Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Fertilization strategies for externally fertilizing fishes

Strategie oplozování u ryb s vnějším oplozením

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Czech Republic, Vodňany, 2016

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Title of thesis: Fertilization strategies for externally fertilizing fishes
Strategie oplozování u ryb s vnějším oplozením

Ph.D. thesis, USB FFPW, RIFCH, Vodňany, Czech Republic, 2016, 88 pages, with the summary in English and Czech.
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz
ISBN 978-80-7514-053-1

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Fertilization strategies of externally fertilizing fishes

Fertilization in water is extremely difficult yet fishes have evolved incredible ways of overcoming the barriers to reproduction. Species have adapted many alternative strategies in order to produce the greatest number of viable offspring to carry genes to subsequent generations. Fishes use a variety of different methods of fertilization, including internal and external fertilization, as well as some unique strategies for parental care such as oral brooding (exhibits in cichlids) and sex role reversal (exhibits in syngnathids) (Mandal, 2015). Several fish species employ parental care to ensure a greater chance of offspring survival for the fewer number of young produced. The majority of fishes engage in external fertilization, which requires both male and female releasing gametes simultaneously or asynchronously (Balon, 1981). External fertilization is often accomplished by broadcasting gametes where many individuals of both sexes come together and release their gametes into the water or on various substrates; this strategy produces greater numbers of offspring, but without parental care. Internal fertilization is strenuous and energetically very costly, yet effective. The three methods of fertilization include oviparity (egg laid outside female body; e.g. hornshark, skates), ovoviparity (eggs retained within female with yolk being the primary nutrition; e.g. guppies, angel sharks, coelacanths), and viviparity (development within female, with some maternally supplied nutrient followed by live birth; e.g. surfperches, splitfins, lemon shark, seahorses and pipefish) (Budy, 2012).

Along with environmental factors (e.g. water pH, osmolality, temperature, hardness etc.), a variety of both male and female gamete traits have been reported to influence fertilization success of fish (Serrão and Havenhand, 2009). Female gametic traits include egg size, number and size of micropyle, structure of egg membranes, and sperm chemo-attractants. Therefore, knowledge of egg morphology, structure of egg membranes, their development and functions, structure of micropyle and their functions in polyspermy block are essential to understanding fertilization mechanisms of fish. The majority of fish eggs are surrounded with a thick proteinaceous envelope or chorion (Cherr and Clark, 1982). The chorionic structure is varied among the families and reflects adaptations to different environmental conditions or reproductive modality (Rizzo et al., 2002). Pelagic eggs usually possess thinner follicular epithelium layer and zona radiata than the demersal eggs and the latter are either adhesive or non-adhesive (Stehr and Hawkes, 1979). The role of egg envelope including protection of the eggs from mechanical forces such as water currents (Riehl and Patzner, 1998; Esmaeili and Johal, 2005), predators (Riehl and Patzner, 1998; Rizzo et al., 2002) and bacterial and fungal infection (Paxton and Willoughby, 2000). The surface egg membrane and structure of micropyle show substantial differences among teleosts (Riehl, 1980; Chen et al., 1999; Breining and Britz, 2000) as well as among acipenserids (Debus et al., 2002; Debus et al., 2008). Although the egg envelope of teleosts and acipenserids are similar, there are notable differences between the two groups in number and structure of micropyles (Debus et al., 2002). Most fishes possess single micropyle, while acipenserids and some other species possess two to several micropyles. The structure of micropyles vary greatly among species and is used for taxonomic description (Riehl, 1993; Chen et al., 1999; Li et al., 2000). The inner opening of the micropylar canal is slightly larger than the diameter of the sperm head, but is not wide enough to allow multiple sperm to enter (Psenicka et al., 2010). Contrary, male gamete traits include structure of sperm, sperm longevity and swimming speed (Rothschild and Swann, 1951; Vogel et al., 1982), and energetic reserves (Levitan, 2000). Moreover, sperm dilution rate and egg to sperm ratio also play an important role in determining fertilization success.

1.2. Three main actors in fertilization

In a fertilization event, there are three main components: sperm, egg and activating medium (Figure 1). For both, natural and controlled reproduction, activating medium is a mediator to provide suitable environment for the activation and encounters of gametes followed by fertilization. Spermatozoa are immotile in the seminal plasma and become motile when they are released in water or any activating media. In general, to be activated, marine fish sperm requires high osmolality, while freshwater fish sperm requires very low osmolality. Mature oocytes remain in an arrested state at meiotic metaphase-II after ovulation. This developmental block of oocytes is removed upon activation in activating media (e.g. water) or at fertilization (Ginzburg, 1972; Lubzens et al., 2010). In the majority of marine fish, egg activation is induced by the fusion of sperm, while in several freshwater fish and salmonids, egg changes can be induced by contact with water or mechanical stimulation (Ginzburg, 1972; Pavlov *et al.,* 2009). During activation of eggs, the influx of intracellular free Ca^{2+} mediates the cortical alveoli to initiate exocytosis in the egg cytosol (Coward et al., 2002; Finn, 2007; Vasilev et al., 2012), which results in formation of a fertilization membrane (Minin and Ozerova, 2008; Govoni and Forward, 2008). However, the role of activation medium is not negligible in a fertilization event.

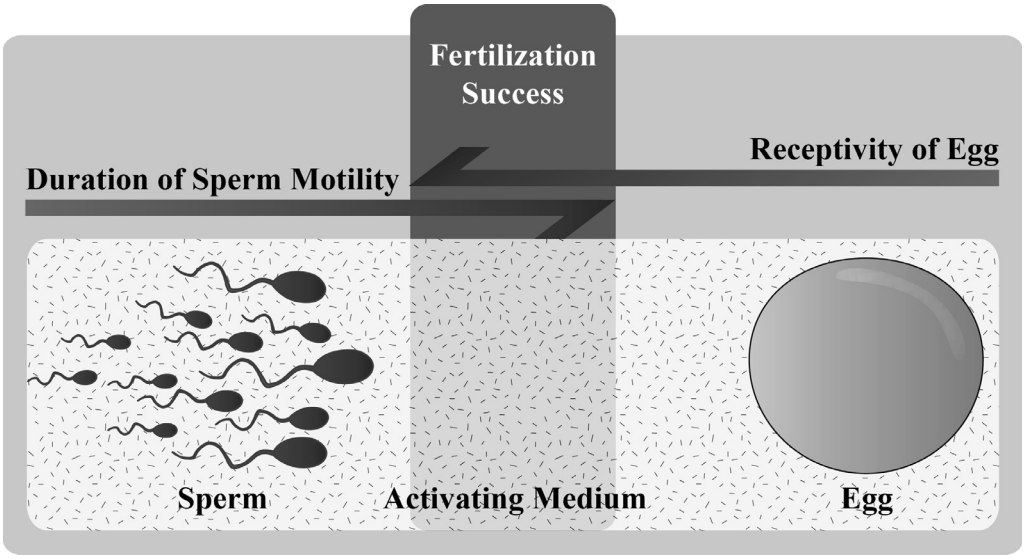

Figure 1. *A conceptual framework of the fertilization event for externally fertilizing fishes.*

1.2.1. Role of sperm longevity and egg receptivity in fertilization

The duration of sperm motility or sperm longevity is greatly varied between marine and freshwater species (reviewed in Browne et al., 2015). The key environmental factors influencing the period of motility of fish spermatozoa are temperature, osmolality, pH and ionic composition of the activating medium. The sperm motility period of freshwater fish species is shorter than marine species (Table 1 and 2) typically lasting for 1 to 2 minutes (Suquet et al., 1994; Browne et al., 2015). On the other hand, the duration of egg receptivity or the fertilization ability of eggs after activation is species-specific and is closely related to different water flow regimes on the spawning ground (Mann, 1996; Merz et al., 2004; Probst

et al., 2009). Additionally, the duration of egg receptivity is usually shorter in freshwater than in marine species (see table 3). For instance, egg receptivity period of sockeye salmon *Oncorhynchus nerka* and rainbow trout *Oncorhynchus mykiss* are up to 40 s after activation (Hoysak and Liley, 2001; Liley et al., 2002), 60 s for crucian carp *Carassius carassius* (Żarski et al., 2014), and 150 s for European perch *Perca fluviatilis* (Żarski et al., 2012). In contrast, eggs of marine fishes like winter flounder *Pseudopleuronectes americanus* and Atlantic cod *Gadus morhua* retain their fertilization ability up to 32 and 120 min, respectively (Butts et al., 2012 and Davenport et al., 1981).

Table 1. *Longevity of marine fish sperm.*

	Common name	Scientific name	Longevity (s)	Reference
Marine species	European sea bass	*Dicentrarchus labrax*	40	Fauvel et al., 1999
	Mediterranean horse mackerel	*Trachurus mediterraneus*	60	Lahnsteiner and Patzner, 1998
	Red mullet	*Mullus barbatus*	90	Lahnsteiner and Patzner, 1998
	Atlantic halibut	*Hippoglossus hippoglossus*	130	Alavi et al., 2011
	Atlantic bluefin tuna	*Thunnus thynnus*	140	Cosson et al., 2008a
	Barramundi	*Lates calcarifer*	300	Palmer et al., 1993
	Atlantic herring	*Clupea harengus*	360	Evans and Geffen, 1998
	European hake	*Merluccius merluccius*	450	Evans and Geffen, 1998
	Atlantic cod	*Gadus morhua*	750	Cosson et al., 2008b
	Bogue	*Boops boops*	900	Lahnsteiner and Patzner, 2008
	Ornate wrasse	*Thalassoma pava*	900	Lahnsteiner and Patzner, 2008
	Turbot	*Scophthalmus maximus*	1020	Suquet et al., 1994
	European conger	*Conger conger*	1200	Cosson et al., 2008a
	Gilthead seabream	*Sparus aurata*	1440	Fabbrocini et al., 2000
	Mediterranean rainbow wrasse	*Coris julis*	7200	Lahnsteiner and Patzner, 2008
	Peacock blenny	*Salaria pavo*	18000	Patzner and Seiwald, 1988

Table 2. *Longevity of freshwater fish sperm.*

	Common name	Scientific name	Longevity (s)	Reference
Freshwater species	African catfish	*Clarias gariepinus*	30	Mansour et al., 2002
	Grayling	*Thymallus thymallus*	31	Hochman et al., 1974
	European perch	*Perca fluviatilis*	35	Lahnsteiner and Patzner, 2008
	Arctic cisco	*Coregonus autumnalis*	36	Breder and Rosen, 1966
	Rainbow trout	*Oncorhynchus mykiss*	40	Billard and Cosson, 2005
	Peled	*Coregonus peled*	43	Breder and Rosen, 1966
	Pink salmon	*Oncorynchus gorbuscha*	47	Breder and Rosen, 1966
	Jundia	*Rhamdia quelen*	48	Borges et al., 2005
	Tench	*Tinca tinca*	54	Breder and Rosen, 1966
	Grass carp	*Ctenopharyngodon idella*	56	Belova, 1981
	Chinook salmon	*Oncorhynchus tshawytscha*	60	Breder and Rosen, 1966
	Brook trout	*Salvelinus frontinalis*	60	Hochman et al., 1974
	Zebra danio	*Brachydanio rerio*	60	Wilson-Leedy and Ingerman, 2007
	Bullhead	*Cottus gubio*	60	Lahnsteiner et al., 1997
	Suruvi	*Steindachneridion scripta*	63	Luz et al., 2001
	White bream	*Blicca bjoerkna*	71	Breder and Rosen, 1966
	Barbel	*Barbus barbus*	75	Alavi et al., 2009a
	Northern pike	*Esox lucius*	75	Alavi et al., 2009b
	European eel	*Anguilla anguilla*	75	Gallego et al., 2014
	Freshwater bream	*Abramis brama*	93	Breder and Rosen, 1966
	Brown trout	*Salmo trutta*	100	Vladic and Jarvi, 1997
	Goldfish	*Carassius auratus*	100	Zheng et al., 1997
	European whitefish	*Coregonus lavaretus*	113	Breder and Rosen, 1966
	Lake whitefish	*Coregonus clupeaformis*	120	Hochman et al., 1974
	Common carp	*Cyprinus carpio*	120	Belova, 1981
	Bunni	*Barbus sharpeyi*	120	Alavi et al., 2010
	Long whiskers catfish	*Mystus gulio*	150	Sunitha and Jayaprakas, 1997
	Crucian carp	*Carassius carassius*	169	Breder and Rosen, 1966
	Persian sturgeon	*Acipenser persicus*	200	Alavi et al., 2004
	Pejerrey	*Odontesthes bonariensis*	240	Strussmann et al., 1994
	White sturgeon	*Acipenser transmontanus*	240	Cheer and Clark, 1985
	Russian sturgeon	*Acipenser guldenstadti*	245	Cheer and Clark, 1985
	Blue tilapia	*Oreochromis aureus*	270	Chao et al., 1987
	Mozambique tilapia	*Oreochromis mossambicus*	270	Chao et al., 1987
	Atlantic salmon	*Salmo salar*	300	Vladic and Jarvi, 1997
	Paddlefish	*Polyodon spathula*	300	Brown et al., 1995
	Nile tilapia	*Oreochromis niloticus*	300	Chao et al., 1987

Sperm longevity, the swimming velocity and the duration of egg receptivity impact the success of a fertilization event (Trippel and Morgan, 1994; Butts et al., 2009). Is has been predicted that when duration of sperm motility period and duration of egg receptivity increase, the chance of fertilization success also increase (see the conceptual model in Figure 1). For instance, if sperm are viable for longer periods of time in the activation medium, then the potential of contacting and fertilizing an egg increases (Butts et al., 2009). In contrast, when longevity of sperm is very short, than egg receptivity may be necessary to increase fertility, since longer periods of egg receptivity are predicted to increase the probability of a successful fertilization event (Trippel, 2003). Therefore, duration of egg receptivity provides valuable insights on reproductive behavior for any fish species (Butts et al., 2012). Fish which have short periods of sperm longevity and egg receptivity, show different fertilization strategies. In this case, males and females release their gametes at the same time or males release their milt on the eggs to facilitate the fertilization process.

Table 3. *Duration of egg receptivity of marine and freshwater fishes.*

Species	Scientific name	Duration of egg receptivity	Reference
Silver carp	*Hypophthalmichthys molitrix*	30–40 s	Mikodina and Makeyeva, 1980
Sockeye salmon	*Oncorhynchus nerka*	40 s	Hoysak and Liley, 2001
Rainbow trout	*Oncorhynchus mykiss*	40 s	Liley et al., 2002
Crucian carp	*Carassius carassius*	1 min	Żarski et al., 2014
Goldfish	*Carassius auratus*	<1 min	Hamano, 1951
European perch	*Perca fluviatilis*	2.5 min	Żarski et al., 2012
Vendace	*Coregonus albula*	4 min	Lindroth, 1947
Japanese rice fish	*Oryzias latipes*	4 min	Yamamoto, 1944
Pond loach	*Misgurnus anguillicaudatus*	5 min	Gamo et al., 1960
European weatherfish	*Misgurnus fossilis*	10 min	Minin and Ozerova, 2008
Mummichog	*Fundulus heteroclitus*	10–30 min	Kagan, 1935
Chum salmon	*Oncorhynchus keta*	15–30 min	Yamamoto, 1951
Winter flounder	*Pseudopleuronectes americanus*	32 min	Butts et al., 2012
Atlantic cod	*Gadus morhua*	2 h	Davenport et al., 1981
Pontic shad	*Alosa immaculate*	>2 h	Kryzhanovskii, 1956
Atlantic herring	*Clupea harengus*	4 h	Kryzhanovskii, 1956
Russian sturgeon	*Acipenser gueldenstaedtii*	6 h	Ginzburg, 1972

1.2.2. Role of activating medium in fertilization

The main role of an activating medium is to initiate motility of spermatozoa and to activate the eggs during fertilization. In controlled reproduction, hatchery water is commonly used as an activating medium for the majority of freshwater fishes, while seawater is used for marine fishes. In freshwater fishes, sperm are usually activated in lower osmotic pressure,

where different ions, pH, temperature, and osmolality of the activating medium affect motility parameters (Alavi and Cosson, 2006; Butts et al., 2013). Sarosiek et al. (2012) found Lahnsteiner's buffer (100-mM NaCl, 10-mM Tris, 0.5% albumin, pH 9.0, and 199 mOsm kg^{-1}) is the most effective medium for activating the sperm of golden ide *Leuciscus idus*. Another activating medium containing 0.4% NaCl, 0.3% urea, 0.5% albumin and characterized by pH 7.7 and 181 mOsm kg^{-1} (Babiak et al., 1997) was found to increase the sperm motility period and velocity for the same species (Sarosiek et al., 2012). The durations of sperm motility of two freshwater fish, rainbow trout and northern pike *Esox lucius*, were found to lengthen as osmolality increased from that of freshwater, but this trend is not apparent in paddlefish *Polyodon spathula*, vimba bream *Vimba vimba*, and common carp *Cyprinus carpio* (Browne et al., 2015).

Most eggs of teleosts (cichlids, catfish, cyprinids, blenniids, gobiids, and percids) and acipenserids possess adhesive properties upon contact with water. In hatchery practice, different egg de-sticking solutions (e.g. tannic acid, Woynarovich solution, saline solution, urea, milk, proteolytic enzymes etc.) have been using to remove egg adhesiveness for common carp, tench *Tinca tinca*, European catfish *Silurus glanis*, African catfish *Clarias gariepinus*, pikeperch *Sander lucioperca*, and sturgeon eggs (reviewed in Siddique et al., 2016). Moreover, it has been found that duration of fertilization capacity of eggs is largely dependent of the activating solution used for fertilization (Saad and Billard, 1987; Żarski et al., 2012; Żarski et al., 2014). For instance, Żarski et al. (2015) reported that osmolality of the activating medium is one of the most important parameters affecting egg receptivity in common carp. In cyprinid species, activation medium with osmolality of 100 to 200 mOsm/kg prolonged the duration of egg receptivity and increased fertilization success (Żarski et al., 2015).

1.2.2. Role of pre-incubation of eggs before fertilization

In controlled reproduction, pre-incubation of eggs before fertilization is a new concept. Pre-incubation of egg is a very simple way to measure the duration of egg receptivity. By pre-incubation of eggs for different time gradients, we can easily measure the duration of egg receptivity in terms of fertilization rate. As we discussed before, to know the exact duration of egg receptivity or how long the eggs are able to be fertilized is very important to standardize the fertilization protocol for any fish species (Butts et al., 2012). In acipenserids and some other species, when a large volume of ovarian or coelomic fluid remains with the eggs after stripping, fertilization success can be reduced (Dettlaff et al., 1993). Therefore, pre-incubation of eggs before fertilization can remove excess ovarian fluid and can initiate egg activation process. Knowledge about pre-incubation of eggs before fertilization is very limited, therefore, there is a necessity to study the effects of pre-incubation of eggs for different fish species.

1.3. Parthenogenetic development of eggs

Parthenogenesis is a common form of asexual reproduction amongst arthropods and rotifers, but can also be found in fish (Chapman et al., 2007, Chapman et al., 2008; Robinson et al., 2011). In facultative parthenogenesis, organisms produce offspring either parthenogenetically or via sexual reproduction (Bell, 1982). Facultative parthenogenesis has been documented in a variety of multi-cellular organisms, including fishes (Chapman et al., 2008; Booth et al., 2010; Booth et al., 2012). The first parthenogenetic reproduction in vertebrates was described in 1932 in the Amazon molly *Poecilia formosa.* In fish, parthenogenesis is mostly exhibited in

the blacktip shark *Carcharhinus limbatus*, hammerhead shark *Sphyrna lewini*, and Zebra shark *Stegostoma fasciatum* (Chapman et al., 2007, Chapman et al., 2008; Robinson et al., 2011). Facultative parthenogenetic cleavage patterns in non-ovulated eggs of Starry sturgeon *Acipenser stellatus* were first reported by Dettlaff and Ginsburg (1950) and later documented by Dettlaff et al. (1993). Thereafter, very few studies have been conducted to document and quantify the percentages of parthenogenetic cleavage development in sturgeon. Thus, there is a need to further study this phenomenon.

1.4. Aims of the thesis

In this thesis, I address issues relating to fertilizing strategies of both freshwater and marine fishes, more specifically, the effects of pre-incubation on the fertilization success of ovulated eggs, determination of egg receptivity period, understanding the role of different activating media, manipulation of egg receptivity period and to document a unique development mode of eggs in Acipenserid species. Chapter 2 starts with a review providing information on the morphology and the developmental stages of the egg membranes of acipenserid species and also standardizing the terminology of the egg membranes to minimize the confusion for future work on acipenserid eggs. Chapter 3 assesses the number of sperm required to fertilize sterlet eggs and explore how pre-incubation of eggs in freshwater interacts with different sperm ratios. Chapter 4 assesses the effects of pre-incubation of fresh and overripe sea bass eggs in seawater and also determined the duration of egg receptivity for this species. In Chapter 5, I investigated the effects of pre-incubation of eggs and activation medium on the percentage of eyed embryos for ide. Duration of egg receptivity period was measured and manipulated by different activating media. Chapter 6 documented facultative parthenogenetic cleavage development of sterlet eggs and quantified the percentage of parthenogenetically developed eggs in relation to the fertilization ability of different females. Finally, a general discussion and summary on my published results, acknowledgements, list of publications, training and supervision plan during my doctoral study and my curriculum vitae are included in Chapter 7.

In brief, the aims of this thesis were:

1. To review the structure of sturgeon egg membranes and of associated terminology.
2. To study the duration of egg receptivity period and the effects of pre-incubation of eggs in freshwater fishes.
3. To examine the duration of egg receptivity period and the effects of pre-incubation of eggs in marine fishes.
4. To understand the effects of activation medium to manipulate egg receptivity period for externally fertilizing fishes.
5. To document an unique development mode, parthenogenesis in Acipenserid species.

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CHAPTER 2

A REVIEW OF THE STRUCTURE OF STURGEON EGG MEMBRANES AND OF THE ASSOCIATED TERMINOLOGY

Siddique, M.A.M., Cosson, J., Psenicka, M., Linhart, O., 2014. A review of the structure of sturgeon egg membranes and of the associated terminology. Journal of Applied Ichthyology 30: 1246–1255.

It was allowed by publishers on 14th March, 2016 to include the paper in this Ph.D. thesis.

Journal of Applied Ichthyology

J. Appl. Ichthyol. 30 (2014), 1246–1255
© 2014 Blackwell Verlag GmbH
ISSN 0175–8659

Received: November 9, 2013
Accepted: August 13, 2014
doi: 10.1111/jai.12604

A review of the structure of sturgeon egg membranes and of the associated terminology

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Summary

Knowledge of the morphological properties, species specific differences, development, and function of the sturgeon egg envelope is of importance for a better understanding of sturgeon reproduction as well as for improvement of the caviar quality. The structure of the egg envelope is similar among the various species, comprising an external envelope (thecal cells, basal lamina, and follicular epithelium), a five-layered oocyte envelope (adhesive layer, alveolar layer, epilayer, and zona radiata externa and interna) and a layer of oocyte matrix and cortical granules. Egg diameters (1.5–4.9 mm), structure and number of the micropyles (2–52), and the size of the micropylar field distinguish eggs of the various species. The development of the egg envelope within the ovary comprises five stages, with further changes following fertilization with the formation of a fertilization cone. The mechanism of polyspermy block in sturgeon eggs and the role of the micropyle and cortical granules in polyspermy block are also described in this review. This review provides information on the morphology and the developmental stages of the egg envelope that will further the understanding of egg biology of acipenserid species. Standardization of terminology describing the egg envelope would minimize confusion and be helpful for future work on acipenserid eggs.

Introduction

Sturgeons are the source of the world-renowned black caviar (Gessner et al., 2008; Bronzi et al., 2011), and sturgeon aquaculture is expanding worldwide mainly for caviar production and conservation re-stocking programmes (Graham et al., 1995; Duke et al., 1999; Chebanov and Billard, 2001; Williot et al., 2001; Bronzi et al., 2011). In aquaculture, the reproductive success of any species is dependent on the production of good quality eggs (Brooks et al., 1997; Bobe and Labbé, 2010). Studies of the morphology and structure of the egg envelope of sturgeon are crucial for better understanding of the reproductive strategies of the fish as well as improving the quality of the caviar (Debus et al., 2002, 2008).

The surface of the egg envelope and the micropylar structure show substantial differences among teleosts (Riehl, 1980; Chen et al., 1999; Vorob'eva and Markov, 1999; Breining and Britz, 2000) as well as among sturgeon species (Riehl and Schulte, 1978; Li et al., 2000; Debus et al., 2002). Although there are some similarities in the egg envelope of teleosts and acipenserids, notable differences between the two groups exist in the micropyle structure (Debus et al., 2002). The number of micropyle in the eggs, as well as their fine-structure, is used for taxonomic description (Riehl, 1993; Chen et al., 1999; Li et al., 2000) and can be considered general phylogenetic characteristics of fish (Esmaeili and Johal, 2005).

Several light microscopy studies have contributed to the description of the external structure of the sturgeon egg envelope (Ginsburg, 1959; Sadov, 1963; Dettlaff and Skoblina, 1969; Caloianu-Iordachel, 1971; Markov, 1975; Doroshov et al., 1991, 1997). The envelope and the micropylar structure of acipenserid eggs have been described in the species: *Acipenser gueldenstaedtii*, *Acipenser stellatus*, *Acipenser baerii*, *Huso huso*, and *A. ruthenus* (Markov, 1975; Le Menn and Pelissero, 1991; Dettlaff et al., 1993; Spinaci et al., 1999; Vorob'eva and Markov, 1999; Debus et al., 2002; Psenicka et al., 2010; Zelazowska, 2010), *Acipenser transmontanus* and *Acipenser naccarii* (Cherr and Clark, 1982; Doroshov et al., 1991), and the paddlefish *Polyodon spathula* (Linhart and Kudo, 1997). The terminology used for the layers of the egg envelope in these studies is not consistent, providing the potential for misunderstanding and misinterpretation. There is an increased demand for further information on the structure and development of the sturgeon egg envelope, because it is closely related to the improvement of the caviar quality. The aim of the review was to describe the morphology and function of layers of the sturgeon egg envelope and to suggest standardized terminology.

Maturation, fecundity, and egg size

Sturgeon eggs are mostly spherical or somewhat elongated and brownish-grey in colour (Dettlaff et al., 1993). The size of the mature eggs is inconsistent among females even of the same species (Dettlaff et al., 1993). However, the difference in size among the eggs does not influence the viability of the pre-larvae. Fecundity and diameter of mature unfertilized eggs of selected sturgeon species are presented in Table 1.

Table 1
Fecundity of female and diameter of ovulated unfertilized eggs of selected sturgeon species

Scientific name	Common name	Fecundity ($\times 10^3$ eggs per individual)	Fecundity eggs per kg $\times 10^3$	Ovulated egg diameter (mm)
Acipenser baerii Brandt, 1869	Siberian sturgeon	0–420[a] 200–800[c]	13.6–16.5 [d]	3.7–4.0[b] 2.4–4.9[d]
A. brevirostrum Lesueur, 1818	Shortnose sturgeon	48–99[a]	na	3.0–3.2[d]
A. dabryanus Duméril, 1868	Yangtze sturgeon	60–130[a]	na	na
A. fulvescens Rafinesque, 1817	Lake sturgeon	50–700[a]	na	2.7–3.5[d]
A. gueldenstaedtii Brandt and Ratzeberg, 1833	Russian sturgeon	50–1165[a] 80–85 [c]	10.8–14.8[d]	2.4–3.9[b] 2.8–3.2[d]
A. schrenckii Brandt, 1869	Amur sturgeon	27.6–1292[a]	na	na
A. nudiventris Lovetzky, 1828	Fringebarbel sturgeon	28–1300[a] 280–1000[c]	9.1–21.7[d]	1.5–3.0[d] 2.5[c]
A. oxyrinchus Mitchell, 1814	Atlantic sturgeon	1030–3756[a]	16.0[d]	2.1–3.0[a]
A. ruthenus L. 1758	Sterlet	8–199[a] 5–100[c]	na	1.9–2.0[e] 2.0–2.5[c]
A. sinensis Gray, 1834	Chinese sturgeon	306–1303[a]	na	3.7–4.9[d]
A. stellatus Pallas, 1771	Starry sturgeon	20–360[a] 35–630[c]	na	2.1–2.8[b] 2.8–3.0[c]
A. sturio L. 1758	Sturio	200–2500[a]	18.0–23.0[d]	2.6–3.0[e] 2.4–2.8[d]
A. transmontanus Richardson, 1836	White sturgeon	98.2–4000[a]	na	3.5–4.0 [f]
Huso huso (L. 1758)	Beluga	360–7700[a] 1000–8000[c]	3.3[d]	2.8–4.5[b] 3.3–4.0[d]
H. dauricus (Georgi, 1775)	Kaluga	186–4868[a]	3.2–15.0[d]	3.6–4.0[e] 2.5–3.5[d]
Polyodon spathula (Walbaum, 1792)	Paddlefish	66–609[a]	9.1–2.6[d]	2.21–3.22[b] 2.0–3.1[d]
Psephurus gladius (Martens, 1862)	Chinese swordfish	0–970[a]	na	na
Scaphirhynchus platorynchus (Rafinesque, 1820)	Shovelnose sturgeon	10–51[a]	na	na

[a]Froese and Pauly (2013); [b]Debus et al. (2002); [c]Vorob'eva and Markov (1999); [d]Billard and Lecointre (2001); [e]Berg et al. (1949); [f]Cherr and Clark (1982); na, data not available.

Egg morphology

The distribution of yolk and lipid inclusions and the structure of the cortical granules in a mature egg do not differ significantly from those of oocytes (Dettlaff et al., 1993). The oocyte exhibits a distinctly polarized structure with a vegetal region containing yolk granules and large lipid inclusions and an animal region containing the cytoplasm, at a lower concentration than that of yolk, and a nucleus or germinal vesicle. The cytoplasm contains a large amount of reserve nutrients that support embryonic development. In mature sturgeon oocytes, the nucleus is many times larger than the somatic cells and is conventionally called the germinal vesicle. At the conclusion of gonadal maturity stage IV, the nucleus shifts towards the animal pole and becomes surrounded by the fine-grained yolk (Kazanskii et al., 1978). The nucleus is filled with nucleoplasm and numerous stained nucleoli (Dettlaff et al., 1993).

At stage IV of maturity, intra-ovarian oocytes possess envelope layers similar to those of ovulated eggs except for the thickness of layers and the shape of the follicular cells. Mature oocytes are covered with five layers of oocyte envelope, with the external envelope encased in three layers (thecal cells, basement lamina, follicular epithelium) (Dettlaff et al., 1993; Debus et al., 2008). The internal surface of the follicular epithelium is in close contact with the adhesive layer. In most sturgeon species, this adhesive layer or jelly-like membrane originates in the intra-ovarian follicular epithelium (Ginzburg, 1972; Markov, 1978; Cherr and Clark, 1982; Dettlaff et al., 1993; Vorob'eva and Markov, 1999) and lies between the follicular epithelium and alveolar layer. The main component of this layer is an 110 kDa glycoprotein containing sialic acid (Cherr and Clark, 1982). This layer of the egg becomes hydrated upon contact with water and gives rise to egg adhesiveness (Dettlaff et al., 1993).

Several micropylar canals (1–13 in *A. stellatus*, up to 33 in *H. huso*, up to 52 in *A. gueldenstaedti*, 5–13 in *A. ruthenus*, 3–9 in *Acipenser sturio*, 3–15 in *A. transmontanus,* 4–8 in *P. spathula*, and 3–16 in *A. baerii*) in the egg membrane are located at the center of the animal pole near the oocyte nucleus (Salensky, 1878; Ginzburg, 1972; Cherr and Clark, 1982; Dettlaff et al., 1993; Linhart and Kudo, 1997; Debus et al., 2008). A layer beneath the zona radiata interna (ZRI) consists of a cytoplasmic membrane up to 1 μm thick that contains microvilli and numerous outgrowths of the cytoplasmic cortex (Aizenshtadt and Dettlaff, 1972). The cortical granules are spherical lysosome-like structures located immediately beneath the plasma membrane (Aizenshtadt and Vasetskii, 1984).

Structure of the egg envelope and inter-species differences

Authors have described the oocyte envelope without following a standardized system of terms or most commonly used

names for identical structures (Debus et al., 2008). A membrane present consistently in the majority of sturgeon eggs proximal to the oolemma is variously called the zona pellucida (Caloianu-Iordachel, 1971), the vitelline envelope (Ginsburg, 1959; Korniyenko, 1975; Altuf'yev et al., 1980), or the zona radiata (Sadov, 1963; Ginzburg, 1972; Markov, 1975; Le Menn and Pelissero, 1991; Vorob'eva and Markov, 1999; Debus et al., 2008; Zelazowska, 2010). The ultrastructure of this membrane has been described as being composed of two regions or zones: the zona radiata interna (ZRI) and zona radiata externa (ZRE) (Ginzburg, 1972; Le Menn and Pelissero, 1991; Vorob'eva and Markov, 1999; Debus et al., 2008; Zelazowska, 2010). In the present study, we have described these layers separately and used the term 'vitelline envelope' to refer the entire zona radiata membrane. Layers of the external envelope and oocyte envelope are illustrated in Fig. 1.

External envelope

The layer of thecal cells is located at the periphery of the follicle, separated from the follicular epithelium by a basement lamina. Caloianu-Iordachel (1971) and Dettlaff et al. (1993) proposed differentiating thecal cells into two parts: the external and the internal theca. The thecal cells, basement lamina, and follicular epithelium have been observed in both ovulated and non-ovulated oocytes of *H. huso, A. baerii, A. gueldenstaedtii*, and *A. stellatus* (Le Menn and Pelissero, 1991; Debus et al., 2008). Helical or screw-like projections originating in the follicular epithelium cells penetrate the ductules of the alveolar layer, and extend into the ZRE of pre-ovulated and ovulated eggs (Debus et al., 2008).

Oocyte envelope

The presence of four distinct layers (adhesive layer, alveolar layer, ZRE and ZRE) of the oocyte envelope has been confirmed and described by several authors (Cherr and Clark, 1982; Le Menn and Pelissero, 1991; Debus et al., 2008; Zelazowska, 2010).

Adhesive layer. The adhesive layer of the sturgeon egg envelope is the outermost layer that closely links and partially penetrates into the pores or ductules of the alveolar layer (Debus et al., 2008). This homogenous jelly coat is composed of less dense and finely flocculent materials and acts as a barrier over the micropyle during sperm penetration (Psenicka et al., 2010). The thickness of this adhesive layer was reported as 0.3–0.64 μm in *A. transmontanus* (Cherr and Clark, 1982), and 0.5–0.9 μm in *A. baerii* (Le Menn and Pelissero, 1991).

Alveolar layer. The alveolar layer is a thick external envelope on the ZRE and often referred to as the jelly coat or extra-chorion. A number of studies have referred to the outermost layer as 'layer three' (Cherr and Clark, 1982; Doroshov et al., 1991), while other authors have described the egg envelope beginning with the outside and designating it as 'layer one' (Linhart and Kudo, 1997). Debus et al. (2008) called it the 'alveolar layer'

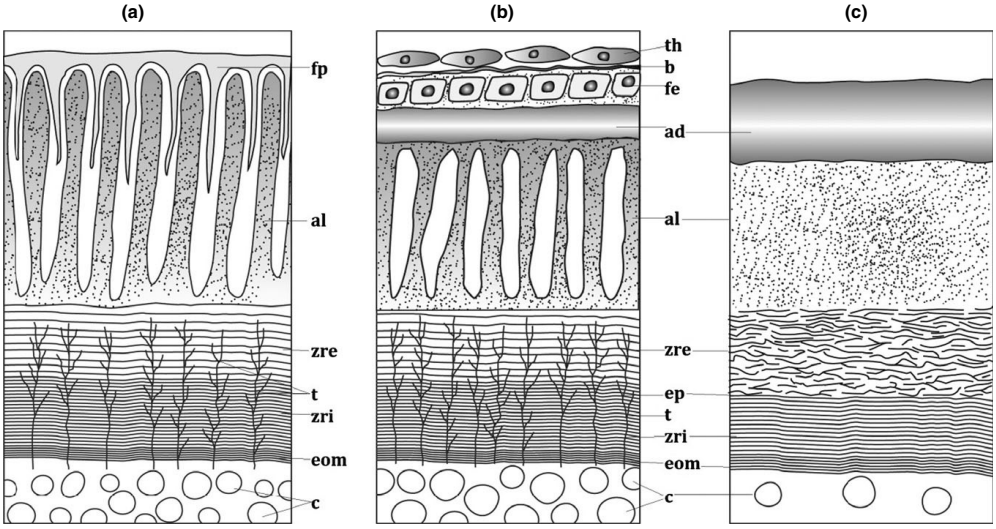

Fig. 1. Schematic of egg envelope of acipenserids at different developmental stages; (a) maturing oocyte (intra-follicular); (b) mature ovulated oocyte; (c) fertilized egg after swelling with water; fp, follicular protrusions; th, thecal cells; b, basement lamina; fe, follicular epithelium; ad, adhesive layer; al, alveolar layer; zre, zona radiata externa; ep, epilayer; t, tubules; zri, zona radiata interna; eom, extra-oocyte matrix; c, cortical granules

Table 2
Different nomenclatures used for the egg envelopes of sturgeon

Proposed nomenclature	Commonly used terminology	Location
Thecal cells	Thecal cells[j,n], external theca and internal theca[c,k]	Periphery of the follicle
Basement lamina	Membrane perifolliculaire[c], basement lamina[j,n], membrane[b]	Between the thecal cells and follicular epithelium
Follicular epithelium	Follicular cells[c,o], follicular envelope[b], follicular epithelium[g,j,k]	Located in the external envelope of the eggs
Adhesive layer	Jelly coat[j,k], adhesive layer[h,n]; jelly envelope[o]	Proximal to the alveolar layer, originating in follicular cells
Alveolar layer	Gelatinous envelope[a,f,g], external envelope[b], gelatinous zone[e], layer 3[h], chorion 3[i], secondary external gelatinous envelope[f], chorion[o]	Outermost layer of oocyte envelope
Zona radiata externa	External vitelline envelope[a], outer vitelline zone[e], external vitelline membrane[g], layer 2[h,l] chorion layer 2[i], zona pellucida interna[c]; zona radiata externa[b,d,f,j,k,n]	Zona radiata externa is the outer part of vitelline envelope, immediately beneath the alveolar layer
Epilayer	Epilayer 1[m], layer 4[m]	Separates zona radiata interna from zona radiata externa
Zona radiata interna	Internal vitelline envelope[a], inner vitelline zone[e], internal vitelline membrane[g], chorion layer 1[i], zona pellucida interna[c]; zona radiata interna[b,d,f,j,k,n]	Inner part of the zona radiata, closely associated with zona radiata externa
Extra-oocyte matrix	Extra-oocyte matrix[j,n], slit-like space[d]	Zona radiata interna is separated from the ooplasma by a thin space, the extra-oocyte matrix, into which the oolemma projects numerous microvilli

[a]Ginsburg (1959); [b]Sadov (1963); [c]Caloianu-Iordachel (1971); [d]Ginzburg (1972); [e]Korniyenko (1975); [f]Markov (1975); [g]Altuf'yev et al. (1980); [h]Cherr and Clark (1982); [i]Doroshov et al. (1991); [j]Le Menn and Pelissero (1991); [k]Dettlaff et al. (1993); [l]Linhart and Kudo (1997); [m]Spinaci et al. (1999); [n]Debus et al. (2008); [o]Zelazowska (2010).

due to its alveolar structure. Nomenclature used by other authors to describe the sturgeon egg envelope is presented in Table 2. Most authors have not differentiated between the adhesive and the alveolar layer and have designated the layers together as the 'gelatinous envelope' (Ginsburg, 1959; Korniyenko, 1975; Markov, 1975; Altuf'yev et al., 1980). The homogenous alveolar layer is in close contact with the ZRE and appears as a granular and 24–63 μm thick structure penetrated by many perpendicularly oriented ductules (0.35–0.5 μm in diameter) (Cherr and Clark, 1982; Debus et al., 2008). The thickness of the alveolar layer was reported as 18 μm in *A. transmontanus* (Cherr and Clark, 1982, 1984) and 45 μm in *A. naccarii* (Spinaci et al., 1999).

Zona radiata externa (ZRE). The ZRE is often called the external vitelline envelope (Ginsburg, 1959; Altuf'yev et al., 1980) or the outer vitelline zone (Korniyenko, 1975). The ZRE layer is approximately 25–59 μm thick and consists of a network of longitudinal filamentous fibrils that terminate in screw-like helical projections on the surface of this layer (Cherr and Clark, 1982; Debus et al., 2008). The fibrils are groups of filaments that are often arranged and joined together inside the vitelline envelope to form the fibril.

Epilayer. A thin epilayer containing fibres with granulose organization has been reported between the ZRE and ZRI of sturgeon eggs in *A. gueldenstaedtii* (Markov, 1975) and *A. naccarii* (0.5 μm thick) (Spinaci et al., 1999). Debus et al. (2008) also confirmed the presence of this layer in *H. huso, A. baerii, A. gueldenstaedtii*, and *A. stellatus* by transmission electron microscopy.

Zona radiata interna (ZRI). The ZRI or internal vitelline envelope is the innermost layer of the egg envelope and is closely associated with the oolemma (Cherr and Clark, 1982). The ZRI is usually 14–25 μm thick (Debus et al., 2008). The thickness of this layer was reported as 12 μm in *A. transmontanus* (Cherr and Clark, 1982, 1984), and 20 μm in *A. naccarii* (Spinaci et al., 1999). This layer consists of several longitudinal fibrils, smaller in diameter than those of the ZRE and packed more tightly. As in the ZRE, helical projections of ductile were not observed inside the ZRI (Debus et al., 2008).

Extra-oocyte matrix, cortical and pigmented granules. The ZRI under electron microscopy appears separated from the ooplasma by a thin electron-lucent membrane into which numerous microvilli project from the oolemma. Several authors have reported the presence of microvilli and numerous outgrowths of the cytoplasmic cortex filling the space between the oolemma and the ZRI. This material is designated the 'extra-oocyte matrix' (Aizenshtadt and Dettlaff, 1972; Le Menn and Pelissero, 1991; Dettlaff et al., 1993; Debus et al., 2008). As the extra-oocyte matrix is proximal to the ZRI, the microvilli enter its ductules. The cortical granules are closely engaged with the oolemma and located just beneath the extra-oocyte matrix throughout the oocyte surface, while the pigmented granules are deposited near the cortical granules (Dettlaff et al., 1993). The cortical granules

and pigmented granules are round, ellipsoid, often irregular, and usually arranged in one or two rows.

Developmental stages of egg envelope formation

During the second half of the maturation period, sturgeon oocytes enter a succession of meiotic phases that result in the reduction of the number of chromosomes. The meiotic phases begin with prophase I (meiosis I) and proceed until metaphase II (meiosis II) when ovulation begins (Flajshans et al., 2013). The subsequent phases of meiosis II (anaphase II and telophase II) are completed only after fertilization (Dettlaff et al., 1993). Vitellogenin and choriogenin are serum precursor proteins of the egg yolk and vitelline envelope, respectively. During oogenesis, the vitellogenin and choriogenin are synthesized in the liver of female fish and accumulate in the yolk to meet the nutritional requirements of the embryo and in the egg envelope, providing protection to the embryo from biological and environmental challenges (Yamagami et al., 1994). Development of the egg envelope in sturgeon species usually occurs in consecutive stages. The egg envelope has been described and compared in several species of *Acipenser* and *Huso*. However, detailed histological observations of egg envelope formation and development are rare for most Acipenseridae species. Linhart and Kudo (1997) observed the surface ultrastructure of paddlefish eggs before and after fertilization. Zelazowska (2010) described the formation and development of the *A. gueldenstaedtii* egg envelope in five consecutive stages:

Stage I of egg envelope formation

The egg envelope is usually secreted into the peri-oocytic space, mainly from the microvilli zone and extra-oocyte matrix, to form two types of envelope: the vitelline envelope and the chorion (Zelazowska, 2010). The vitelline envelope is composed of filaments that are secreted by the oocyte, and the chorion is composed of a single layer of homogeneous material that is secreted by follicular cells. The oocyte plasma membrane forms numerous microvilli directed towards the follicular cells, which penetrate into the egg envelope at a further stage of egg envelope formation (Zelazowska, 2010).

Stage II of egg envelope formation

During stage II of the egg envelope formation, an additional internal layer of the vitelline envelope is secreted and deposited underlying the first vitelline envelope, and, similarly, an extra-chorion layer is also deposited on the surface of the chorion (Zelazowska, 2010). The ZRI is composed of thin and short filaments, while the ZRE is composed of fibrils (more tightly arranged filaments) and trabecules (Zelazowska, 2010). At a later stage, fibrils from the ZRE convert to trabecules, which constitute the alveolar layer of the egg envelope.

Stage III of egg envelope formation

At stage III, secretion and deposition of the vitelline envelope continues. Newly secreted materials are composed of short filaments attached together to form fibrils, and, subsequently, the ZRI of the vitelline envelope develops on the oocyte surface (Zelazowska, 2010). At this stage, a thin and densely packed homogeneous layer is formed between the ZRE and ZRI. Debus et al. (2008) termed this the epilayer 1 (Debus et al., 2008). During the developmental stages of egg envelope formation (from stage II to stage V), the follicular cells change shape, with the primary follicular cells changing from a rectangular to a cuboid shape (Zelazowska, 2010).

Stage IV of egg envelope formation

The vitelline envelope layer is deposited on the oocyte surface during stage IV. The ZRE and ZRI acquire a homogeneous appearance due to the reduction of space between filaments. The follicular cells of the external envelope of the eggs also change shape at this stage, becoming cubical or cylindrical (Zelazowska, 2010).

Stage V of egg envelope formation

During this stage, the composition of the vitelline envelope is similar to previous stages (Zelazowska, 2010). However, the extra-chorion (adhesive layer and follicular epithelium) extends over the alveolar chorion. Numerous canals containing the oocyte microvilli and follicular cell processes perforate the alveolar chorion. Canals that perforate the ZRE are filled with a homogenous material. At this stage, the peripheral ooplasm contains numerous pigment granules, yolk platelets, and cortical alveoli (Zelazowska, 2010).

Micropylar structure and inter-specific differences

Sturgeon eggs contain numerous micropyles with a double-tapered, often funnel shaped structure usually located in the region of the animal pole in the surface of the egg chorion. The number of micropyles varies among specimens of the same species, with inter-specific variability also being common. The number of micropyles is never fewer than two (Vorob'eva and Markov, 1999). The presence of numerous micropyles on the chorion surface could appear to increase the chances of polyspermy in sturgeon eggs. However, the increased risk of polyspermy is compensated for by the double-tapered spermatozoa entry canal (Cherr and Clark, 1982). Each micropyle on the surface of the chorion of sturgeon eggs, except in *H. huso*, consists of a wide funnel that passes through the membranes and the inner opening of the micropylar canal (Vorob'eva and Markov, 1999). Such a complex structure of micropyles may serve to limit sperm access to the oolemma or previtelline space (Cherr and Clark, 1982).

The micropylar canals are restricted to a small area near the sperm entry site in the animal pole region. The dimension of the area in which the micropyles are located is referred to as the 'size of the micropylar field' (Debus et al., 2002). In *A. stellatus*, *H. huso*, and *A. gueldenstaedtii*, the distance between neighboring outer micropylar openings is usually between 20 and 80 μm, less frequently up to 100 μm. When 5–10 micropyles are present, they are located in a region

about 120–130 μm in diameter. The diameter of the micropylar field depends on the number of micropyles on the egg surface (Debus et al., 2002). For example, in *A. transmontanus* (3–15 micropyles), the size of micropylar field is up to 100–200 μm (Cherr and Clark, 1982). An exception to the norm is seen in the size of micropylar field observed in *A. gueldenstaedtii*, with more than 30 micropyles at 1000–1100 μm (Dettlaff et al., 1993). The diameter of the micropylar field of eggs of *H. huso* is 100–826.2 μm, *A. stellatus* is 61.9–173.5 μm, *Apersicus* is 114.3–276.5 μm, *A. baerii* is 142.6–569.9 μm, and *A. nudiventris* is 117.7–143.4 μm (Debus et al., 2002). The diameter of the external opening of the micropyle funnel is 20 μm in *H. huso* and *A. ruthenus*, up to 50 μm in *A. stellatus,* and 50–60 μm in *A. nudiventris* (Vorob'eva and Markov, 1999). The inner opening of the micropylar canal is much narrower than the opening of the micropylar funnel. The inner opening of the micropylar canal is slightly larger than the diameter of the acrosome and is not wide enough to allow multiple sperm to enter the micropyle (Psenicka et al., 2010). Diameter of the inner opening of the micropylar canal is 2.5–3.0 μm in *A. ruthenus*, 5 μm in *A. gueldenstaedtii* and *A. Baerii*, and 10 μm in *A. persicus* (Vorob'eva and Markov, 1999). After critical point drying of the eggs for scanning electron micoscopy the micropylar opening diameters were 8.3–21.5 μm in *H. huso*, 4.6–16.0 μm in *A. gueldenstaedtii*, 7.1–13.6 μm in *A. stellatus*, 9.7–21.2 μm in *A. persicus*, 9.94–17.8 μm in *A. baerii*, and 12.4–13.0 in *A. nudiventris* (Debus et al., 2002).

Fertilization of the egg

Before and during the fertilization process, the transformation of the vitelline envelope or zona radiata into the fertilization envelope constitutes morphological changes common to many fish species (Kudo, 1982a,b). The thickness of the vitelline envelope in the egg animal pole region decreases following ovulation. In sturgeon, the vitelline envelope is thinner at the animal pole (approx. 20 μm for *A. stellatus* and 20–30 μm for *A. gueldenstaedti*) than other areas of the envelope (Dettlaff and Ginsburg, 1954). Mature eggs of sturgeon species possess several micropyles that perforate the surface of the vitelline envelope at the animal pole region. The ZRE forms a small number of dome-like structures consisting of tufts of microvilli under each of the micropyles. The number of spermatozoa entry sites usually corresponds to the number of micropyles on the egg surface (Linhart and Kudo, 1997). The ultrastructure of the spermatozoa entry site in sturgeon eggs is similar to that observed in most other fish species (Linhart and Kudo, 1997). The ultrastructural features of the spermatozoa entry site and egg fertilization cone have been investigated in carp (Kudo, 1982a,b), European catfish (Kudo et al., 1994), and a few sturgeon species (Vorob'eva and Markov, 1999).

Several fish species respond to initial stages of spermatozoa penetration by forming a *cytoplasmic process* or early *fertilization cone* (Kudo, 1980, 1991; Kobayashi and Yamamoto, 1981; Kudo and Sato, 1985). The formation of the cytoplasmic process is initiated prior to the cortical reaction (Kudo, 1982a; Linhart and Kudo, 1997). At 60 s post-fertilization, Linhart and Kudo (1997) observed a cytoplasmic process or a mature fertilization cone extending to several micropyle canals in the animal pole region of paddlefish eggs. A few smaller cytoplasmic processes or flocculent materials were found in other micropylar canals of the same egg. The plasma membrane of the fertilization cone showed no fusion with the plasma membrane of supernumerary spermatozoa (Kudo, 1980, 1991; Kobayashi and Yamamoto, 1981; Kudo and Sato, 1985). At 60 s post-fertilization the fertilization cone enlarged to approx. 20–30 μm in diameter and appeared as a spherical shape on the egg envelope (Linhart and Kudo, 1997; Psenicka et al., 2010). The proteases of the vitelline envelope are responsible for the elevation and transformation of the vitelline layer to the fertilization envelope (Murata, 2003). The alteration of the vitelline layer by substances released from the cortical granules and the formation of the elevated fertilization membrane is a feature of the sea urchin *Strongylocentrotus purpuratus* (Foerder and Shapiro, 1977; Murata, 2003).

Subsequently, 3, 10, and 20 min post-fertilization, the uneven surface of the cortical cytoplasm of paddlefish eggs at the animal pole region was slightly elevated with less numerous microvilli than in the area around the secondary polar body (Linhart and Kudo, 1997). The cytoplasmic process of the egg envelope plays an important role in the prevention of polyspermy (Kudo, 1980; Kobayashi and Yamamoto, 1981). When a spermatozoon has penetrated the egg, the second polar body is formed (20 min post-fertilization at 20°C room temperature) (Linhart and Kudo, 1997).

Blocking of polyspermy in eggs

Oviparous fishes spawn a huge number of eggs; fertilization thereof must be completed between one maternal and one paternal gamete. In mono-spermic eggs, if more than one spermatozoon fuses with the oocyte, the embryo will die at an early stage (Ginzburg, 1972; Murata, 2003). During fertilization, huge numbers of spermatozoa compete to fertilize an oocyte, moving toward the opening of the micropylar canal in the animal pole region of the oocyte. However, only a single spermatozoon can penetrate into the cytoplasm of the oocyte, and the spermatozoa nucleus fuses with the oocyte nucleus. Mono-spermic eggs usually possess several mechanisms to prevent supernumerary spermatozoa penetration into the oocyte (Murata, 2003). The nature of this defense mechanism is a protective reaction by the egg envelope called 'polyspermy block'. Sturgeon eggs are mono-spermic and possess numerous micropyles or spermatozoa entry sites on the surface of the egg envelope, creating a high potential for polyspermy. However, the structure of the micropyle and the response of cortical granules to fertilization inhibit polyspermy in the sturgeon eggs (Ginsburg, 1961; Dettlaff, 1962; Psenicka et al., 2010).

Mechanisms of 'polyspermy block' in sturgeon eggs

Although sturgeon eggs possess numerous micropyles, the structure of the micropyle and the cortical granules play a significant role in polyspermy block; this mechanism can be briefly described in the following steps:

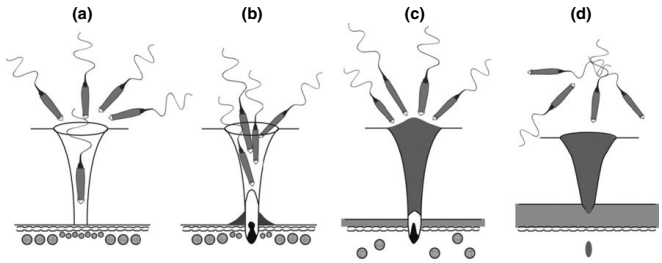

Fig. 2. Mechanism of polyspermy block in sturgeon eggs (a) narrow inner opening of micropylar canal preventing polyspermy, (b) fertilization cone forms usually within 60 s of fertilization preventing passage of multiple spermatozoa through the micropyle canal (Linhart and Kudo, 1997; Psenicka et al., 2010), (c) content of cortical granules repels and immobilizes supernumerary spermatozoa; at 3-min post-fertilization, external apertures of most micropyle are sealed with flocculent material (Linhart and Kudo, 1997), (d) after complete formation of the vitelline space, enzymes secreted from the cortical granules provoke hardening of egg chorion surface and block spermatozoa entry sites (Murata, 2003)

The structure of the micropyle restricts the number of sperm entering the oocyte plasma membrane (Fig. 2). The diameter of the inner aperture of the micropyle of sturgeon eggs is slightly larger than the acrosome diameter, limiting access to a single spermatozoon, and has been described in teleost species (Billard et al., 1986; Dettlaff et al., 1993; Linhart and Kudo, 1997).

After fusion with the spermatozoon, a fertilization cone originating from the cytoplasm of the egg appears on the spermatozoon entry sites. Growth of the fertilization cone starts from the top of the fused sperm head (Kudo, 1980; Kudo and Sato, 1985). The cytoplasmic process is formed beneath several micropyles on the egg. Psenicka et al. (2010) found only a single fertilization cone per fertilized egg. A few micropyles did not form a fertilization cone, but exhibited smaller cytoplasmic processes (Linhart and Kudo, 1997). The fertilization cone plugs the inner opening of the micropyle and prevents multiple spermatozoa from attaching to the oocyte plasma membrane (Linhart and Kudo, 1997).

Immediately after fertilization, a perivitelline space is formed from the egg cortical granules (Ginzburg, 1972; Linhart and Kudo, 1997). During fertilization, protein released from the cortical granules is converted to lectin (Grey et al., 1974). Cherr and Clark (1985a,b) reported the presence of lectin in sturgeon eggs. The content of fish egg cortical granules (especially cortical granule lectins) immobilizes and repels supernumerary spermatozoa (Murata, 2003).

The content of the cortical granules become the vitelline fluid after fertilization when water imbibement is complete. This vitelline fluid is responsible for the hardening of the egg envelope after cortical reaction (Zotin, 1958; Ha et al., 1995; Shibata et al., 2000, 2012; Murata, 2003). Dettlaff (1962) reported the hardening of the sturgeon egg envelope after fertilization, which could be induced by vitelline fluid.

Role of the micropyle

In general, the role of the micropyle is to aid in the fertilization process or the connection of gametes. Water in natural spawning grounds may be more turbid than hatchery water. In turbid water, micropyles effectively guide the spermatozoon into the oocyte. Micropyles are situated exclusively in the animal pole region, which is in proximity to the egg genome, so that after entering the micropyle canal the spermatozoon can easily penetrate the oocyte. The multiple micropyles in sturgeon eggs increase the chances of fertilization. Sturgeon eggs need only one percent of the spermatozoa to fertilize each oocyte in salmonids (Ginzburg, 1972).

The micropylar cell determines the structure of the micropyle and its position on the egg surface during oogenesis (Riehl, 1978). Beneath the micropyle opening on the egg surface, there is a tuft of microvilli (Linhart and Kudo, 1997). As mentioned, the number of micropyles varies markedly among acipenserid species and even among the eggs from the same female. The size of the micropylar field is positively correlated to the number of micropyles in each egg. The distance between any two micropyles is not more than 100 μm. The micropylar canal traverses the inner layer of the egg envelope (ZRI), and the diameter of the inner end of the micropylar canal is slightly larger than the width of the spermatozoa head. As in most fish species, the base of the micropylar canal in sturgeon eggs is just wide enough for the passage of a single spermatozoon (Ginzburg, 1972; Billard et al., 1986; Linhart et al., 1995). The micropylar canal in a carp *Cyprinus carpio* egg is wider than the spermatozoon head, but the carp egg does not possess multiple micropyles as do sturgeon and paddlefish (Ginzburg, 1972; Kudo, 1980; Cherr and Clark, 1982; Linhart and Kudo, 1997). As in most fish species, in the mammalian egg there is no micropyle or sperm entry canal on the egg surface. In mammals, spermatozoa need to bind with the egg envelope or zona radiata externa. After attaching to the egg envelope, the spermatozoa acrosome secretes a substance containing enzymes such as acrosin to digest the egg envelope glycoproteins, making possible fusion between the spermatozoa plasma membrane and the oocyte plasma membrane (Murata, 2003).

At 60 s post-fertilization, outgrowths or cytoplasmic projections were observed on the sturgeon egg envelope, extending to the external aperture of the micropylar canal (Ginsburg, 1959; Linhart and Kudo, 1997). Similar out-

growths from the egg ooplasm have also been observed in carp (Kudo, 1980) and zebrafish *Danio rerio* eggs (Hart et al., 1992). This outgrowth has been called the *fertilization cone* (Kudo, 1980; Kobayashi and Yamamoto, 1981; Hart et al., 1992; Psenicka et al., 2010).

Micropyles likely aid in the fertilization process and also provide an effective system for blocking polyspermy. When a spermatozoon penetrates the oocyte, the fertilization cone is developed in the micropyle (Linhart and Kudo, 1997; Psenicka et al., 2010). The accompanying release of cytoplasmic fluid not only penetrates the active micropyle but also other micropyles. The fertilization cone functions as an eruption, distributing cytoplasm around several micropyles. Linhart and Kudo (1997) observed fertilization cones without spermatozoa in paddlefish eggs.

Role of cortical granules in blocking polyspermy

When the first spermatozoon reaches the oocyte plasma membrane through the micropylar canal, the egg envelope responds by producing the fertilization wave, also referred to as the Ca^{2+} wave. This fertilization wave moves through the cortex of the oocyte from the spermatozoon entry site to the opposite point of the cortex surface (Murata, 2003; Psenicka et al., 2010). Following this fertilization wave, the cortical granules rapidly break down to form a perivitelline space (Ginsburg, 1961; Dettlaff, 1962; Psenicka et al., 2010). The content of the cortical granules is released into the perivitelline space between the oocyte plasma membrane and the egg envelope. The perivitelline space fills with perivitelline fluid, which is formed by imbibed water and the substances released from the cortical granules. Subsequently, the egg envelope separates from the oocyte plasma membrane (Dettlaff, 1962; Murata, 2003; Psenicka et al., 2010; Liu, 2011).

The cortical granules are specialized Golgi-derived secretory granules, which contain glycoproteins collectively called lectins (Grey et al., 1974; Krajhanzl, 1990; Dong et al., 2004) usually found in the perivitelline space. The presence of lectin has been reported in eggs of sturgeon (Cherr and Clark, 1984, 1985a), Chinook salmon (Janna and Yamamoto, 1984; Murata, 2003), medaka (Iwamatsu et al., 1997), trout (Ginzburg, 1972), and many other fish species. Following fertilization, the cortical granule lectins interact with the spermatozoa and agglutinate with spermatozoa immobilizing activity. The content of the cortical granules also repels supernumerary spermatozoa from the micropylar vestibule, due to the increase of osmotic pressure in the perivitelline space (Murata, 2003).

It has long been hypothesized that, post-fertilization, the content of the cortical granules is involved in hardening of the egg envelope (Shibata et al., 2012). A number of studies argued that the enzymes alveolin and transglutaminase, secreted from the cortical granules, trigger the envelope hardening process during the cortical reaction in many fish species (Zotin, 1958; Ha et al., 1995; Shibata et al., 2000, 2012; Murata, 2003). The inter-molecular cross-linking between egg envelope proteins is catalyzed by transglutaminase, which is involved in the hardening process. Transglutaminase has been shown to be embedded in the egg envelope in species of fish including rainbow trout (Ha and Iuchi, 1998), medaka (Iuchi et al., 1995) and carp (Kudo et al., 2000; Chang et al., 2002), but remains inactive until the cortical reaction occurs in the egg (Shibata et al., 2012).

A second chorion hardening inducing agent, alveolin, is also reported by many authors (Shibata et al., 2000, 2012; Murata, 2003). In sea urchin eggs, within 30 s of first spermatozoa attachment to the oocyte, an electrically mediated block to further spermatozoon entry is immediate induced by depolarization of the egg plasma membrane (Jaffe, 1977; Murata, 2003). After depolarization, trypsin-like protease is released from the cortical granules to cleave spermatozoon receptors from the eggs and act as a second block to further the entry of spermatozoa (Foerder and Shapiro, 1977). The presence of alveolin and transglutaminase has not been studied in sturgeon eggs, but Dettlaff (1962) reported hardening of the sturgeon egg envelope after fertilization. The aspect of chorion hardening in sturgeon eggs by alveolin and transglutaminase warrants further investigation.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (LO1205 under the NPU I program), GAJU 114/2013/Z, and GACR P502/13/26952S. The authors would like to thanks Md. Khurshid Alam Bhuiyan for his kind help during drawing the figures. Our appreciation is extended to the Lucidus Consultancy for their suggestions and improvements English.

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CHAPTER 3

EFFECTS OF PRE-INCUBATION OF EGGS IN FRESHWATER AND VARYING SPERM CONCENTRATION ON FERTILIZATION RATE IN STERLET STURGEON, *ACIPENSER RUTHENUS*

Siddique, M.A.M., Butts, I.A.E., Psenicka, M., Linhart, O., 2015. Effects of pre-incubation of eggs in freshwater and varying sperm concentration on fertilization rate in sterlet sturgeon, *Acipenser ruthenus*. Animal Reproduction Science 159: 141–147.

Animal Reproduction Science 159 (2015) 141–147

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effects of pre-incubation of eggs in fresh water and varying sperm concentration on fertilization rate in sterlet sturgeon, *Acipenser ruthenus*

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ARTICLE INFO

Article history:
Received 5 February 2015
Received in revised form 2 June 2015
Accepted 8 June 2015
Available online 12 June 2015

Keywords:
Artificial reproduction
Sperm to egg ratio
Effects of pre-incubation
Fertilization success

ABSTRACT

Standardization of fertilization protocols for sterlet *Acipenser ruthenus* is crucial for improving reproductive techniques and for conservation purposes. Our objectives were to determine the number of sperm (tested 430,000:1, 43,000:1, 4300:1, 430:1 sperm to egg) required to fertilize eggs and explore how pre-incubation of eggs in freshwater for 0 min, 0.5 min, 1 min, and 10 min interacts with different sperm ratios. Fertilization success ranged from 29.7% at 430:1 to 84.2% at 430,000:1. Pre-incubation time had no effect on fertilization success at 430,000:1 and 43,000:1 sperm to egg ratios, while it was significant at the 4300:1 and 430:1 ratios. The use of adequate experimental suboptimal sperm to egg ratio revealed a positive effect of pre-incubation time, such that at the 430:1 ratio, 0.5 min pre-incubation increased the fertilization rate than 10 min. At 0 min pre-incubation the proportion of fertilized eggs increased at the 430,000:1 ratio, while at 1 min fertilization increased at the 4300:1 ratio. At the 10 min pre-incubation time, fertilization increased at the 43,000:1 ratio. Moreover, at the 0.5 min pre-incubation time, the 43,000:1 ratio increased the fertilization rate than the 430:1 ratio. Generally, for 430:1 ratio, the fertilization rate is lower than in control. Transmission electron microscopy showed that pre-incubation of eggs in water for <10 min does not trigger a cortical reaction or the formation of a perivitelline space. Results suggest that with a low sperm to egg ratio 0.5 to 1 min pre-incubation of eggs in freshwater prior to fertilization can enhance fertilization rate of sterlet.

1. Introduction

Sterlet, *Acipenser ruthenus* is a small acipenserid widely distributed in central-eastern Europe and Asia (Sokolov and Vasilév, 1989; Bemis and Kynard, 1997). Due to early sexual maturity and its economic importance sterlet has become one of the most commonly cultured sturgeon species in many countries (Nikolskij, 1971; Chebanov and Billard, 2001; Williot et al., 2005). Although basic procedures for artificial fertilization have been described (Dettlaff et al., 1993; Chebanov and Galich, 2011), very few empirical studies have been conducted with respect to standardization of fertilization protocols by pre-incubation of eggs for this species.

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http://dx.doi.org/10.1016/j.anireprosci.2015.06.008
0378-4320/

In general, sturgeon eggs contain numerous micropyles (reviewed in Siddique et al., 2014a). Furthermore, egg morphology, the structure of egg envelopes, as well as the adhesive properties of the eggs (when in contact with fresh water) are similar among all sturgeon species (Siddique et al., 2014a). Additionally, the ultrastructure of sperm, sperm density, and osmolality of the seminal plasma are similar amongst males (Dettlaff et al., 1993; Li et al., 2011). In the hatchery, sturgeon gametes are collected into dry containers and then sperm is diluted with water at a 1:200 ratio, to provide the highest probability of monospermic insemination (Gela et al., 2008). Thus, fertilization strategies and rearing techniques during artificial spawning are comparable amongst all species. As a result, standardization of the fertilization protocol for sterlet can be useful for other hatchery-reared sturgeon.

Male fertility is predicted to increase when more sperm are incorporated into the fertilization environment. However, in case of sturgeon, an excessive volume of sperm increases the chance of polyspermy, which can impair embryonic development (Bergh et al., 1992; Psenicka et al., 2010). Therefore, quantification of sperm density is important for standardizing fertilization protocols under controlled conditions for any species (Butts et al., 2009, 2014). Unfortunately, there is currently no data on the optimal sperm to egg ratio for sterlet; limited data is available for sturgeon species, in general (reviewed in Butts et al., 2012).

The fertilizing capacity of mature ovulated fish eggs gradually decreases in coelomic fluid and in fresh water. Loss of fertilization ability occurs rapidly, within 1 min in northern Pike *Esox lucius* and vimba Bream *Vimba vimba* and within several min in chum salmon *Oncorhynchus keta*, chinook salmon *Oncorhynchus tschawytscha*, Atlantic salmon *Salmo salar*, lake trout *Salvelinus namaycush*, and vendace *Coregonus albula* (Ginzburg, 1972; Dettlaff et al., 1993). Sockeye salmon *Oncorhynchus nerka* and Rainbow trout *Oncorhynchus mykiss* eggs lost their fertilization ability within 40 s (Hoysak and Liley, 2001; Liley et al., 2002), crucian carp *Carassius carassius* within 1 min (Żarski et al., 2014), European perch *Perca fluviatilis* within 2.5 min (Żarski et al., 2012), European weatherfish *Misgurnus fossilis* within 10 min (Minin and Ozerova, 2008) and winter flounder *Pseudopleuronectes americanus* within 32 min (Butts et al., 2012). Sturgeon eggs are considerably more resistant to the effects of fresh water (Ginzburg, 1972). When sterlet eggs are collected by hand stripping, a large volume (50 to 70%) of coelomic fluid remains with the eggs. Dettlaff et al. (1993) reported that coelomic fluid can hinder fertilization, but there have been no studies of pre-incubation in fresh water of sterlet eggs to increase fertilization success.

Thus, the objective of this study was to estimate the adequate sperm to egg ratio for sterlet fertilization and to minimize the sperm to egg ratio by modifications of fertilization environment in order to save sperm and prevent polyspermy.

2. Materials and methods

2.1. Broodstock handling and gamete collection

Six mature males and five females were kept in two separate tanks (volume 0.8 m^3) with a water temperature of 14 to 15 °C. To induce spermiation, males were administered a single intramuscular injection of carp pituitary extract (CPE) at 4 mg kg^{-1} body weight. Sperm were collected 48 h after injection by a plastic catheter (4 mm diameter) (Gela et al., 2008). Sperm were collected before stripping the females and stored in an icebox at 4 °C. Ovulation was induced with CPE by an initial injection of 0.5 mg kg^{-1} body weight and a second injection of 4.5 mg kg^{-1} body weight 12 h after the first injection. Eggs were collected 18 to 20 h after the second injection (Gela et al., 2008). Pooled eggs from the five females were used for experiments; eggs from these females were collected within 30 min.

2.2. Quantification of sperm density and motility

Prior to analysing sperm concentration, sperm was diluted 1000 times with an immobilizing solution (20 mM Tris, 10 mM KCl, pH 8). A 10 μL sample of diluted sperm was placed onto a Burker cell haemocytometer, and sperm were counted at 200× magnification (Olympus, BX41, Tokyo, Japan). The number of sperm were counted in 20 squares (depth 0.1 mm × length 0.2 mm) of the Burker cell haemocytometer (Meopta, Czech Republic).

To trigger sperm motility, Tris–HCl buffer (10 mM, pH 8.0) was used as the activation medium. Sperm were diluted in this activation solution with a dilution rate of 1:1000. Motility of sperm was recorded within 10–15 s after adding the activating solution using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (Olympus BX50, 200) and illuminated with a stroboscopic lamp (Chadwick-Helmut, 9630, USA). Video records were analyzed to estimate the percent of motile cells (motility rate) by micro-image analyzer (Olympus Micro Image 4.0.1. for Windows, Japan) on five successive overlapping video frames (Alavi et al., 2011). Triplicate samples were measured for each male.

2.3. Effects of pre-incubation time and sperm to egg ratio on fertilization success

Eggs and sperm from individual fish were pooled for this study. Mean (±SD) sperm motility for the six males was 63.33 ± 12.11%, while mean sperm density was $0.43 \pm 0.03 \times 10^9$ sperm per mL. Eggs (2 g) were first placed in 48 × 50 mL plastic dishes. The plastic dishes were then placed on a shaker, with a constant rotation of 200 min^{-1} for 3 min, and then 8 mL of fresh water from the hatchery was added to each dish. The eggs remained in the fresh water for 0 min (no incubation to serve as the experimental control), 0.5 min, 1 min, and 10 min before sperm was added for fertilization; there were 12 plastic dishes for each allotted pre-incubation period. Immediately following pre-incubation, sperm were added to the eggs using a micropipette at sperm to egg ratios of 430,000:1, 43,000:1,

4300:1, or 430:1. For the 4300:1 and 430:1 sperm to egg ratios, milt was diluted with seminal plasma at 1:10 and 1:100, respectively. Seminal plasma was obtained by centrifugation at 400 × *g* for 6 min at 20 °C. Within each pre-incubation time there were three replicate dishes for each sperm to egg ratio. For the experimental control, with no pre-incubation, the fresh water and sperm were added simultaneously. Following fertilization, the eggs were transferred to plastic Petri dishes for incubation at 20 °C. At 3 h post-fertilization, first cleavage development was observed. Polyspermic eggs are usually divided into an excessive number of blastomeres from the second cleavage division. This unusual cleavage pattern of polyspermic eggs can be easily distinguished from the normal monospermic eggs. Therefore, only eggs that had developed >32 cells after 4 h incubation were used for analysis. 110 to 130 eggs were observed for each replicate.

2.4. Fixation and preparation of unfertilized eggs for transmission electron microscopy

To observe pre-incubated eggs by transmission electron microscopy (TEM), 10 to 12 eggs pre-incubated in fresh water for 0 min, 0.5 min, 1 min, and 10 min were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and stored for 2 weeks at 4 °C. The samples were post-fixed in osmium tetroxide for 2 h at 4 °C and dehydrated in a graded concentration (30, 50, 70, 90, 95, and 100%) of acetone for 15 min. Samples for TEM were embedded in resin (Polybed 812; Polysciences, Inc.). A series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Microsystems), counterstained with uranyl acetate and lead citrate, and examined in a TEM JEOL 1010 (JEOL Ltd., Tokyo, Japan). The size of the cortical granules was measured using ImageJ v 1.48 software. Three eggs from each pre-incubation treatment were then observed using TEM.

2.5. Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). If data violated ANOVA assumptions fertilization success was arcsin square-root transformed. Alpha was set at 0.05 for main effects and interactions. A-posteriori analyses were performed using Tukey's multiple comparisons procedure.

Fertilization success was analyzed using a two-way ANOVA model (PROC GLM; SAS Institute, 2003) containing the pre-incubation time (0 min, 0.5 min, 1 min, and 10 min) and sperm to egg ratio main effects (430,000:1, 43,000:1, 4300:1, and 430:1 sperm:egg) as well as the pre-incubation time × sperm to egg ratio interaction term. When a significant pre-incubation time × sperm to egg ratio interaction was detected the saturated model was decomposed into a series of one-way ANOVA models following Keppel (1991). These decomposed one-way ANOVA models were run to determine the effect of pre-incubation time for each sperm to egg ratio and of sperm to egg ratio for each pre-incubation time. The reduced models involved only pre-planned comparisons and did not include repeated use of the same data, so alpha-level corrections for a posteriori comparisons were not necessary.

3. Results

3.1. Effects of pre-incubation time and sperm to egg ratio on fertilization success

The saturated two-way ANOVA model revealed a significant pre-incubation time × sperm to egg ratio interaction ($P = 0.002$); therefore, the model was reran into a series of one-way ANOVAs to determine the effect of pre-incubation time for each sperm to egg ratio and of sperm to egg ratio for each pre-incubation time.

When the saturated model was decomposed to determine the effect of pre-incubation time for each sperm to egg ratio fertilization success ranged from 67.3 to 84.2% at 430,000 sperm to egg, 67.4 to 72.3% at 43,000 sperm to egg, 45.2% to 62.3% at 4300 sperm to egg, and 29.7 to 57.0% at 430 sperm to egg (Fig. 1A). Pre-incubation time had no effect on fertilization success at the 430,000:1 ($P = 0.101$) and 43,000:1 ($P = 0.773$) sperm to egg ratios. On the contrary, pre-incubation time was significant at the 4300:1 sperm to egg ratio, such that fertilization success followed a dome-shaped function across the pre-incubation time gradient ($P = 0.006$). Additionally, pre-incubation time had a significant effect at the 430:1 sperm to egg ratio, such that the 0.5 min incubation time generated more fertilized eggs then the 10 min incubation time ($P = 0.045$).

When the saturated model was decomposed to determine the effect of sperm to egg ratio for each pre-incubation time a significant effect was detected at all pre-incubation times; 0 min ($P = 0.003$), 0.5 min ($P = 0.047$), 1 min ($P = 0.003$), and 10 min ($P < 0.001$) (Fig. 1B). More specifically, at the 0 min pre-incubation time (experimental control) the proportion of fertilized eggs increased at the 430,000:1 sperm to egg ratio, while at the 1 min pre-incubation time fertilization increased at the 4300:1 sperm to egg ratio. At the 10 min pre-incubation time, fertilization success increased at the 43,000:1 sperm to egg ratio. Moreover, at the 0.5 min pre-incubation time, the 43,000:1 sperm to egg ratio generated more fertilized eggs than the 430:1 sperm to egg ratio. In general, the 430:1 sperm to egg ratio produced the least amount of fertilized eggs.

3.2. Fixation and preparation of unfertilized eggs for transmission electron microscopy

There were no significant differences in cortical granules or in pigmented granules among the four incubation periods ($P > 0.05$). Several cytoplasmic protrusions and microvilli were observed between zona radiata interna (ZRI) and oolemma after 1 and 10 min pre-incubation but no exocytosis of cortical granules or perivitelline space were observed (Fig. 2). The microvilli are retracting in the space between oolemma and ZRI. The absence of a perivitelline space indicated that pre-incubation in water for up to 10 min did not initiate a cortical reaction before insemination. During pre-incubation the thickness of ZRI was

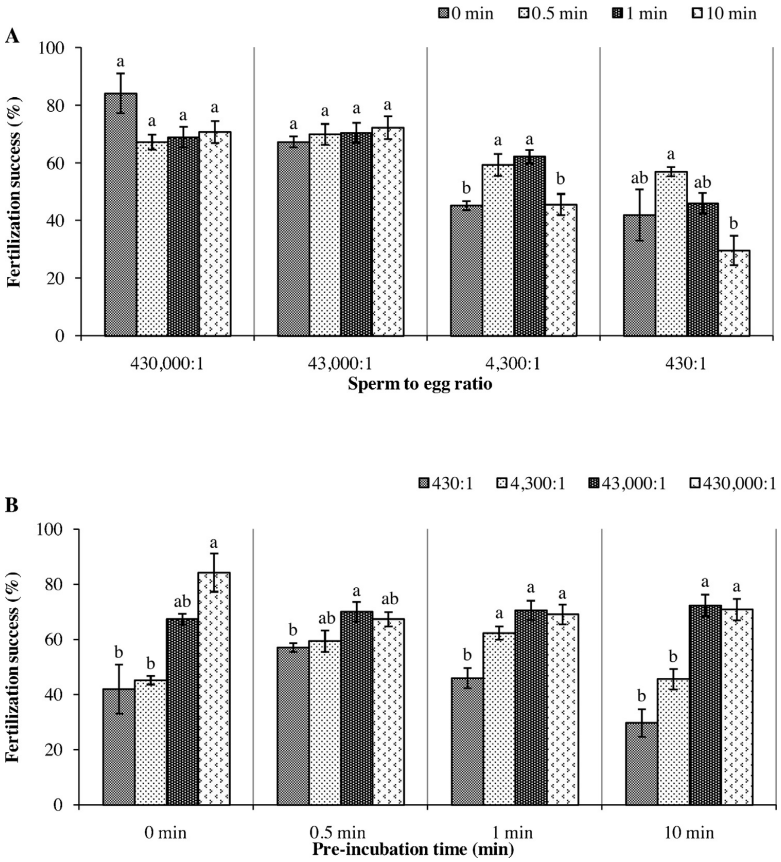

Fig. 1. (A) Effect of pre-incubation time (0 min, 0.5 min, 1 min and 10 min) on fertilization rate of sterlet *Acipenser ruthenus* eggs for each sperm to egg ratio. Treatments without a common superscript differ significantly ($P < 0.05$). Error bars represent least square means standard error. (B) Effect of sperm to egg ratio (430,000:1, 43,000:1, 4300:1 and 430:1) on fertilization rate of sterlet *Acipenser ruthenus* eggs for each pre-incubation time. Treatments without a common superscript differ significantly ($P < 0.05$). Error bars represent least square means standard error.

slightly increased (5 to 10%), but no significant changes were seen in mitochondria and lipid droplets.

4. Discussion

In fish the number of sperm required to fertilize a single ovum is high and species dependent (reviewed in Butts et al., 2012), such that the "optimal" number of sperm required to fertilize an egg depends on the quantity and composition of the activation solution as well as gamete quality at the time of stripping (among others factors). The sperm of sturgeon *sp*. is significantly different from that of other freshwater fish in that they have a longer duration of sperm motility (Dettlaff et al., 1993; Alavi and Cosson, 2005), possess an acrosome (Cherr and Clark, 1984b; Dettlaff et al., 1993; Ciereszko and Dabrowski, 1994; Psenicka et al., 2010), and have low osmolality seminal plasma (Gallis et al., 1991; Li et al., 2011).

In this study, a relatively high fertilization rate ($84.24 \pm 6.94\%$) was detected with the 430,000 sperm per egg ratio. Additionally, even at the lowest sperm to egg ratios (i.e. 430 sperm per egg) we still obtained a noticeable proportion of fertilized eggs ($42.01 \pm 8.83\%$). This phenomenon may be related to the number of micropyles in sturgeon eggs. For instance, the number of micropyles in sterlet eggs varies between 5 and 13 (reviewed in Siddique et al., 2014a). Although, the presence of numerous micropyles on the chorion surface could increase the chances of polyspermy (Siddique et al., 2014a), there seems to be a trade-off with eggs having a higher probability to be fertilized at lower sperm to egg ratios in comparison to fishes having only one micropyle (reviewed in Butts et al., 2012). Previous studies have also shown that

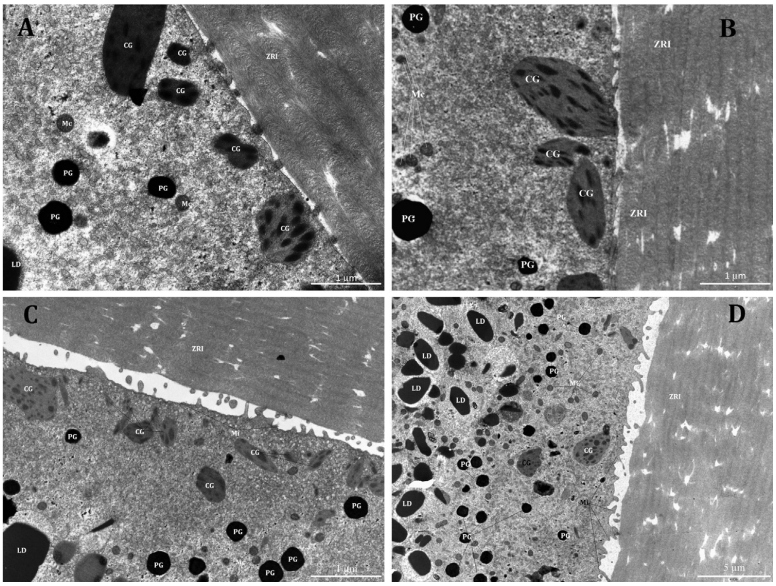

Fig. 2. Transmission electron micrographs of activated eggs (animal pole) of sterlet *Acipenser ruthenus* at various pre-incubation times. (A) 0 min of pre-incubation; (B) 0.5 min of pre-incubation; (C) 1 min of pre-incubation; (D) 10 min of pre-incubation; CG—cortical granules; PG—pigmented granules; Mi—microvilli; LD—lipid droplet; Mc—mitochondria; ZRI—zona radiata interna.

sturgeon sperm become motile at a low dilution rate of sperm (Gallis et al., 1991; Linhart et al., 1995; Toth et al., 1997). The duration and intensity of sperm motility in *Acipenser baerii* increased when the dilution rate was increased from 1:6 to 1:100 (Gallis et al., 1991). Therefore, sperm dilution is a major factor in the maintenance of fertilizing ability of diluted fish sperm (Billard, 1983).

Osmolality of sterlet seminal plasma has been reported as mean (±SD) of 69.7 ± 1.8 mOsmol kg^{-1} (fish from the Danube) and 79.1 ± 4.8 mOsmol kg^{-1} (fish from the Odra) (Piros et al., 2002). In captive condition, osmolality of sterlet seminal plasma was reported at 50.7 to 79.1 mOsmol kg^{-1} (Li et al., 2011). Coelomic fluid of sterlet was found as mean (±SD) of 192.17 ± 10.51 mOsmol kg^{-1} (Siddique et al., Unpublished data). Therefore, osmolality of ovarian fluid of sterlet is higher than that of the seminal plasma. Spermatozoa are inactive in the seminal plasma, so they need an activation medium to be activated before fertilization. In artificial fertilization, sperm is activated in water and added to the eggs within seconds (Ginzburg, 1972). Cherr and Clark (1984a) reported that ovarian fluid inhibited the motility of white sturgeon *A. transmontanus* sperm. Ovarian fluid acts to strongly agglutinate sperm, inhibiting their movement toward the micropyles. It is inferred that the agglutination properties of ovarian fluid may be due to compounds in the ovarian fluid or to proteins released from damaged eggs (Lahnsteiner et al., 2004).

Results from our experiments indicated a significant interaction between pre-incubation time and sperm to egg ratio. In particular, pre-incubation time had a significant effect on fertilization success at the low sperm to egg ratios (i.e. 4300:1 and 430:1 sperm to egg ratio), while the sperm to egg ratio effect was significant at all pre-incubation times. In the present study, pre-incubation of eggs prior to fertilization partially removed the ovarian fluid and helped to decrease the osmolality of the activation solution. Feledi et al. (2011) observed that for eggs of Siberian sturgeon *A. baerii*, rinsing the eggs in fresh water for 15 s prior to fertilization improved fertilization rates. Our current results concur with those reported by Van Eenennaam et al. (2008), where the authors reported that removal of ovarian fluid from the eggs by rinsing in fresh water enhances the fertilization rate of green sturgeon *A. medirostris*. Sturgeon eggs can be fertilized after many hours in water, but their fertilization ability decreases after 1 h in Russian sturgeon *A. gueldenstaedtii* and in less time in stellate sturgeon *A. stellatus* (Dettlaff et al., 1993).

Under hatchery conditions, one of the most important factors that should be taken into account for pre-incubation of eggs in fresh water is the adhesive properties of sturgeon eggs. Eggs of sterlet are strongly adhesive (reviewed in Siddique et al., 2014b). Sturgeon eggs develop adhesiveness within a few min of contact with fresh water and strongly adhere to one another, forming a solid mass that interferes with gas exchange and imposes oxidative stress on the eggs (Siddique et al., 2014b). Therefore, pre-incubation of sturgeon eggs for >3 to 4 min is not recommended as it might sharply decline the fertilization rate (Siddique et al., 2014b), especially when a large volume of eggs are used. Although, a small amount of eggs (2 g) was used for each

treatment we observed that the 10 min pre-incubation time of sterlet eggs significantly decreased the fertilization rate for 4300:1 and 430:1 sperm to egg ratio.

Micrographs from TEM confirmed that there were no substantial changes in the cortical and pigmented granules and there were no indications of initiation of cortical reactions or forming of perivitelline space in the pre-incubated eggs. Compared to the structure of the cortical layer in mature unfertilized eggs of stellate sturgeon, our pre-incubated eggs of sterlet were similar (Dettlaff et al., 1993). Dettlaff et al. (1993) found that exocytosis of cortical granules initiated 3 s after insemination and after 180 s the contents of the cortical granules discharge from the cytoplasm to merge in a single layer in the fertilized egg. In our study, secreted contents of the cortical granules were not found in the cortical layer of pre-incubated eggs. Together, these results suggest that sturgeon eggs can be pre-incubated in water up to 10 min without initiating any exocytosis of cortical granules in the eggs.

In conclusion, our results indicate that higher sperm to egg ratios 430,000:1 and 43,000:1 are better for improving fertilization success of sterlet. The interaction between pre-incubation time and sperm to egg ratio was highly significant, suggesting that the optimal sperm to egg ratio depends on pre-incubation time. Moreover, with a low quantity of sperm 30 s to 1 min pre-incubation of eggs in fresh water prior to fertilization can enhance the fertilization rate and control the polyspermy.

Conflict of interest statement

None declared.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic—projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (LO1205 under the NPU I program), GAJU 134/2013/Z, and GACR P502/13/26952S.

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CHAPTER 4

FERTILIZATION STRATEGIES FOR SEA BASS *DICENTRARCHUS LABRAX* (LINNAEUS, 1758): EFFECTS OF PRE-INCUBATION AND DURATION OF EGG RECEPTIVITY IN SEAWATER

Siddique, M.A.M., Butts, I.A.E., Linhart, O., Macias, A.D., Fauvel, C., 2015. Fertilization strategies for Sea Bass *Dicentrarchus labrax* (Linnaeus, 1758): Effects of pre-incubation and duration of egg receptivity in seawater. Aquaculture Research, early view, doi: 10.1111/are.12887).

It was allowed by publishers on 13th March, 2016 to include the paper in this Ph.D. thesis.

Aquaculture Research

Aquaculture Research, 2015, 1–9 doi: 10.1111/are.12887

Fertilization strategies for Sea Bass *Dicentrarchus labrax* (Linnaeus, 1758): effects of pre-incubation and duration of egg receptivity in seawater

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Abstract

Studying gamete biology can provide important information about a species fertilization strategy as well as their reproductive ecology. Currently, there is a lack of knowledge about how long sea bass *Dicentrarchus labrax* eggs can remain viable after being activated in seawater. The objectives of this study were to understand the effects of pre-incubation of fresh and overripe sea bass eggs in seawater and to determine the duration of egg receptivity. Pooled eggs (fresh and overripe) from four females were pre-incubated in seawater for 0 min (control), 0.5 min, 1 min, 3 min, 10 min and 30 min and then fertilized by pooled sperm from four males. The fresh eggs had a higher fertilization success than overripe eggs. Our results revealed a significant effect of pre-incubation time for both the fresh ($P < 0.01$) and overripe eggs ($P < 0.01$). Fertilization success of eggs significantly declined for both these treatments after 3 min of pre-incubation, which clearly indicates that sea bass eggs are able to be fertilized by sperm for up to 3 min after release into seawater. This study has particular importance for understanding fertilization strategies, reproductive potential, as well as reproductive ecology of sea bass.

Keywords: egg longevity, overripe eggs, reproductive ecology, Mediterranean sea bass, *Dicentrarchus labrax*

Introduction

Marine and freshwater teleosts use different reproductive strategies to adapt with diverse aquatic habitats. Mature eggs of most teleost fish are enclosed in an acellular multilayered egg envelope (Dumont & Brummet 1980; Yamagami, Hamazaki, Yasumasu, Masuda & Iuchi 1992; Scapigliati, Carcupino, Taddei & Mazzini 1994; Baldacci, Taddei, Mazzini, Fausto, Buonocore & Scapigliati 2001). The morphology of an egg depends on species and reflects adaptations to different ecological conditions (Fausto, Picchietti, Taddei, Zeni, Scapigliati, Mazzini & Abelli 2004). The main functions of the egg envelope are to fixate deposited eggs to substratum (for demersal eggs), sperm chemo-attraction, prevent polyspermy, and antibacterial and mechanical protection (Hart 1990; Zelazowska 2010; Siddique, Cosson, Psenicka & Linhart 2014). The egg envelope also enables gas exchange, as it aids in the excretion and transport of nutrients from the external environment for developing embryos (Riehl 1999).

The duration of egg receptivity of marine and freshwater fish is species specific and is closely related to different water flow regimes on the spawning ground (Mann 1996; Merz, Setka, Pasternack & Weathon 2004; Probst, Stoll, Hofmann, Fisher & Eckmann 2009). Sperm longevity, the velocity of sperm and the duration of egg

receptivity impact the success of a fertilization event (see Trippel & Morgan 1994; Butts, Trippel & Litvak 2009 among others). For instance, if sperm are viable for longer periods of time in the activation medium, then the potential of contacting and fertilizing an egg increases (Butts *et al.* 2009). On the other hand, when longevity of sperm is very short, than egg receptivity may be necessary to increase fertility, since longer periods of egg receptivity are predicted to increase the probability of a successful fertilization event (Trippel 2003). Therefore, duration of egg receptivity provides valuable insights on reproductive behaviour for any fish species (Butts, Roustaian & Litvak 2012).

The activation process for fish eggs represents several complex changes including the release of the developmental block of meiosis at metaphase (II), consecutive breakdown of cortical granules and formation of the perivitelline space (Pavlov, Emel'yanova & Novikov 2009). Egg activation is induced by the fusion of sperm in the majority of marine fish, while in freshwater fish and salmonids it is induced by contact with water or mechanical stimulation (Dettlaff 1962; Ginzburg 1972; Pavlov *et al.* 2009). In general, the influx of intracellular free Ca^{2+} in eggs mediates the cortical alveoli to initiate exocytosis (Finn 2007; Vasilev, Chun, Gragnaniello, Garante & Santella 2012). Following this Ca^{2+} wave, cortical glycoproteins are then broken into smaller units by proteolysis and form the osmotic gradient that facilitates uptake of ambient seawater across the egg membrane (Lønning & Davenport 1980; Govoni & Forward 2008). The perivitelline space between the oocyte plasma membrane and egg envelopes fills with perivitelline fluid, which is formed by imbibed water and the substances released from the cortical granules (Siddique *et al.* 2014). Until now, there is a knowledge gap on activation mechanisms and formation of perivitelline space of sea bass eggs.

The European sea bass *Dicentrarchus labrax* (L.) is a leading species for aquaculture in the Mediterranean due to its emerging economic importance in the Mediterranean and North East Atlantic regions (Vandeputte, Dupont-Nivet, Haffray, Chavanne, Cenadelli, Parati, Vidal, Vergnet & Chatain 2009; Colléter, Penman, Lallement, Fauvel, Hanebrekke, Osvik, Eilertsen, D'Cotta, Chatain & Peruzzi 2014). Adults usually exhibit demersal behaviour and inhabit coastal waters down to 100 m depth but are more common in the littoral zone on various kinds of bottoms in estuaries, lagoons and occasionally in rivers. In the Mediterranean, first sexual maturity occurs at 2–4 years and fish spawn once a year in groups (Froese & Pauly 2015). The egg envelope of sea bass consists of three distinct layers with a funnel shaped micropylar canal (Fausto, Carcupino, Scapigliati, Taddei & Mazzini 1994; Scapigliati *et al.* 1994). Sea bass eggs are pelagic, small in size (1.1–1.5 mm diameter), freely floating in seawater and fertilized externally (Froese & Pauly 2015). In teleost fish, eggs can be activated by contact with water or by the penetration of sperm (Pavlov *et al.* 2009). In case of sea bass, eggs can be activated in seawater and after a few seconds of activation, a thin perivitelline space is formed beneath the egg membrane. Currently, there is lack of knowledge about how long sea bass eggs can remain viable after being activated in seawater within the natural environment.

In controlled reproduction, the major problem encountered for this species is over ripening of eggs. After ovulation, sea bass eggs over ripen very quickly and they can even start to over-ripen in the ovary before stripping. Very little is known about the fertilization ability of overripe eggs of sea bass. However, knowledge about gamete biology, longevity of eggs and effects of pre-incubation is crucial for standardization of fertilization protocol for any fish species. Here, we conducted a laboratory experiment to determine the duration of egg receptivity in seawater and effect of pre-incubation of sea bass eggs (fresh and overripe) on fertilization success.

Materials and methods

Broodstock husbandry and gamete collection

Sea bass broodstock (aged 4–6 years and weighted 2–5 kg) were kept at the Ifremer Experimental Aquaculture Station (Palavas-les-Flots, France). Males and females were kept separately in recirculation systems (8 m^3 volume). Mature males were recognized by gentle abdominal pressure and females were selected by assessing the maturation stage with ovarian biopsies. Maturation stages were determined based on oocyte diameter and migration of the germinal vesicle using a light microscope (4× magnification). Females at 'stage B' of development (when the germinal vesicle started its migration to the animal pole) were

selected for hormonal induction (Fauvel & Suquet 1988). Each female received a single dose (10 μg kg^{-1} body weight mixed with physiological solution) of Luteinizing Hormone Releasing Hormone analogue (LHRHa; Sigma-Aldrich, Lyon, France) in order to induce final maturation and ovulation (Fauvel & Suquet 1988). The treated females were isolated in individual tanks (1.5 m^3, 17 L h^{-1} water renewal, and low air flow) at 13°C water temperature. Ovulated oocytes were collected from females after 72 h of hormonal stimulation by abdominal pressure. Sperm were collected from the male's genital papilla by applying pressure to the abdominal region. Samples were drawn into 5 ml syringes. Before collection, the genital papilla was wiped dry and extra care was taken to avoid contamination with urine. Sperm were then held at 4°C until use. The males and females both were treated without anaesthetics and immediately wrapped in a dark wet towel to limit stress and fish movement during sperm and oocyte collection.

Quantification of sperm density and motility

Sperm concentration was measured before pooling using a Thoma hemocytometer (depth 0.1 mm × length 0.05 mm) after dilution of sperm by 1:2000 in distilled water. After allowing 10 min for sedimentation of the sperm, three frames of 24 squares were recorded randomly through a video camera (Axiolab, Zeiss + SSC-D50AP video camera; Sony Corporations, NY, USA). The cells were then automatically counted using image analysis software (Image J; National Institutes of Health (NIH), Bethesda, MD, USA). Sperm motility was measured according to the procedure by Fauvel, Boryshpolets, Cosson, Leedy, Labbe, Haffray and Suquet (2012).

We determined the effects of pre-incubation time for fresh and overripe sea bass eggs in seawater as well as the duration of egg receptivity. For this experiment we used proportionally pooled sperm from four males. The mean (±SD) sperm density of pooled sperm was $5.3 \pm 0.03 \times 10^9$ sperm mL^{-1}, while sperm motility was 60 ± 8.16%, and mean sperm longevity was 42 ± 6 s.

Effects of pre-incubation time on fertilization and determination of egg receptivity period

Pooled eggs from four females were used for this experiment. The difference between egg collection from the first female to fourth female was 20 min. Freshly stripped eggs (10 mL) were first placed in 18 × 250 mL plastic dishes. Then 5 mL of seawater (pH 8.22, salinity 37.4 psu) was added to each dish. The eggs were pre-incubated in seawater for 0 min (no incubation to serve as the experimental control), 0.5 min, 1 min, 3 min, 10 min and 30 min before sperm was added for fertilization. There were three plastic dishes for each allotted pre-incubation period for replication. Immediately following pre-incubation, 5 mL of sperm were added to the eggs using a micropipette and the plastic dishes were rotated by hand for 30 s to facilitate fertilization. For the experimental control, with no pre-incubation, the seawater and sperm were added simultaneously.

Following fertilization, 150–200 mL of additional seawater was added to each plastic dish and kept at 13°C for incubation. For the overripe eggs (1 h of storage at 13°C), these eggs (10 mL) were placed in 18 × 250 mL plastic dishes and the same procedure was applied as for the freshly stripped eggs (see above).

At 3 h post-fertilization, cleavage was observed. After removing water from the plastic dishes, eggs were mixed properly with a plastic spoon and 300 eggs were randomly chosen for observation. Only eggs that had developed to 2–4 cells after 3 h incubation were used for analysis. Additionally, we took pictures for the unfertilized eggs under light microscope at 0, 60, 100, 220, 300, 450 and 500 s post activation by water to observe the swelling process and formation of perivitelline space.

Statistical analyses

All data were analysed using SAS statistical analysis software (SAS Institute, 2003). Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals versus predicted values). If data violated ANOVA assumptions fertilization success was arcsin square-root transformed. Alpha was set at 0.05 for main effects and interactions. A-posteriori analyses were performed using Tukey's multiple comparisons procedure.

Fertilization success was analysed using a two-way repeated measures ANOVA model containing the pre-incubation time (0 min, 0.5 min, 1 min, 3 min, 10 min and 30 min; fixed repeated factor) and egg status (fresh and overripe; fixed factor) main effects as well as the pre-incubation

time × egg status interaction term. When a significant pre-incubation time × egg status interaction was detected the saturated model was decomposed into a series of lower-order statistical models following Keppel (1991). Here, the decomposed ANOVA models were run to (i) determine the effect of pre-incubation time for each egg status category using a series of one-way repeated measures ANOVA models and (ii) determine the effect of egg status category for each pre-incubation time using a series of *t*-tests. These reduced models involved only pre-planned comparisons, so alpha-level corrections for *a posteriori* comparisons were not necessary.

Results

The saturated two-way repeated measures ANOVA model revealed a significant pre-incubation time × egg status interaction term ($P < 0.05$); therefore, the model was decomposed into a series of lower-order statistical models. When the saturated model was decomposed to determine the effect of pre-incubation time for each egg status category a significant pre-incubation time effect was detected for both the fresh ($P < 0.01$; Fig. 1a) and overripe eggs ($P < 0.01$; Fig. 1b), such that egg fertilization success significantly declined for both these treatments after 3 min of pre-incubation. Together, this means that sea bass eggs have the capability to be fertilized within 3 min post-activation. After that window of receptivity the eggs lose their ability to be fertilized. Moreover, when the saturated model was decomposed to determine the effect of egg status for each pre-incubation time a significant egg status effect was detected at the 0.5 min ($P < 0.01$; Fig. 1d), 1 min ($P < 0.05$;

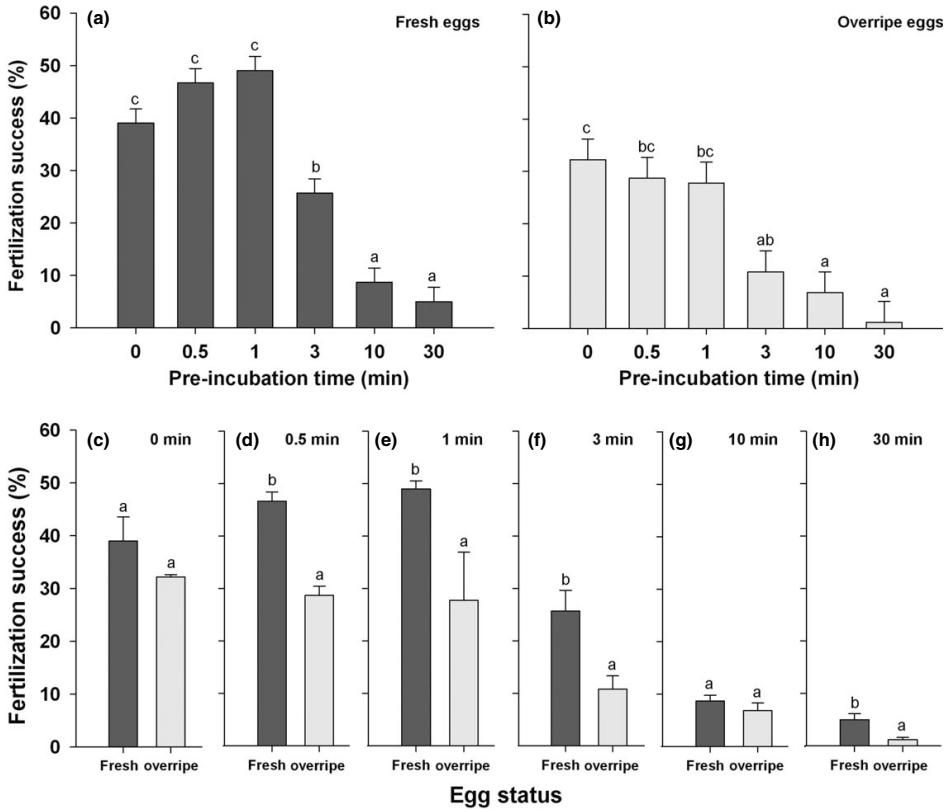

Figure 1 Effects of pre-incubation time (0 min, 0.5 min, 1 min, 3 min, 10 min and 30 min) on fertilization rate of (a) fresh eggs and (b) overripe eggs of sea bass *Dicentrarchus labrax*; and effects of egg status on fertilization rate for (c) 0 min, (d) 0.5 min, (e) 1 min, (f) 3 min, (g) 10 min and (h) 30 min pre-incubation time.

Fig. 1e), 3 min ($P < 0.05$; Fig. 1f), and 30 min pre-incubation times ($P < 0.05$; Fig. 1h); here, the fresh eggs had a higher fertilization success than overripe eggs. This means that fertilization ability is decreased in overripe eggs; therefore, short-term storage of sea bass eggs is not feasible due to their fast over ripening process. On the contrary, no significant effect was detected between the fresh and overripe eggs at the 0 min ($P > 0.05$; Fig. 1c) and 10 min pre-incubation times ($P > 0.05$; Fig. 1g).

Sea bass eggs showed a very rapid swelling process after releasing into seawater (Fig. 2a and b). During activation of eggs in seawater, a small perivitelline space was observed in several eggs across the females. After 60 s of activation, the perivitelline space became clearly visible (Fig. 2c) and there were no substantial changes in perivitelline space after 100 s (Fig. 2d) and 220 s (Fig. 2e) of activation. Then, the perivitelline space became large after 300 s (Fig. 2f) to 450 and 500 s (Fig. 2g and h) of activation.

Discussion

Egg activation is a key process in early embryonic development of fish, but not fully understood (Webb & Miller 2013). Fish eggs are activated upon contact with water and this activation mechanism is initiated by the release of intracellular stored Ca^{2+} in the egg cytosol (Coward, Bromage,

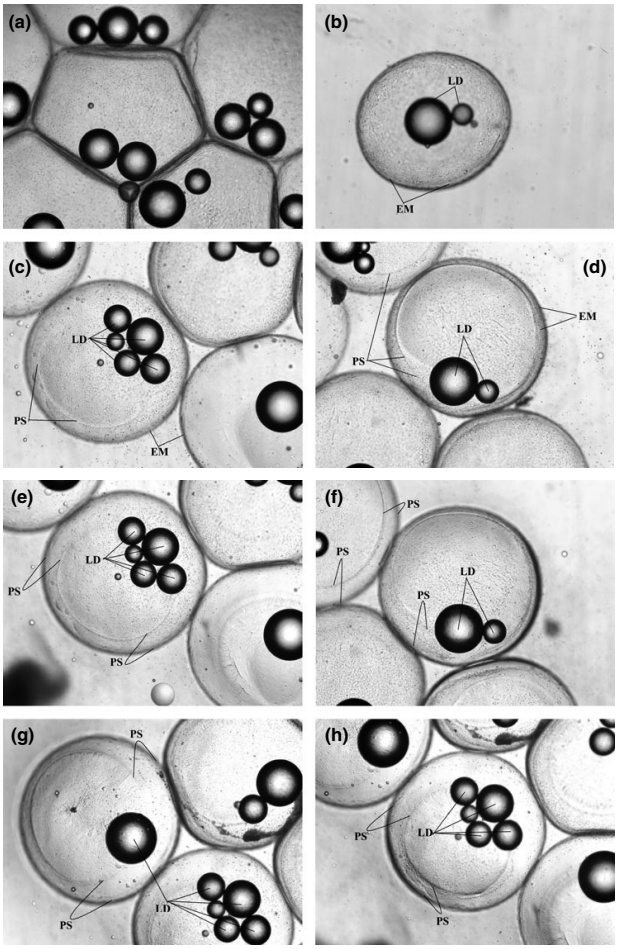

Figure 2 Micrograph of sea bass *Dicentrarchus labrax* eggs; ovulated, non-activated eggs (a); unfertilized eggs 20 s after activation in seawater (b); unfertilized eggs developing perivitelline space at 60 s (c), 100 s (d), 220 s (e), 300 s (f), 450 s (g), and 500 s (h) after activation in seawater. PS, perivitelline space; LD, lipid droplet; EM, egg membrane. Micrographs were taken at 4× magnification.

Hibbitt & Parrington 2002; Finn 2007), which resulted in formation of the fertilization membrane (Minin & Ozerova 2008). As seen in our study, sea bass eggs swell very rapidly (within 10–20 s) upon activation by contact with seawater. However, when in contact with seawater the egg only takes a few seconds to achieve flexible size and structure, and to acquire fertilization ability. In this study, mean fertilization rate of fresh eggs slightly increased for 30 s and 1 min of pre-incubation in seawater, but it was not statistically significant. In our decomposed statistical models, a significant pre-incubation time effect was detected for both the fresh and overripe eggs, where a significant decline of egg fertilization rate was observed for both these treatments after 3 min of pre-incubation. This means that sea bass eggs have the capability to be fertilized within 3 min after activation in seawater; after that period the eggs lose their fertilization ability. Moreover, in the decomposed model, fertilization success of the fresh eggs was significantly higher than overripe eggs at the 0.5 min, 1 min, 3 min and 30 min pre-incubation times. Thus, our results reveal that the fertilization ability of overripe eggs are less than the fresh eggs, but they are still capable of being fertilized for up to 3 min after being released into seawater.

We determined the egg receptivity period of sea bass for the first time. Pre-incubation of eggs in water prior to fertilization is the simplest way to determine the egg receptivity period. Although, sea bass is a marine species, the duration of egg receptivity of sea bass is close to many freshwater species like Rainbow trout *Oncorhynchus mykiss*, crucian carp *Carassius carassius* and European perch *Perca fluviatilis* (see Table 1). The spermatozoa longevity of sea bass is also very short (less than 1 min). Fish, which have short periods of sperm longevity and egg receptivity, show different fertilization strategies. In this case, male and females release their gametes at the same time or males release their milt on the eggs to facilitate the fertilization process. For sea bass, we showed that the eggs were receptive to be fertilized for 3 min. Compared with other marine species like winter flounder *Pseudopleuronectes americanus* and Atlantic cod *Gadus morhua*, the duration of egg receptivity of sea bass eggs is much shorter (Table 1).

For unfertilized sea bass eggs, formation of the perivitelline space initiates within 30 s after activation in seawater, but not for all eggs. Formation of the perivitelline space upon activation in seawater is a common feature for many marine fish species; including European eel *Anguilla anguilla* and Japanese eel *Anguilla japonica* (Govoni & Forward 2008; Sørensen, Butts, Munk & Tomkiewicz 2015). In acipenserids, the perivitelline space is only formed after fertilization of eggs by sperm (Dettlaff, Ginsburg & Schmalhausen 1993; Linhart & Kudo 1997; Siddique *et al.* 2014). Generally when eggs are fertilized by sperm, the cortical reaction and formation of perivitelline space is faster (Iwamatsu & Ito 1986). In sea bass eggs, when they are released in seawater, this process starts within a few seconds after activation without sperm but takes several minutes to complete. At the initial stage of forming the perivitelline space, eggs are capable to be fertilized by sperm, but in the later space when the perivitelline space become larger, the perivitelline fluid blocks the micropylar canal or sperm entry site. This is the mechanism of polyspermy block for acipenserids (Linhart & Kudo 1997; Siddique *et al.* 2014).

For sea bass eggs, it is difficult to control post-ovulatory ageing. Therefore, hand stripping is needed to understand ovulatory rhythms in females and to minimize the impact of over-ripening. Following ovulation, sea bass oocytes remain viable during a short window before they undergo a natural breakdown process. We observed that the over ripening period of sea bass is <1 h which is similar to striped bass *Morone saxatilis* (<1 h; Stevens 1966) but two-fold higher than white bass *Morone chrysops* (15–30 min; Mylonas, Magnus, Gissis, Klebanov & Zohar 1996). The fertilization ability of over-ripe oocytes sharply declines and totally depends on the storage temperature and the time interval between ovulation and stripping. All oocytes in an ovary are not ovulated at the same time; therefore, the percentage of over-ripe eggs in each individual is also important to consider. In our study, we obtained 32.5% fertilization rate from the overripe eggs (control group), which was not significantly different from the fresh eggs. This is only possible when eggs are collected immediately after ovulation.

In conclusion, the information provided here is pertinent to fisheries ecologists and also has implications for domestication and controlled reproduction of sea bass. Further studies to observe the changes of egg membranes and how long the micropyle remains open during activation in seawater are encouraged.

Table 1 Duration of egg receptivity and spawning habitat of marine and freshwater fish

Species	Duration of egg receptivity	Natural spawning habitat*	Reference
Silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844)	30–40 s	Rivers and tributaries	Mikodina and Makeyeva (1980)
Sockeye salmon *Oncorhynchus nerka* (Walbaum, 1792)	40 s	Stream	Hoysak and Liley (2001)
Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)	40 s	Lake and streams	Liley, Tamkee, Tsai and Hoysak (2002)
Crucian carp *Carassius carassius* (Linnaeus, 1758)	1 min	Shallow pond, lake and rivers	Żarski, Horváth, Bernáth, Palińska-Żarska, Krejszeff, Müller and Kucharczyk (2014)
Goldfish *Carassius auratus* (Linnaeus, 1758)	<1 min	Shallow water, river, and lakes	Hamano (1951)
European perch *Perca fluviatilis* (Linnaeus, 1758)	2.5 min	Lake and rivers	Żarski, Horvath, Kotrik, Targon′ska, Palin′ska, Krejszeff, Bokor, Urbanyi and Kucharczyk (2012)
Sea bass *Dicentrarchus labrax* (Linnaeus, 1758)	3 min	Sea	Present study
Vendace *Coregonus albula* (Linnaeus, 1758)	4 min	Lakes and shallow waters	Lindroth (1947)
Japanese rice fish *Oryzias latipes* (Temminck & Schlegel, 1846)	4 min	Pond, marsh, paddy field, and small streams	Yamamoto (1944)
Pond loach *Misgurnus anguillicaudatus* (Cantor, 1842)	5 min	Stream and pond	Gamo, Yamauchi and Suzuki (1960)
European weatherfish *Misgurnus fossilis* (Linnaeus, 1758)	10 min	Open water, lake, and streams	Minin and Ozerova (2008)
Mummichog *Fundulus heteroclitus* (Linnaeus, 1766)	10–30 min	Salt marsh and tidal creeks	Kagan (1935)
Chum salmon *Oncorhynchus keta* (Walbaaum, 1792)	15–30 min	River	Yamamoto (1951)
Winter flounder *Pseudopleuronectes americanus* (Walbaum, 1792)	32 min	Sea	Butts *et al.* (2012)
Atlantic cod *Gadus morhua* (Linnaeus, 1758)	2 h	Offshore water	Davenport, Lønning and Kjørsvik (1981)
Pontic shad *Alosa immaculate* (Bennett, 1835)	>2 h	Large river	Kryzhanovskii (1956)
Atlantic herring *Cluoea harengus* (Linnaeus, 1758)	4 h	Shallow coastal areas or offshore banks	Kryzhanovskii (1956)
Russian sturgeon *Acipenser gueldenstaedtii* (Brandt and Ratzeburg, 1833)	6 h	River	Ginzburg (1972)

*Data are retrieved from FishBase (Froese & Pauly 2015).

Acknowledgments

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (LO1205 under the NPU I program), the Grant Agency of the University of South Bohemia (No. 134/2013/Z) and the Grant Agency of the Czech Republic (No. 15-12034S). M.A.M. Siddique highly appreciated the grant from COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE) for his research stay at IFREMER, Station de Palavas, France. Technical help from Gilbert Dutto, Marie-Odile Vidal and Stéphane Lallement during experiment is highly appreciated.

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CHAPTER 5

EFFECTS OF PREINCUBATION OF EGGS AND ACTIVATION MEDIUM ON THE PERCENTAGE OF EYED EMBRYOS IN IDE (*LEUCISCUS IDUS*), AN EXTERNALLY FERTILIZING FISH

Siddique, M.A.M., Linhart, O., Krejszeff, S., Żarski, D., Król, J., Butts, I.A.E., 2016. Effects of preincubation of eggs and activation medium on the percentage of eyed embryos in ide (*Leuciscus idus*), an externally fertilizing fish. Theriogenology 85: 849–855.

Theriogenology 85 (2016) 849–855

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effects of preincubation of eggs and activation medium on the percentage of eyed embryos in ide (*Leuciscus idus*), an externally fertilizing fish

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ARTICLE INFO

Article history:
Received 21 May 2015
Received in revised form 25 October 2015
Accepted 26 October 2015

Keywords:
Sperm motility
Saline water
Woynarovich solution
Egg receptivity
Artificial reproduction
In vitro fertilization

ABSTRACT

Standardization of fertilization protocols is crucial for improving reproductive techniques for externally fertilizing fish in captive breeding. Therefore, the objectives of this study were to determine the effects of preincubation of eggs and activation medium on the percentage of eyed embryos for ide (*Leuciscus idus*). Pooled eggs from five females were preincubated in three different activating media for 0, 30, 60, 90, and 120 seconds and then fertilized by pooled sperm from five males. At the eyed-egg stage, the percentage of viable embryos was later calculated. Results showed that preincubation time was significant for the freshwater activation medium ($P < 0.001$), such that the percentage of eyed embryos declined across the preincubation time gradient. Additionally, there was an effect on the percentage of eyed embryos when eggs were incubated with Woynarovich solution ($P < 0.001$), such that a decline was detected at 90 seconds, whereas no effect was detected for the saline water medium. Activating medium had a significant effect on the percentage of eyed embryos for each preincubation time ($P < 0.05$). More precisely, freshwater produced the lowest percentage of eyed embryos at all preincubation times (ranged from 1.9% at 120 seconds to 43.6% at 0 seconds), whereas saline water and Woynarovich solution produced the highest percentage of eyed embryos at 0 seconds and 30 seconds before incubation. Woynarovich solution produced the highest percentage of eyed embryos at 60 seconds (65.26%), whereas saline water produced the highest percentage at 90 seconds (68.37%). No difference was detected between saline water and Woynarovich solution at 120 seconds. Examination of sperm traits showed no impact of activating medium on computer assisted sperm analysis parameters. Together, these results suggest that saline water or Woynarovich solution improve fertilization rate in ide during IVF; thus, these media are useful for standardizing fertilization protocols and controlled reproduction for this species.

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http://dx.doi.org/10.1016/j.theriogenology.2015.10.032

1. Introduction

The motility period of sperm and the duration of egg receptivity (defined as the length of time an egg is receptive to be fertilized by a sperm cell) can both impact natural and artificial fertilization environments [1–3]. The sperm motility period of freshwater fish species is shorter than marine species and typically persists for 1 to 2 minutes [4,5]. Additionally, the fertilization ability of eggs after activation is species-specific and is usually shorter in freshwater than in marine species. For instance, sockeye salmon (*Oncorhynchus nerka*) and rainbow trout (*Oncorhynchus mykiss*) eggs can be fertilized within the first 40 seconds after activation [6,7], crucian carp (*Carassius carassius*) within 60 seconds [8], and European perch (*Perca fluviatilis*) within 150 seconds [9]. On the contrary, eggs of marine fishes like winter flounder (*Pseudopleuronectes americanus*) and Atlantic cod (*Gadus morhua*) can be fertilized by a spermatozoon within 32 and 120 minutes, respectively [10,11]. During captive breeding, different activating media are often used to facilitate the gamete activation process; i.e. to increase fertilization success. Most of these activating media are designed to improve motility parameters of sperm, while few studies have been conducted to manipulate the duration of egg fertilizing ability [8,9,12]. A thorough investigation of egg receptivity is critical, for any given externally fertilizing fish species, because longer periods of receptivity are predicted to increase the probability of a successful fertilization event [13].

Ide (*Leuciscus idus* L.) is a rheophileous cyprinid widely distributed in Europe, especially in rivers, lower reaches, and lakes [14–16]. During the past decade, ide production has increased, especially in Eastern Europe [17]. For instance, from 2000 to 2002, Poland produced ~4.7 million summer fry and 27 tons of 1-year-old fish, comprising 69% and 91% of the total production of rheophilic cyprinids [17,18]. In recent years, reproduction of ide in captivity has received increasing attention to meet the demand of high-quality juveniles for restocking [17–19], sport fishing, and as an ornamental fish [14,15,20]. Ide is also considered a model species for toxicity studies [21,22], biomonitoring [23,24], as well as for development of reproductive protocols of riverine cyprinids [16,17,25].

Ide females spawn only once during the spawning season, which occurs from April to May [26,27]. This species spawn naturally in ponds, lakes, or rivers. In captivity, hormonal induction is necessary for obtaining high-quality gametes and viable offspring. The physiological temperature for this species, during the spawning season, ranges from 8 °C to 15 °C [28]. In their native environments, individual females will spawn with several males. These males are usually assembled on the spawning grounds and follow ripe females. Ide eggs are strongly adhesive and require 90 to 120 days for hatching [28]. Females attach their sticky eggs to gravel or a submerged plant material or their eggs may be found sticking to stones or weeds in shallow waters [29]. Previous studies have mainly focused on developing artificial spawning and larval rearing techniques [15]. Although an attempt to understand the role of hatchery water on the survivability of embryos has been undertaken [30], information regarding gamete biology and fertilization mechanisms of ide is limited.

During captive breeding, egg stickiness is a major problem when a large number of eggs are incubated [31]. Ide eggs become adhesive within seconds after activation in freshwater or in distilled water and make a solid mass or "bulk of eggs", which may compromise the chances of sperm reaching the egg micropyle. Thus, it is necessary to determine the best activating medium for these strongly adhesive eggs, which can facilitate gamete interactions during a fertilization event. Therefore, the objectives of this study were to understand the effects of preincubation of eggs (0, 30, 60, 90, and 120 seconds) and activation medium (freshwater, saline water, and Woynarovich solution) on the percentage of eyed embryos for ide. Additionally, sperm activity parameters were quantified to determine the effects of activating medium on male fertility.

2. Materials and methods

2.1. Broodstock husbandry and gamete collection

All experimental procedures involving animals were performed in accordance with National and institutional guidelines and were approved by the Local Ethical Committee, Olsztyn, Poland. Wild-caught ide broodstock (aged 1–3 years and weighted 700 g to 2 kg) were collected in the middle of April 2015 from a lake in Olsztyn, Poland. In total, 13 males and 12 females were kept separately in recirculation systems (1000 L volume) with controlled temperature (10 °C) and photoperiod (14 hours light and 10 hours dark) [32]. Mature males were recognized by releasing sperm by gentle abdominal pressure, and females were selected by assessing the maturation stage with ovarian biopsies according to Krejszeff et al. [17]. Males and females were stimulated with Ovopel (Interfish, Hungary) containing mammalian GnRH analogue and metoclopramide as a dopamine antagonist. One Ovopel pellet (25 mg) contains a mammalian GnRH analogue (D-ala^6, Pro^{9}NetmGnRH at dose 18–20 mg) and dopamine antagonist: metoclopramide (dose: 8–10 mg) [33]. Before administration, Ovopel pellets were homogenized in a mortar and dissolved in 0.9% NaCl solution (10-mL physiological solution for ten pellets).

For spermiation, each male received a single dose of 1-mL Ovopel solution kg^{-1} body weight, intraperitoneally under the left pectoral fin. Ovulation was induced by an initial injection of 0.2-mL Ovopel solution kg^{-1} body weight and a second injection of 1-mL Ovopel solution kg^{-1} body weight 12 hours after the first injection. Ripe gamete donors were anesthetized in a solution of 2-phenoxyethanol (0.5 mL L^{-1}). Milt was collected from the genital papilla under abdominal pressure avoiding contamination with urine, using a 5-mL plastic syringe. After collection, stripped milt was stored at 4 °C. Females were checked every 2 hours between 30 and 36 hours after injection. After 36 hours of hormonal stimulation, ovulated oocytes were collected by abdominal pressure and stored in 250-mL plastic containers at 4 °C.

2.2. Measurements of pH and osmolality for seminal plasma, ovarian fluid, and activating medium

Seminal plasma and ovarian fluid were obtained by centrifugation at 3000 rpm for 15 minutes at 20 °C. Osmolality and pH of the seminal plasma, ovarian fluid, and activating medium were measured by vapor pressure osmometer (Model: 5600, Wescor. Inc., USA) and a microprocessor pH meter (Model: WTW 320, WTW GmbH, Germany), respectively. Triplicate samples were measured for each male, female, and activating medium. The mean (±standard error of the mean [SEM]) values of pH and osmolality of the three different activating media were 7.25 ± 0.04 and 77.67 ± 1.45 for freshwater, 7.29 ± 0.03 and 148.0 ± 0.58 for saline water, and 7.59 ± 0.01 and 195.0 ± 1.00 for Woynarovich solution, respectively. There were significant differences in osmolality ($P < 0.001$), but no significant differences were detected for pH among the three activating media.

2.3. Sperm analysis

To determine sperm motility and concentration, milt from five males were analyzed with a computer assisted sperm analysis system (Sperm Class Analyzer v. 4.0.0. by Microptic S.L., Barcelona, Spain). The concentration of sperm was determined using the spectrophotometric method [34]. To measure motility, a 1-μL milt sample was activated by the selected activating solution (1 mL) and then placed on a Leja slide. The durations of total motility period of sperm from each male were recorded first for each activating medium. Then, the motility parameters of sperm were recorded ~8 seconds after activation of motility using a Basler 202K digital camera integrated with an Olympus BX51 microscope. Analyses were done separately for each male and activating medium. Two replicates were done for each activating medium.

2.4. Experimental design

Five batches of eggs from five females were proportionally pooled and used for this experiment. For three different treatments, 5 mL of freshwater, saline water (4-g NaCl in 1 L distilled water), and Woynarovich solution (4-g NaCl + 3-g urea in 1 L distilled water) were first placed in 3 × 15 glass petri dishes (40 mL). Then pooled eggs (0.15 g equaling ~100–110 eggs) were gently added to each activating medium so that eggs could be spread throughout the petri dish. The eggs were preincubated in different activating media for 0 (for experimental control), 30, 60, 90, and 120 seconds before milt was added. There were three petri dishes for each allotted preincubation period. Milt from five males was proportionally pooled where the mean (±SEM) concentration of sperm was 10.76 ± 1.6 × 10^9 sperm mL^{-1}. Immediately after the allotted preincubation time, 10 μL of pooled milt was added to the eggs using a micropipette (at ~1 million sperm per egg), and petri dishes were shaken for 20 seconds to facilitate fertilization. For the experimental control, with no preincubation, the activating medium and milt were added simultaneously. After fertilization, all the petri dishes were kept in a 300-L incubation tank at 15.6 °C with continuous water flow for 5 days. After 5 days of incubation, all the petri dishes were gently removed from the incubation tank, and digital images were taken under a stereoscopic microscope at 4.7 × mag to count the number of eyed embryos. The percentage of eyed embryos (E_r) was then calculated for each treatment from the total number of eggs (E_t) placed in the petri dish minus dead eggs (E_d) by the following equation:

$$E_r = [(E_t - E_d)/E_t] \times 100$$

2.5. Statistical analyses

All data are analyzed using SAS statistical analysis software [35]. Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). If data violated ANOVA assumptions, the percentage of eyed embryos was arcsine square root–transformed. Alpha was set at 0.05 for main effects and interactions. A posteriori analyses were performed using Tukey's multiple comparisons procedure. A series of one-way ANOVAs were performed to determine whether pH or osmolality differed between seminal plasma, ovarian fluid, and different activation media. In addition, one-way ANOVAs were performed to determine whether sperm quality traits differed between the activation media.

The percentage of eyed embryos was analyzed using a two-way repeated measures ANOVA model containing the preincubation time (i.e. 0, 30, 60, 90, and 120 seconds) and activating medium (freshwater, saline water, and Woynarovich solution) main effects as well as the preincubation time × activating medium interaction. When a significant interaction was detected, the model was decomposed into a series of lower-order models following Keppel [36]. Here, the decomposed ANOVA models were run to (i) determine the effect of preincubation time for each activating medium using a series of one-way repeated measures ANOVA models, and (ii) determine the effect of activating medium for each preincubation time. These reduced models involved only preplanned comparisons, so alpha-level corrections for a posteriori comparisons were not necessary.

3. Results

The mean (±SEM) sperm concentration of the five males was 10.76 ± 1.61 × 10^9 cells mL^{-1} with values ranging from 6.37 to 14.89 × 10^9 cells mL^{-1}. The mean values (±SEM) of pH and osmolality were 8.04 ± 0.03 (range: 7.93–8.13) and 248.3 ± 8.60 mOsm/kg (range: 236–281 mOsm/kg) for seminal plasma and 8.15 ± 0.03 (range: 8.05–8.22) and 321.7 ± 6.47 mOsm/kg (range: 307–343 mOsm/kg) for ovarian fluid, respectively. Duration of sperm motility for freshwater, saline water, and Woynarovich solution was recorded at 48 ± 5 seconds, 53 ± 8 seconds, and 51 ± 6 seconds, respectively. The motility parameters of sperm from all the males for each activating medium were

not significantly different. The mean (±SEM) percentage of motile spermatozoa for freshwater, saline water, and Woynarovich solution was 40.75 ± 7.21%, 42.95 ± 8.29%, and 45.34 ± 5.91%, respectively. The mean (±SEM) values of curvilinear, straight line, and average path velocity of the sperm from all the males were 31.83 ± 1.32 (range: 29.19–33.20), 22.49 ± 1.17 (range: 20.15–23.68), and 26.89 ± 1.44 (range: 24.0–28.39) μm s^{-1} for these three activation media. Here, activating medium had no significant effect on any of the sperm motility parameters.

The two-way ANOVA model revealed a significant pre-incubation time × activating medium interaction ($P < 0.001$). The factorial model was therefore decomposed into a series of one-way ANOVAs to determine the effect of preincubation time for each activating medium and of activating medium for each preincubation time (see methods above). When the saturated model was decomposed to determine the effect of preincubation time for each activating medium, the percentage of eyed embryos (mean ± SEM) ranged from 1.9 ± 0.26% at 120 seconds to 43.6 ± 9.85% at 0 seconds for freshwater, 56.1 ± 6.33% at 120 seconds to 68.4 ± 4.31% at 90 seconds for saline water, and 42.2 ± 7.91% at 90 seconds to 73.8 ± 1.0% at 0 seconds for Woynarovich solution (Fig. 1). Preincubation time was significant for freshwater, such that the percentage of eyed embryos declined across the preincubation time ($P < 0.001$; Fig. 1A). On the contrary, preincubation time had no effect on the percentage of eyed embryos when the eggs were incubated in saline water (Fig. 1B), whereas there was an effect for the Woynarovich solution ($P < 0.001$), such that a significant decline was detected at 90 seconds (Fig. 1C).

When the saturated model was decomposed to determine the activating medium for each preincubation time, a significant effect was detected at all preincubation times; 0 ($P < 0.05$; Fig. 1D), 30 ($P < 0.0001$; Fig. 1E), 60 ($P < 0.0001$; Fig. 1F), 90 ($P < 0.001$; Fig. 1G), and 120 seconds ($P < 0.001$; Fig. 1H). More precisely, freshwater produced the lowest percentage of eyed embryos at all preincubation times (Fig. 1D–H). Saline water and

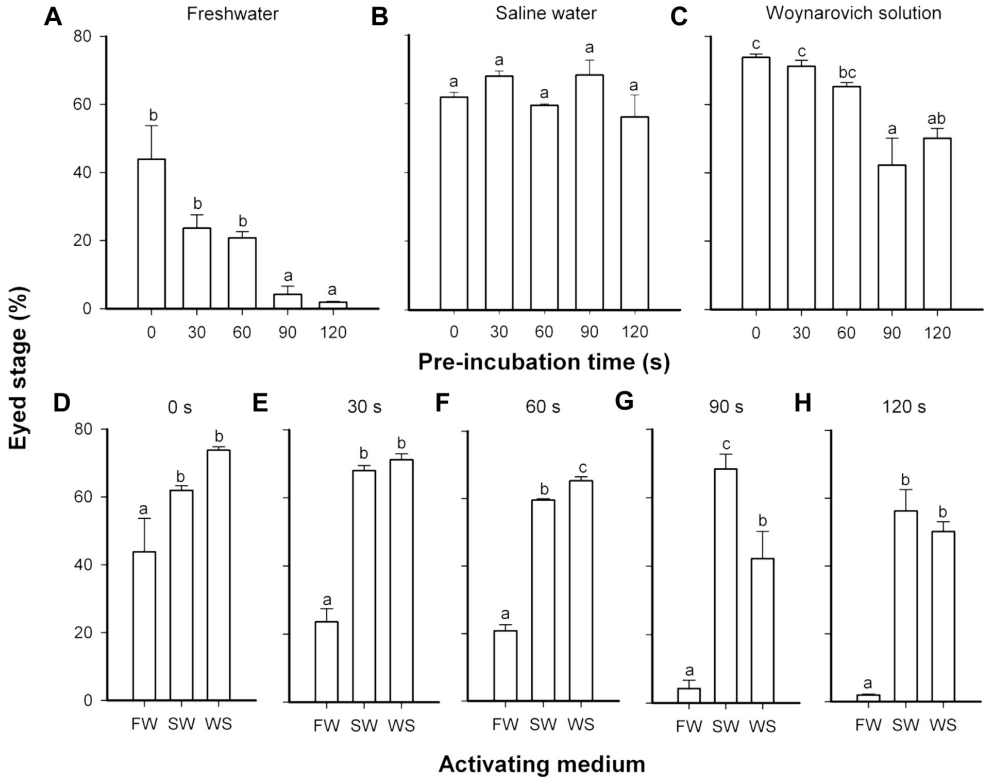

Fig. 1. Effects of preincubation time (0, 30, 60, 90, and 120 seconds) of eggs before insemination on the percentage of eyed embryos in three different activating media: freshwater (A), saline water (B), and Woynarovich solution (C). Triplicate samples were used for each treatments. Additionally, the effect of different activating media on the percentage of eyed embryos is shown at 0 (D), 30 (E), 60 (F), 90 (G), and 120 seconds (H) of preincubation time. Error bars represent standard errors. Bars without common superscript differed significantly ($P < 0.05$). FW, freshwater; SW, saline water; WS, Woynarovich solution.

Woynarovich solution produced the highest percentage of eyed embryos at 0 seconds (Fig. 1D) and 30 seconds (Fig. 1E) before incubation. Furthermore, Woynarovich solution produced the highest percentage of eyed embryos at 60 seconds (Fig. 1F), whereas saline water produced the highest percentage at 90 seconds (Fig. 1G). No significant difference was detected between saline water and Woynarovich solution at 120 seconds (Fig. 1H).

4. Discussion

During captive breeding, it is important to know how long eggs can be fertilized in a given activating medium. For majority of freshwater fish species, hatchery water is used as an activating medium. However, in some cases, the viability of eggs is suboptimal in freshwater. Thus, in these cases, it may be necessary to find a better alternative, especially if it is necessary to prolong gamete receptivity. For instance, strongly adhesive eggs of common carp (*Cyprinus carpio*), tench (*Tinca tinca*), European catfish (*Silurus glanis*), African catfish (*Clarias gariepinus*), pikeperch (*Sander lucioperca*), and sturgeons are activated in freshwater, but after fertilization, different egg desticking solutions (e.g. tannic acid, Woynarovich solution, saline solution, urea, milk, proteolytic enzymes etc.) have been used to remove egg adhesiveness [31]. Therefore, using egg desticking solution as an activating medium for strongly adhesive eggs would be more suitable than the hatchery water, especially if there are adverse effects of that medium on sperm motility parameters.

In the present study, the osmolality and pH values of ovarian fluid of ide are reported for the first time. The osmolality of ovarian fluid of ornamental common carp was reported to be as high as 290 ± 13 mOsm/kg [37], which is very close to results obtained in the present study. On the contrary, the osmolality of seminal plasma (ranging from 236.0 to 281.67 mOsm/kg) was higher as compared to the previous reports for this species (118.31–203.19 mOsm/kg; [15]). Osmotic pressure of the seminal plasma can be decreased by contamination of urine during milt collection and it is frequently observed in many species [38]. However, like many freshwater fish species, the mean osmolality of seminal plasma of ide was lower than that of the ovarian fluid. In freshwater fishes, sperm are usually activated in lower osmotic pressure, where different ions, pH, temperature, and osmolality of the activating medium affect motility parameters [39,40]. The osmolality of different activating media used in this study were less than that of seminal plasma. Sarosiek et al. [41] found Lahnsteiner's buffer (100-mM NaCl, 10-mM Tris, 0.5% albumin, pH 9.0, and 199 mOsm kg^{-1}) is the most effective medium for activating the sperm of golden ide. Another activating medium containing 0.4% NaCl, 0.3% urea, 0.5% albumin and characterized by pH 7.7 and 181 mOsm kg^{-1} [42] was found to increase the sperm motility period and velocity for the same species [41]. The durations of sperm motility of two freshwater fish, rainbow trout (*Oncorhynchus mykiss*) and northern pike (*Esox lucius*), were found to lengthen as osmolality increased from that of freshwater, but this trend is not apparent in paddlefish (*Polyodon spathula*), vimba bream (*Vimba vimba*), and common carp [5]. The osmolality of saline water and Woynarovich solution was higher than that of the freshwater treatment, and we did not use any buffer to adjust pH. Results from our sperm analysis showed that activating medium had no impact on sperm motility parameters. Thus, the effect of different activating medium on eggs was not related to changes in sperm activity.

Here, our results indicated that preincubation and activating medium influenced embryo viability at the eyed-stage. The decomposed model showed that preincubation time significantly effects the percentage of eyed embryos for freshwater and Woynarovich solution. The eggs preincubated in freshwater for 0, 30, 60, 90, and 120 seconds produced a lower percentage of eyed embryos. In freshwater, the percentage of eyed embryos sharply declined to 4.23% after 60 seconds of preincubation, meaning that ide eggs lose their fertilizing ability after 60 seconds of preincubation in freshwater. In saline water, the percentage of eyed embryos of ide increased at 30 and 90 seconds before incubation, but it was not statistically significant than the other preincubation time gradients. On the contrary, preincubation of i.e. sterlet eggs for 30 to 60 seconds in freshwater increased fertilization rates, especially when a large volume of coelomic fluid remains with the eggs [43]. For ide, the amount of ovarian fluid that remains with the eggs is very little and therefore, no positive effects of preincubation were observed in freshwater, saline water, or even in Woynarovich solution. Except for the use of saline water, the best results in each treatment were obtained with the shorter periods of preincubation. From 0 to 60 seconds, preincubation of eggs in Woynarovich solution presented results similar or better than in saline water. Therefore, there is no necessity of preincubation of eggs before fertilization for this species.

In a natural spawning environment, the duration of egg viability or fertilizing ability is likely stable, but in an artificially produced environment, this "critical" fertilization window can be easily manipulated. For instance, we reported that eggs preincubated for 120 seconds in saline water or Woynarovich solution produced over 50% of eyed embryos, which clearly indicates that the fertilizing ability of ide eggs increases in saline water or Woynarovich solution, as compared to freshwater. Duration of fertilization capability of eggs is largely dependent on the activating solution used for fertilization [8,9,12,30]. For instance, Żarski et al. [44] reported that osmolality of the activating medium is one of the most important parameters affecting egg receptivity in common carp. In cyprinid species, activation medium with osmolality of 100 to 200 mOsm/kg prolonged the duration of egg receptivity and increased fertilization success [44]. Osmolality of saline water (148.0 mOsm/kg) and Woynarovich solution (195.0 mOsm/kg) used in this study were significantly higher than the freshwater activation treatment (77.67 mOsm/kg) and within the range of suggested osmolality for common carp [44]. Saline solution was found to increase the fertilization rate of Northern pike eggs [45]. Leifritz & Lewis [46] reported that during artificial fertilization, saline solution opens the micropyle of eggs for a longer period as compared to freshwater. When

we activated the eggs in saline water or Woynarovich solution, the swelling process of eggs should be slower than in freshwater due to higher osmolality. However, for the marine species long rough dab, *Hippoglossoides platessoides limandoides*, salinity and osmotic gradient had no effects on the degree of egg swelling [47]. Therefore, further studies should address the effects of osmolality on egg swelling process for freshwater species.

Ide eggs are strongly adhesive in freshwater, but the adhesiveness is usually decreasing when the eggs are activated by medium characterized by a higher osmolality, such as saline water or Woynarovich solution [44]. Therefore, the size and shape of the micropyle and the egg surface chorion remain unchanged for an extended period in saline or Woynarovich solution than when activated in freshwater. During artificial reproduction, strongly adhesive eggs adhere to one another and make a solid mass that interferes with gas exchange and imposes oxidative stress on eggs [31]. This adhesive nature of the egg, apart from possibilities to attach the egg to the substrate, is agglutinating the sperm to the surface of egg chorion and hindering sperm movement toward the micropyle. Additionally, Woynarovich solution has been used for elimination of egg adhesiveness for common carp [48], white sturgeon (*Acipenser transmontanus*) [49], lake sturgeon (*Acipenser fulvescens*) [50] and many other freshwater species. Therefore, Woynarovich solution could be an effective activation medium for strongly adhesive eggs for artificial fertilization.

4.1. Conclusions

In the present study, we reported for the first time the fertilizing ability of ide eggs in different activating media. Although the duration of fertilizing ability of ide eggs in freshwater was found to be similar to the other cyprinid species (about 60 seconds), it has to be emphasized that it can be manipulated by application of saline water or Woynarovich solution. Together, this proves that the duration of fertilizing ability is not fixed for ide and it is strictly dependent on the activating medium. Results from this study also suggest that pre-incubation of eggs is not necessary for this species and that saline water or Woynarovich solution could be the best activating medium for ide eggs during captive breeding.

Acknowledgments

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (LO1205 under the NPU I program), the Grant agency of the University of South Bohemia (no. 134/2013/Z), and the Grant agency of the Czech Republic (no. 15-12034S). Partial financial support from the University of Warmia and Mazury, Poland (grant No. 0808.0801) is highly appreciated.

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CHAPTER 6

FIRST REPORT ON FACULTATIVE PARTHENOGENETIC ACTIVATION OF EGGS IN STERLET STURGEON, *ACIPENSER RUTHENUS*

Siddique, M.A.M., Butts, I.A.E., Cosson, J., Linhart, O., 2016. First report on facultative parthenogenetic activation of eggs in sterlet sturgeon, *Acipenser ruthenus.* Animal Reproduction Science 168: 110–115.

Animal Reproduction Science 168 (2016) 110–115

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

First report on facultative parthenogenetic activation of eggs in sterlet sturgeon, *Acipenser ruthenus*

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ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form 29 February 2016
Accepted 29 February 2016
Available online 2 March 2016

Keywords:
Cleavage development
Fertilization ability
Acipenserid

ABSTRACT

This study reported facultative parthenogenetic cleavage development of sterlet sturgeon *Acipenser ruthenus* eggs and quantified the percentage of parthenogenetically developed eggs in relation to the fertilization ability of different females. When eggs were activated in freshwater, 5.1–13.7% of eggs developed parthenogenetically, while among those activated eggs 3.6–9.4% developed to 2 cells, 0.4–4.5% developed to 4 cells, and 0–0.8% developed to 8 cells. The mean percentage of fertilized and parthenogenetically activated eggs among the females was negatively correlated ($R^2 = 0.71$, $p = 0.036$), which indicates that parthenogenetic activation rate of sterlet eggs depends on the quality of eggs in terms of fertilization rate.

1. Introduction

Parthenogenesis is a common form of asexual reproduction amongst arthropods and rotifers in which the offspring develop from unfertilized eggs. It can also be found in fish (Hubbs and Hubbs, 1932; Chapman et al., 2007, 2008; Robinson et al., 2011), amphibians, and reptiles (Vrijenhoek et al., 1989; Price, 1992; Watts et al., 2006; Booth et al., 2012), as well as in crustaceans and decapods (Scholtz et al., 2003; Buřič et al., 2011). Most organisms that reproduce parthenogenetically also exhibit a phase of sexual reproduction. More specifically for facultative parthenogenesis, asexual reproduction usually occurs in bisexual species (Booth et al., 2012) and this form of parthenogenesis has been documented in a variety of multi-cellular organisms, including fishes (Chapman et al., 2008; Booth et al., 2010).

In fish, parthenogenesis is only exhibited in the blacktip shark *Carcharhinus limbatus*, hammerhead shark *Sphyrna lewini*, and Zebra shark *Stegostoma fasciatum* (Chapman et al., 2007, 2008; Robinson et al., 2011). Facultative parthenogenetic cleavage patterns in non-ovulated eggs of Starry sturgeon *Acipenser stellatus* were first reported by Dettlaff and Ginsburg (1950). Thereafter, no research has been conducted on parthenogenetic cleavage development in sturgeon. Thus, there is a need to further study this phenomenon so that we can quantify unisexual development in sterlet, as well as in other Chondrostei.

Ovulated eggs of sturgeon are deposited in their body cavity. In some cases, these eggs can be activated in the body cavity and parthenogenetic cleavage development can be initiated before the eggs are released (Dettlaff et al., 1993); thus, negatively affecting fertilization rate. Esti-

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http://dx.doi.org/10.1016/j.anireprosci.2016.02.033
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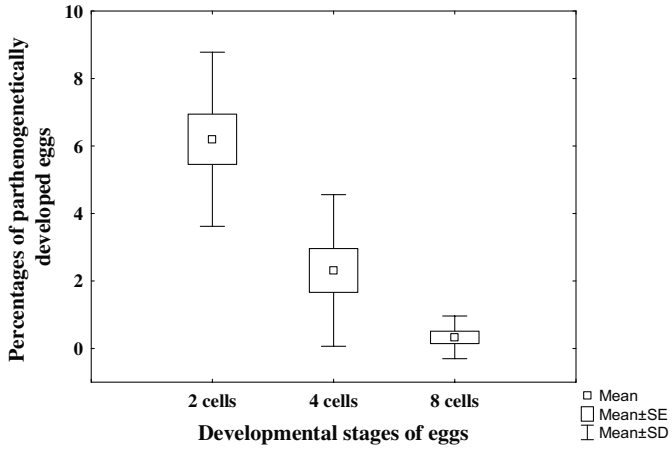

Fig. 1. Percentage of parthenogenetically developed eggs of sterlet sturgeon, *Acipenser ruthenus* activated in freshwater.

mation of fertilization rate for acipenserid eggs is very difficult because they possess 2–52 micropyles (reviewed in Siddique et al., 2014). Due to having several microplyes, polyspermic fertilization in acipenserid eggs is very common and can also be problematic for sustaining embryo viability. Polyspermic eggs show unusual cleavage development like parthenogenetically activated eggs, therefore, there is a need to document parthenogenetically activated eggs to distinguish their cleavage pattern from polyspermic eggs.

Although the mechanism for parthenogenetic cleavage development in acipenserid eggs is unknown, we speculate that egg quality should have an influence as eggs released from supportive tissue during ovulation undergo a decrease in fertilizing ability with storage time when reared *in vivo* or *in vitro* (Rothbard et al., 1996; Bobe and Labbé, 2010; Linhart et al., 2016), which may produce embryonic malformations (Bonnet et al., 2007). Thus, in this study, our objectives were to examine parthenogenetic cleavage development of sterlet sturgeon *A. ruthenus* eggs and quantify the percentages of parthenogenetically developed eggs in relation to egg quality, as evident by fertilization percentage, from different females.

2. Materials and methods

Four mature males and six females were kept in two separate tanks (volume 0.8 m^3) with a water temperature of 14–15 °C. To induce spermiation, males were administered a single intramuscular injection of carp pituitary extract (CPE) at 4 mg kg^{-1} body weight. Milt was collected 48 h after injection by a plastic catheter (4 mm diameter); milt was collected before stripping the females and stored in an icebox at 4 °C. Ovulation was induced with CPE by an initial injection of 0.5 mg kg^{-1} body weight and a second injection of 4.5 mg kg^{-1} body weight, 12 h after the first injection. Eggs were collected by applying pressure to the abdominal region 18–20 h after the second injection (Gela et al., 2008; Chebanov and Galich, 2011). Pooled milt from four males was used for experimentation.

Table 1
pH and osmolality of coelomic fluid and percentages (mean ± SD) of fertilized and parthenogenetically developed eggs in sterlet sturgeon, *Acipenser ruthenus*.

Female	pH	Osmolality (mOsmol kg^{-1})	Percentages of fertilized eggs	Percentages of Parthenogenetically activated eggs
1	7.9 ± 0.2^a	155 ± 2.6^a	73.1 ± 4.3^a	5.1 ± 0.2^a
2	7.9 ± 0.1^a	199 ± 3.5^b	44.8 ± 12.8^b	13.7 ± 0.6^b
3	8.0 ± 0.3^a	202 ± 3.6^b	72.7 ± 1.3^a	5.8 ± 2.9^a
4	8.0 ± 0.2^a	232 ± 5.2^c	28.7 ± 12.3^b	12.9 ± 1.0^b
5	7.9 ± 0.2^a	180 ± 3.1^d	92.7 ± 6.0^a	6.4 ± 4.2^{ab}
6	8.0 ± 0.2^a	185 ± 0.6^d	74.1 ± 13.4^a	9.1 ± 4.8^{ab}

Note: Different letters depict significant differences within a column ($p < 0.05$).

Sperm density and motility were analyzed according to Siddique et al. (2015a). Mean (±SD) sperm motility for the four males was 86.7 ± 6.7%, while mean sperm density was $480 \pm 51 \times 10^6$ sperm per mL. Eggs from six females were used in this study. Coelomic fluid from the females was collected by a plastic dropper to measure osmolality and pH with three replicates. Osmolality and pH of the coelomic fluid were measured by a vapor pressure osmometer (Model: 5600, Wescor. Inc., USA) and microprocessor pH meter (Model: WTW 320, WTW GmbH, Germany), respectively.

For each female, 2 g of eggs were placed in 6 × 50 mL plastic dishes; 3 dishes were used for the control (fertilization with sperm) and 3 dishes were used for observation of parthenogenetic cleavage development (without sperm). For the control, 8 mL of UV treated fresh water (pH 7.3) and 50 μL of sperm were added simultaneously, while for the parthenogenetic development treatment, eggs were only activated with 8 mL of fresh water. The plastic dishes

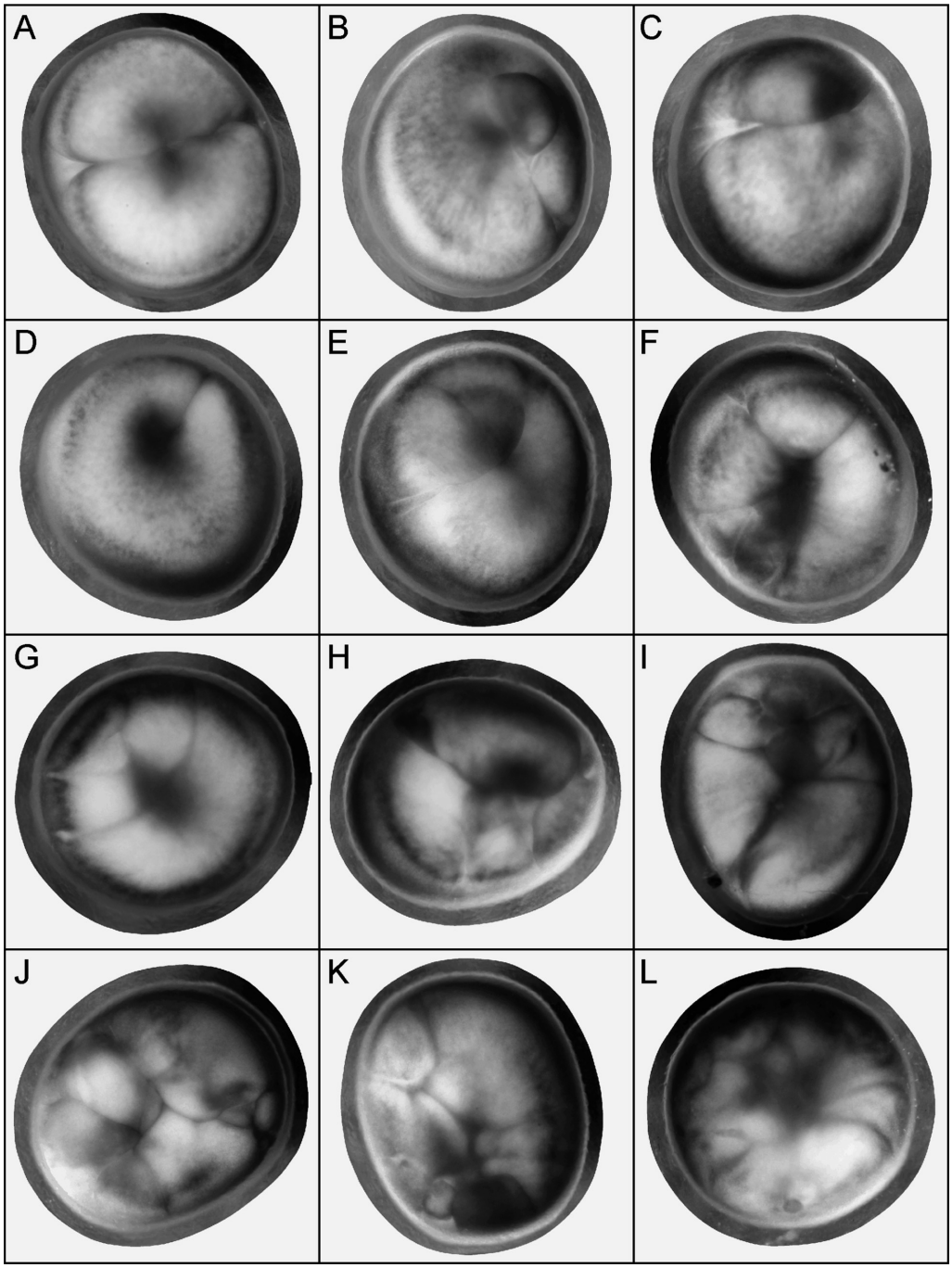

Fig. 2. Form and distribution patterns of furrows in parthenogenetically developed eggs of sterlet sturgeon, *Acipenser ruthenus*. (A–D) first cleavage to form

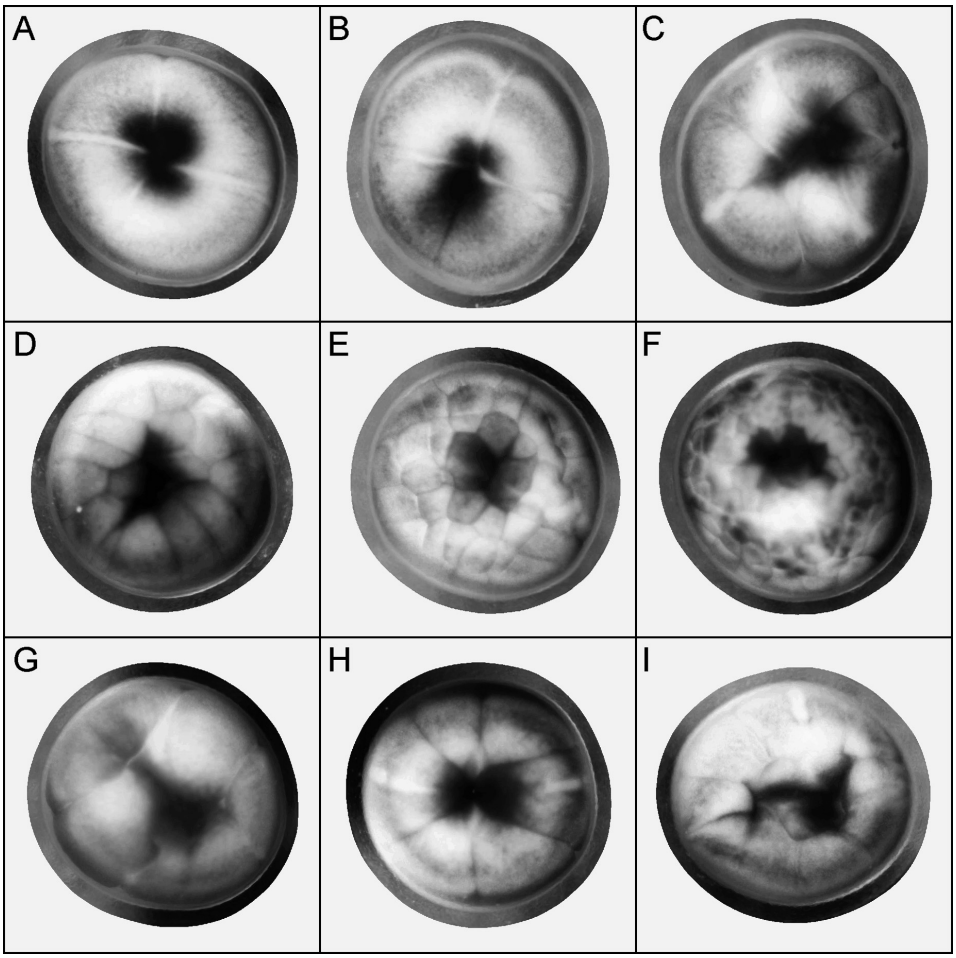

Fig. 3. Form and distribution patterns of furrows in normally developed (A- 2 cells, B- 4 cells, C- 8 cells, D- 16 cells, E- 32 cells and F- 64 cells) and polyspermic (G, H- 8 cells, and I- 16 cells) eggs of sterlet sturgeon, *Acipenser ruthenus*. Photographs were taken at 3× magnification.

were then placed on a shaker, with a constant rotation of 200 min^{-1} for 2 min. Following fertilization, the eggs were transferred to plastic Petri dishes for incubation at 20 °C.

Cleavage of all control (inseminated and fertilized) and activated (non-inseminated) eggs were observed under a light microscope (3× magnification) at 3 h post-fertilization or post-activation; cleavage for all the groups was counted at 4 h post-fertilization or post-activation. Sturgeon eggs can develop pathenogenetically even within inseminated groups. At the early blastula stage of cleavage development, fertilized eggs are difficult to separate from the polyspermic and parthenogenetically activated eggs. Therefore, for the control group, only eggs that developed >64 cells after 4 h incubation were considered as fertilized eggs and rest of the eggs that developed <64 cells were counted as unfertilized (including polyspermic and parthenogenetically activated eggs). For the parthenogenetic development treatment, the number of cells and also the number of undeveloped eggs were counted and quantified for 24 h (at 4, 6, 12 and 24 h post activation) under a light microscope. For each replicate, 110–130 eggs/embryos were observed.

All data were analyzed using Statistica V 12.0 (Statsoft Inc, Tulsa, OK, USA). Data were tested for normality

2 cells; (E–H) second cleavage to form 4 cells; (I–L) third cleavage to form 8 cells in the animal hemisphere. Photographs were taken at 3× magnification.

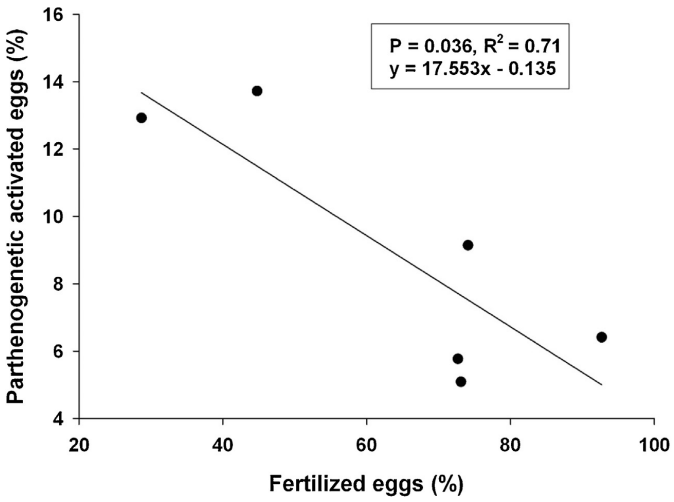

Fig. 4. Relationship between fertilized and parthenogenetically activated eggs from the six females of sterlet sturgeon, *Acipenser ruthenus.*

and homogenous variance using a Shapiro-Wilk and Levene's test, respectively. All proportional data were arcsin square-root transformed prior to analyses. Osmolality, pH, fertilization rate, and parthenogenetic activation rate of the different females were analyzed using a series of one-way ANOVA models followed by Fisher's LSD test. Additionally, linear regression was used to examine the relationship between the mean percentage of fertilized and parthenogenetically activated eggs among the females. Data were presented as mean ± SD. Alpha was set at $p < 0.05$.

3. Results and discussion

Osmolality and pH of coelomic fluid from six females are presented in Table 1 . Osmolality, the percentage of fertilized eggs, and parthenogenetically activated eggs were significantly different among the females ($p < 0.05$; Table 1). When the eggs were activated in freshwater, 5.1–13.7% of eggs developed parthenogenetically, while among the activated eggs (in freshwater) 3.6–9.4% developed to 2 cells, 0.4–4.5% developed to 4 cells and 0–0.8% developed to 8 cells (Fig. 1). Compared to fertilized eggs, parthenogenetic cleavage development of the activated eggs in fresh water started ~40 to 60 min later. When we observed parthenogenetic cleavage in activated eggs, at the same time, fertilized eggs had developed several blastomeres (>64 cells). In parthenogenetically activated eggs, the development of cleavages was unusual and often exhibited an odd number of cells (e.g. 3 cells, 5 cells, or 7 cells). The developmental patterns of parthenogenetically activated eggs are shown in Fig. 2. The arrangement of furrows in parthenogenetically cleaving eggs was similar to polyspermic fertilized eggs, but completely distinguishable from normally fertilized eggs. Polyspermic eggs usually develop abnormally and were not viable (Dettlaff et al., 1993). The arrangement of furrows in normally fertilized and polyspermic eggs are shown in Fig. 3. If embryos develop from polyspermic eggs, most die during the incubation period before gastrulation or few produce inviable larvae with abnormal structures (Dettlaff et al., 1993). In the case of parthenogenetically activated eggs, the development of cleavage was limited to several blastomeres (up to 8 cells) and then the embryo slowly died.

The mechanism for parthenogenetic activation of sterlet sturgeon eggs remains an enigma. The membrane of Acipenserid eggs is very sensitive and they can initiate the activation process by "pricking" with a glass needle at the micropylar field or even at the vegetal pole of the egg (Dettlaff, 1962). In the present study, we avoided touching the eggs by hand or by any other means. A universal feature of initiating egg activation at fertilization is a substantial increase in intercellular free Ca^{2+} in the egg cytosol. In physiological conditions, intracellular Ca^{2+} levels can be increased either by release from intracellular stores or by influx from extracellular space (Vasilev et al., 2012). In echinoderm, ascidian, and vertebrate eggs, the Ca^{2+} rise occurs as a result of inositol trisphosphate mediated release of Ca^{2+} from the endoplasmic reticulum (Runft et al., 2002). Eggs activated by artificially increasing the intracellular Ca^{2+} levels have suggested that Ca^{2+} serves as a signal to initiate a number of metabolic and cytological changes in fertilized eggs (Vasilev et al., 2012). The activation of oocyte metabolism during the process of fertilization is shown through a series of responses such as exocytosis of cortical granules (Siddique et al., 2015a,b), involving modifications in the glycoproteins of the vitelline layers, resumption of meiosis, emission of the second polar body, formation and migration of pronuclei, initiation of maternal mRNA translation and the synthesis of new DNA (Nuccitelli, 1991). All these reactions originate due to a repetitive and transient

rise in intracellular concentration of Ca^{2+} (Kline and Kline, 1992; Uranga et al., 1996). In the case of sterlet sturgeon, we hypothesize that free Ca^{2+} in coelomic fluid may increase free Ca^{2+} levels in eggs, and ultimately influence parthenogenetic development.

In the present study, the mean percentage of fertilized and parthenogenetically activated eggs among the females was negatively correlated ($R^2 = 0.71$, $p = 0.036$; Fig. 4). Thus, parthenogenetic activation rate of sterlet eggs depends on the quality of eggs in terms of fertilization rate; eggs having less fertilizing ability have a higher chance to develop parthenogenetically. In sterlet sturgeon, the occurrence of polyspermic fertilization is reported ~6.7% (Chebanov and Galich, 2011). Polyspermic fertilization in acipenserid eggs is common due to possessing several micropyles on the animal polar region. However, beside polyspermic fertilization, parthenogenetic activation of sterlet sturgeon eggs is also a barrier for achieving fertilization success and should be explored in further details.

In conclusion, facultative parthenogenetic activation of eggs in sterlet sturgeon is frequent and accounts for 5.1–13.7% of the total eggs when they are activated in freshwater. Additionally, the severity of this phenomenon is highly dependent on the quality of eggs.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic—projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program), the Grant agency of the University of South Bohemia (No. 134/2013/Z), and the Grant agency of the Czech Republic (No. P502/15-12034S).

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CHAPTER 7

GENERAL DISCUSSION

Fertilization strategies for externally fertilizing fishes

The fertilizing capacity of both marine and freshwater fish eggs gradually decrease in ovarian or coelomic fluid and in water (Ginzburg, 1972; Dettlaff et al., 1993; Hoysak and Liley, 2001; Liley et al., 2002; Żarski et al., 2012, 2014). Therefore, estimation of egg receptivity is crucial for standardization of fertilization protocol for any fish species. We estimated egg receptivity period for two freshwater species sterlet sturgeon *Acipenser ruthenus* and Ide *Leuciscus idus,* and for a marine species sea bass *Dicentrarchus labrax.* We pre-incubated the eggs for different time gradients and fertilized them by adding sperm to estimate the egg receptivity period.

Sturgeon eggs can be fertilized after many hours in water (Dettlaff et al., 1993), and are considerably more resistant to the effects of fresh water (Ginzburg, 1972). Further, the duration of sperm motility for sturgeon species are longer than other freshwater species (Dettlaff et al., 1993; Alavi and Cosson, 2005). In our first experiment, when sterlet eggs were pre-incubated for 10 min in freshwater, there were no substantial changes in the cortical and pigmented granules. Micrographs from transmission electron microscope confirmed that there were no indications of initiation of cortical reactions or the formation of perivitelline space in the pre-incubated eggs. Our results also showed that the interaction between pre-incubation time and sperm to egg ratio was highly significant, suggesting that the optimal sperm to egg ratio depends on pre-incubation time; higher sperm to egg ratios 430,000:1 and 43,000:1 were better for obtaining high fertilization rates of sterlet. Polyspermic fertilization is very common in sturgeons and it might occur with adding high quantity of sperm as sturgeon eggs possess several micropyles (Dettlaff et al., 1993; Vorob'eva and Markov, 1999; Debus et al., 2002). Therefore, a low quantity of sperm and, 30 s to 1 min pre-incubation of sterlet eggs in fresh water prior to fertilization can enhance the fertilization rate and control the polyspermic fertilization.

In the second experiment on sea bass, pre-incubation of fresh and over-ripe eggs did not increase the fertilization rate due to forming perivitelline space in the egg cortisol within 30 s after activation in seawater. Although, initiation of perivitelline space is only formed after fertilization in acipenserids (Dettlaff et al., 1993; Linhart and Kudo, 1997), formation of the perivitelline space upon activation in seawater is a common feature for several marine fish species (Govoni and Forward, 2008; Sørensen et al., 2016). The activation of sea bass eggs started within a few seconds after activation without sperm and proceeded slowly. At the initial stage of forming a perivitelline space, eggs were capable of being fertilized by sperm, but as the perivitelline space became larger in the later stages, it blocked the micropylar canal. The fertilization ability of over-ripe oocytes sharply declines and totally depends on the storage temperature and the time interval between ovulation and stripping (Migaud et al., 2013; Linhart et al., 2016). All oocytes in an ovary are not ovulated at the same time, therefore, over-ripe eggs can also be fertilized with a lower fertilization rate than the freshly ovulated eggs. However, the duration of egg receptivity period are the same for both freshly ovulated and over-ripe eggs.

In a natural spawning environment, the duration of egg viability or fertilizing ability is likely to remain stable. During captive breeding, different activating media are often used to increase fertilization success. Most of these activating media such as Billard solution (0.45 g NaCl, 0.1211 g Tris, 0.1126 g glycine, 0.0056 g $CaCl^2$, 0.5% albumin, (50 mL), pH 9.0; 344 $Osmkg^{-1}$; Billard et al., 1995), Lahnsteiner's buffer (100-mM NaCl, 10-mM Tris, 0.5% albumin, pH 9.0, and 199 mOsm kg^{-1}; Lahnsteiner et al., 1998) and some other media are designed to improve

motility parameters of sperm, while few studies have been conducted to manipulate the duration of egg fertilizing ability (Saad and Billard, 1987; Żarski et al., 2014). Therefore, the activating medium should also be suitable for the eggs by increase of fertilizing ability and duration of egg receptivity. Our results showed that in an artificially produced environment, this "critical" fertilization window for Ide eggs can be easily manipulated by using different activating solution. This manipulation of egg receptive period is also possible for other cyprinids to achieve higher fertilization success when the motility period of spermatozoa is short. For instance, saline solution was found to increase the fertilization rate of Northern pike eggs (Rieniets and Millard, 1987) by keeping the micropyle open for a longer period (Leifritz and Lewis, 1986). On the other hand, using of de-sticking solutions such as saline water, Woynarovich solution etc. as an activating medium for strongly adhesive eggs can facilitate gamete interactions during fertilization (Siddique et al., 2016).

We examined parthenogenetic cleavage development of sterlet eggs and quantified the percentages of parthenogenetically developed eggs. The unusual cleavage patterns of parthenogenetically activated eggs was similar to polyspermic fertilized eggs, but completely distinguishable from normally fertilized eggs. Like polyspermic eggs, parthenogenetically activated eggs usually develop abnormally and were not viable (Dettlaff et al., 1993). The mechanism for parthenogenetic activation of sterlet eggs remains an enigma. Dettlaff et al. (1993) reported that sturgeon eggs can be activated in the body cavity and parthenogenetic cleavage development can be initiated before the eggs are released. The parthenogenetic activation of sterlet eggs might be due to a substantial increase in intercellular free Ca^{2+} in the egg cytosol (Runft et al., 2002; Coward et al., 2003; Vasilev et al., 2012) or due to poor quality of eggs. Our results indicated that parthenogenetic activation rate of sterlet eggs depends on the quality of eggs in terms of fertilization rate. It means that eggs with lower fertilizing ability have a higher chance to develop parthenogenetically.

In summary, the duration of spermatozoa motility and egg receptivity period are varied among species. Egg receptivity period or fertilizing ability of eggs is closely related to the duration of sperm motility for any species. Pre-incubation of eggs before fertilization can enhance fertilization rate when a large volume of ovarian fluid remains with the eggs. In control reproduction, manipulation of egg receptivity period is possible using different activating solutions to facilitate the fertilization event. This thesis provided some basic knowledge about pre-incubation, egg receptivity period for few species and manipulation of egg receptivity period and documented a different mode of egg activation strategy, parthenogenetic activation of egg which can be useful for understanding of fertilization strategies of marine and freshwater fish species, their gamete biology and as well as to standardized fertilization protocol for these species in captivity.

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ENGLISH SUMMARY

Fertilization strategies for externally fertilizing fishes

Mohammad Abdul Momin Siddique

Morphological properties, species specific differences, development, and function of egg envelopes are of importance for a better understanding of fertilization strategies as well as for captive reproduction. We reviewed morphology and the developmental stages of egg envelopes, mechanism of polyspermy block, and also the role of micropyle and cortical granules in polyspermy block for acipenserid eggs. The structure of the egg envelope is similar among the acipenserids, comprising an external envelope (thecal cells, basal lamina, and follicular epithelium), a five-layered oocyte envelope (adhesive layer, alveolar layer, epilayer, and zona radiata externa and interna) and a layer of oocyte matrix and cortical granules. The development of acipenserid egg envelope within the ovary comprises five stages, with further changes following fertilization. Moreover, we standardized the terminology used to describe the egg membranes which can minimize the confusions and be helpful for future work on acipenserids eggs as well as for other fish species.

The sperm to egg ratio (required to fertilize eggs) and effects of pre-incubation of eggs in freshwater before fertilization were studied to standardized fertilization protocols for sterlet *Acipenser ruthenus.* Pre-incubation time had no effect on fertilization success at 430,000:1 and 43,000:1 sperm to egg ratios, while it was significant at the 4300:1 and 430:1 ratios. The use of adequate experimental suboptimal sperm to egg ratio revealed a positive effect of pre-incubation time, such that at the 430:1 ratio, 0.5 min pre-incubation increased the fertilization rate more than 10 min. Transmission electron microscopy showed that pre-incubation of eggs in water for <10 min did not trigger a cortical reaction, suggesting that a low sperm to egg ratio 0.5 to 1 min pre-incubation of eggs in freshwater prior to fertilization can enhance fertilization rate of sterlet (minimally do not change fertilization).

The effects of pre-incubation in seawater and the duration of egg receptivity were determined for fresh and over-ripe sea bass eggs. Our results revealed a significant effect of pre-incubation time for both the fresh ($P < 0.01$) and over-ripe eggs ($P < 0.01$). The fresh eggs had a higher fertilization success than over-ripe eggs. Fertilization success of eggs significantly declined for both of these treatments after 3 min of pre-incubation, clearly indicating that sea bass eggs are able to be fertilized by sperm for up to 3 min after release into seawater.

Effects of pre-incubation of eggs and activation medium on the percentage of eyed embryos for Ide *Leuciscus idus* were examined. At the eyed-egg stage, pre-incubation time was significant for the freshwater activation medium ($P < 0.001$), such that the percentage of eyed embryos declined across the pre-incubation time gradient. Activating medium had a significant effect on the percentage of eyed embryos for each pre-incubation time ($P < 0.05$). More precisely, freshwater produced the lowest percentage of eyed embryos at all pre-incubation times, whereas saline water and Woynarovich solution produced the highest percentage of eyed embryos at 0 s and 30 s before incubation. Examination of sperm traits showed no impact of activating medium on computer assisted sperm analysis parameters. Our results suggested that saline water or Woynarovich solution improve fertilization rate in Ide during fertilization.

We reported facultative parthenogenetic cleavage development of sterlet eggs and quantified the percentage of parthenogenetically developed eggs in relation to the fertilization ability of different females. When eggs were activated in freshwater (without adding sperm), 5.1–13.7% of eggs developed parthenogenetically, while among those activated eggs

3.6–9.4% developed to 2 cells, 0.4–4.5% developed to 4 cells, and 0–0.8% developed to 8 cells. We documented the cleavage pattern of parthenogenetically activated and polyspermic eggs to distinguish them from normally fertilized eggs. The mean percentages of fertilized and parthenogenetically activated eggs among the females were negatively correlated, which indicates that parthenogenetic activation rate of sterlet eggs depends on the quality of eggs in terms of fertilization rate.

In conclusion, this thesis provides basic knowledge on gamete biology, role of egg receptivity period, effects of pre-incubation of eggs and different activating medium which can be useful to understand the fertilization strategies of different externally fertilizing fishes as well as standardizing their fertilization protocol for captive reproduction.

CZECH SUMMARY

Strategie oplozování u ryb s vnějším oplozením

Mohammad Abdul Momin Siddique

Informace o morfologických vlastnostech, druhově specifických rozdílech, vývoji a funkci obalů jikry mají velký význam pro pochopení oplozovacích strategií u ryb a jejich umělou reprodukci. Ve své práci jsem se zaměřil na zhodnocení morfologie a vývojových stadií obalů jiker, mechanizmus blokování polyspermie a také úlohu mikropyle a kortikálních granulí při eliminaci polyspermie jeseterovitých ryb. Struktury obalů jiker jsou u této čeledi ryb velmi podobné. Skládají se z vnějšího obalu (thekální buňky, bazální lamina, folikulární epitel), pětivrstevného obalu oocytu (adhesivní vrstva, alveolární vrstva, epiteliální vrstva a zona radiata externa a interna), vrstvy matrixu oocytu a kortikálních granulí. Vývoj obalů jiker jeseterovitých ryb uvnitř ovarií probíhá v pěti fázích. Další změny pak následují po oplození. Věnovali jsme se i standardizaci terminologie používané pro popis membrán jiker. Jednotná terminologie může pomoci minimalizovat nejasnosti a může být využita v budoucích pracech zaměřených na výzkum jiker nejen jeseterovitých ryb, ale i dalších rybích druhů.

Tato práce se dále věnuje hledání vhodného množství spermií potřebného k oplodnění jikry a vlivu předinkubace jiker ve sladké vodě před jejich oplozením u sladkovodních druhů ryb. Tyto informace poslouží u jesetera malého (*Acipenser ruthenus*) k standardizaci reprodukčních protokolů. Z našich výsledků vyplývá, že při poměru spermií k jikře 430 000 : 1 a 43 000 : 1 nemá předinkubační doba žádný vliv na úspěch při oplození. Doba předinkubace je naopak důležitá v případě použití nízkého počtu spermií, a to 4 300 : 1 a 430 : 1. Při použití suboptimálního poměru spermie-jikra 430 : 1 s využitím předinkubační doby v délce 0,5 minuty bylo zjištěno vyšší procento oplozenosti jiker v porovnání s předinkubací o délce 10 minut. Transmisní elektronová mikroskopie odhalila, že předinkubace jiker ve sladké vodě po dobu kratší než 10 minut nespouští kortikální reakci. Z toho logicky vyplývá, že při nízkém poměru spermie-jikra můžeme pomocí předinkubace jiker jesetera malého ve sladké vodě v délce 0,5–1 min zvýšit jejich oplozenost (v nejhorším případě se oplozenost nesníží).

Vliv předinkubace v mořské vodě a délka trvání vnímavosti k oplození (receptivity) jiker byla určována pro čerstvě vytřené i přezrálé jikry u morčáka evropského. Naše výsledky odhalily významný vliv předinkubační doby u čerstvě vytřených ($P < 0,01$) i přezrálých jiker ($P < 0,01$). Čerstvé jikry se vyznačovaly vyšší oplozeností než přezrálé jikry. Oplozenost signifikantně klesala v obou případech po 3 minutách předinkubace, což nasvědčuje tomu, že si jikry morčáka evropského zachovávají schopnost oplodnění spermatem do 3 minut po jejich uvolnění do mořské vody.

Dále byl určován i vliv předinkubace jiker a aktivačních medií na procento oplozenosti jiker v očních bodech u jelce jesena (*Leuciscus idus*). V tomto případě neměla předinkubační doba významný vliv na procento oplozenosti jiker v očních bodech při použití sladkovodního aktivačního media ($P < 0,001$). Procento oplozenosti jiker v tomto stadiu vývoje postupně klesalo s prodlužováním předinkubační doby. Druh aktivačního media měl signifikantní vliv na procento oplozenosti jiker v očních bodech pro každou předinkubační dobu ($P < 0,05$). Při použití sladké vody bylo zaznamenáno nižší procento oplozenosti jiker v očních bodech bez ohledu na testovanou délku předinkubační doby, zatímco při použití slané vody a Woynarovichova roztoku bylo pozorováno vyšší procento oplozenosti jiker v očních bodech bez předinkubace a s předinkubací v délce 30 s, což bylo prokázáno analýzou parametrů spermií pomocí CASA software.

U jesetera malého jsme zaznamenali a kvantifikovali fakultativní partenogenetické rýhování jiker v souvislosti se schopností oplození jiker u různých samic. Pokud byly jikry jesetera malého aktivovány ve sladké vodě (bez přidání spermatu), objevilo se partenogenetické rýhování u 5,1–13,7 % jiker. Mezi jikrami aktivovanými oplozovacím médiem bez spermií se 3,6–9,4 % z nich partenogeneticky vyvinulo do stadia 2 buněk, 0,4–4,5 % z nich se vyvinulo do stadia 4 buněk a 0–0,8 % z nich do stadia 8 buněk. Zdokumentován byl rovněž charakter rýhování jiker vyvíjejících se partenogeneticky nebo po polyspermickém oplození s cílem odlišit je od normálně oplozených jiker. Byla zaznamenána negativní korelace mezi procentem oplozených jiker a partenogeneticky se vyvíjejících jiker u jednotlivých jikernaček, což nasvědčuje tomu, že míra partenogenetického vývoje jiker u jesetera malého závisí na jejich kvalitě, je-li posuzována mírou oplození.

Tato dizertační práce rozšiřuje základní znalosti o biologii gamet, době vnímavosti jiker k oplození a vlivu předinkubace jiker v různých aktivačních médiích před oplozením. Tyto informace mohou být užitečné nejen pro pochopení oplozovacích strategií u několika druhů ryb s vnějším oplozením, ale i pro standardizaci jejich oplodňovacích protokolů při umělé reprodukci.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor Prof. Otomar Linhart for his support, encouraging and personal guidance, patience, wide knowledge, constructive criticism, understanding, and prompt feedback. Prof. Linhart gave me a lot of academic freedom during my study, for this, I am very grateful to him. It has been an honor for me being his student. I will always remember Prof. Linhart for his never-ending effort to help me succeed and have a fulfilling Ph.D. experience. I would like to express my deep and sincere gratitude to my advisor, Dr. Martin Pšenička, for the continuous support of my research and for his patience, and motivation.

My warm appreciation is extended to my colleagues Prof. Jacky Cosson, Prof. William Lee Shelton, Dr. Borys Dzuba, Dr. Marek Rodina and Prof. Martin Flajšhans for their fruitful discussion, helpful suggestions and inspiration during my Ph.D. research. I would also like to thank Lucie Kačerová for her academic supports, Vladimíra Tučková for her excellent help and support during experiments and all of my colleagues from the Faculty of Fisheries and Protection of Waters in Vodňany, who helped me with my experiments and laboratory works and also to the staffs of electron microscopy lab, Institute of Parasitology, Czech Academy of Science in České Budějovice for their valuable helps during experiments.

I would like to thank Dr. Ian Anthony Ernest Butts (DTU Aqua, Technical University of Denmark, Denmark) for his guidance, encouragement and continuous support during my Ph.D. research. I gratefully acknowledge Dr. Christian Fauvel (IFEMER, Station Expérimentale d'Aquaculture, Palavas Les Flots, France) and Dr. Daniel Żarski and Dr. Sławomir Krejszeff (Warmia and Mazury University in Olsztyn, Poland) for their hospitality, inspiration, extensive discussions and personal attention during my stay in their laboratories. Special thanks goes to my elder brother Md. Khurshid Alam Bhuiyan (University of Cadiz, Spain) for his nice illustrations and cover page of this thesis.

Finally, my deepest gratitude goes to my beloved wife Mahbuba Aktar Badhan, my mom Hazera Bashir and all of my brothers and sisters for their endless love, patient, prayers and encouragement. My Ph.D. thesis is dedicated to my dad Mohammad Bashir and to my lovely son Yasin bin Siddique who passed away during my study.

This Ph.D. thesis was financially supported by

- The Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (LO1205 under the NPU I program)
- The Grant agency of the University of South Bohemia (No. 125/2016/Z)
- The Grant agency of the Czech Republic (No. 15-12034S).
- COST (No. LD14119) and COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE).

LIST OF PUBLICATIONS

PEER – REVIEWED JOURNALS WITH IF

Dadras, H., Dzyuba, B., Cosson, J., Golpour. A., **Siddique, M.A.M.**, Linhart, O., 2016. Effect of water temperature on the physiology of fish spermatozoon function: a brief review. Aquaculture Research, early view, doi: 10.1111/are.13049. (IF 2015 = 1.606)

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Khan, M.S.K., **Siddique, M.A.M.**, Zamal, H., 2013. Replacement of fish meal by plant protein sources in Nile tilapia (*Oreochromis niloticus*) diet: growth performance and utilization. Iranian Journal of Fisheries Sciences 12: 864–872. (IF 2013 = 0.320)

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ABSTRACTS AND CONFERENCE PROCEEDINGS

Siddique, MA.M., Linhart, O., Krejszeff, S., Żarski, D., Butts, I.A.E., 2015. Duration of egg receptivity in different activating media for an externally fertilizing freshwater fish, Ide (*Leuciscus idus).* In: Aquaculture Europe 2015, Rotterdam, Netherlands, 20–24 October 2015. (Oral Presentation)

Siddique, M.A.M., Linhart, O., Krejszeff, S., Żarski, D., Pitcher, T.E., Butts, I.A.E., 2015. Gamete biochemistry and genetic architecture on the rate of eyed embryos in an externally fertilizing freshwater fish, Ide *Leuciscus idus.* In: The 5th International Workshop on the Biology of Fish Gametes, Ancona, Italy, 7–11 September 2015. (Oral Presentation)

Siddique, M.A.M., Linhart, O., 2014. Fertilize ability of sterlet (*Acipenser ruthenus*) eggs using different quantity of sperm during pre-incubation with hatchery water. In: Aquaculture Europe 2014, Donostia-San Sebastian, Spain, 14–17 October 2014. (Oral Presentation)

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	**Mohammad Abdul Momin Siddique**
Research department	Laboratory of Reproductive Physiology, FFPW, USB
Supervisor	Prof. Otomar Linhart
Period	October 2013 until September 2016

Ph.D. courses	**Year**
Fish reproduction	2014
Pond aquacultures	2014
Applied hydrobiology	2014
English language (IELTS)	2014
Basics of scientific communication	2015

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2013
Seminar days of RIFCH and FFPW	2014
Seminar days of RIFCH and FFPW	2015

Foreign stays during Ph.D.	**Year**
Dr. Daniel Żarski, Department of Lake and River Fisheries, Warmia and Mazury University in Olsztyn, Poland (March – May, 2 months)	2015
Dr. Christian Fauvel, IFEMER, Station Expérimentale d'Aquaculture, Palavas Les Flots, France (January – February, 1.5 months)	2015

International conferences	**Year**
Aquaculture Europe 2015, 20–24 October 2015, Rotterdam, Netherlands (Oral Presentation)	2015
The 5th International Workshop on the Biology of Fish Gametes, 7–11 September, 2015, Ancona, Italy (Oral Presentation)	2015
Aquaculture Europe 2014, 14–17 October 2014, Donostia San Sebastian, Spain (Oral Presentation)	2014

Name of the course/training/seminar	**Year**
The 5th Aquagamete Training School "Cryopreservation of fish germ cells", Universitat Politècnica de València, Valencia, Spain	7–11 March, 2016 Certificate
Course on Biological Specimen Preparation for Electron Microscopy, Biology Centre, Academy of Sciences, Laboratory of Electron Microscopy, České Budějovice, Czech Republic	16–20 June, 2014 Certificate
Microscopy and Image Analysis Workshop, Faculty of Fisheries and Protection of Waters, Vodnany, Czech Republic	7–10 October, 2013 Certificate

CURRICULUM VITAE

PERSONAL INFORMATION

Name: Mohammad Abdul Momin
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PRESENT POSITION

Ph.D. student at the University of South Bohemia in České Budějovice (USB), Faculty of Fisheries and Protection of Waters (FFPW), Research Institute of Fish Culture and Hydrobiology (RIFCH), South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA), Laboratory of Reproductive Physiology, Vodňany, Czech Republic

EDUCATION

2013–2016	Ph.D. student in Fishery, Laboratory of reproductive physiology, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic
2009–2011	M.Sc. in Fisheries, Aquaculture Economics and Management, University of Tromsø, Tromsø, Norway
2005–2006	M.Sc. in Marine Science, University of Chittagong, Chittagong, Bangladesh
2001–2004	B.Sc. (Honours) in Marine Science, University of Chittagong, Chittagong, Bangladesh

Ph.D. COURSES

Fish reproduction, Pond aquaculture, Applied hydrobiology, English language, and Basics of scientific communication

KNOWLEDGE OF LANGUAGES

Bengali, English, Hindi

RESEARCH STAY AND COLLABORATIONS

March – May, 2015	Dr. Daniel Żarski, Department of Lake and River Fisheries, Warmia and Mazury University in Olsztyn, Poland
January – February, 2015	Dr. Christian Fauvel, IFEMER, Station Expérimentale d'Aquaculture, Palavas Les Flots, France

SUPERVISION OF STUDENT

Bachelor student Marek Let (Co-supervising with Prof. Otomar Linhart), 2015–2016.
Thesis title: Short-term storage of eggs of Sterlet, *Acipenser ruthenus* in artificial coelomic fluid *in vitro*.