRYO: Sperm was diluted 1:1 (v/v) with DMSO (DMSO-CRYO) and EG extenders (EG-CRYO). The diluted samples were equilibrated on ice for 15 min, then loaded into 0.5-ml plastic straws and kept in liquid nitrogen vapor by floating on a Styrofoam tray (3-cm thickness) on liquid nitrogen for 20 min. Subsequently, straws were plunged directly into liquid nitrogen. After 2 wk, sperm was thawed by immersing straws in a 40ºC water bath for 6 sec.

Results

No significant differences from group SB were observed for levels of TBARS and CP in DMSO and EG groups, whereas significantly higher levels of both oxidative products were found in groups DMSO-CRYO and EG-CRYO. No significant differences from controls were found for SOD activity in any experimental group. GR activity in group DMSO was significantly lower than that of controls during the equilibration period. In the EG group, significantly lower GR activity was observed only after 30 min. The GR activity in DMSO-CRYO group was strongly inhibited in comparison with SB group, whereas no significant differences were found with group EG-CRYO. The GPx activity was significantly higher than controls in group DMSO immediately after exposure, but there was no significant difference for GPx activity in group

EG during the equilibration period. Compared with controls, GPx activity in both DMSO-CRYO and EG-CRYO groups was significantly induced, especially for EG-CRYO.

Discussion and conclusions

The results of this study provide new information about effects on oxidative damage of DMSO and EG in common carp spermatozoa induced by cryoprotective agent (CPA) equilibration and freeze/thaw processes. Both CPA effect and the freeze/thaw process can induce oxidative stress in spermatozoa, and DMSO showed more adverse effects than EG with respect to GR and GPx activity. In addition, the antioxidant response of spermatozoa is mainly due to the capacity of GPx to counteract ROS stress of spermatozoa and minimize cryopreservation damages. All data suggest that an alternative CPA, such as EG, should be considered for carp spermatozoa, and that more research is required to gain a better understanding of osmotic and oxidative stress before it is applied to cryopreservation of fish sperm on a large scale.

Acknowlegements

Present study was financially supported by grants CZ.1.05/2.1.00/01.0024, MSM 6007665809, LC06073, IAA608030801, ME10015, QH92308, QH82118, QH82119, GACR P502/11/0090, 046/2010/Z.

References

Li Z.H., Zlabek V., Velisek J., Grabic R., Machova J., Randak T., 2010: Modulation of antioxidant defence system in brain of rainbow trout (***Oncorhynchus mykiss***) after chronic carbamazepine treatment. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 151, 137-141.

Kopeika E.F., 1986: Instruction on low temperature preservation of carp sperm [in Russian]. Russia: VNIPRKh; Moscow.

Kurokura H., Hirano R., Tomita M., Iwahashi M., 1984: Cryopreservation of carp sperm. Aquaculture 37, 267-273.

O-5/04
TREATMENT BY HYPOTONIC SOLUTIONS OF COMMON CARP (*CYPRINUS CARPIO*) SPERMATOZOA PRIOR TO FREEZING IMPROVES SURVIVAL TO CRYOINJURIES

Boris Dzyuba[1, 2], Gunes Yamaner[3], Olga Bondarenko[1] and Otomar Linhart[1]

[1] *Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic*
[2] *Institute for Problems of Cryobiology and Cryomedicine, NAS, Kharkiv, Ukraine*
[3] *Istanbul University, Faculty of Fisheries, Beyazit/Eminonu, 34452 Istanbul, Turkey*

E-mail: gyamaner@istanbul.edu.tr

Introduction

The methods of fish sperm cryopreservation reveal several common problems, such as considerable variability in spermatozoa motility and fertility after thawing (Kopeika et.al. 2008). These circumstances determine the necessity in improvement of fish sperm cryopreservation methods. Ability of cells to survive the osmotic pressure changes occurring during freezing-thawing is one of prerequisite for successful results of cryopreservation (Mazur,1984). Treatment of cells by solutions of different osmolality could increase their tolerance to osmotic stress possibly by membrane reorganisation (Bondarenko,1993). In our study we check hypothesis that sperm volume changes appearing in response to incubation in solutions of different osmolality could positively influence on sperm cryoresistance.

We used carp sperm as a model cell, which is able to reversibly change its total volume in accordance with environmental osmolality (Dzyuba et al, 2001).

Material and methods

Fish and sperm sampling. Sperm from 5 males of common carp *Cyprinus carpio* was stripped by abdominal massage and collected into plastic syringes, 24h after injection with carp pituitary extract in the dose of 1 mg kg-1 of body weight. *Experimental design (treatments before freezing).*Before cryopreservation sperm was diluted with 5 different solutions of osmolality 100, 150, 200, 250 and 300 mOsmoll^{-1} NaCl containing 10mM tris-HCl buffer ph 8.0, at a dilution rate 1:4. Ten sec after dilution, 2M KCL was added to adjust final osmolality at 300mOsmoll^{-1}. The resultant sperm suspension was diluted 1:1 with two cryoprotective media and frozen.

Croprotective media composition and procedure of freeze-thawing

Two croprotective media are used in our experiments: 1) K: 59mM NaCl, 6.3 mM KCL, 0.68mM CaCl2 , 2.1 mM MgCl2 mM, 27mM NaHCO3 , 3.4mM sucrose, 69mM manitol, mM Tris, pH 8.2 containing, 16% ethylene glycol (Kopeika, 1987); 2) KK: 62mM NaCL, 134mM KCL, 1.5mM CaCl2 , 3.2mM MgCl2, 2.4mM NaHCO3 containing, 10% DMSO (Kurokura, 1984). 10 min after dilution sperm samples were frozen in 0.5 ml straws, 3 cm above liquid nitrogen level for 20 min, then followed by plunging into liquid nitrogen. Control group was frozen after sperm 1:1 dilution with cryprotective medium. Sperm samples were thawed at 40 ^{o}C for 6 seconds.

Evaluation of motility percentage; sperm velocity and motility duration

A saline solution composed of 45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.2 (AM) was used as hypotonic activating media. Sperm was added to the AM using the tip of a dissecting needle, thereafter the sperm suspensions were thoroughly dispersed for 2 s. Motility was recorded for 1–2 min post-activation using video microscopy techniques until motility cessation. Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL), percentage of motile cells (motility) by microimage analyzer (Olympus Micro Image 4.0.1. for Windows).

Estimation of osmolalities of media used in experiment

Osmolalites of media used in experiments before and after KCL addition were evaluated using a Vapor Pressure Osmometer 5520 (Wescor, USA), and expressed in mOsmolkg^{-1}.

Data presentation and statistical analysis

For the comparison of motility percentage Kruskal- Wallis test was used with following application of Mann–Whitney U-test and Tukey's honest significant difference (HSD) test was applied ($P < 0.05$ were considered to be significant. The mean values with standard deviation are used to present results in text and in figure 1.

Results

The highest post-thaw motility (44±10%) was obtained after sperm treatment by 200 mOsmol NaCl with following application of cryoprotective media K and after sperm treatment by 150 mOsmol with subsequent application of cryoprotective media KK (44±2%). The longest motilitiy duration (60 ± 24 s) was found after sperm treatment by 250 mOsmol NaCl with subsequent application of cryoprotective media K and after sperm treatment by 200 mOsmol with following application of cryoprotective media KK (49 ±12 s). The use of cryoprotective media KK resulted in high variation of post-thaw motility (0-40%) and no significant differences between treatments were found. However significant increase of motility percentage($p < 0.05$, Mann–Whitney U-test, fig. 1A), VCL($p < 0.05$, HSD test, fig1B) and motility duration (fig 1C) were observed after sperm treatment by media of 100, 150 and 200 mOsmoll^{-1} NaCl with subsequent application of cryoprotective media K.

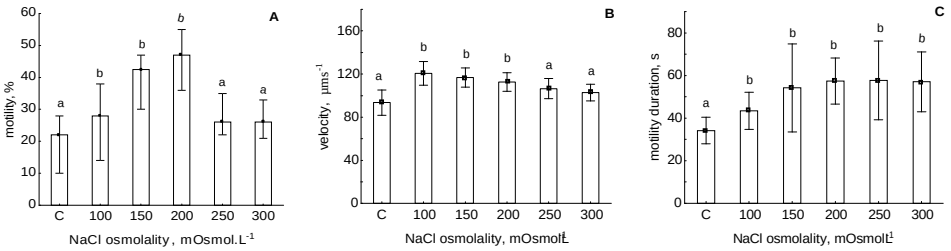


Figure 1. Post-thaw motility (A), VCL (B) and motility duration (C) in samples treated with NaCl solutions of different osmolality before cryopreservation. Values with different letters are significantly different ($p < 0.05$, Mann–Whitney U-test)

Discussion

To our knowledge, we report for the first time the possibility of improvement of post-thaw sperm motility parameters by hypotonic treatment of spermatozoa prior to freezing. We hypothesize, that sperm volume changes occurring in proposed mode of treatment lead to sperm membrane modification, improving the ability of spermatozoa to better survive freeze-thawing processes. Future studies about the possibility of this methodology application in fish artificial reproduction are required.

Aknowledgements

Special thanks are expressed to the projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

References

Bondarenko T.P., 1993: The combined effect of medium temperature and osmolality on the resistance of erythrocytes to hypertonic stress. Problems of cryobiology. 2, 11-17.

Dzuba, B.B.; Bozhok, G.A.; Rudenko, S.V., 2001: A study of the dynamics of volume changes during the period of active motility in carp, Cyprinus carpio L., spermatozoa. Aquaculture Research. 32, 51-56.

Kopeika, E.F., 1986: Instruction on low temperature preservation of carp sperm. VNIPRKh. Moscow.

Kopeika, E,; Kopeika, J., 2008: Variability of sperm quality after cryopreservation infish. In: *Fish Spermatology*, Alavi, S.M.H., Cosson, J., Coward, K., eds. Alfa Science Oxford, UK. pp. 347–396.

Kurokura, H.; Hirano, R,; Iwahashi, M., 1984: Cryopreservation of carp sperm. Aquaculture. 37, 267–273.

Mazur, P., 1984: Freezing of living cells: mechanisms and implications. American Journal of Physiology. 247, 125–142.

O-5/05
NEW TOOLS FOR GENOME PRESERVATION: GRAFTING SPERMATOGONIA IN BROWN TROUT (*SALMO TRUTTA*)

C. Fernández-Díez[1], R. Pérez-Sanchiz[1], C. Sarasquete[2], E. Cabrita[2], M.P. Herráez[1], José Beirao[1]

[1] *Dept. of Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain*
[2] *ICMAN-Institute of Marine Science of Andalusia, Spanish National Research Council, Avda. República Saharaui 2, 11510 Puerto Real, Cádiz, Spain*

E-mail: paz.herraez@unileon.es

INTRODUCTION

The potential of primordial germ cells to resume gametogenesis after intra or interspecific grafting has been reported for a limited number of species during the last years (Takeuchi et al 2004). These findings opened a big expectation for the development of new biotechnologies in aquaculture, the possibility to generate germ-line quimeras for surrogate production being one of the most suggestive. Recent studies showed that this ability is maintained in more differentiated cells of the germinal line, the spermatogonia (Okutsu et al 2006, Lacerda et al 2010, Yazawa et al 2010), which are easier to obtain from immature testes. The objective of the present study was to evaluate the use of these tools in the conservation programs of brown trout populations originating from the Northwest of Spain, which are threatened by extinction. This report focuses on the first steps: the optimization of the protocols for spermatogonia isolation and the ability of cells to colonize the host gonads after intraspecific grafting.

MATERIALS AND METHODS

Spermatogonia isolation: Immature testicles were obtained by dissection from *Salmo trutta* fries 4 to 13 g weight (12-14 months of age). Testicles were cleaned, cut in small pieces and dissociated by incubation, with gentle stirring, in different solutions containing DNAse, and tripsine or collagenase in L15 culture medium. Dissociation was assessed every 30min at light and fluorescence microscopy, using propidium iodide (IP) and SYBR-14 to evaluate the degree of cell packaging and the percentage of live cells. At the appropriate time the cell suspension was filtered with a 180 μm mesh, washed in Leibovitz's L15 and resuspended in the same medium. Homogenate was enriched in spermatogonia incubating the cell suspension in gelatin coated plates for 3h, and collecting the non-adherent cells.

Transplantation: Spermatogonia suspensions were labeled with PKH26, a red-fluorescent lipophilic tracer which incorporates into the plasma membrane, their fluorescence lasting for weeks. Labeled cells were intraperitoneally microinjected in 23 *Salmo trutta* embryos at hatch. Larvae were incubated and, throughout the development up to 45 days after grafting, larvae were fixed, embedded in paraffin, sectioned at 15m, counterstained with haematoxilin and observed at fluorescence and confocal microscopy.

RESULTS

Collagenase provided better results than tripsine for the testicle dissociation (Fig 1). Time required for dissociation was reduced and the percentage of live cells increased. After 3h the average of live cells was 41.50% and the percentage of spermatogonia, identified by morphology, was 6.12%. After incubation in gelatin coated plates the percentage of spermatogonia slightly increased to 10.63%.

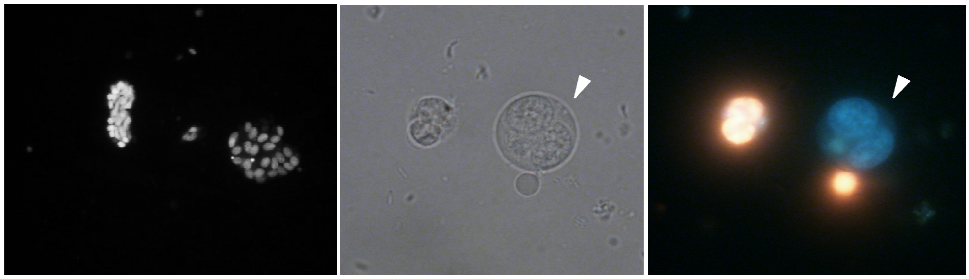


Fig. 1 Live (green) and dead (red) cells during the process of dissociation with collagenase medium. Left, 30min treatment 400X; Center and right, 3h treatment 1000X. Arrowhead spermatogonia A after mitosis

Three microinjected larvae died during the first week post-grafting (14%). Labeled cells appeared in the body cavity of host after grafting. From day 35 onwards 50% of the evaluated receptors showed from 1 to 18 fluorescent cells incorporated into the germinal ridge (Fig. 2).

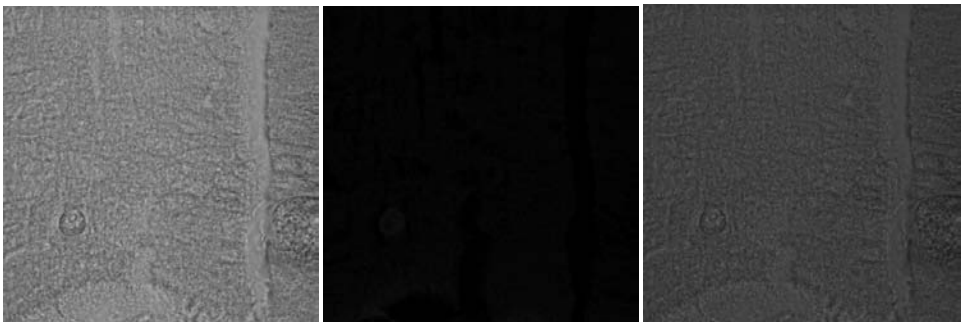


Fig 2 Germinal cell labeled with PKH26 in the gonadal tissue of a receptor 35 days after transplantation. Left, transmission; center, red fluorescence; right merge.

DISCUSSION AND CONCLUSIONS

Dissociation of testicular tissue was successfully achieved, but low percentages of spermatogonia were obtained. According to Shikina et al (2008) the elimination of somatic cells can be achieved in rainbow trout testicles homogenates after two or three passages in culture, 30-40h each. Culture conditions are not optimized for brown trout germinal cells and this enrichment method cannot be applied at present. Nevertheless, in spite of the low percentage of injected cells, colonization of the host gonad was positively achieved, demonstrating the ability of spermatogonia to migrate to the germinal ridge and to incorporate to the host tissue after allogenic transplantation. These results encourage proceeding with xenogenic transplantation to rainbow trout, the next step in order to develop a useful methodology for surrogate production with conservation purposes.

ACKNOWLEDGEMENTS

Research supported by the Spanish Ministry of Science and Innovation, PGCFISH (AGL2008-02172) project. The authors are grateful to the Regional Environmental Service from the Castilla y León Council at León at their Ichtiological Center at Vegas del Condado (León) for providing the biological material.

References

Lacerda, S.M.S.N.; Batlouni, S.R.; Costa, G.M.J.; Segatelli, T.M.; Quirino, B.R; Queiroz, B.M.; Kalapothakis, E.; França, L.R., 2010: A New and Fast Technique to Generate Offspring after Germ Cells Transplantation in Adult Fish: The Nile Tilapia (Oreochromis niloticus) Model. PLoS ONE 5, 1-9.

Okutsu T.; Suzuki K.; Takeuchi Y.; Takeuchi Y.; Yoshizaki, G, 2006: Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. PNAS 103, 2725–2729.

Shikina S.; Ihara S.; Yoshizaki G., 2008: Culture conditions for maintaining the survival and mitotic activity of rainbow trout transplantable type A spermatogonia. Molecular Reproduction and Development 75: 529-537.

Takeuchi, Y.; Yoshizaki, G.; Takeuchi T., 2004: Surrogate broodstock produces salmonids. Nature 430, 629–630.

Yazawa, Y.; Takeuchi, K.; Higuchi, T.; Yatabe, N.; Kabeya, Y.; Yoshizaki, G., 2010: Chub mackerel gonads support colonization, survival, and proliferation of intraperitoneally transplanted xenogenic germ cells. Biology of Reproduction 82, 896–904.

O-5/06
QUANTITATIVE DNA DAMAGE EVALUATION IN CRYOPRESERVED ZEBRAFISH PRIMORDIAL GERM CELLS

M.F. Riesco, V. Robles

INDEGSAL and Molecular Biology, University of León, León, Spain

E-mail: v.robles@unileon.es

Introduction
Primordial germ cells (PGCs) are important cell sources for cryobanking in fish, since they can colonize sexually undifferentiated embryonic gonads and resume gametogenesis after transplantation of a single PGC from a donor species (Saito *et al.*, 2008). During cryopreservation, reactive oxygen species (ROS) can produce DNA damage. An adequate cryopreservation protocol must guarantee not only high survival rates but also absence of genetic damage. In this study we present a successful protocol for zebrafish PGC cryopreservation. Using this protocol we obtained high survival rates after the process and absence of genetic damage (using the technique of comet assay). In order to study more in detail the potential damage at the DNA level caused by cryopreservation, we have done a quantitative evaluation using qPCR. This study represents an important step forward in DNA damage evaluation after cryopreservation.

Materials and methods
Zebrafish *(Danio rerio)* vasa EGFP ZF45 transgenic line was used. Samples (embryos, genital ridges –GRs- or single cells) were exposed, in a step-wise manner, to one of the following cryoprotectant solutions: (1) DMSO 5 M, EG 1 M, PVP 4% (CPAs) for 10 min, (2) CPAs+AFP 10 mg/mL or (3) CPAs+ AFP 20 mg/mL). PGCs were cryopreserved and vitrified using 0.5 mL straws, cryovials, microdrops and microcapsules. For PGC viability assessment trypan blue was used and DNA damage was determined using comet assay following the technique described by Peggy and Bánath (2006) with some modifications. DNA damage was quantitatively evaluated by qPCR in fresh GRs, GRs cryopreserved with CPAs and frozen GRs without cryoprotectants. Total DNA was purified using phenol-chloroform, quantify and stored at 4ºC. DNA lesion frequencies were compared in different regions of the mitochondrial and nuclear genome. The DNA damage was calculated as lesion per 10 kb.

Results and discussion
Our results showed that zebrafish PGCs can be successfully cryopreserved using DMSO 5 M, EG 1 M, PVP 4%. Survival rates were close to 90% in samples cryopreserved in 0.5 mL straws (GRs and embryos) and microdrops (GRs). When PGCs were microencapsulated, survival decreased significantly to 25% (Table 1). DNA damage determined by comet assay (percentage of DNA in tail) showed no differences in fresh samples and those cryopreserved in 0,5ml straws, but showed increased fragmentation levels when samples were microencapsulated (Table 2). The addition of AFP 20mg/ml to the cryoprotectant solution significantly decrease survival but does not affect DNA fragmentation (Tables 1 and 2). In this work we have employed for the first time a quantitative method for the evaluation of DNA damage caused by cryopreservation. Our results showed that qPCR can be successfully used for determination of genetic damage in cryopreserved samples. Moreover, this technique was significantly more sensitive than comet assay, allowing us to detect some differences between fresh and cryoporeserved PGCs (Fig.1).

Acknowledgements
Authors would like to thank MICINN AGL2009-06994, Fundación Ramón Areces, Ramón y Cajal program (RYC-2008-02339), JCyL(E-24-2009-0036681), S. Martinez and C. Miranda

References

Saito T, Goto-Kazeto R, Arai K, Yamaha E. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol Reprod. 2008;78(1):159-66.

Olive PL, Banáth JP. The comet assay: a method to measure DNA damage in individual cells. Nat Protoc. 2006;1(1):23-9.

Table 1. PGC survival rates (%) after Embryo, GR and PGC cryopreservation using different loading containers

Container	CPAs solution	Sample	PGC survival rate (%)
Straw	CPAs	Embryo	92.3±13.9
		GRs	91.2±25.1
	AFP10	GRs	89.3±19.8
	AFP20	GRs	49.0±15.9*
Cryovial	CPAs	Embryo	76.7±25.9
		GRs	72.1±21.7
Microdrop	CPAs	GRs	95.9±19.2
Microcapsule	CPAs	Dissociated cells	25.3±3.82**

Embryos or GRs per trial=10, n=3. Significant differences among different cryoprotectant solutions used in 0.5 straws are represented with one asterisk. Significant differences among different cryopreservation protocols are represented with two asterisks.

Table 2.Percentage of DNA in tail, (%DNAt) in control and cryopreserved PGCs

Container	Treatment	Percentage of tail DNA (%DNAt)
Control (fresh)		3.7±0.6^{c}
0.5 Straws	CPAs	3.6±1.3^{c}
	CPAs AFP 10	8.5±0.5^{c}
	CPAs AFP 20	5.9±3.3^{c}
	Without CPAs	40.0±5.0^{b}
Microcapsule	CPAs	26.1±2.9^{a}

Samples were cryopreserved in 0.5 mL straws and microcapsule, using DMSO 5 M, EG 1M, PVP 4% (CPAs) with or without AFP at 10 mg/ml (AFP 10) or 20 mg/ml (AFP 20). GRs per trial=20, n=3. Letters represent significant differences among treatments.

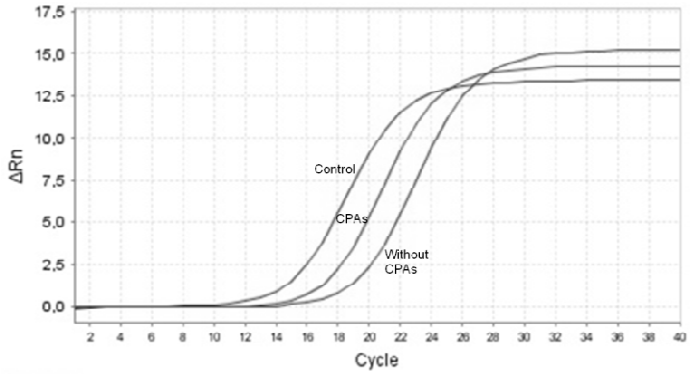


Figure 1. Amplification plots of control (fresh) and cryopreserved (with and without CPAs) samples

O-5/07
VITRIFICATION OF PRIMORDIAL GERM CELLS USING WHOLE EMBRYOS FOR GENE-BANKING IN LOACH, *MISGURNUS ANGUILLICAUDATUS*

Daisuke Inoue[1], Takafumi Fujimoto[1], Yutaka Kawakami[2], George Shigueki Yasui[1], Etsuro Yamaha[2] and Katsutoshi Arai[1]

[1] *Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido, 041-8611, Japan*

[2] *Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, 2-9-1 Sakura, Nanae, Kameda, Hokkaido, 041-1105, Japan*

E-mail: motchan@fish.hokudai.ac.jp

Introduction

Preservation of genetic resources is important in aquaculture to maintain valuable strains established in long-term breeding programs. Sperm cryopreservation is the most convenient method for gene-banking in fish. In contrast, it is hard to cryopreserve eggs and embryos in teleost because of the complex structure of embryos including cells, yolk syncytial layer and yolk, low membrane permeability and chilling sensitivity (Chao and Liao, 2001). However, primordial germ cells (PGCs), which are embryonic precursors of germ cells, are regarded as a new source for gene-banking (Yamaha et al., 2007). Germline chimera is required to produce functional gametes from PGCs. Both sperm and eggs have been successfully produced from the transplanted single PGC via germline chimera (Saito et al., 2008). Cryopreservation of PGCs in fish was established in rainbow trout, in which isolated genital ridges containing PGCs were cryopreserved (Kobayashi et al., 2007). Recently, Higaki et al (2010) developed a new method to cryopreserve PGCs in zebrafish, in which whole embryos including PGCs were vitrified, and eggs and sperm were differentiated from cryopreserved PGCs in germline chimera. In this method, PGCs were selectively picked up after thawing, although embryos could not develop, and then the PGCs were transplanted to produce germline chimera. In loach, *Misgurnus anguillicaudatus*, germline chimeras were successfully produced by the transplantation of PGCs derived from blastomeres which had been cryopreserved at blastula stage (Yasui et al., 2011). In the present study, in order to apply the vitrification method to the loach, four cryoprotectants, three cryopreservation media and two types of equilibration procedures were investigated. In addition to this, we also tried to transplant the PGCs recovered from the vitrified embryos.

Materials and methods

Mature eggs and sperm of loach and artificial fertilization were conducted according to the previous study (Fujimoto et al., 2004). Embryos which developed at 12-17 somite stage were used for our experiments and their yolks were removed manually before each experiment.

In order to evaluate toxicities of reagents and cryopreservation media, two experiments were conducted in the present study. First, toxicities of dimethyl sulfoxide (DMSO), methanol (MeOH), ethylene glycol (EG) and propylene glycol (PG) were tested by immersion of embryos for 15 min at room temperature. Survival of cells was examined by staining the dead cells with propidium iodide. Subsequently, the toxicities of three cryopreservation media, DE (15% DMSO and 15% EG), DMP (2M DMSO, 2M MeOH and 2M PG) and DP (2M DMSO and 4M PG), which were produced based on the previous results, were tested in terms of immersion time (2, 5, 10, 15 and 20 min) at room temperature.

In the equilibration of embryos with cryopreservation media, two types of procedures were tested in each medium using embryos with PGCs visualized by green fluorescent protein translated from artificial mRNA injected at one cell stage. The first procedure was performed by stepwise equilibration: exposure to a half concentration of cryopreservation medium for 15 min, and then immersion in full concentration of the medium for 5 min (two-step method). The second procedure was the immersion of embryos in full concentration of the medium for 20 min (one-step method). The procedures were evaluated in terms of viability of embryonic cells, and the detection of PGCs with green fluorescence after vitrification and thawing, which was conducted according to the previous study (Higaki et al., 2010). Finally, PGCs derived

from cryopreserved embryos using DE were transplanted into host embryos at the blastula stage whose PGCs were labeled with red fluorescence, and transplanted PGCs were observed under a fluorescent microscope.

Results

Due to the toxicity of each reagent used for vitrification, the viability of embryonic cells decreased as concentration increase. In contrast, survival rates in treatment batches showed more than 80% except for 5M DMSO. Survival rates of 5M DMSO indicated about 60%, which were significantly lower than others. In the results of the toxicities of the three cryopreservation media, all of the media examined showed 90% of viability until 5min after immersion. DMP demonstrated more than 90% of the survival rates, even after 20 min of immersion, and DE also showed high survival rates after 20 min of immersion. In contrast, 10 min after immersion, DP survival rates decreased. The survival rate of DP after 20 min of immersion was significantly decreased when compared with the survival rate at 5 min after immersion. After the vitrification of embryos which was performed in two procedures with three kinds of media, the frozen embryos were thawed to evaluate the efficiency of the procedures by viability of cells, the presence of PGCs detected with green fluorescence, and the migration ability of transplanted PGCs. Viable cells were observed in all of the cryopreservation procedures we tried. Among these procedures, the cell viabilities of DE with 1 step and DP with 1 step methods were significantly higher than that of the control method including no cryoprotectants. Embryos possessing PGCs with green fluorescence after thawing were obtained from procedures using DP and DE. When PGCs recovered from vitrified embryos, they were transplanted into host embryos at the blastula stage. Then, the transplanted PGCs could migrate to a host genital ridge as endogenous PGCs did.

Discussion and conclusions

In the present study, we evaluated the toxicity of reagents used for vitrification in each concentration and cryopreservation media containing multiple reagents, and assessed their availability for vitrification. Although DMP, which was newly synthesized in this study, showed low toxicity, it could not recover any PGCs. In contrast, another newly developed medium, DP, showed higher survival rates of embryonic cells after thawing than DE, which was used for vitrification in previous studies (Higaki et al., 2010, Kawakami et al., 2010). Furthermore, the one-step method to equilibrate embryos with cryopreservation media resulted in high survival rates of embryonic cells after thawing and successful recovery of PGCs from frozen embryos as in the two-step method which is traditionally used for vitrification. The DP and one-step method investigated in this study are helpful for the handling of embryos for vitrification. Transplanted PGCs recovered from embryos vitrified by the one-step method successfully reached a genital ridge in host embryos. This suggests that PGCs that were vitrified by our methods could be used to create a germline chimera for the production of gametes from PGCs of frozen embryos.

Acknowledgements

This work was supported in part by Grant-in-Aid for the Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) from the Bio-oriented Technology Research Advancement Institution (BRAIN) of Japan.

References

Chao N.H.; Liao I.C., 2001: Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture 197, 161-189.

Fujimoto T.; Kataoka T.; Otani S.; Saito T.; Aita T.; Yamaha E.; Arai K., 2004: Embryonic stages from cleavage to gastrula in the loach Misgurnus anguillicaudatus. Zool. Sci. 21, 747-755.

Higaki S.; Eto Y.; Kawakami Y.; Yamaha E.; Kagawa N.; Kuwayama M.; Nagano M.; Katagiri S.; Takahashi Y., 2010 Production of fertile zebrafish (Danio rerio) possessing germ cells (gametes) originated from primordial germ cells recovered from vitrified embryos. Reproduction 139, 733-740.

Kakami Y.; Goto-Kazeto R.; Saito T.; Fujimoito T.; Higaki S.; Takahashi Y.; Arai K.; Yamaha, E., 2010: Generation of germ-line chimera zebrafish using primordial germ cells isolated from cultured blastomeres and cryopreserved embryoids. Int. J. Dev. Biol. 54, 1493-1501.

Kobayashi T.; Takeuchi Y.; Takeuchi T.; Yoshizaki G., 2007: Generation of viable fish from cryopreserved primordial germ cells. Mol. Reprod. Dev. 74, 207-213.

Saito T.; Goto-Kazeto R.; Arai K.; Yamaha E., 2008: Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol. Reprod. 78, 159-166.

Yamaha E.; Saito T.; Goto-Kazeto R.; Arai K., 2007: Developmental biotechnology for aquaculture, with special reference to surrogate production in teleost fishes. J. Sea Res. 58, 8-22.

Yasui G.S.; Fujimoto T.; Sakao S.; Yamaha E.; Arai K., 2011: Production of loach (Misgurnus anguillicaudatus) germ-line chimera using transplantation of primordial germ cells isolated from cryopreserved blastomeres. J. Anim. Sci. in press

O-5/08
EFFECT OF OXYGENE ATMOSPHERE AND ANTIOXIDANTS ON THE COMMON CARP (*CYPRINUS CARPIO* L.) MILT SHORT TERM STORAGE

R. K. Kowalski[1], B. I. Cejko[1], B. Sarosiek[1], K. Targońska[2], D. Żarski[2], D. Kucharczyk[2], J. Glogowski[1,2]

[1] *Institute of Animal Reproduction and Food Research PAS, Poland*
[2] *University of Warmia and Mazury in Olsztyn*

E-mail: b.cejko@pan.olsztyn.pl

Introduction
Storage of milt at temperature above the freezing point of water is an method widely used in the aquaculture practice (McNiven et al. 1993). Oxygen atmosphere is believed to have positive effect on sperm survival during this process (Cabrita et al., 2008). However some authors suggest that oxidative stress during short-term storage might be a problem limiting survival of spermatozoa, especially when free oxygen atmosphere is applied (Bencic et al., 2000). Presented study concern effect of oxygen atmosphere and various antioxidants addition into immobilizing buffer on the short-term preservation success of common carp sperm.

Materials and methods
Sperm of common carp was collected from five individuals. As an immobilizing solution we used buffer TLP as described by Bavister (1989) supplemented with 100mM trehalose; pH 8.6. One portion of milt (50µl) from each male was subjected into short term preservation without dilution (K) or mixed (1:20) with immobilizing buffer alone (A) or with addition of 5 mM glutation (B), 5 mM ascorbic acid (C), 1.5 mg ml^{-1} vitamin E (D) and mix of antioxidants (E). Spermatozoa motility was activated in Woynarovich solution supplemented with 0.5% BSA. Computer Assisted Sperm Analysis (CASA) was used to evaluate curvilinear speed (VCL), and spermatozoa motility. Sperm membrane integrity was tested with use of Live/Dead double staining kit (Merc). After 10 day of preservation fertility trial was conducted (50 000 of spermatozoa/egg). As the control of eggs fertility potential, freshly collected milt was used (n=3). The significance of differences between all analyzed groups were tested by analysis of variance (ANOVA) followed by post hoc Tukey test.

Results
During 10 days of milt preservation spermatozoa without addition of oxygen VCL remained at initial level in variants A, B and D. Oxygen atmosphere altered motility and VCL of diluted samples compared to samples preserved in anoxia condition. Motility of spermatozoa decreased during preservation time from 90% to about 80% (Fig. 1) in the best variants (A and D). Samples supplemented with ascorbic acid and mixture of antioxidants loss their viability during 6 days of preservation and therefore were not used in fertilization trial. Sperm membrane integrity in samples A, B and D preserved in anaerobic condition remain unaffected at 10th day of storage. Fertilization success measured as a hatching rate showed that diluted with immobilizing solution common carp milt during 10 days of preservation in refrigerator in aerobic condition maintained its fertilizing potential (Fig. 2).

Discussion and conclusions
We found that oxygen atmosphere is not required for successful short-term preservation of common carp sperm. Undiluted common carp sperm stored in oxygen atmosphere had higher VCL than sample preserved in anoxia condition up to 8 days from collection. Saad et al. (1988) observed beneficial effect of oxygen atmosphere up to 6 days of undiluted milt preservation. In the case of diluted and exposed to atmosphere of oxygen milt of common carp, 30% of motile spermatozoa were found up to 85 hours from the time of collection (Ravinder et al., 1997). In our experiment sperm motility remain unaffected until 9 days of storing in anoxia condition. Samples preserved with use of oxygen atmosphere gradually loose their motility potential from 5th day from collection (Fig 1). Antioxidant addition did not improve preservation in both, oxygen and anoxia condition. Moreover addition of ascorbic acid and mixture of antioxidants showed negative effect on sperm viability. Sperm fertilization potential and membrane integrity remain unaffected in

samples diluted with immobilizing solution and preserved in anoxia condition up to 10th day from collection (Fig. 2).

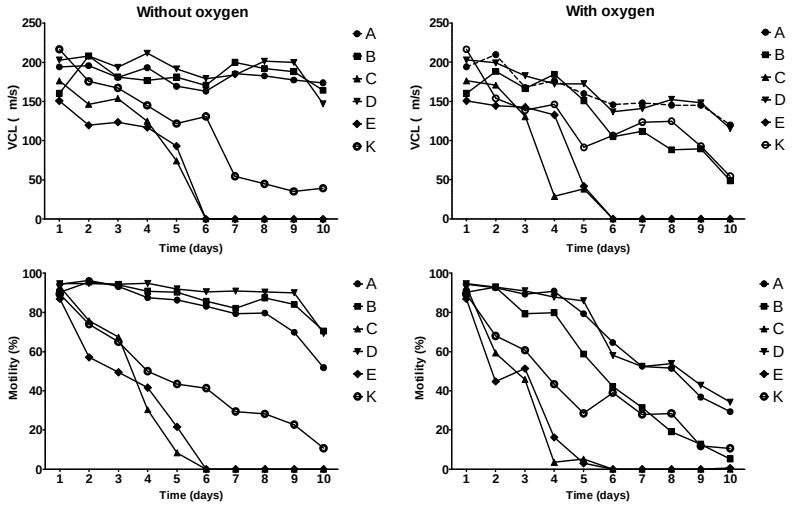


Figure 1. Effect of oxygen atmosphere and antioxidant on common carp sperm motility parameters during short term preservation. A control (●), B with glutathione (■), C with ascorbic acid (▲), D with vitamin E (▼), E with all antioxidants (♦), K without dilution (○)

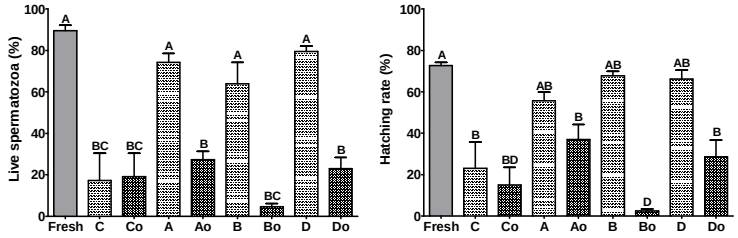


Figure 2. Effect of oxygen atmosphere and antioxidant addition on sperm membrane integrity and fertilization success after 10 days of preservation. Data represent mean value and SEM ($p<0.05$). Fresh – sperm collected before fertilization, C – undiluted sperm, A sperm diluted with immobilizing buffer, B – addition of glutathione, D – addition of vitamin E. Letter "o" indicated samples preserved with use of oxygen atmosphere

Acknowledgments

Authors would like to thanks Ms Katarzyna Dryl and dr Wiesław Demianowicz for excellent technical assistance.

References

Bavister, B.D.; 1989: A consistently successful procedure for *in vitro* fertilization of golden hamster eggs. Gamete Res., 23, 139–158.

Bencic, D.C.; Krisfalusi, M.; Cloud, J.G.; and Ingermann, R.L.; 2000: Short-term storage of salmonid sperm in air versus oxygen, N. Am. J. Aquacult., 62, 19.

Cabrita, E; Robles, V; Herráez P; 2008: Methods in Reproductive Aquaculture: Marine and Freshwater Species. Cabrita, E, Robles, V, Herráez, MP (Eds). CRC Press, Boca Raton.

Mcniven, M.A.; Gallant, R.K.; Richardson, G.F.; 1993: Fresh storage of rainbow trout (*Oncorhynchus mykiss*) milt using a non-aqueous medium. Aquaculture 109, 1971-1982.

Ravinder, K.; Nasaruddin, S.; Majumdar Shivaji, K.C; 1997: Computerized analysis of motility, motility patterns and motility parameters of spermatozoa of carp following short-term storage of milt. J. Fish Biol. 50, 1309 -1328.

Saad, A.; Billard, R.; Theron, M.C.; and Hollebecq, M.G.; 1988: Short-term preservation of carp (*Cyprinus carpio*) milt, Aquaculture, 71, 133.

O-2/01
IDENTIFICATION OF SEMINAL PROTEINS IN FISH: FROM TRADITIONAL APPROACH TO PROTEOMICS

Andrzej Ciereszko, Mariola A. Dietrich, Joanna Nynca

Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-747 Olsztyn, Tuwima 10, Poland

E-mail: a.ciereszko@pan.olsztyn.pl

Identification of seminal proteins using the traditional approach

The traditional approach for protein characterization in fish semen is based on two major steps: the purification of proteins with the use of numerous liquid preparative chromatographic methods and the identification of proteins using sequencing. Chromatographic methods include ion-exchange chromatography, gel filtration or size-exclusion chromatography, affinity chromatography and hydrophobic interaction chromatography. Modern column chromatographic systems for protein purification are computer-controlled and integrated into fast protein/performance liquid chromatography operating at low or medium pressure. The final purification is usually obtained using reversed-phase chromatography in high performance/pressure liquid chromatography systems and preparative electrophoresis. Sequencing of purified proteins is performed on automated sequencers using Edman degradation and allows to decode the sequence of 20-50 amino acids. Major carp and rainbow trout seminal plasma proteins identified so far are shown in Fig. 1.

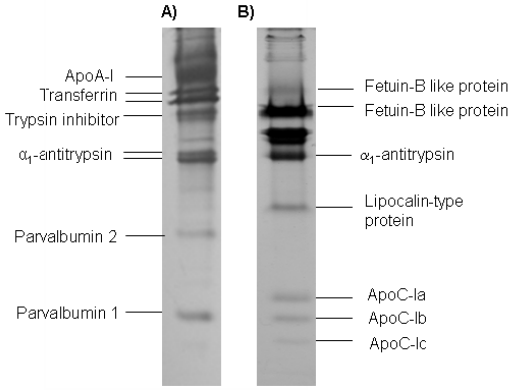


Figure 1. One-dimensional electrophoresis (native PAGE) of seminal plasma of carp (A) and rainbow trout (B). Identified so far major proteins are shown.

The 'OMICS revolution

For most of its development biological chemistry was based on the studies of a few or several genes or proteins at the same time. Such science has been reductionistic, i.e. focused on understanding complex things by reducing them to simpler or more fundamental things. The successful execution of the Human Genome Project and unraveling the structure of the human genome made it possible to study hundreds or thousands of genes, proteins and metabolites at the same time. This was possible through combining the knowledge of the human genome together with new methodical approaches, including bioinformatics, with the traditional knowledge already established. This is referred to as the '"omic" revolution. ***Genomics*** concerns the quantitative study of genes, regulatory and non-coding sequences; ***transcriptomics*** - RNA and gene expression; ***proteomics*** - protein expression, and ***metabolomics*** - metabolites and metabolic networks.

Proteomics

Proteomics concerns the study of protein products expressed by the genome, in other words and the term "proteome" originating from ***PROTEin complement of the genome*** (Brewis, 1999). Proteomics emerged as a leading technology in the postgenomic era because of a central role of proteins and protein-protein interactions in cell physiology. It is now known that an astonishingly large number of proteins (300000 and probably more) originates from a far lower number of protein-encoding-genes (22000 – 25000 in humans).

Basic techniques for proteomic research

Most proteomic research involves three basic steps: (i) separation and isolation of proteins from biological materials; (ii) determination of protein primary structure (sequence of amino acids); and (iii) identification of proteins utilizing databases (Graves and Haystead (2002). The separations of hundreds or thousands of proteins together with their identification using mass spectrometry are core principles of proteomics. For the separation of complex protein mixtures two-dimensional electrophoresis (2-DE) is used. During 2-DE proteins are resolved according to their net charge in the first dimension and according to their size/molecular mass in the second dimension. Identification of protein spots is performed with the use of protein staining methods, such as silver staining, Coomassie staining, or by using fluorescent dyes. A recent advance on protein detection is the introduction of difference gel electrophoresis (DIGE) which is based on utilizing different fluorescent tagging for control and experimental samples. This way both samples can be run and visualized in the same 2-D gel (this technique is also important for quantitative protein profiling).

Mass spectrometry (MS) is the central technological basis for proteomics regarding protein identification and quantification. Samples analyzed by MS are converted to desolvated ions and analyzed by either using electrospray ionization (ESI) or matrix-assisted desorption/ionization (MALDI). In both methods, peptides are converted to ions by the addition or loss of one or more protons. Mass spectrometers allow to measure molecular mass of peptides and this information is used for identification by peptide mass fingerprinting. Most importantly, mass spectrometry allows to determine the short amino acid sequences of the peptides– the core information necessary for protein identification. In order to identify proteins the use of powerful biostatistics methods with the aid of numerous databases and search engines is necessary.

Proteomic studies of the fish male reproductive system

During the last decade enormous advances have been made regarding proteomic studies of vertebrate male reproduction, especially mammals. First applications of proteomic research into studies of fish reproduction have already been made, and include the effects of cryopreservation on semen proteome, molecular mechanisms determining sperm motility initiation and the effects of domestication on semen quality (Forne et al., 2010). Comparative analysis of sturgeon sperm proteome has also been performed (Li et al., 2010). The introduction of 'omics into studies of male reproduction in fish creates unique opportunity to unravel, using integrative approach, the physiological mechanisms important for sperm function, such as motility and fertilizing ability. Proteomic studies would greatly increase the chances for the identification of new biomarkers of male fertility. Such knowledge can contribute to the perfection of controlling of reproduction in aquaculture conditions, especially the efficacy of fertilization procedures and semen storage, including cryopreservation. Selected proteins can further be purified and characterized (using the traditional approach) in order to better understand their specific functions.

References

Brewis, I.A., 1999: Proteomics in reproductive research - The potential importance of proteomics to research in reproduction. Hum. Reprod. 14, 2927-2929.

Forne, I.; Abian, J.; Cerda, J., 2010. Fish proteome analysis: Model organisms and non-sequenced species. Proteomics 10, 858-872.

Graves, P.R.; Haystead, T.A.J., 2002: Molecular biologist's guide to proteomics. Microbiol. Mol. Biol. R. 66, 39-63.

Li, P.; Hulak, M.; Rodina, M.; Sulc, M.; Li, Z.H.; Linhart, O., 2010: Comparative protein profiles: potential molecular markers from spermatozoa of Acipenseriformes (Chondrostei, Pisces). Comp. Biochem. Phys. D 5, 302-307.

O-2/02
MARATHON *VS* SPRINT RACERS: AN ADAPTATION OF SPERM CHARACTERISTICS TO THE REPRODUCTIVE STRATEGY OF PACIFIC OYSTER, TURBOT AND SEABASS

M. Suquet[1], J. Cosson[2], A. Donval[3], C. Labbé[4], P. Haffray[5], I. Bernard[1], C. Fauvel[6]

[1] *Ifremer, UMR 100, Station Expérimentale d'Argenton, Argenton, France*
[2] *CNRS, UMR 7009, Université Paris VI, Station Marine de Villefranche sur Mer, France; University of South Bohemia, Vodnany, Czech Republic.*
[3] *IUEM, Lemar, Technopole Brest Iroise, Plouzané, France*
[4] *INRA, UR 1037 Scribe, Rennes, France*
[5] *SYSAAF, Scribe, Rennes, France*
[6] *Ifremer, Station Expérimentale d'Aquaculture, Palavas les flots, France*

E-mail: msuquet@ifremer.fr

Introduction
The basic characteristics of Pacific oyster (*Crassostrea gigas*) sperm remain badly known. The aim of this paper is to review the present knowledge and to compare the equivalent recorded in two marine fish species (turbot, *Psetta maxima* and seabass, *Dicentrarchus labrax*), which sperm characteristics have been widely described and which reproduction strategy differs from oyster.

Material and methods
Gametes were stripped during the spawning period, avoiding seawater contamination of semen of the three species but also urine in fish. The analytical methods used were previously described (oysters: Faure 1996, fish: Dreanno 1998).

Results
The general morphology of spermatozoa mainly differs between species by the presence of an acrosome in the oyster giving an ovoid shape, while fish sperm has no acrosome and presents a round head. The movement characteristics presents low values for spermatozoa collected in the testes as compared to those collected at the gonopore for turbot or released after a 10mM serotonin stimulation for oysters (Fig. 1).

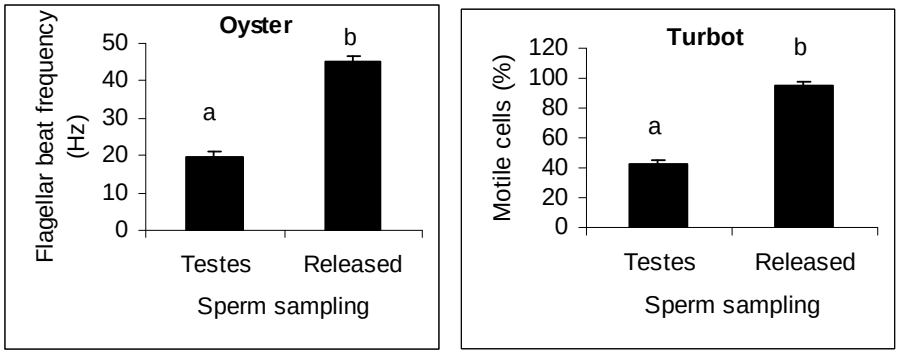


Fig. 1. Effect of sampling localization on the movement characteristics of turbot (from Dreanno 1998) and Pacific oyster sperm (mean±SEM).

This suggests a 'maturation process' along the genital duct in these species which can be *in vitro* simulated, adding 10mM caffeine or 0.3mM Dibutyril cAMP to intratesticular oyster spermatozoa.

Hypertonicity (from 300-320 mosm kg^{-1} to osmotic pressure of seawater) is the main factor triggering the motility of spermatozoa in both marine fish. On the other hand, an increase in the pH value of the seminal fluid (6.2±0.1; Faure 1996) up to seawater value (8.2) could play a major role in Pacific oyster sperm activation.

Intracellular ATP content of non activated spermatozoa is low in oyster (45±2 nmoles 10^9 spermatozoa), compared to values observed in seabass (75±3) and turbot (221±14). However, initial ATP content drops rapidly to 25% of its initial value, 10s after seabass sperm activation, mitochondrial oxidative phosphorylation remaining inefficient to compensate ATP hydrolysis. In turbot, the supply of energy required for sperm motility is partly provided by mitochondrial activity of spermatozoa: 54% of the initial ATP content was measured 10s *post* activation. Then, ATP content of oyster sperm decreases only to 93% of its initial value after a 24h movement, suggesting a high ATP metabolism compensating for its hydrolysis by dinein motors and sustaining a long term sperm movement duration in oysters. This high capacity of ATP regeneration during oyster sperm movement is sustained by the limited changes observed in the shape and structure of mitochondria after a 24h movement period. In contrast, shrinkage of mitochondria was observed in seabass and turbot sperm at the end of the motility period.

After activation, sperm swimming speed (VSL) is higher in fish species (turbot: 233±7µms^{-1}, seabass: 142±8µms^{-1}) compared to that in oyster (13±2µms^{-1}). Sperm movement duration is quite different between species: 40-50s in seabass, 3 to 5min in turbot and 20 to 24h in oyster. These swimming characteristics result in highly different total distances covered by spermatozoa during their movement period: 2mm for seabass, 12mm for turbot and up to 1m for Pacific oyster. Furthermore, the numbers of spermatozoa required for optimal fertilization is very different between the three species (Fig. 2).

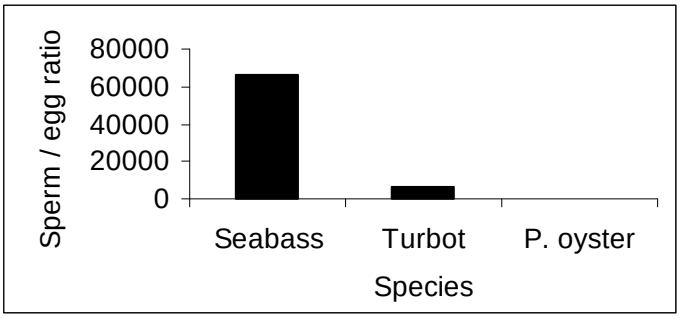


Fig. 2. Minimum sperm to egg ratio required for optimum fertilization rate in the three studied species.

Discussion and conclusions

The success of fertilization requires synchrony of male and female gamete release and is promoted by the proximity of individuals of both sexes. This was observed in both fish species, which spawning determinism is controlled by the release of pheromones followed by a mating behaviour: isolated seabass females do not release their eggs while heterosexual or only female couples spontaneously spawn. When ready to spawn, the females approach the males face to face and then escape, inducing the chase resulting in a fast swimming of both sexes. Then, ova and spermatozoa are released simultaneously, insuring the proximity of gametes during fertilization. As a compensative strategy of the absence of mating in molluscs, breeder individual proximity is increased by the formation of oyster beds and fertilization is promoted since spawns are mainly observed during the slack tide, a low current period.

In conclusion, the three studied species can be opposed in relation to their sperm behaviour into sprint racers (seabass: high velocity and short distance covered) and marathon ones (oyster: low velocity and long distance). The case of turbot can be considered as intermediate, but close to the seabass model. Furthermore, the long distance covered by Pacific oyster sperm may be considered as a compensative strategy to the absence of mating in oyster breeders in opposition to the reproductive behaviour recorded in fish.

References

Dreanno C., 1998. Régulation de la mobilité des spermatozoïdes de turbot (Psetta maxima) et de bar (Dicentrarchus labrax). Thesis, Univ. Rennes, 81pp.

Faure C., 1996. Paramètres physiologiques de l'émission et de l'activation des gamètes mâles de deux mollusques bivalves, la coquille Saint Jacques Pecten maximus (L.) et l'huître creuse Crassostrea gigas (Thunberg). Thesis, Univ. Paris VII, 255pp.

O-2/03
SPERM MOTILITY MULTIPLE ACTIVATIONS: PHYSIOLOGICAL BACKGROUND AND PRACTICAL USE IN AQUACULTURE

S. Boryshpolets[1], B. Dzyuba[1,2], M. Rodina[1] and O. Linhart[1]

[1] *University of South Bohemia, Faculty of Fisheries and Protection of Waters, CENAKVA, Zatisi 728/II, 389 25 Vodnany, Czech Republic*
[2] *Institute for Problems of Cryobology & Cryomedicin of National Academy of Sciences of the Ukraine, Kharkov, Ukraine*

E-mail: sboryshpolets@frov.jcu.cz

Introduction
In freshwater fish, spawning in fresh water, sperm motility activation related to low osmolality of an environment, its duration lasts for short periods and the lost motility is associated with disappearance of fertilizing ability (Ginzburg 1972). During artificial reproduction there is the possibility to increase the duration of sperm motility (Cosson 2004) and even restore the motility of spermatozoa being immotile (Linhart et al. 2008). In this report we summarized our results on studying the physiological backgrounds for sperm motility multiple activation in fish spermatozoa and appearance of spontaneous activations during cryopreservation. Further, we discuss the possible advantage of this phenomenon use when necessary to improve the results of artificial reproduction in fish.

Materials and methods
Fish and sperm sampling

Mature males of Eurasian perch and Common carp were obtained after fish farm pond harvesting and kept in laboratory aquatic systems. Sperm samples were obtained during natural spawning period.

Sperm motility parameters

Sperm velocity and motility (percentage of motile spermatozoa) were estimated using an analysis of video records obtained by applying CCD video camera mounted on a dark-field microscope and illuminated with a stroboscopic lamp. Motility was initiated either by sperm dilution in hypotonic media or by freeze-thawing.

Models of sperm multiple activation

(1) motility of carp sperm was initiated in hypotonic activating media (100-150 mOsm), after motility stop KCl was added to increase the osmolality up to 300 mOsm and after 20 min the second motility activation by osmolality decrease was initiated; (2) motility of perch spermatozoa was initiated 3 times using stepwise reduction of osmolality of activating solution; (3) motility arisen from freeze-thawing process was observed in carp and perch without transferring them into activating media.

Sperm cryopreservation

Sperm samples were cryopreserved using specific cryoprotective media and freezing methods described previously.

Sperm ATP content measurements

ATP content was evaluated by bioluminescence using Bioluminescence Assay Kit and multifunctional microplate reader.

Results and discussion
The different modes of multiple sperm motility activation were investigated. (1) After motility stop following the transferring of carp sperm into isotonic condition the ATP level and cellular volume can be recovered together with ability for the second motility activation in hypotonic condition. During the second activation the gradual increase of motility percentage was observed, while in firstly activated spermatozoa the maximum motility observed right after the start of movement. The rate of motility percentage in

secondly activated spermatozoa is associated with individual properties of sperm samples (fig 1, Boryshpolets et al. 2009a). These secondly activated spermatozoa have the fertilizing ability (Linhart et al. 2008) and survived cryopreservation demonstrating up to 20% of motility after thawing. The respiration rate during resting period was significantly higher then in immotile sperm and not different from respiration rate during motility or uncoupling condition (Boryshpolets et al. 2009a). That is why we suppose that at list part of ATP produced during reactivation were supported by oxidative phosphorylation.

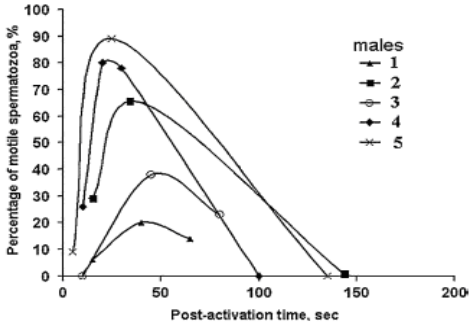


Fig. 1. Graph showing percentage of motile spermatozoa (%) at the second activation obtained for five different males

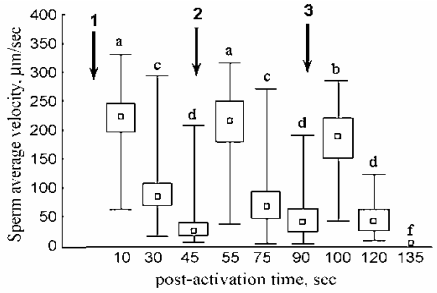


Fig. 2. Perch sperm average velocity during motility activation) in the model of multiple activation.
Arrows – first, second and firth motility activation correspondingly.

Second mode (2) consists of stepwise decreasing the osmolality of the activating medium. During this kind of multiple activation the total duration of motility is around 3 times longer if compared with activation by sharp osmolality decrease. This phenomenon is based on prolonged and slow ATP consumption during several "waves" of motility arisen due to stepwise environment osmolality decrease (fig.2, Boryshpolets et al. 2009b). Third mode (3) of multiple activation was observed in carp and perch, when procedure of freeze - thawing itself leads to spontaneous sperm motility activation and endogenous ATP level decrease. Afterwards the sperm could be reinitiated in hypotonic conditions. During this useless activation spermatozoa lost the main part of ATP but still able to be secondly activated and preserve ability for fertilization (Boryshpolets et al. 2009c).

Conclusion

The phenomenon of multiple sperm activation could be the base for the elaboration of the most optimal sperm use because the prolongation of total sperm motility duration potentially and more likely makes the fertilization successful. Spontaneous sperm motility activation during freeze-thawing should be taken into account during cryopreserved sperm use. Finally, conjunction the ability of sperm for multiple activation and its cryopreservation could be at the base of multiple sperm use for fertilization if there is a deficit of valuable individuals' sperm.

Acknowledgements

Special thanks are expressed to the projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

References

Boryshpolets S.; Dzyuba B. and Drokin S., 2009a: Pre-spawning water temperature affects sperm respiration and reactivation parameters in male carps. Fish Physiol. Biochem. 5, 661–668.

Boryshpolets S.; Dzyuba B.; Stejskal V.; Linhart O., 2009b: Dynamics of ATP and movement in Eurasian perch (Perca fluviatilis L.) sperm in conditions of decreasing osmolality. Theriogenology 72, 851–859.

Boryshpolets S.; Dzyuba B.; Rodina M.; Li P.; Hulak M.; Gela D.; Linhart O., 2009c: Freeze-thawing as the factor of spontaneous activation of spermatozoa motility in common carp (Cyprinus carpio L.). Cryobiology 59, 291–296.

Cosson J., 2004: The ionic and osmotic factors controlling motility of fish spermatozoa. Aquacult. Int. 12, 69–85.

Ginzburg, A.S., 1972: Fertilization in fishes and problem of polyspermia. US Dep Comm. Nat. Tech. Inf. Ser. Springfield. p366.

Linhart O.; Alavi SMH.; Rodina M.; Gela D.; Cosson J., 2008: Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (Cyprinus carpio). J. Appl. Ichthyol. 24, 386–92.

O-2/04
STERLET SPERMOTOZOA BEAHAVIOUR AT DIFFERENT LEVELS INSIDE THE DROP

S. Boryshpolets[1], V. Bondorenko[1], M. Rodina[1], B. Dzyuba[1,2], J. Cosson[1] and O. Linhart[1]

[1] *University of South Bohemia, Faculty of Fisheries and Protection of Waters, CENAKVA, Zatisi 728/II, 389 25 Vodnany, Czech Republic*

[2] *Institute for Problems of Cryobology & Cryomedicin of National Academy of Sciences of the Ukraine, Kharkov, Ukraine*

E-mail: sboryshpolets@frov.jcu.cz

INTRODUCTION

A spermatozoon has the crucial function to transport the male haploid chromosomes set into the oocyte. Velocity, percentage of moving and in addition flagella beat frequency are the commonly used parameters of sperm quality characteristics. Microscopic methods of sperm motility analysis associated with observation of motility inside of drop of activation media under microscope. According to data in literature, spermatozoa could change motility behavior when moving closed to surface (Woolley 2003). Fish spermatozoa as well as sperm of many other species have the tendency to move in vicinity of liquid surface (Cosson et al. 2003). During observation of fish spermatozoa motility under microscope spermatozoa are moving at water-glass or water-air interfaces which contrasts with natural fish reproduction where spermatozoa moving in the water column. That is why in this study possible influence of vicinity to surface on motility of sterlet (*A. ruthenus*) spermatozoa was studied.

Materials and Methods

Fish and sperm sampling

Five mature males of sterlet were kept in laboratory aquatic systems of Faculty of Fisheries and Protection of Waters (FFPW). Sperm samples were obtained during natural spawning period after hormonal treatment.

Sperm motility recording

Sperm motility recording by CCD video camera mounted on a dark-field microscope (20x lens) and illuminated with a stroboscopic lamp. Motility was initiated by sperm dilution in pond water.

Sperm motility analysis

To compute sperm velocities CASA plugin for ImageJ software was used according to manual of Wilson-Leedy and Ingermann (2007). Motility was recorded on top (water-air) and bottom (water-glass) position of open drop. Minimal of six records (3 per treatment) were recorded for sperm sample from one male. Total number of spermatozoa analyzed is 5696.

Results

Curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) were measured in this study. After activation of sperm significant decreasing of sperm velocities at bottom compare to top of drop was observed in all studied males. The same behavior of moving sperm was observed in different time after activation except 180 s after activation where is changes in velocity were not significant (Fig. 1).

Discussion and conclusion

Observed decrease of velocities at bottom of the drop as compared to the top could be related to confinement of sperm movement in the vicinity to the glass surface or to the different properties of water-glass interfaces. For better understanding of that phenomenon further studies are required.

The sterlet spermatozoa behave similarly during whole motility period except from 180 s after activation, probably because in the end of motility period, number of motile spermatozoa was too low. The fact that sperm motility could be influenced by vicinity of different interfaces in all of the studied parameters (VCL, VAP, VSL) during mostly whole motility period assumes existence of some coefficient of "slowing" induced by surface. We determined it as ratio between velocity at bottom and velocity at top at different

time after activation (excluded 180 s) and take mean ± standard deviation. In our study those coefficients were: 0.84 ± 0.04 (for VCL), 0.85 ± 0.04 (for VAP) and 0.85 ± 0.04 (for VSL).

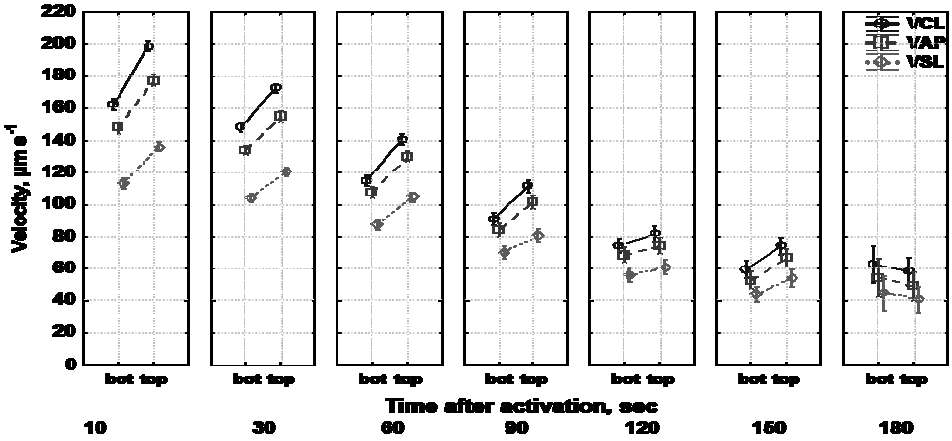


Fig. 1 Sterlet sperm velocities at bottom and top of drop at different time after activation

Similar value of the coefficients for different categories of velocities confirmed the fact that linearity of movement which in average was 0.897 ± 0.014 was not influenced by surface.

Differences in velocities appearing at different levels of a drop could be one of the reasons why different systems used for sperm motility analysis show variability in velocities of sperm movement. That is why we suppose that this phenomenon should be taking on account during processing of sperm samples with automatic systems of motility analyses and even during simple observation under the microscope.

Acknowledgements
Special thanks are expressed to the projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

References

Woolley, D. M., 2003: Motility of spermatozoa at surfaces. Reproduction 126, 259–270.

Cosson, J., Huitorel, P. and Gagnon, C., 2003: How spermatozoa come to be confined to surfaces. Cell Mot. & Cytoskel. **54**: 56-63.

Wilson-Leedy, J. G. and Ingermann, R. L., 2007: Development of a system based on open source software for characterization of zebrafish sperm motility parameters. Therioginology **67**, 661-672.

O-2/05
VARIATIONS AND DETERMINISM OF SPERMATOZOA QUALITY IN THE COURSE OF SEABASS (*DICENTRARCHUS LABRAX* L.) SEMEN CHILLED STORAGE AND AFTER CRYOPRESERVATION

C. Fauvel[1], S. Boryshpolets[2], B. Marmey[1], M. Suquet[3]

[1] *UMR 5119 CNRS-Université Montpellier II-IRD- Ifremer, Université Montpellier II, CC093, 34095 Montpellier Cedex 05, France*
[2] *University of South Bohemia, Faculty of Fisheries and Protection of Water, Research Institute of Fish Culture and Hydrobiology, 38925 Vodnany, Czech Republic*
[3] *Ifremer, UMR 100, Station Expérimentale d'Argenton, Argenton, France*

E-mail: cfauvel@ifremer.fr

Introduction
Contrary to freshwater fish like salmonids and sturgeon sperm, seabass sperm quickly loses its ability to move and fertilize after stripping and conservation in non activating saline media (Sansone, 2001, Peñaranda et al. 2008). Since it may be interesting to maintain sperm for several days in order to transport it without cryopreservation, it was interesting to study the determinism of sperm quality during chilled conservation. According to Bobe and Labbé, 2009 the diluant composition is very important. They emphasized the necessity of inhibition of sperm motility generally triggered by the change of environment at ejaculation and the role of antibiotics in the dilution medium. In order to better understand the quick loss of fertility of seabass sperm during conservation as chilled, we studied the main features of sperm biology during this period and in different media.

Material and methods
Spermatozoa quality was analysed through respiration capacity, ATP content, Membrane integrity, DNA integrity, motility and fertility at different times after stripping. Moreover, cryopreservation according to standardized protocol was attempted and the success was checked through CASA analysis and fertility assessment.

Results and discussion
The decrease of fertility of seabass sperm with conservation time is clearly linked to the decrease of the percentage of motile sperm whereas the shape of velocity curve is similar. Concomitantly, the membrane of spermatozoa is affected and disturbs sperm activation. DNA integrity is still under investigation.

The use of culture media such as leibovitz L15 improved conservation compared to the use of saline diluant. The analysis of both oxygen consumption and ATP showed the necessity of respiration during conservation of seabass sperm.

Conclusions
The use of diluents designed to fulfil the requirements of sperm for survival and fertility, improved sperm chilled conservation. It already allowed collecting large numbers of sperm samples the day before female stripping hence it allowed to perform large factorial cross getting rid of cryopreservation.

References

Sansone, G et al., 2001, Inactivator media of seabass(Dicentrarchus labrax L.) spermatozoa motility. Aquaculture 202, 257-268

Peñaranda, D. et al 2008, Effects of extenders and cryoprotectant combinationson motility and morphometry of seabass (Dicentrarchus labrax) spermatozoa.

Bobe, J. and Labbé, C. 2009, Chilled storage of sperm and eggs. Methods in reproductive aquaculture, marine and freshwater species. Cabrita, Robles and Herraez eds. CRC press, Boca Raton, Fl., USA. 219-236

O-2/06
GRADING FISH SPERM QUALITY FOR EVALUATION OF ITS POTENT SWIMMING PERFORMANCES: TOWARDS NEW APPROACHES

Jacky Cosson[1,2], Volodymyr Bondarenko[2]

[1] *UMR 7009 CNRS, Marine Station, Univ. P. M. Curie, BP 28, 06230, Villefranche sur mer, France*

[2] *University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, 389 25, Vodnany, Czech Republic*

E-mail: jacosson@gmail.com

Introduction

In fish reproduction, evaluation of sperm quality, especially motility, varies broadly depending on technologies used for such goal and on needs of fish farmers or laboratory requirements.

Methods

In the past, rustic technology regarding motility appreciation have used a simple microscope, in which case grading is mainly relying on eye evaluation of the proportion of moving cells in the microscopic observation field.

Two obvious limitations occur when using such approach: 1- fish spermatozoa performances drop down rapidly during the short period following motility activation (Morisawa 1985; Cosson 2010), 2- the quantification of percentage of moving cells is approximate by "eye" method. Therefore, a first improvement has consisted of using recording tools such as photography, cinematography and more recently video-microscopy. In all three cases, such records allow analysis of still frames or visualization on slow-motion sequences.

An additional improvement, mostly resulting from digitized video records, is the possibility to apply automatic analysis to get information about the swimming performances of spermatozoa, by use of the so-called CASA (Computer Aided Sperm Analysis) after adaptation to situations or constraints specific to fish sperm (Kime et al. 2001; Hobson 1996), specially related to the high velocity of fish spermatozoa (Wilson-Leedy & Ingerman 2007).

In contrast to the above mentioned technologies which are mostly focused on movement of sperm head, a complementary approach is appealing to the use of higher microscopic magnification allowing visualization of flagella and leading to possibility of quantifying flagella parameters in terms of geometrical shape, wave length or amplitude, but also to detect local damages responsible for decrease of their swimming performances (Cosson et al. 1997). An additional facility resulting from the use of stroboscopic illumination is the possibility of measuring a very accurate parameter, the flagellar beat frequency or FBF (Cosson et al. 1985).

More recently, access to high-speed video-microscopy records represents a step further, well suited to sperm cells having high velocity and decreasing rapidly their swimming performances (Cosson et al. 1997) during their motility period. This approach allows precise quantification of flagellar shape and its evolution within one single beat cycle (such as wave length or amplitude, velocity of waves or curvature) but also comparison of such parameters between sperm samples exposed to various physiological conditions (Cosson 2008).

Discussion

One should be aware of the fact that this inflation of swimming parameters generated by more and more sophisticated new technologies does not reflect necessarily a corresponding increase of information, because some of these parameters are not independent between each other but rather redundant. For instance, flagellar beat frequency is mainly responsible of and in direct relationship with forward velocity, meaning that both parameters are tightly linked. Therefore selection of truly discriminative parameters should be carefully applied.

Also, only a restricted number among those parameters should be adopted to validate comparatively results between laboratories or species or environmental situations. So far, also, motility criteria are truly discriminative mainly when one needs to counter-select any problematic sample.

In addition, motility parameters constitute only one set of criteria in the attempt to predict best success of fertilization by these spermatozoa. Many studies attempt to identify factors that would explain why one apparently normal and fertile semen is, in fact, more capable of fertilizing eggs than another. Few of the semen tests that are currently available appear to be able to provide mechanistic explanations for this phenomenon.

Selection of spermatozoa by females or by eggs themselves at various stages of the fertilization process could be partly responsible for such lack of predictive and accurate ability. One example of sperm selection by eggs is occurring through the process so-called "chemo-attraction". This chemotaxis process was proved to be active in quite a large diversity of species, including fish such as herring or trout (Yanagimachi et al. 1992). There is an emerging set of data about chemotaxis and corresponding sperm plasma membrane receptors, in vertebrates (as well as invertebrates) that provides strong indications to allow them to respond appropriately to chemotactic signals. It is not clear why only a subset of spermatozoa would possess the requisite receptors and adequate signaling systems in the sperm population of a given sample.

In similar respects, heterogeneity in a sperm population is frequently suspected but rather difficult to demonstrate. There are only some published pieces of information suggesting this important feature (see Harrison 1998, for review ; Kaspar et al. 2008). Gaining an understanding that a minority of sperm cells is the functionally significant population should, in theory, provide a warning that most spermatozoa judged as representative because described by averaging a population are somehow functionally defective regarding their ultimate duty, fertilization (Holt & Van Look 2004).

Conclusions

The more and more refined possibilities of defining the so-called "quality" of spermatozoa in terms of motility appear in response to an increasing demand in the field of aquaculture and cryo-conservation of fish spermatozoa but should be carefully selected for their truly discriminative abilities.

Acknowledgements

We are grateful to Dr O. Linhart for giving us hospitality in his institute and thus, full access to facilities, including sperm from a large variety of fish species. We are thankful to CNRS as well as Czech grant agencies for the funding of projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

References

Cosson J., 2010: Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J. Fish Biol. 76, 240-279

Cosson J.; Billard R.; Cibert C.; Dreanno C., Linhart, O.; Suquet M., 1997: Movements of fish sperm flagella studied by high speed videomicroscopy coupled to computer assisted image analysis. Polish Arch. Hydrobiol. 44, 103 – 113.

Cosson J., 2008: Methods to analyse the movements of fish spermatozoa and their flagella In Fish Spermatology (Alavi, S. M. H., Cosson, J. J., Coward, K. & Rafiee, G., eds.), pp. 63 – 101. Oxford: Alpha Science.

Cosson M-P.; Billard R.; Gatti J-L.; Christen R., 1985: Rapid and quantitative assessment of trout spermatozoa motility using stroboscopy. Aquaculture 46, 71-75.

Harrison R. A., 1998: Sperm evaluation: what should we be testing? In "The 6th MAFF International Workshop on Genetic Resources". Genetic Diversity and Conservation of Animal Genetic Resources, pp 135–154. Tsukuba, Ibaraki, Japan: National Institute of Agro- biological Resources.

Hobson G., 1996: Hobson Tracker User Manual, Hobson Tracking Systems.

Holt V. W. ; Van Look K. J. W., 2004: Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. Reproduction 127, 527–535.

Kaspar V.; Vandeputte M.; Kohlman M.; Hulak M.; Rodina M.; Gela D.; Kocour M.; Linhart O., 2008: A proposal and case study towards a conceptual approach of validating sperm competition in common

carp (Cyprinus carpio L.) with practical implications for hatchery procedures. J. Appl. Ichthyol. 24, 406-409.

Kime D. E.; Van Look K. J. W.; McAllister B. G.; Huyskens G.; Rurangwa E.; Ollevier F., 2001: Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp. Biochem. and Physiol., C Toxicol. and Pharmacol. 130, 425–433.

Morisawa M., 1985: Initiation mechanism of sperm motility at spawning in teleost. Zool. Sci. 2, 605-615.

Wilson-Leedy J. G.; Ingermann R. L., 2007: Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenol. 67, 661–672.

Yanagimachi R.; Cherr, G. N.; Pillai, M. C.; Baldwin, J. D., 1992: Factors controlling sperm entry into the micropyles of salmonid and herring eggs. Develop. Growth & Differ. , 34(4), 447-461.

O-2/07
ENZYMATIC ACTIVITY OF TURBOT SPERMATOZOA IN RELATION TO SAMPLING DAY DURING SPAWNING

Edyta Gosz[1,2] and Jan Horbowy[2]

[1] *Sea Fisheries Institute in Gdynia, Department of Fish Resources, 1 Kołłątaja, Gdynia 81-332, Poland*
[2] *University of Gdańsk, Faculty of Biology, 24 Kładki, Gdańsk 80-952, Poland*

E-mail: goszed@mir.gdynia.pl

Introduction
Sperm quality varies during the spawning period as was demonstrated for Atlantic cod (Rouxel et al., 2008) and some of these changes are related to the sperm ageing. The ageing phenomenon of spermatozoa was previously illustrated by variations of chromatin condensation and a decreasing quantity of midpiece (Suquet et al.,1998) and nucleus (Alavi et al., 2008) vesicles, as well as decrease of morphometric parameters of sperm cell (Gosz et al., 2010). However, information concerning activity variation of spermatozoal enzymes during the spawning season have not been evaluated. Therefore, we investigated the activity of selected spermatozoa enzymes (NAD^+- and NADP-dependent dehydrogenases and creatine kinase (CK)) in relation to the sampling day during spawning. As a model fish, we used the Baltic turbot, *Psetta maxima*.

Materials and methods
Mature male turbot were caught in the Pomeranian Bay (POM) and the Gulf of Gdańsk (GDA) during the spawning season through May and June of 2009. All examined fish (n = 99) were at the same maturity stage and in full breeding condition. Individuals were maintained at 14°C in an indoor seawater tank and analyzed within two days after capture.

Fresh undiluted milt from each male was frozen and stored at -80°C until analysis. The following enzymes were measured: L-lactate dehydrogenase (LDH, lactate:NAD+ oxidoreductase EC 1.1.1.27), malate dehydrogenase (MDH, malate:NAD+ oxidoreductase EC 1.1.1.37), malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) (ME, malate:NADP+ oxidoreductase (oxaloacetate-decarboxylating) EC 1.1.1.40), glucose-6-phosphate dehydrogenase (G-6-PDH, D-glucose-6-phosphate:NADP+ 1-oxidoreductase EC 1.1.1.49) and creatine kinase (CK, ATP:creatine N-phosphotransferase EC 2.7.3.2). Assay conditions were previously described by Rurangwa et al. (2002). The protein concentration was determined using the Coomassie blue method (Spector, 1978). The biochemical assays were performed using a UV-1601 UV-visible spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany).

The following general linear model (GLM) was applied to analyze the effects of the sampling day and location on enzymatic activities:

$$\text{Enzyme} = \text{sampling\_day} + \text{location} + \text{interactions} + \text{error}, \qquad (1)$$

where, *Enzyme* is the enzymatic activity of the individual enzyme, *sampling_day* is the day of sampling with May 28 as the first day and *location* is GDA or POM.

Results
For all analyzed enzymes, *sampling_day* was highly significant in model (1) ($P < 0.003$), and a decline in enzymatic activity was shown with spawning time. The model explained 13-52% of the variance of the *Enzyme*. The *location* was significant at 5% level for MDH and CK, at 10% level for LDH and ME, and insignificant for G6PDH. The interactions of *sampling_day* with *location* were not significant; therefore, the decline in *Enzyme* could be considered to be similar in both locations and amounted to 1.4-2.2% per day for each enzyme.

Discussion and conclusions

In the current work, the enzymes of spermatozoal metabolic pathways such as MDH (citric acid cycle, gluconeogenesis), LDH (glycolysis), G6PDH (pentose phosphate pathway), ME and CK (pyruvate and ATP metabolism) showed decrease with the sampling day during the spawning season.

At the end of the reproductive season turbot spermatozoa show decreasing quantity of midpiece vesicles (Suquet et al.,1998) and midpiece shrinkage was observed for this species (Gosz et al., 2010). According to this, we suggest that the decrease of enzymatic activity is associated with spermatozoa ageing.

The ageing of spermatozoa is an interesting feature in sperm production in many marine fish species (Trippel, 2003) and the change of the enzymatic activity during reproductive season could be reliable as indicator of spawning stage.

References

Alavi, S. M. H.; Psenicka, M.; Rodina, M.; Policar, T.; Linhart, O., 2008: Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Teleostei: Cyprinidae) during the reproductive season. Aquat. Living Resour. 21, 75–80.

Christ, S. A.; Toth, G. P.; McCarthy, H. W.; Torsella, J. A.; Smith, M. K., 1996: Monthly variation in sperm motility in common carp assessed using computer-assisted sperm analysis (CASA). J. Fish Biol. 48, 1210–1222.

Gosz, E.; Mirny, Z.; Horbowy, J.; Ziętara, M.S., 2010: Morphometry of turbot spermatozoa in relation to the location and time of capture during the spawning season. J. Appl. Ichthyol. 26, 784–788.

Rouxel, C.; Suquet, M.; Cosson, J.; Severe, A.; Quemener, L.; Fauvel, C., 2008: Changes in Atlantic cod (*Gadus morhua* L.) sperm quality during the spawning season. Aquacult. Res. 39, 434-440.

Suquet, M.; Dreanno, C.; Dorange, G.; Normant, Y.; Quemener, L.; Gaignon, J. L.; Billard, R., 1998: The ageing phenomenon of turbot spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization and storage capacities. J. Fish Biol. 52, 31–41.

Trippel, E. A., 2003: Estimation of male reproductive success of marine fishes. J. Northw. Atl. Fish. Sci. 33, 81-113.

Wang, Z.; Crim, L. W., 1997: Seasonal changes in the biochemistry of seminal plasma and sperm motility in the ocean pout, *Macrozoarces americanus.* Fish Physiol. Biochem. 16, 77-83.

O-2/08
EGG QUALITY CRITERIA IN THE PACIFIC RED SNAPPER *LUTJANUS PERU*

I. Moguel-Hernández[1], R. Peña[1], H. Nolasco-Soria[2], S. Dumas[1], M. Contreras-Olguín[1]

[1] *Unidad Piloto de Maricultivos. CICIMAR-IPN. P.O. Box 592. La Paz, B.C.S. 23090. México*
[2] *Laboratorio de Fisiología. CIBNOR. La Paz, B.C.S. 23090. México*

E-mail: blacklarvae@hotmail.com

Introduction
Due to its economical importance along the Pacific coast of Mexico, there has been a high interest for development of a culture program of the Pacific red snapper (*Lutjanus peru*). However, high mortality rates have been observed during early developmental stages. Egg quality has been considered as a major factor affecting larval survival (1). Many studies have identified the utility of morphological and biochemical parameters as indicators of egg quality (3, 4). The aim of the present study was to evaluate biochemical parameters like proteins and energetic metabolites, and activity of metabolic and digestive enzymes, in order to be used as possible determinants of egg quality in *L. peru*.

Materials and methods
Fertilized eggs of eight spawns were obtained by hormonal induction and were maintained in 120 L conical tanks filled with filtered and sterilized seawater at 26º C and constant aeration. Egg quality criteria like abnormal cleavage (%A), hatching rate (%H) and survival at first feeding (%S) were recorded in each spawn. Additionally, samples were taken during the embryonic and early larval development (newly-fertilized egg, early blastula, blastula, gastrula, newly-hatched larvae, 24 and 48 h after hatching). The concentration of protein and the energy metabolites glucose, fructose and glucose-6-phosphate were determined. Enzymatic activity of acid phosphatase (E.C. 3.1.3.2.), alcaline phosphatase (E.C. 3.1.3.1), lactate dehydrogenase (E.C. 1.1.1.27), transaldolase (E.C. 2.2.1.2.), glucose-6-phosphatase (E.C. 3.1.3.9) amylase (E.C. 3.2.1.1.), lipase (E.C. 3.1.1.3.), trypsin (E.C. 3.4.21.4), chymotrypsin (E.C. 3.4.21.1) and cathepsin-like was evaluated by colorimetric methods adjusted for the sample size and quantity. Pearson correlation and simple and multiple regressions models were adjusted using the biochemical parameters as independent variables and the criteria of egg quality as dependant variables.

Results
Most of the energy metabolites and enzymatic activities evaluated were present in all developmental stages, with the exception of chymotrypsin and glucose-6-phosphate. The concentration of protein, energy metabolites, and the enzymatic activities had a similar pattern during the embryonic development and yolk-sac larvae in all spawns.

Positive and negative correlations were detected between enzymatic activities, proteins, and metabolites with abnormal cleavage, hatching rate, and survival rate. The activity of glucose-6-phosphatase (%A r=0.87; %H r=-0.65; %S r=-0.67) and the concentration of fructose (%A r=-0.64; %H r=0.54; %S r=0.64) were the only biochemical parameters with a high correlation with the three egg quality criteria.

The simple linear regression models for the biochemical parameters, whose significance level was $p \leq 0.05$, are shown in Table I.

Table I. Simple linear regression models using biochemical parameters in different developmental stage to predict abnormal-cleaved embryos (%A), hatching rate (%H) and survival at first feeding (%S) of the Pacific red snapper L. peru.

	Stages	Independent variables	Model	r^2	p
% A	I	Glucose	y = 18.5762 + 0.0451x	0.54	0.04
		Fructose	y = 4.0297 + 0.0441x	0.76	0.01
% H	I	Acid phosphatase	y = 120.0903 – 1.5326E5x	0.55	0.04
		Trypsin	y = 115.2254 – 98.5772x	0.50	0.05
% S	IV	Trypsin	y = -7.8618 + 282.8434x	0.51	0.05

Development stages: I, newly-fertilized egg; IV, gastrula

The use of multiple regression models increased the coefficient of regression in the three quality criteria. Multiple regression models with a level of significance $p<0.05$ and the regression coefficient $R^2> 0.75$ are shown on Table II.

Table II. *Multiple regression models using biochemical parameters to predict abnormal-cleaved embryos (%A), hatching rate (%H) and survival at first feeding (%S) of the Pacific red snapper L. peru.*

Dependant variable	Independent variable	Model	R^2	P
%A	x_1 = F; x_2 = G6	$y=0.729x_1-0.330x_2+21.424$	0.85	<0.009
%A	x_1 = G; x_2 = F; x_3 = G6	$y=-0.616x_1+1.265x_2-0.397x_3+20.889$	0.91	<0.016
%H	x_1 = F; x_2 = T	$y=-0.543x_1+0.604x_2+138.306$	0.78	<0.022
%H	x_1 = AF; x_2 = G6	$y=-0.757x_1+0.564x_2+100$	0.87	<0.006
%H	x_1 = F; x_2 = AF; x_3 = G6	$y=-0.133x_1-0.696x_2+0.506x_3+106$	0.88	<0.027
%H	x_1 = AF; x_2 = G6; x_3 = T	$y=-0.737x_1+0.559x_2-0.025x_3+101$	0.87	<0.032
%H	x_1 = P; x_2 = AF; x_3 = G6	$y=-0.123x_1-0.704x_2+0.524x_3+110$	0.88	<0.027
%S	x_1 = G; x_2 = F; x_3 = G6	$y=1.485x_1-1.975x_2+0.603x_3+82.573$	0.92	<0.013
%S	x_1 = G; x_2 = F; x_3 = G6; x_4 = T	$y=1.722x_1-2.335x_2+0.725x_3-0.243x_4+109.093$	0.93	<0.04

P, proteins; G, glucose; F, fructose; G6, glucose-6-phosphatase; T, trypsin; AF, acid phosphatase.

Discussion and Conclusions

Biochemical parameters during different stages of early development in different fish species have been evaluated in an attempt to establish, predict and/or improve egg quality, however the results may differ for every species (1).

In our study, fructose explained more than 70% of the variation of the abnormalities during early cleavage ($r^2 = 0.76$), which suggests an important role of carbohydrates during the early development (3). Acid phosphatase and trypsin explained around 50% of the hatching rate ($r^2 = 0.55$ and 0.50, respectively). And the only biochemical parameter related to survival at first feeding was trypsin activity ($r^2 = 0.51$). This regression coefficients were higher than the ones reported in *Sparus aurata* $r^2 < 0.450$ (3), *Puntazzo puntazzo* $r^2 < 0.445$ (4) and *Dentex dentex* $r^2 < 0.5$ (2); although different biochemical variables were considered in these studies.

The multiple regression models increase the R^2 for all the quality criteria. Similar multiple regression coefficients reported in *Sparus aurata* ($R^2 = 0.741$) (3) and *Puntazzo puntazzo* ($R^2 = 0.535$) (4) were lower than the ones calculated for the Pacific red snapper.

Fructose, glucose and glucose-6-phosphatase were involved in all the multiple regression models, suggesting an important role of carbohydrate metabolism during the early development of the Pacific red snapper. Similar results were reported in embryos and larvae of *Sparus aurata* (3).

The models proposed in this study may predict the egg quality of the Pacific red snapper with a high significance level. However the models with fewer variables are recommended since less variables reduces the analysis cost and effort.

Acknowledgements
Fellowship and funding was provided by SIP, PIFI and COFAA at the IPN of Mexico and also by the CONACYT of Mexico (SEP-CONACYT 60803).

References

[1] Faulk, C.K.; Holt, G.J., 2008: Biochemical composition and quality of captive-spawned cobia Rachycentron canadum eggs. Aquaculture. 279, 70–76.

[2] Giménez, G.; Estévez, A,; Lahnsteiner, F.; Zecevic, B.; Bell, J.G.; Henderson, R.J.; Piñera, J.A.; Sanchez-Prado, J.A., 2006: Egg quality criteria in common dentex (Dentex dentex). Aquaculture. 260, 232-243.

[3] Lahnsteiner, F.; Patarnello, P., 2004: Egg quality determination in the gilthead seabream, Sparus aurata, with biochemical parameters. Aquaculture. 237, 443-459.

[4] Lahnsteiner, F.; Patarnello, P., 2004: Biochemical egg quality determination in the gilthead seabream, Sparus aurata: reproducibility of the method and its application for sharpsnout seabream, Puntazzo puntazzo. Aquaculture. 237, 433-442.

O-2/09
DNA METHYLATION PATTERN IN SPERM AND SOMATIC CELLS AFTER CRYOPRESERVATION: INFLUENCE OF THE CRYOPROTECTANT

Laurent Gautier, Alexandra Depince, Nathalie Chenais, Lucie Marandel, Pierre-Yves LeBail, Catherine Labbe

INRA, UR 1037 SCRIBE, Campus de Beaulieu, F-35000 RENNES, France

E-mail: Catherine.Labbe@rennes.inra.fr

Introduction

DNA methylation of cytosine residues in CpG sites in the promoter region of some genes is one epigenetic modification known to repress gene expression. It was shown in zebrafish that after fertilization, methylation level decreases and then increases again at later stages, around the embryonic genome activation (Mhanni and McGowan, 2004). Such changes are thought to enable the reprogramming of gamete chromatin in order to induce the expression of genes involved in early development. The erasure-establishment of DNA methylation is therefore important for the quality of fish embryo development (Yamakoshi and Shimoda, 2003). No relationship was described however between the initial DNA methylation level in sperm and oocyte and the quality of the reprogramming during early development. Yet, this issue is important with regards to fertilization with frozen-thawed sperm, especially when dimethyl sulfoxide (DMSO) is used as cryoprotectant. Indeed, DMSO, the most widely used cryoprotectant for fish spermatozoa and cultured cells, is a very active chemical whose methylation induction properties were described in other systems (Kawai et al., 2010).

In this work, we address the question as how the cryopreservation technology may affect the global DNA methylation level in sperm and in somatic cells in goldfish (Carassius auratus). The erasure-establishment of DNA methylation during goldfish development was explored additionally. The obtained profile will serve as a reference to assess the risk of methylation profile changes after fertilization with cryopreserved sperm, or after nuclear transfer with cryopreserved somatic cells.

Materials and methods

Sperm was collected on 2 years old goldfish after hormonal induction as in (Le Bail et al., 2010). Sperm was cryopreserved in CryoFish (IMV L'Aigle) with 10 % DMSO according to (Labbe et al., 2001). Fin cells were cultured from caudal fin explants and cryopreserved with 7.5 % DMSO in a -80°C chest freezer as in (Moritz and Labbe, 2008). All experiments were performed on 3 independent samples.

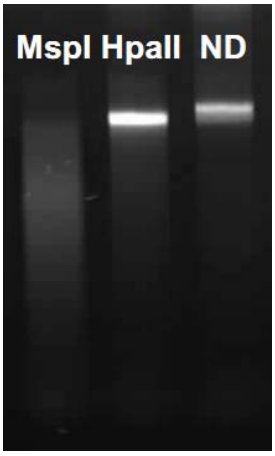


Figure 1. Example of sperm genomic DNA digestion in the MspI/HpaII system. ND: not digested.

Genomic DNA was isolated from sperm, oocytes, embryo at different stages from fertilization to hatching, and from fin pieces and cultured fin cells. For each sample, global methyl-cytosine content was analyzed by the restriction enzyme assay according to (Mhanni and McGowan, 2004), using the MspI/HpaII enzyme digestion for 1h at 37°C.

Results

The enzymes MspI and HpaII are isoschiomeres specific of the CCGG restriction site. While MspI will cut all CCGG sites, HpaII will cut only when the cytosine bases are unmethylated. As shown Figure 1, the extent of the digestion was assessed after migration of the Gel Red stained DNA on agarose gel. DNA methylation was deduced from the difference in smear intensity between the 2 digestions products. The lesser the HpaII smear, the higher the DNA methylation of the corresponding sample on the CCGG restriction site.

Sperm DNA methylation level assessed with this method showed 68% ± 17% of methylated sites. Oocytes had a much lower methylation level (36% ± 15%).

After fertilization, methylation level at each division stayed very low (29% ± 19%) up to the mid-blastula stage. At mid blastula, methylation increased (54% ± 18%) up to hatching (80 % ± 13%). Methylation of fin

pieces was at the same level as that of whole embryos at hatching. By contrast, methylation of the cultured fin cells was low and little variable (42 % ± 4%).

After cryopreservation in DMSO, methylation level increased almost twice in fin cell samples (72% ± 9%) compared to the untreated ones (42%). Changes in sperm after cryopreservation, and changes after cryopreservation with 1-2,propanediol are currently under study and will be presented.

Discussion and conclusion

In this work, global methylation was assessed with the MspI/HpaII system. It should not be overlooked that this method gives a picture of the methylation level only at cytosine bases belonging to the CCGG sequence. Besides, such global methylation does not reflect the gene by gene response to the demethylation-remethylation process. This approach was chosen however because it suits well the analysis of a global feature which may be altered at large by specific technologies such as cryopreservation.

We demonstrated that goldfish embryo does undergo a demethylation-remethylation sequence after fertilization. The sequence reported in zebrafish (Mhanni and McGowan, 2004) was similar in shape to the one obtained here on goldfish. Differences in development rate between the two species however required that such profile be drawn for goldfish, as it will be necessary for every new fish species. The precision of this sequence should give a valuable tool to assess whether the use of cryopreserved cells will modify epigenetic changes upon fertilization.

The use of DMSO to cryopreserve cells which had a low initial methylation level induced a dramatic increase of the DNA methylation level. How this applies to a more methylated cell that is spermatozoa is under question. Special attention should be paid now to the consequence of these alterations on the déméthylation-remethylation sequence after fertilization or after nuclear transfer.

It cannot be excluded indeed that some of the developmental failures observed after fertilization with cryopreserved cells (Horvath and Urbanyi, 2000) may find their roots in aberrant epigenetic reprogramming.

References

Horvath,A. and Urbanyi,B. (2000). The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish Clarias gariepinus (Burchell 1822) sperm. Aquacult. Res. 2000. 31, 317-324.

Kawai,K., Li,Y.-S., Song,M.-F., and Kasai,H. (2010). DNA methylation by dimethyl sulfoxide and methionine sulfoxide triggered by hydroxyl radical and implications for epigenetic modifications. Bioorg Med Chem Lett 20, 260-265.

Labbe,C., Martoriati,A., Devaux,A., and Maisse,G. (2001). Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Mol Reprod Dev 60, 397-404.

Le Bail,P.Y., Depince,A., Chenais,N., Mahe,S., Maisse,G., and Labbe,C. (2010). Optimization of somatic cell injection in the perspective of nuclear transfer in goldfish. BMC Dev Biol 10, 64.

Mhanni,A.A. and McGowan,R.A. (2004). Global changes in genomic methylation levels during early development of the zebrafish embryo. Dev Genes Evol. 214, 412-417.

Moritz,C. and Labbe,C. (2008). Cryopreservation of goldfish fins and optimization for field scale cryobanking. Cryobiology 56, 181-188.

Yamakoshi,K. and Shimoda,N. (2003). De novo DNA methylation at the CpG island of the zebrafish no tail gene. Genesis. 37, 195-202.

O-2/10
SPERM MOTILITY TRAITS IN MALE GOLDFISH EXPOSED TO BISPHENOL A, *IN VIVO*

Azadeh Hatef, Sayyed Mohammad Hadi Alavi, Marek Rodina, Otomar Linhart

South Bohemia Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodnany, Czech Republic

E-mail: ahatef@frov.jcu.cz

Introduction

Bisphenol A (BPA) is one of many diphenylalkanes that are raw materials for the production of polymers such as polycarbonates, epoxy, phenolic resins, polyesters, and polyacrylate (Kang et al. 2007). Its levels in river water were reported between 0.02–21 μg/L (Kang et al. 2007). BPA is known as an estrogen mimicking compound; but there are a few evidences for its anti-androgenic activity depending on dose and period of exposure (Akingbemi et al. 2004; Vandenberg et al. 2009). Literature have demonstrated testicular dysfunctions in fish exposed to BPA such as delay of sperm maturation, histopathological abnormalities, formation of intersex (testis-ova) and decrease of sperm production in male fish exposed to BPA (see review by Kang et al. 2007; Vandenberg et al. 2009). However, effect of BPA on sperm motility that is a key parameter for successful fetrilization of eggs is still unclear. Lahnsteiner et al. (2005) observed lower sperm motility and velocity in males brown trout exposed to 1.75–2.4 μg/L BPA at the beginning and middle of spawning. Our previous studies showed the effects of BPA on sperm motility traits and morphology, *in vitro* (Hatef et al. 2010). We also observed decrease of androgens in male goldfish exposed for 1 month to BPA at 0.5 and 1.5 μg/L (Hatef et al. unpublished data). The present study shows the effects of BPA on sperm motility traits in mature male goldfish (*Carassius auratus*) that have been exposed to BPA (0.1, 0.5 and 1.5 g/L) for one month during the spawning season.

Materials and methods

Mature males of goldfish were exposed to BPA at 0.1, 0.5 and 1.5 g/L for 30 days during the spawning season. BPA was dissolved in DMSO and added into aquaria. Body weight and total length of male goldfish were not significantly differed among treated and control groups. At 10, 20 and 30 days after exposure, sperm samples were collected from each individual using a syringe and kept on ice during analysis. Motility of sperm was activated in an activation medium composed of NaCl 50 mM, Kcl 5 mM, Tris 20 mM, pH 8.5 (110 mOsmol/kg). Sperm motility was recorded using a 3 CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) equipped with a stroboscopic lamp. A micro image analyzer (Olympus Micro Image 4.0.1 for Windows) was used to measure % sperm motility and sperm velocity from 5 successive video frames captured from a DVD-recorder (SONY DVO-1000 MD, Japan) according to Hatef et al. (2010). Analysis of sperm motility was performed at 15, 30, 60 and 90 s post-activation. Repeated measure ANOVA was used to study the effects of BPA concentration, exposure time and their interaction on sperm motility traits (Hatef et al., 2011).

Results

For repeated measures ANOVA, there was a significant BPA concentration × exposure time interaction effect for sperm motility and velocity at 15, 30, 60 and 90 s post-activation. Therefore, for each post-activation time, the models were revised into separate one-way ANOVAs to examine the effect of BPA concentration (0 to 1.5 μg/L) at each exposure time. Sperm motility at any time post-activation did not differ in males exposed for 10 days to different concentrations of BPA. At 20 days after exposure, sperm motility showed significant decrease at all examined time post-activation (15, 30, 60 and 90 s) in males exposed to 0.5 or 1.5 μg/L BPA. At 30 days after exposure, sperm motility was affected at all treated groups (0.1, 05 and 1.5 μg/L BPA). Revised model for sperm velocity showed no significant difference in sperm velocity at 15 s post-activation at any exposure time (10, 20 or 30 days). At 10 days after exposure, sperm velocity was affected at 0.5 μg/L at 30 s post-activation and 0.1 μg/L at 60 and 90 s post activation. After 20 and 30 days exposure, significant decrease of sperm velocity was observed at 0.1 or 0.5 μg/L at 30, 60 and 90 s post-activation.

Discussion and conclusions

The present study demonstrated the negative impact of BPA on sperm motility traits at environmentally relevant concentrations. Similar to results published by Lahnsteiner et al. (2005), both sperm motility and velocity are affected. Further studies are required to study the mechanisms of action of BPA on sperm motility. One of mode of action might be through modulation of sperm maturation, which is regulated by sex steroids such as androgens (Nagahama 1994). On the other hand, observed anti-androgenic mode of action of BPA (Akingbemi et al. 2004) causes testicular dysfunctions in sex steroid synthesis (Mandich et al. 2007; Hatef et al. unpublished data) and lead to decrease of mature sperm. Another mode of action might be regulated through modulations of metabolism and energetics of sperm. Initial ATP content of sperm is a key factor determining sperm velocity (Cosson 2010). Therefore, BPA may decrease ATP contents of sperm and lead to decrease of sperm velocity. In conclusion, the present study showed negative impact of BPA on sperm motility traits in fish exposed to BPA at environmentally relevant concentrations, which might be addressed to decrease of androgens or disordering of sperm maturation.

Acknowledgements

This study was supported by GACR 523/09/1793, CZ.1.05/2.1.00/01.0024, GAJU 046/2010/Z, GAJU 047/2010/Z, IAA 608030801, ME10015, QH82119, LC06073, and GACR P502/11/0090.

References

Akingbemi, B.T.; Sottas, C.M.; Koulova, A.I.; Klinefelter, G.R.; Hardy, M.P., 2004; Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology 145, 592–603.

Cosson, J., 2010: Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J. Fish Biol. 76, 240–279.

Hatef, A.; Alavi, S.M.H.; Butts, I.A.E.; Policar, T.; Linhart, O., 2011: Mechanism of action of mercury on sperm morphology, adenosine triphosphate content, and motility in Perca fluviatilis (Percidae; Teleostei). Environ. Toxicol. Chem. 30, 905–914.

Hatef, A.; Alavi, S.M.H.; Linhartova, Z.; Rodina, M.; Policar, T.; Linhart, O., 2010: In vitro effects of Bisphenol A on sperm motility characteristics in Perca fluviatilis L. (Perciade; Teleostei). J. Appl. Ichthyol. 26, 696–701.

Kang, J.H.; Aasi, D.; Katayama, Y., 2007: Bishenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. Crit. Rev. Toxicol. 37, 607–625.

Lahnsteiner, F.; Berger, B.; Kletzl, M.; Weismann, T., 2005: Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, Salmo trutta f. fario. Aquat. Toxicol. 75, 213–224.

Mandich, A.; Bottero, S.; Benfenati, E.; Cevasco, A.; Erratico, C.; Maggioni, S., 2007: In vivo exposure of carp to graded concentrations of bisphenol A. Gen. Comp. Endocrinol. 153, 15–24.

Nagahama, Y., 1994: Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217–229.

Vandenberg, L.N.; Maffini, M.V.; Sonnenschein, C.; Rubin, B.S.; Soto, A.M., 2009: Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. Endocrine Rev. 30, 75–95.

O-2/11
THE PERIOD OF EURASIAN PERCH, *PERCA FLUVIATILIS* L., EGGS ABILITY TO FERTILIZATION

Daniel Żarski[1], Akos Horvath[2], Laszlo Kotrik[2], Katarzyna Targońska[1], Katarzyna Palińska[1,3], Sławomir Krejszeff[1], Zoltan Bokor[2], Bela Urbanyi[2], Dariusz Kucharczyk[1]

[1] *Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn, Poland*
[2] *Department of Aquaculture, Institute of Environmental and Landscape Management, Faculty of Agriculture and Environmental Science, Szent István University, Páter Károly u. 1. H-2103 Gödöllő, Hungary*
[3] *Department of Zoology, University of Warmia and Mazury in Olsztyn, Poland*

E-mail: danielzarski@interia.pl

Introduction

Eurasian perch, *Perca fluviatilis* (L.), is one of the most promising species for freshwater aquaculture diversification. One of the obstacles in artificial reproduction of the species is the fact that perch females are able to release the eggs into the tank in which they were kept (Żarski et al. 2011) or during manipulation. This creates many problems for breeding programs and for general aquaculture of this species. In addition, some problems with high variability of fertilization rates during artificial insemination have been observed (Kucharczyk D., personal information).

Clean hatchery water is very often used in hatcheries as an activating solution for perch egg fertilization. Eggs exposed to water are activated and lose their capacity to be fertilized within a short period of time, depending on the species (Coward et al. 2002, Minin and Ozerova 2008). Knowledge of this limited exposure time can be crucial for aquaculturists because eggs released into the water retain their fertilizing capacity for only a few minutes following exposure. To date, no information is available on the time limits of fertilization of water-activated perch eggs.

The aim of this study was to evaluate the period of Eurasian perch eggs ability to fertilization following exposure to hatchery water and compare the results with sperm parameters.

Materials and methods

Wild perch spawners were selected for use in the study. The fish were caught with gillnets in Lake Dejguny (northeastern Poland) at the end of April. Eggs were obtained after induced spawning and HCG (500 IU kg^{-1}) was used for stimulation. Eggs (from 3 females) were collected by stripping into dry containers and each ribbon was cut into 27 samples. Each sample (about 200 eggs each) was placed on a separate Petri dish and covered prior to further procedures.

Sperm (from 4 males) was collected into dry syringes. One ml of sperm samples from each male was subjected to further analysis of sperm motility (percentage of motile spermatozoa, percentage of progressively motile spermatozoa) (using the CASA system) and motility duration (subjectively under the microscope). The same hatchery water was used for sperm activation.

Insemination of eggs (with 0.05 ml of pooled sperm) was proceeded by exposition of eggs to hatchery water for 15, 30, 45, 60, 90, 120, 150 and 180 seconds and compared to the control group inseminated at the moment of egg activation (0 s). The same procedure was done in triplicate for each female. Egg samples were incubated separately in a closed water system at 14°C. At the eyed-egg-stage, the embryo survival rate was recorded.

Results

The results indicate that Eurasian perch eggs are able to be fertilized for at least 2.5 minutes in hatchery water (Fig. 1). After this time, a significant decrease ($P<0.05$) in embryo survival was observed. In the control group (0 s), a lower survival rate of embryos was recorded ($P<0.05$) as compared to the groups where insemination was applied within the range of 15 to 150 s after egg exposure to hatchery water.

Sperm analysis showed that hatchery water affected motility in over 75.25% (±9.17 SD) of spermatozoa. However, only 27.55% (±7.94 SD) of all spermatozoa were moved progressively. The duration of motility ranged from 26 to 37 seconds.

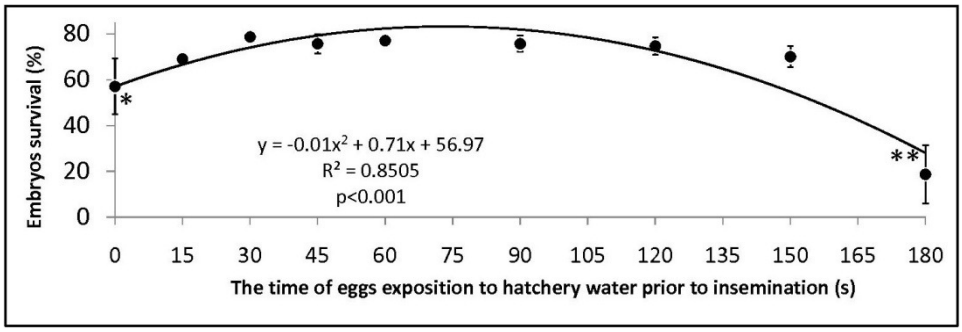


Fig. 1. Results obtained after Eurasian perch egg insemination after exposing eggs to hatchery water for different time periods. Data are expressed as mean ±SD. Data marked with an asterisk or double asterisk were statistically different (P<0.05)

Discussion and conclusions

Fish eggs after contact with water are usually able to be fertilized for a certain period (Coward et al. 2002, Minin and Ozerova 2008). However, this aspect was not previously considered to be an important part of artificial insemination. The results indicate that the common practice of artificial insemination (as was done in the control group) gives unsatisfactory (variable) results.

Kucharczyk et al. (2010) suggested that in ide *Leuciscus idus* (L.) a more important aspect in artificial insemination may be the duration of spermatozoa motility than the percentage of motile spermatozoa. Because spermatozoa were motile for at least 26 seconds in the activating solution it may be suggested that Eurasian perch eggs acquire the ability to be fertilized after a certain period. However, it could not be excluded that in the case of Eurasian perch it was also caused by an incompletely distributed ribbon in the hatchery water and some of the eggs were not exposed to sperm (were covered by other eggs). In the present study, very small parts of ribbon were used and this effect was minimized. Thus, this effect should be studied more closely.

The results also indicate that it is possible to fertilize the Eurasian perch eggs within 2.5 minutes. It creates the possibility to successfully fertilize the eggs released by females into the tank or during the manipulations. Also, it could be recommended to perform the artificial insemination after about 30 s following the Eurasian perch egg distribution in the water. Despite the fact that the mechanism of fertilization failure (when the standard procedure of artificial insemination was applied) is not yet clear, such a procedure may improve fertilization rates and, consequently, production outcome.

Acknowledgements

This study was partially financed under the auspices of the project "Innovations in finfish aquaculture with special reference to reproduction", Operational Programme Sustainable Development of the Fisheries Sector and Coastal Fishing Areas 2007-2013" (OR14-61724-OR1400003/09/10/11).

References

Kucharczyk, D.; Gomułka, P.; Krejszeff, S.; Żarski, D.; Targońska, K., 2010: The effect of prepared activating liquid on the survivability of ide Leuciscus idus (L.) embryos. Pol. J. Nat. Sci. 25, 200-208.

Minin, A.A.; Ozerova, S.G., 2008: Spontaneous activation of fish eggs is abolished by protease inhibitors. Rus. J. Dev. Biol. 38, 293-296.

Żarski, D.; Palińska, K.; Targońska, K.; Bokor, Z.; Kotrik, L.; Krejszeff, S.; Kupren, K.; Horvath, A.; Urbanyi, B.; Kucharczyk, D., 2011: Oocyte quality indicators in Eurasian perch, Perca fluviatilis L., during reproduction under controlled conditions. Aquaculture 313, 84-91.

O-2/12
ROLE OF INTRACELLULAR CHANGES IN CALCIUM, POTASSIUM AND PH IN THE INITIATION OF EUROPEAN EEL SPERM MOTILITY

Víctor Gallego[1], Felipe Martínez-Pastor[2], Ilaria Mazzeo[1], David S. Peñaranda[1], M. Paz Herráez[2], Juan F. Asturiano[1], Luz Pérez[1]

[1] *Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain*

[2] *Grupo de Investigación en Técnicas de Reproducción Asistida, Departamento de Biología Celular, Universidad de León, Campus de la Vegazana s/n, 24071 León, Spain*

E-mail: mlpereig@dca.upv.es

Introduction

In marine teleosts, such as the European eel (*Anguilla anguilla*), the increase of environmental osmolality is the main factor determining the activation of sperm motility (Cosson et al. 2004). The osmolality change faced by the spermatozoa when discharged into the marine environment leads to a rapid influx/efflux of ions/water between intracellular and extracellular space. The changes of ion concentration in that process has been demonstrated: in common carp, the influx/efflux of Ca^{2+} and K^+ plays an important role in the initiation on sperm motility (Krasznai et al. 1995); in Japanese eel it was proposed the existence of Ca^{2+} channels and a K^+/H^+ exchanger (Tanaka et al. 2004). While on mammals the functions of these ions on sperm motility are well studied, there are not many reports on teleost fish, and there are no studies on European eel. In this work we have measured by the first time the intracellular concentrations of Ca^{2+}, K^+, and H^+ on eel spermatozoa by flow citometry, evaluating concentrations before and after activation.

Materials and methods

Fifteen adult eel males (100±2 g; 40±5 cm) were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of hCG (1.5 IU g^{-1} fish). Sperm samples were collected by abdominal pressure, motility was assessed subjectively for triplicate and samples having >50% of motile cells were selected for its study. Sperm was activated with artificial seawater (37‰, pH 8.2). The relative amounts of different ions on pre and postactivation times were calculated by different ion indicators: $[Ca^{2+}]_i$, spermatozoa were loaded with Fluo-4 AM indicator (Invitrogen F14201) for a final concentration of 1 μM during 30 min; $[K^+]_i$, spermatozoa were loaded with PBFI AM indicator (Invitrogen P1267) for a final concentration of 5 μM during 90 min; $[H^+]_i$, spermatozoa were loaded with Snarf-5F AM indicator (Invitrogen S23923) for a final concentration of 5 μM during 45 min, all of them at room temperature. In all the cases, sperm cells were also loaded with TO-PRO®-3 (Invitrogen T7596) for a final concentration of 2 μM to exclude dead cells from the analysis. Spermatozoa were analyzed on a Beckman Coulter CyAn ADP Flow Cytometer where 5000 sperm events were selected. Weasel software (WEHI, Victoria, Australia) and SPSS were used for citometry data and statistical analysis, respectively. Microphotographies were obtained using a Nikon Eclipse E600 microscope with UV-2A and B-2A filters.

Results

The intracellular $[Ca^{2+}]$, $[K^+]$, and $[H^+]$ of pre and post-activation sperm cells were estimated (Figure 1a,b,c). Both Ca^{2+} and K^+ intracellular concentrations increased significantly after starting sperm motility, being the Ca^{2+} increase higher than the K^+ increase. Also, elevated $[K^+]$ and $[Ca^{2+}]$ levels remained in this way until 120 s. Conversely, pH showed a progressive decrease after sperm activation, with significant differences with baseline levels at 60 and 120 s.

It was observed by the first time that intracellular calcium and potassium stores (areas with high fluorescence intensity) occurred on the apical zone of the European eel spermatozoa (Figure 1d,e).

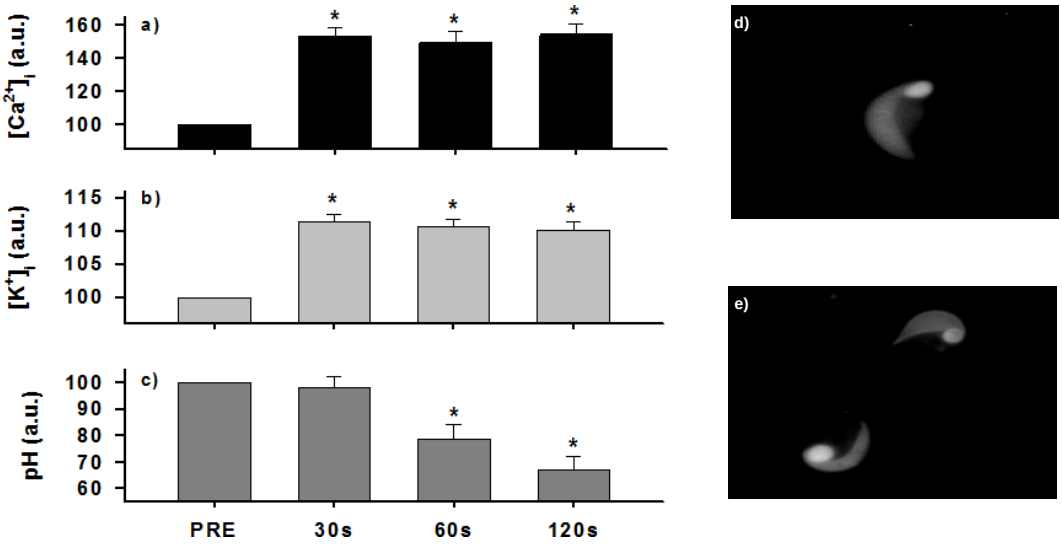


Figure 1. Intracellular ion concentrations on pre and post activation (30, 60 and 120 s) in eel sperm: a) calcium concentration; b) potassium concentration; c) pH. Pictures show (d) calcium and (e) potassium distribution on quiescent eel spermatozoa. Asterisks indicate significance differences with baseline levels

Discussion and conclusions

Our results showed that $[Ca^{2+}]_i$ and $[K^+]_i$ increase upon activation, and possibly have an important role in the initiation on European eel spermatozoa motility, as occur in other marine and freshwater teleosts. The increase in intracellular concentration of both ions can be due to three factors: a) influx of Ca^{2+} and K^+ through ion channels from extracellular medium, b) release of Ca^{2+} and K^+ from intracellular stores or c) decrease in the cell volume by aquaporin-mediated water outflux (Zilli et al. 2009). Our results strongly agree with the second hypothesis, suggesting that a membrane depolarization causes a Ca^{2+} and K^+ release from intracellular reserves, according to the model of marine fish sperm motility proposed by Morisawa (2008). In that sense, spermatozoa fluorescence images showed that calcium and potassium are stored on the mitochondria (located in the apix of the spermatozoon head), suggesting an important role of this cellular compartment on the activation mechanism on eel sperm. Therefore, more studies (using ion channels blockers, fluorescence imaging of ion stores, pre and post-activation, and changes in cell volume) may be necessary to determine the complete mechanism of the activation on European eel sperm.

Acknowledgements

Funded from the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology", grant agreement n°245257 (Pro-Eel), Generalitat Valenciana (ACOMP/2011/ 229) and Spanish Ministry of Science and Innovation (AGL2010-16009). D.S.P. has postdoc grant from UPV. I.M. and V.G. have predoctoral grants from GV and MICINN, respectively.

References

Cosson, J.; Dreanno, C.; Billard, R.; Suquet, M.; Cibert, C., 1999: Regulation of axonemal wave parameters of fish spermatozoa by ionic factors. In: The Male Gamete: From Basic Knowledge to Clinical Applications. Montreal: Cache River Press. pp. 161-186.

Krasznai, Z.; Morisawa, M.; Morisawa, S.; Krasznai, Z.T.; Tron, L.; Márián, T., 2003: Role of ion channel and membrane potential in the initiation of carp sperm motility. Aquat. Living. Resour. 16, 445-449.

Morisawa, M., 2008: Adaptation and strategy for fertilization in the sperm of teleost fish. J. Appl. Ichtiol. 24, 362-370.

Tanaka, S.; Utoh, T.; Yamada, Y.; Horie, N.; Okamura, A.; Akazawa, A.; Mikawa, N.; Oka, H.P.; Kurokura, H., 2004: Role of sodium bicarbonate on the initiation of sperm motility in the Japanese eel. Fish. Sci. 70, 780-787.

Zilli, L.; Schiavone, R.; Sauvigné, F.; Cerdá, J.; Storelli, C.; Vilella, S., 2009: Evidence for the involvement of aquaporins in sperm motility activation of the teleost gilthead sea bream (Sparus aurata). Biol. Reprod. 81, 880-888.

O-2/13
REVIEW OF CONCEPTS USEFUL FOR MAINTAINING QUALITY OF MALE REPRODUCTIVE FIELD SAMPLES FOR LABORATORY STUDY

Jill A. Jenkins

U.S. Geological Survey, National Wetlands Research Center, 700 Cajundome Boulevard, Lafayette, LA 70506, USA

E-mail: jenkinsj@usgs.gov

Introduction

Investigations into cellular and molecular characteristics of male gametes obtained from fish in natural ecosystems require careful sample handling and shipping in order to minimize artifacts. Maintaining sample integrity engenders confident assessments of ecosystem health, whereby animal condition is often reflected by gamete biomarkers - indicators that respond in measurable ways to changes. A number of our investigations have addressed the hypothesis that biomarkers from fish along a pollution gradient are reflective of site location. Species biology and the selected biological endpoints direct choice of parameters such as: temperature, buffer osmolality, time in transit, fixation, cryoprotectants, protease inhibition, and antibiotic inclusion in extender. This paper will highlight case studies, and outline parameters and thoughts on approaches for use by field and laboratory researchers.

Materials and methods

Testes and milt were collected by field crews from largescale suckers (Catostomus macrocheilus), studied as a surrogate for federally listed salmonids in the Columbia River Basin. Milt was also collected from endangered pallid sturgeon (Scaphirhynchus albus) from the Missouri River and razorback suckers (Xyrauchen texanus) from Lake Mead National Recreation Area. Sample size for all sites was 13 or more. Biomarker data collected from sperm from either shipped testes or milt included DNA integrity by nuclei outside the main population (NOMP) (Fig. 1), apoptosis, computer-assisted sperm motion analysis, cell morphology, viability, mitochondrial function, ATP concentration, and cell counts.

Results

Prevalent bacterial contamination of largescale sucker (LSS) samples from collection sites necessitated antibiotic inclusion in shipping buffer with testes in order to optimize shipping conditions. Results using 7 extender treatments with penicillin and/or streptomycin at two concentrations with three LSS individuals and a koi carp (Cyprinus carpio) model due to a lack of field-available LLS showed that HBSS at 320 mosm/kg containing 100 μg/mL of streptomycin was a suitable shipping buffer (Fig. 2). For each treatment, mitochondrial function was higher on day two than day 6 ($P = 0.0251$), and NOMP was lower ($P < 0.0001$). Macrocephalus, the primary LSS sperm cell abnormality, was derived from measures of head widths of 2.5 μm (SE 0.05), head lengths of 5.0 μm (0.07), and tail length of 47.8 μm (1.2).

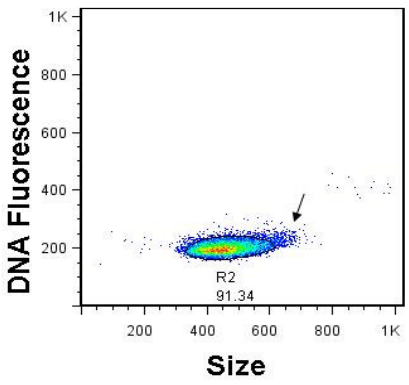


Fig. 1.) Largescale sucker sperm nuclei outside the main population (NOMP) [gated] indicate fragmented DNA [arrow]. Use of cells fixed in buffered formalin is a convenience in this assay, which is comparable to the sperm chromatin structure assay. Exposure to heat and acid present strand breaks accessibly for staining. Flow cytometric data are presented in a density plot showing 10K nuclei

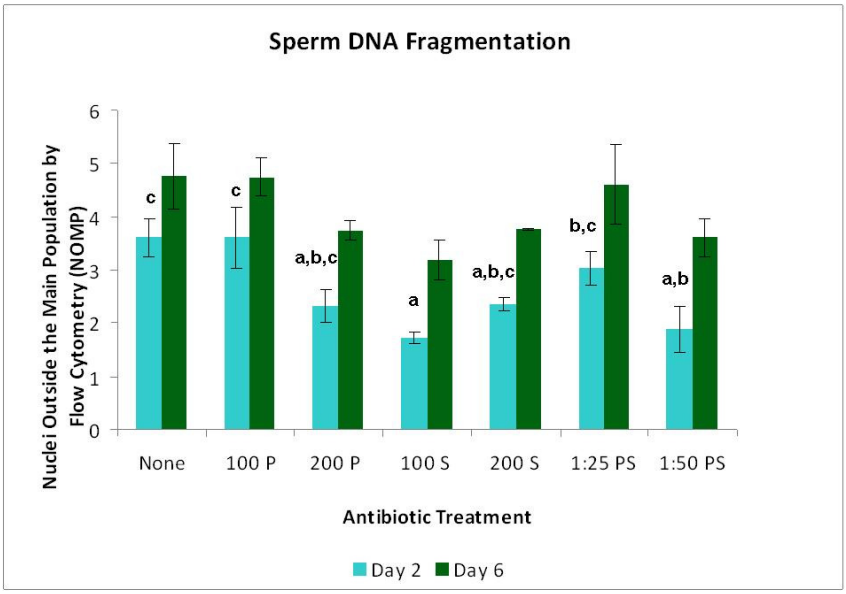


Fig. 2.) Flow cytometric analysis of DNA integrity (NOMP) from koi sperm stored for 2 and 6 days in extender treatments including penicillin (effective against Gram positive bacteria) or streptomycin (effective against both Gram positives and negatives). Statistics on day 2 data were performed; different letters indicate significant differences by Tukey's (P = 0.0014). [S = streptomycin; P = penicillin]

Results (i.e., motility, mitochondrial function, and NOMP) from the antibiotic preliminary study done prior to shipping LSS testes from the 3 field sites along the Columbia River pointed to the inclusion of 100 μg/mL streptomycin in the extender.

Pallid sturgeon milt shipped in modified Tsvetkova solution showed minimal vibrational motility, but when shipped from the field in modified HBSS, none was observed. Therefore, HBSS was used in the field for milt collection. Razorback sucker milt collections employed extender that was hyperosmotic (500 mOsm/kg) to remedy any lowering of osmolality because of inadvertent urine inclusion which artificially activates sperm (Jenkins et al. 2011).

Discussion and conclusions

Resource management decisions with ecological relevance depend on reliable data. Taking sample integrity into account has allowed for consistent, accurate assessments of fish reproductive condition among sites. Minimizing the occurrence of unwanted, degradative microorganisms is beneficial (Jenkins and Tiersch 1997; Jenkins 2011). Reducing potassium ion concentrations in investigations of sperm physiological variables has enhanced the science of milt handling for pallid sturgeon (Wayman 2003), and continued progress with other quality control studies will advance recovery efforts for listed species. Relationships among biomarkers such as cell count and spermatogenic stage within testis are relevant information for life history studies, as well. Gamete cell characters necessitate accommodating methods to obtain best shipping and handling of reproductive samples, crucial for obtaining precise biomarker data.

Acknowledgements

Funding for this project was provided by the Southern Nevada Public Land Management Act, administered by the US Bureau of Land Management through sponsorship of the National Park Service; the USGS Columbia River Contaminants and Habitat Characterization Project; the USFWS contract number 843207H459; the USGS CERC Comprehensive Sturgeon Research Project.

References

Jenkins, J.A.; Eilts, B.E.; Guitreau, A.M.; Figiel, C.R.; Draugelis-Dale, R.O.; Tiersch, T.R., 2011: Sperm quality assessments for endangered razorback suckers Xyrauchen texanus. Reproduction 141, 55-65. http://dx.doi.org/10.1530/REP-10-0153

Jenkins, J.A., Tiersch, T.R., 1997: A preliminary bacteriological study of refrigerated channel catfish sperm. J. World Aquacult. Soc. 28, 282-288.

Jenkins, J. A. 2011. Minimizing Microbial Contamination of Sperm Samples, Chapter 53. In: Cryopreservation in Aquatic Species, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 684-687.

Wayman, W.R., 2003: From Gamete Collection to Database Development: Development of a Model Cryopreserved Germplasm Repository for Aquatic Species with Emphasis on Sturgeon. PhD. dissertation. Louisiana State University, Baton Rouge, LA.

O-2/14
INFORMATION GAINED FROM HIGH SPEED VIDEO IMAGES OF SWIMMING FISH SPERMATOZOA

Volodymyr Bondarenko[1], Jacky Cosson[1,2]

[1] *Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, 389 25 Vodnany, Czech Republic*

[2] *UMR 7009 CNRS, Marine Station, University P. M. Curie, BP 28, 06230 Villefranche sur mer, France*

E-mail: vbondarenko@frov.jcu.cz

Introduction

Fish spermatozoa swim for very short periods right after being shed in the surrounding medium where fertilization occurs in most fish species, but during this brief period, their motility characteristics change in many respects. In the present study, sturgeon spermatozoa are taken as a model example. Methods for analyzing sperm motility currently used mainly allow getting information such as number of motile sperm head and their velocity. Much more information can be gained from analysis of flagella swimming parameters (Cosson 2008).

Materials and methods

Successive positions of sperm heads were measured from video frames using a video-recorder (SONY SVHS, SVO-9500 MDP, Japan) and computed from five successive frames by a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Sperm motility parameters included velocity (mean value of motile sperm cells) and percentage of motile sperm. For regular sperm observations, an Olympus BX50 microscope, with dark-field optics (objective 20×) illuminated by pulsed light from a stroboscopic lamp (Chadwick-Helmut, 9630, USA) was combined with video recording by 3 CCD video camera (SONY DXC-970MD, Japan). Such video records provide 50 frames/sec. with 720x540 pixels spatial resolution per frame. Detailed images of moving spermatozoa were obtained using 100x Phase contrast optics (Zeiss Ph 3 NeoFluar 100x, Oil) and recorded with a high-speed video camera (Olympus i-speed TR) providing 848x688 pixels spatial resolution, 1000 frames /sec.

Sperm samples were collected during natural spawning period from 10 mature and hormonally injected sterlet (Acipenser ruthenus) males by catheterisation of urogenital tract. Sperm was diluted 1:1000 in swimming solution containing 10 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris-Cl pH 8.5 (Dzyuba et al. 2010).

Results

Current records of fish sperm movement using a regular video camera show some limitations mostly because their flagellar Beat Frequency is high (BF up to 70 Hz) as well as their velocity (up to 200 μm/sec). An image example obtained by use of a regular video camera and stroboscopic illumination is shown in fig 1: individual flagella can be observed but with an overlay between images partly due to high BF.

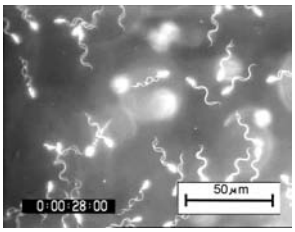


Fig. 1: Example of video image obtained by a regular video camera

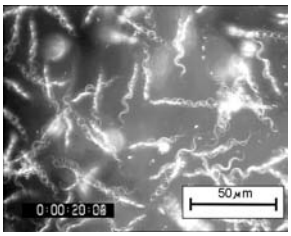


Fig.2: CASA analysis uses group of five images to obtain velocity of sperm)

Analysis of such video records (fig.2) mostly leads to information regarding percentage of motile cells, velocity or linearity of sperm head tracks by use of CASA (Computer Aided Sperm Analysis) (Kime et al.

2001; Hobson 1996), but does not allow to access to information regarding detailed movement of individual flagellum.

Therefore, for better analysis of fish sperm flagella having high-speed mobility and short motility period, we used high-speed video recording. (fig. 3).

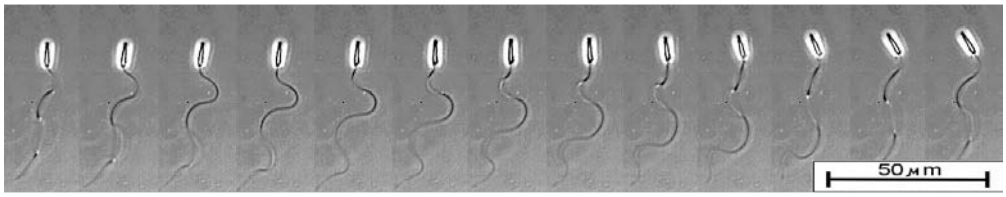


Fig. 3: Example of high resolution video-images (1000 frames per second, time interval between two images = 2 msec., time duration of one full beat cycle = 26 msec., corresponding to 13 above images)

Analysis of such images provides qualitative information about orientation of head, rotation of whole cell in 3D or defects occurring to flagellar membrane. In addition, quantitative data can be extracted from such series of images. Fig. 4 shows some of these parameters: wave velocity, wave amplitude, wave-length, wave dampening or curvature vs distance along the flagellum. Moreover, successive images in a series can cover a full beat cycle or more (fig. 3).

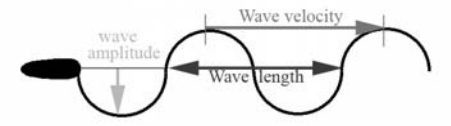


fig 4. Some of the flagellar parameters obtained by using high-speed video recording

In case of fish sperm, there are indications that such parameters greatly evolve during motility period or according to swimming solution composition (Cosson 2008) but also depending on "history" of the sperm cells such as damage generated at collection or during freeze-thawing process.

Discussion and conclusions

Use of high-speed video allows to record individual positions of flagellum during one full beat cycle. Such data will lead to more accurate modeling of flagellum behavior during fish sperm motility period and provide a deeper understanding of basis of sperm motility. In addition, these data also allow to develop new approaches for simulating fish sperm flagellum movement by computerization methods (Dzyuba et al. 2010).

Acknowledgements

We are grateful to Dr O. Linhart for giving us hospitality in his institute and thus, full access to facilities, including sperm from a large variety of fish species. We are thankful to CNRS as well as Czech grant agencies for the funding of projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

REFERENCES

Dzyuba B.; Boryshpolets S.; Rodina M.; Gela D.; Linhart O., 2010: Spontaneous activation of spermatozoa motility by routine freeze-thawing in different fish species. J. Appl. Ichthyol. 26, 720–725

Cosson J., 2008: Methods to analyse the movements of fish spermatozoa and their flagella In Fish Spermatology (Alavi, S. M. H., Cosson, J. J., Coward, K. & Rafiee, G., eds.), pp. 63 – 101. Oxford: Alpha Science.

Kime D. E.; Van Look K. J. W.; McAllister B. G.; Huyskens G.; Rurangwa E.; Ollevier F., 2001: Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp. Biochem. and Physiol., C Toxicol. and Pharmacol. 130, 425–433.

Manual on sturgeon reproduction. Coppens International bv, 5700 AM, 21-23.

O-2/15
EFFECT OF DIETS ON GAMETE QUALITY IN COD: THE COD BROODSTOCK NUTRITION PROJECT

D. Hamoutene[1], L. Lush[1], K. Burt[1], M. Webb[1], J. Perez-Casanova[1], N. Camarillo-Sepulveda[1], A. Walsh[2] and J. Moir[3]

[1] *Fisheries and Oceans Canada, Northwest Atlantic Fisheries Center, 80 East White Hills Road St John's NL, Canada A1C 5X1*
[2] *Sapphire Sea Farms P.O. Box 234, Bay Bulls, NL, Canada A0A 1C0*
[3] *Newfoundland Cod Broodstock Company, PO Box 296, St John's, NL, A1C 5J2*

E-mail: dounia.hamoutene@dfo-mpo.gc.ca

Introduction
Broodstock nutrition affects the viability and health of offspring and that of the broodstock fish (Pavlov et al. 2004). A study aimed at determining the influence of diet on spawning and growth of first generation (F1) cod broodstock was initiated in 2008 by testing three diets on farmed broodstock hatched in 2006. One group was fed a commercial on-growing pellet, the second group a commercial pellet formulated for marine finfish broodstock (not currently available in Canada), and the third a diet of baitfish supplemented with vitamins. Results show that fish fed baitfish have better growth and condition factor than fish fed pelleted diets. Moreover, the baitfish diet provided the best results in terms of reproductive output (fertilization/hatch rates) over pelleted on-growing and broodstock feeds (Hamoutene et al., submitted). To further explore differences between groups receiving different diets, some biochemical parameters of sperm quality as well as egg lipid composition were evaluated.

Material and methods
Three diets were tested in duplicate tanks with 20 two year old F1 broodstock (10 males and 10 females): 1) on-growing diet (OG) 2) commercial broodstock diet (Vitalis CAL, Skretting España S.A.) (BR) 3) baitfish diet (herring, mackerel and squid) enriched with vitamin premix (BA). Fish were regularly monitored for growth and Fulton's condition factor (CF) calculated. Manual stripping of gametes occurred twice weekly during two spawning periods.

Sperm quality was monitored by evaluating motility microscopically and establishing a ranking of samples according to the following scale: no motility detected (0); little motility and slow movement (1); over half motile with non-vigorous movement (1.5); vast majority motile and swimming actively (2); all cells are vigorously motile (2.5). The following sperm parameters were also investigated, specifically: ATP, respiration, protein, glycolytic metabolism enzymes (lactate dehydrogenase and citrate Synthase), lipid metabolism enzyme (lipase) and an anti-oxidant enzyme (catalase) as well as sperm density.

Eggs were 'dry fertilized' and fertilization rates and hatch rates were calculated. Egg lipid extractions were performed using a modified Folch method by Parrish (1999). Lipid analysis was performed using a modified Parrish (1983) method via thin-layer chromatography with flame ionization detection. Lipids were separated into classes using a four solvent development system and scanned after each development using an Iatroscan. The resulting chromatograms were compared with a 9-component standard for class identification ([Hydrocarbons (HC), Steryl Esters (SE), Ketones (KET), Triacylglycerols (TAG), Free Fatty Acids (FFA), Alcohols (ALC), Sterols (ST), Acetone Mobile Polar Lipids (AMPL) and Phospholipids (PL).

Results
No initial differences in CF were detected (not shown in table). After 20 months of using separate diets, CF revealed significant differences, with baitfish and broodstock diets having the highest CF (Table 1). Differences were shown to be mostly due to the effect of diets on males. The baitfish diet also produced the highest fertilization and hatch rates.

Table 1- Condition factor (CF) as well as fertilization and hatch rates in cod broodstock in the three diet groups

(different letters denote statistical differences)

	CF- All	CF- Males	CF- Females	Fertilization rates (%)	Hatch rates (%)
On-growing	0.99 ± 0.03[a]	0.95 ± 0.07[a]	1.03 ± 0.08[a]	14.72 ± 3.03[a]	10.37 ± 3.56[ab]
Broodstock	1.14 ± 0.05[ab]	1.14 ± 0.09[ab]	1.11 ± 0.00[a]	24.40 ± 4.08[ab]	9.09 ± 2.10[a]
Baitfish	1.28 ± 0.07[a]	1.33 ± 0.08[b]	1.24 ± 0.09[a]	36.22 ± 5.84[b]	22.87 ± 5.20[b]

The ranking of sperm according to their motility show significant differences between males fed the 3 diets with those fed the broodstock and baitfish diets having higher scores. Broodstock fed males had sperm with higher ATP (first season p=0.029) levels than other males. Significant differences in sperm enzymes of baitfish fed males are found with higher lipase activity (second season p=0.027), and protein amounts (first season p= 0.059). No significant differences were found in the lipid classes in the eggs from the three diets, some trends can be observed. The highest lipid class for all diets was PL. Eggs from the broodstock diets exhibited the highest TAG while PL was found higher in the eggs of fish from the baitfish diet.

Discussion and conclusions

This study demonstrates that male Atlantic cod fed baitfish exhibit significantly enhanced reproductive performance as compared to those fed pelleted on-growing diets. The increased sperm quality measurements of baitfish fed males may be due to differences observed in CF. Similar to our observations, Yoneda and Wright (2005) found that reproductive investment was related to body condition in male cod. Reduced sperm motility in on-growing fed males lends evidence to diet's role in sperm quality. However, Trippel and Neilson (1992) observed that sperm motility alone lacks predictive ability for overall sperm quality. Enzymatic results reveal significant differences in lipase and protein concentration in baitfish fed males over on-growing fed males, and in ATP levels of broodstock diet fed males. Energy levels (ATP) and enzymes involved in metabolism can provide markers of sperm general status; however, literature data show conflicting results in correlations with fertilization, as most studies link metabolism with motility rather than fertilizing capacity (e.g. Perchec et al. 1995). Fertilization rates were significantly higher in the baitfish group, providing further evidence that superior motility and higher enzymatic activity increase the sperm's fertilization ability. However, lack of differences in hatch rates indicate a limitation of sperm quality influence. No significant differences in egg lipid analyses were found between the three groups of fish. Further analysis of fatty acid contained in eggs is required to determine dietary effects on egg quality.

References

Pavlov, D., E. Kjorsvik, T. Refsti, and O. Andersen. 2004. Broodstock and egg production. Pages 129-204 in Moksness, E., E. Kjorsvik, and Y. Olsen. Culture of cold-water marine fish. Blackwell Publishing, Oxford.

Parrish, CC; Wells, JS; Yang, Z; Dabinett, P. 1999. Growth and lipid composition of scallop juveniles, Placopecten magellanicus, fed the flagellate Isochrysis galbana with varying lipid composition and the diatom Chaetoceros muelleri. Marine Biology 133(3):461-471.

Perchec, G., C. Jeulin, J. Cosson, F. André, and R. Billard. 1995. Relationship between sperm ATP content and motility of carp spermatozoa. Journal of Cell Science 108: 747-753.

Trippel, E.A., and J.D. Neilson. 1992. Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (Gadus morhua) and associated hatching success. Canadian Journal of Fisheries and Aquatic Sciences 49:2118-2127.

Yoneda, M., and P.J Wright. 2005. Effect of temperature and food availability on reproductive investment of first-time spawning male Atlantic cod, Gadus morhua. ICES Journal of Marine Sciences 62:1387-1393.

O-2/16
MOTILITY PARAMETERS AND REPRODUCTIVE SUCCESS OF CRYOPRESERVED SPERM OF SIBERIAN STURGEON (*ACIPENSER BAERII* BRANDT) AFTER SHORT-TIME EXPOSURE OF FRESH MILT TO MERCURY AND CADMIUM

G.J. Dietrich[1], A. Ciereszko[1], R.K. Kowalski[1], A. Rzemieniecki[2], E. Bogdan[4], W. Demianowicz[1], M. Dietrich[1], R. Kujawa[3], J. Glogowski[1]

[1] *Department of Gamet and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-747 Olsztyn, Poland*
[2] *Department of General Zoology, University of Szczecin*
[3] *Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn*
[4] *Fish Breeding Farm "Ryba", Oleśnica, 64-800 Chodzież*

E-mail: g.dietrich@pan.olsztyn.pl

Introduction
Toxicity of heavy metals to male reproduction may affect sperm motility, trajectory and fertilization capability. Computer-assisted sperm analysis (CASA) allows to distinguish sublethal effects of heavy metals on spermatozoa including changes in sperm velocity and trajectory of sperm movement (Dietrich et al 2010; Dietrich et al. 2011). Moreover, it has been proven that CASA parameters are well correlated with fertilization (Gage et al., 2004). The aims of the present study were to investigate how cryopreserved sperm quality, including motility parameters, viability and fertilizing capacity, were affected by 4h in vitro exposure of Siberian sturgeon fresh sperm to mercury and cadmium ions (0-100 mg/l) diluted in seminal plasma.

Materials and methods
The mercuric chloride or cadmium chloride was diluted in sturgeon seminal plasma. Milt samples (n=4) were then diluted 2:1 either with seminal plasma containing final Hg^{2+} and Cd^{2+} concentrations in a milt suspension of 1, 10, 100 mg/l or with the control (no heavy metals added) and stored for 4h at 4ºC. Since eggs were not available at the time of experiment, we decided to cryopreserve samples in order to perform fertilization experiment in the later time. Consequently, all analyses (motility and viability) were also performed on cryopreserved sperm. Samples were cryopreserved using freezing solution containing Tris-sucrose-KCl medium with 10% methanol. Just before fertilization, straws were thawed and adequate volume of diluted milt (200,000 spermatozoa/egg) was added to 100 eggs. Fertilization rates were calculated at hatching. The motility of thawed spermatozoa was measured and analyzed with Computer Assisted Sperm Analysis (CASA) using the Hobson Sperm Cell Tracker. CASA parameters included percentage of motile sperm (MOT) and straight-line velocity (VSL). Sperm viability was assessed by supravital staining by applying a combination of the fluorophores SYBR-14 and propidium iodide. Data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's test for post hoc comparisons of means. Fertilization rates were compared using nonparametric Friedman's test, followed by Dunn's test. The level of significance was set at 0.05.

Results
The mercury and cadmium ions significantly affected MOT in dose-dependent manner (Fig. 1). Only the highest concentrations of heavy metals (100 mg/l) caused significant decrease of VSL. There was a decrease in sperm viability for 10 and 100 mg /l of 10 Hg^{2+} or Cd^{2+}. Mercury ions at 10 mg Hg^{2+}/l caused a ten-fold decrease of hatching rate compared to control and none of the embryos survived to the hatching stage after exposure to 100 mg Hg^{2+}/l concentration. On the other hand cadmium ions at tested concentrations did not significantly affect hatching rates.

Discussion and conclusions
The influence of mercury and cadmium ions on sperm motility was similar for both heavy metals. However, there were heavy metal-specific differences in sperm fertilization ability which suggests that mercury ions but not cadmium appeared to be more detrimental for sturgeon sperm. These results suggest that other

sturgeon sperm structures important for fertilizing ability such as an acrosome could be affected by mercury ions (Psenicka at al. 2010).

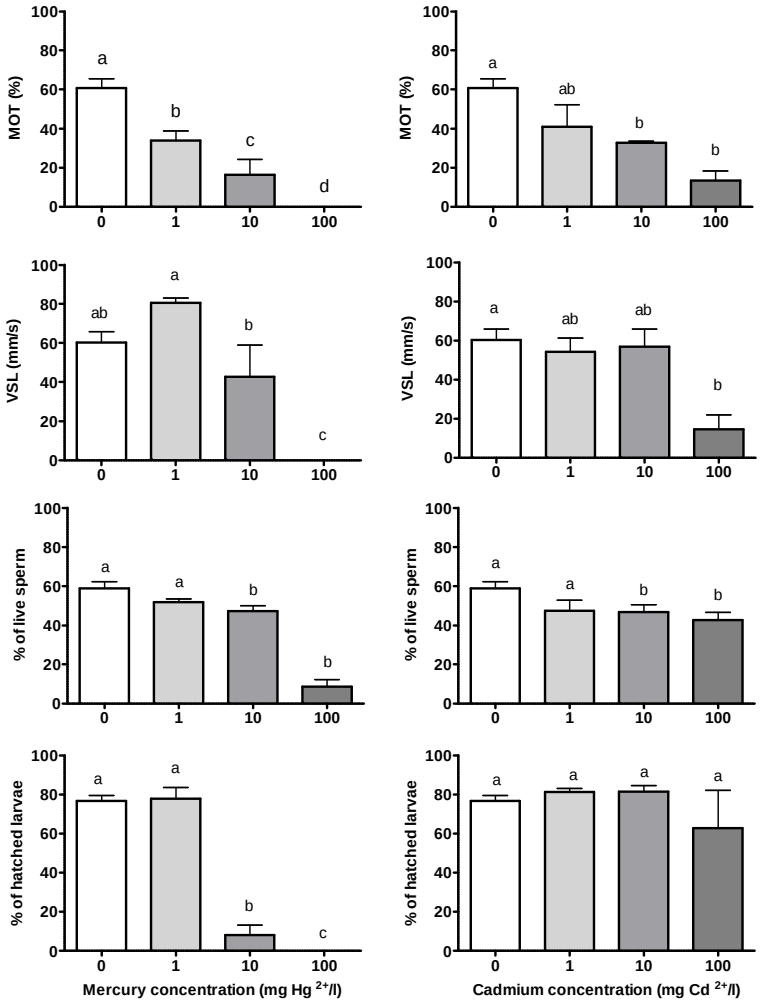


Fig. 1. The effects of 4h exposure of Siberian sturgeon milt to mercury and cadmium ions on cryopreserved sperm motility parameters, sperm viability and hatching success (n=4). Different letters indicate statistical significance at $P < 0.05$

Acknowledgment

This study was supported by the State Committee for Scientific Research (KBN) as Solicited Project PBZ-KBN-084/P06/ 2002/5.8.

References

Dietrich, GJ.; Dietrich, M.; Kowalski, R.K.; Dobosz, S.; Karol, H.; Demianowicz, W.; Glogowski, J., 2010. Exposure of rainbow trout milt to mercury and cadmium alters sperm motility parameters and reproductive success. Aquat Toxicol; 97: 277-284.

Dietrich, M.A.; Dietrich, G.J.; Hliwa, P.; Ciereszko, A., 2011. Carp transferrin can protect spermatozoa against toxic effects of cadmium ions. Comp. Biochem. Physiol. C 153, 422–429.

Gage, M.J.G.; Macfarlane, C.P.; Yeates, S.; Ward, R.G.; Searle, J.B.; Parker, G.A., 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14, 44–47.

Psenicka, M., G.J. Dietrich, M. Wojtczak, J. Nynca, M. Rodina, O. Linhart, J. Cosson, A. Ciereszko., 2010. Acrosome staining and motility characteristics of sterlet spermatozoa after cryopreservation with use of methanol and DMSO. Cryobiology 56, 251-253.

Abstracts of poster presentations

P-101
SOME PROPERTIES OF MALIC ENZYME FROM HERRING (*CLUPEAHARENGUS*) SPERMATOZOA

N. Niedźwiecka and E.F. Skorkowski

Gdańsk University Biological Station, Laboratory of Comparative Biochemistry and Ecotoxicology, 80-680 Gdańsk-Sobieszewo, Poland

E-mail: skorkows@biotech.ug.gda.pl

Summary
Herring (*Clupeaharengus*) spermatozoa exhibit a higher activity of malic enzyme (ME) than salmon, trout, carp and catfish spermatozoa. This enzyme in herring spermatozoa is involve in the generation of NADPH in the reversible decarboxylation of malate to form pyruvate in the presence of NADP and requires some divalent cations to express activity. The spermatozoa from herring show the presence of two molecular forms of malic enzyme (ME), one specific for NADP alone as the coenzyme (NADP-dependent ME) and the other utilizing both coenzymes but preferring NAD (NAD(P)-dependent ME). Regulation of NAD(P)-ME from herring spermatozoa could respond to changing levels of ATP. The effect of concentration of some divalent cations such as Mn^{2+}, Mg^{2+} and Cd^{2+} on the reaction catalyzed by NAD(P)-ME from herring spermatozoa has been examined. The Cd^{2+} ion shows strong inhibition of the NAD(P)-ME activity *in vitro*. The high level of cadmium concentration in the water in the Gdansk Bay, region were herring have spawning season could influence viability and motility of spermatozoa.

Keywords: Fish, herring, Clupeaharengus, spermatozoa, malic enzyme, ATP, cadmium

P-102
ELABORATION OF A RELIABLE METHOD FOR QUANTITIVE DESCRIPTION OF FISH SPERMATOZOA VOLUME CHANGIES

Olga Bondarenko[1], Boris Dzyuba[1, 2], Gunes Yamaner[3] and Otomar Linhart[1]

[1] *Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic*
[2] *Institute for problem of Cryobiology and Cryomedecine of National Academy of Science of Ukraine, 23 Pereyaslavskaya Str., 61015 Kharkov, Ukraine*
[3] *Istanbul University, Faculty of Fisheries, Beyazit/Eminonu, 34452 Istanbul, Turkey*

E-mail: Bondao00@frov.jcu.cz

Introduction
It is well known that fish sperm undergo osmotic stress after release from testes at spawning due to the differences between seminal plasma and environment osmolalities. For some species this change of osmotic pressure represents a main signal for motility activation. We suggest that volume change, generated from osmotic pressure, would be involved in this signaling. In previous researches, several methods of measurement of spermatozoa volume changes were proposed, among which: spermatocrit (Perchec Poupard et al, 1997), spectrophotometry (Dzuba et. al, 2001) or video microscopy (Perchec Poupard et al, 1997). However, quantitive description of spermatozoa volume changes still requires additional studies. The goal of our study was the elaboration of the method which would be the most informative and sensitive to quantify changes of spermatozoa volume, such method should also allow us to work with any osmolalitiys and observe dynamics of spermatozoa volume changes for future understanding of its impact on sperm motility parameters.

Materials and methods
Males of Carp (*Cyprinus Carpio*) and Sterlet (*Acipenser ruthenus*) were intramuscularly injected with carp pituitary extract (1 and 4 $mgkg^{-1}$ respectively). In case of Rainbow trout (*Onocorhynchus* mykiss), sperm was collected during the spawning season. Sperm from carp and rainbow trout was collected directly into plastic syringes by abdominal massage, while sterlet sperm was collected by catheterisation of urogenital tract.

Three methods of cell volume changes described previously were used in the study.

1. Nephelometry method. The measurements of cell absorbance were accomplished by means of a recording spectrophotometer SPECORD 210 (Analytic Jena AG, Germany) equipped with a thermostat-controlled cell chamber in a 1 cm cuvette at 500 nm, sperm dilution rate into the studied media was 1:1000. The value of relative change in absorbance (A1) was considered as a parameter, which could describe the volume changes (Dzyuba et al, 2001) according to the relation: $A1 = (A_0-A_1)/A_0$, where A_0 is the value of absorbance just after adding sperm to solution and A_1 is the value of absorbance in time period used to reach plateau (60 s approximately).

2. Spermatocrit method. Sperm of carp and rainbow trout was diluted 1:1 with studied medium. For sturgeon, sperm was centrifuged for 30 min at 300 g to increase sperm density. Resultant pellet was diluted 1:1 by supernatant. Obtained suspension was diluted 1:3 with studied media. The diluted semen was introduced into a hematocrite tube and than centrifuged for 10 min at 1500 g (Perchec Poupard et al, 1997). Relative sperm volume evaluated by spermatocrit method was calculated by formula: $V/V_{0(SpC)}=SpC_m/SpC_{300}$,

where SpC_m is spermatocrit in studied media, SpC_{300} – spermatocrit in 300mOsm solution.
3. Light microscopy. Sperm was added to the drop of studied medium on microscope slide using the tip of dissecting needle whereafter the sperm suspension was thoroughly mixed for 2 s. Directly after dilution, motility was recorded for 2 min post-activation using phase contrast microscope (Olympus BX50, 200) equipped with CCD video camera (Sony, SSCDC50AP). At 60 s of post-dilution, video frames were analyzed (by Olympus Micro-Image 4.0.1. for Windows) to estimate diameter of spermatozoa's heads (from

which volume was calculated) in all studied solutions. Relative sperm head volume (V/V0 (micro)) was calculated: $V/V_{0(micro)}=V_m/V_{300}$, where V_m is the volume of head in studied media and V_{300} is the volume of head in 300 mOsm solution.

For each method, media of different osmolality were used. NaCl media of 0, 50, 100, 150, 200, 250 and 300 mOsm were used for carp and rainbow trout while 0, 25, 50, 75, 100 and 300 mOsm were used for sterlet. All solutions contained 10 mM Tris-HCl, pH 8.0.

Values of $V/V_{0\ (micro)}$ and $V/V_{0(SpC)}$ in media of different osmolalities are presented in figure 1 as means with standard deviation bars. Figure 2 represent the Diagram of dispersions for A1 and $V/V_{0\ (micro)}$. Curves of linear regressions (solid lines) and 95% confidence interval (doted lines) for linear regression, linear regression equations with R^2 and p are presented in figures 1, 2. All analysis was performed using STATISTICA V9.1(Statsoft Inc.,USA).

Results

Regarding A1, $V/V_{0(SpC)}$ evaluated in each tested medium no differences were observed in rainbow trout and sterlet sperm. Carp sperm showed negative correlations of all three parameters with respect to osmolality of medium. Relative sperm volume measured by spermatocrit and relative sperm head volume measured by microscopy in media of different osmolality are presented in figure 1.

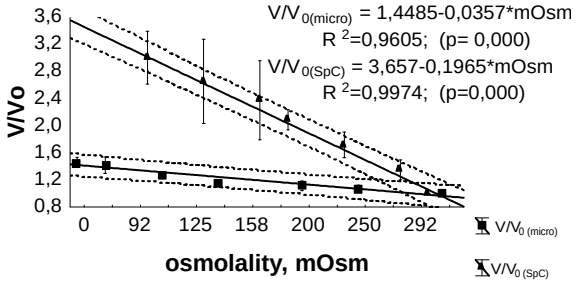


Fig.1 Relative volume of carp spermatozoa measured by Spermatocrit ($V/V_{0(SpC)}$) and relative sperm head volume measured by microscopy method ($V/V_{0\ (micro)}$) in media of different osmolalities.

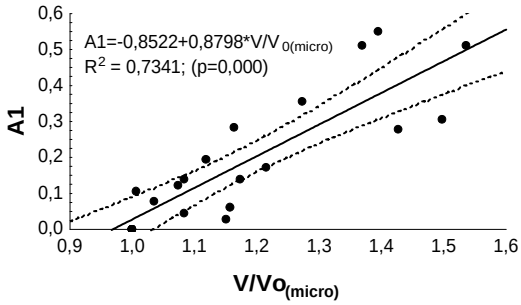


Fig.2 Diagram of dispersions for relative change in absorbance (A1) and relative sperm head volume measured by microscopy method ($V/V_{0\ (micro)}$).

Values of $V/V_{0(SpC)}$ were increased to larger values with lowering osmolality in comparison with $V/V_{0\ (micro)}$ and significantly higher coefficients of linear regressions for these values were determined (see 95% confidence interval area for regression line). Diagram of dispersions for relative sperm head volume ($V/V_{0\ (micro)}$) and relative change in absorbance (A1) values is presented in figure 2, showing a significantly high correlation ($R^2=0.734$, $p<0.001$) between these two parameters.

Discussion and conclusions

Sperm ability for swelling is species specific and understanding of role of this parameter in sperm physiology is required. The spermatocrit method of relative sperm volume measurement allows to estimate this parameter in a narrow range of osmolality because of low dilution rate of sperm with studied medium is required. The reasons why such differences in relative volumes estimated by spermatocrit and microscopy methods appear require future studies. Quite strong correlation between A1 and $V/V_{0\ (micro)}$ is promising for quantitive description of volume changes by nephelometry method. We consider this method as best appropriate for future studies of the kinetics of sperm volume changes in different experimental conditions.

Aknowledgements

Special thanks are expressed to the projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

References

Dzuba B B, Bozhok G A & Rudenko S V, 2001: A study of the dynamics of volume changes during the period of active motility in carp, Cyprinus Carpio L., spermatozoa. Aquaculture Research, pp. 51-56.

Perchec Poupard G., Gatti J. L., Cosson J., Jeulin C., Frierville F. and Billard R.,1997 : Effects of extracellular environment on the osmotic signal transduction involved in activation of motility of carp spermatozoa. Journal of Reproduction and Fertility, pp. 315 – 327.

P-103
APPLICATION OF SWIM-UP AND GRADIENT CENTRIFUGATION METHODS IN THE SEMEN OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) DISINFECTION

Aygül Ekici[1], Didem Ercan[2]

[1] *Istanbul University, Faculty of Fisheries, Aquaculture Department, 34470 Laleli/Istanbul Turkey*
[2] *Muğla University, Faculty of Fisheries, Aquaculture Department, 48000 Kötekli/Muğla Turkey*

E-mail: dican@mu.edu.tr

Introduction
In last years, a variety of assisted reproductive technologies have been developed for preservation of livestock genetics. For this purpose, main objective is preventation of disease transmission in farm animals (Bielanski, 2007). Some pathogenic organisms have been identified in association with semen (Russell et al, 1997; Pasquier et al, 2000). In humans, washing by the gradient centrifugation and swim-up methods appears to be effective for reducing the microbial population in semen. In this study disinfection procedures were used to decrease bacterial contamination of fish semen.

Materials and methods
The semen was collected by abdominal massage from 3-4 year old male rainbow trout into pre-cooled test tubes. Experiments were realized at 2-4°C. Morphologically good-looking fish were used as a control group. Apparently at the experiment diseased fish have shown lesions on the skin.

At the study two different washing methods, swim-up and gradient centrifugation, were used. Before and after washing of semen samples they were inoculated onto Tryptic Soy Agar (TSA), Modified Anacker and Ordal Agar (MAOA) and Brain Heart Infusion (BHI) Agar.

Results
After washing of the semen by swim-up; some bacteria colonies were grown on those mediums. But after the semen washing by gradient centrifugation method, no bacteria were grown on TSA, MAOA and BHI.

In this study, found that the gradient centrifugation method was more reduced bacterial contamination than swim-up method.

Discussion and conclusions
Some sperm washing procedures may not completely remove the infectious agent, but may reduce the microbial contamination (Bielanski, 2007). Washing of human and animal spermatozoa has become the most important procedure for the control and elimination of microorganisms. A variety of methods have been used to separate the motile spermatozoa from the seminal plasma (Drobnis et al., 1991) and to prepare pure sperm. In this study two washing procedures were used for reduce microbial contamination in rainbow trout spermatozoa. To the our knowledge this is the first report semen washing on fish.

Our results showed that the gradient centrifugation method is reduced bacterial contamination as in mammalian and no found motile spermatozoa for the fertilization after washing procedures; whereas in mammals, motile spermatozoa are collected by washing methods. For further research, It will developed method for reducing bacterial contamination and to get motile spermatozoa after sperm washing process.

Acknowledgements
This work was supported by the Istanbul University Research Fund. Project Number: 2732.

References

Bielanski, A., 2007: Disinfection prosedures for controlling microorganisms in the semen and embryos of humans and farm animals. Theriogenolgoy. 68, 1-22.

Drobnis, E.Z.; Zhong, C.Q.; Overstreet. J.W., 1991: Separation of cryopreserved human semen using Sephadex columns, washing or Percoll gradients. Journal of Andrology 12:201-208.

Pasquier, C., Daudin, M.; Righi, L.; Berges, L.; Thauvin, L.; Berrebi, A.; Massip, P.; Pue,l J.; Bujan, L.; Izopet, J., 2000: Sperm washing and virus nucleic acid detection to reduce HIV and hepatitis C virus transmission in serodiscordant couples wishing to have children. AIDS. 29;14(14):2093-2099.

Russell, P.H;, Lyaruu, V.H.; Millar, J.D., 1997: The potential transmission of infectious agents by semen packaging during storage for artificial insemination. Animal Reprod. Sci., 47, 337–342.

P-104
ULTRASTRUCTURAL ANÁLYSES OF SPERMATOGENESIS IN THE NEOTROPICAL CICHLID *CICHLA KELBERI* (PERCIFORMES: CICHLIDAE)

Diógenes Henrique de Siqueira Silva[1], Raphael da Silva Costa[1], Alexandre Ninhaus-Silveira[2], Carlos Alberto Vicentini[3], Rosicleire Veríssimo-Silveira[2]

[1] *Neotropical Ictiology Laboratory (L.I.NEO) - PPG Animal Biology –IBILCE/UNESP, S.J. Rio Preto, SP, Brazil*
[2] *Neotropical Ictiology Laboratory (L.I.NEO) – Dep. Biology and Zootecnia, FE/UNESP, Ilha Solteira, SP, Brazil*
[3] *Laboratory of Morphology of Aquatic Organisms - FC/UNESP, Bauru, SP, Brazil*

E-mail: siqueira.diogenes@gmail.com

Introduction
Cichla kelberi, known as yellow peacock bass is a Neotropical cichlid much appreciate in the culinary and, principally, as sporting fish, being considered the fishery national symbol. Considering that the ultrastructural analyses of spermatogenesis, principally of the spermatozoon, can show phylogenetic traces, the ultrastructural description of the males' germinative cells of *C. kelberi* will make available many characters that can be useful in future phylogenetic analyses of the Cichlidae family.

Materials and methods
Eleven mature males of *C. kelberi* were collected from the Jupiá Reservoir, Paraná river, Ilha Solteira, SP, Brazil, from mar/2009 to feb/2010.Testes fragments were fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1M Sorensen phosphate buffer, pH 7.4. The material was dehydrated in alcohol, embedded in historesin (Technovit 7100). The material was post-fixed for two hours in the dark in 1% osmium tetroxide in the same buffer, contrasted in block with aqueous solution of 5% uranyl acetate for two hours, dehydrated in acetone, embedded in araldite, sectioned and stained with a saturated solution of uranyl acetate in 50% alcohol and lead citrate. Electron micrographs were obtained by using Philips – CM 100 transmission electron microscope.

Results
In *C. kelberi* the spermatogenesis occurs in cysts presents in the germinative compartment. Each cyst is formed by a group of germinative cells, in synchronic development, involved by Sertoli cells cytoplasmic extensions. The primary spermatogonia are isolated; the nucleus is large, spherical and central with uncompressed chromatin and uniformly distributed and eccentric nucleolus (Fig1.A). In the cytoplasm is observed electron-dense material, associated or not to agglomerates of spherical mitochondria (Fig1.B). The secondary spermatogonia originated from mitotic divisions of primary spermatogonia are connected by cytoplasmic bridges; has nucleus circular and central and chromatin more compacted that the prior phase; in the cytoplasm there are few electron-dense material and mitochondria of variable forms (Fig1.C). The secondary spermatogonia differentiate in primary spermatocytes, characterized by the presence of synaptonemal complex, starting the meiotic phase (Fig1.D). In the end of Meiosis I are formed the secondary spermatocytes, fewer and rarely observed in the seminiferous tubules (Fig1.E). The spermiogenesis is characterized by the lateral flagellum development, complete nuclear rotation, fossa nuclear formation and cytoplasmic channel; the chromatin compaction progresses to filamentous bundles juxtaposed (Fig1.F-I) and end in the juxtaposed cluster form in the spermatozoon. The spermatozoon has circular head and absence of acrosome. The nuclear fossa is shallow in unique arc form and slightly eccentric by the nuclear base, where is house of part of the centriolar complex. The proximal centriole is anterior, lateral and oblique to the distal (Fig1.H). The midpiece is short and has circular mitochondria, separated from the flagellum by a cytoplasmic channel; shows one long and strait cytoplasmic projection, dilated in its distal portion, called cytoplasmic sheath. The long flagellum possess perpendicular axis and slightly eccentric to the nuclear base, contains two fins and the classic axonem (9+2) (Fig1.J).

Discussion and conclusions

The spermatozoa in Teleostei can be classified as Type I or II based on the perpendicular or parallel position of the flagellar axis to the nucleus that depends of the presence or absence of nuclear rotation during spermiogenesis, respectively (MATTEI, 1970; JAMIESON, 1991). *C. kelberi* presents the Type I, typical of the Cichlidae family (MATTEI, 1991). Besides of this classification the spermatozoon in *C. kelberi* is considered the basic type of the spermatozoa in Teleostei, known as anacrosomal aquasperm spermatozoa (JAMIESON, 1991). Other characteristics of the *C. kelberi*'s spermatozoon seems common to the genus, like the compaction of the chromatin in filamentous clusters and the slightly eccentric position of nuclear fossa, also found in *C. intermedia* (Quagio-Grassiotto *et al.,* 2003). To know if the spermatozoon in *Cichla* has similar characteristic and to help clarify its relation with other Cichlidae genus, spermatozoal ultrastructure of other *Cichla* have to be studied.

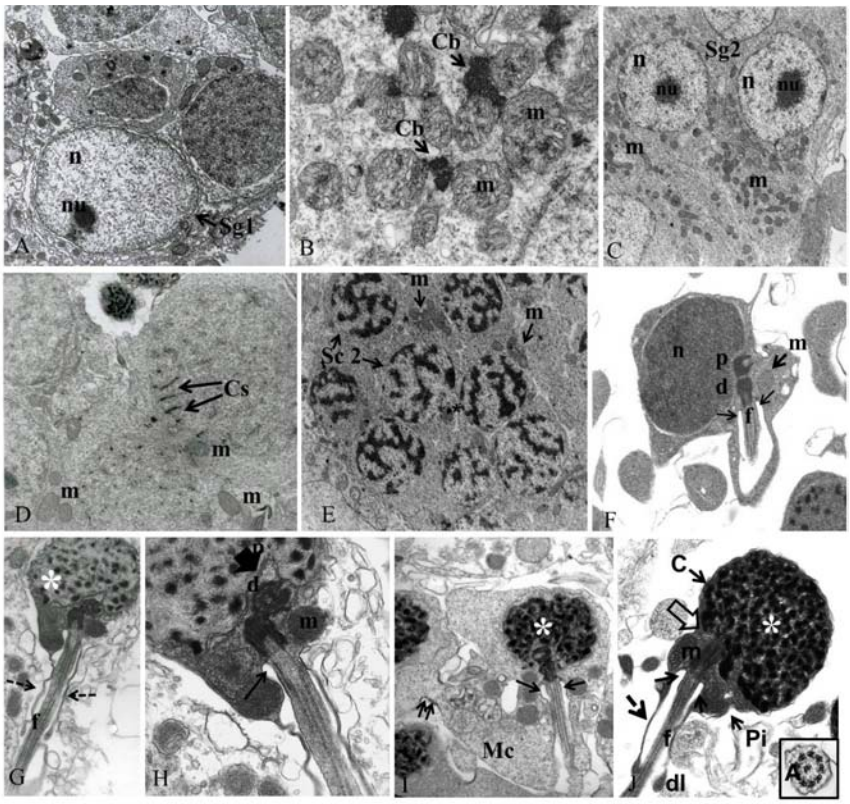


Fig 1. A, B – Primary spermatogonia (Sg1); C – Secondary spermatogonia (Sg2); D – Primary spermatocyte; E – Secondary spermatocyte (Sc2); F-I – Spermatid; J – Spermatozoon. A: axonem; c: Spermatozoon head; Cb: Chromatoid bodies; Cs: Synaptonemal complex; d: distal centriole; dl: cytoplasmyc sheat dilatation; f: flagellum; m: mitochondria; Mc: Cytoplasmic mass; n: nucleus; nu: nucleolus; Pi: Intermediate piece; ➧ *nuclear fossa;* ➪ *flagellum insert in nuclear base;* ⇉ *cytoplasmic bridge;* → *cytoplasmic channel;* ➔ *cytoplasmic sheath.*

References

Jamieson, B.G.M. 1991: Fish Evolution and Systematics: Evidence from Spermatozoa. Cambridge: Cambridge University Press.319p.

Mattei, X. 1970: Spermiogenèse comparée des poisson. In: BACCETTI, B. Comparative Spermatology. Academic Press, New York, 57-70.

Mattei, X. 1991: Spermatozoon ultrastructure and its systematic implications in fishes. Can. J. Zool. 69, 3038-55,

Quagio-Grassiotto, I.; Antoneli, F. N.; Oliveira, C. 2003: Spermiogenesis and sperm ultrastructure in Cichla intermedia with some considerations about Labroidei spermatozoa (Teleostei, Perciformes, Cichlidae). Tissue & Cell 35, 441–446.

P-105
SPERMATIC PARAMETERS OF *STEINDACHNERIDION PARAHYBAE* AMONG 10 AT 20 SECONDS POS-ACTIVATION USING OPEN SOURCE CASA

Eduardo Antônio Sanches[1], Renan Yoshiharu Okawara[2], Danilo Caneppele[3], Robie Allan Bombardelli[4], Elizabeth Romagosa[2]

[1] *Aquaculture Centre. State University of São Paulo, Jaboticabal, SP, Brazil*
[2] *Fishery Institute – APTA, São Paulo, SP, Brazil*
[3] *Hydrobiology and Aquaculture Station - CESP, Paraibuna, SP, Brazil*
[4] *State University of West Paraná, Toledo, PR, Brazil*

E-mail: eromagosa@pesca.sp.gov.br

Introduction
Fish sperm quality is often assessed by subjective methods, among which progressive sperm motility and sperm vigour are the ones most frequently observed. However, these measurements have raised doubts regarding their validation because the values obtained vary considerably, and basically depend on the experience of a single observer (Sanches et al., 2010). Lately, by means of specific software, fish sperm analyses have been conducted with computer programmes such as the *computer-assisted sperm analysis* (CASA) (Wilson-Leedy & Ingermann, 2007). However, for this method to be efficient is necessary basics knowledge about parameters utilized in the analysis such as, frame rate, number of frames, time of the video, and instant of evaluation. The present study was carried out with the aim of evaluate spermatic parameters of the method of CASA using open-source software among 10 the 20 seconds pos-activation in fish species threatened of extinction surubim-do-paraiba, *Steindachneridion parahybae*

Materials and methods
Thirteen *S. parahybae* males (639.6±104.2 g) which released semen under slight abdominal pressure were used. The semen collected (1.0 mL of each male) was used to capture video and further evaluation in software CASA. The semen was activated (distilled water) at a proportion of 1:50. Afterwards, 10 μL of the mixture were placed in a mirrored Neubauer chamber (100 μm deep), which was transferred to the light microscope for the capture of the videos (400x of the magnification). A Basler 602fc camera attached to a trinocular Nikon microscope was used. The videos were captured by the software AMCAP (Basler Vision Technologies) at a rate of 100 fps (656x490 pixels). The videos were captured in format *.avi, edited in the software VIRTUALDUB-1.9.0 (virtualdub.org), and exported as a sequence of images in format *.jpg. The images corresponding 0.5 second of video were opened, edited in the software IMAGEJ (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) and compiled using the application CASA (University of California and Howard Hughes Medical Institute, USA) according to Wilson-Leedy & Ingermann (2007) and Sanches et al. (2010). The analyses of sperm motility by application CASA were conducted after 10s of sperm activation, with 1s of interval until 20s pos-activation. For the semen originated from each male, the analyses were performed in triplicate. The sperm motility (MOT), curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) were submitted at ANOVA and linear regression analysis at 5% of significance.

Results
The spermatic parameters were different among time of 10 at 20s pos-activation (P<0,05), showed higher in 10s and lower in 20s, with inversely proportional relation (P<0,05) for all parameters evaluated (Figure 1). The number mean of spermatozoa evaluated was 61 for field visualization, this number is adequate for computed of fish sperm analysis (Wilson-Leedy & Ingermann, 2007). In the time of 10s after activation the values observed (means ± standard error) were 89.11 ± 1.25%, 107.23 ± 2.35 $\mu m.s^{-1}$, 83.58 ± 3.48 $\mu m.s^{-1}$ and 77.08 ± 3.56 $\mu m.s^{-1}$ for MOT, VCL, VAP and VSL respectively.

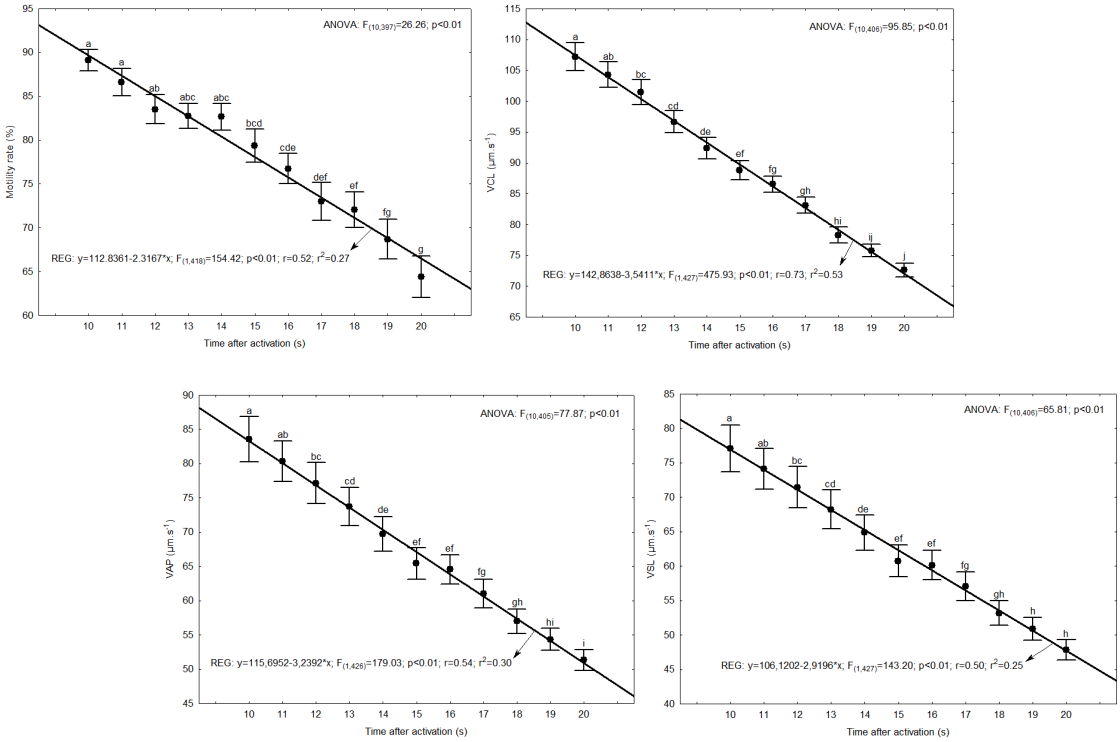


Figure 1. Sperm motility, curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) in S. parahybae among 10 at 20s pos-activation. Variance analysis (ANOVA). Linear regression analysis (REG). Different letters indicated (P<0,05) according Tukey test.

Discussion and conclusions

The behavior in *S. parahybae* sperms among 10 at 20s pos-activation (Figure 1) indicated continued reductions in sperm quality due to reduction of energy reserves (Cosson et al., 2010). These results are similar to those observed by Sanches et al. (2010) for a South American catfish, these authors observed reduction in sperm motility and velocity of *Rhamdia quelen* immediately after its activation. Given this we suggested that assessments of sperm parameters in *S. parahybae* immediately after activation, therefore this time may occur the highest rates of motility and velocity. However, lower times can be obtained with the experience of the person who is reviewing. Assessments for fish sperm in the short time that show activation (Billard & Cosson, 1992) is a limiting factor, especially when it comes to computer analysis that require standardization of several parameters (Sanches et al., 2010). The implementation of computerized sperm analysis presents an important tool for describing the behavior of sperm (Wilson-Leedy & Ingermann, 2007). The CASA open source software is rapid, exact and objective method for assessing to spermatic parameters of *S. parahybae*.

Acknowledgements

CNPq (number 478347/2009-0), FAPESP (number 2009/18609-6) and CESP.

References

Billard, R. and Cosson, M. P. 1992: Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 22-31.

Cosson, J., 2010: Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J. Fish Biol. 76, 240-279.

Sanches, E.A., Bombardelli, R.A., Marcos, R.M., Neumann, G., Toledo, C.P.R., Romagosa, E. 2010: Sperm motility oh Rhamdia quelen studied using computer-assisted analysis by open-souce software. Aqua. Res. 42, 153-156.

Wilson-Leedy, J. G. and Ingermann, R. L., 2007: Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology, 67, 661-672.

P-106
CRUDE PROTEIN CAN AFFECT THE OOCYTES EVOLUTION OF PACU, *PIARACTUS MESOPOTAMICUS,* KEPT IN CAGES

Fábio Bittencourt[1], Tatiane Andressa Lui[2], Bruno Estevão de Souza[3], Wilson Rogério Boscolo[2], Aldi Feiden[2], Elizabeth Romagosa[4]

[1] *Universidade Estadual de São Paulo – Centro de Aquicultura – Jaboticabal, Brazil*
[2] *Universidade Estadual do Oeste do Paraná – Toledo, Brazil*
[3] *Instituto Federal do Paraná – Foz do Iguaçu, Brazil*
[4] *Instituto de Pesca – São Paulo, Brazil*

E-mail: fabio_gemaq@yahoo.com.br

Introduction
The pacu, *Piaractus mesopotamicus* is a migratory indigenous fish of great economic interest for Brazilian aquaculture because it adapts easily to artificial diets and, is possible, optimizing egg and fry production, considering a limiting factor in the development of the aquaculture industry (Bittencourt, 2008). The pacu do not reproduce in captivity and the choice of hormonal therapy for the induction of maturation (mainly OM in the females) depend on whether the fish presents single-batch group-synchronous fishes reproduce only once during every annual reproductive period. The stripping of eggs and artificial insemination may be used in annually spawning to obtain the total amount of available eggs from each female (Romagosa, 2008). Nevertheless, there exist some problems concerning its nutritional demands and information on its reproductive performance, egg and sperm production during the reproductive season, and gamete quality when maintained in floating cages (Andrade et al., 2010; Reidel et al., 2010). The present study evaluated the oocytes evolution of pacu, *Piaractus mesopotamicus*, kept in cages fed with four diets with different compositions (4 protein levels, and one level of energy).

Materials and methods
The fish adults were kept in 16 cages (5,0 m^3 each) with 14 fish, for 153 days (pre-spawning: Ago.-Dec./09, in the reservoir of Itaipu Power Station, in Santa Helena, Paraná, Brazil with 4 treatments and 4 replications: (T1) 18% of crude protein (C.P.); (T2) 24% C.P.; (T3) 30% C.P.; (T4) 36% C.P., and 3,300 $Kcal.g^{-1}$ all treatments (NRC, 1993). The diets were offered twice a day until animal satiation. In the reproductive period 11 females broodfish of each treatment (average weight 2.9 to 3.0kg) were selected during their 1st ovarian maturation according to the association between the external anatomic characteristics (swollen abdomen and enlarged urogenital papilla) and internal, based on development of the intra-ovarian oocytes degree that sampled with a plastic catheter and viewed macroscopically and showing 60.0 % of the progressive change in the position of the nucleus to the oocyte periphery (re-start of the meiosis). After that, the females were maintained into a 1000L fiberglass tank with current water. The females have presented the germinative vesicle shift were induced spawning with two doses of CPE, 0.5 and 5.0 mg $kg,^{-1}$ intramuscular injections, at 12-h intervals. Two different samples were taken before the 1st and 2nd doses and maintained in Gilson solution for determining of oocytes diameters (stereomicroscopy, 10X31).

Results and discussion
The patterns of the oocytes diameters distribution have initial modesof the 1st samples: T1 (18%) unimodal (719,9 μm) but, the another three treatments presented polimodal tendency with distinct modals, T2 (24%) 782,5; 845,1 and 970,3 μm; T3 (30%) 719,9; 845,1, 970,3 μm and T4 (36%) 782,5, 845,1 and 970,3 μm., respectively. The 2nd oocytes samples (ovulation) showed similar distributions (polimodal) to the four treatments with approximately values of 845,1; 907,7 and 970,3 μm (18%), 845,1 and 970,3 μm (24%), 845,1 and 907,7 μm (30%) and, 845,1 e 970,3 μm (36%).The females that received treatments containing the highest protein levels showed evidence of the ovarian regression process (Romagosa, 1998). The same was evaluated in *Brycon opalinus*, that no spawning indicated the presence of whitish oocytes disperse with gray and garnet (Narahara et al., 2002).

Conclusions

It was evident that the treatments 1 and 2, responded positively, demonstrating the importance of using oocyte colour, the position of germinative vesicle and diameter as described here. The T4 showed those oocytes with diameters greater than the ones at ovulation and which are released were termed "overripe". Similar results were obtained by Romagosa et al. (1982). The present method uses a smaller number of fish as well as a reduced quantity of hormone. This will result in a cost decrease which is an important element in the production of fish.

Acknowledgements
The first author is grateful to FAPESP (nº 2010/00013-7), São Paulo, Brazil and ITAIPU BINATIONAL, Paraná, Brazil and UNIOESTE, Paraná, Brazil

References

Andrade, V.X.L.; Honji, R.M.; Romagosa, E. 2010. Processo de maturação das gônadas de *Pseudoplatystoma corruscans* alimentados com dietas contendo níveis protéicos e suplementada com óleo de milho. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* (prelo).

Bittencourt, F. 2008. Cultivo de pacu *Piaractus mesopotamicus* sob diferentes densidades em tanque-rede no reservatório de Itaipu. Universidade Estadual do Oeste do Paraná, Marechal Cândido Rondon. 46p.

Narahara, M.Y.; Andrade-Talmelli, E.F.; Kavamoto, E.T.; Godinho, H.M. 2002. Reprodução Induzida da Pirapitinga-do-Sul, *Brycon opalinus* (Cuvier, 1819), Mantida em Condições de Confinamento. Revista Brasileira de Zootecnia, v.31, n.3, p.1070-1075.

NRC (National Research Council). 1993. Nutrient requirement of warmwater fishes and shellfishes. *National Academy Press*, Washington, DC, pg.114

Reidel, A.; Boscolo, W.R.; Feiden, A.; Romagosa, E. 2010. The effect of diets with different levels of protein and energy on the process of final maturation of the gametes of *Rhamdia quelen* stocked in cages. Aquaculture 298: 354-359.

Romagosa, E. 2008. Avanços na reprodução de peixes migradores avanços in the reproduction of migratory fish. AquaCiência 2008 (2008: Maringá). Tópicos especiais em biologia aquática e aquicultura III/Edição J.E.P. Cyrino, J.D. Scorvo Filho, L.A.Sampaio, R.O. Cavalli. Jaboticabal: Sociedade Brasileira de Aqüicultura e Biologia Aquática, 2010.

Romagosa, E. 1998. Mudanças morfológicas (microscopia de luz e eletrônica) das gônadas do pacu, *Piaractus mesopotamicus* (Holmberg, 1887), durante o ciclo reprodutivo, em condições de confinamento. Rio Claro, SP. Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Rio Claro, SP. Tese de Doutorado.

P-107
ULTRASTRUCTURAL CHARACTERISTICS OF THE *NANNOSTOMUS* UNIFASCIATUS SPERMATOZOA (CHARACIFORMES, LEBIASINIDAE)

Rosicleire Veríssimo-Silveira[1], Elisa Aparecida Gregório[2], Irani Quagio-Grassiotto[2]

[1] *Department of Biology and Animal Science, FE/UNESP, Ilha Solteira, SP, Brazil*
[2] *Department of Morphology - IBB/UNESP, Botucatu, SP, Brazil*

E-mail: rosiverissimo@bio.feis.unesp.br

Introduction
Several studies have focused the ultrastructure of spermatozoa as an additional and complementary tool for morphological and molecular studies in order to improve the phylogenetic relationships among groups (JAMIESON, 1991). Therefore, to contribute with the systematics and phylogeny of the order Characiformes through ultrastructural morphology, the description of the ultrastructure of spermatozoa in *Nannostomus unifasciatus* can be useful in future phylogenetic analyses of the Lebiasenidae family.

Materials and methods
Ten specimens (mature males) of *N. unifasciatus* were obtained from ornamental fish store, Botucatu, SP, Brazil. Fragments of testes were fixed overnight in a solution of 4% paraformaldehyde and 2% glutaraldehyde in a Sorensen phosphate buffer 0.1M, pH 7.4. The material was post-fixed for 2 hours in the dark in 1% osmium tetroxide in the same buffer, contrasted in block with aqueous solution of 5% uranyl acetate for two hours, dehydrated in acetone, embedded in araldite, and sectioned and stained with a saturated solution of uranyl acetate in 50% alcohol and lead citrate. The electron micrographs were obtained using a Phillips – CM 100 transmission electron microscope.

Results
N unifasciatus spermatozoa have a head, midpiece and flagellum (Fig. 1A). The head exhibits no acrosomal vesicle. It contains a spherical nucleus, with 2,0 μm in diameter and highly condensed, homogeneous granular chromatin with occasional electron lucent areas (Figs. 1C-D). The nuclear fossa is absent. The centriolar complex stays lateral to the nucleus, the proximal centriole is anterior, lateral and oblique to the distal, are observed anchoring fibers attach the distal centriole to the plasma membrane (Figs. 1A-F). The midpiece contains several elongate mitochondria near to nucleus and separated from the inicial flagellum segment by the short cytoplasmic canal; abundant elongate vesicles are present in the periphery of the midpiece (Figs. 1A-C; 1E-I). The flagellum contains a classic 9+2 axonema (all microtubules are electron luced), elongate vesicles and no fins (Fig. 1J-N).

Discussion and conclusions
In *N. unifasciatus* spermatozoa, the nuclear rotation does not occur and, as a consequence, the flagellum remains lateral to the nucleus, resemble the Type II aquasperm of Mattei (1970). This characteristic isn't found in none *Nannostomus* species described (VERISSIMO-SILVEIRA, 2007), except in other Lebiasinidae *Phyrrulina australis* (BURNS et al., 2009). The sperm ultrastructure in *N. unifasciatus*, compared with other Nannostomus (VERISSIMO-SILVEIRA, 2007), shows a high degree of variability, however this fact is not usually observed within a single genus.

Acknowledgements
The authors are grateful to the Brazilian fostering Agency FAPESP (03/05570-8; 2010/00988-8) for funding support and Electron Microscopy Laboratory of IBB-UNESP for allowing us the use of their facilities

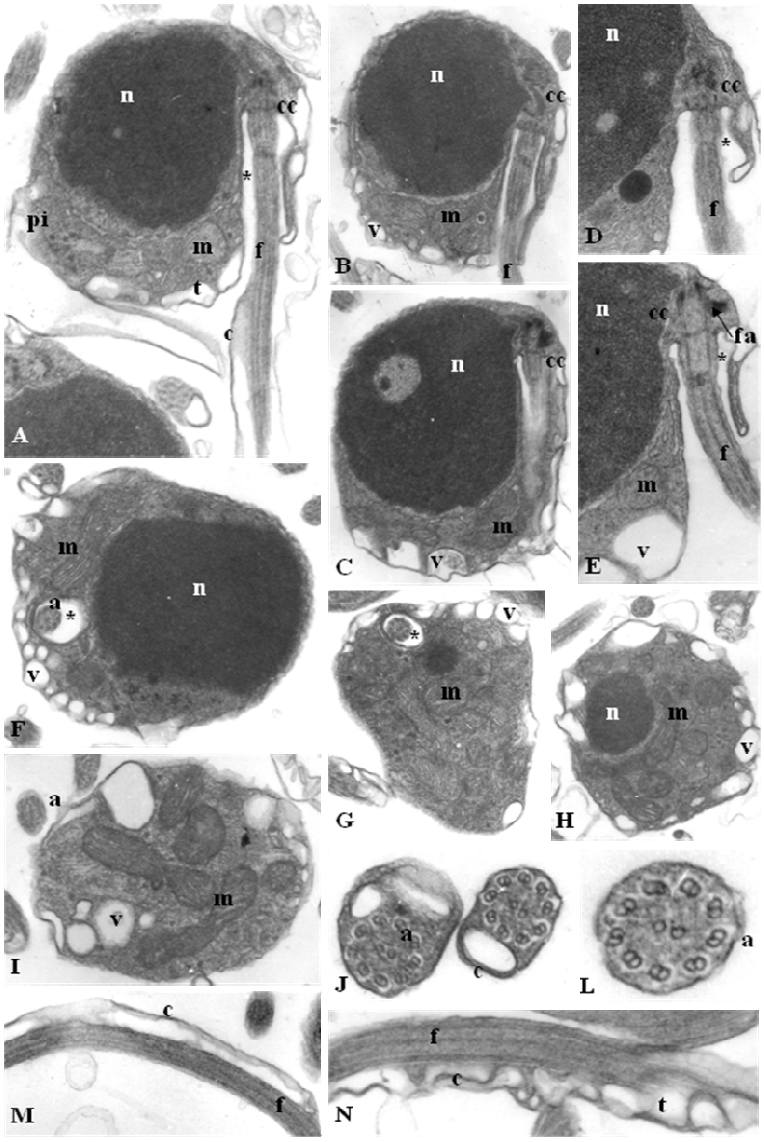


Fig 1 - N. unifasciatus spermatozoa. ***A-C:****.Cross sections of the spermatozoa head, midpiece and flagellum. A: 27.500X; Fig. B: 23.750X; Fig. C: 23.000X.* ***D e E:*** *Longitudinal sections of the initial portion of the flagellum, showing the ancoring fibers around the distal centriole. D: 32.150X; E: 31.500X.* ***F:*** *Spermatozoa head and midpiece (cross section) 28.650X.* ***G-I:*** *Cross section of midpiece. G: 23.750X; H: 18.700X; I: 25.900X.* ***J e L:*** *Flagelum (cross sections). J: 82.150X; L: 13.270X.* ***M e N:*** *Elongate vesicles in flagellum (Longitudinal sections). M: 28.140X; N: 47.250X.* ***n:*** *nucleus;* ***cc:*** *centriolar complex;* ***fa:*** *anchoring fibers;* ***m:*** *mitochondria;* ***pi:*** *midpiece; asterisk: cytoplasmic canal;* ***v:*** *vesicles;* ***c****: elongate vesicles;* ***a****: axonema;* ***f****: flagellum*

References

JAMIESON, B.G.M. Fish Evolution and Systematics: Evidence from Spermatozoa. Cambridge: Cambridge University Press. 1991. 319p.

MATTEI, X. Spermiogenèse comparée des poisson. In: BACCETTI, B. Comparative Spermatology. Academic Press, New York, 1970. p. 57-70.

BURNS J.R.; QUAGIO-GRASSIOTTO, I.; JAMIESON, B.G.M. Ultrastructure of Spermatozoa: Ostariophysi. In: B.G.M.Jamieson. (Org.). Reproductive Biology and Phylogeny of Fishes (Agnathans and Neotelestomi). 1ed ed. New Hampshire: Science Publishers, 2009, v. 8A, p. 287-387.

VERÍSSIMO-SILVEIRA, R. Estudo filogenético da sub-ordem Characoidei (Teleostei, Ostariphysi, Characiformes) com base na ultraestrutura dos espermatozóides. Doctoral thesis, Universidade Estadual Paulista, Botucatu, São Paulo, Brasil.

P-108
FEEDING RAINBOW TROUT WITH A PLANT BASED DIET FROM FIRST FEEDING TO SPAWNING DOES NOT AFFECT SPERM QUALITY

Catherine Labbe[1], Florence Le Gac[1], Edwige Quillet[2], Jean-Michel Lecalvez[3], Lionel Goardon[3], Laurent Labbé[3], Françoise Medale[4], Mathilde Dupont-Nivet[2]

[1] *INRA, UR 1037 SCRIBE, Campus de Beaulieu, F-35000 RENNES, France*
[2] *INRA, UMR1313 GABI, F-78350 Jouy-en-Josas, France*
[3] *INRA, UE 937 PEIMA, F-29450 Sizun, France*
[4] *INRA, UMR 1067 NuaGe, F-64310 St Pée-sur-Nivelle, France*

E-mail: Catherine.Labbe@rennes.inra.fr

Introduction
Salmonids in aquaculture have always been considered as highly demanding fish with regards to the amount of fish oil and fish meal in their diet. Nowadays however, the decrease in fisheries resources prompted the search for a substitution of fish meal and fish oil by plant products. In rainbow trout, earlier work showed that replacement of fish oil by corn oil in the diet of for one year prior to spawning does not affect sperm fertilizing ability after cryopreservation (Labbe et al., 1993). In this work however, fish meal was still present in the diet.

The authors showed that a basic sperm plasma membrane composition was maintained which may explain the maintenance of a high sperm quality.

The objective of the present work was to test whether feeding rainbow trout from the first feeding stage to spawning with a diet devoid of any fish oil and meal could still allow the production of a normal sperm quality and could maintain sperm ability to cryopreservation.

Methods
Rainbow trout (Oncorhynchus mykiss) were fed two different diets from the first feeding stage (420°C.day). The marine (M) diet, based on fish meal and fish oil, and the plant based diet (P) totally devoid of fish products were both isoproteic, isolipidic and isoenergetic. After 2 years, sperm from 12 males in each diet group was collected. Sperm was cryopreserved in Cryofish with 10 % DMSO (IMV, L'Aigle) according to Labbe and Maisse (2001). Quality of the fresh sperm and that of the frozen-thawed sperm was estimated from the development rates at 100°C.day, after fertilization of eggs from conv entionally fed females.

Results
As shown Figure 1A, fertilization rates of fresh sperm from the males fed the plant based diet were

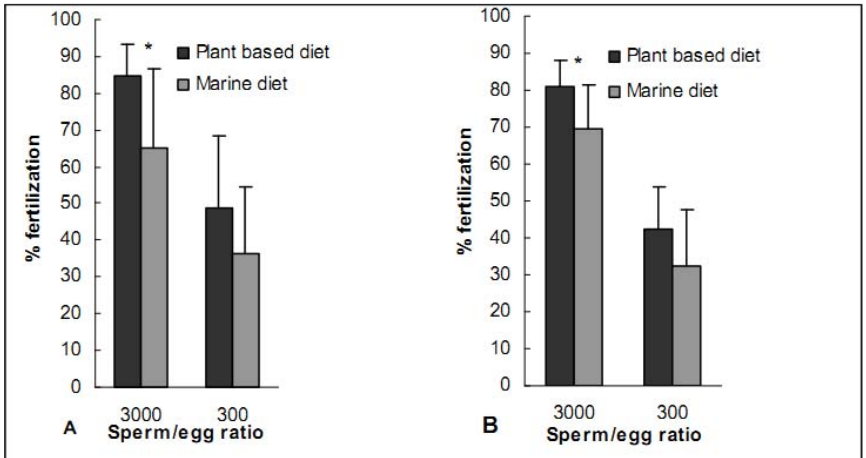


Figure 1. Fresh sperm (A) and frozen-thawed sperm (B) quality after fish were fed from the first feeding with Plant based diet or Marine diet. * p<0,05 (U test).

significantly higher than those of the marine diet, at least at the highest sperm/egg ratio (3000). A lower sperm/egg ratio (300) reduced the fertilization rates, and no significant diet effect was detected although a favorable tendency was observed for the plant based diet. Moreover, fish feeding with the plant based diet did not alter sperm ability toward cryopreservation. Fertilization rates were even higher in the plant based group than in the marine one at the highest sperm/egg ratio (Figure 1B). Additionally, we observed that sperm from both diet groups had the same average sperm concentration upon stripping.

Discussion and conclusion

This experiment demonstrated that feeding fish with a diet totally devoid of fish resource did not affect sperm quality after 2 years of rearing in these conditions.

Sperm quality was estimated from the development rates obtained more than 10 days after fertilization. Older stages were not assessed, because we consider that the initial sperm quality should no longer affect development after this stage (Labbe et al, 2001), although long term effect of the diet during development was observed with sea bass sperm (Asturiano et al, 2001). We were surprised nevertheless to observe that the marine diet did not produce fertilization rates as high as those obtained with the plant based diet. We infer that the marine diet formulated in this experiment was not optimal.

Because fertilization rate is the most integrative test to assess sperm quality, we did not explore further other cellular sperm parameters which could have been modified by the plant based diet. It will however be interesting to analyze whether the conservation of a basic sperm plasma membrane composition, as proposed by Labbe et al (1993), will still operate in sperm from fish reared in these extreme conditions.

In conclusion, total substitution of fish oil and meal in fish diet is not deleterious to sperm quality, even after cryopreservation. The question should now be raised for egg quality of females reared in the same conditions, as the quality of the yolk is of major importance all over embryo development.

Acknowledgements

The authors whish to thank Frederic Borel and Cécile Melin from IE SCRIBE for their efficient help in the sperm fertilization experiments. Experimental diets were produced at the INRA Donzacq experimental farm.

References

Asturiano,J.F., Sorbera,L.A., Carillo,M., Zanuy,S., Ramos,J., Navarro,J.C., and Bromage,N. (2001). Reproductive performance in male European sea bass (Dicentrarchus labrax, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture 194, 173-190.

Labbe,C., Loir,M., Kaushik,S., and Maisse,G. (1993). The influence of both rearing temperature and dietary lipid origin on fatty acid composition of spermatozoan polar lipids in rainbow trout (Oncorhynchus mykiss). Effect on sperm cryopreservation tolerance. In Fish Nutrition in Practise, Les Colloques (Paris: INRA), pp. 49-59.

Labbe,C. and Maisse,G. (2001). Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity. Aquaculture 201, 287-299.

Labbe,C., Martoriati,A., Devaux,A., and Maisse,G. (2001). Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Mol Reprod Dev 60, 397-404.

P-109
ARTIFICIAL REPRODUCTION OF THE CRUCIAN CARP *CARASSIUS CARASSIUS* (L.) UNDER CONTROLLED CONDITIONS

Katarzyna Targońska[1], Daniel Żarski[1], Tamás Müller[2], Sławomir Krejszeff[1], Krzysztof Kozłowski[3], Ferenc Demény[2], Béla Urbányi[2], Dariusz Kucharczyk[1]

[1] *Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn, Poland*
[2] *Department of Aquaculture, Institute of Environmental and Landscape Management, Faculty of Agriculture and Environmental Science, Szent István University, Páter Károly u. 1. H-2103 Gödöllő, Hungary*
[3] *Department of Fish Biology and Pisciculture, University of Warmia and Mazury in Olsztyn, Poland*

E-mail: s.krejszeff@wp.pl

Introduction

In many countries, the crucian carp is an endemic species whose status is endangered (Copp et al. 2008). Thus, effective controlled reproduction protocol of this species is needed for both aquacultural and sustainable purposes.The results of preliminary studies, conducted by Targońska et al. (2009), have pointed to the need for using hormonal agents in controlled reproduction of the crucian carp. The effectiveness of reproduction protocols (expressed as ovulation rate, relative fecundity as well as gametes and larvae quality) is affected by many factors. A combination of these aspects should form the basis of a protocol which may be successfully used in further artificial reproduction activities. The aim of the study was to find the optimal conditions for artificial spawning in a wild origin crucian carp under controlled conditions.

Materials and methods

Crucian carp spawners (weight 100 to 150 g each) were caught during the reproductive period (May/June) and were kept (males and females separately) in 1000 L tanks. The tanks were equipped with devices which maintained a constant and fully controlled temperature and photoperiod conditions. The water temperature after the fish were introduced was maintained at 17°C. The spawning agents and doses of preparations used in the experiment are shown in table 1. Each female group comprised 10 fish. After two days of keeping the fish at 17ºC, the females were given hormonal preparations. All the preparations were injected intraperitoneally, at the ventral fin base. The water temperature was raised to 21ºC after the resolving injection has been administered. Ovulation was checked between 14 and 22 hours after the injection. During the experiment the ovulation rate and latency time were recorded (tab.1). The relative fecundity was calculated (number of eggs per gram of female body weight). The gamete quality was determined as embryo survival rate to the eyed-egg stage. To this end, three egg samples (100 – 150 eggs each) from each female were fertilised with pooled sperm collected from at least 5 males with a minimum motility of 80% (0.05 ml per eggs sample). Eggs were incubated in Petri dishes at 21°C in a closed water circulation.

Statistical differences between the groups with respect to embryo survival to the eyed-egg stage and relative working fecundity were analysed with an analysis of variance (ANOVA) followed by Tukey's *post-hoc* test ($P<0,05$). All the data expressed in percentage were subjected to the arcsine transformation before their statistical analysis.

Results

The results of testing the usability of different hormonal preparations in artificial reproduction of the crucian carp has shown that application of spawning agents is necessary. No ovulation was observed in any female in the control group (tab. 1). The highest ovulation rate was observed in the groups stimulated with

preparations containing LHRH analogues and dopamine antagonists (Ovopel, Ovaprim). The highest relative working fecundity was observed in those groups and in the CPH group ($P<0.05$). The highest embryo survival to the eyed-egg stage was observed in the groups stimulated with hCG and Ovaprim ($P<0.05$).

Table 1. Hormonal doses and the results of reproduction of the crucian carp following the application of different hormonal agents

Groups	Control	Ovopel (pellet kg^{-1})	hCG (IU kg^{-1})	CPH (mg kg^{-1})	Ovaprim (cm^3 kg^{-1})	GnRHa (µg kg^{-1})
Injection I	-	0.2	100	0.3	0.1	20
Injection II	-	1.0	600	2.7	0.5	100
Female weight (g)	169±21^a	170±20^a	175±19^a	169±22^a	172±23^a	171±21^a
Ovulation (%)	0	90	50	70	90	50
Latency time (hrs)	-	16	16-20	12-14	14-18	16-20
Relative fecundity (eggs/female BW)	-	159±8^a	90± 8^c	162±11^a	160± 9^a	122± 8^b
Embryos survival to the eyed-egg stage (%)	-	80.1±3.6^b	92.4±4.3^a	77.8±4.3^b	90.1±2.3^a	78.9±3.2^b

Discussion

The effects of using combinations of hormonal agents in artificial reproduction of fish has been frequently unexpectedly positive. A very good outcome of using a combination of hCG and CPE in cyprinids has been reported e.g. by Kucharczyk et al. (1997). In addition, a combination of Ovopel (injection I) and CPH or Ovaprim (injection II) has proven successful in other species, for example, in the ide and the dace (Żarski et al. 2009). Very good results have been achieved by using a combination of Ovopel with CPH and Ovaprim in the crucian carp. The highest percentage of ovulating females (100%) and the highest embryo survival to the eyed-egg stage was observed when Ovopel and Ovaprim were used in combination. The same relationship was observed by Żarski et al. (2009) in a study with ide and dace.

Successful reproduction of fish under controlled conditions, i.e. high ovulation and spermiation, is the basis for planning further cultures. But an effective procedure which takes into account many factors affecting the outcome of the measures taken and employs technical resources and time efficiently, will provide a strong basis for the further development of a culture. The data presented in this paper provide many important recommendations for further improvements in the procedures and methods of artificial reproduction of the crucian carp. They should be taken under the consideration in further studies into the reproduction of wild populations of the species.

Acknowledgements

This study was partially financed under the auspices of the project "Innovations in finfish aquaculture with special references to reproduction", Operational Programme Sustainable Development of the Fisheries Sector and Coastal Fishing Areas 2007-2013" (OR14-61724-OR1400003/09/10/11).

References

Copp GH, Warrington S, Wesley KJ, 2008: Management of an ornamental pond as a conservation site for a threatened native fish species, crucian carp Carassius carassius. Hydrobiologia, 597, 149–155.

Kucharczyk D, Kujawa R, Luczynski M, Glogowski J, Babiak I, Wyszomirska E, 1997: Induced spawning in bream, Abramis brama (L.), using carp and bream pituitary extract and hCG. Aquacult Res, 28, 139–144.

Targońska K, Żarski D, Krejszeff S, Kucharczyk D, 2009: Artificial reproduction of crucian carp, Carassius carassius (L.) – preliminary results. Proc. Aquaculture Europe, Trondheim, 14-17 August 2009, Europ. Aquacult. Soc. 602-603.

Żarski D, Kucharczyk D, Targońska K, Jamróz M, Krejszeff S, Mamcarz A, 2009: Application of Ovopel, Ovaprim and their combination in artificial reproduction of two rheophilic cyprinid fishes. Pol J Nat Sc, 24, 235–244.

P-110
INFLUENCE OF TIME OF CONTACT SPEM WITH WATER TO FERTILIZATION ABILITY IN IDE *LEUCISCUS IDUS* L.

Katarzyna Targońska[1], Sławomir Krejszeff[1], Daniel Żarski[1], Radosław K. Kowalski[2], Beata I. Cejko[2], Beata Sarosiek[2], Katarzyna Palińska[1,3], Jan Glogowski[2], Dariusz Kucharczyk[1]

[1] *Department of Lake and River Fisheries, University of Warmia and Mazury, Olsztyn, Poland*
[2] *Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn*
[3] *Department of Zoology, University of Warmia and Mazury, Olsztyn, Poland*

E-mail: s.krejszeff@wp.pl

Introduction
Fertilization rate is one of the most important factors in sustainable and commercial aquaculture. In wild cyprinids, such as asp *Aspius aspius* L., ide *Leuciscus idus* L. or dace *Leuciscus leuciscus* L. problems with high embryo survival were noted (Targońska et al. 2008, 2010, Żarski et al. 2009). Thus, there is high need to work out the most effective insemination procedures. The aim of this study was impact of fertilization ability of ide spermatozoa in relationship to time after their activation.

Materials and methods
Ide spawners were collected from pond culture in early spring. After collection fish were transported to the hatchery. Fish were artificially reproduced using the method described by Żarski et al. (2009). Sperm motility parameters were determined using a CASA system (computer assay sperm analysis). For activation hatchery water was used. Sperm movement was recorded at activation moment and next after 15, 30, 45, 60, 90, 120, 150 and 180 s. Insemination the portions (~100) of eggs was proceeded by adding the eggs to water mixed with 0.05 ml of pooled sperm for 30, 60, 90, 120, 150 and 180 s and compared to the control group inseminated at the moment of sperm activation (0 s). The same procedure was done in triplicates. At the eyed-egg stage embryo survival was recorded.

Results and discussion
The ability of spermatozoa to egg fertilization was depended from many factors: e.g. water temperature, spermatozoa:egg ratio and spermatozoa motility (MOT) (Targońska et al. 2008, Kucharczyk et al. 2010). In reophilic fishes, like asp or ide which spawn in floating water, time of gametes contact is usually short. But in artificial reproduction this contact is much longer. Ide spermatozoa motility decreased significantly within 45 s after activation (Fig. 1). However, embryos survival exceeded 70% sixty seconds after sperm activation where only 6.8% MOT was recorded.

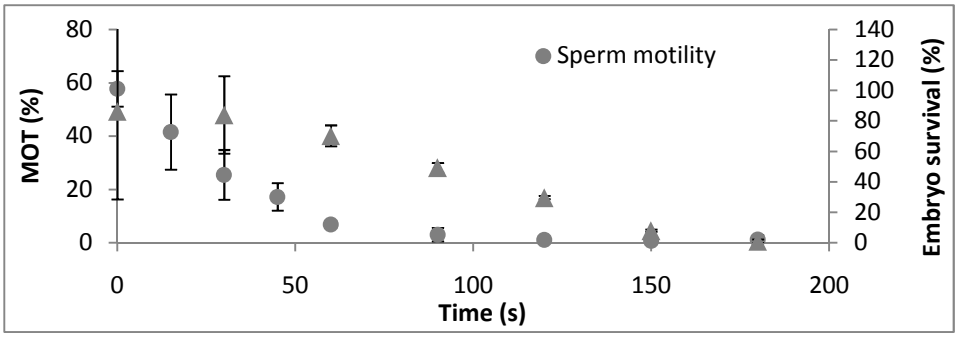


Fig. 1. Spermatozoa motility (MOT) and embryos survival in relation to time after sperm activation in hatchery water

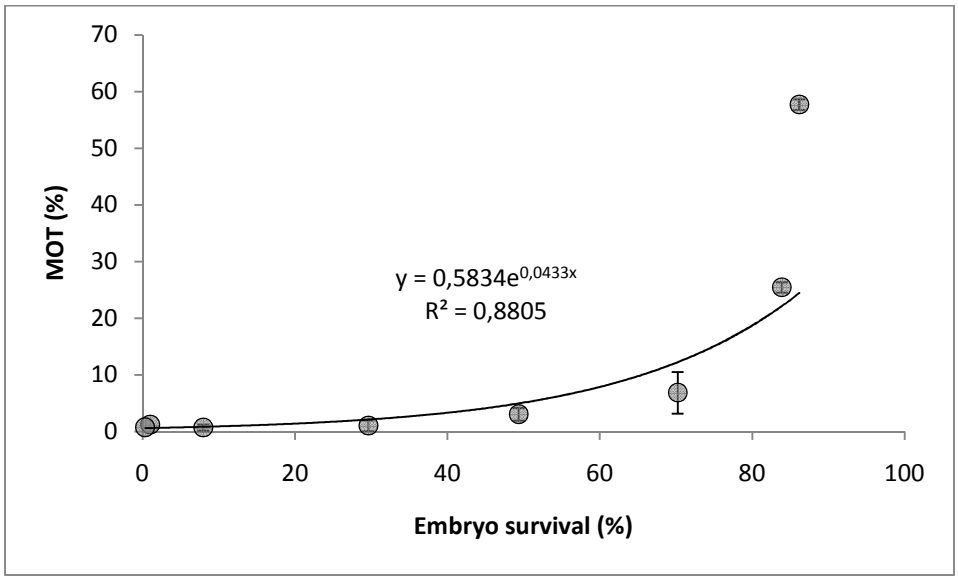


Fig. 2. The relationship between percentage spermatozoa motility (MOT) and ide embryos survival to the eyed-egg-stage

The relationship between MOT and ide embryos survival to the eyed-egg-stage (Fig. 2) showed that embryos survival was much higher than expected in comparison to MOT. Only 5% of MOT caused over 50% survival rate of embryos. Earlier works concerning the influence of MOT and time of motility in ide and asp showed, that time of spermatozoa motility is much more important for eggs fertilization than percentage of motility (Targońska et al. 2008, Kucharczyk et al. 2010). These suggestions are confirmed in the present work.

Acknowledgements

This study was partially financed under the auspices of the project "Innovations in finfish aquaculture with special references to reproduction", Operational Programme Sustainable Development of the Fisheries Sector and Coastal Fishing Areas 2007-2013" (OR14-61724-OR1400003/09/10/11).

References

Kucharczyk, D.; Gomułka, P.; Krejszeff, S.; Żarski, D.; Targońska, K., 2010: The effect of prepared activating liquid on the survivability of ide Leuciscus idus (L.) embryos. Pol. J. Nat. Sci. 25, 200-208.

Targonska K., Kucharczyk D., Mamcarz A., GLOGOWSKI J., KREJSZEFF S., PRUSIŃSKA M., KUPREN K. 2008. Influence of individual variability in the percentage of motile spermatozoa and motility time on the survival of embryos of chosen fish species. Pol. J. Natur. Sc., 23: 178-187.

TARGOŃSKA K., KUCHARCZYK D., KUJAWA R., MAMCARZ A., ŻARSKI D. 2010. Controlled reproduction of asp, *Aspius aspius* (L.) using luteinizing hormone releasing hormone (LHRH) analogues with dopamine inhibitors. Aquaculture 306, 407-410.

Woynarovich, E.,Woynarovich, A., 1980. Modified technology for elimination of stickiness of common carp *Cyprinus carpio* eggs. Aquac. Hung. 2, 19–21.

Żarski D, Kucharczyk D, Targońska K, Jamróz M, Krejszeff S, Mamcarz A, 2009: Application of Ovopel, Ovaprim and their combination in artificial reproduction of two rheophilic cyprinid fishes. Pol J Nat Sc, 24, 235–244.

P-111
CHARACTERISTIC OF BURBOT (*LOTA LOTA* L.) SPERM AFTER STIMULATION OF SPERMIATION WITH MAMALIAN GONADOLIBERINE ANALOGUE

Dariusz Kucharczyk[1], Roman Kujawa[1], Andrzej Mamcarz[1], Katarzyna Targońska[1], Daniel Żarski[1], Jan Glogowski[2], Radosław K. Kowalski[2], Beata I. Cejko[2], Katarzyna Palińska[1,3]

[1] *Department of Lake & River Fisheries, Warmia & Mazury University in Olsztyn, Poland*
[2] *Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn*
[3] *Department of Zoology, University of Warmia and Mazury, Olsztyn, Poland*

E-mail: darekk@uwm.edu.pl

Introduction

Burbot (*Lota lota* L.) is one of the endangered fish species in Poland and in many others European countries (Maitland and Lyle, 1991). The data on spawning of burbot in captivity (Żarski et al. 2010), as well as from observation in natural environment are very limited (Linhart, 1995). Developing methods of artificial spawning of burbot under controlled conditions might be useful in many programmes of restoration of this species (Kucharczyk et al., 1998). Additionally, burbot is one of the most perspective fish species for diversification of freshwater aquaculture. Thus, all aspects of controlled reproduction, including hormonal stimulation, would be very useful for aquaculturists.

To date, there is no information regarding effectiveness of hormonal stimulation on sperm characteristics in burbot. In this paper the results of inducing spermiation in burbot males using GnRH analogue with dopamine antagonist were presented.

Material and methods

Burbot breeders were captured in the late September from Vistula River near Gdansk (northern Poland) and in Odra river system and transported to the hatchery. Fish were kept in 1000dm^3 tanks with controlled temperature and photoperiod. Spawners were fed with commercial trout pellets and sporadically with frozen freshwater forage fish. After photo-thermal manipulations (beginning of January) fish from treated groups were injected with GnRH-analogue (Ovopel) (Table 1). One ovopel pellet (average weight - 25 mg) contains a mammalian GnRH analogue (D-Ala^6, Pro^{9}Net-mGnRH at a dose 18-20 g) and metoclopramide (dose 8-10 mg) (Horvath et al., 1997). The milt was sampled using syringes and kept under 4 °C before further treatment. The quantity of sampled milt, as well as spermatozoa motility, motility duration, spermatozoa conentration and time of spermatozoa motility were recognized subjectively.

Data were statistically analyzed with Duncan's ***post-hoc*** test (α=0.05).

Table 1. Description of experimental groups and used doses of hormones (dose of applied Ovopel pellets).

Time between injections was 24 hrs. N = 10 in each group

Description	Control	Group 1	Group 2	Group 3
I injection	0.9% NaCl	-	0.1 pellet	-
II injection	0.9% NaCl	1 pellet	1 pellet	2 pellets

Results and discussion

Characteristics of burbot milt was presented in Table 2. In all measured parameters high variable was noted. All males from treated groups were spermiated, whereas in control group only 75%. In treated groups males produced more milt, which characterized with better parameters. The best results were obtained when males were treated with double injections (group 3) and with highest dose of hormones (group 4). However,

due to high variability (SD values) no statistical differences were found (P>0.05). Similar results (also highly variable) were reported by Linhart (1995) for wild matured males and by Kucharczyk et al. (1998) for artificially matured males.

Table 2. The results obtained after hormonal stimulation of burbot males. Groups were described in Table 1. No differences were found between the groups (Duncan's post-hoc test, P>0.05)

Sperm characteristics	Control	Group 1	Group 2	Group 3
Quantity [ml/kg]	8.2 ± 4.3	11.1 ± 5.3	13.9 ± 4.8	12.7 ± 5.0
Spermatozoa motility [%]	65 ± 9	77 ± 14	79 ± 12	78 ± 13
Time of motility [s]	38 ± 12	34 ± 18	43 ± 20	40 ± 18

Hormonal stimulation was found to affect quantity and quality (e.g. spermatozoa motility) positively (e.g. Cejko et al. 2010). However, the results obtained in the present study indicate that in the case of burbot hormonal stimulation with mammalian GnRH analogue did not affect sperm parameters significantly even in groups were highest dose of hormonal preparations were applied. It could have great importance for aquaculturists and affect economic profitability of artificial reproduction of burbot because costs related with hormonal stimulation may be limited (Hakuć-Błażowska et al. 2010).

Aknowledgments
This study was partially financed under the auspices of the project "Innovations in finfish aquaculture with special references to reproduction", Operational Programme Sustainable Development of the Fisheries Sector and Coastal Fishing Areas 2007-2013" (OR14-61724-OR1400003/09/10/11).

References

Cejko, B.I., Kowalski, R.K., Kucharczyk, D., Targońska, K., Krejszeff, S., Żarski, D., Glogowski, J., 2010a: Influence of the length of time after hormonal stimulation on selected parameters of milt of ide Leuciscus idus L. Aquacult. Res. 41, 804-813.

Hakuć-Błazowska, A., Kupren, K., Turkowski, K., Targońska, K., Żarski, D., Kucharczyk, D. 2010: A comparison of the economic effectiveness of various spawning agents for stimulating the reproduction of the cultured and wild forms of the common barbel Barbus barbus (L.). Pol. J. Nat. Sci. 25, 272–286.

Horvath, L., Szabo, T., Burke, J. 1997: Hatchery testing of GnRH analogue-containing pellets on ovulation in four cyprinid species. Pol. Arch. Hydrobiol., 44, 221-226.

Kucharczyk, D., Kujawa, R., Mamcarz, A., Skrzypczak, A., and Babiak, I., 1998: Artificial spawning of burbot (*Lota lota* L.) under controlled conditions. European Aquaculture Society, Special Publication, 26, 149-150.

Linhart, O., 1995: Spermiation. semen quality and semen quantity in some species of fish. Prace VUHR Vodnany, 31, 112-123. (In Czech with English summary).

Maitland, P.S., and Lyle, A.A., 1991: Conservation of freshwater fish in the British Isles: The current status and biology of threatened species. Aquatic Conservation: Mar. Freshwat. Ecosyst., 1, 25-54.

Pullianen, E., and Korhonen, K., 1993: Does the burbot, *Lota lota*, rest years between normal spawning seasons. J. Fish Biol., 43, 355-362.

Żarski, D., Kucharczyk, D., Sasinowski, W., Targońska, K., Mamcarz, A., 2010: The influence of temperature on successful reproductions of Burbot, Lota lota (L.) under hatchery conditions. Pol. J. Nat. Sc. 25, 93-105.

P-112
GONAD DEVELOPMENT AND GAMETOGENESIS IN THE ASIAN SEA BASS *(LATES CALCARIFER)* GROWN IN AN INTENSIVE AQUACULTURE SYSTEM

Katalin Szentes[1], Erika Mészáros[1], Tamás Szabó[1], Balázs Csorbai[2], Gyula Borbély[2], Béla Urbányi[1], Ákos Horváth[1]

[1] *Department of Aquaculture, Szent István University, H-2100 Gödöllő, Páter Károly u. 1.*
[2] *Jászkiséri Halas Kft. H-5137 Jászkisér, Vágóhíd út 15.*

E-mail: Szentes.Katalin@mkk.szie.hu

Introduction
Asian sea bass or barramundi (*Lates calcarifer*) is a catadromous tropical fish species that inhabits the Indo-West Pacific region. It is cultured for its fast growth and excellent boneless meat. In addition to its culture in its native habitat, the species has been introduced to warm-water intensive aquaculture systems throughout the world.

The Asian seabass is a protandrous hermaphrodite that inhabits rivers but needs saltwater for its reproduction. The dynamics of its reproductive cycle and sex inversion have been described in detail earlier (Guiguen et al., 1994). According to the available sources, the species becomes sexually mature at around 3 years of age. However, no information is available on early sexual maturation and the development of its gonads in intensive freshwater aquaculture systems.

The objective of this study was to investigate early gonad development and gametogenesis in Asian sea bass cultured in a recirculating aquaculture system using conventional histology methods.

Materials and methods
Asian sea bass individuals aged 9-15 months were cultured at a warm-water recirculation system in Jászkisér, Hungary. Each month 5-12 fish were sacrificed and their gonads fixed in 8% buffered formalin for histology. Total length and weight of the fish were recorded. Gonad samples were dehydrated, embedded in paraffin and sections of 4-7 μm were prepared and stained with hematoxylin and eosin. Following microscopic observations testis maturity was categorized according to Guiguen et al. (1994): M0 - immature fish with no visible gonad differentiation; M1 – testis containing mostly spermatogonia; M2 – testis in active spermatogenesis contanining mostly spermatocytes and spermatids, M3 – testis in spermiation, containing mostly spermatozoa in the lumina of testicular lobules.

Correlations of testis maturity stages with total body length and weight were analysed using the statistical software R version 2.13.0 for Mac.

Results
Initial body weight and total length of fish were 453 ± 167 g and 28 ± 9 cm (9-month old fish) while the final were 1127 ± 499 g and 41 ± 17 cm (15-month old fish). Immature individuals were found in 9-, 12- and 13-month old fish at low percentages (Fig. 1). In mature fish all stages of spermatogenesis were found regardles of their age, thus, mature spermatozoa were present even in testes that were otherwise categorized as M1. During the experimental period a continuous decrease of testes in stage M1 (from 50% in 9-month old to 10% in 15-month old fish) was observed with a subsequent increase of testes in stages M2 and M3. Previtellogenic oocytes were not detected in any of the samples. Statistically significant but weak correlations were found between testis maturity stages and body length (r=0.2977) or body weigth (r=3206).

Discussion and conclusions
According to our findings, sexual maturation of Asian sea bass starts much earlier in the recirculation system than it was previously described (Davis et al. 1982, Toledo et al., 1991). The youngest fish studied in these experiments already had mature spermatozoa which indicates that spermatogenesis in these fish starts earlier than 9 month of age. The fact that mature spermatozoa were present in testes that contained mostly spermatogonia indicates highly asynchronous spermatogenesis. The lack of previtellogenetic oocytes in the samples suggests that the process of oogenesis starts at a more advanced age and female germ cells in these fish were probably present in the form of oogonia indistinguishable from spermatogonia. Correlations

found between testicular development stages and body length or weight confirm previous observations that maturity primarily depends on the size rather than the age of fish (Moore, 1979), however, the weakness of these correlations also indicates that size alone is not a reliable predictor of testicular development stages.

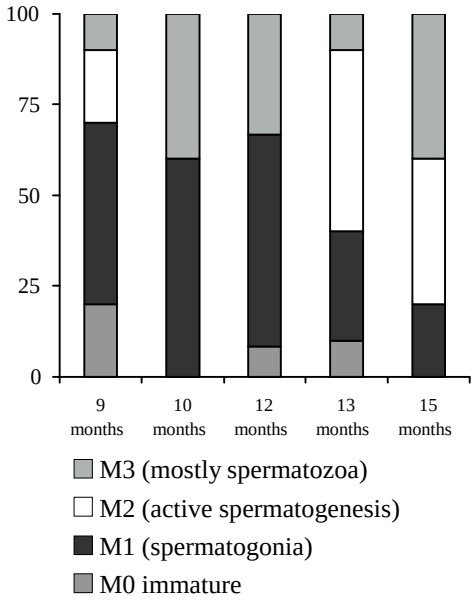


Figure 1. Distribution of testicular development stages in different age groups of the Asian sea bass (Lates calcarifer) reared in a recirculating aquaculture system

Acknowledgements

The study has been supported by the NTP project number TECH_09-A3-2009-0235 provided by the National Innovation Office of Hungary.

References

Davis, TLO. 1982: Maturity and sexuality in barramundi, Lates calcarifer (Bloch), in the Northern-Territory and Southeastern Guf Carpentaria. Australian Journal of Marine and Freshwater Research 33, 529-545.

Guiguen, Y., Cauty, C., Fostier, A., Fuchs, J., Jalabert. B. 1994: Reproductive cycle and sex inversion of the seabass, Lates cacarifer, reared in sea cages in French Polynesia: histological and morphometric description. Environmental Biology of Fishes 39, 231-247.

Moore, R. 1979: Natural sex inversion in the giant perch (Lates calcarifer). Australian Journal of Marine and Freshwater Research 30, 803-813.

Toledo, JD., Marte, CL., Castillo, AR. 1991: Spontaneous maturation and spawning of sea bass – Lates calcarifer – in floating net cages. Journal of Applied Ichthyology – Zeitschrift Fur Angewandte Ichthyologie 7, 217-222.

P-113
STAGES OF OOCYTE DEVELOPMENT IN *CICHLA PIQUITI* (PERCIFORMES: CICLIDAE)

Rosicleire Veríssimo-Silveira[1], Cristiane Bashiyo-Silva[1], Raphael da Silva Costa[2], Alexandre Ninhaus-Silveira[1]

[1] *Department of Biology and Animal Science, FE/UNESP, Ilha Solteira, SP, Brazil*
[2] *Laboratório de Ictiologia Neotropical (L.I.NEO) - PPG Biologia Animal –IBILCE/UNESP, Sâo José do Rio Preto, SP, Brazil*

E-mail: rosiverissimo@bio.feis.unesp.br

Introduction
The oocyte growth and maturation in fish are processes used to determine the phase of ovarian development during the spawning cycle (SELMAM & WALLACE,1989; GRIER et al., 2007). Therefore, this study aims to characterize the morphology of female germ cells of blue peacock bass, Cichla piquiti.

Materials and methods
Sexually mature females of C. piquiti were collected monthly (mar/2009 to feb/2010) in Paraná river (S20°28'23" W51°25'49"), SP, Brazil. The specimes were anaesthetized (0,1% benzocaine) and fragments of ovaries were fixed overnight in a solution of 4% paraformaldehyde and 2% glutaraldehyde in a Sorensen phosphate buffer 0.1M, pH 7.4, processed according to an usual methods for light microscopy (Technovit 7100/historesin) and stained with hematoxylin/eosin and reticulin.

Results
In C. piquiti, germ cells were observed at different stages of maturation within the same gonad, divided into: a)folliculogenesis: 1) oogonia - smaller germline cell, is ovoid and slightly basophilic nucleus and a clear cytoplasm (Fig1.A). 2) leptotene oocyte - the presence of small basophilic granules and evident nucleus (Fig1.B). 3) diplotene Oocyte – are observed a basophilic ring along the nuclear envelop and multiple nucleoli, prefollicle cells increasingly surround the oocytes and a basement membranes form around, separating them one from another – ovarian follicle (Fig1.C); b) Oocyte primary growth stage: 1) initial previtellogenic oocyte - nucleus with numerous nucleoli arranged in the nuclear periphery, is observed a organization of the thecal layers and follicular cells, both are squamous (Fig1.D). 2) pre-vitellogenic oocyte final - has as its main characteristic is the appearance of zona pellucida and the cortical alveoli, follicular cells become cuboidal and thecal cells are arranged in more layers (Fig1.E and inset).c) Oocyte secondary growth stage: 1) early vitellogenic oocytes - has as its main characteristic is the appearance of yolk globules, the zone pellucida becomes a little thicker than the previous stage (Fig1.F and inset). 4) late vitellogenic oocyte - deposition of yolk globules and proceeds until the oocyte is full-grown and the ooplasm is filled with yolk globules, large zona pellucida and a thick columnar follicular and presence of the micropyle (Fig1.G and inset).

Discussion and conclusions
The origin and development of oocytes in C. piquiti follows the same pattern of Perciformes (GRIER et al., 2007) and Gymnotiformes Gymnotus sylvius (FRANÇA, et al., 2010), and can be divided into folliculogenesis: proliferation of oogonia, initiating meiosis and forming nests of germ cells; oocyte previtellogenic stage or primary growth: follicular complex is formed, and Oocyte secondary growth stage or vitellogenesis. In C. piquiti revealed the presence of germ cells at different stages in the gonad of fish species characteristic of multiple spawning (Vazzoler, 1996).

Acknowledgements
The authors are grateful to the Brazilian fostering Agency FAPESP (2008/01083-9; 2010/00988-8) for funding support.

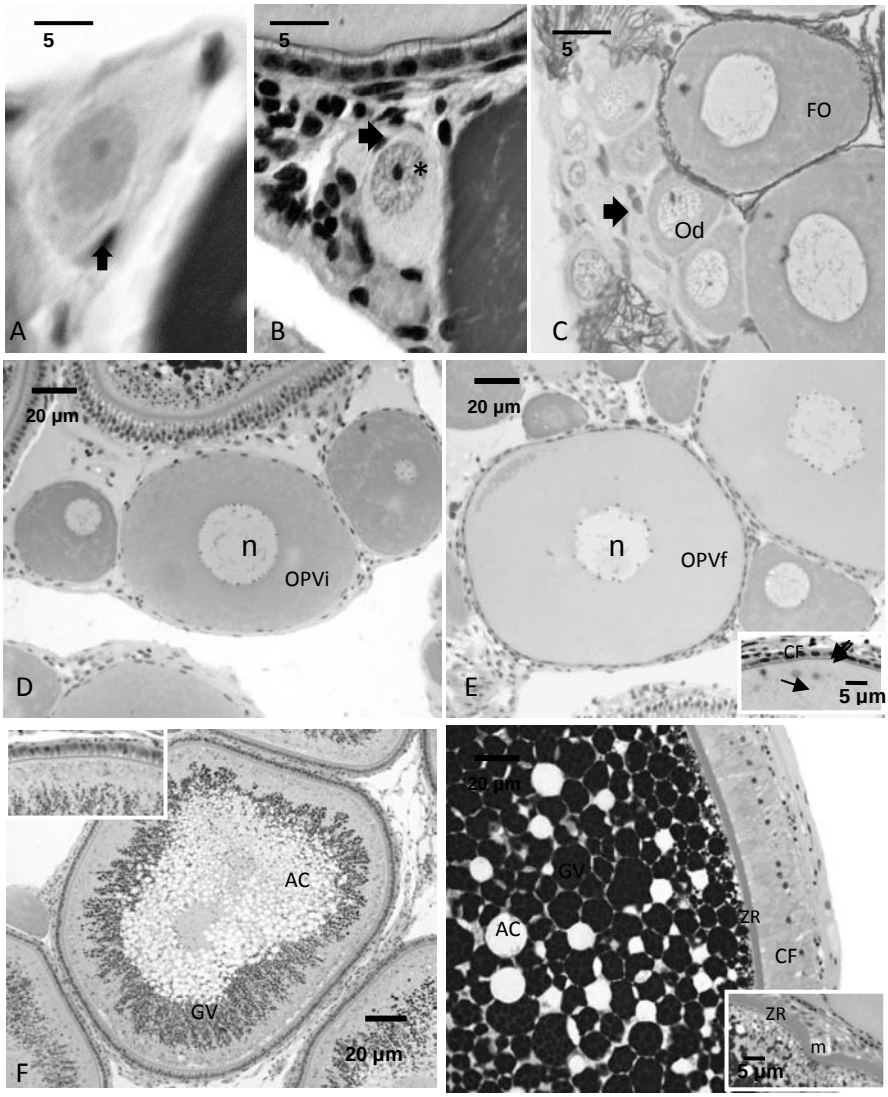


Fig 1 - Folliculogenesis in Cichla piquiti. A – oogonia; B – leptotene oocyte (*); C- Nests diplotene oocytes (Od) and ovarian follicle (FO). Oocyte primary growth stage D – Initial previtellogenic oocyte (OPVi); E- final previtellogenic oocyte OPVf; F- initial vitellogenic oocyte; G- final vitellogenic oocyte. E-inset- follicular layer paving (CF), thin zona pellucida (two arrow) and cortical alveoli (thin arrow). F-inset- follicular layer cubic (CF), thick zona pellucida (two arrow). G-inset – micropile (m) and thicker zona pellucida. Legends – n- nucleo; AC – cortical alveoli; GV – yolk globules; CF- follicular layer; thick arrow – prefolliculogenics cell.H.E. reticulin

References

França, G. F., Grier, H. J., Quagio-Grassiotto, I.2010. A new vision of the origin and the oocyte development in the Ostariophysi applied to Gymnotus sylvius (Teleostei: Gymnotiformes). Neotropical Ichthyology, 8(4):787-804.

Grier, H.J., Uribe, M.C., Parenti, L.R. 2007. Germinal epithelium, folliculogenesis and postovulatory follicles in ovaries of rainbow trout, Oncorhynchus mykiss (Walbaum, 1792) (Teleostei, Protacanthopterygii, Salmoniformes). Journal of Morphology, 268:293-310.

Selman K, Wallace RA. 1989. Cellular aspects of oocyte growth in teleosts. Zool Sci 6:211–231.

Vazzoler, A.E.A.M. 1996. Biologia da reprodução de peixes teleósteos: teoria e prática. Maringá: EDUEM/NUPÉLIA,169p.

P-114
INDUCTION OF OVULATION IN THE PIKEPERCH (*SANDER LUCIOPERCA*) BY OVARIAN LAVAGE

Ádám Németh[1], Kornél Orbán[2], Petra Faidt[2], Ákos Horváth[3], Tamás Müller[3], László Horváth[2,3]

[1] *Tógazda Halászati Zrt. H-2240 Százhalombatta, Arany János u. 7., Hungary*
[2] *Attalai Hal Kft. H-7252 Attala, Halászház, Hungary*
[3] *Department of Aquaculture, Szent István University, H-2100 Gödöllő, Páter Károly u. 1., Hungary*

E-mail: Horvath.Akos@mkk.szie.hu

Introduction
The pikeperch (*Sander lucioperca*) is a predatorial percid species farmed in Central European pond aquaculture in order to control the stocks of smaller cyprinids that would represent food competition for the cultured common carp. Due to its high sensitivity to handling, induced hatchery spawning was considered problematic until recently. Several studies have been published on the hormonally induced spawning of this species (Zakes and Szczepkowski, 2004; Zarski et al., 2011) which will facilitate the adoption of these methods into the hatchery practice.To avoid injuries related to injection of fish alternative hormone administration methods are investigated that could later be used for hatchery spawning of the species.

The objective of this study was to investigate the possibility of ovarian lavage, a non-invasive direct hormonal injection into the ovary of the pikeperch through a catheter to induce ovulation.

Materials and methods
Sexually mature pikeperch were maintained at the hatchery of Attalai Hal Kft. (Attala, Hungary). Females were distributed into three groups: 10 were injected intramuscularly with 4 mg per kg of body weight of carp pituitary; 11 fish were injected with the same dosage of carp pituitary delivered through a silicone catheter (1 mm internal and 2 mm external diameter) directly into the ovary via the oviduct; 11 fish received no hormonal treatment only intramuscular saline solution and served as the control. Groups were placed into 12 m^2 concrete tanks with 2 male individuals in each tank to stimulate spawning and to help display spawning behavior. Ovulation (appearance of eggs) was checked in two-hour intervals by a gentle pressure to the abdomen. Ovulated eggs were collected into dry plastic bowls and the weight of eggs collected from each individual was recorded. The initial weight of spawners and the weight of collected eggs was used to calculate the Pseudo-Gonado-Somatic Index (PGSI) using the following formula: PGSI = weight of eggs / weight of fish prior to spawning × 100. Relationships of body length and stripped egg weight were also investigated.

Results
The total body length of fish used in the experiments was 55 ± 10 cm, while their body weight was 2187 ± 616 g (n = 32). Of the 10 fish injected intramuscularly, 7 (70%) produced eggs within 76 hours post-injection, while of the 11 individuals that received ovarian lavage eggs were collected from 10 fish (91%). In the control group spontaneous ovulation (without hormonal treatment) occurred in one fish (9%). PGSI of ovulated fish in the group injected inramuscularly was 8.8 ± 2.6 % while that of individuals treated with an intra-ovarian hormonal administration was 7.4 ± 3.5 %, with no significant difference (t-test, $P = 0.306$) between the two results. Overall a significant and relatively strong correlation was found between the standard length of fish and the weight of stripped eggs in this experiment (Fig. 1).

Discussion
Ovarian lavage has successfully been applied to species of fish that do not respond to conventional hormonal injection, such as *Tetraodon nigroviridis* and *Mastacembelus erythrotaenia* (Watson et al., 2009). The current study shows that the presented non-invasive method of hormonal injection is similarly suitable for the induction of ovulation in the pikeperch as the traditionally used intramuscular injection of hormonal products. Injected hormones need to enter the blood circulation of fish even if they are applied in the site

of action. Our results indicate that this can successfully be achieved without compromising the effectiveness of the hormonal treatment. Administration of hormone products or other liquid chemicals directly into the ovary carries further possibilities, such as the local application of medicaments.

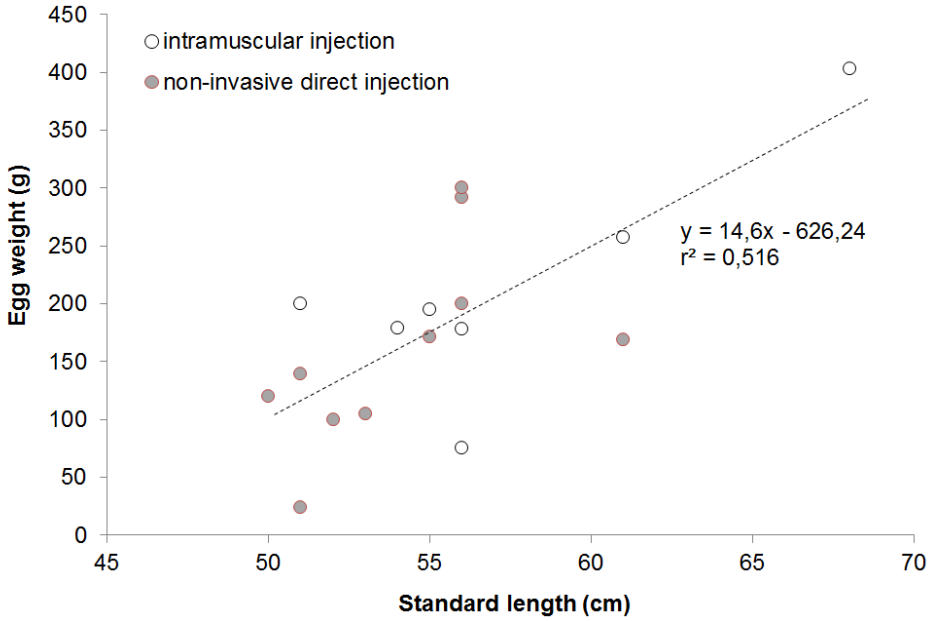


Figure 1. The relationship of fish length and weight of stripped eggs of the pikeperch

REFERENCES

Zakes, Z., Szczepkowski, M. 2004: Induction of out-season spawning of pikeperch, *Sander lucioperca*(L.), Aquac. Int. 12, 11–18.

Żarski, D., Kucharczyk, D., Targońska, K., Palińska, K., Kupren, K., Fontaine, P., Kestemont, P. 2011: A new classification of pre-ovulatory oocyte maturation stages in pikeperch, Sander lucioperca (L.), and its application during artificial reproduction Aqua Res (in press) DOI: 10.1111/j.1365-2109.2011.02879.x

Watson, C., Wood, A., Graves, JS. 2009: New technique for administration of human chorionic gonadotropin – Ovarian lavage. Aquaculture America, Book of abstracts, p. 394.

P-115
FATTY ACID COMPOSITION OF THE HEAD MEMBRANE AND FLAGELLA AFFECTS *SPARUS AURATA* SPERM QUALITY

J. Beirão[1], L. Zilli[2], S. Vilella[2], E. Cabrita[3], C. Fernández-Díez[1], R. Schiavone[2], M.P. Herráez[1]

[1] *Dept. of Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain*
[2] *Dept. of Biological and Envir. Sciences and Tech., Lab. of Comparative Physiology, University of Salento, Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy*
[3] *ICMAN-Institute of Marine Science of Andalusia, Spanish National Research Council, Avda. República Saharaui 2, 11510 Puerto Real, Cádiz, Spain*

E-mail paz.herraez@unileon.es

Introduction

Plasma membrane of sperm cells is richer in polyunsaturated fatty acids (PUFA) than that of somatic cells, providing higher membrane fluidity. Lipids are also present in seminal plasma and the sperm content on fatty acids affect functions such as motility and fertilizing capacity (Lahnsteiner *et al.*, 2009). Different studies with mammalian sperm have positively correlated the PUFA amount of the total sperm with different quality parameters (Aksoy et al., 2006; Am-in et al., 2011). Lipids from plasma membrane are related to their specific functions and could be differentially distributed in the membrane domains with differential characteristics. In the present study the lipid composition of plasma membrane from head and flagella will be analyzed and correlated to sperm function.

Material and methods

Samples collection and quality evaluation

Individual sperm samples from *Sparus aurata* (n=10) were obtained from the Aquaculture Research Center of Acquatina (University of Salento, Italy) in November. Samples were checked for motility using the CASA system Hobson Sperm Tracker and associated software (Hobson Vision Ltd., Baslow, UK) and viability under a fluorescent microscope using the fluorescent "live/dead Kit" from Molecular Probes (Invitrogen, Italy).

Head plasma membrane (HM) and flagella isolation

Samples were washed with 1% NaCl to eliminate the seminal plasma. Head and flagella were mechanically separated passing ten times through a 50 cm × 0.5 mm i.d. capillary. Samples were layered over sucrose gradient 0.5 – 2 M and ultracentrifuged at 28 000 × g for 45 min. The upper band (0.5M sucrose) consisting of flagella and a lower band (between 1.5 and 2M sucrose) containing spermatozoa heads were recovered. Flagella were pelleted and used for lipid extraction. Head suspensions were pelleted and exposed to a hypoosmotic shock with distilled water allowing us to lyse the heads. Head suspensions were centrifuged to sediment the cellular debris and the supernatants containing the HM were pelleted by ultracentrifuge and used for lipid extraction.

Fatty acids analysis

Lipids were extracted from flagella and HM samples according to Folch *et al.* (1957). Samples were transmethylated following the protocol by Berry *et al.* (1965) and the fatty acids analyzed by gas chromatography.

Results and discussion

Flagella had higher proportion of unsaturated fatty acids, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) both n3 and n6 compared with the HM, on the other hand HM presented higher proportion of saturated fatty acids (Table 1). According to Connor et al. (1998) studying the monkey sperm, the higher content in unsaturated fatty acids of the flagella is related with the need of higher fluidity for the flagellar beating.

Head membrane fatty acids affected mainly the sperm viability whereas flagella fatty acid composition affected specially the motility (Table 2). The proportion of saturated fatty acids was negatively correlated with the sperm quality (viability and motility), conversely unsaturated fatty acids proportion was positively correlated with the sperm quality parameters. In the case of PUFA, n3 PUFA were more relevant in the flagella composition affecting both motility and viability while n6 were more relevant in the HM. According to most authors working with mammalian sperm (Am-in et al., 20011) an increase in the n3/n6 ratio affects positively the sperm quality. Furthermore in the study by Connor et al. (1998) this ratio was higher in the flagella, while in our case no difference was observed. In any case both n3 and n6 PUFAS and their balance are important for the correct function of the sperm.

Table 1 – Mean fatty acid composition of the Flagella and HM.

	Flagella	Head Membranes
Saturated	**31.3 ± 2.5***	**66.4 ± 5.0**
MUFA	**17.3 ± 0.7***	**9.6 ± 1.4**
PUFA	**51.4 ± 2.4***	**23.9 ± 3.7**
n3	**42.1 ± 2.0***	**19.3 ± 3.0**
n6	**9.3 ± 0.5***	**4.7 ± 0.8**
n3/n6	4.6 ± 0.2	5.1 ± 1.5
EPA/AA	55.7 ± 7.6	90.0 ± 18.0
DHA/EPA	2.2 ± 0.1	1.9 ± 0.2

Significant differences detected with the non-parametric test Mann-Whitney U (P<0.05) are signed with * and bolt (n=10).

Table 2 – Significant correlations obtained between the percentage of motile and viable cells and the fatty acid composition of the HM and Flagella.

	% Motile		% Viable	
	HM	Flagella	HM	Flagella
Saturated	-0.747	**-0.670(*)**	**-0.793(*)**	-0.622
MUFA	0.742	-0.023	**0.792(*)**	-0.045
PUFA	0.733	**0.707(*)**	**0.776(*)**	**0.663(*)**
n3	0.680	**0.752(*)**	0.724	**0.723(*)**
n6	**0.805(*)**	0.316	**0.839(*)**	0.235
n3/n6	-0.480	0.314	-0.505	0.389
EPA/AA	0.535	0.309	0.549	0.351
DHA/EPA	**-0.842(*)**	0.021	**-0.894(**)**	0.01

Pearson's correlation P<0.05 signed with *, P<0.01 signed with **.

Conclusions

Flagella and HM presented different fatty acid composition according their function. Flagella fatty acid composition seems to be more related with the need of fluidity for the flagellar beating On the other hand, HM fatty acid composition seems to be more related with preservation of its membrane integrity. Differences between males on fatty acid composition affect both sperm motility and viability which will probably have effects on fertilization ability.

Acknowledgments

The authors are grateful to Dr Catherine Labbé from INRA (France) for providing the protocols for lipids analysis and to Francisca Martínez from the ULE-LTI (Spain) for the technical work with the fatty acids analysis. This work was supported by the project Petri (PTR 95.1026.OP.01). J. Beirão was supported by a PhD grant from the Portuguese Ministry of Science and Technology (SFRH/BD/31990/2006) co-founded by POPH - QREN (FEDER and MCTES).

References

Aksoy, Y.; Aksoy, H.; Altinkaynak, K.; Aydin, H.; Ozkan, A., 2006: Sperm fatty acid composition in subfertile men. Prostaglandins Leukot. Essent. Fatty Acids 75, 75-79.

Am-In, N.; Kirkwood, R.; Techakumphu, M.; Tantasuparuk, W., 2011: Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology 75, 897-903.

Berry, J.; Cevallos, W.; Wade, R., 1965: Lipid class and fatty acid composition of intact peripheral nerve and during wallerian degeneration. J. Am. Oil Chem. Soc. 42, 492-500.

Connor, W.; Lin, D.; Wolf, D.; Alexander, M., 1998: Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. J. Lipid Res. 39, 1404-1411.

Folch, J.; Lees, M.; Stanley, G., 1957: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Lahnsteiner, F.; Mansour, N.; McNiven, M.; Richardson, G., 2009: Fatty acids of rainbow trout (Oncorhynchus mykiss) semen: Composition and effects on sperm functionality. Aquaculture 298, 118-124.

P-116
COMMON CARP (CYPRINUS CARPIO) MALE FERTILIZATION POTENCY WITH SECURE NUMBER OF SPERMATOZOA PER OVA

Otomar Linhart, Marek Rodina, S. M. Hadi Alavi, Vojtěch Kašpar

University of South Bohemia České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, 38925 Vodňany, Czech Republic

E-mail: linhart@frov.jcu.cz

Introduction

Genetic consequences of sperm competition have been studied in salmonids (McKay and McMillan, 1991) and in common carp (Cyprinus carpio) (Linhart et al., 2005, Kaspar et al., 2007, 2008). One of the main practical problems caused by sperm competition is unequal representation of males in hatchery progeny, when pooled sperm is used for fertilization. In case of common carp, it results in reduction of the effective size of the populations and a dramatic loss of genetic variability (28.4-42.2 %; Linhart et al., 2005; Kašpar et al., 2007, 2008).

A possible way to eliminate the consequences of sperm competition in common carp is to divide eggs into batches equal to the number of males used and to fertilize themseparately, before combining them for incubation (Vandeputte et al., 2004, Kocour et al., 2007). By this methodology, reduction of number of effective males in fertilization trials was finally lower, meaning 4.2 % (Vandeputte et al., 2004) and 14.9 % (Kocour et al., 2007) reductions. If separate fertilization of egg batches is used, it is possible to obtain male representations in progeny, that are close to the optimum of male distributions. However, there is still quite large loss of genetic variability. The above mentioned approach could be recommended for the propagation of carp brood stocks in hatcheries in the absence of a good predictor of male fertilization potency, with emphasis on secure number of spermatozoa per ova.

Therefore, the present study was conducted on male common carp fertilization potency using in vitro separate fertilization tests, which are close to fundamental characteristics of the natural fertilization (Linhart et al., 2003). The main objective was to look for fertilization potency of different males when different sperm quantity per ova of single female was examined.

Materials and methods

For experiment, 10 males were injected with carp pituitary (CP) at 1 $mg.kg^{-1}$, 24 h before stripping at 21°C. Among them, sperm of 5 males representing very good sperm motility were used after checing the motility of sperm (Linhart et al. 2005). Five females were also injected with CP at the dose of 0.4 $mg.kg^{-1}$ and then at 2.1 $mg.kg^{-1}$, 24 h and 12 h before stripping, respectively (Linhart et al. 2003). For the experiment, the ova from one female exhibiting the best apparent quality with visual inspection were used. Sperm concentration was calculated 25.7, 20.3, 27.3, 26.1 and 23.3 x 10^9 spermatozoa per ml for the 5 males. The sperm of each male at volumes 5, 10, 20, 40 and 400 μl were pre-diluted with 995, 990, 980, 960 and 600 μl of Kurokura solution (Linhart et al., 2000), respectively. Then, 4 000 eggs and pre-diluted spermatozoa from each male, one by one, were simultaneously added with 1 000, 5 000, 10 000, 20 000 and 200 000 spermatozoa and activated with hatchery water.

Results

Sperm motility and velocity

After activation of sperm motility in distilled water, sperm motility and velocity in all five males decreased at 15, 30, 45 and 60 s post-activation. Initial sperm motility was in range of 89.5 - 97.2 % at 15 s and decreased to 19.1 - 30.2 % at 60 s post-activation. In all evaluated times post-activation, sperm motility did not differ significantly among the males. Sperm velocity decreased from 126.1 - 161.2 $\mu m.s^{-1}$ at 15 s to 11.9 - 35.2 $\mu m.s^{-1}$ at 60 s post-activation. Sperm velocity was significantly different among males at 15 s post-activation; males 1 represent the highest sperm velocity and males 2 and 5 showed the lowest sperm velocity.

Effects of different spermatozoa per ova and different males

Fertilization and hatching rates were progressively decreased from 200 000 to 10 000, 5 000 and 1 000 spermatozoa per ova. But, at 20 000 spermatozoa per ova similar results were observed compared to 200 000 and/or 10 000 spermatozoa per ova. Fertilization and hatching rates were 95 and 86 %, respectively, when 200 000 spermatozoa per ova was examined. At 10 000 spermatozoa per ova, fertilization and hatching were 84 and 78 %, respectively. The lowest fertilization and hatching rates (28 and 23 %) were observed at 1000 spermatozoa per ova.

Multiple effects of different spermatozoa per ova and different males

Fertilization and hatching rates were similar in all males at higher examined number of spermatozoa per ova (20 000 and 200 000). Similar fertilization and hatching rates were observed in four males out of five at 10 000 spermatozoa per ova. Lower spermatozoa per ova (5 000) induced very different results from 48 to 82 % for fertilization rate and from 42 to 72 % for hatching rate. At 1 000 spermatozoa per ova, very high variability was observed; 10 to 50 % for fertilization rate and 8 to 43 % for hatching rates. These results did not correspond with sperm velocity among males.

Discussion and conclusion

The extent of sperm competition is very important for the logistics of genetic programs, because the common practice of pooling sperm from different males exhibits very detrimental effects if sperm competition levels are high (McKey and McMillan, 1991). Consequently, pooling sperm from different males resulted in much lower number of males being effectively represented in offspring. Using separate fertilizations of egg batches to obtain male representations in progeny is close to the optimum of males distribution as has been demonstrated by Vandeputte et al. (2004) and Kocour et al. (2007), but still there was quite large loss of genetic variability. This study suggests that 20 000 spermatozoa per ova may partly avoid such loss of genetic variability and such level of sperm can be recommended for artificial propagation of carp broodstocks in hatcheries. To our knowledge, this is the first observation showing that 20 000 spermatozoa is consider as secure number of spermatozoa to reach high fertilization in common carp. The result may guarantee secure fertilization during factorial designs of fertilization for carp broodstocks in hatcheries. The results of present study are important for hatcheries, where further broodstock for next generation will be generated to avoid loss of genetic variability, but not necessarily for the mass production of fingerlings for stocking grow-out ponds.

Acknowledgements

This study was supported by CZ.1.05/2.1.00/01.0024, GAJU 046/2010/Z, GAJU 047/2010/Z, IAA 608030801, ME10015, QH82119, LC06073.

References

Kaspar, V.; Kohlmann, K.; Vandeputte, M.; Rodina, M.; Gela, D.; Kocour, M.; Alavi, S.M.H.; Hulak, M.; Linhart, O., 2007: Equalization of sperm concentrations in a pool of sperm does not prevent large variance in males contribution in common carp progeny. Aquaculture 272, S204–S209.

Kaspar, V.; Vandeputte, M.; Kohlmann, K.; Hulak, M.; Rodina, M.; Gela, D.; Kocour, M.; Linhart, O., 2008: A proposal and case study towards a conceptual approach of validating sperm competition in common carp (Cyprinus carpio L.), with practical implications for hatchery procedures. J. Appl. Ichthyol. 24, 406-409.

Kocour, M.; Mauger, S.; Rodina, M.; Gela, D.; Linhart, O.; Flajshans, M.; Vandeputte, M., 2007: Heritability estimates for processing traits in common carp (Cyprinus carpio L.) using a molecular pedigree. Aquaculture 270, 43–50

Linhart, O., Rodina, M. and Cosson, J., 2000. Cryopreservation of sperm in common carp Cyprinus carpio: Sperm motility and hatching success of embryos. Cryobiology 41, 241-250

Linhart, O.; Rodina, M.Gela, D.; Kocour, M.; Rodriguez, M., 2003: Improvement of common carp artificial reproduction using enzyme for elimination of eggs stickiness. Aquat. Liv. Resour. 16, 450-456.

Linhart, O.; Rodina, M.; Gela, D.; Kocour, M.; Vandeputte, M., 2005: Spermatozoal competition in common carp (Cyprinus carpio): what is the primary determinant of competition success? Reproduction 130, 1–8.

McKay, L.R.; McMillan, I., 1991: Effect of sperm pooling on inbreeding levels, Bull. Aquac. Assoc. Can. 3, 16–18.

Vandeputte, M.; Kocour, M.; Mauger, S.; Dupont-Nivet, M.; De Guerry, D.; Rodina, M.; Gela, D.; Vallod, D.; Chevassus, B.; Linhart, O., 2004: Heritability estimates for growth-related traits using microsatellite parentage assignment in juvenile common carp (Cyprinus carpio L.). Aquaculture 235, 223–236.

P-117
MOTILITY AND VIABILITY PARAMETERS OF SHORT-TERM STORED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) SEMEN IN RELATION TO THE SPERM MOTILITY RATE AT PH 6.5

J. Nynca[1], G.J. Dietrich[1], S. Dobosz[2], H. Kuźmiński[2], A. Ciereszko[1]

[1] *Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-747 Olsztyn, Poland*

[2] *Inland Fisheries Institute, Department of Salmonid Research, Rutki 83-330 Żukowo, Poland*

E-mail: j.nynca@pan.olsztyn.pl

Introduction

The assessment of the quality of fish spermatozoa, comprising the evaluation of motility and viability, is important for the improvement of artificial fertilization procedure in hatcheries. However, the relationship between these parameters is unclear. Previously, we demonstrated that rainbow trout spermatozoa can be activated at pH 6.5 (Ciereszko et al., 2010). We suggested, that motility at pH 6.5 is affected by urine contamination of semen. If this suggestion is correct, samples of semen that cannot be activated at pH 6.5 are of poor quality. In this study, we described simultaneous changes in sperm viability and motility parameters during an 8–day liquid storage of rainbow trout semen. The relationship between these parameters was evaluated and related to the sperm motility rate at pH 6.5 measured in freshly collected semen. We sought to determine if sperm motility at pH 6.5 can be an indicator of semen quality and possibly its usefulness during short-time storage of semen.

Materials and methods

Milt was collected during spring spawning from 16 rainbow trout males. Individual milt samples (0.5 cm depth) were stored without antibiotics at 4°C for eight days under oxygen atmosphere. At the beginning of the experiment, the percentage of spermatozoa which can be activated at pH 6.5 of each fresh semen sample was evaluated. Every day of storage, the motility and viability parameters for each sperm sample were examined under standard conditions for rainbow trout semen (pH 9.0). The motility of the spermatozoa was measured and analyzed using Hobson Sperm Tracker (Hobson Vision Ltd, Baslow, UK) as previously described (Dietrich et al., 2005). The viability of spermatozoa was measured using NucleoCounter SP-100 (Chemometec, Denmark) as described by Nynca and Ciereszko (2009). The sperm samples were centrifuged at 8000×g to obtain seminal plasma for osmolality measurement (Vapor Pressure Osmometer 5520; WESCOR, Logan, USA). All analyses were performed at a significance level of 0.05 using GraphPad Prism software (GraphPad Software Inc, San Diego, CA, USA).

Results

The percentage of viability and motility of rainbow trout spermatozoa decreased in relation to the time of storage ($p<0.001$, Fig. 1). From the second day of storage the percentage of viability was consistently higher compared to the percentage of motility. These parameters significantly correlated from day 6 of storage ($r=0.67$, $p<0.01$; $r=0.57$, $p<0.05$; $r=0.77$, $p<0.001$ for day 6, 7, 8, respectively).

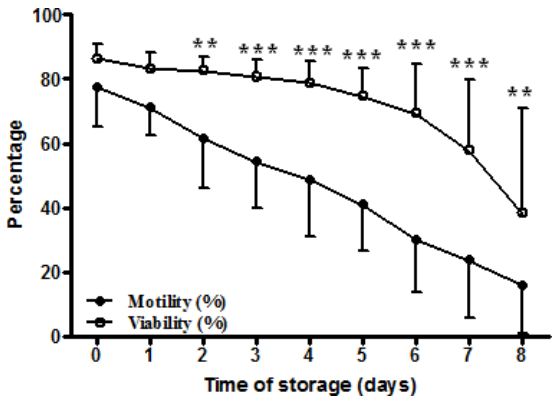


Fig. 1. *The effect of storage time on the percentage of rainbow trout sperm motility and viability (n=16). The general effect of storage time was found ($p<0.001$). The statistical interaction between sperm viability and motility values was recorded. Asterisks indicate significant differences between motility and viability during storage (**$p<0.01$; ***$p<0.001$).*

After day 8, the final viability (38.2 ± 32.5%) was 56% lower compared to the initial value (86.4 ± 4.5%), whereas the average percentage of motility decreased about 80% (from 77.4 ± 12.1% to 16.0 ± 15.2%). The motility rate which can be activated at pH 6.5 was related to the higher sperm motility during storage (Fig. 2A), nevertheless such an effect on viability was found only on the last day of storage (Fig. 2B). Semen samples with the motility at pH 6.5 were characterized by higher seminal plasma osmolality (245.4 ± 36.6 mOsm kg^{-1}) compared to immotile ones (184.9 ± 42.2 mOsm kg^{-1}).

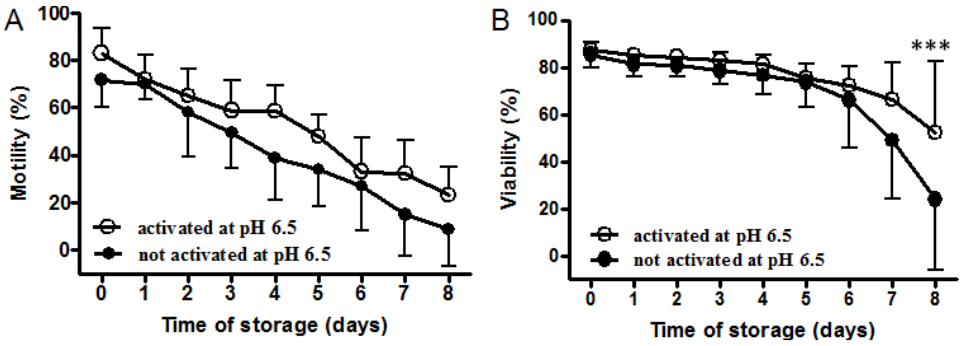


*Fig. 2. Sperm motility (A) and viability (B) during the 8-day storage in relation to the sperm motility rate activated at pH 6.5. The general effect of storage time ($p<0.001$) and sperm motility at pH 6.5 ($p<0.05$) were found. Asterisks indicate significant differences between the viability of immotile and motile sperm at pH 6.5 during storage (***$p<0.001$).*

Discussion and conclusions

In this study, within the same milt samples the viability was always higher than motility parameters during storage. It can be assumed that short-time storage has no distinct negative effect on the sperm membranes, contrary to motility. Possibly, sperm storage can lead to the exhaustion of endogenous ATP stores resulted in the decrease in sperm motility, but membrane integrity was preserved at the same time. Further studies focused on the monitoring of ATP reduction in spermatozoa during storage together with changes in motility and viability should be conducted in order to elucidate the relationship between motility and viability of rainbow trout spermatozoa during short-time storage. In our opinion sperm motility is more appropriate compared to viability in the evaluation of sperm quality in hatchery conditions, when a quick evaluation of milt for fertilization is required. Sperm motility rate at pH 6.5 was related to the higher sperm motility during the 8-day storage. The sperm motility rate at pH 6.5 is likely associated with low contamination of milt samples with urine. These results indicate better usefulness of sperm activated at pH 6.5 for short-time storage. Therefore, it can be proposed that the testing of rainbow trout spermatozoa activation at pH 6.5 can be used in practical hatchery conditions for the evaluation of rainbow trout sperm quality and to predict semen usefulness for short-time storage.

Acknowledgements

This study was supported by funds appropriated to Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences.

References

Ciereszko, A.; Dietrich, G.J.; Dietrich, M.; Nynca, J.; Kuźmiński, H.; Dobosz, S.; Grudniewska, J., 2010: Effects of pH on sperm motility in several Salmoniformes species: Oncorhynchus mykiss, Salvelinus fontinalis, Salmo trutta, Salmo salar and Thymallus thymallus. J. Appl. Ichthyol. 26, 665-667.

Dietrich, G.J.; Kowalski, R.; Wojtczak, M.; Dobosz, S.; Goryczko, K.; Ciereszko, A., 2005: Motility parameters of rainbow trout (Oncorhynchus mykiss) spermatozoa in relation to sequential collection of milt, time of post-mortem storage and anesthesia. Fish Physiol. Biochem. 31, 1-9.

Nynca, J.; Ciereszko, A., 2009: Measurement of concentration and viability of brook trout (Salvelinus fontinalis) spermatozoa using computer-aided fluorescent microscopy. Aquaculture 292, 256-258.

P-118
ISOLATION OF STURGEON SPERMATOGONIA AS MATERIAL FOR BIOTECHNOLOGICAL APPLICATIONS

Martin Psenicka[1], Taiju Saito[2,4], Shinji Adachi[3], Katsutoshi Arai[4], Etsuro Yamaha[2]

[1] *University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, Zatisi 728/II, 389 25 Vodnany, Czech Republic*
[2] *Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Nanae 041-1105, Japan*
[3] *Laboratory of Aquaculture Biology, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan*
[4] *Laboratory of Aquaculture Genetics & Genomics, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan*

E-mail: psenicka@frov.jcu.cz

Introduction
Most of sturgeons belong to endangered species mostly due to caviar and complications with their reproduction characteristics such as late maturation. Micromanipulations with germ line cells and embryos could provide us with a more efficient system of sturgeon reproduction. Primordial germ cells (PGCs) or subsequently spermatogonia are the only cells in developing embryos with potential to transmit genetic information to the next generation. Therefore they have a potential to be of value for gene banking particularly via the production of donor gametes with germ–line chimeras (Yamaha et al., 2003).

Visualization of PGCs in sturgeon using injection of GFP-zebrafish nos1 3'UTR mRNA, description of their migration and transplantation into goldfish embryos was done by Saito et al. (this proceedings). Nevertheless the yield of PGCs from one embryo is 23.5 on average. Spermatogonia Type A, having the ability of multiplication and differentiation into both types of gametes, could be an alternative of PGCs manipulations. The yield of spermatogonia from one animal can be huge. Okutsu. et al. (2007) described applying of spermatogonial transplantation in fish. Spermatogonia isolated from the testes of vasa-green fluorescent protein transgenic rainbow trout (*Oncorhynchus mykiss*) were transplanted into the peritoneal cavity of triploid masu salmon (*Oncorhynchus masou*) hatchlings of both genders. Nevertheless isolation of sturgeon spermatogonia has not been elaborated.

Materials and methods
The testes were collected from 4 years old Siberian sturgeon males. It was cut into small pieces and incubated in 0.2% collagenase for 2 hours. The obtained homogeneous suspension was filtered through 50 μm filter to remove large debris. The cell suspension was then loaded onto a discontinuous Percoll gradient (10, 15, 20, 25, 30, 35, 40, and 50% in PBS) and centrifuged at 800 × g for 30 min. Each cell fraction were removed from the gradient and transferred to a test tube and washed two times with PBS. The cells were re-suspended in PBS and stained with antispermatogonia-specific antigen 1 originally made for Japanese eel by Kobayashi et al. (1998) combined with Anti-Rabbit IgG–FITC antibody.

Results
The cells obtained from layer 10-30% of percoll solution showed the specific fluorescent signal and shape of spermatogonia. The layers below contained cells without almost any signal with spermatocyte-like shape. Isolation of spermatogonia from mature testes was contaminated with high amount of spermatozoa, although most spermatozoa were collected from layer of 50% percoll solution. Therefore the isolation from mature testes is not recommended.

Discussion
The isolated germ-line cells can be then cryopreserved according to Okutsu et al. (2007) and use for production of germ-line chimera. Donor genotypes can be restored in the next generation. We suggested that the long generation interval of sturgeon might be extremely shortened when species that mature earlier are used as the surrogate host and produce donor gametes.

Acknowledgements
I am grateful to all colleagues from Faculty of Fisheries Sciences and Nanae Fresh Water Laboratory of Hokkaido University and JSPS (P09325), who supported me during my fellowship in Japan.

References

Kobayashi T.; Kajiura-Kobayashi H.; Nagahama Y., 1998: A novel stage-specific antigen is expressed only in early stages of spermatogonia in Japanese eel, Anguilla japonica testis. Mol. Reprod. Dev. 51, 355–361.

Okutsu T.; Shikina S.; Kanno M.; Takeuchi Y.; Yoshizaki G., 2007: Production of trout offspring from triploid salmon parents. Science 317, 1517.

Yamaha E.; Murakami M.; Hada K.; Otani S.; Fujimoto T.; Tanaka M.; Sakao S.; Kimura S.; Sato S.; Arai, K., 2003. Recovery of fertility in male hybrids of a cross between goldfish and common carp by transplantation of PGC (primordial germ cell)-containing graft. Genetica 119, 121-131.

P-119
EFFECTS OF PH, SMOLARITY AND TEMPERATURE ON BLACK CONGER EEL SPERM MOTILITY (*GENYPTERUS MACULATUS*) (TSCHUDI, 1846) CULTURED UNDER LAB CONDITIONS

Iván Valdebenito[1], Cristian Lozano[1], Cristian Moreno[1], Juan M. Estrada[2], Alejandro Ramirez[2], Diego Ramirez[2], Andrea Ubilla[1]

[1] *Escuela de Acuicultura, Universidad Católica de Temuco, Rudecindo Ortega 02950 Casilla 15-D, Temuco-Chile*
[2] *CIMARQ, Universidad Andrés Bello, Santiago de Chile*

E-mail: ivisler@uct.cl

Introduction
Tascheri *et al,* (2003) refers to reproductive biology of eel species found on Chilean coasts. They define black conger eel as a spawner of asynchronous or partial kind, determining that the maximum maturity stadiums and spawning were observed in spring and mainly in summer, with a size over 65 cm, for females.

Yang and Tiersch (2009), claim that the knowledge of gametes of a species is necessary for artificial fertilization and cryopreservation of spermatozoa. There are no references in the literature regarding spermatology of black conger eel. There is just some information about the sperm ultrastructure in Japanese eel *(Conger myriaster)* (Okamura and Motonobu, 1999).

The aim of this study was to research the effect of pH, osmolarity and temperature in sperm motility of black eel (*Genypterus maculatus*), cultured under lab conditions.

Materials and methods
Four mature black conger eel males *(G. maculatus)* were used, cultured under lab conditions in the Center of Marine Research of Quintay (CIMARQ) of University Andrés Bello, located in the city of Quintay, 33°11'36.41"S and 71°42'9.12"O, Valparaíso Region, Chile. Once the maximum maturity of the subjects was determined, their gametes were extracted through stripping. Sperm was diluted (SD) in a relation 1:2 in a physiological solution 0,98% and later it was transferred to the Reproduction Laboratory of the Catholic University of Temuco (UCT), using oxygenated containers, maintaining a constant temperature of 7ºC.

For sperm activation, three solutions with different salinity proportions were used; a treatment (T) control© with 100% of sea water (AM) (928 mOsm Kg^{-1}); T1 with 90% of AM (754 mOsm Kg^{-1}); T2 with 75% AM (571 mOsm Kg^{-1}) and T3 with distilled water (mOsm Kg^{-1}). Osmolarities were determined by a Fiske Micro-Osmometer model 210, used on each activating solution. Additionally, these solutions were tested with three different temperatures (4º, 10º and 16º C). Besides, the pH of the control solution was adjusted at three different values (5, 7 and 9) estimating the effect on motility at an environmental temperature of 12-13ºC.

Results
The observations showed primitive spermatozoa with a big and slightly elongated head, lack of acrosome, a small midpiece with scarce mitochondrias and a long flagella. Regarding measurements for sperm structure, the head measured 1,87 ± 0,17µm long and 1,44 ± 0,21µm wide. Its midpiece was 0,89 ± 0,23µm long and the flagella measured an average of 69,17 ± 13,27µm long. The total length of spermatozoa was 71,93 ± 13,23µm. The sperm density from the semen pool was 7,49 ± 0,47 x 10^9 spermatozoa/ml and the average percentage of spermatocrit was 96,7 ± 5,8%.

Sperm motility
pH effect: The observed times were 1062 ± 119s for pH 5; 1457 ± 144s in pH 7 and 1260 ± 79s for pH 9. Statistically significant differences were found ($p<0,05$) in all the treatments.

Temperature and Osmolarity Effect: In T3, no sperm motility was registered in any medium. In the treatments with different osmolarities at different temperatures, T1 obtained a maximum time of 1470 ± 132s at 4ºC. Statistically significant differences were found with other osmolarities at these temperatures. While in the other temperatures, the higher averages were registered in control with 1244 ± 87s at 10ºC and 1207 ± 71s at 16ºC. Thus, there are statistically significant differences ($p<0,05$), in both temperatures between Control and T1 with T2 and T3.

Discussion and conclusions

Spermatozoa total length was 71,93 ± 13,23μm. This data is lower than the one described in red conger eel (*G. chilensis*) by Valdebenito (2011, unpublished data), who registered a spermatozoa total length of 89,46μm. The head measured 2,01 ± 0,15μm long and 1,59 ± 0,15 μm wide. As for the midpiece, it measured 0,85 ± 0,15μm and 85,01 ± 7,16μm for the flagella.

In the test of ph and temperature the maximum period of flagellar activity registered was up to 1400s, does not differ from the records by Valdebenito *et al.* (2011, unpublished data), in diluted and undiluted semen of red conger eel (*G. chilensis).* These results exceed those reported in the literature for marine species such as halibut (110-120s), Turbot (600s), sea bass (50-60s), salt cod (7-800s) and tuna (140s) (Cosson *et al.*, 2008a; 2008b)

This initial background is the first reported in sperm literature of *Genypterus maculatus,* thus more studies should be carried out to search for more biological information that will allow performing a more adequate "in vitro" management of gametes of this species.

Acknowledgments

Research funded by the projects **FONDEF D06I1020, D06I1024** and **D06I1028.**

References

Cosson, J.; Groison, A.; Suquet, M.; Fauvel, C.; Dreanno, C.; Billard, R., 2008a: Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460-486.

Cosson, J.; Groison, A.; Suquet, M.; Fauvel, C.; Dreanno, C.; Billard, R., 2008b: Marine fish spermatozoa: racing ephemeral swimmers. Reproduction 136, 277-294.

Okamura, A.; Motonobu, T., 1999: Spermatozoa of *Conger myriaster* observed by electron microscopy. Zool. Sci. 16, 927-933.

Tascheri, R.; Sateler, J.; Merino, J.; Díaz, E.; Ojeda, V.; Montecinos, M., 2003: Estudio biológico-pesquero congrio colorado, congrio negro y congrio dorado en la zona centro-sur. Instituto de Fomento Pesquero. Informe Final FIP Nº 2001-15. 356 pp.

Yang, H.; Tiersch, T., 2009: Sperm motility initiation and maduration in a euryhaline fish, medaka (*Oryzias latipes*). Theriogenology 72, 386-399.

P-120
FUNCTIONAL PRIMORDIAL GERM CELLS DIFFERENTIATE FROM EGG FRAGMENT IN GOLDFISH *CARASSIUS AURATUS*

Tomoko Kitauchi-Sekiguchi[1], Taiju Saito[1,3], Taizo Motomura[2], Katsutoshi Arai[3] and Etsuro Yamaha[1]

[1] *Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, 2-9-1 Sakura, Nanae, Kameda, Hokkaido, 041-1105, Japan*
[2] *Muroran Marine Station, Field Science Center for Northern Biosphere, Hokkaido University, 1-13 Bokoi-Minami-Machi, Muroran, Hokkaido, 051-0003, Japan*
[3] *Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido, 041-8611, Japan*

E-mail: taiju@hokudai-nanae.hokudai.ac.jp

Introduction

In teleost, primordial germ cells (PGCs), the precursors of germ cells, have a predetermination pattern of development (reviewed by Raz 2002). PGCs originate from blastomeres inherited maternal cytoplasm aggregated at the ends of the early cleavage furrows in several fish species (reviewed in Yamaha et al., 2010). Several maternal mRNAs, such as *vasa*, *nanos*1, *H1M, brul* and *zDazl*, are included in the cytoplasm. Ultrastructurally, a unique cytoplasmic structure is observed at the ends of cleavage in zebrafish (Knaut *et al*., 2000) and ice goby (Miyake *et al*., 2006). In zebrafish, several mRNAs show different special distribution in egg and aggregation pattern during blastodisc formation (Theusch et al., 2006). These mRNAs distribute the cortical part of egg. There are, however, many suspended particles in its cytoplasm. It is still unknown that factors responsible for PGC specification distribute only around the egg cortex. In goldfish, we can experimentally get the cytoplasmic fragment of fertilized egg without suspended particles. In the present study, we show in goldfish that functional PGCs can differentiate from the cytoplasmic fragment derived from centrifuged eggs.

Materials and methods

Eggs and sperm were obtained from mature goldfish, *Carassius auratus*, and fertilized in fertilization solution (0.2% urea and 0.25% NaCl in tap water).

Centrifugation of fertilized eggs was performed as follows. Eggs were fertilized in plastic dishes. The plastic dishes were placed in the swing rotor of a centrifuge apparatus (KUBOTA; KN-70). After 10 min at room temperature, the eggs were centrifuged at 700 x g for 10 min. After centrifugation, chorion was removed by trypsin solution (0.1% trypsin and 0.4% urea in Ringer's solution, pH. 7). For labeling of cytoplasm, artificial mRNA (GFP-zebrafish *nos*1 3'UTR) and biotin-dextran in 0.2M KCl were co-injected into egg cytoplasm.

Transparent cytoplasm of centrifuged eggs and cytoplasm of normal eggs were cut off from the centrifuged egg by using fine glass wool, avoiding injury. The excised blastodiscs from eggs developed into spherical embryos when the control embryos were blastula stage. A spherical embryo was cut to two hemispheres and transplanted onto animal part of a normal blastula. PGCs in the operated embryos were observed under fluorescent microscope, and detected histo-chemically on paraffin sections at 10 days after fertilization.

A part of subsequent embryos without labeling are fixed with glutaraldehyde followed by osmium tetraoxide for transparent electron microscopy (TEM), or with paraformaldehyde for whole mount *in situ* hybridization (WMISH) at early cleavage stage. TEM samples were dehydrated through an acetone series, and finally embedded in Spurr's resin (TAAB). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEOL 1011 electron microscope. WMISH for *vasa* mRNA and histological analysis of PGC are also performed by the method according to Otani et al. (2005).

Results

The cytoplasmic fragment excised from centrifuged eggs clearly had fewer granules, while that from denuded control eggs contained many granules and yolk. Cleavage was observed in most excised fragments from both centrifuged and control eggs. The cleavage occurred holoblastically in cytoplasmic fragment excised from centrifuged eggs. Notably, the sizes of the blastomeres in the fragments of centrifuged eggs showed considerable variation. In excised fragments without granules, histological analysis revealed that cytoplasmic bridges connected the blastomeres during early cleavage stages. Clear nuclei were detected in the blastomeres produced by fragments from centrifuged eggs, but not in those from denuded control eggs. The fragments developed into spherical embryos by about 9 hours post-fertilization; at this time control embryos were at the late blastula stage.

A few *vasa* signals by WMISH were detected at junctions between the blastomeres produced by holoblastic segmentation of the fragments. The signal in the excised fragment from the centrifuged eggs was larger in size than that observed at the ends of early cleavage furrows in normal development.

Electron-dense, amorphous structures were detected at the position which *vasa* mRNA signals were detected, when ultrastructural observation was performed at two- to four-stage embryos derived from excised fragments of centrifuged eggs. The amorphous structures did not have a membrane, and were composed of aggregations of elliptical objects and showed a speckled pattern with electron-dense and light regions.

The embryos transplanted with spherical embryos developed normally. Many PGCs were located on the normal route of migration of PGCs to the genital ridge and a few were also present around the otic vesicles. Biotin-labeled PGCs were observed histologically around the genital anrage in 10 days post-fertilization larvae. Larger numbers of labeled-PGCs were observed around the gonadal anlage in larvae resulting from transplantation of spherical embryos from centrifuged eggs (mean=12.7, N=19) than from normal eggs (mean=6.2, N=12)

Discussion and conclusions

WMISH and ultrastructural analyses of the fragments from the centrifuged eggs showed that *vasa* signals and electron-dense structures were present at the same position between blastomeres that developed from excised cytoplasmic fragments of centrifuged eggs. These electron-dense structures were similar to those reported previously in fish and *Drosophila* germ plasm (zebrafish, Knaut *et al*., 2002, ice goby, Miyake *et al*., 2006; *Drosophila*, Mahowald, 1968). Therefore, all factors responsible for PGC specification distribute in egg cytoplasm without suspended granules.

Acknowledgements

This work was supported in part by Grant-in-Aid for the Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) from the Bio-oriented Technology Research Advancement Institution (BRAIN) of Japan.

References

Knaut, H., Pelegri, F., Bohmann, K., Schwarz, H. and Nüsslein-Volhard, D. (2000) Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. J. Cell Biol. 149: 875-888.

Knaut, H., Steinbeisser, H., Schwwarz, H., and Nüsslein-Volhard, C. (2002) An evolutionary conserved region in the vasa 3'UTR targets RNA translation to the germ cells in the zebrafish. Curr. Biol. 12: 454-466.

Mahowald, A.P. (1968) Polar granules of Drosophila. Ⅱ. Ultrastructural changes during early embryogenesis. J. Exp. Zool. 167: 237-61.

Miyake, A., Saito, T., Kashiwagi, T., Ando, D., Yamamoto, A., Suzuki, T., Nakatsuji, N. and Nakatsuji, T. 2006. Cloning and pattern of expression of the shiro-uo vasa gene during embryogenesis and its roles in PGC development. Int. J. Dev. Biol. 50: 619-625.

Otani, S., Kitauchi, T., Sakao, S., Saito, T., Maegawa, S., Inoue, K., Arai, K. and Yamaha, E. (2005) The formation of PGCs from the germline cells in spherical embryos derived from the blastodisc of 2-cell embryos in goldfish, Carassius auratus. Int. J. Dev. Biol. 49: 843-850.

Raz, E (2002) Primordial germ cell development in zebrafish. Semin Cell Dev Biol 13: 489-495.

Theusch, E.V., Brown, K.J. and Pelegri, F. (2006) Separate pathways of RNA recruitment lead to the compartmentalization of the zebrafish germ plasm. Dev. Biol. 292:129-41.

Yamaha, E., Goto-Kazeto, R., Saito, T., Kawakami, T., Fujimoto, T., Adachi, S. and Arai, K. (2010) Primordial germ cell in teleost fish with special references to its specification and migration. J. Appl. Ichthyol. 26: 816-822.

P-121
THE EFFECTIVNESS OF SELECTED HORMONAL PREPARATIONS IN STIMULATING SPERMIATION IN THE COMMON DACE *LEUCISCUS LEUCISCUS* (L.)

Beata Irena Cejko[1], Katarzyna Targońska[2], Radosław Kajetan Kowalski[1], Daniel Żarski[2], Beata Sarosiek[1], Dariusz Kucharczyk[2], Jan Glogowski[1,3]

[1] *Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Ford Research, Polish Academy of Science, Olsztyn, Poland*
[2] *Department of Lake and River Fisheries, Faculty of Environmental Sciences and Fisheries, University of Warmia and Mazury, Olsztyn, Poland*
[3] *Department of Ichthyology, Faculty of Environmental Sciences and Fisheries, University of Warmia and Mazury, Olsztyn, Poland*

E-mail: cejko@pan.olsztyn.pl

Introduction
In wild populations of rheophilic fish, hormonal stimulation is necessary and entirely responsible for ovulation in females (Targońska et al. 2010). An appropriately chosen and applied hormonal preparation also significantly increases the volume of milt obtained and sperm count, as well as leads to an increase in the percentage of sperm motility in males taken from their natural environment (Cejko et al. 2010). The available literature lacks detailed information regarding the possibility of increasing the effectiveness of common dace *Leuciscus leuciscus* (L.) reproduction in a controlled environment (Kucharczyk et al. 2008). There is also an insufficient amount of data regarding the effectiveness of various hormonal preparations in stimulating spermiation in this species of fish. The purpose of the study was the comparison of the effectiveness of Ovopel [(D-Ala^6,Pro^{9}NEt)-mGnRH+metocropramide], Ovaprim [(D-Arg^6,Pro^{9}NEt)-sGnRH+domperidone], LHRHa, human chorionic gonadotropin (hCG), and carp pituitary extract (CPE) in stimulating spermiation in the common dace. The effectiveness of the selected hormonal preparations was verified based on the volume of milt obtained, sperm count, and their motility, as determined by means of the CASA system.

Materials and methods
Milt was obtained from the common dace 48 hours following the hormonal stimulation of fish by means of Ovopel (1 granule per kg^{-1} b.w.), Ovaprim (0.5 ml per kg^{-1} b.w.), LHRHa (100 μg per kg^{-1} b.w.), hCG (500 UI per kg^{-1} b.w.), and CPE (3 mg per kg^{-1} b.w.). Each group of fish consisted of 12 specimens, along with a control group of males which received 0.9% NaCl (0.5 ml per kg^{-1} b.w.) instead of a hormonal preparation. The amount of milt obtained was measured at each time that the milt was collected, thus enabling the estimation of the total volume of milt obtained in milliliters (TVM, ml). Possessing values regarding the males' weight and TVM made it possible to calculate the volume of milt per kg of body weight (VOM, ml per kg^{-1} b.w.). Sperm concentration in milt (x10^9 ml^{-1}) was determined by means of the spectrophotometric method, whilst the total sperm production in billions (TSP, x10^9) was calculated based on values of sperm concentration and TVM. Total motility (MOT, %), progressive motility (PRG, %), curvilinear velocity (VCL, μm s^{-1}) and straightlinear velocity (VSL, μm s^{-1}), the linearity of movement (LIN=100xVSL/VCL, %), wobbling (WOB=100xVAP/VCL, %), the amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz) were all determined using the CASA system (Computer Assisted Sperm Analysis). Values of the CASA parameters, TVM, VOM, sperm concentration, and TVP were characterized by arithmetic mean and standard deviation (±SD). The significance of differences in values of the analyzed parameters was verified by means of the analysis of variance (One-way ANOVA) and post hoc Tukey's test (GraphPad Software Inc., San Diego, CA, USA).

Results
The highest values of TVM and VOM were confirmed following stimulation with Ovaprim (1.01±0.46 ml and 11.50±4.43 ml per kg^{-1} b.w. respectively), with the lowest noted following the application of hCG

(0.41±0.16 mi and 4.78± ml per kg^{-1} b.w. respectively). The increase in values of TVM after administering Ovaprim was statistically significant when compared to values obtained following stimulation with Ovopel ($P<0.05$), LHRHa ($P<0.001$), hCG ($P<0.001$) and the control group ($P<0.001$). Sperm concentration was highest in the control group ($6.29\pm0.94x10^9ml^{-1}$), whereas the highest significant decrease, to a value of approx. $3.5x10^9$ ml^{-1} ($P<0.001$), was noted after stimulation with CPE. A significantly lower sperm concentration in relation to the control group was also confirmed following the application of Ovaprim ($4.13\pm0.85x10^9$ ml^{-1}, $P<0.001$) and LHRHa ($4.91\pm1.06x10^9$ ml^{-1}, $P<0.05$). TSP values following stimulation with hCG, CPE, and LHRHa were shown to be within the range of $2.1\text{-}2.3x10^9$ and did not differ significantly from values of the control group ($1.4x10^9$), ($P>0.05$), whereas stimulation with Ovaprim resulted in a significant increase in the value of TSP ($4.07\pm1.70x10^9$) when compared to LHRHa ($P<0.05$), hCG ($P<0.01$), CPE ($P<0.05$), and the control group ($P<0.001$). MOT (72-84%) and PRG (36-45%) values amounted to comparable levels in all groups and did not differ significantly ($P>0.05$). The highest values of VCL and VSL were confirmed after stimulation with LHRHa (133.4±26.7 and 107.8±23.4 μm s^{-1} respectively), with the lowest values recorded following stimulation with hCG (86.9±18.9 and 68.2±15.9 μm s^{-1} respectively) and Ovopel (95.9±11.9 μm s^{-1} and 76.5±12.1 μm s^{-1} respectively), ($P<0.01$). Hormonal stimulation was not shown to bring about a significant increase in the values of LIN, WOB, and BCF, ($P>0.05$). The highest values for ALH were noted following stimulation with LHRHa (1.11±0.11 μm s^{-1}), with the lowest recorded after stimulation with hCG (0.89±0.89 μm s^{-1}).

Discussion and conclusions

Hormonal stimulation applied in the biotechnology of fish reproduction often aims to speed up the maturation of gametes and increase the amount of oocytes or spermocytes obtained (Mylonas et al. 2010). Our studies led to the observation that, in the case of common dace males, a significant increase in TVM when compared to the control group can occur 48 hours after stimulation with Ovaprim and CPE. The positive effect of Ovaprim in stimulating spermiation in the common dace is also signified by the highest VOM value, which differs significantly from values obtained in the remaining groups. The total sperm production during spawning season is of no small importance when it comes to the ability to fertilize as many eggs as possible (Lahnsteiner et al. 2003). The highest and most significant increase in the value of TSP in relation to the control group was recorded following stimulation with Ovaprim, which confirms its high effectiveness in stimulating spermiation in common dace males.

References

Cejko B.I., Kowalski R.K., Kucharczyk D., Targońska K., Krejszeff S., Żarski D., Glogowski J. (2010): Influence of the lenght of time after hormonal stimulation on selected parameters of milt of ide *Leuciscus idus* L. Aquacult. Res. 41: 804-813.

Kucharczyk, D.; Targońska, K.; Żarski, D.; Kujawa, R.; Mamcarz, A.; (2008) A review of the reproduction biotechnology for fish from the genus *Leuciscus*. Arch. Pol. Fish. 16 (4): 319-340.

Lahnsteiner F.; Berger B.; Weismann T. (2003): Effects of media, fertilization technique, extender, straw volume, and sperm to egg ratio on chatchability of cyprinid embryos, using cryopreserved sperm. Theriogenology 60: 829-841.

Mylonas C.C.; Fostier A.; Zanuy S. (2010) Broodstock management and hormonal manipulations of fish reproduction. Gen. Com. Endocrinol. 156: 516-534.

Targońska K.; Kucharczyk D.; Kujawa R.; Mamcarz A.; Żarski D. (2010): Controlled reproduction of asp *Aspius aspius* (L.) using luteinizing hormone releasing hormone (LHRHa) analogues with dopamine inhibitors. Aquaculture 306: 407-410.

P-122
ISOLATION AND CHARACTERISTICS OF -N-ACETYLGLUCOSAMINIDASE PRESENT IN THE SPERM OF RAINBOW TROUT (*ONCOTHYNCHUS MYKISS*) AND SIBERIAN STURGEON (*ACIPENSER BAERII*)

Beata Sarosiek[1], Radosław Kajetan Kowalski[1], Katarzyna Dryl[1], Jan Glogowski[1,2]

[1] *Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10; 10-747 Olsztyn, Poland*
[2] *Department of Ichthyology, Faculty of Environmental Sciences and Fisheries, University of Warmia and Mazury, Olsztyn, Poland*

E-mail: b.sarosiek@pan.olsztyn.pl

Introduction

-N-acetylglucosaminidase (-NAGase) is an enzyme commonly found in animal tissues and bodily fluids. It has been described, e.g. in human sperm (Miranda et al. 2000), or in hamster sperm (Zitta et al. 2004). Its presence has been also confirmed in the acrosome of sturgeon spermatozoa (Piros et al. 2002). The presence of this enzyme in rainbow trout sperm is astonishing, since spermatozoa of this species of fish do not have acrosome (Stanley 1969), due to the presence of a micropyle on the surface of the oocyte. However, our observations indicate that the activity of the enzyme present in the trout sperm is much higher than in sturgeon sperm. For comparison, no activity of another acrosomal enzyme, namely arylsulphatase, was found in trout sperm (unpublished data). It still remains an open question why trout gametes are equipped with one acrosomal enzyme, while the others are absent. To date, -NAGase has been described in the semen of sturgeon and trout, but that research concerned characteristics of unpurified enzymes (Sarosiek et al. 2008). The present study describes the properties of purified -N-acetylglucosaminidase from seminal plasma and sperm extract of rainbow trout and Siberian sturgeon.

Materials and methods

The sturgeon and Rainbow trout milt were obtained in aquaculture facilities, then after transport to the laboratory on ice (+4^{o}C), milt was centrifuged to obtain seminal plasma and spermatozoa, which was used to isolation -NAGase. We used the FPLC system, Q Sepharose (ion exchange chromatography) and Superdex 200 (gel filtration) columns. The kinetic properties of purified enzymes (obtained from milt plasma and spermatozoa extracts) were also determined.

Results

After applying ion exchanging chromatography to -NAGase from rainbow trout sperm, two peaks of -NAGase activity were obtained for plasma material and one for sperm material. After gel filtration, 133 and 195-fold purification degree was obtained for peak I and II, respectively. The molecular weight was 74 kDa for both forms of the enzyme obtained from the seminal plasma. The enzyme present in spermatozoa had a higher molecular weight than plasma forms (127 kDa).

During chromatographic separations of the sturgeon seminal plasma, one peak of -NAGase activity was washed out. During gel filtration, two peaks of -NAGase activity were washed out. The molecular weight of the enzyme originating from sturgeon seminal plasma was about 265 and 113kDa for peak I and II. The first stage of purifying -NAGase originating from spermatozoa was to treat the sperm extracts with N-butanol. This resulted in about a 10-fold reduction of the protein content in the extract. Ion exchanging chromatography of sturgeon sperm extracts resulted in obtaining one peak of -NAGase activity. During gel filtration, one peak of -NAGase activity was also washed out. Molecular weight of the enzyme was about 271 kDa.

Optimum pH was determined for purified peak I of trout seminal plasma, amounting to 4.6. Michaelis-Menten constant was 11.59x10^{-4}M. Purified peak II from trout seminal plasma was characterized by the following kinetic parameters: optimum pH of 4.6; K_m=11.48x10^{-4}M. Incubation of purified peak I and II at 56^{o}C resulted in about 45-50% decrease in activity of both forms. Optimum pH for the purified enzyme

from rainbow trout sperm extract ranged from 4.6 to 5.0. The Michaelis-Menten constant was $5.4x10^{-4}$M. Determination of the effect of enzyme incubation at high temperature proved this form of the enzyme to be most resistant to high temperatures, since its activity after 20 minutes of incubation at 56°C decreased by only 25%.

Optimum pH for purified peak I of sturgeon seminal plasma ranged from 3.8 to 5.0. The Michaelis-Menten constant was $5.12x10^{-4}$M. 20-minute incubation at 56°C resulted in a 64% decrease in the activity of -NAGase from sturgeon seminal plasma. Optimum pH for purified peak II of sturgeon seminal plasma ranged from 3.8 to 4.6. The K_m constant was $5.28x10^{-4}$M and the loss of -NAGase activity at 56°C was 58%. Purified enzyme from sturgeon sperm extract had optimum pH ranging from 3.8 to 4.6. The K_m constant was $5.28x10^{-4}$M. Enzyme incubation at 56°C resulted in 35% loss of enzyme activity. The above data indicate that the forms of enzyme present in trout and sturgeon semen are thermostable.

Discussion and conclusion

The data available in the literature indicate that numerous techniques can be useful for purification of -NAGase originating from various sources (Giemba et al. 1998, Martinez et al. 2000). While isolating -NAGase from seminal plasma and sperm extracts of rainbow trout and Siberian sturgeon, ion exchanging chromatography and gel filtration proved most useful. Attempts to apply other techniques for protein separation, namely hydrophobic chromatography and separation on Concavalin A Sepharose, were unsuccessful. The kinetic parameters, especially thermostability of -NAGase was similar to the homologous enzymes observed in mammals semen (Miranda et al. 2000).

Acknowledgements

The present study was supported by Ministry of Science and Higher Education Grant No N 308 018 31.

References

Giemba M.P., Riffel A., Brandelli A. 1998: Purification and characterization of -N-acetylhexosaminidase from phytopathogenic fungus Biopolaris sorokiniana. J. Appl. Microbiol. 85: 708-714.

Martinez M. L., Martelotto L., Cabada M. O. 2000: Purification and biological characterization of N-acetyl -D glucosaminidase from Bufo arenarum spermatozoa. Mol. Reprod. Dev. 57: 194-203.

Miranda P. V., Gonzales-Echeverria F., Blaquier J. A., Mahuran D. J., Tezon J. G. 2000 : Evidence for the participation of -hexosaminidase in human sperm-zona pellucida interaction in vitro. Mol. Hum. Reprod. 6(8): 699-706.

Piros B., Glogowski J., Kolman R., Krzemieniecki A., Domagała J., Ciereszko A. 2002: Biochemical characterization of Siberian sturgeon Acipenser baerii and sterlet Acipenser ruthenus milt. Fish Physiol. Biochem. 26: 289-295.

Prody G. A., Greve L. C., Hedrick J. L. 1985: Purification and characterization of an N-acetyl--D-glucosaminidase from cortical granules of Xenopus laevis eggs. J. Exp. Zool. 235: 335-340.

Sarosiek B., Kowalski R., Glogowski J. 2008: Isolation and preliminary characteristics of -N-acetylglucosaminidase in the sperm of Siberian sturgeon (Acipenser baerii) and rainbow trout (Oncorhynchus mykiss). J. Appl. Ichthyol. 24(4): 492-496.

Stanley H.P. 1969: An electron microscope study of spermiogenesis in the teleost fish Oligocottus maculosus. J. Ultrasruc. 27: 230-243.

Zitta K., Wertheimer E.V., Miranda P.V. 2006: Sperm N-acetylglucosaminidase is involved in primary binding to the zona pellucida. Mol. Hum. Reprod. 12(9): 557-63.

P-123
BIOTECHNOLOGICAL USE OF GERM CELLS FROM THE COMMERCIAL FLATFISH *SOLEA SENEGALENSIS*

Elsa Cabrita[1], Tiziana Pacchiarini[1], Carmen Sarasquete[1], Pedro Pousão-Ferreira[2], M. Teresa Dinis[3], M. Paz Herráez[4]

[1] *ICMAN.CSIC - Institute of Marine Sciences of Andalusía, Av. Republica Saharaui 2, 11510 Puerto Real, Cádiz, Spain*
[2] *INRB I.P.-IPIMAR, Av. 5 Outubro s/n 8700 Olhão, Portugal*
[3] *CCMAR – Centre for Marine Sciences, University of Algarve, 8005-139 Faro, Portugal*
[4] *Dept. of Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain*

E-mail: elsa.cabrita@icman.csic.es

Introduction
Cryopreservation of testicular germ cells offers a tool for the conservation and production of species with potential for aquaculture, due to the capacity of spermatogonia differentiating into gametes. These cells have the ability to proliferate in a host individual after transplantation producing, in their gonad, functional gametes depending on the host sex determination (Yoshizaki *et al.,* 2011). Cryopreservation and transplantation of testicular germ cells have been attempted in several mammalian species (Redden *et al.,* 2009) and more recently in some fish (Takeuchi *et al.* 2009; Yoshizaki *et al.,* 2011). In the present study cryopreservation protocols were developed for testicular germ cells from Senegalese sole (*Solea senegalensis*), a species with several reproductive problems, with the aim to transplant into newly hatched larvae and test their efficiency.

Materials and methods
Cryopreservation Experiments: Several testes were extracted from 12-months old *Solea senegalensis* juveniles (weight: 44±14 g, length: 16.54±1.3 cm). Fish were anesthetized with a lethal dose of phenoxyethanol (2,000 ppm, 5 min) and the small testes were chirurgical removed. Each testis was divided in 2-3 fragments and several fragments (around 8 per cryovial) were cryopreserved in PBS or L15 based medium supplemented with 0.5% BSA and 5.5 mM glucose and 1.5 M DMSO or 1.5 M glycerol (modified from Yoshizaki *et al.,* 2007). Testes fragments were frozen in cryovials using a nitrogen-free programmed biofreezer (Grant Asymptote, UK), or introducing the cryovials at 6.5cm above liquid nitrogen in a Styrofoam box. Freezing and thawing rates were monitorized using a thermocouple inside the cryovials. A total of four cryovials were frozen per treatments. Cell integrity, viability and the number of recovered cells/ spermatogonia were determined in post-thaw samples using the dual stain IP/SYBR-14 (fluorescent microscopy), calcein (fluorimetry) and microscopy observations.

Cell Transplantation: Testes fragments cryopreserved with PBS+glycerol were used in this experiment. After thawing (40ºC, 2.30 min), fragments were washed in L15 and trypsinized (0.25% trypsin in L15 + 0.5% FBS + DNAse-I 200 units, 2 h, 22ºC). Cell suspension was filtered (180 μm mesh), washed to eliminate trypsin and cells were stained with PKH26 for visualization, according to manufacturer instructions. Stained cells were microinjected (13.7nl) intraperitoneally into anaesthetized (0.01% MS-222) *S. senegalensis* larvae from 6 to 20 days post-hatching (dph) (length range: 4 - 8.5 mm).

Larval rearing: Larvae were reared in circular tanks, feed with rotifers from 2 to 6 dph and with *Artemia* nauplii from 6 to 14 dph. Frozen *Artemia* was introduced at 10 dph until the end of the experiment. Transplanted larvae (n=60 per day) were acclimatized in 0.04% BSA prepared in seawater during 30 min before being transferred into controlled conditions in small incubator compartments and feed as described before. Survival at 1 day post-transplantation (1dpt) was recorded in 6, 10, 16 and 20 dph transplanted larvae.

Results and discussion
No significant differences were obtained between cryopreservation protocols and freezing methods in terms of cell integrity (SYBR/IP). Therefore, in the subsequent trails, cryopreservation was performed using a

nitrogen-free controlled biofreezer since the freezing procedure was more stable and reproducible and the transportation of nitrogen to fish farms was avoided. When comparing the number of recovered cells, DMSO was better tolerated than glycerol, since it allowed to recover a higher number of testicular cells after freezing/thawing. However, the percentage of spermatogonia present in cell suspension was higher in treatments containing glycerol (14.7-18.2%) than the ones with DMSO (9.9-10.6%), showing that glycerol is more suitable for the cryopreservation of these cells, protecting spermatogonia better than the rest of testicular cells. PBS resulted in higher protection to cells than L15 in terms of cell viability, determined by calcein incorporation into metabolic active cells, although a higher percentage of spermatogonia was obtained with L15+Glycerol. Regarding transplantation, *S. senegalensis* larvae were very resistant to microinjection procedure obtaining 89.16%-100% survival rate at 1dpt. The survival rate increased with larval age, with 20 dph larvae being more resistant than 6 dph larvae. Further experiments need to be conducted in order to see if transplanted spermatogonia are incorporated into recipient gonad, as demonstrated by Takeuchi *et al.,* (2009) in Nibe croaker larvae.

Conclusions

Spermatogonia from *Solea senegalensis* testes fragments can be cryopreserved using PBS or L15 supplemented with glucose and BSA and glycerol as cryoprotectant. After thawing, these cells can be successfully transplanted into 6-20 dph larvae with no effects on the survival rate.

Acknowledgements

The authors thank to the fish farm A. Coelho e Castro, Lda (Povoa de Varzim, Portugal) for providing part of the biological material and to IPIMAR staff and CRYOSPERM team for helping during larval rearing. This research was supported by the Spanish Ministry of Science and Innovation through project PGCFISH (AGL2008-02172) and ASSEMBLE call; E. Cabrita was supported by a research contract (RYC-2007-01650); T. Pacchiarini was supported by a JAE-DOC fellow (CSIC).

References

Redden, E., Davey, R., Borjigin, U., Hutton, K., Hinch, G., Hope, S., Hill, J., Herrid, M. 2009. Large quantity cryopreservation of bovine testicular cells and its effect on enrichment of type A spermatogonia. Cryobiology 58, 190-195.

Takeuchi, Y., Higuchi, K., Yatabe, T., Miwa, M., Yoshizaki, G. 2009. Development of spermatogonial cell transplantation in Nibe croaker, Nibea mitsukurii (Perciformes, Sciaenidae). Biology of Reproduction 81:1055-1063.

Yoshizaki, G., Fujinuma, K., Iwasaki, Y., Okutsu, T., Shikina, S., Yazawa, R., Takeuchi, Y. 2011. Spermatogonial transplantation in fish: A novel method for the preservation of genetic resources. Comparative Biochemistry and Physiology Part D 6, 55-61.

P-124
STABILIZATION OF SPERM MEMBRANE LIPIDS DURING CRYOPRESERVATION BY THE ADDITION OF AFPS

J. Beirão[1], L. Zilli[2], S. Vilella[2], E. Cabrita[3], C. Fernandez-Díez[1], R. Schiavone[2], M.P. Herráez[1]

[1] *Dept. of Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain*
[2] *Dept. of Biological and Envir. Sciences and Tech., Lab. of Comparative Physiology, University of Salento, Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy*
[3] *ICMAN-Institute of Marine Science of Andalusia, Spanish National Research Council, Avda. República Saharaui 2, 11510 Puerto Real, Cádiz, Spain*

E-mail paz.herraez@unileon.es

Introduction
Changes in the plasma membrane lipid composition during cryopreservation have been related to a decrease in sperm quality (Muller et al., 2008). In the present work we tested the addition of antifreeze proteins (AFPs) to the cryopreservation extender because of their ability to depress the freezing point and their potential interaction with membranes.

Material and methods
Samples collection and cryopreservation
Sperm samples from *Sparus aurata* (8 pools of 7 mL) were obtained from the Aquaculture Research Center of Acquatina (University of Salento, Italy), between November and January. Aliquots of each pool were cryopreserved with 5% DMSO in 1% NaCl with and without AFPI or AFPIII (1μg/mL) in 0.5 French straws (IMV). An equilibration time of 4 min at room temperature was applied plus 10 min at 2cm from the liquid nitrogen surface, after which were directly plunged into the liquid nitrogen. Samples were thawed in a water bath at 25ºC for 30 sec.

Motility and Viability evaluation
Fresh and thawed samples were checked for motility using the CASA system Hobson Sperm Tracker and associated software (Hobson Vision Ltd., Baslow, UK) and viability under a fluorescent microscope using the fluorescent "live/dead Kit" from Molecular Probes (Invitrogen, Italy).

Head plasma membrane (HM) and flagella isolation
Samples were washed with 1% NaCl to eliminate the seminal plasma. For head and flagella separation, samples resuspended in 1% NaCl were passed ten times through a 50 cm × 0.5 mm i.d. capillary. Samples were layered over sucrose gradient 0.5 – 2 M and ultracentrifuge at 28 000 × g for 45 min. The upper band (0.5M sucrose) consisting of flagella and a lower band (between 1.5 and 2M sucrose) containing spermatozoa heads were recovered. Flagella were pelleted and used for lipid extraction. Head suspensions were pelleted and exposed to a hypoosmotic shock with distilled water allowing us to lyse the heads. Head suspensions were centrifuged to sediment the cellular debris, and the supernatants containing the HM were pelleted by ultracentrifuge and used for lipid extraction.

Lipid analysis
Lipids were extracted from flagella and HM samples according to Folch *et al.* (1957). Cholesterol was measured with a CHOD PAP Kit (Biolabo, France). Total phospholipids were quantified with the procedure by Rouser *et al.* (1970). After phospholipids purification with SEP-PAK silica gel cartridges (Waters, Spain), detection and quantification was performed by high pressure liquid chromatography. For fatty acids analysis samples were transmethylated following the protocol by Berry *et al.* (1965) and analyzed by gas chromatography.

Results
The analysis of fresh sperm reveals differences in the composition of HM and flagella in fresh sperm. Phosphatidyl-choline (PC) was the most represented phospholipid in both domains. Phosphatidyl-ethanolamine (PE) and phosphatidyl-serine (PS) were significantly higher in the flagella and HM showed a higher amount of saturated fatty acids (C18 and total saturated). After cryopreservation sperm quality

decreases in all treatments but, compared with the control cryopreserved with 5% DMSO alone, the addition of AFPIII increased the straight line velocity (VSL) and percentage of viable cells. The addition of AFPI did not significantly improve these parameters. Cholesterol/phospholipids ratio did not show any significant difference between treatments. Respect to the phospholipid classes, only phosphatidyl-serine (PS) from the HM was affected by freezing/thawing procedure with significantly higher values in samples frozen with DMSO than in fresh samples. Cryopreservation with DMSO also increased the saturated fatty acids (palmitic and stearic) and decreased the unsaturated fatty acids (mainly polyunsaturated (PUFA)) both in HM and flagella. HM was more affected by cryopreservation without AFPs, suffering decreases in C20:3n3, C20:5n3 and C22:5n3. The addition of AFPIII provided similar composition than fresh sperm, avoiding most of the changes promoted by cryopreservation with DMSO alone. The amount of unsaturated fatty acids in HM was positively correlated with the quality parameters whereas the saturated ones correlated negatively.

Discussion and conclusions

AFPs, especially AFPIII, seem to have interacted with unsaturated fatty acids avoiding changes in lipidic profiles during cryopreservation and stabilizing the plasma membrane organization both in head and flagella. This effect had contributed to improve sperm quality (VSL and percentage of viable cells) after cryopreservation. AFPs effect was different in HM and flagella probably because of the different lipid arrangement of these domains. AFPIII seem to have interacted preferentially with unsaturated fatty acids, this fact is in accordance with the hypothesis supported by (Tomczak et al., 2002b; Inglis et al., 2006). According to these authors AFPs will interact with membranes by the insertion of a hydrophobic segment of the protein, being this reaction highly dependent on the membrane phospholipids fatty acids composition (interacting preferentially with membrane domains made with unsaturated fatty acids rather than with saturated ones). The higher proportion of PE and PS in the flagella could be related also with the higher proportion of unsaturated fatty acids since as observed in other fish sperm studies (Labbé et al., 1995; Bell et al., 1997) both PE and PS were the highly unsaturated phospholipids. The higher content on unsaturated fatty acids presented by the flagella might be related with its need for higher fluidity for the flagellar beating as suggested Connor et al. (1998) in monkey sperm.

Our results have shown that the use of AFPIII in the cryopreservation solution minimizes the loss of sperm quality after freezing/thawing maintaining the lipid composition of the plasma membrane of the analyzed domains (head and flagella) and exerting a stabilizing effect on gilthead seabream sperm membrane.

Acknowledgments

The authors are grateful to Dr Catherine Labbé from INRA (France) for providing the protocols for lipids analysis and to Francisca Martínez from the ULE-LTI (Spain) for the technical work with the fatty acids analysis. This work was supported by the project Petri (PTR 95.1026.OP.01). J. Beirão was supported by a PhD grant from the Portuguese Ministry of Science and Technology (SFRH/BD/31990/2006) co-founded by POPH - QREN (FEDER and MCTES).

References

Bell, M.; Dick, J.; Buda, C., 1997: Molecular speciation of fish sperm phospholipids: Large amounts of dipolyunsaturated phosphatidylserine. Lipids 32, 1085-1091.

Connor, W.; Lin, D.; Wolf, D.; Alexander, M., 1998: Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. J. Lipid Res. 39, 1404-1411.

Inglis, S.; Turner, J.; Harding, M., 2006: Applications of type I antifreeze proteins: Studies with model membranes & cryoprotectant properties. Curr. Protein Pept. Sci. 7, 509-522.

Labbé, C.; Maisse, G.; Muller, K.; Zachowski, A.; Kaushik, S.; Loir, M., 1995: Thermal-acclimation and dietary lipids alter the composition, but not fluidity, of trout sperm plasma-membrane. Lipids 30, 23-33.

Muller, K.; Muller, P.; Pincemy, G.; Kurz, A.; Labbé, C., 2008: Characterization of sperm plasma membrane properties after cholesterol modification: Consequences for cryopreservation of rainbow trout spermatozoal. Biology of Reproduction 78, 390-399.

Tomczak, M.; Vigh, L.; Meyer, J.; Manning, M.; Hincha, D.; Crowe, J., 2002: Lipid unsaturation determines the interaction of AFP type I with model membranes during thermotropic phase transitions. Cryobiology 45, 135-142.

P-201
COULD CALCIUM ANTAGONIST PROTECT STURGEON SPERM FROM CD-INDUCED STRESS?

Zhi-Hua Li[1,2], Ping Li[1,2], Marek Rodina[1], Tomas Randak[1]

[1] *University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 389 25 Vodnany, Czech Republic*

[2] *Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Jingzhou 434000, China*

E-mail: zhihuali06@yahoo.com

Introduction

Since the quality of fish sperm is a major factor contributing to successful production, measurement of its motility could provide a sensitive and accurate bio-indicator of aquatic pollution. Besides, the sperm are rich in polyunsaturated fatty acids (PUFA) and have a low concentration of scavenging enzymes, so they are very susceptible to oxidative damage when attacked by reactive oxygen species (ROS) (Li *et al.*, 2010b). Published data suggested that several cellular Ca^{2+} acceptors are Cd^{2+} targets as well, and indicated that the blockage of voltage sensitive Ca^{2+} channels could inhibit cadmium uptake and decrease cadmium-induced toxicity (Craig et al., 1999). However, the roles of Ca^{2+} channels on cadmium-induced stress in fish sperm are not well known. The aims of this study include, 1) how does short-time (2 h) *in vitro* exposure of Cd affect motility parameters and oxidative stress in sturgeon sperm; 2) what is the role of Ca^{2+} channel blocker VRP on cadmium-induced stress in sturgeon sperm.

Materials and methods

Sperm of Russian sturgeon was obtained from five different males (age: 7-8 years old; body weight: 5.1 ± 0.9 kg; body length: 102.5 ± 19.3 cm) raised in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology, Czech Republic. Sturgeon sperm was diluted with an immobilization medium (20 mM Tris, 10 mM KCl, pH=8). The sperm suspension (n=5) were then exposed for 2 h to 50 μg/L VRP, 5.0 μg/L Cd, the mixture of 50 μg/L VRP + 5.0 μg/L Cd, 50 μg/L Cd and the mixture of 50 μg/L VRP + 50 μg/L Cd. A group exposed to immobilization medium was used as control. Each experimental condition was duplicated once.

Sperm velocity (μm/s, measured only motile sperm) and percentage of motile sperm cells (%) were determined according to the methods described by Li *et al.,* (2010b). Oxidative stress indices and antioxidant responses were investigated, including the thiobarbituric acid-reactive substances (TBARS), carbonyl derivatives of proteins (CP), superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx). All values were expressed as mean ± SD and analyzed by SPSS for Win 13.0 software. One-way ANOVA with Dunnett's test was used to determine whether results of treatments were significantly different from the control group ($p<0.05$).

Results and discussion

Compared to the control, sturgeon sperm motility significantly decreased ($\boldsymbol{p}<0.05$ or $\boldsymbol{p}<0.01$) in the two Cd-treated groups, with a reduction rate of 15.63 % at 5.0 μg/L Cd and 30.65 % at 50 μg/L Cd, respectively. The velocity of sturgeon sperm decreased with a rate 32.11 % at 5.0 μg/L Cd and 38.75 % at 50 μg/L Cd, respectively. The present results maybe caused by Cd-induced structural impairments to fish sperm (Au et al., 2001). With the presence of VRP, a trend to overcome Cd-induced stress in both of two sperm motility parameters was observed after 2 h exposure at the higher concentration of Cd, but there was statistical significance ($\boldsymbol{p}<0.05$ or $\boldsymbol{p}<0.01$). Consistent with the findings of previous studies, this suggests that VRP, a Ca^{2+} channel blocker, performs a protective role in Cd-induced stress (Craig et al., 1999).

LPO levels significantly increased upon exposure to 5.0 μg/L Cd ($p<0.05$) and above, but the CP levels were only significantly induced at higher concentration of Cd. The results suggest an increased oxidative stress and LPO was more sensitive than CP as an indicator of evaluating oxidative stress of fish sperm

exposed to Cd. Furthermore, all antioxidant enzymes activities (SOD, GR and GPx) were significantly inhibited in Cd-treated groups with higher concentration, indicating that over production of ROS and relatively low antioxidant system in sturgeon sperm (Li *et al.*, 2010a). With the presence of VRP, all antioxidant parameters of sturgeon sperm exposed to the lower concentration of Cd returned to control, however, there was still statistical significance between those exposed to the higher concentration of Cd and the control, except for CP. The obtained data show protective properties of VRP in sturgeon sperm under Cd-induced stress. The possible reason is the presence of similar behavior between Ca^{2+} and Cd^{2+}, which is attributed to a comparable charge and ionic radius of two ions (Verbost et al., 1989). To our knowledge there is a lack of information concerning Ca^{2+} channel in sturgeon sperm, even though it has been found in sperm of some teleost species (Cosson, 2004), and therefore could be expected in sturgeon sperm.

Conclusion

In summary, this present results report a protective role of VRP in Russian sturgeon sperm under Cd-induced stress by analyzing motility parameters and antioxidant responses and provide support to the possibility that a Ca^{2+} channel is present in sturgeon sperm.

Acknowlegements

This study was supported by the CENAQUA No. CZ.1.05/2.1.00/01.0024, the USB RIFCH No. MSM 6007665809, the Ministry of the Environment of the Czech Republic No. SP/2e7/229/07, GACR P502/11/0090, IAA 608030801, ME10015, QH82119, LC06073, the GAJU Nos. 046/2010/Z, 047/2010/Z and 007/2010/Z.

References

Au, D.W.T., Lee, C.Y., Chan, K.L., Wu, R.S.S., 2001. Reproductive impairment of sea urchins upon chronic exposure to cadmium. Part I: Effects on gamete quality. Environ. Pollut. 111, 1-9.

Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquacult Int 12, 69-85.

Craig, A., Hare, L., Tessier, A., 1999. Experimental evidence for cadmium uptake via calcium channels in the aquatic insect Chironomus staegeri. Aquat. Toxicol. 44, 255-262.

Li, Z.H., Li, P., Dzyuba, B., Randak, T., 2010a. Influence of environmental related concentrations of heavy metals on motility parameters and antioxidant responses in sturgeon sperm. Chem-Biol Interact 188, 473-477.

Li, Z.H., Li, P., Rodina, M., Randak, T., 2010b. Effect of human pharmaceutical Carbamazepine on the quality parameters and oxidative stress in common carp (Cyprinus carpio L.) spermatozoa. Chemosphere 80, 530-534.

Verbost, P.M., Flik, G., Pang, P.K.T., Lock, R.A.C., Bonga, S.E.W., 1989. Cadmium inhibition of the erythrocyte Ca2+ Pump- A molecular interpretation. J. Biol. Chem. 264, 5613-5615.

P-202
SUCCESSIVE ACTIVATIONS OF STURGEON SPERM MOTILITY BY OSMOLALITY AND CA^{2+} CONCENTRATION

Boris Dzyuba[1,2], Jacky Cosson[1], Victoria Dzyuba [1,3], Marek Rodina[1], David Gela[1] and Otomar Linhart[1]

[1] *Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic*
[2] *Institute for Problems of Cryobiology and Cryomedicine, NAS, Kharkiv, Ukraine*
[3] *Institute of Biology, V.N. Karazin Kharkiv National University, Kharkiv, Ukraine*

E-mail: bdzyuba@frov.jcu.cz

Introduction
In chondrostean fish, both sperm motility activation and motility duration depend on osmolality and ionic composition of environmental medium. While increased osmolality (Ginzburg, 1972) and presence of Ca^{2+} ions (Alavi et al. 2004) can prolong motility duration, the presence of Ca^{2+} ions in any swimming medium is supposed to be the prerequisite of motility activation itself (Linhart et al. 2002). However interrelationship between impacts of osmolalilty and Ca^{2+} on sperm motility in sturgeons needs future studies.

The aim of the present study was the investigation of sperm motility responses on sequential changes of osmolality and Ca^{2+} concentration in swimming media using sterlet *Acipenser ruthenus* as experimental model of chondrostean fish.

Material and methods
Fish and sperm sampling. Sperm samples were collected during natural spawning period from 10 mature sterlet males of 1-2 kg body weight (bw) by catheterisation of urogenital tract 24-36h after injection of carp pituitary extract at dosage of 4 mg kg^{-1} bw.

Experimental design. Sperm motility was initiated in solution of various osmolality created by 10, 40, 80 mM sucrose (S10, S40, S80 correspondingly) dissolved in 10 mM tris-HCl buffer pH8.5 (first step of activation). After sperm movement arrest, two modes of next treatment were applied: 1) $CaCl_2$ concentration was increased up to 1mM (activation step 2) and after motility arrest the osmolality of swimming medium was decreased two fold by addition of distilled water (activation step 3, figure Ia); 2) the osmolality of swimming media was decreased two fold by addition of distilled water (activation step 2) and after motility arrest, $CaCl_2$ concentration was increased up to 1mM (activation step 3, figure Ib).

Evaluation of motility percentage and sperm velocity. Sperm was diluted 1 : 50 into activating media S10-S80. Directly after dilution, motility was recorded for 1–2 min post-activation using video microscopy techniques. Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) and percentage of motile cells (motility) by microimage analyzer (Olympus Micro Image 4.0.1. for Windows) as described earlier (Rodina et al., 2008).

Estimation of seminal fluids and activating media osmolalities. Seminal fluids (SF) were obtained after sperm samples centrifugation at 16,000g for 10 min. Osmolalites of SF and media used in experiments were evaluated using a Vapor Pressure Osmometer 5520 (Wescor, USA), and expressed in $mOsmolkg^{-1}$.

*Data presentation and statistical analysis.*For the comparison of VCL Tukey's honest significant difference (HSD) test was applied ($P < 0.05$ were considered to be significant). The mean values with bars of 95% confidence interval are presented in figure 1.

Results
Osmolality values of SF were ranging 52±4 $mOsml^{-1}$ and osmolality of S10,S40 and S80 were 30±1, 52±1, 86±3 $mOsml^{-1}$ correspondingly. Sterlet spermatozoa are able to be activated for the first time in each medium studied. Activation steps 2 and 3 of motility activation were dependent on activation mode applied

and osmolality of medium used at step 1 of activation (Fig.1). Activation step 2 by $CaCl_2$ concentration increase was observed in cases where S10 and S40

(S40 results not included in fig.1) were applied after step 1 motility was completed, while no motility were observed in case of S80, no activation at step 3 was observed in case of Ia mode of activation (Fig.1, Ia). Activation steps 2 and 3 occurred when 1b mode of sequential activation was applied using S80 medium and only activation step 2 was observed after reduction of activating medium osmolality with subsequent increase of CaCl2 concentration in case of S10 and S40 application (Fig.1,Ib).

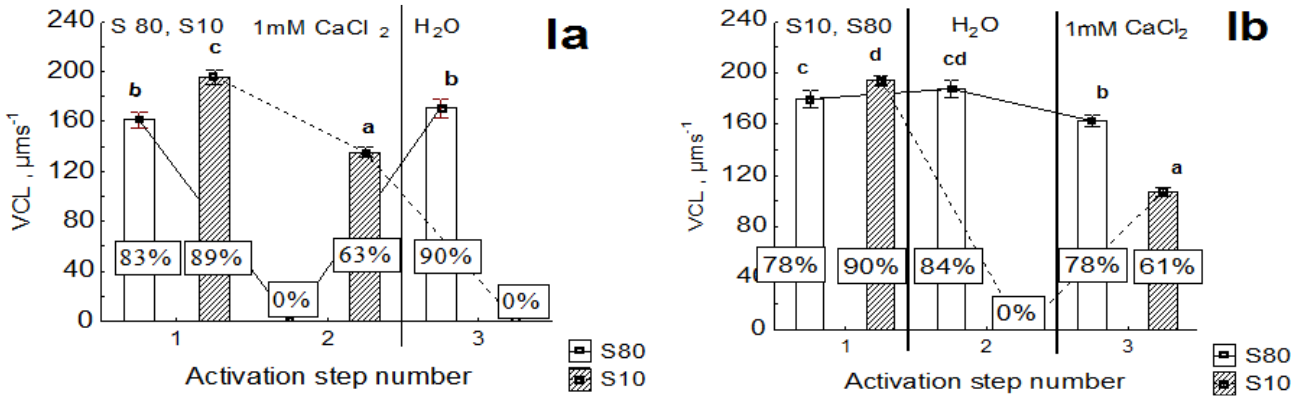


Figure 1. Curvilinear sperm velocity (columns) and corresponding motility percentage (values in boxes) in conditions of sequential activations. Ia – sperm motility was initiated in 10 and 80 mM sucrose solutions (S10, S80 correspondingly, activation step 1), after sperm movement arrest CaCl2 concentration was increased up to 1mM (activation step 2) and after motility arrest the osmolality of swimming medium was decreased two fold by distilled water (activation step 3); Ib - sperm motility was initiated in sucrose solutions 10 and 80 mM (S10, S80 correspondingly, activation step1), after sperm movement arrest the osmolality of swimming media was decreased two fold by distilled water (activation step 2) and after motility arrest, CaCl2 concentration was increased up to 1mM (activation step 3). Values having different letters are significantly different (p<0.05,Tukey's test)

Discussion and conclusions

According to our knowledge, our study reports for the first time in sturgeons spermatozoa the possibility of sequential motility activation by changes of osmolality or/and Ca2+ concentration. As soon as first motility activation was observed in all investigated solutions (S10 being hypotonic, S40 isotonic and S80 hypertonic in comparison with seminal fluid) we consider this step of activation as osmotically independent and resulting only from seminal fluid K+ dilution. It was shown, that increased K+ concentration in seminal fluid plays the crucial role in prevention of motility initiation (Linhart et al. 2002). That is why sperm dilution with K+ -free solutions plays such a key role as activating factor. Second motility activation can be achieved by Ca2+ concentration increase under hypotonic condition or by osmolality decrease from hypertonic level down to isotonic one. It is precisely in this condition (activation step 3) that motility was observed. As described phenomena are dealing with the main fractions of sperm population (more than 60%) and motility initiation on each step occurs immediately after environmental change, it seems that they are not related to energy source changes and could arise from intrinsic regulation of flagellar activity by each activating factor (osmolality, K+ or Ca2+) . We conclude that sequential sperm activation in sturgeons could be an attractive model for future understanding of motility activation mechanisms and application of these phenomena to artificial sturgeon reproduction requires more studies in future.

Acknowledgements

Special thanks are expressed to the projects: IAA 608030801, ME10015, QH82119, LC06073, CZ.1.05/2.1.00/01.0024, GACR P502/11/0090, GAJU 046/2010/Z.

References

Alavi S.M.H.; Cosson J. and Kazemi R., 2006: Semen characteristics in Acipenser persicus in relation to sequential stripping. J. Appl. Ichthyol. 22 (Suppl. 1), 400-405

Ginzburg, A. S. 1972. Fertilization in fishes and the problem of polyspermy. US Dep Comm.Nat. Tech. Inf. Ser. Springfield. p366.

Linhart O.; Cosson J.; Mims S.D.; Shelton W.L.; Rodina M., 2002: Effects of ions on the motility of fresh and demembranated Paddlefish, Polyodon spathula spermatozoa. Reproduction.124,713-7199

P-203
MOTILITY, MORPHOLOGY, MITOCHONDRIA AND ATP CONTENT OF DIPLOID SPERMATOZOA FROM SEX-REVERSED CLONAL DIPLOID AND NEO-TETRAPLOID LOACH, *MISGURNUS ANGUILLICAUDATUS*

Yan Zhao[1], Martin Psenicka[2], Takafumi Fujimoto[1], Taiju Saito[3], George Shigueki Yasui[1], Etsuro Yamaha[3], Katsutoshi Arai[1]

[1] *Hokkaido University, Faculty and Graduate School of Fisheries Sciences, Hakodate, Hokkaido, 041-8611, Japan*
[2] *University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodňany, Zátiší 728/II, 389 25, Czech Republic*
[3] *Hokkaido University, Nanae Fresh-Water Laboratory, Nanae, Hokkaido, 041-1105, Japan*

E-mail: zhuzizhaodahai@hotmail.com

Introduction

Most loach (*Misgurnus anguillicaudatus*) individuals are sexually reproducing diploids and they generate haploid gametes (eggs and sperms) by meiosis. However, all-female clonal lineages have been found in the wild population, Japan, and they spawn unreduced diploid eggs which develop by gynogenesis (Morishima et al., 2002). When clonal diploid males are induced by sex-reversal using 17-α methyltestosterone administration, they produce genetically identical clonal diploid sperm (Yoshikawa et al., 2007). The other source of diploid sperm is neo-tetraploid males, which have been induced by fertilizing eggs from a normal diploid with diploid sperm of a natural tetraploid, followed by inhibition of the second polar body release shortly after fertilization (Fujimoto et al., 2010). Previous studies reported that sex-reversed clonal diploids generated clonal diploid sperm with very poor motility and the fertilization rate was very low when used clonal diploid sperm, while diploid sperm of neo-tetraploid gave considerably active motility like haploid sperm of normal diploid loach. Mechanisms underlying such a difference in motility between two kinds of diploid sperm have not been examined yet. In the present study, morphological characteristics including sperm-head size, flagellum length, location and number of mitochondria were investigated in two kinds of diploid sperm and normal haploid sperm by scanning and transmission electron microscopy (SEM and TEM). Then, volume of mitochondrial mass per spermatozoon was also estimated in two kinds of diploid and normal haploid sperm by MitoTracker Green staining and flow-cytometry. Finally, content of ATP (adenosine tri-phosphate) was measured among three kinds of sperm with different ploidy level and genotypes.

Materials and methods

Normal diploid males (n=3), sex-reversed clonal diploid males (n=3), and neo-tetraploid male (n=1) were used as specimens in this study. Ploidy status of somatic cells and sperm of each specimen was assayed by flow cytometry using a ploidy analyzer (Partec PA, Germany). Motility was assessed using our previous procedures (Fujimoto et al., 2008). Briefly, total motility (%) and progressive motility (%) were obtained from video sequences from subjective visualization of sperm movement with a video recorder (Sharp VHS VC-HF920). The total motility corresponds to the percentage of cells with any type of movement. Progressive motility corresponds to the percentage of motile cells with a straight movement along a linear track. Ultrastructure of the spermatozoa was observed by SEM and TEM, according to the procedures in carp sperm (Psenicka et al., 2009). To evaluate the volume of mitochondrial mass per spermatozoon, spermatozoa were stained with the mitochondria-specific MitoTracker Green (Molecular Probes, USA) and then fluorescence emission was analyzed by excitation with argon laser at 488 nm wave length with a 525nM filter using EPICS ALTRA Cell Sorter (Beckman-Coulter, USA). ATP content was measured using a Bioluminescence Assay Kit HS II (Roche Diagnostics GmbH, Germany).

Results

Poor total motility (<5%) and no progressive motility of diploid sperm from the sex-reversed clonal diploid were confirmed, while haploid sperm from normal diploid males and diploid sperm from neo-tetraploid

male demonstrated active progressive motility (80 to 92%). Diploid spermatozoa from sex-reversed clonal diploid and neo-tetraploid males had normal shape similar to haploid spermatozoa, but head size (length / width of head, results were presented as means ± SD) of two kinds of diploid spermatozoa (2.24 ± 0.09 / 2.23 ± 0.11 μm, there is no significant difference between these two kinds of diploid spermatozoa) was approximately 1.24 times larger than that of haploid spermatozoa (1.8 ± 0.07 / 1.8 ± 0.06 μm). Flagellum was longer in two kinds of diploid spermatozoa (31.13 ± 5.37 μm), when compared with haploid spermatozoa (23.84 ± 2.26 μm). According to the observation by TEM, the number of mitochondria per spermatozoon was 7 to 14 both in haploid spermatozoa from normal diploids and diploid spermatozoa from sex-reversed clonal diploids. In contrast, diploid spermatozoa from a neo-tetraploid gave 14 to 22 mitochondria per spermatozoon. Total volume of mitochondrial mass per spermatozoon was not different between diploid spermatozoa from sex-reversed clonal diploid and those from neo-tetraploid, but larger than haploid spermatozoa from normal diploid. Significant differences of ATP content were recorded among haploid spermatozoa from normal diploid (79.02 ± 5.3 nmol/10^9spermatozoa), diploid spermatozoa from sex-reversed clonal diploid (179.05 ± 4.68 nmol/10^9spermatozoa) and diploid spermatozoa from neo-tetraploid (360.53 ± 5.88 nmol/10^9spermatozoa) (one-way analysis of variance, $P<0.05$).

Discussion and conclusions

Spermatozoa size and flagellum length are positively depended on ploidy level and thus diploid spermatozoa show larger sperm-head size and longer flagellum. Similar findings were observed for sperm of oyster (Dong et al., 2005). Volume of mitochondrial mass per spermatozoon is also increasing parallel to the elevation of ploidy level from haploid to diploid. In contrast, number of mitochondria per spermatozoon is same between haploid spermatozoa from normal diploid and diploid spermatozoa from sex-reversed clonal diploid. However, diploid spermatozoa from neo-teraploid showed increased number of mitochondria. Larger size of diploid spermatozoa may require much more energy to assure active motility for successful fertilization. The quantity of stored ATP is positively correlated to sperm motility (Igermann et al., 2003), and thus perhaps initial ATP content predict overall sperm quality. ATP content of clonal diploid spermatozoa is higher than that of haploid spermatozoa, but is lower than that of diploid spermatozoa from neo-tetraploid. In conclusion, poor motility of clonal diploid sperm may be explained in part by lower ATP content than that of diploid sperm from neo-tetraploid, because there is no difference in sperm-head size and flagellum length between two kinds of diploid spermatozoa. Thus active motility of normal diploid spermatozoa from neo-tetraploid may relate to the increase of mitochondrial number per spermatozoon.

Acknowledgements

This work was supported in part by Grants-in-Aid from JSPS (No.21380114, P9325), BRAIN (Promotion of Basic Research Activities for Innovative Bioscience), and JAMBIO (No.22-75, 23-11).

References

Dong Q.; Huang C.;Tiersch T.R., 2005: Spermatozoal ultrastructure of diploid and tetraploid Pacific oysters. Aquaculture 249, 487– 496.

Fujimoto T.; Yasui G.S.; Yoshikawa H.; Yamaha E.; Arai K., 2008: Genetic and reproductive potential of spermatozoa of diploid and triploid males obtained from interspecific hybridization of *Misgurnus anguillicaudatus* female with *M.mizolepis* male J. Appl. Ichthyol.24, 430-437.

Fujimoto T.; Yasui G.S.; Hayakawa M.; Sakao S.; Yamaha E.; Arai K., 2010: Reproductive capacity of neo-tetraploid loaches produced using diploid spermatozoa from a natural tetraploid male. Aquaculture 308, 133–139.

Ingermann R.L.; Robinson M.L.; Cloud J.G., 2003: Respiration of steelhead trout sperm: sensitivity to pH and carbon dioxide and carbon dioxide. J.Fish Biol.62, 13-23.

Psenicka M.; Rodina M.; Flajshans M.; Kaspar V.; Linhart O., 2009: Structural abnormalities of common carp *Cyprinus carpio* Spermatozoa. Fish Physiol Biochem. 35, 591–597.

Morishima K.; Horie S.; Yamaha E.; Arai, K., 2002: A cryptic clonal line of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) evidenced by induced gynogenesis, interspecific hybridization, microsatellite genotyping and multilocus DNA fingerprinting. Zool. Sci.19, 565-575.

Yoshikawa H.; Morishima K.; Kusuda S.; Yamaha E.; and Arai K., 2007: Diploid Sperm Produced by Artificially Sex-Reversed Clone Loaches. J. Exp. Zool.307A, 75–83.

P-204
POST-THAWED SPERM MOTILITY AND CURVILINEAR VELOCITY OF FOUR MIGRATORY FISH SPECIES NATIVE TO THE GRANDE RIVER BASIN, BRAZIL

Marcelo C. Leal[1], Antônio C.S. Gonçalves[2], Thatijanne S.G. Carvalho[2], Ana T.M. Viveiros[2]

[1] *R. Topázio 524, Condomínio Stone Village, Lavras, 372000-000, MG, Brazil*
[2] *Dept Animal Science, Federal University of Lavras, Lavras, 372000-000, MG, Brazil*

E-mail: anatmviveiros@hotmail.com

Introduction
The Brazilian freshwater fish fauna has the highest diversity in the world with almost 2,500 formally described species (Buckup et al. 2007) and many of these species migrate up-stream or down-stream to find an appropriate environment and spawn (Godinho & Godinho 1994). Brazilian migratory fishes are a highly valuable natural resource with an amazing diversity of life histories (Carolsfeld et al. 2003). However, changes in the river stream due to construction of hydroelectric dam blocks the migratory route of these species. The hydroelectric companies are thus responsible for reproducing these fishes artificially and restore the population. The use of cryopreserved sperm for restoration is highly appreciated as sperm becomes available during the whole spawning season and for exchange purpose among hatcheries. The aim of the present study was to evaluate post-thawed sperm motility and curvilinear velocity (VCL) of four migratory characiform species (piracanjuba *Brycon orbignyanus*, piapara *Leporinus elongatus*, streaked prochilod *Prochilodus lineatus* and dorado S*alminus brasiliensis*) native to the Grande River basin and artificially reproduced at Hydroelectric Company of Furnas in the Southeastern Brazil.

Material and methods
Males were selected from earthen ponds at the Fish Culture Unit of the Electric Company of Furnas S. A., São José da Barra, MG, Brazil, during the spawning season (December and January). Males with detectable running sperm under soft abdominal pressure received a single (3 mg/kg; *S. brasiliensis*) or double (0.3 and 3 mg/kg; *B. orbignyanus*, *L. elongatus and P. lineatus*) intramuscular doses of carp pituitary extract. After 7-10 hours at ~25 °C, the urogenital papilla was carefully dried and sperm was hand-stripped. Sperm was diluted in a freezing medium comprising the combination of an extender (5% glucose for *L. elongatus*, *P. lineatus* and *S. brasiliensis,* or 5% BTS™ for *B. orbignyanus*) and methyl glycol as cryoprotectant, according to previous report (Godinho & Viveiros 2011). Diluted sperm was aspirated into 0.5-mL straws (n = 2-4 straws/male) and frozen in a nitrogen vapor vessel (dry-shipper) at -170 °C. Straws were transported ~260 km by car from Furnas to the Laboratory of Semen Technology at the Federal University of Lavras (UFLA), where straws were transferred to liquid nitrogen within 20-24 hours for storage. After 2-3 months, straws were thawed in a water bath at 60 °C for 8 sec (Viveiros & Godinho 2009) and sperm was immediately evaluated for progressive motility and VCL using a computer-assisted sperm analyzer (CASA). Post-thaw sperm was activated in a Leja™ counting chamber placed on a phase contrast microscope (Nikon™ E200, Japan), 100 x magnification, green filter, and pH1 position. The microscope was connected to a video camera (Basler Vision Technologies™ 602FC, Ahrensburg, Germany) which generated 100 frames/sec. Video recording was started at ~10 sec post-activation in NaCl 50 mM. Each image was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™, Microptics, S.L. Version 5.1, Barcelona, Spain). Spermatozoon was considered immotile when velocity was slower than 20 μm/sec.

Results
The highest progressive motility (57%) was observed for *P. lineatus* while the lowest motility was observed for *B. orbignyanus* and *L. elongatus* (18%; Table 1). The highest VCL (192 μm/sec) was observed for *P. lineatus*, which was almost twice as much compared to *B. orbignyanus* (95 μm/sec) and three times or more compared to *S. brasiliensis* (64 μm/sec) or *L. elongatus* (52 μm/sec), respectively.

Table 1. Post-thawed sperm motility and curvilinear velocity (VCL; mean ± SD) evaluated using CASA of four migratory characiform species originated from the Grande River basin, Brazil

Species	number of males	Progressive motility (%)	VCL (μm/sec)
Brycon orbignyanus	5	18 ± 8	95 ± 17
Leporinus elongatus	2	18 ± 12	52 ± 14
Prochilodus lineatus	4	57 ± 8	192 ± 51
Salminus brasiliensis	3	33 ± 12	64 ± 7

Discussion and conclusions

During the last decade, the use of CASA to evaluate sperm quality in fish species has become popular, but this technology has only recently been introduced in Brazil (Godinho & Viveiros 2011), and thus sperm of only few species has been evaluated using CASA. Although the CASA settings are different among studies, post-thawed sperm of *P. lineatus* always yields motility above 50%. Furthermore, post-thawed sperm VCL in *P. lineatus* (192 μm/sec) was very high compared to the other three characiform species evaluated here (52-95 μm/sec), and also compared to the same species in another study (49 μm/sec; Viveiros et al. 2010). In that study, *P. lineatus* sperm was frozen in the same medium (glucose and methyl glycol) but CASA settings were different: the video camera generated only 25 frames/sec, and spermatozoon was considered immotile when velocity was < 10 μm/sec. As reported before (Wilson-Leedy & Ingermann 2007) and also observed in the present study, increasing the number of frames/sec increases VCL (the recommended rate is 100 frames/sec), and immotile spermatozoa should be considered those which velocity is < 20 μm/sec (or some immotile but vibrating spermatozoa are counted as motile). In our laboratory we standardized the setting described above so that we can compare differences among species.

Acknowledgements

ANEEL P&D Furnas (017965), CNPq (554950/2009-0; 506679/2010-1) and FAPEMIG (PPM CVZ 00040/09).

References

Buckup, P.A.; Menezes, N.A.; Ghazzi, M.S., 2007: Catálogo das espécies de peixes de água doce do Brasil. Museu Nacional, Rio de Janeiro.

Carolsfeld, J.; Godinho, H.P.; Zaniboni Filho, E.; Harvey, B.J., 2003: Cryopreservation of sperm in Brazilian migratory fish conservation. J. Fish Biol. 63, 472-489.

Godinho, H.P.; Godinho, A.L., 1994: Ecology and conservation of fish in southeastern Brazilian river basins submitted to hydroelectric impoundments. Acta Limnol. Bras. 5, 187-197.

Godinho, H.P.; Viveiros, A.T.M., 2011: Current status of sperm cryopreservation of Brazilian characiform fishes. In: Cryopreservation in Aquatic Species. 2nd edition. Tiersch T.R.; Green C.C., eds. Baton Rouge: World Aquaculture Society. pp. 875-884.

Viveiros, A.T.M.; Godinho, H.P., 2009: Sperm quality and cryopreservation of Brazilian freshwater fish species: a review. Fish Physiol. Biochem. 35, 137-150.

Viveiros, A.T.M.; Nascimento, A.F.; Orfão, L.H.; Isaú, Z.A., 2010: Motility and fertility of the subtropical freshwater fish streaked prochilod (*Prochilodus lineatus*) sperm cryopreserved in powdered coconut water. Theriogenology 74, 551-556.

Wilson-Leedy, J.G.; Ingermann, R.L., 2007: Development of a novel CASA system based on open source software for characterization of zebrafish motility parameters. Theriogenology 67, 661-672.

P-205
STANDARDIZATION OF EUROPEAN EEL SPERM MOTILITY EVALUATION BY CASA SOFTWARE

Víctor Gallego[1], Paulo C.F. Carneiro[2], Ilaria Mazzeo[1], María C. Vílchez[1], Rosa Baeza[1], David S. Peñaranda[1], Luz Pérez[1], Juan F. Asturiano[1]

[1] *Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universidad Politécnica de Valencia. Camino de Vera s/n, 46022 Valencia, Spain*
[2] *Embrapa Tabuleiros Costeiros, Av. Beira Mar 3250, 49025-040 Aracaju, Brazil*

E-mail: jfastu@dca.upv.es

Introduction
Conventional methods used for sperm quality analysis, including sperm motility assessment, are subjective and great variations have been reported in non-objective evaluations of the same samples (Coetzee et al. 1999). Studies of spermatozoa kinetics are useful tools that have been possible by the development of powerful CASA software. These objective and accurate methods has been used and validated for several species (Verstegen et al. 2002), but some questions have emerged in relation to sample evaluation (i.e., frame rates, sperm dilution, analysis chamber, etc). Therefore, a systematic standardization of analytical settings is a fundamental requirement for its correct application (Contri et al. 2010; Rosenthal et al. 2010). In the present study, we evaluated the effects of different settings as time of acquisition, model of analysis chamber and microscopy magnification on the kinetic results of eel sperm.

Materials and methods
Sixty adult eel males (body weight 100±2 g; length 40±5cm) were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of hCG (1.5 IU g^{-1} fish). Sperm samples were collected by abdominal pressure, motility was assessed for triplicate using ISAS® system and samples were classified into four motility classes (I: <25%, II: 25-50%, III: 50-75%, and IV: >75%; n=15 samples for each class). Two chamber models (10 µm Makler® reusable and 20 µm ISAS® disposable counting chambers) and two magnification lenses (10 and 20x) were tested on each class. Sperm motility parameters were recorded at different post-activation times (30, 60 and 90 s).

Results
Motility values obtained on all sperm classes showed a progressive decrease over time. Differences resulted significant on classes III and IV, in which motility recorded at 90 s was lower than motility obtained at 30 and 60 s (Fig. 1).

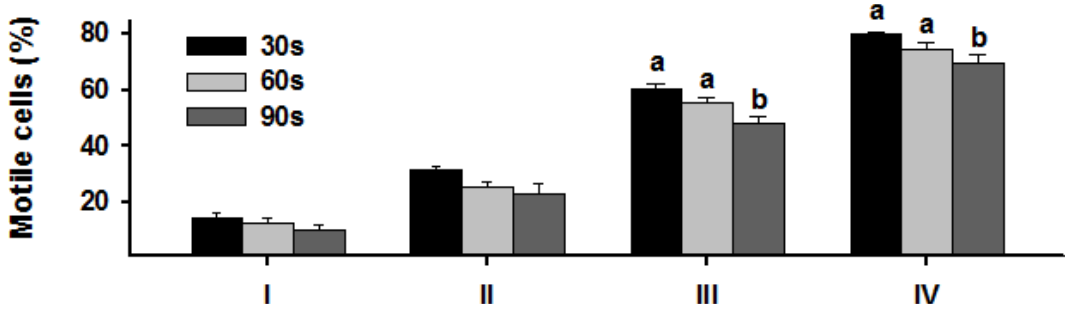


Figure 1. *Sperm motility at different post-activation times (30, 60 and 90s) on different sperm classes (I-IV). Data are expressed as mean ± SEM and different letters indicate significant differences between times*

On the other hand, different magnifications did not affect significantly sperm parameters on any motility class (Table 1). However, samples analyzed by 20x magnification lens showed higher coefficients of dispersion than the same samples analyzed by 10x lens.

Chamber model, Makler® and ISAS®, neither affected significantly sperm parameters on any motility class (Table 1).

Table 1. Mean of sperm parameters: motile spermatozoa (MOT; %), progressive motile spermatozoa (PM; ≥80% straightness; %) and sperm kinetic parameters (curvilinear velocity, VCL; straight/line velocity, VSL; and average path velocity, VAP; μm s⁻¹) for different sperm classes, chamber models and microscopy magnifications

Class	Camera	Lens	MOT	PM	VCL	VSL	VAP
I	Proiser	10X	10.9 ±	20.8 ±	100.5 ± 9.3	34.7 ±	53.9 ±
		20X	14.3 ±	23.3 ±	79.8 ± 11.4	35.2 ±	48.4 ±
	Makler	10X	14.8 ±	21.9 ±	81.0 ± 11.6	29.9 ±	45.2 ±
		20X	19.6 ±	26.4 ±	89.2 ± 13.0	34.7 ±	48.7 ±
II	Proiser	10X	24.9 ±	32.1 ±	121.5 ± 8.0	52.8 ±	72.4 ±
		20X	33.9 ±	33.6 ±	100.0 ±	47.1 ±	62.7 ±
	Makler	10X	17.9 ±	39.8 ±	114.9 ±	53.2 ±	70.3 ±
		20X	23.7 ±	36.7 ±	88.1 ± 12.0	40.1 ±	54.0 ±
III	Proiser	10X	53.3 ±	40.9 ±	152.9 ± 7.4	70.8 ±	93.9 ±
		20X	60.6 ±	43.5 ±	123.8 ±	61.3 ±	79.2 ±
	Makler	10X	46.6 ±	46.3 ±	146.7 ± 6.8	71.3 ±	91.8 ±
		20X	46.7 ±	24.9 ±	114.0 ±	47.7 ±	69.3 ±
IV	Proiser	10X	70.7 ±	46.5 ±	141.0 ±	70.8 ±	93.5 ±
		20X	79.0 ±	36.0 ±	154.4 ±	70.2 ±	99.6 ±
	Makler	10X	63.9 ±	46.7 ±	166.4 ±	80.9 ±	105.7 ±
		20X	78.8 ±	46.1 ±	137.8 ±	68.7 ±	90.6 ±

Discussion and conclusions

Motility is considered as an important parameter on sperm quality evaluation and it is essential to standardize techniques used to estimate it (Contri et al. 2010). In our study we evaluated, firstly, different post-activation times; the decreases in sperm motility and velocity over time suggest that sperm analysis should be performed within the first 60s after activation.

Secondly, two magnification lenses were tested, and the most accurate measurements were obtained with the 10x lens. This result is probably due to the bigger sample size, which involves a higher number of captured spermatozoa by the 10x lens, resulting in a lower variation on the acquired data.

Finally, two chamber models with different shapes, depths and handling were tested, and not significant differences were found between them.

In conclusion, protocol variables on the sperm quality analysis by this CASA software (ISAS®) did not show a significant effect on eel sperm parameters on any motility class, meaning that results obtained by different laboratories could be compared directly.

Acknowledgements

Funded from the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology", grant agreement n°245257 (Pro-Eel) and Generalitat Valenciana (ACOMP/2011/ 229). D.S.P. and P.C.F.C. have postdoc grants from UPV and PAC-EMBRAPA, respectively. I.M. and V.G. have predoctoral grants from GVand Spanish MICINN, respectively.

References

Coetzee, K.; Kruger, T.F.; Lombard, C.J., 1999: Repeatability and variance analysis on multiple computer-assisted (IVOS) sperm morphology readings. Andrologia 31, 163-168.

Contri, A.; Valorz, C.; Faustini, M.; Wegher, L.; Carluccio, A., 2010: Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa. Theriogenology 74, 424-435.

Rosenthal, H.; Asturiano, J.F.; Linhart, O.; Horvath, A., 2010: On the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. J. Appl. Ichthyol. 26, 621-622.

Verstegen, J.; Iguer-Ouada, M.; Onclin, K., 2002: Computer assisted semen analyzers in andrology research and veterinary practice.Theriogenology 57, 149-179.

P-206
COMPARISON OF TECHNIQUES FOR THE MORPHOMETRY STUDY OF GILTHEAD SEABREAM (*SPARUS AURATA*) SPERMATOZOA AND CHANGES INDUCED BY CRYOPRESERVATION

Víctor Gallego, David S. Peñaranda, Francisco Marco-Jiménez, Ilaria Mazzeo, Luz Pérez, Juan F. Asturiano

Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

E-mail: jfastu@dca.upv.es

Introduction

The use of ASMA software to estimate the spermatozoa morphology has allowed the development of objective measurements and the increase of accuracy. However, some problems have emerged in relation to sample preparation, i.e.: protocol variables as fixation techniques or staining process, can influence spermatozoa morphometry (Maree et al. 2010). Therefore, to find an optimal (standardized) technique, producing the minimal variation, should be useful to make possible the comparison of studies between research groups.

Moreover, sperm cryopreservation has several applications in aquaculture, such as synchronization of gamete availability or preservation of genetic resources. However, it is known that this process causes negative effects on spermatozoa morphometry, motility and viability in teleost fish, but actually there are scarce reports on gilthead seabream sperm. The main objectives of the present study were: first, the assessment of a good technique for morphometry analysis of gilthead seabream spermatozoa; and second, to know the effect that cryopreservation process has on sperm cells morphology, motility and viability.

Materials and methods

Sperm was collected during natural spermiation period and motility was subjectively evaluated for triplicate. Fifteen pools were formed with sperm from at least 3 males having >90% of motile cells and an aliquot from each pool was cryopreserved using the protocol described by Cabrita et al. (2005). Fluorescence stain analysis Live/Dead Sperm Viability Kit (Invitrogen) was used to evaluate cell viability, while morphometry parameters were evaluated using the morphometry module of ISAS®. Two experiments were carried out:

Experiment 1. Evaluation of two techniques for the spermatozoa morphometry analysis. At least 100 cells/pool were analyzed using two different techniques. Briefly, first was a staining method (HH) in which sperm was fixed with methanol across a slide during 10 min, stained in Harri´s Hematoxylin solution during 30 min and analyzed by non-phase contrast lens; alternatively a negative phase contrast technique (NPC) was used, fixing sperm with glutaraldehyde (2.5%), that was later analyzed using an Improved Neubauer hemocytometer.

Experiment 2. Effects of cryopreservation process on sperm quality parameters. Parameters of morphometry, motility and viability were recorded for fresh, diluted (in freezing medium containing 5% DMSO as cryoprotectant) and frozen-thawed sperm.

Results

Experiment 1. HH staining caused larger cells, showing significant differences in all the parameters (Table 1). Coefficients of variation obtained with NPC technique were lower than those obtained with HH technique.

Table 1. *Mean morphometry parameters and coefficients of variation (CV, %) obtained with NPC or HH techniques. Asterisks mean significant differences*

	NPC	**HH**	**CV_{NPC}**	**CV_{HH}**
Area (μm²)	4.89*	6.98	4.02*	7.00
Perimeter(μm)	8.08*	10.31	2.13*	3.37
Length (μm)	2.57*	3.14	2.09*	1.95
Width (μm)	2.34*	2.75	2.29*	4.57

Experiment 2. Fresh sperm cells showed significantly higher width than diluted and frozen-thawed cells (Table 2). Fresh sperm showed significantly higher motility and viability than frozen-thawed samples (Figure 1).

Table 2. Size parameters of gilthead seabream spermatozoa on fresh sperm (FRESH), diluted sperm (DMSO) and frozen-thawed sperm (CRYO). Data are expressed as mean ± SEM and different letters indicate significant differences

	FRESH	DMSO	CRYO
Area (μm^2)	4.89 ± 0.05	4.78 ± 0.03	4.79 ± 0.04
Perimeter	8.08 ± 0.04	8.04 ± 0.04	8.08 ± 0.04
Length (μm)	2.57 ± 0.01	2.57 ± 0.02	2.58 ± 0.01
Width (μm)	2.34 ± 0.01a	2.28 ± 0.01b	2.28 ± 0.02b

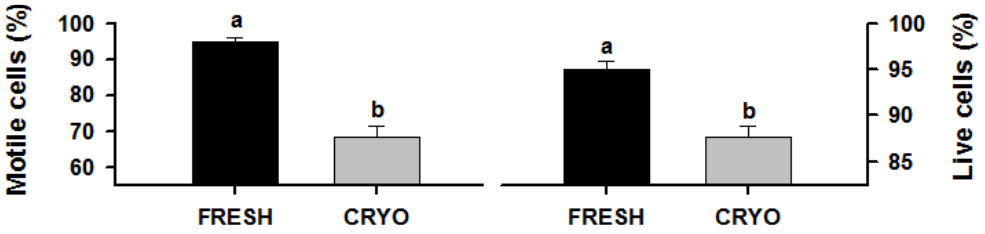


Figure 1. Motile cells and spermatozoa viability in fresh and frozen-thawed (CRYO) gilthead seabream sperm. Data are expressed as mean ± SEM and different letters indicate significant differences

Discussion and conclusions

Our results evidenced the importance of the choice of an accurate and simple technique for the morphometry characterization of fish spermatozoa. A right election can make easier to compare results from different research groups and to establish standardizations (Rosenthal et al. 2010). In this sense, NPC technique resulted more accurate for measuring head spermatozoa, as probed their smaller CVs, being a useful and simple method, without the need of staining process, and then minimizing the possibility that spermatozoa head dimensions would be influenced by protocol steps.

This is the first report on the effects of the cryopreservation process on the morphology of gilthead seabream spermatozoa. Diluted and frozen-thawed sperm showed a little decrease of cells head area and more elongated shape, being the addition of cryoprotectant (DMSO) the most important factor that caused these changes. Also, significant decreases in motility (27%) and viability (8%) were detected, as possible results of multiple factors such as the loss of membrane function or the decrease in ATP production (Peñaranda et al. 2008).

Acknowledgements

This work was financed by Spanish Ministry of Science and Innovation (MICINN; AGL2007-64040-C03-00). V.G. and I.M. were supported by FPI scholarships financed by MICINN and Generalitat Valenciana, respectively. D.S.P. has a postdoctoral grant from UPV.

References

Cabrita, E.; Robles, V.; Cuñado, S.; Wallace, J.C.; Sarasquete, C.; Herráez, M.P., 2005: Evaluation of gilthead seabream, *Sparus aurata*, sperm quality after cryopreservation in 5 ml macrotubes. Cryobiology 50, 273-284.

Maree, L.; du Plessis, S.S.; Menkveld, R.; Van der Horst, G., 2010: Morphometric dimensions of the human sperm head depend on the staining method used. Hum. Reprod.25, 1369-1382.

Peñaranda, D.S.; Pérez, L.; Fakriadis, G.; Mylonas, C.C.; Asturiano, J.F., 2008: Assay on motility and morphometry effects of extenders and cryoprotectant combinations on sea bass (*Dicentrarchus labrax*) spermatozoa. J. Appl. Ichthyol. 24, 450-455.

Rosenthal, H.; Asturiano, J.F.; Linhart, O.; Horvath, A., 2010: On the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. J. Appl. Ichthyol. 26, 621-622.

P-207
EFFECT OF SHORT-TERM CONSERVATION AND EXPOSURE TO FIVE DIFFERENT CRYOPROTECTANTS ON SPERM MOTILITY OF *PAGELLUS ERYTHRINUS* (L.)

Valentina Vitiello[1], Antonio Luca Langellotti[1], Francesco Del Prete[1], Fausto Silvestri[1,2], Francesca Rinna[1], Giovanni Sansone[1,3]

[1] *Interdepartmental Research Center for Hydrobiological Resources Management and for Aquaculture, CRIAcq, University of Naples Federico II, Via Università, Naples, Italy*
[2] *The CAPES Foundation – Ministry of Education of Brazil, Brazil*
[3] *Department of Biological Sciences - University of Naples Federico II, Naples, Italy*

E-mail: valentina.vitiello@unina.it

Introduction
The common pandora, *Pagellus erythrinus* (Linnaeus, 1758), is an important species for fisheries and one of the most promising species for diversification of marine aquaculture in the Mediterranean Sea (Klaoudatos et al. 2004).

Successful storage of fish sperm in liquid nitrogen has been achieved in several fish species, but the procedures for fish sperm cryopreservation differ from species to species (Tanaka et al. 2002).

Although there are works on the morphology and seasonal fluctuations in sperm quality of common pandora (Maricchiolo et al. 2004; Lechekhab 2007), there are not studies on semen cryopreservation of this specie. In the present study, the effects on sperm motility after short-term conservation (until 72 hours) and after incubation in five different cryoprotectants were examined; this experimentation represents a preliminary step for the development of a cryopreservation protocol for sperm of common pandora.

Materials and methods
Seminal fluid was obtained by abdomen stripping of at least 10 adult mature males, previously anesthetized, bred in a Mediterranean fish farm (Panittica Pugliese, Apulia, Italy). The semen was collected individually and maintained at a temperature of 3±1°C. Individual semen samples showing better motility were mixed in homogeneous pools. All activations were made by dilution 1:100 with filtered seawater (0.2 μm, 36‰, 8.1 pH) at 18±1°C, evaluating the percentage of motile spermatozoa and the quality of their movement, as described in Fabbrocini et al. (2000). Four values were recorded to describe the motility sperm curve: time to reach the maximum motility (activation time), maximum motility value, duration of maximum motility and total time of motility (until class 0). To evaluate short-term conservation, aliquots of dry semen and diluted semen (1:6 in 1% NaCl - inhibitor of motility) were placed at 3±1°C in the dark and activated 6, 24, 48 and 72h after sampling. The maximum class and the total duration of sperm motility were recorded. For cryoprotectants toxicity evaluation, the sperm diluted 1:6 with a solution of 1% NaCl (v/v) and containing the cryoprotectants, was incubated at 18±1°C. The following cryoprotectants were tested: dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (GlOH) at 5-7-10-15-20 % v/v and methanol (MetOH) at 2-4-6-8-10 % v/v. After 10, 20 and 30 minutes, aliquots of sperm suspensions were activated and the maximum motility classes recorded.

Results
The sperm motility values were reported in Table 1. After collection, the *P. erythrinus* sperm is characterized by a very fast activation time (1 min), a maximum motility corresponding to percentage of RVL sperm (rapid, vigorous and linear) over 80% (class 4), the maintenance of sperm motility over class 3 for 10 min and 30 min of total total duration of motility. Dry semen kept at 3±1°C showed a significant ($p<0.05$) reduction in sperm motility already after 24 h, with decay in maximum class, duration of maximum class and total duration of motility. The dilution with 1% NaCl induced a reduction in maximum class of motility already after 6 h of incubation, while it allowed a better duration of the highest class and the total duration of motility until 72 h. The activation time did not show any significant modification (data not shown).

Table 1. Motility parameters of P. erythrinus semen: [a] maximum motility value (motility class, mean ± sd), [b] duration of maximum motility values (min) and [c] total time of motility (until class 0) (min)

Motility parameters	Activation after pool formation (0h)	Dry conservation at 3±1°C				1% NaCl dilution (1:6) conservation at 3±1°C			
		6h	24h	48h	72h	6h	24h	48h	72h
[a] (class)	4.2±0.2	4.0±0.2	3.5±0.2	3.5±0.3	3.5±0.3	3.3± 0.2	3.1±0.3	3.0±0.3	3.0±0.2
[b] (min)	10	10	5	5	2	10	10	7	3
[c] (min)	30	30	25	25	20	30	30	30	15

DMSO and EG were found to be the less toxic cryoprotectants (Table 2): their concentrations up to 10% did not induce significant differences in sperm motility compared to the control, even after 30 min of incubation. The other cryoprotectants tested (PG, GlOH, MetOH) showed higher levels of toxicity already at 10 min of incubation time.

Table 2. Maximum percentage (v/v) of cryoprotectants that gave values of sperm motility statistically not different from control (p>0.05) after 10, 20 and 30 min of incubation

	DMSO (%v/v)	EG (%v/v)	PG (%v/v)	GlOH (%v/v)	MetOH (%v/v)
10 min	15	15	10	10	6
20 min	15	15	10	10	4
30 min	10	10	5	7	4

Discussion and conclusions

Common pandora showed a motility sperm pattern similar to other marine fish, with a high initial value and a decline in few minutes after activation (Cosson et al, 2008); differences were recorded in total time of sperm motility respect to data of Lechekhab (2007), that reported total motility duration ranging from 35 (in August) to 60 min (in July) in common pandora adults males bred in Algeria. The best short-term conservation procedure until 24 h after the sperm collection was obtained by diluting the semen with 1% NaCl and kept at 3±1°C.

The effects on sperm motility obtained after exposure to cryoprotectants showed considerable differences respect to the results reported for seabream in Fabbrocini et al. (2000) and for seabass in Sansone et al. (2002). In particular, common pandora showed lower effects on sperm motility after exposure to all cryoprotectants tested. The results obtained in this work will be useful to develop an effective cryopreservation protocol for *P. erythrinus* spermatozoa.

References

Cosson, J.; Groison, A.-L.; Suquet, M.; Fauvel, C.; Dreanno, C.; Billard, R, 2008: Studyng sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460-486.

Fabbrocini, A.; Lubrano Lavadera, S.; Rispoli, S.; Sansone, G., 2000: Cryopreservation of sea bream (*Sparus aurata*) spermatozoa. Cryobiology 40, 46-53.

Klaoudatos, S.D.; Iakovopoulos, G.; Klaoudatos, D.S., 2004: *Pagellus erythrinus* (common pandora): a promising candidate species for enlarging the diversity of aquaculture production. Aquaculture Inter. 12, 299–320.

Lechekhab, S., 2007: Contribution à l'étude de la fertilité chez *Pagellus erythrinus*: spermogramme et spermocytogramme. Cybium 31(2), 245-249.

Maricchiolo, G.; Genovese, L.; Laurà, R.; Micale, V.; Muglia, U., 2004: Fine structure of spermatozoa in the common pandora (*Pagellus erythrinus* Linnaeus, 1758) (Perciformes, Sparidae). Histol. Histopathol. 19, 1237-1240.

Sansone, G.; Fabbrocini, A.; Ieropoli, S.; Langellotti, A.L.; Occidente, M.; Matassino, D., 2002: Effects of extender composition, cooling rate, and freezing on the motility of sea bass (*Dicentrarchus labrax*, L.) spermatozoa after thawing. Cryobiology 44, 229–239.

Tanaka, S.; Zhang, H.; Horie, N.; Yamada, Y.; Okamura, A.; Utoh, T.; Mikawa, N.; Oka, H.P.; Kurokura, H., 2002: Long-term cryopreservation of sperm of Japanese eel. J. of Fish Biol. 60, 139–146.

P-208
TOXICITY OF *STEINDACHNERIDION PARAHYBAE* EMBRYOS AT DIFFERENT CRYOPROTECTANTS SOLUTIONS

Eduardo Antônio Sanches[1], Taís da Silva Lopes[2], Renan Yoshiharu Okawara[3], Danilo Caneppele[4], Elizabeth Romagosa[3]

[1] *Aquaculture Centre, State University of São Paulo, Jaboticabal, SP, Brazil*
[2] *Animal Science Department, State University of São Paulo, Jaboticabal, SP, Brazil*
[3] *Fishery Institute – APTA, São Paulo, SP, Brazil*
[4] *Hydrobiology and Aquaculture Station - CESP, Paraibuna, SP, Brazil*

E-mail: eromagosa@pesca.sp.gov.br

Introduction
The Surubim-of-Paraíba, *Steindachneridion parahybae* (Siluriformes: Pimelodidae), is a catfish gray with dark spots along the body, endemic to the basin of the Paraíba do Sul River, and currently is in the list of Brazilian fauna threatened of extinction with critical condition (Honji et al. 2009). Recovery programs of local fish fauna, biodiversity conservation programs in genetic studies, gamete characterizing, cryopreservation and reproductive biotechnologies should be conducted (Caneppele et al., 2009). The possibility of cooling and cryopreservation of embryos is an alternative to be applied to the conservation of genetic material (Lopes et al., 2011). However, to be effective need the prior knowledge of factors affecting their viability, including the use of an extender solution, consisting of intracellular and extracellular agents, which prevents the formation of ice crystals (Streit Jr. et al., 2007). Thus, the aim was to evaluate the toxicity of six solutions cryoprotectant to embryos of *S. parahybae*.

Materials and methods
The experiment was carried in the Hydrobiology and Aquaculture Estation - CESP, Paraibuna/SP/BR. Were used 1,680 embryos in closing the blastopore (10h post-fertilization in water at 22.9 ± 0.4°C) from three females (733.3 ± 115.5 g) and one male (950g) of *S. parahybae*. After counting, the embryos were subjected to a bath for 30 minutes with the cryoprotectant solutions composed of intracellular and extracellular cryoprotectants, respectively: (Met + Lac) 10% methanol + 0.5 M sucrose, (Met + Suc) methanol + 10% lactose 0.5 M; (Ethyl + Suc) 10% ethylene glycol + 0.5 M sucrose, (Ethyl + Lac) 10% ethylene glycol + 0.5 M lactose, (Dmso + Suc) 10% dimethil sulphoxide + 0.5 M sucrose, (Dmso + Lac) 10% dimethil sulphoxide + 0.5 M lactose. The control treatment was free bath. The cryoprotectant solutions were subjected to -10°C to verify the formation of ice crystals. Then, 420 embryos (60 per treatment) were fixed in buffered formalin (10%) to measure the diameter of the embryo and chorion in stereomicroscope (20x magnification). After 1,260 were incubated (60 embryos each) in 21 conical incubators in 1.5 L of water recirculation system. After hatching was the counting of larvae, and classified as normal or defective. Data were subjected to analysis of variance (P<5%).

Results
There was no effect of cryoprotectant solutions on the percentage of larvae, size of the embryo and chorion (P>0.05), however, with values (means ± standard error) of 84.74 ± 2.30%, 0.27 ± 0.01mm and 3.26 ± 0.01mm, respectively. The percentage of normal larvae was different (P<0.05) among the solutions tested, with the lowest values found in the use of extender solution counting Met+Lac (Figure 1). We observed the formation of ice crystals in solutions Met+Lac, Ethyl+Lac, Dmso+Suc and Dmso+Lac.

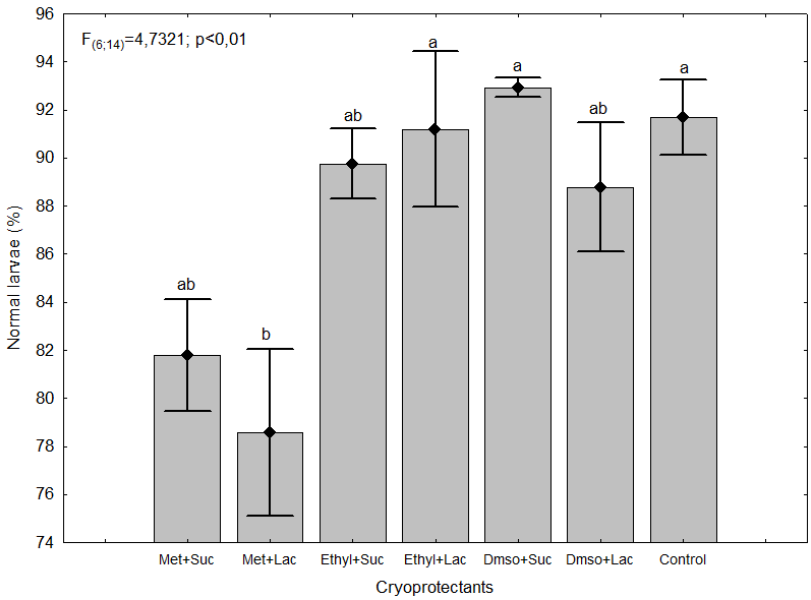


Figure 1. Percentage of normal larvae (mean ± standard error) of S. parahybae underwent 30 minutes of bathing. Different letters indicate P<0.05 according to Tukey test

Discussion and conclusions

The use of solutions Met+Suc, Ethyl+Suc, Ethyl+Lac, DMSO+Suc and DMSO+Lac larvae showed values closer to controls, giving an indication that these cryoprotectants are less toxic for embryos of S. parahybae. Among these, only Met+Suc, Ethyl+Suc could be tested in cryopreservation protocols, since they are not formed ice crystals, when exposed to temperatures below zero (-10°C). These results are fundamental for future studies related to embryo cryopreservation S. parahybae, as well as contribute to the preservation of this species.

Acknowledgements

CNPq (n° 478347/2009-0) and CESP.

References

Caneppele, D.; Honji, R. M.; Hilsdorf, A. W. S.; Moreira, R. 2009: Induced spawing of the endangered neotropical species *Steindachneridion parahybae* (Siluriforme:Pimelodidae). Neotrop. Ichth., 7 (4), p.759-762.

Honji, R. M.; Caneppele, D.; Hilsdorf, A. W. S.; Moreira R. G. 2009: Threatened fishes of the world: Steindachneridion parahybae (Steindachner, 1877) (Siluriformes: Pimelodidae). Environ Biol Fish, 85, 207–208.

Lopes, T.S., Romagosa, E., Streit JR. D.P., Ribeiro R.P., Digmayer, M. 2011: Chilling curves for pacu, *Piaractus mesopotamicus* (Holmberg, 1887) embryos stored at -8 °C. Theriogenology, 75 (3): 570-576.

Streit Jr. D.P., Digmayer, M., Ribeiro, R.P., Sirol, R.N., Moraes, G.V., Galo, J.M. 2007: Embriões de pacu submetidos à diferentes protocolos de resfriamento. Pesq. Agrop. Bras. 42 (8). 1199-1202.

P-209
SPERM QUALITY OF ENDEMIC CATFISH, STEINDACHNERIDION PARAHYBAE (SILURIFORMES, PIMELODIDAE) CRITICALLY ENDANGERED IN BRAZIL

Elizabeth Romagosa[1], Eduardo Antônio Sanches[2], Renan Yoshiharu Okawara[1], Cesar Pereira Rebechi de Toledo[3], Giovano Neumann[3], Danilo Caneppele[4], Robie Allan Bombardelli[3]

[1] *Fishery Institute – APTA, São Paulo, SP, Brazil*
[2] *Aquaculture Centre. State University of São Paulo, Jaboticabal, SP, Brazil*
[3] *State University of West Paraná, Toledo, PR, Brazil*
[4] *Hydrobiology and Aquaculture Station - CESP, Paraibuna, SP, Brazil*

E-mail: eromagosa@pesca.sp.gov.br

Introduction
The use of high quality gametes from captive fish broodstock is a great importance for ensuring the production of valuable offspring for Brazilian aquaculture (Romagosa, 2008). The S. parahybae is a fish migratory freshwater endemic species regionally extinct (Canepelle et al., 2009). This specie require hormonal induction and the rational use of gametes may affect the future genetic integrity of the stock (Romagosa et al., 2010) and, preservation it is an indispensable alternative in fish selection and synchronization of gamete availability. While viability and motility are scored relatively easily, the usefulness of motility measurements has long been questioned since subjective scoring methods are used and have produced variable results. In spite of the recent introduction of computer-assisted sperm analysis (CASA) systems which have provided and objective assessment of sperm quality in fish, their application improve the culture condition of male broodstock in fish farms (ponds or cages) and the success of artificial fertilization in hatcheries is still in its infancy in Brazil (Sanches et al., 2010). The assessment of the quality of fish spermatozoa using computer-assisted techniques is a novel field and little practical progress has been achieved in aquaculture science (Sanches et al., 2010). Our experiments (2009/2011) has been focused: (1) to define sperm quality during the different steps of used or no artificial insemination and, (2) to give an overview of some the more traditional methods used for assessment of sperm quality and to higlight the advantages of using computer-aided analyses of sperm motility as measure of sperm quality.

Materials and methods
These experiments were realized males (511.84 ± 123.53g) during the reproduction (October - April), of the CESP, Paraibuna, SP, BR, during two years: (1) The production capacity and the characteristics of the semen were to examine during the one reproductive cycle and, the effect of induced of CHE. During six collected, with 26 fishes, were divided in two treatments (no induced and induced). (2) In 13 males was collected the semen and used to capture video and further evaluation in CASA according Sanches et al. (2010). A Basler 602fc camera attached to a trinocular Nikon microscope was used (400x magnification). The videos were captured by the software AMCAP (Basler Vision Technologies) at a rate of 100 fps (656 x 490 pixels). The videos were captured in format *.avi, edited in the software VIRTUALDUB-1.9.0 (virtualdub.org), and exported as a sequence of images in format *.jpg. The images corresponding to 0.5 second of video were opened, edited in the software IMAGEJ (National Institutes of Health, USA, *http://rsb.info.nih.gov/ij*) and compiled using the application CASA (*University of California and Howard Hughes Medical Institute, USA*). The analyses of sperm motility by application CASA was conducted after 10s of sperm activation.

Results and discussion
(1)**Traditional method** - The induced hormonal did not affect the seminal parameters (P>0.05) and the parameters of two treatments showed: the semen volume (9.39 ± 1.19 and 9.87 ± 1.03 mL kg^{-1}); motility rate (79.05 ± 1.85 and 81.77 ± 1.54%); activity time (38.76 ± 0.74 and 39.94 ± 0.80 s); spermatic time (9.24 ± 0.88 and 8.75 ± 0.58 $x10^9$ sptz mL^{-1}) and in the seminal plasma (pH: 7.23 ± 0.06 and 7.32 ± 0.05 and osmolarity: 263.21 ± 2.40 and 259.46 ± 3.54 mOSM kg^{-1}), respectively. The spermatic production indicated

is continued, but it is variable during this period, and the hormonal induced no showed better performance in the producing capacity or seminal characteristics of *S. parahybae*.

(2) **CASA method** - The sperm motility parameters evaluated in *S. parahybae* with the application CASA (Table 1). The data suggest that the fertility of *S.paraybae* is being adversely affected by domestic, industrial and agricultural pollution and that CASA is a valuable methodology for monitoring reproductive health status.

Table1*. Sperm computerized parameters of S. parahybae.*

Parameters	Mean	Median	SD	SE	VC
MOT (%)	85.81	88.00	12.12	1.94	14.12
VCL (μm/s)	106.39	104.70	14.83	2.37	13.94
VAP (μm/s)	83.43	79.80	21.30	3.41	25.53
VSL (μm/s)	76.96	75.42	21.74	3.48	28.25
LIN (%)	91.69	92.11	3.77	0.60	4.11
WOB (%)	77.58	76.72	11.40	1.83	14.70
PROG (μm)	1,221.43	1,188.12	331.91	53.15	27.17
BCF (Hz)	25.22	25.91	4.64	0.74	18.41
NSPZ	45.92	47.00	23.79	3.81	51.80

MOT – motility, VCL – curvilinear velocity, VAP - average path velocity, VSL – straight line velocity, LIN – linearity, WOB – wobble, PROG – progression, BCF - beat cross frequency, NSPZ - number of monitored spermatozoa, S.D. –standard deviation, SE – standard error, VC –variation coefficient (%).

These preliminary results on productivity and quality of semen are similar to other species of tropical fish with satisfactory characteristics for the artificial reproduction (Viveiros & Godinho, 2009). Also, are crucial for future studies related to the quality of gametes, reproductive management in captivity and specie preservation.

Acknowledgements
The authors are indebeted to CNPq (nº 478347/2009-0) for support of this project and the fellowship of FAPESP (nº 2009/18609-6) and, CESP, Paraibuna, SP/BR.

References

Caneppele, D., Honji, R., Hilsdorf, A., Moreira, R.G. 2009: Induced spawning of the endangered Neotropical species Steindachneridion parahybae. Neotrop Ichthyol 7 (4), 759-762.

Sanches, EA; Bombardelli, RA; Marcos, RM; Neumann, G; Toledo, CPR; Romagosa, E. 2010: Sperm motility of Rhamdia quelen studied using computer-assisted analysis by open-source software. Aqua Res 42, 153-156.

Romagosa, E.; Souza, B.E.; Sanches, EA; Baggio, D. M.; Bombardelli, RA. 2010: Sperm motility of Prochilodus lineatus in relation to dilution rate and temperature of the activating medium. J Appl Ichthyol 26, 678–681.

Romagosa, E. 2008: Avanços na reprodução de peixes migradores. In: Tópicos especiais em biologia aquática e aquicultura. São Paulo: Jaboticabal, cap. 3, p.1-16.

Viveiros, A.T.M., Godinho, H.P. 2009: Sperm quality and cryopreservation of Brazilian freshwater fish species: a review. Fish Physiol Biochem 35, 137-150

P-210
CRYOGENIC SEMEN PRESERVATION AND MOTILITY ACTIVATION OF THE *BRYCON ORBIGNYANUS* SPERM

Alexandre Ninhaus-Silveira[1], Rosicleire Veríssimo-Silveira[1], José Augusto Senhorine[2], Hélio Takachi Okuda[1]

[1] *Laboratório de Ictiologia Neotropical – L.I.NEO, Departamento de Biologia e Zootecnia – UNESP, Faculdade de Engenharia; Avenida Brasil, 56,CEP 15385-000, Ilha Solteira, São Paulo, Brasil*

[2] *Centro Nacional de Pesquisa e Conservação de Peixes Continentais - CEPTA, Pirassununga, São Paulo, Brasil*

E-mail: ninhaus@bio.feis.unesp.br

Introduction

The study of the seminal characteristics and the techniques of freezing of germ cells of the teleosts species it could extend the perspectives of its use in fishculture programs, with interesting implications for the technician-scientific development and of production in these areas of activities. As well as for the maintenance of the genetic variability of the species in the preservation "in situ", that it will be of basic importance when of the necessity to restore of the environment that is being degraded and modified for the action of the man. This work aimed at to the magnifying of the knowledge of the reproductive biology of *Brycon orbignyanus* through the study of its seminal characteristics and the development of techniques of freezing of germ cells of this species.

Materials and Methods

Adult specimens of *Brycon orbignyanus* had been induced to the spermiation by one injection of 1mg of carp pituitary extract (CPE) per kg of body weight. After 6 h, semen was collected by abdominal pressing and stored in sterilized test tubes. Assessment of semen characteristic semen was collected from ten males. The semen samples were evaluated for volume (mL); color; subjective motility rate (%) and, sperm concentration (spermatozoa/mm^3), obtained by using a Neubauer cell counting chamber. Semen was diluted in a cryoprotective solution composed basically for glucose (5.4%), hen egg yolk (20mL), a cryoprotective substance (CPS) and distilled water (Ninhaus-Silveira, 2006). Some CPSs will be tested: dimetil sulfoxide ($MeSO_4$) and propilene glycol (PG) in four different concentrations: 5%, 10%, 15% and 20%, in tree different semen:diluente ratios 1:3, 1:7 and 1:10. The diluted semen was filled into 0.5mL straw at the ambient temperature and immediately frozen in liquid nitrogen vapors in a "dry container" of CryoPac/CP-300 (Taylor-Warton) to the temperature of -150ºC, without previous period of balance of the semen to the CPS.. After the freezing, the straws with semen had been transferred to a type "liquid container" (- 195ºC).To verify the spermatic viability of the cryopreserved semen, 5 motility activators solutions were tested: tap water, $NaHCO_3$ 59,5 mM, $NaHCO_3$ 119 mM (Fogli da Silveira, 1990), 25 NaCL mM and NaCL 50 mM and two thawing methods had been evaluated: 1) immersing the straw in a 36ºC water bath to 10s or; 2) immersing for 70ºC to 5 s.

Results

The semen of *B. orbignyanus*, was presented dense, milt coloration, with an average spermatic concentration of $17{,}29x10^9$ sptz/mL. After hormonal induction, the gotten average volume through abdominal massage was 4,0mL. In all the analyzed individuals the spermatic motility presented 80% of mobile cells, with duration of about 60 seconds until the total stop of cellular movement. The statistics analysis of the data did not demonstrate significant effect ($\alpha = 0.01$) for activation of the spermatozoa of the fresh semen, as for the ones of the semen diluted and freezing in liquid nitrogen vapor (-185ºC), between salts tested and the defined concentrations for the respective saline solutions. As well as significant interactions had not been demonstrated ($\alpha = 0.01$) between the type of salt and saline concentration with cryoprotector (dimetil sulfoxide, propilene glycol). The analysis of the data indicates a significant correlation to the increase of the mobility of the sperm, the type of cryprotector and its concentration and a positive correlation for the use of propilene glycol in the 5% concentration or dimetil sulfoxide in 5 and 20%, not having significant

difference between these and a negative correlation for the use of the concentrations of 10 and 15% to $MeSO_4$ (Table 1).

Table 1: Relation between cryoprotector, cryoprotector concentration (%) and sperm motility(%)

	Cryoprotector concentration			
	5%	10%	15%	20%
Propilene Glycol	70,36Aa	50Bb	47,14BCa	30Cb
Dimetil Sulfoxide	67,50Aa	56,07Ba	47,14Ba	71,78Aa
Dimetil Acetamide	67,52Aa	64,28Aa	52,86 Ba	32,52Cb
Etilene Glycol	70,36Aa	68,57Aa	35,36Ba	64,28Aa
	Spermatic Motility			

Analysis of the data demonstrated that it did not have a significant effect ($\alpha = 0.01$) in the correlation between the dilution of the semen in cryoprotective solution (1:3 and 1:7) and the subjective motility. Although not to have significant correlation ($\alpha = 0.01$) in relation to the temperatures of unfreeze (36º and 70ºC) and the spermatic motility, the temperature of 36ºC can be considered most favorable, therefore a more homogeneous unfreeze with lesser probability of cellular death for exposition to the high temperature.

Discussion and conclusion

In this study, the cryosolutions with 5 and 20% were capable of protecting sperm cells during freezing and thawing when motility and the percentage of live spermatozoa were estimated. Similar results were obtained (Viveiros et al., 2006) to the percentage of live sperm were estimated in *B.orbignyanus* using methylglycol as cryoprotectant. Ribeiro & Godinho (2003) with *Leporinus macrocephalus,* suggests that the solution that better active the spermatozoa after the unfreeze is $NaHCO_3$ 119 mM. What it differs from our results with B. orbygnianus where did not have significant difference between the tested solutions, suggesting that this factor has that to be tested for each worked species.

Acknowledgements

This work was supported by Centro Nacional de Pesquisa e Conservação de Peixes Continentais - CEPTA/ICMBIO, which provided the fish and the facilities used in this study, and also by CNPq (Conselho Nacional de Pesquisa) and FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo).

References

Fogli Da Silveira, W.; Kavamoto, E.T.; Cestarolli, M.A.; Godinho, H.M.; Ramos, S.M.; Silveira, A.N. 1990. Avaliação espermática, preservação criogênica e fertilidade do sêmen do pacu, Piaractus mesopotâmicus (Holmberg, 1887), proveniente de reprodução induzida. Bol. Inst. Pesca São Paulo, 17:1-13.

Ninhaus- Silveira, A., Veríssimo-Silveira, R., Senhorini, J. A., Okuda, H. T. 2006. Seminal Analysis, Cryogenic Preservation, and Fertility in Matrinxã Fish, Brycon cephalus (Günther, 1869). Braz. Arch. Biol. Tech., 49(4): 651-659.

Ribeiro, R. I. M. A; Godinho, H. P. 2003. Testicular sperm cryopreservation of the teleost 'piau-açu' Leporinus macrocephalus. Arq. bras. med. vet. zootec;55(1):75-79

Maria, A.N., Viveiros, A. T. M., Freitas, R. T. F., Oliveira A. V. 2006. Extenders and cryoprotectants for cooling and freezing of Brycon orbignyanus semen, an endangered Brazili an teleost fish. Aquaculture 260: 298–306.

P-211
SHORT-TERM STORAGE OF PIKEPERCH SPERM

Jiri Kristan, Azadeh Hatef, Sayyed Mohammad Hadi Alavi, Tomas Policar

University of South Bohemia, Faculty of Fisheries and Protection of Waters, South Bohemia Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany 389 25, Czech Republic

E-mail: kristj01@frov.jcu.cz

Introduction
Pikeperch is a carnivorous fish and its culture has been recently considered in aquaculture (Lappalainen et al., 2003). Similar to tench (*Tinca tinca*) (Linhart et al. 2003; Rodina et al., 2004) and pike (*Esox lucius*) (Alavi et al. 2009), spontaneous contamination of milt occurs in pikeperch during stripping, because of the presence of urinary bladder in genital papilla. Therefore, the present study was conducted to develop methods for sperm manipulation this species. The main objective was to investigate changes of sperm motility during short-term storage in different immobilizing medium. An additional goal was to improve activation of incubated sperm using different activation medium.

Materials and methods
Broodfish and sperm collection: The sperm of 4 males (Body weight: 868 g ± 98 g and Total length: 464 mm ± 16 mm) were collected in syringes, 2 – 3 days after hormonal stimulation of spermiation by single intramuscular injection of HCG at dosage of 750 IU per kg body weight and kept on ice during incubation.

Sperm incubation and activation: Immediately after sperm collection, the sperm samples of each male was separately diluted in two different immobilizing media (IM) at ratio 1: 2 (sperm: IM). Non-incubated sperm was used as control. The composition of IM were 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2.2H_2O$, 2.38 mM $NaHCO_3$, pH 8.0 (IM # 1) and 180 mM NaCl, 2.68 mM KCl, 2.38 mM $NaHCO_3$, pH 8.0 (IM # 2). Three different activation media (AM) were used to test the motility of sperm: AM # 1, – an ionic solution composed of ions (NaCl 45 mM, KCl 5 mM and Tris 20 mM, pH 8.5, Osmoality 100 mOsml/kg), AM # 2 – a non-ionic solution composed of 100 mM Sucrose, 20 mM Tris, pH 8.5, Osmolality 100 mOsmol/kg) and AM # 3 composed of 100 mM sucrose, 1 mM $CaCl_2$, 20 mM Tris, pH 8.5.

Sperm motility assessment: Sperm motility was examined at 0, 6, 24 and 48 h after incubation after activation in different AM according to Hatef et al., (2010). Briefly, sperm motility was recorded using a 3 CCD video camera mounted on a dark-field microscope equipped with a stroboscopic light at a frequency 50 Hz after activation in the activation medium. Then, the analyses of sperm motility and velocity were based on the successive positions of sperm heads using a micro-image analyzer (Olympus Micro Image 4.0.1. for Windows, CASA.

Results
Motility of sperm was induced in all different kinds of AM at 0, 6 and 24 h after incubation. Sperm motility and velocity were significantly decreased at 24 h after incubation. At 48 h after incubation, motility of only incubated sperm in IM was induced in all different kinds of AM, but the motility of non-incubated sperm was not induced. At this time, motility of incubated sperm in IM # 1 was higher than those samples incubated in IM # 2. Also, motility of sperm and velocity was higher after activation in AM # 3 containing Ca^{2+} compared to those of AM # 1 or # 2.

Discussion and conclusions
In freshwater fish, hypo-osmolality induces sperm activation (Alavi & Cosson 2006). The motility of sperm decreases after activation due to decline of ATP contents (Cosson 2010). There are several studies that show recovery of ATP content after incubation of sperm in IM with osmolality similar or slightly higher than seminal plasma (Billard et al. 1995; Perchec et al. 1995). Therefore, better sperm motility observed in incubated sperm in IM than non-incubated sperm can be addressed to osmolality effects, which lead to recovery of ATP contents in sperm cells (Billard et al. 1995).

The present study also showed enhancement of sperm activation in incubated sperm by adding small amount of Ca^{2+} into the AM. On the other hand, sperm cells are still alive and have potentiality for triggering their activation. But, Ca^{2+} is necessary for inducing sperm activation probably due to modifications of sperm Ca^{2+} signaling during incubation period. It was frequently shown that Ca^{2+} is a key factor for inducing flagellar beating in sperm (Morisawa 2008). In most freshwater fish, sperm activation is a Ca^{2+}-dependent mechanism, which is regulated by hypo-osmolality (Krasznai et al., 2000; Morisawa 2008).

In conclusion, the present study suggests using IM and AM containing Ca^{2+} for short-term storage of sperm and for activation of sperm.

Acknowledgments
This study was supported by CENAKVA CZ.1.05/2.1.00/01.0024, GACR 523/09/1793, QI101C033, QH71305 and GAJU 047/2010/Z.

References

Alavi, S.M.H.; Cosson, J., 2006: Sperm motility in fishes: (II) Effects of ions and osmotic pressure. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H.; Rodina, M.; Viveiros, A.T.M.; Cosson, J.; Gela, D.; Boryshpolets, S.; Linhart, O., 2009: Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (Esox lucius L.). Theriogenology 72, 32–43.

Billard, R.; Cosson, J.; Perchec, G.; Linhart, O., 1995: Biology of sperm and artificial reproduction in carp. Aquaculture 129, 95-112.

Cosson, J., 2010: Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J. Fish Biol. 76, 240–279.

Hatef, A.; Alavi, S.M.H.; Linhartova, Z.; Rodina, M.; Policar, T.; Linhart, O., 2010: In vitro effects of Bisphenol A on sperm motility characteristics in Perca fluviatilis L. (Percidae; Teleostei). J. Appl. Ichthyol. 26, 696–701.

Krasznai, Z.; Marian, T.; Izumi, H.; Damjanovich, S.; Balkay, L.; Tron, L.; Morisawa, M., 2000: Membrane hyperpolarization removes inactivation of Ca2+ channels leading to Ca2+ influx and initiation of sperm motility in the common carp. PNAS 97, 2052–2067.

Lappalainen, J.; Dorner, H.; Wysujak, K., 2003: Reproduction biology of pikeperch Sander lucioperca (L.) – a review. Ecol. Fresh. Fish 12, 95-106.

Linhart, O.; Rodina, M.; Bastl, J.; Cosson, J., 2003: Urinary bladder, ionic composition of seminal fluid and urine with characterization of sperm motility in tench (Tinca tinca). J. Appl. Ichthyol. 19, 177 – 181.

Morisawa, M., 2008: Adaptation and strategy for fertilization in the sperm of teleost fish. J. Appl. Ichthyol. 24, 362–370.

Perchec, G.; Jeulin, C.; Cosson, J.; Andre, F.; Billard, R., 1995: Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747 – 753.

Rodina, M.; Cosson, J.; Gela, D.; Linhart, O., 2004: Kurokura solution as immobilizing medium for spermatozoa of tench (Tinca tinca L.). Aquacult. Int. 12, 119–131.

P-212
EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) SPERM MODULATION TO IMPROVE ANTIOXIDANT SYSTEM FOR CRYOPRESERVATION

Sonia Martínez-Páramo[1], Marco Cerqueira[1], Patricia Diogo[1], Maria Teresa Dinis[1], Carmen Sarasquete[2], Elsa Cabrita[2]

[1] *CCMAR – Centre for Marine Sciences, University of Algarve, 8005-139 Faro, Portugal*
[2] *ICMAN.CSIC - Institute of Marine Sciences of Andalusía, Av. Republica Saharaui 2, 11510 Puerto Real, Cádiz, Spain*

E-mail: sparamo@ualg.pt

Introduction
Oxidative stress, as a result of the imbalance between the reactive oxygen species (ROS) and the sperm antioxidant system, promotes sperm morphological and physiological damage, since all cellular components (lipids, proteins and nucleic acids) are potential targets for ROS. Although antioxidant defense system is active in semen, its activity is limited as the amount of spermatozoa cytoplasm is low (Shiva et al. 2011). Moreover, dilution in the extender media for cryopreservation reduces the seminal plasma constituents being sperm more vulnerable to oxidative stress. Oxidative damage can be reduced by sperm modulation through breeders' diet (Cerolini et al. 2006) or by antioxidant addition in the freezing media (Cabrita et al. 2011). Thus, in the present work, these two ways of modulation were tested to improve the antioxidant system of seabass sperm by enrichment of HUFAs, vitamin E and Selenium (Se), with the aim to reduce cryodamage.

Materials and methods
Two European seabass (Dicentrarchus labrax) broodstocks, maintained at ICMAN.CSIC (Cádiz, Spain) facilities, were feed with two different diets (SPAROS, SA). Fish were feed with a control diet or with an experimental diet supplemented with vitamin E (700% over control diet), Se (150% over control diet) and unsaturated fatty acids: oleic acid, arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (19%, 15%, 38% and 151% respectively, over the control diet).

Sperm and blood collection

Males from both tanks were anesthetized to obtain sperm and blood samples (N=15). Blood was collected from the caudal vein and centrifuged (3,000xg, 5min, 4ºC) to obtain plasma. Sperm samples were divided in two aliquots. One was diluted (1:3, v/v) in non-activating medium (NAM) (Fauvel et al. 1998) and the other one was centrifuged (10,000xg, 10min, 4ºC) to obtain seminal plasma.

Sperm cryopreservation and thawing

Two extenders were used for sperm cryopreservation: NAM 10% DMSO and NAM 10% DMSO containing 0.25mM vitamin E, 5mM vitamin C, 0.25mM DHA and 12mM Se (final concentration). For cryopreservation, diluted sperm was diluted again (1:3, v/v) in each extender, loaded into 0.5mL straws and placed at 6.5cm over the liquid nitrogen surface for 15min. Straws were plugged in the nitrogen and stored until used. For thawing, straws were immersed in a water bath at 35ºC for 15s.

Evaluation of sperm quality and antioxidant status

Motility (CASA) and lipid peroxidation (Bioxitech MDA-586 kit, Oxis, USA) were determined in sperm, before and after cryopreservation.

Total antioxidant status (TAS; RANDOX, UK) was determined in seminal and blood plasma.

Glutathione peroxidase (GPX), glutathione reductase (GSR) and superoxide dismutase (SOD) activity were determined in spermatozoa extracts, before and after cryopreservation (RANDOX, UK).

Results
Fish feed with the experimental diet had higher level of antioxidants in blood plasma that the control group. However, this increase was not significantly different in seminal plasma. Motility of fresh sperm was similar

in both groups, but, despite of the significant decrease after cryopreservation, the supplemented extender increased the percentage of progressive spermatozoa respect to the extender control. Experimental diet produced a slightly increase in lipid peroxidation in fresh sperm. After cryopreservation, lipid peroxidation significantly increased in all treatments. Regarding enzymatic analysis, the supplemented extender increased GPX activity, whereas GRS and SOD activities were similar in both extenders.

Discussion and conclusions

In the present work, two different ways to modulate sperm were tested.

Experimental diet increased blood antioxidant status, whereas this improvement was not reflected in seminal plasma, maybe because longer periods of treatment were needed. Sperm from males feed with the experimental diet showed slightly higher level of lipid peroxidation, due to the incorporation of HUFAs into plasma membrane phospholipids, increasing the targets for ROS (Cerolini et al. 2006).

Cryopreservation with the supplemented extender increased the percentage of progressive spermatozoa and the GPX activity due to the presence of the cofactor Se in the freezing media (Castellini et al. 2002). However, despite this increase, lipid peroxidation remained very high after cryopreservation, as was observed in carp sperm (Li et al. 2010). This fact could be due to an imbalance with others enzymatic antioxidant systems (GSR and SOD), which activity did not increase with the extender supplements. SOD scavengers the superoxide radical to prevent lipid peroxidation (Shiva et al. 2011), thus its activity is important to neutralize ROS generation during the freezing/thawing process. In conclusion, higher percentage of progressive spermatozoa demonstrated that it is possible to reduce cryodamage through the extender, although sperm modulation through the diet is not enough to improve sperm antioxidant system.

Longer periods of feeding with the experimental diet, could improve these results.

Acknowledgements

The authors thank to Jose Luis Coello for maintaining the fish broodstock and to Jorge Dias for diet formulation support. Source of funding: Project CRYOSPERM (PTDC/MAR/64533/2006) founded by FCT national funding; S. Martínez-Páramo (SFRH/BPD/48520/2008) co-founded by POPH-QREN, Tipologia 4.1 (FEDER and MCTES) and E. Cabrita (RYC-2007-01650).

References

Cabrita, E.; Ma, S.; Diogo, P.; Martínez-Páramo, S.; Sarasquete, C.; Dinis, M.T., 2011: The influence of certain aminoacids and vitamins in post-thaw fish sperm motility, viability and DNA fragmentation. Anim. Reprod. Sci. (doi: 10.1016/j.anireprosci.2011.03.003.).

Castellini, C.; Lattaioli, P.; Bosco, A.D.; Beghelli, D., 2002: Effect of supranutritional level of dietary [alpha]-tocopheryl acetate and selenium on rabbit semen. Theriogenology 58, 1723-1732.

Cerolini, S.; Zaniboni, L.; Maldjian, A.; Gliozzi, T., 2006: Effect of docosahexaenoic acid and [alpha]-tocopherol enrichment in chicken sperm on semen quality, sperm lipid composition and susceptibility to peroxidation. Theriogenology 66, 877-886.

Fauvel, C.; Suquet, M.; Dreanno, C.; Zonno, V.; Menu, B., 1998: Cryopreservation of sea bass (Dicentrarchus labrax) spermatozoa in experimental and production simulating conditions. Aquat. Living Resour. 11, 387-394.

Li, P.; Li, Z.-H.; Dzyuba, B.; Hulak, M.; Rodina, M.; Linhart, O., 2010: Evaluating the impacts of osmotic and oxidative stress on common carp (Cyprinus carpio, L.) sperm caused by cryopreservation techniques. Biol. Reprod. 83, 852-858.

Shiva, M.; Gautam, A.K.; Verma, Y.; Shivgotra, V.; Doshi, H.; Kumar, S., 2011: Association between sperm quality, oxidative stress, and seminal antioxidant activity. Clinical Biochem. 44, 319-324.

P-213
ARTIFICIAL HYBRIDISATION OF EEL SPECIES BY USING CRYOPRESERVED SPERM FROM FRESHWATER REARED MALES (*ANGUILLA JAPONICA* ♀×*A. ANGUILLA*♂)

Tamás Müller[1], Ákos Horváth[1], Eisuke Takahashi[2], Balázs Kolics[3], Kincső Szaszkóné Decsi[3], Katalin Bakos[1], Balázs Kovács[1], János Taller[3], Miklós Bercsényi[3], László Horváth[1], Béla Urbányi[1], Shinji Adachi[2], Katsutoshi Arai[2], Etsuro Yamaha[2]

[1] *Department of Aquaculture, Szent István University, 2100-Gödöllő, Páter K. u. 1., Hungary*
[2] *Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Japan*
[3] *Georgikon Faculty, University of Pannonia, 8360-Keszthely, Deák F. u. 16., Hungary*

E-mail: Muller.Tamas@mkk.szie.hu; eyamaha@fsc.hokudai.ac.jp

Introduction

European eel is a catadromous fish species, which means that after living in freshwater premature individuals adapt to sea water, and migrate to the Sargasso Sea to spawn. The annual production of eels in Europe has been restricted by the yield of glass eel captured during entering rivers. This yield has dropped drastically since the 1980's due to overfishing of glass eels and mature adults as much as due to persistent pollution of eel habitats, spread of parasites and increase in ocean temperature. Contrary to many other farmed fish species only the initial steps of successful artificial propagation of European eel are known and we have only little information on larval rearing (Palstra and Thillart 2009). Okamura et al. (2004). managed to obtain interspecific hybrids between male European eels and female Japanese eels by artificial insemination.

There are several methods reported on European eel sperm cryopreservation (Asturiano, 2008) but no data are available about successful fertilisation tests using European eel cryopreserved sperm so far.

The objective of this study was to investigate the effect of cryopreserved sperm form freshwater reared European eel males on fertilisation using Japanese eel eggs.

Materials and methods

Full sexual maturation and long term spermiation of farmed European eel males were induced by weekly hCG injections (100-250 IU) in freshwater in 2001, 2005, 2007. A freezing diluent was prepared in a test tube composed of 3.2 mL of modified Kurokura or Tanaka solution and 400 μL of methanol, and finally 400 μL of sperm was added to it. Thus sperm was diluted in a 1:9 ratio and the final concentration of cryoprotectant was 10%. Sperm was loaded into 250- or 500-μL straws immediately after dilution. (Müller et al., 2004). Artificial sexual maturation and ovulation of Japanese eel was conducted by the method according to Ohta et al (1997). The fertilisation test with Japanese eel eggs and European eel cryopreserved sperm was performed at Hokkaido University, Japan by using dry fertilisation method. Normal (not deformed) *A. japonica* and *hybrid* larvae were used for genetic investigations.

Results

Full sexual maturation and long term spermiation of farmed European eel males in freshwater could be induced. These experiments using hCG injections lead to similar results as other's experiments in seawater (Müller et al., 2005).

Our main summarised results:

1. Successful fertilisation tests were carried out using European eel cryopreserved sperm (males were maturated in freshwater in 2005).
2. There were no differences between the embryonic development and rhythm of Japanese eel and hybrids.
3. The fact of hybridisation was proved by genetic analyses as well.

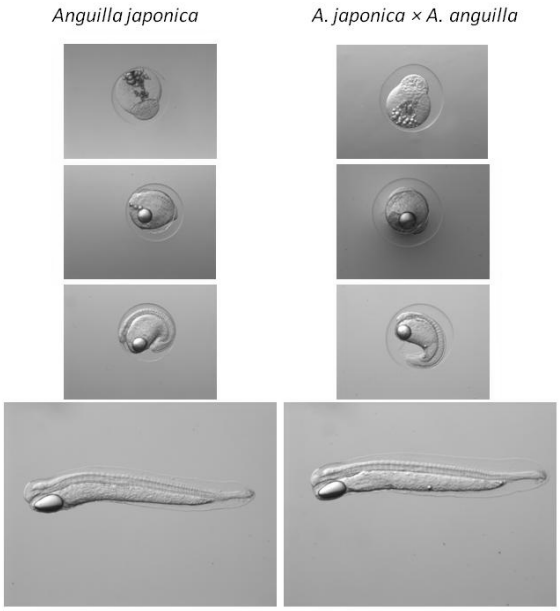


Figure 1. Embryogenesis and larvae of native A. japonica and hybrid

Discussion

Since there is no significant difference between the fertilisation capability of the freshwater and seawater spermatozoa, it is supposed that freshwater rearing of males is not a barrier factor for the artificial propagation of *A. anguilla*. Our experiments on the cryopreservation of eel sperm show that the extender originally developed for the common carp sperm (modified Kurokura extender) is suitable for freezing eel sperm together with methanol as cryoprotectant. Besides developmental biology applications, sperm cryopreservation may assist in developing tools for androgenesis between the two species as well.

Acknowledgements

This work was supported by the Hungarian Scientific Research Fund (project No. OTKA PD 73466), Hungarian-Japanese Bilateral project and Bolyai Research Fellowship of the Hungarian Academy of Sciences.

References

Asturiano JF, 2008: Different protocols for the cryopreservation of European eel (Anguilla anguilla) sperm. pp. 415-420. In :Methods in reproductive aquaculture: marine and freshwater species (eds.:Cabrita, E., Robles, V., Herráez, P.). CRC Press, Taylor and Francis Group, Boca Raton, London, New York. pp. 1-549.

Müller T, Urbányi B, Váradi B, Binder T, Horn P, Bercsényi M, Horváth Á, 2004: Cryopreservation of sperm of farmed European eel Anguilla anguilla. J World Aqua Soc, 35, 225-231

Müller T, Baska F, Váradi B, Horn P, Bercsényi M, 2005: Testis histology in artificially maturated European eel (Anguilla anguilla L.) at the end of sexual maturation and spermatozoa ultrastructure in freshwater rearing. Acta Biol Hung 56 (1), 169-172.

Ohta H, Kagawa H, Tanaka H, Okuzawa K, Iinuma N, Hirose K, 1997: Artificial induction of maturation and fertilization in the Japanese eel, Anguilla japonica. Fish Physiol Biochem, 17, 163-169.

Okamura A, Zhang H, Utoh T, Akazawa A, Yamada Y, Horie N, Mikawa N, Tanaka S, Oka HP, 2004: Artificial hybrid between Anguilla anguilla and A. japonica. J Fish Biol, 64, 1450-1454.

Palstra A, Van den Thillart G, 2009: Artificial maturation and reproduction of the European eel. In: Van den Thillart, G., Dufour, S., Rankin, C. (eds) Spawning migration of the European eel. Springer Sci+Bussiness Media B, New York Inc., pp. 309-331.

P-214
EFFECTS OF EXTENDER, INDIVIDUAL MALE AND SPERM-TO-EGG RATIO ON CRYOPRESERVATION SUCCESS OF ATLANTIC COD (*GADUS MORHUA* L.) SPERM

Vegard Marschhäuser[1], Oddvar Ottesen[1], Geir Rudolfsen[2], Bjørnar Eggen[1], Joanna Babiak[1] and Igor Babiak[1]

[1] *Reproductive Biology Group, Faculty of Biosciences and Aquaculture, University of Nordland, N-8049 Bodø, Norway*
[2] *Depatment of Biology, University of Tromsø, 9037 Tromsø, Norway*

E-mail: igor.babiak@uin.no

Introduction
Cryopreservation of sperm brings the opportunity to hold available sperm of optimal quality in perpetuity, and thus can reduce the cost of these manipulations (Suquet et al. 2000) particularly that sperm quality in Atlantic cod has been shown to decrease throughout the spawning season (Rideout et al. 2004). Moreover, sperm cryopreservation can be a useful tool for exchange genetic resources between cod hatcheries and allow for designed crossings necessary in selective breeding programmes, which currently are ongoing in Norway, Iceland and Canada. The present study aimed at improving the protocol for cryopreservation of Atlantic cod sperm, focusing on extender composition, paternal effects and sperm-to-egg ratio. The study was expanded by quantitative assessment of cod sperm using computer–assisted sperm analysis (CASA).

Material and methods
Sperm and eggs were collected from Atlantic cod broodstock kept at the University of Nordland, Bodø, Norway. Fish were held in 6 m diameter circular flow though tanks. Spermatozoa motility was estimated subjectively using counting chambers coated with albumin (Leja products BV) within 5 s after activation. CASA was used for measurement of different motility parameters as described by Babiak et al. (2006). Activated spermatozoa from 12 males were video-recorded before and after cryopreservation. The following parameters were investigated: straight-line velocity of spermatozoa (VSL), curvilinear velocity of spermatozoa (VCL), linearity of the movement (LIN), and percentage of motile spermatozoa (MOT). CASA parameters were quantified for 0.5 s at 15, 30, 45, 60, 75, 90, 105 and 120 s post-activation for each sample. Sperm samples were frozen in liquid nitrogen (-196 ºC, LN_2) vapours as described by Babiak et al. (2008). Portions of ca. 200 eggs were fertilized with cryopreserved sperm of volume depending on spermatozoa concentration and different sperm:egg ratios used in one of the experiments. Eggs were incubated in Petri dishes kept in a cooling room at 5.3 ± 0.3 °C.

In experiment 1, five different diluents and four different cryoprotectants (20 combinations) were examined for their suitability to cryopreserve cod sperm. The equilibration time of sperm diluted with extender prior to freezing was 1 min. After 3 weeks cryopreservation, sperm post-thaw motility was examined subjectively under a microscope immediately after thawing (0 min) and 30 min post-thawing. In experiment 2, the effect of individual male (6 males) and the best extenders selected from experiment 1 (6 extenders) was tested for fertilization efficiency. In experiment 3, CASA parameters of fresh and cryopreserved sperm of 12 males and sperm:to:egg ratio (5 different ratios from 6000:1 to 600000:1) standardization using sperm of 4 males cryopreserved in two extenders were tested.

Results
In experiment 1, HBSS + hen's egg yolk + glycerol was the best combination of diluent and cryoprotectant, significantly better than other combinations. Post-thaw motility in the best variant was on average 60±5% and 65±1%, immediately and 30 min post-thawing, respectively. In 15 of 20 combinations, there was no significant decrease in motility between 0 and 30 min post-thawing.

In experiment 2, there was no significant difference between fertilization and hatching rates achieved with sperm collected at the day of cryopreservation and that stored for 3 days, but the effect of individual male was significant ($F=17$, $p<0.001$ and $F=19$, $p < 0.001$, for fertilization and hatching rates, respectively).

Hatching rates in controls were not significantly different from variants with cryopreserved sperm from 4 of 6 males and not different from those obtained with 2 of 6 extenders tested (HBSS + yolk + DMA and HBSS + yolk + glycerol). Post-thaw motility significantly and positively correlated with fertilization and hatching rates, $r = 0.90$ and 0.78, respectively (n = 39).

In experiment 3, fertilization and hatching rates correlated highly and positively with actual sperm:egg ratios used ($r = 0.78$ and $r = 0.76$, respectively). Hatching rates obtained using sperm cryopreserved with DMA or glycerol decreased between sperm:egg ratios 300,000:1 and 60,000:1, whereas relative hatching rate was not related to the sperm:egg ratio used. The correlation between CASA parameters and survival rates were low in freshly collected sperm, while in cryopreserved sperm it was higher. Correlations between survival of embryos produced with cryopreserved sperm and CASA parameters of cryopreserved sperm were significant regardless of sperm:egg ratio used, but the general tendency was that the lower sperm:egg ratio, the higher value of correlation coefficient was, frequently exceeding r=0.90. The highest correlations between CASA motility parameters for cryopreserved sperm and fertilization and hatching rates were at 30 s post-activation.

Discussion and conclusions

In agreement with Butts et al. (2010), hen's egg yolk has proven to be a valuable extender component for cod sperm cryopreservation. HBSS + hen's egg yolk + glycerol or DMA was the best extender in the present study. A 30 min delay between thawing and activation did not reduce post-thaw spermatozoa motility. Cod sperm could be cryopreserved 3 days post collection. A significant effect of individual male was found on post-thaw spermatozoa motility together with fertilization and hatching rates. Hatching rates obtained when eggs were fertilized with cryopreserved sperm were not reduced compared to control sperm. Fertilization rate was found to be a good indicator for hatching rates. MOT, measured by CASA, was lower in cryopreserved spermatozoa compared to fresh, however high hatching rates were obtained when eggs were fertilized with the cryopreserved sperm. MOT was found to be a good predictor for the fertilization potential of fresh and cryopreserved Atlantic cod sperm. A minimum of 300,000 spermatozoa per egg, when sperm cryopreserved in HBSS + 10% hen's egg yolk + DMA or glycerol is used, is recommended for artificial fertilization in Atlantic cod. The higher sperm:egg ratio, the lower value of correlation coefficient between fertilization and hatching rates and CASA parameters were found.

Acknowledgements

The study was financed from the NorthCod project, financed by EU's regional Northern Periphery Programme.

References

Babiak I.; Bolla S.L.; Ottesen O., 2008: Suitable methods for cryopreservation of semen from Atlantic halibut, Hippoglossus hippoglossus L. Aquacult. Int. 16(6), 561-572.

Babiak I.; Ottesen O.; Rudolfsen G.; Johnsen S., 2006: Quantitative characteristics of Atlantic halibut, Hippoglossus hippoglossus L., semen throughout the reproductive season. Theriogenology 65, 1587-1604.

Butts I.A.E.; Litvak M.K.; Kaspar V.; Trippel E.A., 2010: Cryopreservation of Atlantic cod Gadus morhua L. spermatozoa: Effects of extender composition and freezing rate on sperm motility, velocity and morphology. Cryobiology 61, 174-181.

Rideout R.M.; Trippel E.A.; Litvak M.K., 2004: The development of haddock and Atlantic cod sperm cryopreservation techniques and the effect of sperm age on cryopreservation success. J Fish Biol 65, 299-311.

Suquet M.; Dreanno C.; Fauvel C.; Cosson J.; Billard R., 2000: Cryopreservation of sperm in marine fish. Aquacult Res 31|, 231-243.

P-215
CHRONOBIOLOGY APPLIED TO SPAWNING AND GAMETE WITHDRAWAL: IMPORTANCE OF DAILY RHYTHMS

Catarina Oliveira[1], Natalia Villamizar[2], Borja Blanco Vives[2], Cátia Santos[5], Evaristo Mañanos[3], Elsa Cabrita[4], Florbela Soares[5], Maria Teresa Dinis[1], Javier Sanchez Vazquez[2]

[1] *CCMAR, Algarve University, Campus de Gambelas, 8005-139 Faro, Portugal*
[2] *Physiology Department, Univ. of Murcia, Campus de Espinardo, 30100 Murcia, Spain*
[3] *ICMAN.CSIC, Campus Universitario Rio San Pedro, Puerto Real 11510 Cadiz, Spain*
[4] *IATS/CSIC, Ribera de Cabanes, 12595Castellón, Spain*
[5] *INRB, IP-IPIMAR, Av. 5 de Outubro, s/n, 8700-305 Olhão, Portugal*

E-mail: ccoliveira@ualg.pt

Introduction
Chronobiology is the field of science which studies the biological rhythms and the mechanisms by which the organisms adapt to a constantly changing environment. Animals synchronize their rhythms with environmental cycles choosing the most suitable moment to feed, reproduce or be active, thereby optimizing biological processes. In fish, the study of circadian activity and feeding rhythms has been profusely investigated lately; however, reproduction rhythms are often neglected. Fish species reproduce once a year around a species specific season, but many of them can also present lunar reproduction rhythms and may even select the best moment of the day to spawn (Oliveira & Sánchez-Vázquez 2009). Knowing the moment of the day spawning occurs can improve egg collection protocols and provide insides about time of ovulation. This kind of knowledge can be very useful for gamete collection and in vitro fertilization procedures. In fact, for species like red snapper, red sea bream or bambooleaf wrasse, daily maturation rhythms have been observed (Matsuyama et al. 1988, 1998; Jackson et al. 2006). Thus the objective of this work is to review the evidences of daily rhythms of reproduction in teleost species (e.g. Senegal sole, *Solea senegalensis*, gilthead seabream, *Sparus aurata*, European sea bass, *Dicentrarchus labrax*, white seabream, *Diplodus sargus,* and zebrafish, *Danio rerio*), highlighting the importance of the time of day in gamete studies.

Material and methods
Spawning and sex steroids rhythms were studied. **Spawning** rhythms were monitored using an automatic rotative egg-collector which allowed the hourly collection of the eggs and therefore identifying the moment of the day they were laid (Meseguer et al. 2008). Egg quality parameters such as fertilization and hatching rate were assessed for each time interval, in order to identify a fluctuation through the spawning hours. Furthermore, modified photoperiods and constant lighting conditions were applied to study the plasticity of the rhythm and its endogenous origin, respectively. Once more, egg quality parameters were measured hourly. In some cases, locomotor activity was also monitored in the breeders, using infrared photocells to detect fish movements, relating behavior with spawning timing. For **sex steroids** rhythm assessment, blood samples were withdrawn every 3 hours until a 24h cycle was completed, in order to determine daily oscillations of these hormones concentration in plasma throughout the day. In all cases, the significance of the rhythms was tested using the COSINOR analysis.

Results
All five species presented very marked daily spawning rhythms, with different timings of **spawning**, according to each species. Senegal sole, white seabream and European sea bass spawned at night, between 20:00-00:00h, 20:00-04:00h and 20:00–00:00h, respectively. Gilthead seabream preferred the late hours of the afternoon and the beginning of the night (16:00–22:00h) while zebrafish spawned early in the morning (08:00-12:00h). Egg quality parameters did not show differences between different hours of spawning for Senegal sole and gilthead seabream, however, in white seabream hatching rates of first eggs (20:00-22:00h) were significantly lower than for the ones laid later in the night (02:00-04:00h). Under conditions of modified photoperiod, both Senegal sole and gilthead seabream had the ability to gradually re-synchronize daily spawning rhythms to the new photoperiod. When fish were subjected to LL conditions the rhythm persisted during 2 days in the case of Senegal sole and fee-run during several days in the case of zebrafish

and white seabream. No important changes in egg quantity and quality were noticed when the photoperiod was manipulated. In the cases locomotor activity was registered (sole, seabass and zebrafish), a peak of activity was observed at the moment the spawning occurred. Furthermore, in the case of Senegal sole, this species also presented a very market daily rhythm of both estradiol and testosterone concentrations in females.

Discussion and conclusions

Fish do not spawn equally at any time of day. According to each species requirements there is a preference to spawn at a determined moment, suggesting that also ovulation undergoes a daily rhythm, and there is probably an optimal time window for gametes collection. The daily spawning rhythms are apparently strongly entrained by photoperiod (Blanco-Vives et al. 2009), and can gradually resynchronize to a change in this environmental signal and for some of the species studied they persisted under constant lighting conditions evidencing the presence of an endogenous oscillator driving these rhythm (Meseguer et al. 2008; Oliveira & Sánchez-Vázquez 2009). This capacity of the rhythm to adapt to photoperiod changes without alterations in egg quality may be an important tool for scientists and aquaculturists allowing manipulating photoperiod in order to obtain eggs (or gametes) available at the most convenient time of the day. When the locomotor activity was assessed, rhythms where in phase, suggesting a strategy of each species to choose the best moment of the day to be active at all levels. In the case of Senegal sole also a sex steroid rhythm was observed, reinforcing the markedly rhythmic pattern of daily reproduction (Oliveira & Sánchez-Vázquez 2009). With this in mind, we may say that the time of day has a major importance in reproduction studies, and should be carefully considered in studies related with spawning, gametes and hormonal induction.

Aknowledgements

This research was funded by the MEC project Reprosolea, AGL 2004-07984-C02-01, and by SENECA Project 05690/PI/07 both granted to FJSV, as well as by ProSpawn project (FP7/SME/2008/1) conceded to MTD. CO is supported by a Post Doctoral grant from the Portuguese Ministry of Science and Technology (SFRH/BPD/63933/2009) co-founded by POPH - QREN - Tipologia 4.1 (FEDER and MCTES).

References

Jackson M.W.; Nieland D.L.; Cowan J.H., 2006: Diel spawning periodicity of red snapper Lutjanus campechanus in the northern Gulf of Mexico. J. Fish Biol. 68, 695–706.

Matsuyama M.; Adachi S.; Nagahama Y.; Matsuura S., 1988: Diurnal rhythms of oocyte development and plasma steroid hormone levels in the female red sea bream, Pagrus major, during the spawning season. Aquaculture 73, 357–372.

Matsuyama M.; Morita S.; Nasu T.; Kashiwagi M., 1998: Daily spawning and development of sensitivity to gonadotropin and maturation-inducing steroid in the oocytes of the bambooleaf wrasse, Pseudolabrus japonicus. Environ. Biol. Fishes 52, 281–290.

Blanco-Vives B.; Sánchez-Vázquez F.J., 2009: Synchronization to light and feeding time of circadian rhythms of spawning and locomotor activity in zebrafish. Physiol. Behav. 98, 268-75.

Meseguer C.; Ramos J.; Bayarri M.J.; Oliveira C.; Sánchez-Vázquez F.J., 2008: Light synchronisation of the daily spawning rhythms of gilthead seabream (Sparus aurata L) kept under different photoperiod and after shifting the LD cycle. Chronobiol. Int. 25, 666-679.

Oliveira C.; Sánchez-Vázquez F.J., 2009: Reproduction rhythms in fishes. In: Kulczykowska E. (eds.), Biological Clocks in Fish. Sciene Publishes, Enfield., pp. 185-216.

P-216
STUDIES ON THE CRYOPRESERVATION OF SPERM AND LARVAE OF THE EUROPEAN FLAT OYSTER (*OSTREA EDULIS*)

Ákos Horváth[1], Anita Bubalo[2], Ana Čučević[2], Vlasta Bartulović[2], Béla Urbányi[1], Branko Glamuzina[2]

[1] *Department of Aquaculture, Szent István University, H-2100 Gödöllő, Páter Károly u. 1., Hungary*
[2] *Department of Aquaculture, University of Dubrovnik, Ćira Carića 4, 20 000 Dubrovnik, Croatia*

E-mail: Horvath.Akos@mkk.szie.hu

Introduction
The European flat oyster (*Ostrea edulis*) is a sessile bivalvian mollusk which used to be the most common oyster species cultured in Europe, however, its stocks have been reduced drastically, especially due diseases attributed to parasites such as *Bonamia ostreae* (Montes et al., 2003). Since then, its production has remained low and has largely been replaced by the Pacific oyster (*Crassostrea gigas*). In spite of low production volumes, the wholesale average price for the flat oyster is 3-5 times higher than that of the Pacific oyster. Croatia is one of the few European countries where *Ostrea edulis* is the only cultured oyster species. The need for cryopreservation of gametes and larvae of oysters has expanded due to potential applications in aquaculture such as improved management and production of seedstock, increased availability and distribution of selected lines and development and maintenance of genetically modified stocks (Paniagua-Chavez and Tiersch, 2001). The objectives of the present study included the development of methods for the cryopreservation of sperm and larvae of the European flat oyster.

Materials and methods
Mature oyster individuals were collected from Bistrina, Mali Ston Bay in Southern Croatia. Sperm was collected from sugically dissected testes and diluted in modified calcium-free Hanks' balanced salt solution (HBSS) with osmolality set at 915mOsmolkg^{-1}. Motility of sperm was assessed in HBSS as well as in sea water at 200× magnification. Cryoprotectants dimethyl-sulfoxide (DMSO) or methanol (MeOH) were added to the sperm at a concentration of either 5 or 10%. Diluted sperm was loaded into 0.5-ml straws and frozen in the vapors of liquid nitrogen at 3 cm above the level of nitrogen for a period of 3 minutes. Following thawing in a 40°C water bath for 13s motility of the sperm samples was assessed again. In addition, viability of fresh and frozen sperm samples was also assessed using fluorescent live-dead staining using the combination of the membrane permeable dye SYBR green to label live cells and membrane impermeable propidium iodide (PI) to label dead spermatozoa.

In the trials on the cryopreservation of oyster embryos and larvae, they were diluted with filtered sea water and DMSO or MeOH were added as cryoprotectats at a final concentration of 10%. An equilibration study was conducted with embryos and larvae exposed to increasing concentrations of DMSO for 30 minutes to determine the developmental stage most suitable for cryopreservation. Cryopreservation was conducted in the vapors of liquid nitrogen at various heights (3-13cm) above the level of nitrogen for varying periods of time to determine the parameters resulting in the highest survival rate of larvae. Survival of larvae was detected by the movement of the thawed organisms or at least by the movement of cilia.

Results
The sperm of the European flat oyster is released in the form of spermatozeugmata from which individual spermatozoa are released, however, the bulk of cells remain attached to the spermatozeugma. The progressive motility of freshly extracted *O. edulis* sperm was low (13±6%) with a higher percentage of cells (63±23%) displaying local vibrating movement. Following thawing both progressive and local movement was drastically reduced to less than 10% regardless of the cryoprotectant or its concentration. The viability of freshly extracted sperm was 77±9% whereas the highest viability of cryopreserved sperm (60±6%) was observed with 10% DMSO as cryoprotectant. Application of live-dead staining has also shown that

spermatozoa attached to spermatozeugmata had lower viability than free flowing sperm cells. A disintegration of spermatozeugmata was also observed following thawing.

The equilibration study showed that embryos were more sensitive to cryoprotectant toxicity than later larval stages. Generally, trochophores and D-veligers had the highest tolerance towards long-term exposure to DMSO. Highest post-thaw survival of trochophores (80±16%) was observed with a two-step freezing method (first 10min at 13cm, then 15min at 10cm) and 10% DMSO as cryoprotectant, whereas, the highest post-thaw survival of veligers (89±3%) was recorded with a similar two-step freezing (first step identical to the previous, second 12min at 10cm) and 5% DMSO. Regardless of the immediate post-thaw movement of larvae, no survival was observed 24 hours post hatching.

Discussion

The European flat oyster uses internal fertilization, thus, males spawn spermatozeugmata which are released into the water and carried to the females who filter them in and spermatozeugma breakdown takes place inside the female's brood chamber where eggs are fertilized by spermatozoa dissociated from the spermatozeugma (Ó Foighil, 1989). According to our observations flagella of spermatozoa exposed to seawater become motile even when attached to the spermatozeugma. This static motility was significantly lowered following cryopreservation which can be associated with damages to the spermatozeugmal stucture during freezing and thawing. Post-thaw reduction of motility has been reported for other oyster species, as well (Dong et al., 2005). Although motility parameters were poor, live-dead staining of sperm showed good survival of individual cells, thus fertilization trials are needed to confirm the efficiency of cryopreservation.

Observations on the cryopreservation of European flat oyster larvae generally correspond to those reported on other species (Gwo, 1995), in particular, that later developmental stages seem to be more resistant to cryoprotectant toxicity and cryopreservation survival than earlier ones. Our results have shown good survival rates immediately following thawing, however, the absence of surviving larvae 24 hours post thaw shows that further research is needed in this topic. One of the possible reasons for the high mortalities can be the low accuracy and repeatability of cooling in a plostyrene box (all previous studies used a computer-controlled freezer).

Acknowledgements

The work was supported by the Croatian-Hungarian Intergovernmental S&T programme and funds by the National Agency for Research and Technology of Hungary.

References

Dong, Q., Eudeline, B., Huang, C., Allen, SK., Tiersch, TR. 2005: Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, Crassostrea gigas. Cryobiology 50, 1-16.

Gwo, JC. 1995: Cryopreservation of oyster (Crassostrea gigas) embryos. Theriogenology 43, 1163-1174.

Montes, J., Ferro-Soto, B., Conchas, RF., Guerra A. 2003: Determining culture strategies in populations of the European flat oyster, Ostrea edulis, affected by bonamiosis. Aquaculture 220, 175-182.

Ó Foighil, D. 1989: Role of spermatozeugmata in the spawning ecology of the brooding oyster Ostrea edulis. Gamete Research 24, 219-228.

Paniagua-Chavez, C.G., Tiersch, TR. 2001: Laboratory studies of cryopreservation of sperm and trocophore larvae of the Eastern oyster. Cryobiology 43, 211-223.

P-217
MEASUREMENT OF SOME SPERM PARAMETERS OF WILD AND FARMED ATLANTIC SALMON (*SALMO SALAR*) IN NEWFOUNDLAND

N. Camarillo-Sepulveda[1], D. Hamoutene[1], J. Murray[1], V. Morris[1], M. Bloom[1], L. Lush[1], K.M. Rideout[2], J. Nichols[2], C. Collier[3]

[1] *Fisheries and Oceans Canada, Northwest Atlantic Fisheries Center, 80 East White Hills Road, St. John's, NL, A1C 5X1*
[2] *School of Fisheries, Fisheries and Marine Institute of Memorial University, P.O. Box 4920, St. John's, NL, A1C 5R3*
[3] *Gray Aqua Group Ltd., PO Box 275, Conne River, NL, A0H 1J0*

E-mail: Dounia.Hamoutene@dfo-mpo.gc.ca

Introduction
Atlantic salmon (*Salmo salar*) farming in open net-cage systems induces the coexistence of farmed and wild fish. The escape of farmed salmon into freshwater streams in the Canadian Atlantic region has been well documented (Carr & Whoriskey, 2006). However, questions remain about whether the wild salmon catch and abundance decline is associated with the increased farming of this species. As part of a study aimed at determining the mating success between farmed and wild salmon spawners by analyzing their reproductive output, we assessed some biochemical quality parameters in wild and farmed salmon sperm. It has been well documented that motility of spermatozoa depends on intracellular adenosine triphosphate levels (ATP) levels; dynein ATP-ase catalyses the hydrolysis of ATP to produce the flagellar beat (Gibbons, 1981; Mahmoud et al., 1994; Perchec et al., 1995; Ziętara et al., 2009) Citrate synthase (CS) is an indicator of aerobic metabolism and is crucial for cellular energy production; it has also been suggested that CS in sperm cells acts as a factor for egg activation (Harada et al., 2007). Lactate dehydrogenase (LDH) is used as a measure of anaerobic metabolism and it has been positively correlated to percent motile sperm and total swimming velocity (Lahnsteineret al., 1996). Lipase (LIP) is an indicator of lipid catabolism; it breaks down triglycerides into fatty acids and glycerol which can be further used to produce energy (Lahnsteiner et al., 1993). Lastly, creatine kinase (CK) is a measure of oxidative phosphorylation; it breaks down ATP into generating energy which can support sperm motility (Saudrais et al., 1998; Ziętara et al., 2009).

Materials and methods
Sperm samples from wild salmon were collected at the North East Placentia River in Newfoundland (Canada) from Oct 28th to Nov 10th, 2010. Sperm samples from farmed salmon (Saint John River strain) were collected from mature broodstock kept at Memorial University of Newfoundland (Nov 5th – Nov 10th, 2010). Samples were stored at 4°C for spermatozoa counts, and at -70°C for biochemical analyses. Scale samples from wild salmon were used to determine their age. Frozen sperm samples were thawed, homogenized, diluted in motility inhibitor buffer (PBS), and centrifuged at 14000g for 10 min. The supernatants were used to measure ATP using a bioluminescent assay, and the activities of CS, LDH, LIP, CK using spectrophotometric assays. Protein concentrations were measured following the Lowry method. Furthermore we measured sperm primary energy sources: triglycerides (TG) and glucose (Lahnsteiner et al., 1993). In order to measure them, the samples were deproteinized by acid precipitation and centrifuged at 10000g for 10 min prior to measuring their concentrations spectrophotometrically. All parameters were compared using t-test; when normality test failed, the data was analyzed by Mann-Whitney rank sum test. For all analyses p values ≤ 0.05 were considered to be significant.

Results
Sperm samples from farmed salmon had significantly higher numbers of spermatozoa ($p \leq 0.001$) and protein concentrations ($p = 0.004$) than their wild counterparts. TG concentrations were not different in both groups of sperm samples; however, glucose concentrations in wild sperm samples were significantly higher at 90% confidence when compared to farmed sperm samples ($p = 0.059$). Energy levels (ATP) were significantly higher ($p \leq 0.001$) in wild than in farmed salmon. Furthermore, sperm samples from wild salmon also showed significantly ($p = 0.004$) higher CK levels. CS, LDH, and LIP showed no difference.

Discussion and conclusions

Motility of spermatozoa has been correlated to the energy released by ATP hydrolysis (Gibbons, 1981). Key pathways contributing to ATP production and hydrolysis in fish sperm are: glycolysis, Krebs cycle, and oxidative phosphorylation.

Our results showed significantly higher numbers of spermatozoa and protein levels in farmed versus wild salmon. Nevertheless, the sperm of wild salmon showed statistically higher levels of ATP and CK than farmed fish, these results imply that sperm samples from wild salmon have increased energy available for motility. There might be a preference for the glycolytic pathway instead of lipid catabolism for energy production as glucose concentrations were higher than TG concentrations in both sperm samples. In addition, wild salmon sperm tended to have higher glucose concentrations (90% confidence); this may indicate that sperm from wild salmon might have more glucose to produce ATP than farmed salmon sperm. Despite the fact that farmed salmon have higher numbers of spermatozoa, sperm biochemical parameters measured in this study show an overall higher sperm energy availability in wild salmon. The completion of crosses between wild and farmed salmon from both origins will have to be performed in order to confirm that the increased energy available in wild sperm results in higher fertilization success.

Acknowledgements

This project is being funded by the Canadian Aquaculture Collaborative Research and Development Program.

References

Carr, J. W., & Whoriskey, F. G. (2006). The escape of juvenile farmed Atlantic salmon from hatcheries into freshwater streams in New Brunswick, Canada. Ices J Mar Sci. J du Conseil. 63(7), 1263 -1268.

Gibbons, I. R. (1981). Cilia and flagella of eukaryotes. J. Cell Biol. 91(3), 107s-124s.

Harada, Y., Matsumoto,T., Hirahara,S., Nakashima, A., Ueno, S., Oda, S., Miyazaki, S., et al. (2007). Characterization of a sperm factor for egg activation at fertilization of the newt Cynops pyrrhogaster. Devel Biol. 306(2), 797-808.

Lahnsteiner,F., Berger,B., Weismann,T., & Patzner,R. A. (1996). Motility of spermatozoa of Alburnus alburnus and its relationship to seminal plasma composition and sperm metabolism. Fish Physiol Biochem. 15(2), 167-179.

Lahnsteiner, F., Patzner, R. A., & Weismann, T. (1993). Energy resources of spermatozoa of the rainbow trout Oncorhynchus mykiss (Pisces, Teleostei). Repro Nutr Devel, 33(4), 349-360.

Mahmoud, A. M., Comhaire, F. H., Vermeulen, L., & Andreou, E. (1994). Comparison of the resazurin test, adenosine triphosphate in semen, and various sperm parameters. Hum Reprod, 9(9), 1688-1693.

Perchec, G., Jeulin, C., Cosson, J., André, F., & Billard, R. (1995). Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci, 108 (Pt 2), 747-753.

Saudrais, C., Fierville, F., Loir, M., Le Rumeur, E., Cibert, C., & Cosson, J. (1998). The use of phosphocreatine plus ADP as energy source for motility of membrane-deprived trout spermatozoa. Cell Mot Cyt. 41(2), 91-106.

Ziętara, M., Biegniewska, A., Rurangwa, E., Swierczynski, J., Ollevier, F., & Skorkowski, E. (2009). Bioenergetics of fish spermatozoa during semen storage. Fish Physiol Biochem 4, 607-614.

P-218
DO MALES MATTER? THE PATERNAL INFLUENCE ON THE MORTALITY AND GROWTH OF EMBRYOS AND LARVAE OF ATLANTIC COD (GADUS MORHUA)

M-M. Kroll[1], M.A. Peck[1], E.A. Trippel[2]

[1] *Institute for Hydrobiology and Fisheries Science, University of Hamburg, Olbersweg 24, 22767 Hamburg, Germany*
[2] *Fisheries and Oceans Canada, St. Andrews Biological Station, 531 Brandy Cove Rd., St. Andrews, New Brunswick, Canada*

E-mail: edward.trippel@dfo-mpo.gc.ca

Introduction
Genetic differences among parents can have large consequences to the fitness of marine fish early life stages. It is particularly important to understand these effects in populations that have been severely reduced due to heavy exploitation (overfishing). Previous research reported large paternal (genetic/family) effects on thermal reaction norms for sperm motility (Purchase *et al.* 2010) and for vital rates of eggs and larvae in winter flounder *(Pseudopleuronectes americanus)* (Butts *et al.* 2007). Evidence exists that paternal effects can play an important role for the survival of larvae in Atlantic herring (*Clupea harengus*) (Hoie *et al.* 1999a), brown trout (Salmo trutta) (Vollestad & Lillehammer, 2000), and haddock (*Melanogrammus aeglefinus*) (Rideout *et al.* 2004). Our objective was to disentangle the impacts of paternity and other parental effects on key traits of eggs and larvae of Atlantic cod.

Materials and methods
Laboratory research was conducted during a three-month period (February through April 2010). Milt from 12 males (47.8 – 62.6 cm, 1.7 – 3.5 kg) and eggs from one female (59.8 cm, 3.8 kg) were strip-spawned at the St. Andrews Biological Station on February 15th (expt #1) and the same males and a smaller female (40.2 cm, 1.4 kg)(expt #2) were strip-spawned two weeks later. In each case, eggs were fertilized to produce 12, half sib families. Fertilization was conducted at ~ 6°C using the wet method (Trippel & Morgan, 1994b; Litvak & Trippel, 1998). Fertilized eggs were held for 12 to 16h at 6°C. Fertilization success was evaluated by determining the proportion of fertilized eggs of viable eggs present in a cross.

The fertilized, viable eggs of each of the 12 half-sibling family were separated into 6 replicate, 250ml beakers using ~320 eggs per replicate (total beakers = 6 *12 = 72) and incubated at 6°C. Non-viable eggs were excluded from the experiments prior to the start of the experiments (0dpf). A 70% water exchange and assessment of embryo mortality was assessed each day in each replicate. Embryonic developmental stage and hatching success were recorded. Larval survival until starvation (end of yolk stage) was also tracked each day. Measurements of morphological traits (standard length, yolk-sac volume, eye diameter) were evaluated from digital images of 0, 5 and 19dph larvae. In experiment #2, larval growth rates (μm/d) were calculated according to Probst *et al.* (2006).

Results and conclusions
Fertilization success of both experiments ranged from 71.6 to 90.5 %. Greater than 90 % cumulative mortality occurred during the first 7 days post-fertilization in both experiments, which represents up to and including the gastrulation phase and strong paternal differences in embryonic mortality to this point were observed (Fig. 1). Peak (modal) hatch of experiment #1 occurred at 22 days and of experiment #2 19 days post fertilization. Hatching success ranged from 0 to 2.5 % in the first experiment and 10.2 to 63.0 % in the second, indicating strong maternal effects in both sets of crosses.

Larval growth rates (standard length) ranged from 0.55 to 1.97 μm/d and exhibited large, among-male differences (Fig. 2). In conclusion, paternity had a strong effect on fertilization success and early embryonic mortality. Therefore, natural mortality could be limited by male-female interactions in embryonic

survivorship, which are determined by mate choice in natural populations. The right mating choice during spawning can play a large role on offspring survivorship (fitness) as well as in selective breeding programs.

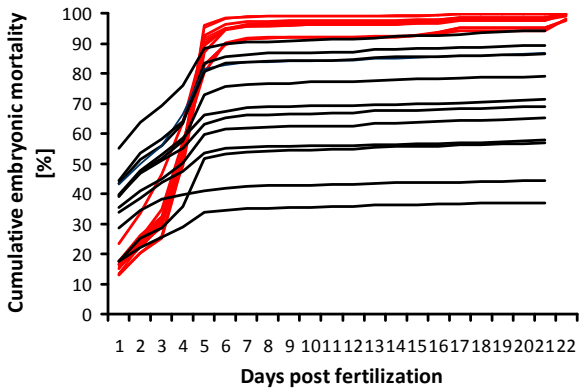


Fig. 1: Mean cumulative embryonic mortality for both females (#1 black, #2 red) over the period of 21 to 22 days

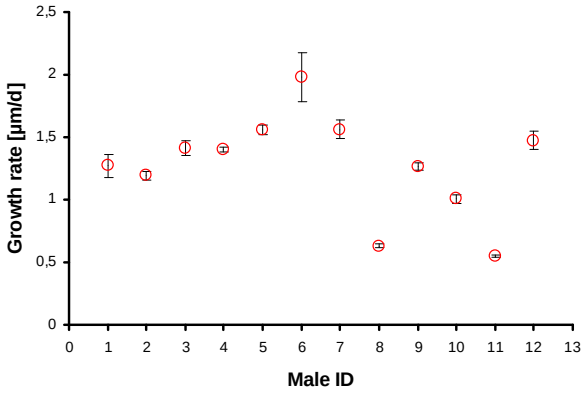


Fig. 2: Growth rate [µm/d] (± SE) of larvae per sire for experiment #2 based on 20 larvae per cross

Acknowledgements

We would like to thank everybody who helped develop this study, namely: Ian Butts, Nathaniel Feindel, Steve Neil, Olivia Puckrin, Maja Walter, Dr. Klaus Huebert and Susan Fordham. Funding was partly provided by the EU-COST Activity FRESH (Fish Reproduction and Fisheries) and the DAAD (Deutsche Akademischer Austauschdienst).

References

Butts, I. A. E. and Litvak, M. K. (2007): Parental and stock effects on larval growth and survival to metamorphosis in winter flounder (Pseuodpleuronectes americanus). Aquaculture 269: 339 – 348.

Høie, H., Folkvord, A. and Johannessen, A. (1999a): The influence of different parental combinations and incubation temperature on the RNA and DNA content of herring larvae at hatching: a pilot study. J. Fish. Biol., 55: 110 – 118.

Litvak, M.K. and Trippel, E.A. (1998): Sperm mortality patterns of Atlantic cod (Gadus morhua) in relation to salinity: effects of ovarian fluid and egg presence. Can. J. Fish. Aquat. Sci., 55: 1871 – 1877.

Probst, W., N., Kraus G., Rideout R., M. and Trippel E.A. (2006): Parental effects on early life histoaitry traits of haddock (Melanogrammus aeglefinus). ICES J. Mar. Sci., 63: 224 – 234.

Rideout, R. M., Trippel, E. A. and Litvak, M. K. (2004): Paternal effects on haddock early life history traits. J. Fish. Biol., 64: 695 – 701.

Trippel, E. A. and Morgan, M. J. (1994b): Sperm longevity in Atlantic cod (Gadus morhua). Copeia, 1025 – 1029.

Vollestad, L.A. and Lillehammer, T. (2000): Individual variation in early life-history traits in brown trout. Ecol. Freshw. Fish., 9: 242 – 247.

P-219
INDIVIDUAL AND POPULATION LEVEL REACTION NORMS FOR BROWN TROUT SPERM PERFORMANCE TO PH

Craig F. Purchase[1], Darek Moreau[1,2]

[1] *Biology Department, Memorial University, St. John's, Canada*
[2] *Current address: Department of Fisheries & Aquaculture, Government of Newfoundland & Labrador, Canada*

E-mail: craig.purchase@mun.ca

Introduction
Phenotypic plasticity occurs when an individual genotype expresses different phenotypes under varying environmental conditions (see Schlichting & Pigliucci 1998). Plasticity has important ecological and evolutionary consequences but is often difficult to assess. Fish sperm represent an ideal system for studying plasticity (Purchase et al. 2010), as they are tightly linked to fitness, are highly sensitive to environmental variation, can be assessed very quickly, and allow one to quantify reaction norms for individual animals (Purchase et al. 2010).

Brown trout were introduced to Newfoundland in the late 1800s and populations have been naturalized for over 100 years. Over this time brown trout have successfully invaded many rivers on the island; displacing native salmonids (Westley and Fleming 2011). Why these fish are able to colonize so many variable river systems is unknown, nor is whether there has been contemporary evolution occurring in the process. Here we investigate potential plasticity of sperm performance to pH in two distinct populations.

Materials And Methods
Adult brown trout were captured from two stream systems near St. John's, Newfoundland in autumn of 2008 and used to create 8 Rennie's River families and 7 Parker's Brook families, which were reared separately under identical conditions in captivity until 8 months post-emergence, upon which they were internally tagged and pooled by population. In November 2010, 20 mature F_1 captive males from Parker's and Rennie's source parents were assessed for sperm quality.

Approximately 1mL of semen was centrifuged at 5°C for 10min at 7500rpm. From this, a 1:50 dilution was made by adding 49.5uL of centrifuged plasma to 5uL of uncentrifuged semen from the same fish. All sperm analyses were conducted at 5°C. 5uL of the diluted semen was placed into a 8mm well of a temperature controlled multi-test slide, followed quickly by 40uL of 5°C "water". Video capture of sperm was instigated instantly on water contact, and usable video was obtained in less than 10s. Swimming sperm were recorded at 200 frames per second for 41 seconds, at which time nearly all sperm in all treatments had stopped moving. Sperm were assessed using water of five different pH (4,5,6,7,8) which was obtained by adding HCl or borax to hatchery sourced freshwater. Each water sample contained 0.1% BSA to prevent sperm from sticking to the slides.

Sperm swimming ability of each procedural replication was quantified at seven time periods post-activation (10,15,20,25,30,35,40s = repeated measures), by analyzing 100 frames of video sequence (0.5s) using the ImageJ CASA plugin. The entire process was repeated 5 times, giving 3500 groups of sperm for analysis (2pops X 10fish X 5pH X 7imes X 5reps), with typically 100-300 sperm in each group. The design is illustrated in Figure 1. Sperm cells within a procedural replicate were averaged, and then the 5 procedural replicates were averaged to get independent units for statistical analysis at the individual fish level.

Results
At this time (April 2011), data for 1 of the 5 procedural replicates are available for analyses and are therefore included in this abstract. However, all data will be presented at the workshop in September.

Swimming data were obtained for 6751 motile sperm at 10s post activation in the first procedural replicate. These were tracked at 7 time periods giving 45,915 sperm movement patterns through time. Individual fish showed marked variability in reaction norms to pH and time post-activation (not shown). On average, sperm movement declined marginally from pH 8 to 5, but dropped substantially below pH 5 (Figure 2). As

expected sperm movement typically slowed dramatically with time, although less so at pH 4; as these sperm were already moving slowly. pH had little effect on sperm movement for cells that had been activated longer than 20s (Figure 2).

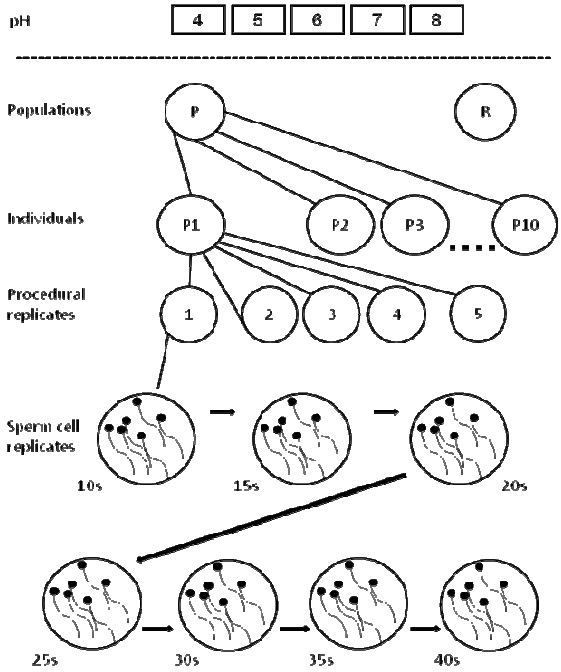


Figure 1: Study design schematic. see text.

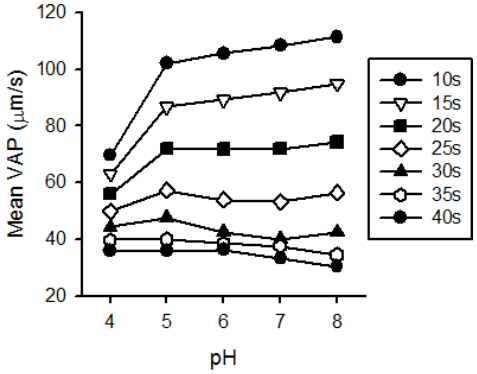


Figure 2: Average reaction norms among 20 individual fish for velocity of the average path traveled.

Discussion and conclusions

From the data analyzed (1 of 5 replicates), it appears that brown trout sperm performance is nearly canalized over a wide range of pH, but falls dramatically below pH 5. This would presumably allow sperm to function normally in most newly colonized river systems of variable pH. There is plasticity (time since activation) in plasticity (response to pH), as sperm that have been activated for longer time periods show less response to pH. Although reaction norms vary markedly among genotypes, there does not appear to be a difference in average response by the two populations.

Acknowledgements

We would like to that Peter Westley and Ian Fleming for kindly providing access to their fish. Funding to CFP was provided by the Natural Sciences & Engineering Research Council of Canada, the Canadian Foundation for Innovation, and the Research & Development Corporation of Newfoundland & Labrador.

References

Purchase, C.F., I.A.E. Butts, A. Alonso-Fernández, and Trippel, E.A., 2010. Thermal reaction norms in sperm performance of Atlantic cod (Gadus morhua). Can. J. Fish. Aquatic. Sci. 67: 498-510.

Schlichting, C.D., and Pigliucci, M. 1998. Phenotypic evolution: a reaction norm perspective. Sinauer Associates, Sunderland, Massachusetts.

Westley, P.A.H., and Fleming, I.A., 2011. Landscape factors that shape a slow and persistent aquatic invasion: brown trout in Newfoundland 1883-2010. Diversity Distrib. 17:566-579.

P-220
EFFECT OF CRYOPROTECTANTS ON THE COLD STORAGE OF STREAKED PROCHILOD *PROCHILODUS LINEATUS* EMBRYOS

Ariane F. Nascimento[1], Mariana Hamaue[2], Danilo P. Streit, Ana T. M. Viveiros[1]

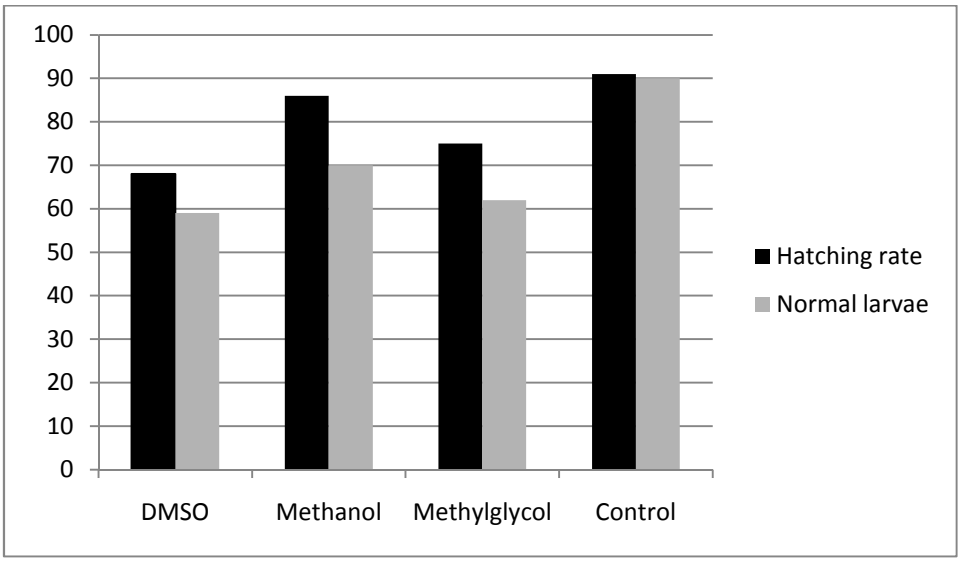
[1] *Department of Animal Science, Federal University of Lavras, MG, 37200-000, Brazil*
[2] *Department of Veterinary, Federal University of Lavras, MG, 37200-000, Brazil*

E-mail: ana.viveiros@dzo.ufla.br; anatmviveiros@hotmail.com

Introduction
The streaked prochilod *Prochilodus lineatus* (Valenciennes1837) is a benthopelagic, detritivorous freshwater fish species (Characiformes order), native to the Paraná -Paraguay and Paraíba do Sul River basins in South America. In Brazil, this species is known as curimba, curimatá or curimbatã, and is highly valued for its culinary aspects in the Northeastern region. Moreover, larvae from the streaked prochilod are used as live feed for endangered carnivorous species, such as the piracanjuba *Brycon orbignyanus* and jaú *Zungaro jahu* (Orfão et al, 2010).Trials on cryopreservation of teleostean oocytes and embryos have remained unsuccessful, despite great effort from several researchers. Fish embryos have a great amount of yolk and represent a complex multicompartmentalized biological class, whose cryopreservation has constantly failed. Thus, the aim of this study was to evaluate the ability of cryoprotectants to maintain the embryo viability during cold storage.

Materials and methods
Streaked prochilods (one male and one female) were selected from earthen ponds at the Hydrobiology and Fish Culture Station of FURNAS São José da Barra, MG, Brazil, during the spawning season. Spawning was induced with carp pituitary extract (cPE) at 4 mg kg $^{-1}$ body weight for the male and at 0.3 and 3 mg kg^{-1} body weight at a 12-h interval for the female. The oocytes (230 g) were divided into four aliquots and fertilized with 1.5 mL of sperm. Embryos were then transferred to four 60-L conical incubators, with controlled water temperature at 28 ºC. Aliquots of 100 embryos of each incubator was randomly collected at the gastrula stage with 90% epibolia and diluted in a cooling media comprising the combination of 0.5 M sucrose solution (Lopes et al, 2011) and one of the three cryoprotectant agents: dimethyl sulphoxide (DMSO), methanol or methyl glycol The remaining embryos inside each incubator were allowed to develop normally and served as fresh control. After dilution, all embryos were stored at -4 °C. Six hours later, cooled embryos were washed in water and then transferred to a PVC basket (10 cm in diameter) with a 0.5 mm mesh bottom and incubated in a flow-through system at 28 °C until hatching. Hatching rate and percentage of normal larvae (those which moved vigorously presenting a normal notochord) were calculated.

Results
The hatching rate was 91% when eggs were incubated without cold storage (fresh control). When eggs were cooled for 6 h, the hatching rate was 68% for embryos refrigerated in DMSO, 86% in methanol, 75% in methyl glycol. The percentage of normal larvae was 90% in control fertilization, 59% for embryos refrigerated in DMSO, 70% in methanol, 62% in methyl glycol.



Discussion and conclusion

The highest hatching rate and percentage of normal larvae among cooled embryos was observed when embryos were stored in methanol, compared to DMSO and methyl glycol. The positive action of methanol as a cryoprotectant for fish embryos, especially in solutions containing sucrose, has been observed in other species: *Labeo rohita*, *Catla catla*, *Cirrhinus Mrigal*, (Ahammad et al. 2003) and *Piaractus mesopotamicus* (Lopes et al. 2011) when cooled in methanol and sucrose. The addition of sucrose to the solution of methanol promotes cell membrane stability, especially with regard to the mechanical deformation caused by water efflux and influx of intracellular cryoprotectant (Ahammad et al. 2003). Although DMSO has been successfully used in the cryopreservation of fish spermatozoa, the percentage of normal larvae was lower than methanol and methyl glycol, in the cooled streaked prochilod embryos. The cryoprotectant methyl glycol has been effectively used for fish sperm, and was first tested for embryos here. Based on this finding, streaked prochilod embryos can be cooled in methanol and transported from one hatchery to the other within a 6-h distance without significant loss of quality.

Acknowledgements:

ANEEL P&D Furnas (Project 017965), CNPq (Project 300994/2008-7; PhD grant 552471-2010-0).

References

Orfão, L.H.; Maria, A.N.; Nascimento, A.F.; Isaú, Z.A.; Viveiros, A.T. M., 2010: Sperm fertility of the subtropical freshwater streaked prochilod Prochilodus lineatus (Characiformes) improved after dilution and cold storage. Aquaculture Research, 41, 679-687.

Ahammad, M.M.; Bhattacharyya, D.; Jana, B.B., 2003: Hatching of common carp (Cyprinus carpio L.) embryos stored at 4 and 2°C in different concentrations of methanol and sucrose. Theriogenology, 60, 1409-1422.

Lopes, T.S.; Romagosa, E;. Streit, D.P.; Ribeiro, R.P.; Digmayer, M., 2011: Cooling of pacu (Piaractus mesopotamicus) embryos at various stages of development for 6 or 10 hours. Theriogenolog,. 75, 570-576.

P-221
BIOCHEMICAL, PHYSIOLOGICAL AND GENETIC CHARACTERISTICS OF FRESH AND CRYOPRESERVED WHITEFISH (*COREGONUS LAVARETUS* L.) SEMEN

J. Nynca[1], G.J. Dietrich[1], D. Fopp-Bayat[2], M.A. Dietrich[1], S. Słowińska[1], E. Liszewska[1], H. Karol[1], A. Martyniak[3], A. Ciereszko[1]

[1] *Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-747 Olsztyn, Poland*
[2] *Department of Ichthyology, University of Warmia and Mazury in Olsztyn*
[3] *Department of Fish Biology and Breeding, University of Warmia and Mazury in Olsztyn*

E-mail: j.nynca@pan.olsztyn.pl

Introduction
The European whitefish (*Coregonus lavaretus* L.) from Lake Łebsko is anadromous species migrating from Baltic Sea water to freshwater Lake Łebsko in order to reproduce. The native population of whitefish from Lake Łebsko is endangered and included in conservation project supervised by Slowiński National Park Authority. The main component of this project is artificial fertilization with the use of collected gametes of wild whitefish and stocking of fingerlings raised in hatcheries. Knowledge regarding characteristics of whitefish fresh and cryopreserved semen is limited to basic sperm motility parameters (Ciereszko et al. 2008) and restricted to small number of observations for cryopreserved semen (n=5). The aims of this study were the comprehensive characteristics of fresh and cryopreserved whitefish semen and the verification of the usefulness of fresh semen parameters in order to predict the quality of cryopreserved semen. The relationship between DNA microsatellite genotyping and quality parameters of fresh and cryopreserved semen was also examined.

Materials and methods
Milt was collected from 41 whitefish males. The cryopreservation procedure was performed using 0.3 M glucose in 10% methanol as the extender (Ciereszko et al. 2008). Sperm concentration was measured using a spectrophotometer (Eppendorf, Hamburg, Germany) according to Ciereszko and Dabrowski (1993) and sperm viability using a NucleoCounter SP-100 (Nynca and Ciereszko 2009). The motility of spermatozoa was measured and analyzed with Computer Assisted Sperm Analysis (CASA) using the Hobson Sperm Cell Tracker as described by Ciereszko et al. (2008). The sperm sample were centrifuged at 8000×g to obtain seminal plasma for osmolality measurement using a Vapor Pressure Osmometer 5520 (WESCOR, Logan, USA) and lactate dehydrogenase (LDH). Genotyping of fin samples at six microsatellite loci (Clav-8, Clav-18, Clav-28, Clav-80, Str-73, Sfo-292) was carried out following Fopp-Bayat and Wiśniewska (2011). The percentage of heterozygotic loci for each male was calculated as 100%×(heterozygotic loci/total loci). Statistical analyses were performed at significance level of 0.05 using Graphpad Prism, v. 4.02 (GraphPad Software Inc, San Diego, USA).

Results
The samples of whitefish semen were characterized by high quality parameters values, mainly the percentage of sperm motility and viability (Tab. 1). After cryopreservation the values of most parameters decreased, but in case of straight line velocity, linearity (LIN) and LDH a significant increase was noticed (Tab. 1). The percentage of sperm motility and viability decreased about 57% and 23%, respectively. Those parameters correlated with each other only in cryopreserved semen ($r=0.47$, $p<0.01$). Low regressions were found between quality parameters for both fresh and cryopreserved semen, except for the regression between the amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL) ($r^2=0.76$, $p<0.0001$ and $r^2=0.82$, $p<0.0001$ for fresh and cryopreserved semen, respectively). Correlations were also found between parameters of fresh and cryopreserved semen, but were characterized by low values.

Table 1. Sperm characteristics of fresh and cryopreserved whitefish semen (results are presented as mean ± SEM)

Parameter	Fresh sperm	Cryopreserved sperm
Viability (%)	86.5 ± 2.4	66.8 ± 9.3***
Motility (%)	81.0 ± 14.0	35.0 ± 13.0***
VCL (μm s^{-1})	148.5 ± 17.8	122.2 ± 27.9***
VAP (μm s^{-1})	55.5 ± 11.5	55.9 ± 14.0
VSL (μm s^{-1})	14.3 ± 6.1	16.9 ± 6.4*
LIN (%)	10.1 ± 3.5	15.9 ± 5.5***
BCF (Hz)	6.2 ± 0.6	4.8 ± 1.0***
ALH (μm)	20.1 ± 1.9	16.5 ± 4.6***
LDH (U/l)	394.0 ± 159.6	613.1 ± 171.6***
Sperm concentration ($\times 10^9$/mL)	11.6 ± 2.7	
Osmolality (mOsmol kg^{1})	253.3 ± 31.4	

*Significantly different from fresh sperm *p<0.05, **p<0.01, ***p<0.001*

A significant, but low regression between the percentage of heterozygotic microsatellite loci and the percentage of sperm motility of fresh semen (r^2=0.11, p<0.05) and seminal plasma osmolality (r^2=0.16, p<0.05) was found. The differences in sperm motility and LIN between homozygotes and heterozygotes at locus Sfo-292A (p<0.05) were noticed for cryopreserved, but not for fresh semen.

Discussion and conclusions

Our results provided the basic characteristics of fresh and cryopreserved semen of whitefish. Discrepancy between sperm motility and viability parameters after cryopreservation compared to fresh semen suggests that cryopreservation of whitefish spermatozoa causes higher levels of injury to motility apparatus and/or energy exhaustion than to the membrane integrity. A significant correlation between motility and viability parameters found only in cryopreserved semen can indicate that viability and motility parameters reflect the separate aspects of quality of fresh sperm. Low correlations between parameters of fresh and cryopreserved semen suggest low usefulness of parameters of fresh semen for predicting the quality of cryopreserved semen. To our knowledge it is the first attempt to correlate microsatellite analysis with quality parameters of fresh and cryopreserved fish semen. The correlation of the percentage heterozygotic loci with the percentage of sperm motility is in agreement with data obtained by Fitzpatrick and Evans (2009) for the semen of endangered mammals. The lack of a relationship between Sfo-292A and parameters of fresh semen suggests that this relationship is not related to semen quality but rather to the ability of spermatozoa to withstand damages caused by freezing-thawing processes. Genetic characterization of whitefish males by microsatellite variability seems to be a promising tool for the studies of genetic background of male reproduction in fish.

Acknowledgements

This study was supported by grant "Ichthyologic biodiversity of lakes – elaborating a model for problem solution: a case study of natural resources of autochthonous common whitefish in Łebsko Lake"(EEA grant PL0468).

References

Ciereszko, A.; Dabrowski, K., 1993: Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using spectrophotometric technique. Aquaculture 109, 1292–1305.

Ciereszko, A.; Dietrich, G.J.; Wojtczak, M.; Sobocki, M.; Hliwa, P.; Kuźmiński, H.; Dobosz, S.; Słowińska, M.; Nynca, J., 2008: Characterization and cryopreservation of whitefish (Coregonus lavaretus L.) semen from Lake Łebsko. Fundamental and Applied Limnology 173, 59-65.

Fitzpatrick, J.L.; Evans, J.P., 2009. Reduced heterozygosity impairs sperm quality in endangered mammals. Biol Lett. 5(3): 320–323.

Fopp-Bayat, D.; Wiśniewska, A.M., 2011: Genetic analysis of whitefish (Coregonus lavaretus) from Łebsko Lake-application of microsatellite DNA. In: Water biodiversity assessment and protection, Jankun, M.; Furgała-Selezniow, G.; Woźniak, M.; Wiśniewska, A.M., eds. pp. 239-245.

P-222
FINE STRUCTURES AND CRYOPRESERVATION OF RIGHTEYE STONE AND LEFTEYE SOUTHERN FLOUNDER SPERMATOZOA

Jin-Chywan Gwo

Department of Aquaculture, Taiwan National Ocean University, Keelung 20224, Taiwan

E-mail: gwojc@hotmail.com

Introduction

Among the spermatozoan structural characters, the loss of the acrosome in most of the neopterygian species is an outstanding feature. Many researchers have found vesicles anterior to the nucleus, which are possibly acrosomal vestiges in mature sperm from several species (Jamieson 2009). These phenomena lead us to expect finding acrosomal vestiges in other fish species. Whether they are vestige of an acrosome, or other type of special structure with or without any important function remains an open question. To find an answer to this question, we studied the fine structures of righteye stone flounder Kareius bicoloratus (Pleuronectidae) and lefteye southern flounder Paralichthys lethostigma (Paralichthyidae) from Japan and USA, respectively. Due to overfishing and pollution, the wild stock is declining. Cryopreservation of semen is a useful method to establish gene banks both for commercial production and for conservation of threatened species (Gwo 2000).

Materials and methods

Mature righteye stone flounder and lefteye southern flounder were obtained from Experimental Station of The University of Tokyo (Hamanako, Shizuoka, Japan) and Marine Science Institute of University of Texas in Austin (Port Aransas, TX, USA) during the spawning reasons, respectively. Sperm was collected by milking the abdomen to extrude semen. Caution was exercised to prevent contamination of the semen with urine, feces, blood, mucus or water. A drop of natural seawater was added to activate sperm to evaluate the effects of osmolality, cryoprotectant, equilibration time, and freezing method on sperm motility. Semen was cryopreserved using a two-step method. Briefly, 1 part of semen was diluted with 3 parts of precooled (4℃) extenders containing cryoprotectants, equilibrated for 3 min, immersed into dry ice-alcohol bath and stored in LN_2. For morphological study (transmission electron microscopy), sperm samples were processed following the procedure of Gwo et al., 2006.

Results and discussion

Both spermatozoa are of the ect-aquasperm type, with a head lacking acrosome, midpiece and tail regions. The spherical, homogeneously electron-dense nucleus has a caudal, medium nuclear fossa. The centriolar complex is situated inside the nuclear fossa (Type I). A midpiece, containing four mitochondria, forms a ring within shallow depressions of the caudal surface of the lefteye southern flounder nucleus. While eight mitochondria in the nucleus of righteye stone flounder. Proximal centriole connects to the anterior ring of the collar around the distal centriole. The complex collar structure is very unique in righteye stone flounder (Pleuronectidae Family). Anterolateral to the nucleus, there is a chromatin free, electron lucent area in lefteye southern flounder (Paralichthyidae Family). These unique features may be synapomorphies for the righteye and lefteye flounders and evidently contribute to the study of phylogenetic relationships in teleosts. Sperm motility was completely blocked when osmolality was lower than 300 mmol/kg. DMSO proved to be the effective cryoprotectant.

References

Gwo, J.-C.; Lin, C.-Y.; Yang, W.-L.; Chou, Y.-C., 2006: Ultrastructure of the sperm of blue sprat, Spratelloides gracilis; teleostei, Clupeiformes, Clupeidae. Tissue & Cell 38,285-291.

Gwo, J.-C.; Jamieson, B.G.M.; Leung, L.K.-P., 2009: Live preservation of fish gametes. In: Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes), Jamieson, B.G.M. (ed.), Reproductive Biology and Phylogeny Volume 8B of Series, Pp.395-484, Science Publishers, Enfield, NH, USA.

Jamieson, B.G.M., 2009: Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes). Phylogeny Reproductive system Viviparity spermatozoa Volume 8A of Series, Science Publishers, Enfield, NH, USA.

P-223
HISTOPATHOLOGICAL ASSESSMENT OF GONADAL TISSUE IN *CLARIAS GARIEPINUS* FROM AN EUTROPHIC IMPOUNDMENT, SOUTH AFRICA

Ina Wagenaar[1], Tarryn Botha[1], Irene Barnhoorn[2]

[1] *Department of Zoology, University of Johannesburg, P.O. Box 524, Auckland Park 2006, Johannesburg, South Africa*
[2] *Andrology, Department of Urology, University of Pretoria, P.O. Box 667, Pretoria 0001, South Africa*

E-mail: inaw@uj.ac.za

Introduction
The Hartbeespoort Dam (HD) is known for its problems with water quality. The HD is characterized by eutrophication and often has severe algal blooms (Koekemoer and Steyn, 2005) due to the high levels of phosphates and nitrates that wash into the impoundment. The primary pollution sources are debatably Sewage Treatment Works located in Gauteng as well as effluent from surrounding agricultural areas. The freshwater indicator fish species, *Clarias gariepinus*, was used to assess and compare the reproductive health status of fish from the HD, known to be polluted and renowned for its extreme eutrophic state and a less impacted reference site, the Marico Bosveld Dam (MD) which receives water from the Marico River and is said to be in an unmodified natural ecological state. The reproductive health status was done by determining the gonadosomatic indices (GSI), staging of the gonadal development, gonadal histopathology and assessing motility parameters of activated sperm using computer analysis (CASA) based on open source software (Wilson-Leedy and Ingermann, 2007).

Material and methods
Adult *C. gariepinus* were sampled using gill nets; during low-flow (n=17) and high-flow (n=20) seasons from the HD, and from the MD (n = 20). Blood was drawn and whole blood was centrifuged and the top layers was pipetted out and placed in Eppendorf. An Estradiol kit (Cayman Chemicals) were used to determine the estradiol. A complete histology based fish health assessment (Humason, 1999) was done using a necropsy based health assessment index and a qualitative and quantitative histological assessment (Bernet et al., 1999) on the testes. Focus was placed on reproductive health aspects by staging the gonads according to their reproductive development and assessing the motility of activated sperm using CASA based on open source software (Wilson-Leedy and Ingermann, 2007). Sperm motility parameters include motility (%MOT); velocity (VCL-μm/s) and the progression (PROG- μm) (Kime et al., 1996; Wilson-Leedy and Ingermann, 2007; Marchand 2010). Water samples were analyzed for selected physical parameters, selected metals (ICP-MS) and endocrine disrupting chemicals (EDCs). The OCs (*o'p-* and *p,p'* of DDT, DDD and DDE), PCBs (Dieldrin, Aldrin, Endrin, α-endosulfan, αendosulfan Heptachlor, Heptachlor epoxide, Lindane and Arochlor) and alkylphenol, nonylphenol (NP) and organochlorine (OC) were determined (detection limit = 0.50μg/L).

Results and discussion
The results showed distinct macroscopic differences in the testes comparing the HD with the MD. Macroscopic morphology of the testes of fish from the HD showed abnormal growths on the surface of the testes. When histologically studied it was distinguished as an increase in connective tissue amongst the lobules. Barse et al. (2006) found there were total loss of architecture with a reduction in the spacing in the interstitium and a disturbance of the lumen when the fish were exposed to endocrine disrupring chemicals There was an increase in estradiol concentration in the blood plasma of the males in the HD. When comparing the HD low-flow and high-flow sampling trips, the fish from the low-flow had more alterations present across all organs. While the CASA results showed that motility, velocity and progression of sperm were lower in fish from the HD for all parameters Results from the MD showed the ideal trend expected from the moment of sperm activation until degeneration. The velocity and progression were significantly ($p < 0.05$) different between HD samples and fish from the MD. The water quality showed increased concentrations of selenium as well as the EDC, NP and di-n-butyl phthalate (DBP). NP (used in detergents, herbicides) has been found to exert estrogenic effects in fish and may influence the fertility of male fish

(Kinnberg et al., 2000). DBP inhibits the binding of estradiol to its receptor and has a weak estrogenic response and can mimic the hormone estradiol in fish (Patyna et al., 2006).

Conclusion

The increase in environmental pollutants in the HD had a greater effect on the reproductive health status of *C. gariepinus* when compared to the reference sit MD. According to the selected parameters assessed, it seemed like the water from the HD has increasing detriment upon fish health.

Acknowledgements

This work was supported by the National Research Foundation, South Africa and the statistical analysis was done by Ms Juliana van Staden, Statkon, University of Johannesburg.

References

Barse A.V.; Chakrabarti T.; Ghosh T.K.; Pal A.K.; Jadhao S.B., 2006. One-tenth dose of LC50 of 4-tert-butylphenol causes endocrine disruption and metabolic changes in Cyprinus carpio. Pestic Biochem. Phys., 86 ;172-179.

Bernet D.; Schmidt H.; Meier W.; Burkhardt-Holm P.; Wahli T., 1999. Histopathology in fish: proposal for protocol to assess aquatic pollution J. Fish Dis 22: 25-34.

Humason G.L., 1979. Animal tissue techniques. 4th Ed. W.H. Freeman, New York.

Kinnberg K.; Korsgaard B.; Bjerregaard P; Jespersen A., 2000. Effects of nonylphenol and 17β-Estradiol on vitellogenin synthesis and testis morphology in male platyfish Xiphophorus maculatus. J. Exp. Biol., 203:171-181.

Kime D.E.; Ebrahimi M.; Nysten K.; Roelants I.; Ruranwa E.; Moore H.D.M.; Ollevier F., 1996 Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish: application to effects of heavy metals. Aquat Toxicol., 36:223-237.

Koekemoer J.H.; Steyn G.J., 2005: Fish community study of Hartbeespoort Dam Final Report. NW Department of Agriculture, Conservation, Environment and Tourism. 67/2004.

Marchand M.J.; Pieterse G.M.; Barnhoorn I.E.J., 2010 Sperm motility and testicular histology as reproductive indicators of fish health of two feral fish species from a currently DDT sprayed area, South Africa. I. Appl. Ichthyol., 26: 707-714.

Patyna PJ.; Brown R.P.; Davi R.A.; Letinski D.J.; Thomas P.E.; Cooper K.R.; Parkerton T.F., 2006. Hazard evaluation of diisononyl phthalate and diisodecyl phthalate in a aJapanese medaka multigenerational essay. Ecotox Environ. Safe., 65:36-47.

Wilson-Leedy J.G.; Ingermann R.L., 2007. Development of a novel CASA system based on open source software for characterization of zebra fish sperm motility parameters. Theriogenology 67: 661-672.

P-224
QUALITY OF CRYOPRESERVED SPERM OF ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) IN RELATION TO FATTY ACID CONTENT WITH EMPHASIS ON N-3/N-6 PUFA

Maren Mommens[1], Bjørnar Eggen[1], Sylvie L. Bolla[1], Geir Rudolfsen[2], Kiron Visvanath[3] and Igor Babiak[1]

[1] *Reproductive Biology Group and [3]Aquatic Animal Health Group, Faculty of Biosciences and Aquaculture, University of Nordland, N-8049 Bodø, Norway*
[2] *Department of Biology, University of Tromsø, 9037 Tromsø, Norway*

E-mail: maren.mommens@uin.no

Introduction
Decrease in quality and quantity of Atlantic halibut, *Hippoglossus hippoglossus*, sperm towards the end of the reproductive season hampers production of good-quality embryos. Cryopreservation of sperm can facilitate controlled reproduction in Atlantic halibut (Babiak et al. 2008). The objective of this study was to examine the effect of sperm FA composition, with emphasis on n-3/n-6 polyunsaturated FAs (PUFAs) on fresh and cryopreserved sperm motility parameters and the ability of thawed sperm to produce surviving Atlantic halibut larvae.

Material and methods
Sperm was collected from 10th of February to 27th of May 2005 from two Atlantic halibut male groups at the University of Nordland (Bodø, Norway). Group 1 (GR1) consisted of males (n = 5) kept under natural photoperiod conditions, fed decapitated and gutted herring (winter herring) stuffed with Fish Breed-M (INVE Aquaculture NV, Dendermonde, Belgium) in approximate proportion 2:1 (v/v). Group 2 (GR2) consisted of males (n = 5) kept under 3-month advanced photoperiod, fed exclusively with Fish Breed-M. Males were hand-stripped and sperm was collected for CASA and cryopreservation according to Babiak et al. (2006). Sperm samples for FA analysis were snap-frozen in liquid nitrogen and stored at -80°C. FA methyl esters (FAMEs) were prepared after Metcalfe et al. (1966). Cryopreservation was performed according to Babiak et al. (2008). Eggs were obtained in 2006 from females held under the same conditions as GR1 males. Before fertilization, straws of cryopreserved sperm were thawed according to Babiak et al. (2008). Fertilized eggs were incubated in Petri-dishes (n = 3) filled with 40ml autoclaved sea-water added antibiotics (Terramycin ®vet, Pfitzer; USA). Fertilization rate was estimated 12-16 h after fertilization. Hatching rates were estimated and hatched larvae were visually classified as normal or abnormal. ANOVA was applied to determine the significance of male group, sampling date and cryopreservation on sperm n-3 and n-6 PUFA concentrations. Principal component analysis (PCA) was performed on PUFA data. Pearson's correlation coefficient (r) was used to estimate linear relationships between n-3 and n-6 PUFAs in sperm from GR1 and GR2 males and CASA motility parameters and rates of fertilization, hatching and normal larval development.

Results
No significant effect of cryopreservation and sampling date on sperm FA concentration was found. Male group; i.e. differences in feed and photoperiod, significantly influenced concentrations of n-3 FAs ($F_{1,10}$ = 29.4, p < 0.001), n-6 FAs ($F_{1,10}$ = 382.7, p < 0.001), -linolenic (18:3n-3, ALA; $F_{1,10}$ = 4.6, p < 0.05), arachidonic acid (20:4n6, ARA; $F_{1,10}$ = 150.3, p < 0.05), eicosapentaenoic acid (20:5n3, EPA; $F_{1,10}$ = 7.1, p < 0.05), ARA/EPA ($F_{1,10}$ = 250.2, p < 0.05) and DHA/EPA (22:6n3, docosahexaenoic acid; $F_{1,10}$ = 5.9, p < 0.05). 66% of the variation in the dataset between male groups was due to concentration differences in ALA, EPA, DHA and n-3 PUFAs (PC1), while 31 % of the variation was due to concentration differences in ARA and n-6 PUFAs (PC2). No significant correlations between thawed sperm n-3 and n-6 PUFA concentrations and rates of fertilization, hatching and normal larvae development were found in GR1 males. In GR2 thawed sperm significant correlations were found for fertilization (n-3/n-6: r = 0.62, p< 0.01; ARA/EPA: r = -0.53, p < 0.05), hatching (n-3/n-6: r = 0.62, p < 0.01; ARA/EPA: r = -0.57, p < 0.05) and normal larvae development (n-3: r = 0.64, p < 0.001; ARA/EPA: r = -0.63, p < 0.001; EPA: r = 0.55, p

< 0.05; DHA: $r = 0.65$, $p < 0.01$). In GR1 fresh sperm negative correlations were found between ARA/EPA and VCL ($r = -0.53$, $p < 0.01$) and VSL ($r = -0.51$, $p < 0.01$), but not in cryopreserved sperm. In GR2 cryopreserved sperm significant negative correlations between total PUFA and VCL (PUFA: $r = -0.70$, $p < 0.01$), VSL (PUFA: $r = -0.76$, $p < 0.01$), LIN (PUFA: $r = -0.68$, $p < 0.01$), and motility (PUFA: $r = -0.59$, $p < 0.01$) were found, but not in fresh sperm.

Discussion

We have shown that cryopreservation and sample date did not influence n-3/n-6 PUFA composition in Atlantic halibut sperm. Diet seemed to influence significantly sperm n-3/n-6 PUFA concentrations in the two male groups but underlying confounding effects with of other factors, e.g. weight and age, may have influenced the results. Differences in sperm n-3 PUFA concentrations explained the majority of the n-3/n-6 PUFA variation in-between the two male groups and influenced the ability of thawed sperm to produce surviving larvae positively towards the end of spawning season. The commercial broodstock feed, offered to GR2 males, had higher n-3 PUFA content than winter herring, and seemed to influence the ability of thawed sperm to produce surviving larvae positively. We have shown that optimizing PUFA concentrations in broodstock feed can help to maintain Atlantic halibut sperm quality after cryopreservation.

Acknowledgements

The authors would like to thank Ellen Sirnes for excellent training in fatty acid analysis. This study was financed through the University of Nordland.

References

Babiak I.; Ottesen O.; Rudolfsen G.; Johnsen S., 2006: Quantitative characteristics of Atlantic halibut, Hippoglossus hippoglossus L., semen throughout the reproductive season. Theriogenology 65, 1587-1604.

Babiak I.; Bolla S.L.; Ottesen O., 2008: Suitable methods for cryopreservation of semen from Atlantic halibut, Hippoglossus hippoglossus L. Aquacult. Int. 16(6), 561-572.

Metcalfe L.D.; Schmitz A.A.; Pelka R.J., 1966: Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chemie. 28, 5104-5105.

Pustowka C.; McNiven M.A.; Richardson G.F.; Lall S.P., 2000: Source of dietary lipid affects sperm plasma membrane integrity and fertility in rainbow trout Oncorhynchus mykiss (Walbaum) after cryopreservation. Aquacult. Res. 31(3), 297-305.

Vassallo-Agius R.; Watanabe T.; Yoshizaki G.; Satoh S.; Takeuchi Y., 2001: Quality of eggs and spermatozoa of rainbow trout fed an n-3 essential fatty acid-deficient diet and its effects on the lipid and fatty acid components of eggs, semen and livers. Fish. Sci. 67(5), 818-827.

Authors' index

Our Sponsors

ISBN 978-963-88019-7-5