DTU Library

On the way to successful European eel larval rearing: Impact of biophysical conditions and gamete quality

Sørensen, Sune Riis

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

On the way to successful European eel larval rearing:

Impact of biophysical conditions and gamete quality

PhD Thesis

Written by Sune Riis Sørensen
Defended 25 February 2014

On the way to successful European eel larval rearing:
Impact of biophysical conditions and gamete quality

PhD Thesis

Written by Sune Riis Sørensen
November 2013

Supervisors:
Senior Researcher Jonna Tomkiewicz, DTU Aqua – main supervisor
Senior Researcher Peter Munk, DTU Aqua – cosupervisor
Professor Dr. ir. Peter Bossier – cosupervisor

Opponents:
Professor Michael St. John, DTU Aqua
Professor Olav Vadstein, NTNU, Norway
Professor Audrey Geffen, University of Bergen, Norway

DTU Aqua
National Institute of Aquatic Resources

Contents

Preface

This PhD project is linked to the Danish research project REEL "Reproduction of European eel in Culture" (1.10.2008-31.3.2010) and the EU FP7 project PRO-EEL "Reproduction of European Eel: Towards a Self-sustained Aquaculture (1.4.2010-31.3.2013). Both of these projects are coordinated by J. Tomkiewicz, DTU Aqua. In the PRO-EEL project, the PhD contributes to tasks related to fertilisation experiments and evaluation of gamete quality and furthermore to experiments on incubation and larval rearing techniques. The research presented in this thesis was conducted between 2010 and 2013 and experimental work carried out at the research facility of DTU Aqua at Lyksvad Eel Farm in the southern Jutland.

Prior to starting my PhD, I had the privilege to get hands on knowledge and insight into the research ongoing in DTU Aqua with respect to induced reproduction and larval culture of European eel. Research experience was obtained during a series of pilot experiments conducted in the DTU lead ROE III (Artificial reproduction of European eel) and REEL projects. Integration in the national and international teamwork including elaboration of proposals and reporting together with experimental research tasks has given me five uttermost exiting years with a wide variety of experiences within practical aquaculture techniques as well as basic research and application development.

The amount of unanswered questions, regarding the biology in this fascinating species have been an inexhaustible source of motivation for this work, and results have only encouraged us to learn more and seek answers to ever arising new questions. The project has given me a unique opportunity to work with a wide group of aquaculture experts and scientists across Europe, and I greatly appreciate having had this opportunity to develop and test ideas.

Acknowledgements

Thesis summary

The European eel is a widely distributed fish species of economic and cultural importance. It inhabits both coastal and freshwater systems, and is targeted by fisheries and treasured as a food item. Although eels are reared in aquaculture, this industry relies solely upon wild-caught juvenile glass eels that arrive to the European coasts after a 6000 km journey from the Sargasso Sea, where they were hatched. The adolescent eels start their long migration from the European continent back to their spawning area in the Sargasso Sea in late autumn as sliver eels. As long as the eels are within the European continent, they are in an immature stage, and they do not start migration and maturation until the *silvering stage*. This stage is however tightly controlled by brain and pituitary hormones, preventing maturation of gonads remote from their natural breeding area.

This hormonal inhibition of maturation is the main reason why it is difficult to reproduce European eel in captivity. Although, attempted since 1930ies, utilizing maturational hormones primarily from other fish species, we only recently succeeded in refining reproduction protocols that enable rich quantities of viable gametes from this species. In view of these obstacles, the last decade's research has shown substantial progress. This PhD has contributed to this progress through new knowledge and development of procedures for successful egg activation and fertilization as well as incubation and larvae culture.

My PhD work addressed biophysical determinants fundamental to producing healthy eggs and larvae. One of my aims was to improve methods and results of *in vitro* fertilization. This research included characterisation of sperm density, "optimal" sperm to egg ratios and gamete mixing. Eel gametes are activated by salt water and incubated in a marine aquatic environment. In this regard, my aim was to identify suited salinities and seawater sources, supporting a good embryonic development. Embryonic development lasts two days from fertilization to hatch. During this time, as well as in early larval stages, mortality is high. Here, my aim was to assess effects of temperature and microbial interference during incubation and larval rearing on order to reduce this mortality in cultures.

The results have provided valuable new insights, contributing to progress of *in vitro* fertilization methods and reduced mortality in egg and larval culture. Our fertilisation procedures initially applied spermatocrit as a sperm quantification technique to standardise sperm:egg ratio. Although being a practical method, it featured moderate precision. Spectrophotometry in contrast, showed high precision in addition to being a fast and practical and subsequently supported experiments that identified optimal sperm:egg ratio. Egg activation and swelling are among the processes often seen to fail in experiments. Activation salinity was found to be a determinant of egg fertilisation, buoyancy, and egg size although egg size effects differed among individual females. Fertilization percent was typically high in the range 30 and 40 psu, while rate of un-activated and dead eggs rose in higher salinities. Egg swelling could be optimized using certain artificial salt types and impeded using others. During egg incubation, microbial interference was found to be a major obstacle for hatch, rather caused by microbial activity than presence. Larval mortality was highly dependent on whether antimicrobial conditions were bacteriostatic or bactericidal. This calls for future technology and microbial management, e.g. by matured water integrated in RAS technology.

The results obtained through these studies have added to Danish progress within artificial reproduction in European eel by improved fertilization protocols and identification of important parameters during the early life stages. Such progress has led to present focus on eel larval culture and feeding, which has brought attention to eel as a potential "new species" in aquaculture.

Dansk resumé

Den europæiske ål er en vidt udbredt art af stor økonomisk og kulturel betydning. Den lever både i ferskvand og kystnære havområder over hele Europa, og er en værdsat art både i fiskeriet og som fødevare. Selvom ål opdrættes i akvakultur, er denne branche helt afhængig af vildfangede glasål. Glasålene fanges, når de ankommer til de europæiske kyster efter en 6000 km lang rejse fra Sargassohavet, som er den Europæiske åls gydeområde. Ålene starter deres lange vandring fra de europæiske kyster tilbage til dette gydeområde som blankål om efteråret. Ålene kønsmodnes ikke så længe de er i Europa, men vil i den tidlige modningsfase skifte farve fra gullig til sølvblank, hvilket indikerer begyndelsen af kønsmodningen og den lange vandring. Udviklingen i blankålsstadiet er imidlertid kontrolleret af hormoner fra hjerne og hypofyse, som sikrer, at udviklingen af sæd og rogn først finder sted, når de nærmer sig Sargassohavet.

Den naturlige hormonale kontrol af modningen er hovedårsagen til at det er vanskeligt at modne ål i fangenskab. Selvom dette har været forsøgt siden midt i trediverne ved brug af hormoner primært fra fisk, er det først for nylig lykkedes at udvikle og forbedre procedurer til kønsmodning ålene og derigennem opnå levedygtige æg fra denne art. Set i lyset af de vanskeligheder, har vi i Danmark gjort bemærkelsesværdige fremskridt indenfor det senest årti. Forskningen i dette PhD projekt har bidraget hertil gennem ny viden og udvikling af procedurer inden for aktivering og befrugtning af æg samt inkubations- og larvekultur.

Mit PhD adresserer fundamentale biofysiske parametre i dannelsen af sunde æg og larver og et af mine mål har været at forbedre metoder og resultater af kunstig befrugtning. Dette har omfattet optimering ad sæd-kvantificeringsmetoder, sæd:æg ratio samt media til blanding af sæd og æg. Både æg og sæd aktiveres ved kontakt med saltvand og æg inkuberes i et saltvandsmiljø. I den henseende var mit mål at sikre en egnet saltholdighed og type af saltvand i befrugtningsprocedurer for at fremme af befrugtningssucces, ægkvalitet og -udviklingsevne. Fra åleægget befrugtes til fosteret er færdigudviklet og klækker, går der to dage. I denne periode samt i det tidlige larvestadie er dødeligheden høj. Jeg undersøgte således også effekt af temperatur og af det mikrobielle miljø i kultur af æg og larver for at reducere denne dødelighed i kulturer.

Denne forskning har bidraget positivt til udviklingen af bedre befrugtningsprocedurer og lavere dødelighed i kultur af æg og larver. Hidtidige procedurer anvendte spermatokrit til måling af sædkoncentration og stan-dardisering af sæd:æg ratio. Metoden er praktisk, men viste sig ikke præcis. Omvendt fandt vi spektro-fotometer-analyse præcis, praktisk og hurtig. Estimering af sædtæthed gennem spektrofotometri blev derfor implementeret i forsøg, hvor optimal sæd:æg forhold til befrugtning blev bestemt i forsøg. Ægakti-vering og efterfølgende svulmning fejler ofte. Saltholdigheden viste sig at være afgørende for befrugtning, flydeevne mens effekten på ægstørrelse varierede mellem hunner. Befrugtningsprocenter var høje i inter-vallet mellem 30 og 40 ‰, mens der var flere døde og uaktiverede æg ved højere saltholdigheder. Desuden sås, at specifikke marine salttyper i aktiveringsvandet forbedrede ægaktiveringen, mens andre gav små æg. Derudover viste forsøg, at bakteriebelæging på æg under inkubering reducerede klækning. Larveover-levelsen var afgørende afhængig af antibakteriel behandling. Disse resultater viser et behov for mikrobiel kontrol i inkubering og larvekultur fx gennem anvendelse af recirkuleringssystemer og modnet vand.

Resultaterne af disse studier har bidraget til de fremskridt, vi har gjort i Danmark indenfor det seneste tiår og har påvist nogle af de forhold, der er afgørende for befrugtningssucces og udviklingen af æg, fostre og larver i europæisk ål. Dette er medvirkende til, at vi i dag fokuserer på larvekultur og fødeindtagelse, og dermed i flere henseender kan karakterisere ål som en ”ny art” i akvakultur.
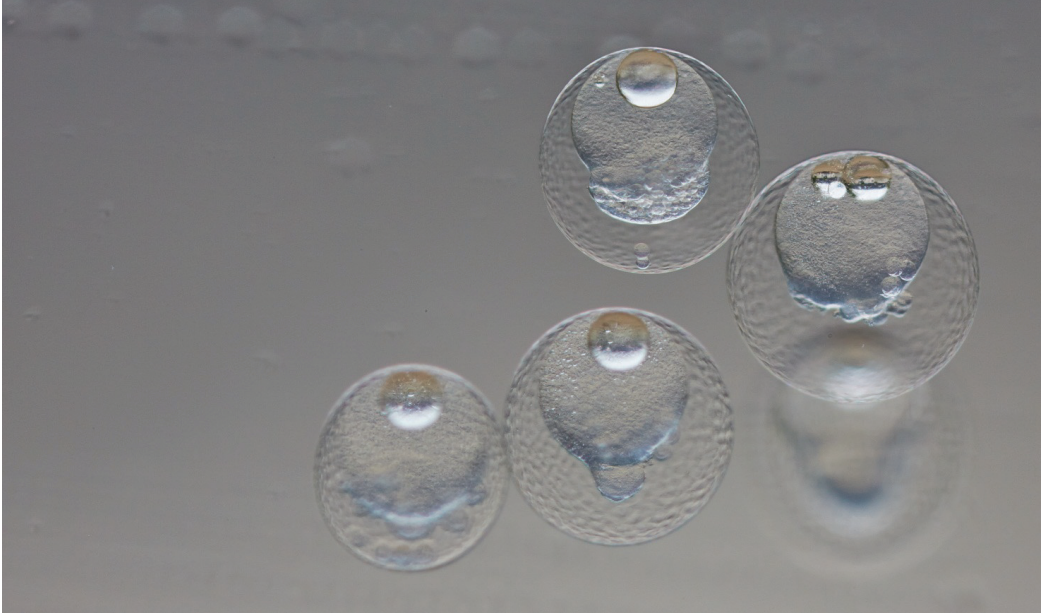
Eggs of European eel in seawater, 36 psu, with buoyancy aided by osmolality of yolk and the clear oildroplet. Eggs on this image are six hours old, counted from time of fertilization, and show various degrees of cell cleavage symmetry reflecting the egg quality which is a focus area of this thesis. While the upper of the eggs features a regular cell cleavage pattern with a gathered blastula, the lower eggs illustrate various degrees of irregular cleavages frequently seen in in vitro fertilization studies. Photo by Sune Riis Sørensen using equipment funded by Elisabeth og Knud Petersens Fond.

Synthesis

1. Introduction

1.1 The European eel

Of thousands of fish species, eel is one of those most familiar to humans. It inhabits both marine and freshwater habitats and is even capable of migrating over land in search for new freshwater habitats. Besides being a highly treasured food item, this species' close connection to humans has triggered curiosity and posed a lot of questions regarding its life cycle. An early question was why eels with mature ovaries or testes were never observed in nature. Not surprisingly, this lead to historical myths and in the work *History of Animals* dating back to 350 B.C. by Aristotle, eels are described not to produce eggs and not to be differentiated

Figure 1. An old eel trap, Klitmøller Ålekiste, in northern Denmark which dates back to 18th century. The eels are caught in limited numbers when migrating as silver-eels in Oct. to Dec. Bypassing the stream through the shed floor enable safe collection. Females shown here were among those used in this thesis. Photo Sune Riis Sørensen

into males and females like other animals. Cresswell wrote in 1883: Their propagation most likely occurs buried in the earth and offspring emerges from muddy and wet soils. Today, we find it amusing to read, however the level of detail given so early in history invoke great respect and furthermore illustrates the intense curiosity and attention eel reproduction evoked at that time and still does today.

Occasional finds of peculiar looking organisms in the upwelling zone near the Messina strait in Italy became the next clue to the eel life cycle. These leaf shaped organisms with a glassy transparent body were called leptocephali and originally considered to be a separate species. In 1896, it was discovered, however that these organisms were indeed a larval stage of the European eel (Grassi, 1896). Grassi gave a detailed description of their morphology and furthermore achieved to culture wild caught leptocephali larvae until they metamorphosed into glass eels. Grassi found it particularly easy to obtain leptochali from stomachs of the ocean sunfish, *Mola mola,* and even managed to culture a few of these. Grassi forwarded the hypothesis of the eel having deep sea spawning, likely in the Mediterranean. Leptocephali larvae were later caught different places in the Atlantic Ocean and early in the 20th century, a Danish marine researcher Johannes Schmidt, identified the Sargasso Sea as the spawning site for European eels by delimiting the area, where the smallest larvae were recorded (Schmidt, 1912, 1923).

Finding the spawning site of European eel 6000 km away from European shores gave reasons why mature eels were not observed in European waters (Ginneken and Maes, 2005). In the century following Schmidt's findings, intensive research gave a lot of exciting answers to eel biology questions, but certainly not to all (Righton et al., 2012). The adult eels leave the European coasts during October to November (Fig. 1) around new moon (Boëtius, 1967) and start their long migration to the Sargasso Sea and, as recently discovered, most likely aided by sensing Earth's magnetic field (Durif, 2013). This migration stage involves a set of morphological changes, where the most obvious is the dorsal part becoming darker, the skin coloration on flanks and abdomen changes from yellowish to silvery, the skin and mucus layers thicken, and the eyes enlarge, all considered adaptations to oceanic life by decreasing visibility to predators and promoting ocean

navigation (Rousseau, 2009). During this silvering process also the digestive tract degenerates and feeding ceases (Boëtius, 1967; Dollerup and Graver, 1985). Silver eels migrate commonly at an age of 6 to 20, but in some cases they may be up to 50 years (Poole and Reynolds, 1998). Eels can grow surprisingly old and a report from Sweden, claims a specimen to be 155 year old. It was introduced into a drinking well in Brantevik, in southern Sweden in 1859, preventing e.g. amphibians from polluting the drinking water. In 1962, the well was emptied and the eel photographed (Fig. 2). The eel was put back again, and followed at regular intervals until it was found dead in august 2014 (Rosén 2014)

Figure 2. European eel claimed to be 155 year old living in a drinking well in Brantevik, in southern Sweden from 1859 to 2014 (Rosén 2014). Photo: Björn Flyckt in Nordberg et al 1985

The maturation of gonads likely occurs during the migration to the Sargasso Sea (Dufour and Fontaine, 1985) and may partly be triggered by swimming (Righton et al., 2012). In swim trials, particular older eels were found to start vitellogenesis and initiate oocyte development (Palstra, 2007), while this was less pronounced in younger eels (van Ginneken, 2007). Maturation is controlled at the brain-pituitary level. In fish, a dual control system exists where gonadotropin release from the pituitary that initiate maturation, is stimulated by the gonadotropin-releasing hormone (GnRH), but inhibited by another hormone, dopamine (Dufour, 2005). The European eel is unusual in the way that a dopamine inhibition sets in during silvering, and arrests development at pre-pubertal stage before the migration (Dufour et al., 2003; Vidal, 2004). For gonadal maturation to occur, this dopamine inhibition has to cease, allowing release of gonadotropins and subsequent synthesis of sex steroids (Dufour et al., 2003). When this occurs, synthesis and release of the two gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) eventually will stimulate oogenesis and spermatogenesis in the ovaries and testis respectively. This pre-pubertal impediment to gonadal maturation is the primary reason for the difficulties experienced in artificial reproduction in European eel and why natural spawning or even maturing eels have never been observed near the European continent (Vidal, 2004).

Ovarian development can be induced in female eels by hormonal treatment with salmon or carp pituitary extract (SPE, CPE) that contain gonadotropins. Injections are given weekly for 12 to 18 weeks until signs of ripeness. By administration of 17, 20 β-dihydroxy-4-pregnen-3-one (DHP) shortly after last SPE treatment final maturation is induced and ovulation occurs 12 to 14h after (Tomkiewicz, 2012). Maturation in male eels is shorter and 5 to 7 weeks of weekly treatments with human chorionic gonadotropin induces spermiation (Tomkiewicz, 2012). This can be done using captive-reared eels as well as wild caught

(Mordenti et al., 2013; Tomkiewicz, 2012). Eels not responding to treatment are common in reproductive studies, and related to the endocrinology stages of the selected broodstock eels (Durif et al., 2006). Silver eels that have initiated migration (J. Boëtius, 1967; Dufour and Fontaine, 1985) and those with the highest condition factor, generally being related to large body size and high age, respond best to induced maturation (Durif et al., 2006). It is hypothesised that the large variability observed in responsiveness to both hormonally induced and naturally triggered maturation, e.g. swimming, is a natural trait among eels. This variability is in nature assumed to account for the unpredictability of oceanic environment (Ginneken and Maes, 2005; Durif et al., 2006).

1.2 Reproduction studies until now

Experimental eel reproduction research has a long history motivated by the curious reproduction biology of this species as well as the potential for aquaculture eel using captive bred fry (Fig. 3). A French group of scientist was the first to publish results on induced maturation in male eels based on maturation using urine from pregnant women containing human chorion gonadotropin (Boucher, 1934; Fontaine, 1936). Improved protocols for male eel maturation was developed in the following years (e.g. Boëtius, 1967; Meske, 1973; Bieniarz, 1977). Maturing female eels proved more difficult and creativity flourished and e.g. organ transplantation of pituitary glands from both ox, swine, rats, garfish, plaice and cod (Bruun et al., 1949). Maturation in the females was studied by the Danish scientists Inge and Jan Boëtius in the 1960'ies with the first production of fertilised eggs (Boëtius, 1962). Later, Japanese researcher further developed methods resulting in successful production of first larvae of Japanese eel in 1974.

Year	Event	Author
2013	Stabile production of European eel larvae from wild broodstock living up to 26 days	PRO-EEL FP7
	European eel larvae living 12 days natural spawned	Mordenti, O. et al
2010	European eel larvae living 12 days though the yolk sac stage.	Tomkiewicz et al
2007	European eel larvae living 5 days through the yolk sac stage	Tomkiewicz & Jarlbaek
2004	European eel larvae 1½ day old	Pedersen, B.
2003		Pedersen, B.
1986	First hatched European eel larvea living 3½ day after hatch	Prokhorchik et al
1983	Successful embryonic development of European eel described	Bezdenezhnykh et al
1974	Japanese eel matured artificially using salmon pituitaries.	Yamamoto & Yamauchi
1972	In 1974 first hatching of japanese eel	Ochiai et al and Yamamoto et al
1967	Protocols establised to mature male eels using HCG	Boëtius & Boëtius
1966	Female maturation procedure described and tested using dry carp pituitaries	Oliwereau & Fontaine
1964	Females matured and unfertilized egg produced based on carp pituitary.	Fontaine et al
1962	Female maturation attempted using human chorion gona-dotropin.	Boëtius et al
1939	Attemts to mature females using frog pituitaries	Hansen et al.
1935	First matured male eel.	Boucher et al.

Figure 3. Time line showing major events in the progress of producing larvae of European eel, Anguilla anguilla in captivity. Grey text indicates milestones in Japanese eel, A. japonica reproduction studies.

Another breakthrough in European eel was in the early 80'ies, where a Belorussian research team managed to successfully rear a batch of larvae, reaching an age of 3½ days post hatch (Bezdenezhnykh et al., 1983; Prokhorchik, 1986, 1987). This achievement became a milestone as no larvae of European eel were hatched for the following 20 years. In 2000, Benedikte Pedersen, University of Copenhagen, successfully applied the protocols developed for Japanese to the European eel and succeeded in producing larvae although limited number and longevity i.e. 1.5 days (Pedersen, 2004, 2003). A European Fisheries Fond project in 2005 was the onset of collaboration between Danish industry and researchers at DTU Aqua. This partnership generated several batches of eel larvae (Fig. 4) of which several lived up to 5 days in 2007 (Tomkiewicz and Jarlbæk, 2008) and succeeded in mass production of larvae of which some lived through the yolk sac phase of ~12 days (Tomkiewicz,

Figure 4. Newly hatched European eel larvae. The clear round oil droplet clearly aids buoyancy. Photo Sune Riis Sørensen

2012). The following years more and frequent batches of larvae were hatched with increasing quality and longevity (Fig. 5). Currently, research and development is ongoing on a European Commission FP7 project PRO-EEL focus on larval culture and up to 25 day old larvae have been reared during experiments. Within the same period, several institutes achieved to mature European eels and in a single case to hatch hybrid larvae of European eel and Short finned eel (*A. australis*) living up to 5 days. However, the Danish and Belorussian hatching of European eel larvae were the only records until very recently, where Italian researchers obtained eggs and fertilization from both stripping and natural spawning of European eel. Although the fertilization percent are around 1.5 to 8 % , they succeeded in obtaining 12 day old larvae (Mordenti et al., 2013).

Figure 5. European eel larvae 14 days post hatch. Photo Sune Riis Sørensen.

Timing of stripping is a crucial factor as matured and ovulated eggs quickly become overripe and this well-known contributing factor to poor egg quality in aquaculture (Bromage et al., 1994). Natural spawning can produce better egg quality as opposed to stripping, mainly due to that timing of stripping requires cautious surveillance of oocyte development and ovulation (Miura et al., 2013; Nomura et al., 2013). Stripping of gametes however, have been used during the Danish project work (Pedersen, 2004; Tomkiewicz and Jarlbæk, 2008; Tomkiewicz, 2012) and with the increasing research-based technology and practical experience, better and better results are obtained with sometimes more than 300.000 eggs in a batch and

close to 100% fertilization. Unlike natural spawning, stripping facilitates a wide range of small scale experiments with controlled environmental parameters, and hence enables us to get important knowledge on how to optimize the reproduction of European eel. Our current ability to produce frequent and large volumes of viable gametes of European eel gives a unique opportunity to study aspects important to successful European offspring production and larval rearing, in particular biophysical factors related to activation and fertilization, incubation and larval culture conditions. Most of these aspects were hardly known when my work started.

1.3 Gaps in knowledge

By the ability to overcome the natural inhibition of maturation in European eels and generate viable gametes, the Danish project REEL enabled a historic opportunity for regular offspring production. As a consequence, research focus shifted from gamete production to studies of gamete fertilisation capacity and biophysical requirement of early life stages needed for culture of larvae. Such larval production opens for new opportunities in aquaculture of this endangered species that presently rely on wild-caught fry. The current project PRO-EEL to which my PhD relates, has as an aim to extend knowledge on factors influencing the fertilisation process and egg quality as well as embryonic survival and development of European eel larvae. This pioneering research is needed to progress offspring production, leading to frequent mass hatching and enhanced survival throughout the yolk sac stages and hence enter the ultimate goal of PRO-EEL with feeding larvae. The research in this thesis addresses factors and processes related to activation, fertilisation, embryogenesis and larval development in order to enhance insight in the biology of early life stages and apply this knowledge for optimising and standardising hatchery protocols.

Particular challenges in breeding experiments include inconsistent results obtained in activation and fertilization trials, embryonic development failure as well as high mortality during incubation and larval culture. No documented protocol exist for optimal sperm:egg ratio and activation conditions for application in standardised *in vitro* fertilisation methods or culture conditions leading to healthy embryonic and larval culture. Advanced research results on Japanese eel, *Anguilla japonica*, being similar in many ways to the European eel (Tsukamoto, 2009) can teach us a lot. Thus, reproduction protocols applied in European eel build on methods developed by Japanese researchers in recent decades. Japanese protocols are however still not ideal and many parameters differ among the two species which calls for basic research and technology specifically for European eel. Since no European

Figure 6. Salinity profile of in the Sargasso Sea in the proposed spawning area, including temperature contours. Data obtained from the Danish Galathea expedition in 2007.

eel spawners and eggs have been obtained from the Sargasso Sea (Tsukamoto et al., 2011), we do not know, at which depth and temperature eggs prevail, or which temperature is optimal for development. Japanese eel eggs and larvae seem to need higher temperatures ~24°C for optimal development than the European eel ~21°C (H. Ohta et al., 1997; Ahn et al., 2012; Tomkiewicz and Jarlbæk, 2008; Tomkiewicz, 2012). Bonhommeau et al (2008) shows that the temperature increase in the Sargasso Sea spawning area from 21.4°C in 1980ies to 22.0°C in 2000 (Bonhommeau et al., 2008) is correlated to the well-known decline in recruitment of European eel described by (Dekker, 2008). The influence of such changes on early life stages can however best be assessed through experimental studies. Similarly, the salinity profile in the Sargasso Sea has been recorded (Fig. 6), however, knowledge about buoyancy of the eggs is lacking as well as the depth at which spawning occurs. The proposed spawning area is believed to be within a depth range of 50 to 200 meters (Castonguay and McCleave, 1987), which entails salinities in the range 36-37 psu (Fig. 6), but, how variation in salinity affects *in vitro* activation, fertilization and incubation, we do not know. Besides the questioned salinity and temperature conditions eggs and larvae develop in a unique microbial environment in the oligotrophic Sargasso Sea (Li et al., 1992). Hatchery rearing likely entails a very different microbial environment (Skjermo and Vadstein, 1999; Blancheton et al., 2013) and how this species will be able to cope with the microbial environment in a hatchery setup is unexplored. These examples emphasise the importance of studying these basic parameters to learn about eel early life history both for application in conservation biology and as well as aquaculture technology development.

2 Objectives of my PhD

Production of European eel larvae requires knowledge about the influence of physical and chemical parameters on embryonic and larval development, including suitable temperature and salinity conditions, as well as information on the potential role of microbial interference. The aim was to address these parameters through a series of controlled experiments. A prerequisite for this work was the development of standardised fertilisation protocols, including determination of e.g. sperm density and optimal egg:sperm ratio. Such studies would contribute to improvement of reproductive output following gamete production in hormonally matured European eels. Three key questions were posed:

Activation and fertilization –how can success be optimized? *(Paper 1 and 2)*

Determination of sperm density and quality is needed to optimise and standardize the mix of gametes in fertilisation protocols. Furthermore, salinity and sea water composition can affect egg activation and fertilization. The objective of this work was to address these variables and develop standardised fertilisation protocols. In this context, methods to evaluate the outcome of egg activation and fertilization, characterisation of egg quality are required. An aim was therefore also to identify egg quality characteristics including information of early cell cleavage quality, size and buoyancy as well as proportions of activated, unactivated, unripe and dead eggs for application as evaluation criteria. Specific objectives included to:

- Establish methods for accurate and practical quantification of sperm density
- Identify the optimal ratio of sperm to eggs for unaffected high fertilization
- Develop criteria to assess egg quality, and early embryonic development capacity
- Assess adequate salinity and sea water type for use in egg activation and fertilization protocols

Incubation conditions –how can egg quality be improved? *(Paper 3, 4 and 5)*

Successful incubation procedures require knowledge about the optimal range of temperature and salinity conditions during the embryonic development as well as efficient water management systems to control microbial interference. In this context, the objective was to evaluate effects of temperature and salinity conditions on embryonic development, as well as effects of microbial communities on incubation success. In addition, the experiments revealed an egg parasite unknown in eel, a syndinian dinoflagellate, and the impact of this species on egg development success was investigated. Hence specific objectives included to:

- Evaluate effects of salinity and sea water type on incubation success
- Identify optimal temperature range for healthy embryonic development
- Assess the role of microbial interaction during incubation on embryonic viability and hatch success
- Describe the syndinian dinoflagellate and its potential effects on embryonic development

Larval culture –how can survival be enhanced? *(Paper 3)*

Oceanic pelagic larvae likely calls for distinct culture conditions that differ from standard rearing systems of a hatchery. In this context, the objective was to identify parameters important for rearing technology in early larval culture, including the microbial conditions and what parameters to address in suitable culture systems. Specific objectives included to:

- Test effects of egg disinfection effects on larval survival
- Assess the effects of microbial interaction on yolk sac larvae

3 Findings of my PhD

A frequent obstacle associated with captive breeding of fish in aquaculture is variable egg quality (Migaud et al., 2013). Often low survival rates reduce offspring production compared to initial output of eggs prevail (Bromage et al., 1994) and many external and environmental causes have been found, but no common link, trait or molecular characteristics across teleost eggs have yet been identified (Migaud et al., 2013). A great deal is known on salinity, temperature, microbial, and water quality effects on early life stages of many fish species, however for the European eel equivalent knowledge does not exist.

In the pioneering work with reproduction in European eel, variability in quality among egg batches is pronounced and although we strive towards stable rearing conditions a big batch of embryos may stop living, while others batches continue embryonic development and hatch. Often, the maturational protocols and variation in female characteristics and responsiveness are assumed to be the reason. Certainly, the maturation process is still sub-optimal and a great deal of improvement lies ahead, as e.g. pituitary extracts (PE) used to induce maturation of females are only analogue to eel hormones or natural triggers. Even so, it is major step forward that we can induce maturation by hormonal treatment and regularly obtain viable gametes, however the further production of competent embryos and larvae needs insight in basic requirements of early life stages in order to develop relevant hatchery technology.

3.1 Activation and fertilization –how can success be optimized? (Paper 1 and 2)

Summary: Spectrophotometry was found to be a precise proxy for sperm density, and supported the identification of 2.5×10^4 sperm cells per egg as being optimal for fertilization protocols. Salinity was found to be a parameter of key importance in egg activation and increase of egg size, however egg batches from individual females proved highly variable regarding tolerance and optimum. A positive relationship was found between activation salinity and neutral buoyancy of eggs, and the salinity independent neutral buoyancy was estimated to 33.8 psu. Artificial sea water was found capable of successful activation and a group of salt types supported large perivitelline space (PVS) formation in contrast to others. These salt types did not differ with respect to fertilization or buoyancy characteristics, besides NaCl treatments being unsuited for activation and fertilization.

Dry fertilisation of stripped eggs is often practiced in captive fish breeding, i.e. stripping fresh semen over the eggs, mixing gametes and adding water. Although, this procedure may be adequate in practical hatchery procedures, such methods may cause variability in fertilization success and zygote development. This is undesirable when the eggs are intended for subsequent controlled experiments to identify effects of external factors, imposing on the fertilised eggs. Therefore, an initial task was to standardise and optimise fertilisation procedures, ensuring homogenous gamete mixtures for my further studies.

Homogeneous mixing of gametes and standardization of fertilisation procedures

Unlike many other fishes, European eel females contain no ovarian fluid (Pedersen, 2004, 2003; Tomkiewicz and Jarlbæk, 2008). Therefore, adequate mixing of gametes is difficult, and accordingly, diluents for sperm storage and mixing of eggs and sperm have been developed for Japanese eels (Hiromi Ohta et al., 1997). Similarly, artificial seminal plasma, mimicking that of high quality males, has been formulated for safe sperm cryopreservation of European eel (Asturiano et al., 2004). This artificial seminal plasma for European

eel was initially tested for applicability as a diluent for use in the development of improved fertilisation protocols.
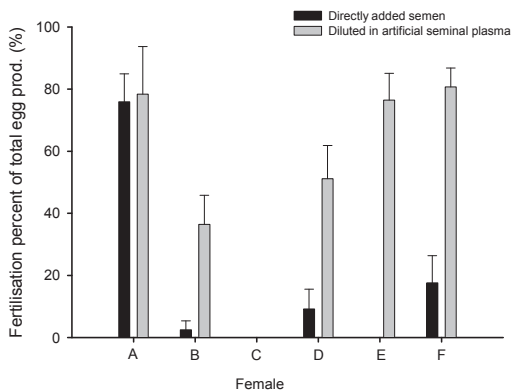

Figure 7. Fertilisation success of eggs of European eel, Anguilla anguilla, fertilised using directly added semen (black bars) and semen diluted in sperm extender P1 (Asturiano et al., 2004) from six different females. Error bars represent STD. (Sørensen et al. unpubl data).

From each of six females, a specific amount of eggs divided into two portions (three replicates) were fertilised by adding semen either in pure or diluted form. Results showed the diluted semen led to a higher and less variable fertilisation success (Fig. 7). The implementation of this artificial sperm plasma as sperm diluent has been instrumental for the development of standardised, optimised fertilisation procedures and the following small scale fertilization experiment. A main advantage is that it ensures a homogenous mixture of gametes and thus representative sampling of gametes and low variation due to uniform egg sperm contact conditions. Furthermore, it facilitated a preparation of sperm prior to experiments, considering standardisation of sperm concentration. Additional positive effects of this medium include sperm immobilisation (Paper 2).

Sperm density

Identification of precise sperm quantification techniques was another aim in the development of fertilization procedures that targeted establishment of a constant sperm to egg ratio in small and large scale experiments. Sperm to egg ratio is known to be important in fish species with external fertilisation (Butts et al., 2009). Initially, I estimated and applied the sperm concentration using the spermatocrit, a method that has proven useful as a proxy for sperm density, e.g. in cod, *Gadus morhua*, (Trippel, 2003).

An evaluation of the accuracy of spermatocrit as well as different alternative methods was conducted in order to improve and document the sperm to egg ratio for a standardised fertilization protocol (Paper 1). The results obtained on the accuracy of spermatocrit for quantification of sperm cells showed high variation between replicates and samples, and relatively low precision of the method. Computer Assisted Sperm Analysis, CASA, using multiple frames/captures and flowcytometry, all providing automated quantification of sperm density were similar in precision to manual sperm counts, using manual hemocytometer. The latter is known for its accuracy and precision and thus useful for evaluation, although being time consuming and demanding with regard to operator training (Paper 1). However, constraints in equipment cost and level of operator training rendered CASA and flowcytometry suboptimal, and instead applicability of spectrophotometry was tested (Paper 2). A spectrophotometer measures light absorbance at chosen wavelengths, and the wavelength best for density approximation corresponds to the highest light absorption from sperm samples due to e.g. cell proteins and lipids. Sperm density was thus approximated using spectrophotometry and the resulting species specific regression analysis revealed a significant

positive relationship between sperm density and absorbance at 350 nm (R2 = 0.94, p < 0.001, y = 2.273e+10x - 2.805e+10) (Paper 2).

Sperm to egg ratio and time before activation

Identification of the minimum amount of sperm needed for high fertilization success, makes it possible to eliminate excess sperm that potentially can serve as substrate for microbial growth (Rosenthal et al., 1988) or increase oxygen consumption from sperm activation (Alavi et al., 2008). We tested the effect of 12 different sperm densities ranging from 1.3×10^3 to 1.0×10^6 sperm cells per egg as well as the time lag from stripping to activation on egg fertilisation success.

The results showed that 2.5×10^4 sperm cells per egg would be sufficient to sustain a high level of fertilization, yet being the lowest number avoiding excess sperm. The ratio was based on dilution of 1:99 in P1 medium as described above and addition of 1ml diluted sperm per 2 gram eggs (Paper 2). Furthermore activation within 10 minutes was found needed in order to avoid a negative impact on fertilization success.

Assessment of fertilization success

Fertilization success is a simple and valid early predictor of egg quality (Bobe and Labbé, 2010). Although, fertilization rate is an important measure, it does not include information about specific characteristics of neither fertilised nor unfertilised eggs. This information was captured through the development of a categorisation system based on specific characteristics of eggs sampled after fertilisation (Paper 4). This included both information about cleavage pattern of fertilised eggs and features pertaining to unfertilised eggs that would otherwise be lost. Such patterns may however possess valuable information identifying e.g. repeated patterns pertaining to females or treatments.

This categorisation system (Fig. 8), made it possible to evaluate results of procedures and treatments in experiments considering the substantial female impact in eel. The system included tree sub-categories of fertilized eggs distinguished by the cell cleavage pattern, which is similar to criteria described for e.g. cod and turbot (Kjørsvik, 1994; Kjørsvik et al., 2003).

The categorisation of eggs was made 3 to 6 h post activation. The fertilisation percentage was as the proportion of overall fertilized eggs, T-1. The categorisation system proved

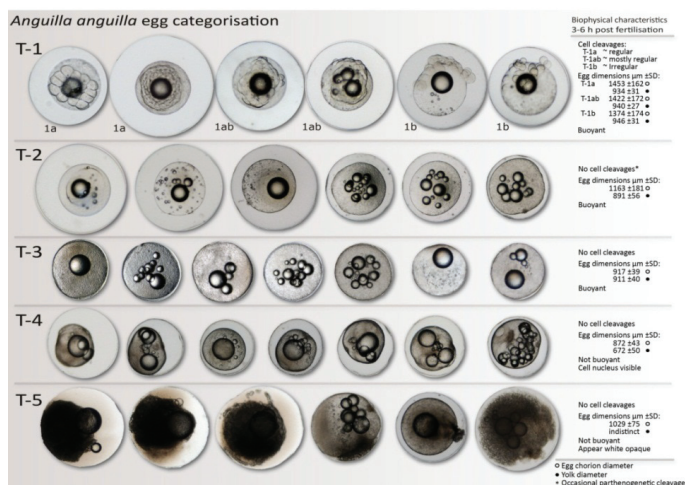*Figure 8. Categorisation of European eel, Anguilla anguilla, eggs fertilized in vitro and incubated at 20 ± 0.5˚C. Five main types of eggs are displayed based on morphology and buoyancy at 35 psu, 3-6 h post fertilization. T-1 are fertilized eggs and subdivided into three sub-categorisations based on cell cleavage pattern: T-1a: regular and symmetric; T-1ab: mostly regular with minor irregularities; T-1b: irregular.*

applicable to every egg batch stripped and useful in evaluation of small scale experiment (Paper 4) as well as in the general egg monitoring program during full scale experiments in project REEL and EU project PRO-EEL.

Salt dependent activation

The Danish reproduction studies during 2005 to 2007 found that, although fertilization of eggs was possible, formation of the characteristic large perivitelline space was often deficient, using artificial sea water i.e. Tropic Marin Sea Salt (Tomkiewicz and Jarlbæk, 2008). Such deficiency of PVS affects embryonic development that in the later stage curl up and die without the ability to hatch (Tomkiewicz and Jarlbæk, 2008) (Fig.9). Application of natural sea water for activation in fertilisation protocols enhanced successful swelling and subsequent hatch. Natural sea water, however, may vary in salinity and quality including microbial communities and in fact, activation salinity may in itself affect swelling as shown in the long rough dab, *Hippoglossoides platessoides limandoides* (Lønning and Davenport, 1980).
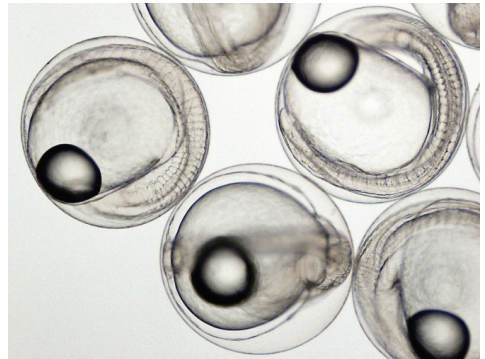
Figure 9. European eel egg with an insufficient PVS.

My aim was to investigate if different artificial sea salt types would be applicable and useful to improve and standardise activation conditions. I compared natural sea water with artificial salt water using different salt types that differ with respect to metal ions and elements (Atkinson and Bingman, 1997) (Paper 4). Many of such metal ions are known to affect the activation process and adequate swelling might still be attained using the proper salt. Therefore, a series of experiments that focussed on effects of different salt types (Fig. 10) was conducted (Paper 4). At same time tolerance of eggs to differences in salinities in the activation medium was tested. In fertilisation procedures addition of different components in particular the sperm diluent affects the final activation salinity. If this this is not considered, the ambient salinity during activation can vary considerably (Paper 4).

Figure 10. Sea salt types tested for activation of gametes of European eel. From the top left: Tropic Marine, Instant Ocean and from bottom left: Red Sea Pro, Red Sea, Reef Crystal, and Tetra Marine

The results showed that the swelling depended on both salinity and salt type, however with substantial variation among females. The degree of swelling and resulting egg size was inconsistent among females, and activation occurred over the entire range of salinities. Also the maximum size obtained differed among females with egg sizes up to ~1642 ± 8 µm at 35ppt (Paper 4). In contract fertilisation success, depended on salinity with highest fertilisation rates in the range 30-40 psu. In relation to salt type, two groups were distinguished; one giving large eggs with a large PVS and another consistently yielding small egg (Paper 4).

Egg size and fertilization obtained using natural sea water was more variable that those obtained from activation by several artificial sea salts. Two artificial salt types, Red Sea (RS) and Reef Crystal (RC) consistently produced large eggs with high fertilization rate. These salts together with natural sea water, Red Sea[pro] and Tropic Marin sea salt formed the group leading to large eggs. The corresponding yolk diameter did not change, probably due to impermeablity of the vitelline membrane during early embryonic development (Paper 4).

The results are discussed in view of mechanisms potentially affecting activation and fertilization of fish eggs and evoking such size differences. Factors that may contribute are passive influx via protein channels, aquaporins, that may be inhibited by certain free metal ions (Haddoub, 2009), enzymatic cleavage of glycoproteins, hyosophorins, released during the cortical reaction, which similarly can be inhibited by various free di- and trivalent metal ions (Eddy, 1983) as well as unsuitable salinities (Finn, 2007). Many of these inhibiting elements occur in the artificial sea salts used (Atkinson and Bingman, 1997; Hovanec and Coshland, 2004). In general, the observed egg chorion diameters were substantially larger than described in several earlier studies of European eel (Boëtius, 1967; Pedersen, 2003, 2004)(Paper 4). Furthermore, the regular cleaved eggs were significant larger than the eggs with irregularities in cleavage pattern. In total, 14% of fertilized eggs from 10 females showed egg chorion diameters ranging from 1600 μm to 1785 μm, which compare to the size range reported for wild-caught Japanese eel eggs (Tsukamoto et al., 2011; Yoshinaga et al., 2011). In addition, egg buoyancy at the blastula stage measured 7 HPF was positively related to the activation salinity. The salinity independent neutral buoyancy was estimated to be 33.8 psu ~ 1.0238 g×ml^{-1} (20˚C),

3.2 Incubation conditions –how can egg quality be improved? (Paper 3, 4 and 5)

Summary: At 30 HPF, a positive relationship between activation salinity in the range 30 to 40 psu and buoyancy of embryos was observed, while the activation salinity at 50 psu had a strong negative effect. Similarly, buoyancy of egg activated by the salt types leading to small eggs differed from the group with large eggs, with the small eggs reaching neutral buoyancy at higher salinities. Egg associated microbial activity had a major negative impact on hatch success and egg disinfection proved an efficient method to reduce microbial coverage on egg chorion surface. Temperature experiments using 16, 20 and 24°C were conducted, however analyses are still ongoing.

Salinity and salt type effects during incubation

The neutral buoyancy measured at the stage of embryo formation following incubation in different salinities, gives an indication of not only the ability for the eggs to maintain buoyancy during incubation but also their tolerance for unsuitable salinities. The ability to maintain buoyant is a prerequisite for hatching (Unuma et al., 2005) and we know that embryo formation is a milestone during the embryonic development in European eel often not possible to achieve in bad quality eggs (Tomkiewicz et al 2012).

The test of buoyancy 30 HPF show a positive relationship between activation salinity and buoyancy, similar to the one observed 7 HPF. However unlike 7HPF, the high salinity (50 psu) attains a clear deviation from the main pattern observed within 30 and 40 psu treatments. Hence this latter range probably supports the salinity limits enabling maintained buoyancy when the embryo is formed. In relation to this is a Japanese study showing egg hatch and survival to decrease in salinity above 42 psu (Okamura et al., 2007) supporting

negative effect at such high salinities. With regards to the buoyancy of eggs incubated in various salt types, the group of salt types supporting big eggs show an evenly and steady buoyancy which is comparable to the corresponding pattern observed at 7HPF. The two salt types resulting in small eggs do however attain their neutral buoyancy at a significantly higher salinity. The two treatments based on pure NaCl lost buoyancy completely. Since the salt types supporting eggs able to maintain a stable buoyancy until embryo formation is coinciding with the salt types resulting in a proper activation and swelling of eggs, it seem best suited for activation and incubation of eel eggs.

Temperature effects on embryology and survival

Natural temperature profiles in the Sargasso Sea indicate that temperatures in the proposed spawning area and depth is about 20-21 degrees (Castonguay and McCleave, 1987; Munk et al., 2010). A first experiment (in collaboration with Elin Kjørsvik, NTNU, integrated in a master thesis) tested embryonic development and hatch at three temperatures (16, 20, and 24 ˚C) in triplicate 5 L conical cylinders in a water bath (Davidsen, 2012). Larvae were hatched from all three temperatures, but survival was highest at 20 ˚C. The differences in embryonic development time are shown in Fig. 11 (Davidsen, 2012). In the lower panel of Fig. 11, these data in day degrees are merged with data from a similar study conducted in Japanese eel, showing that the embryonic development in the two species is alike.

A second series of experiments also in collaboration with Elin Kjørsvik, NTNU, focused on embryonic development, mortality, hatch rates and larval sizes. Egg batches from different females were incubated at 12, 16, 20, and 24 ˚C. Sample and data analysis is in the final phase and a manuscript is being drafted (Additional papers 1). These results will provide useful information regarding optimal rearing temperature and developmental constraints during embryology and the early yolk sac stage. Studies on the Japanese eel showed failure to hatch at 16 ˚C and 31˚C. Results therefore indicate a lower temperature tolerance in European eel compared to the Japanese eel. Preliminary data shows that a temperature about 20-21 degrees used in the different studies of this thesis is close to the optimum for European eel.

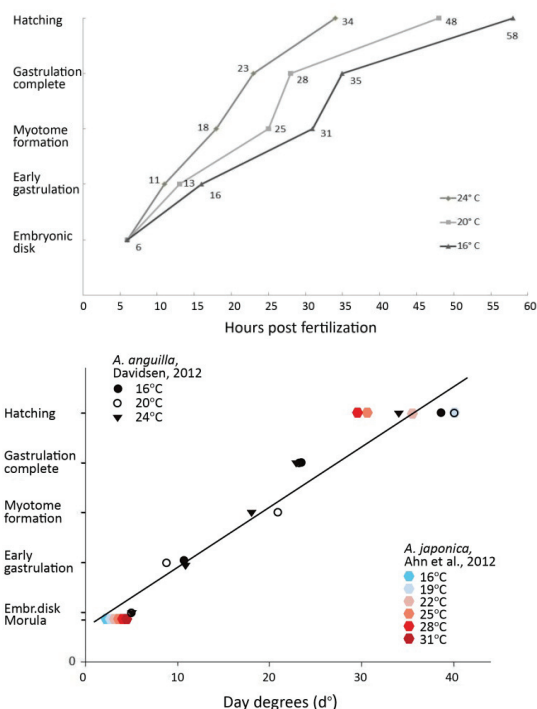Figure 11 Upper graph: Temperature dependent development time to reach successive embryonic stages of European eel A. anguilla at 16, 20 and 24˚C. Y-axis is embryonic stages. Lower graph: Same data in degree days merged and redrawn with results from Japanese eel, A. japonica at 16, 19, 22, 25, 28, and 31˚C at where 16 and 31˚C did not hatch. Data redrawn from Davidsen (2012) and Ahn et al., (2012).

Microbial interference during incubation

Microbial-induced mortality is a well-known factor in aquaculture (Attramadal et al., 2012b) and one of the main causes to mortality and suboptimal development during early life stages (Skjermo and Vadstein, 1999). Fish eggs are usually spawned into an aquatic environment full of pathogens (Swain and Nayak, 2009; Vadstein et al., 2012). During the embryonic phase or incubation, the chorion provides protection against direct contact with the microbial environment (Laale, 1980; Finn, 2007), however microbial activity on the egg chorion surface tends to impede embryonic development (Salvesen and Vadstein, 1995). Effects possible include insufficient gas exchange and waste product secretion across the chorion from the perivitelline fluid (Salvesen et al., 1997).

In European eel, the embryonic stage lasts only 48 h at 20 ˚C, after which the larvae hatch in a fairly undeveloped state (Fig. 12). Effects of microbial interference on embryonic survivorship and larval hatch were studied in detail using different levels of microbial control and egg disinfection (Paper 3). Microbial activity more halved the hatch success. This impact was evident from microbial control in merely the last half part of the incubation period. The observed magnitude of improvement, using antibiotic impediment of microbial activity, indicates that this phase of the embryonic development period is the most vulnerable. The degree to which different antibiotic treatments were able to restrain microbial activity was assessed by measuring growth rates of egg associated bacteria within these treatments. Some antibiotics proved bacteriostatic and some bactericidal, however no difference in hatch was observed between groups.

Egg surface disinfection tests did not show a relationship between the extent of microbial coverage and hatch rate. The physical effects caused by bacterial colony coverage had minor impact compared to microbial activity on the chorion surface. This indicates that eggs tolerate bacterial adhesion, while specific bacterial activity is more important.
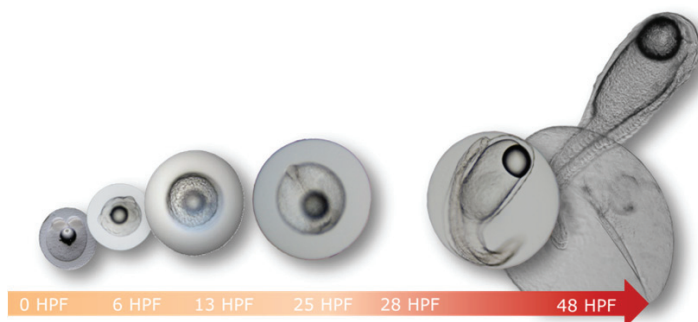
Figure 12. The incubation period of European eel, Anguilla anguilla at 20 ˚C

Disinfection agents are known to differ in efficiency among species and stage at which eggs are treated (Salvesen and Vadstein, 1995; Bergh and Jelmert, 1996; Salvesen et al., 1997). For eel eggs, hydrogen peroxide efficiently removed bacterial coverage on the egg chorion surface and featured good hatch success (Paper 3).

Thus, assessment of microbial growth and impediment from antibiotic on egg-associated microbiota indicated that microbial activity rather than physical coverage led to reduced hatch success. We therefore consider surface disinfection in combination with a controlled microbial environment to be of major importance for future egg incubation.

A new eel parasite

During these experiments, I unexpectedly discovered an endoparasite associated with the eggs and larvae, potentially an Ichthyodinium species that in some species can be of concern for larvae production (Buchmann, 2013). In order to identify the species, infected eggs were sampled and furthermore, a test was made to investigate the infection pathway, i.e. whether this organism was associated with the environment in the hatchery or of maternal origin. Furthermore, occurrence in specific egg batches was registered along with overall hatch success. This study became the first evidence that European eel host a syndinian dinoflagellate *Ichthyodinium,* most likely *I. chabelardi* (Hollande & Cachon, 1952).

The parasite appeared ~15 HPF and prevailed during embryonic development. This tiny organism is positioned on the surface of the vitelline membrane with sizes ranging from 5 to 100 μm. Parasites isolated from sampled eggs were analysed using small subunit ribosomal RNA, which confirmed that the organism was an *Ichthyodinium* species. The inclusion of several GenBank-derived sequences of environmental genes revealed that the parasite *Ichthyodinium sp.* has a cosmopolitan distribution.

Prevalence in the investigated egg batches seemed not to coincide with poor hatch. Lethal effect following proliferation of this parasite described from a few fish species was not observed neither in eggs nor larvae. In this light, European eel does not appear to be seriously affected by the presence of *Ichthyodinium*. Tests to investigate the infection pathway showed no difference in prevalence between sterile and normal stripped fertilized eggs, which indicates a maternally transferred pathway.

These first results indicate that this parasite does not impede captive breeding of European eel. Following inspection of eggs has revealed the parasite to be present in egg batches originating from domestic as well as wild broodstock. Our results suggest that *Ichthyodinium* is a naturally occurring parasite in eel, and likely present also the Sargasso Sea.

3.3 Larval culture –how can survival be enhanced? (Paper 3)

Summary: Disinfection of late egg stages at 25 HPF improved the survival of yolk sac larvae. Microbial activity had a profound impact on larval survival and bactericidal rearing conditions showed higher survival than merely bacteriostatic conditions. Microbial management is concluded vital for the success of larval rearing.

Effects of egg disinfection on yolk sac larvae survival.

Effects of microbial coverage on the egg surface are known to influence survival in fish eggs (Bergh et al., 1992; Morrison et al., 1999), however egg disinfection is often an effective method to reduce mortality and increase viability of hatched larvae, and it may even improve performance of less good quality eggs (Salvesen et al., 1997).

In the present studies, specific egg surface disinfection treatments during the last half of the incubation period significantly improved survival of yolk sac larvae, while others were inadequate or inefficient (Paper 3). Such improved larval survival agrees with several other studies on marine species (Bergh and Jelmert, 1996; Overton et al., 2010; Salvesen and Vadstein, 1995), and surface disinfection of eggs may be a beneficial procedure to adopt in breeding protocols for European eel. However, re-colonisation after

disinfections of eggs will occur and such treatments need to be accompanied by adequate water management procedures. This will be further discussed in Section 4 *Perspectives*.

Microbial activity during yolk sac stages

Upon hatch, larvae are equipped with a maternally transferred and mainly passive immune system (Swain and Nayak, 2009; Magnadottir, 2010) and the larval immune system will gradually develop as it moves from internal to external feeding. However, the degree to which the immune system is expressed differs and microbial interference in the oligotrophic Sargasso Sea will probably be substantially lower than what they experience in our aquaculture facilities. To what extent European eel larvae are capable of coping with this unnatural microbial environment of a hatchery is as yet unexplored, but needs to be addressed as it prerequisite for engineering a rearing system suitable for larval production of this species in captivity. We addressed the bacterial impact on larvae by application of various antibiotic treatments. Larval survival was evaluated, considering the antibiotic treatment's capability to restrain microbial growth.

The study showed that larval survival was significantly improved by impeding microbial activity during the last half of incubation and further during the larval yolk sac growth phase. Results were divided into three groups:

    1: No reduction in microbial growth, resulting in high larval mortality

    2: Bacteriostatic conditions, resulting in intermediate larval survival

    3: Bactericidal conditions, resulting in high larval survival

All together, these finding indicate either high amounts of pathogens in the environment, high sensitivity of European eel larvae, or that the eel larvae possess a specialised immune response adapted to the oceanic environment of the oligotrophic Sargasso Sea. The latter hypothesis seems to be supported by studies on Japanese eel comprising both wild-caught and captive reared larvae (Suzuki and Otake, 2000; Kawabe et al., 2012). They found a special immune adaptation in early eel larval stages. Although the larvae lack spleen and lymphoid tissue of the kidney, their thymus was well developed and lectin producing club cells appeared late in the yolk sac stage and with an exceptional high production of lectins (Suzuki and Otake, 2000; Kawabe et al., 2012). Lectins are known for they ability to act in recognition and neutralization of pathogens (Magnadottir et al., 2005). However, these may be rather specific towards pathogens (Cambi et al., 2005) which may relate to high sensitivity observed in yolk sac larvae reared in our hatchery.

These observations of larvae kept in hatchery water thus resemble often described effects of unsuitable microbial rearing conditions and can likely be greatly improved by implementing well documented techniques i.e. recirculation techniques, RAS, known to create increased stability (Attramadal et al., 2012b) and in particular in combination with microbial management where selection for a matured microbial community is pursued (Skjermo and Vadstein, 1999).

4 Perspectives

Within recent years and during the period of my PhD, our insight and capabilities, producing multiple and large batches of viable larvae have taken a major leap forward and has reached a state, where the challenges in larval culture, we experience, are similar to those often faced for new species in aquaculture. Less than a decade ago very few fertilization experiments had been successful, and the most progressed larval production was that of Prokhorchik and Bezdenezhnykh and their Belorussian research group, which in the mid-1980s makes the famous 3½ days old larvae of European eel (Bezdenezhnykh et al., 1983; Prokhorchik, 1987). Today, we have in the DTU lead research projects, multiple batches of larvae with longevity about 20 days post hatch, viable embryos are shipped by car or airplane for culture experiments in collaborator's labs, and feeding trials are ongoing. All this, I feel privileged to have been part of.

My PhD has addressed many of the obstacles we face in obtaining high quantities of viable European eel larvae. Although the female maturation protocol and timing of stripping is of pivotal importance to gamete quality, working with different parameters that influence gametes after stripping proved beneficial to enhance larvae production. Within these studies, the research I formed, developing standardised techniques for gamete activation and fertilisation as well as categorisation of egg quality to evaluate experiments, improved production of viable embryo and contributed to standardised future reproduction protocols in European eel.

With regard to salinity conditions for application in protocols for *in vitro* fertilization, our results in combination with reported effects on Japanese eel larvae (Okamoto et al., 2009), calls for awareness of conditions during activation. Although the salinity range tested, spans much wider than the range found in the Sargasso Sea, such salinity deviations are not unrealistic in small scale experiments, where e.g. evaporation from test beakers or mixing of media in small quanta can invoke critical salinity deviations.

Our results in relation to increased standardization and stability among fertilizations, using artificial salt water activation and fertilisation medium, have advantages given that the right salt mix is applied. In particular, Red Sea and Reef Crystal sea salts mixes seem to have good activation properties and consistently formed large egg chorion diameters and PVS. These results may prove important in future fertilisation protocols,

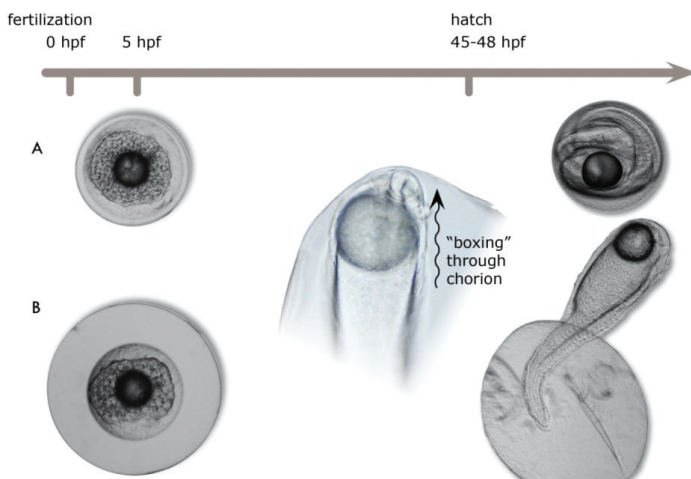*Figure 13: Scenario illustrating the difference in formation of perivitelline space (PVS) in European eel Anguilla anguilla and results for larval hatch that is accomplished through enzymatic activities and larval boxing to break the chorion. A: Eggs with weakly developed PVS and impeded hatch. B: big PVS and thus free movement of embryo to break through the chorion.*

addressing specifically the frequently observed failure in egg swelling that may impair successful hatch. The lack of ability to expand the PVS, pertaining to e.g. salt types IO and TE, is observed to impede embryo movement. Consequently, the embryos are incapable of normal vigorous movements seen in relation to hatch, when repeated "boxing" motion is exerted on the chorion (Fig. 13), which in combination with a thick and elastic chorion may lead to hatching failure.

Figure 14. Above a newly hatched European eel larvae and below picture of Japanese eel larvae close to hatch showing the head region with hatching enzymes stained fluorescent green (Hiroi et al 2004). Scale bar: 200 μm

In contrast, properly swelling of eel eggs appeared to prompt a thin, crisp chorion. During egg activation, the cortical reaction induces egg swelling, but also release chorion hardening proteins like alveolin which together with e.g. Ca^{2+} hardens the chorion (Masuda et al., 1991; Shibata et al., 2000). Such lack of hardening may well be part of the reason why small eggs tend to possess a rubber-like chorion (Kjørsvik and Lønning, 1983). It has been demonstrated that Japanese eel embryos produce and secrete hatching enzymes in the head region at the time of hatch (Hiroi et al., 2004) (Fig. 14). This aids the hatching through proteolytic perforation of the chorion, a process that may be hampered by a thick and rubbery chorion, or the unhardened chorion featuring a different protein structure (Robles et al., 2007). Further insight into the effects of specific chemical components in salts on the activation, swelling and chorion hardening as well as the action of hatching enzymes would promote optimisation of these processes in culture.

Furthermore, the experimental results showed that biotic effects, in particular the interaction between eggs and larvae and microbial communities in the ambient environment, needs to be addressed. Microbial management is fundamental to future success establishing large scale feeding larval culture. The egg as well as the larvae appeared to be highly susceptible to microbial interference in the experiments performed.

The control of the microbial environment was found be of paramount importance to success in the development of future incubation and larval culture systems and practices. The present results show that surface disinfection of eggs may be beneficial in breeding protocols for European eel; however, we did not yet investigate how re-colonisation of eggs and larvae occurred after disinfections. Without proper microbial management, re-colonisation will likely appear fast by microbial adhesion (Hansen and Olafsen, 1989; Morrison et al., 1999). Bacteria produce *adhesins,* which acts to recognise surface receptors of host tissue known as tissue and host tropism (Klemm, 2010). Re-colonisation is thus likely to occur with a similar microbial species composition as before disinfection and selection of opportunistic bacteria may even be promoted. The results obtained, indicate which paths to follow in future incubation and larval culture technology and protocols.

A drawback in any bactericidal treatment in egg and larval culture is that it inevitably leaves a virgin environment of no stability or "Shelter effect" by slow growing bacteria (Salvesen and Vadstein, 1995; Attramadal et al., 2012a; Blancheton et al., 2013). Therefore such treatments often lead to an intensive growth of fast growing opportunist (Hess-Erga et al., 2010), frequently pathogenic species (Wedekind et al., 2010; Attramadal et al., 2012a). Disinfection could prove useful, if treatment of eggs is performed detached from main water and filter systems and with the eggs being passed on to a matured water system. Such

mature water systems can be promoted by controlled nutrient supply to select for slow growing K-strategists (Andrews and Harris, 1986; Skjermo and Vadstein, 1999; Vadstein et al., 2012) or microbial communities can be selected by supporting or supplementing probiotic bacteria (Bjornsdottir et al., 2010). Application of mature water technology not only has the potential to lower the negative microbial impact on egg incubation and survival of hatched larvae, but likely also promote onset of a diverse and stabilizing gut microbiota needed for feeding and on-growing (Tinh et al., 2008). Such stable microbial communities in culture contrast e.g. axenic or antibiotic controlled environments. Although yolk sac larvae may develop adequately in a bacteria free environments, digestion will subsequent be hampered by lack of a bacterial flora in the digestive system, when external feed intake starts (Hansen and Olafsen, 1999).

Although, occurrence of multi-resistant bacteria is increasing, high quantities of antibiotics are still used in today's aquaculture (Cabello, 2006; Defoirdt et al., 2011). However, prophylactic use of antibiotics is not a sustainable way to prevent negative microbial interaction, and evidence is increasing that aquaculture acts as a vector for resistant bacteria to other ecosystems, affecting also humans (Cabello et al., 2013). Finding sustainable and environmentally friendly alternatives is a necessity (De Schryver et al., 2012). Fortunately for future production of eel larvae, the past two decades have given rise to a wide variety of alternatives to counteract pathogenic bacteria, like probiotics, bacteriophages, PHB, disrupted quorum sensing and short chain fatty acid growth inhibitors (Vine et al., 2006; Tinh et al., 2008; Defoirdt et al., 2011; De Schryver et al., 2012). Besides these approaches, reducing virulence or growth rates of microbes using such agents, much effort is also done to refine Recirculated Aquaculture Systems (RAS) to obtain stabile and controllable microbial communities. The basic model of microbial control in such systems is described by Vadstein et al. (1993), where control is obtained either through nonspecific reduction of bacteria by e.g. substrate reduction thus lowering the carrying capacity, or by promoting conditions for beneficial bacteria i.e. probiotic bacteria. Through such selection, a system gradually matures and becomes dominated by high microbial species diversity of slow growing forms, creating a stable microbial community characterised by K-strategists as opposed to r-strategists (Andrews and Harris, 1986; Skjermo et al., 1997).

Such a matured water system may be suited for future European eel larvi-culture, but need also to consider other specific requirements of eel embryos and larvae besides the microbial community management. This includes e.g. the physical systems for supporting an efficient exchange of water without obvious negative effects on larval longevity. A hypothetic RAS system for culture of eel larvae is outlined in Fig. 15. The filter unit serves to remove organic material and contributes to lowering of the carrying capacity by using a pore size gradient in which feed and faeces are retained in the first part (filter strainer) followed by a second filter unit, i.e. a sand-filter and finally a tangential pore filter at 0.2-0.8 µm. Water leaving the filter is treated with Ozone or UV-treatment to reduce build-up of microbes in tubes leading to reservoir and Biofilter.

Matured water RAS system
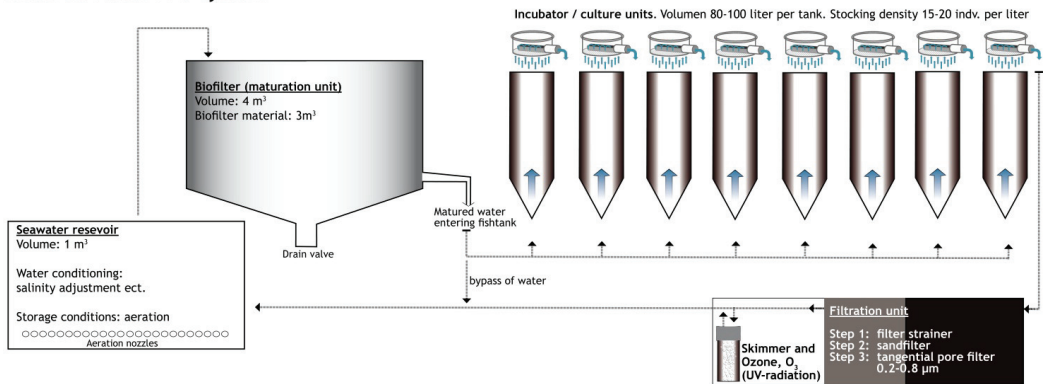
Figure 15. Schematic principle of matured water RAS system for European eel larvae culture. Se description in text.

The choice of UV or ozone would need dedicated tests similar to those of Attramadal et al. (2012a). These two antimicrobial treatments are fundamentally different, ozone being a chemical process and UV a physical radiation destroying unprotected living cells, e.g. bacteria. Ozone was found to give better survival and growth in a cod larvae RAS (Attramadal et al., 2012a) and will likely also prove beneficial in an eel larvae RAS. Ozone administration is done in association with a protein skimmer ensuring, besides important removal of soluble proteins and particles, the most effective reaction within the skimmer airstream (Suantika et al., 2003). Furthermore the air contact within the skimmer prevents leaking reactive radicals into the main system.

5 References

Ahn, H., Yamada, Y., Okamura, A., Horie, N., Mikawa, N., Tanaka, S., Tsukamoto, K., 2012. Effect of water temperature on embryonic development and hatching time of the Japanese eel *Anguilla japonica*. Aquaculture 330–333, 100–105.

Alavi, S.M.H., Linhart, O., Coward, K., Rodina, M., 2008. Fish spermatology: Implications for aquaculture management, in: Fish Spermatology. Alpha Science, pp. 397–460.

Andrews, J.H., Harris, R.F., 1986. r- and K-selection and microbial ecology. Adv. Microb. Ecol. 9, 99–147.

Asturiano, J.F., Pérez, L., Garzón, D.L., Marco-Jiménez, F., Peñaranda, D.S., Vicente, J.S., Jover, M., 2004. Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiol. Biochem. 30, 283–293.

Atkinson, M.J., Bingman, C., 1997. Elemental composition of commercial seasalts. J. Aquaric. Aquat. Sci. VIII, 39–43.

Attramadal, K.J.K., Øie, G., Størseth, T.R., Alver, M.O., Vadstein, O., Olsen, Y., 2012a. The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. Aquaculture 330–333, 121–129.

Attramadal, K.J.K., Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2012b. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac. Eng. 46, 27–39.

Bergh, Ø., Hansen, G.H., Taxt, R.E., 1992. Experimental infection of eggs and yolk sac larvae of halibut, *Hippoglossus hippoglossus* L. J. Fish Dis. 15, 379–391.

Bergh, Øi., Jelmert, A., 1996. Iodophor Disinfection of Eggs of Atlantic Halibut. J. Aquat. Anim. Health 8, 135–145.

Bezdenezhnykh, V.A., Prokhorcik, G.A., Petrikov, A.M., Petukov, V.B., Plyuta, M.V., 1983. Obtaining the larvae of European eel *Anguilla anguilla* L.(Pisces, *Anguillidae*), under experimental conditions, in: Dokl. Akad. Nauk SSSR. pp. 1264–1266.

Bieniarz, K., 1977. Investigations on inducing sexual maturity in the male eel *Anguilla anguilla*. J. Fish Biol. 10, 555–559.

Bjornsdottir, R., Karadottir, E.G., Johannsdottir, J., Thorarinsdottir, E.E., Smaradottir, H., Sigurgisladottir, S., Gudmundsdottir, B.K., 2010. Selection of bacteria and the effects of bacterial treatment of Atlantic halibut (*Hippoglossus hippoglossus* L.) eggs and larvae. Aquaculture 302, 219–227.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d' Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquac. Eng. 53, 30–39.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165, 535–548.

Boëtius, I., 1967. Studies in the European eel, *Anguilla anguilla* (L.) : Experimental induction of the male sexual cycle, its relation to temperature and other factors. C.A. Reitzels Forlag, 1967. Print.

Boëtius, J., 1962. Studies of ovarial growth induced by hormone injections in the European and American eel (*Anguilla anguilla* L. and *Anguilla rostrata* Le Sueur). [s.n.], 1962. Print.

Boëtius, J., 1967. Experimental indication of lunar activity in European Silver eels, *Anguilla anguilla* (L.). Meddelelser Fra Danmarks Fiskeri- Og Havundersogelser — Volume 6, Issue 1-4,

Bonhommeau, S., Chassot, E., Rivot, E., 2008. Fluctuations in European eel (*Anguilla anguilla*) recruitment resulting from environmental changes in the Sargasso Sea. Fish. Oceanogr. 17, 32–44.

Boucher, S., 1934. The provoked maturation of the genital organs of the Eel. C. R. Seances Soc. Biol. Fil. 116, 1284–1286.

Bromage, N., Bruce, M., Basavaraja, N., Rana, K., Shields, R., Young, C., Dye, J., Smith, P., Gillespie, M., Gamble, J., 1994. Egg Quality Determinants in Finfish The Role of Overripening with Special Reference to the Timing of Stripping in the Atlantic Halibut *Hippoglossus hippoglossus*. J. World Aquac. Soc. 25, 13–21.

Bruun, A.F., Hemmingsen, A.M., Møller Christensen, E., 1949. Attempts to induce experimentally maturation of the gonads of the European eel, *Anguilla anguilla* L. Acta Endocrinol. (Copenh.) 2, 212–226.

Buchmann, K., 2013. Impact and control of protozoan parasites in maricultured fishes. Parasitology FirstView, 1–10.

Butts, I.A.E., Trippel, E.A., Litvak, M.K., 2009. The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod *Gadus morhua* L. Aquaculture 286, 89–94.

Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 8, 1137–1144.

Cabello, F.C., Godfrey, H.P., Tomova, A., Ivanova, L., Dölz, H., Millanao, A., Buschmann, A.H., 2013. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environ. Microbiol. 15, 1917–1942.

Cambi, A., Koopman, M., Figdor, C.G., 2005. How C-type lectins detect pathogens. Cell. Microbiol. 7, 481–488.

Castonguay, M., McCleave, J.D., 1987. Vertical distributions, diel and ontogenetic vertical migrations and net avoidance of leptocephali of *Anguilla* and other common species in the Sargasso Sea. J. Plankton Res. 9, 195–214.

Cresswell R., 1883. Aristotle's history of animals : in ten books. London : George Bell.

Davidsen, M., 2012. The effect of incubation temperature on embryonic development and muscle growth in yolk-sac larvae of the European eel (*Anguilla anguilla* L., 1758). Institutt for biologi.

De Schryver, P., Defoirdt, T., Boon, N., Verstraete, W., Bossier, P., 2012. Managing the microbiota in aquaculture systems for disease prevention and control, in: Infectious Disease in Aquaculture : Prevention and Control. Woodhead, pp. 394–418.

Defoirdt, T., Sorgeloos, P., Bossier, P., 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr. Opin. Microbiol. 14, 251–258.

Dekker, W., 2008. Coming to grips with the eel stock slip-sliding away, in: International Governance of Fisheries Ecosystems: Learning from the Past, Finding Solutions for the Future. American Fisheries Society, Symposium. Bethesda, Maryland., pp. 335–355.

Dollerup, J., Graver, C., M., 1985. Repeated induction of testicular maturation and spermiation, alternating with periods of feeding and growth in silver eels, *Anguilla anguilla* (L.). Dana 1985, 19–39.

Dufour, S., 2005. Dopaminergic Inhibition of Reproduction in Teleost Fishes: Ecophysiological and Evolutionary Implications. Ann.- N. Y. Acad. Sci. 1040, 9–21.

Dufour, S., Burzawa-Gerard, E., Belle, N.L., Sbaihi, M., Vidal, B., 2003. Reproductive Endocrinology of the European Eel, *Anguilla anguilla*, in: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer Japan, pp. 373–383.

Dufour, S., Fontaine, Y., 1985. The reproductive migration of the European eel (*Anguilla anguilla*) - the probable importance of hydrostatic-pressure in stimulation of the gonadotropic function. Bull. Soc. Zool. Fr.-Evol. Zool. 110, 291–299.

Durif, C.M.F., 2013. Magnetic compass orientation in the European eel. PloS One 8.

Durif, C.M.F., Dufour, S., Elie, P., 2006. Impact of silvering stage, age, body size and condition on reproductive potential of the European eel. Mar. Ecol. Prog. Ser. 327, 171–181.

Eddy, F.B., 1983. Formation of the perivitelline fluid in Atlantic salmon eggs (*Salmo-salar*) in fresh-water and in solutions of metal-ions. Comp. Biochem. Physiol. Pharmacol. Toxicol. Endocrinol. 75, 1–4.

Finn, R.N., 2007. The physiology and toxicology of salmonid eggs and larvae in relation to water quality criteria. Aquat. Toxicol. 81, 337–354.

Fontaine, M., 1936. Sur la maturation complète des organes genitaux de l'Anguilla male et lémission de ses produits sexuells. Comptes Rendus Académie Sci. 202, 313–314.

Ginneken, V.J.T., Maes, G.E., 2005. The European eel (*Anguilla anguilla*, Linnaeus), its Lifecycle, evolution and reproduction: A literature review. Rev. Fish Biol. Fish. 15, 367–398.

Grassi, G.B., 1896. The Reproduction and Metamorphosis of the Common Eel (*Anguilla vulgaris*). Proc. R. Soc. Lond. 60, 260–271.

Haddoub, R., 2009. Design, Synthesis and Assaying of Potential Aquaporin Inhibitors. Handb. Exp. Pharmacol. 190, 385–402.

Hansen, G.H., Olafsen, J.A., 1989. Bacterial Colonization of Cod (*Gadus morhua* L.) and Halibut (Hippoglossus hippoglossus) Eggs in Marine Aquaculture. Appl. Environ. Microbiol. 55, 1435–1446.

Hansen, G.H., Olafsen, J.A., 1999. Bacterial Interactions in Early Life Stages of Marine Cold Water Fish. Microb. Ecol. 38, 1–26.

Hess-Erga, O.-K., Blomvågnes-Bakke, B., Vadstein, O., 2010. Recolonization by heterotrophic bacteria after UV irradiation or ozonation of seawater; a simulation of ballast water treatment. Water Res. 44, 5439–5449.

Hiroi, J., Maruyama, K., Kawazu, K., Kaneko, T., Ohtani-Kaneko, R., Yasumasu, S., 2004. Structure and developmental expression of hatching enzyme genes of the Japanese eel *Anguilla japonica*: an aspect of the evolution of fish hatching enzyme gene. Dev. Genes Evol. 214, 176–184.

Hovanec, T.A., Coshland, J.L., 2004. A chemical analysis of select trace elements in synthetic sea salts and natural seawater. Sea Scope Aquar. Syst. 21.

Kawabe, M., Suetake, H., Kikuchi, K., Suzuki, Y., 2012. Early T-cell and thymus development in Japanese eel *Anguilla japonica*. Fish. Sci. 78, 539–547.

Kjørsvik, E., 1994. Egg quality in wild and broodstock cod *Gadus morhua* L. J. World Aquac. Soc. 25, 22–29.

Kjørsvik, E., Hoehne-Reitan, K., Reitan, K.I., 2003. Egg and larval quality criteria as predictive measures for juvenile production in turbot (*Scophthalmus maximus* L.). Aquaculture 227, 9–20.

Kjørsvik, E., Lønning, S., 1983. Effects of egg quality on normal fertilization and early development of the cod, *Gadus morhua* L. J. Fish Biol. 23, 1–12.

Klemm, P., 2010. Prevention of bacterial adhesion. Appl. Microbiol. Biotechnol. 88, 451–459.

Laale, H.W., 1980. The perivitelline space and egg envelopes of bony fishes: A review. Copeia 1980, 210–226.

Li, W.K.W., Dickie, P.M., Irwin, B.D., Wood, A.M., 1992. Biomass of bacteria, cyanobacteria, prochlorophytes and photosynthetic eukaryotes in the Sargasso Sea. Deep Sea Res. Part Oceanogr. Res. Pap. 39, 501–519.

Lønning, S., Davenport, J., 1980. The swelling egg of the long rough dab, *Hippoglossoides platessoides* limandoides (Bloch). J. Fish Biol. 17, 359–378.

Magnadottir, B., 2010. Immunological Control of Fish Diseases. Mar. Biotechnol. 12, 361–379.

Magnadottir, B., Lange, S., Gudmundsdottir, S., Bøgwald, J., Dalmo, R.A., 2005. Ontogeny of humoral immune parameters in fish. Fish Shellfish Immunol. 19, 429–439.

Masuda, K., Luchi, I., Yamagami, K., 1991. Analysis of Hardening of the Egg Envelope (Chorion) of the Fish, *Oryzias latipes*. Dev. Growth Differ. 33, 75–83.

Meske, C.H., 1973. Experimentally induced sexual maturity in artificially reared male eels (*Anguilla anguilla* L.) 161–170.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M. p., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Rev. Aquac. 5, S194–S223.

Miura, A., Nomura, K., Imaizumi, H., Jinbo, T., Masuda, Y., Tanaka, H., Ohta, H., 2013. Administration of 17α-hydroxyprogesterone into mature male Japanese eel reduces sperm motility by decreasing potassium ion concentrations in the seminal plasma. Aquaculture 414–415, 217–223.

Mordenti, O., Biase, A.D., Bastone, G., Sirri, R., Zaccaroni, A., Parmeggiani, A., 2013. Controlled reproduction in the wild European eel (*Anguilla anguilla*): two populations compared. Aquac. Int. 21, 1045–1063.

Morrison, C., Bird, C., O'Neil, D., Leggiadro, C., Martin-Robichaud, D., Rommens, M., Waiwood, K., 1999. Structure of the egg envelope of the haddock, Melanogrammus aeglefinus, and effects of microbial colonization during incubation. Can. J. Zool. 77, 890–901.

Munk, P., Hansen, M.M., Maes, G.E., Nielsen, T.G., Castonguay, M., Riemann, L., Sparholt, H., Als, T.D., Aarestrup, K., Andersen, N.G., Bachler, M., 2010. Oceanic fronts in the Sargasso Sea control the early life and drift of Atlantic eels. Proc. R. Soc. B Biol. Sci. 277, 3593–3599.

Nomura, K., Takeda, Y., Unuma, T., Morishima, K., Tanaka, H., Arai, K., Ohta, H., 2013. Post-ovulatory oocyte aging induces spontaneous occurrence of polyploids and mosaics in artificial fertilization of Japanese eel, *Anguilla japonica*. Aquaculture 404–405, 15–21.

Nordberg, P., Gelin, C., Broman, A., Bringéus, N.-A., 1985. Ål -från Sargasso till gillesbord, 1985. Settern, Ystad, Sweden.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Iinuma, N., Hirose, K., 1997. Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. Fish Physiol. Biochem. Fish Physiol Biochem Vol 17 163–169.

Ohta, Hiromi, Tanaka, H., Kagawa, H., Okuzawa, K., Iinuma, N., 1997. Artificial fertilization using testicular spermatozoa in the Japanese eel *Anguilla japonica*. Fish. Sci. Tokyo Fish Sci Vol 63 393–396.

Okamoto, T., Kurokawa, T., Gen, K., Murashita, K., Nomura, K., Kim, S.-K., Matsubara, H., Ohta, H., Tanaka, H., 2009. Influence of salinity on morphological deformities in cultured larvae of Japanese eel, *Anguilla japonica*, at completion of yolk resorption. Aquaculture 293, 113–118.

Overton, J.L., Bruun, M.S., Dalsgaard, I., 2010. Chemical surface disinfection of eggs of Baltic cod, Gadus morhua L. J. Fish Dis. 33, 707–716.

Palstra, A., 2007. Swimming stimulates oocyte development in European eel. Aquaculture 270, 321–332.

Pedersen, B.H., 2003. Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation of the eggs. Aquaculture 224, 323–338.

Pedersen, B.H., 2004. Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. Aquaculture 237, 461–473.

Poole, W.R., Reynolds, J.D., 1998. Variability in growth rate in European eel *Anguilla anguilla* (L.) in a western Irish catchment, in: Biology and Environment: Proceedings of the Royal Irish Academy. JSTOR, pp. 141–145.

Prokhorchik, G.A., 1986. Postembryonic development of European eel, *Anguilla anguilla*, under experimental conditions. J Ichthyol 26, 121–127.

Prokhorchik, G.A., 1987. Embryonic development of European eel, *Anguilla anguilla*, under experimental conditions. J. Ichthyol. 27, 124–131.

Righton, D., Aarestrup, K., Jellyman, D., Sébert, P., van den Thillart, G., Tsukamoto, K., 2012. The *Anguilla spp*. migration problem: 40 million years of evolution and two millennia of speculation. J. Fish Biol. 81, 365–386.

Robles, V., Cabrita, E., de Paz, P., Herráez, M.P., 2007. Studies on chorion hardening inhibition and dechorionization in turbot embryos. Aquaculture 262, 535–540.

Rosén, M., 2014. Världens äldsta ål död. Ystads allehanda, Simrishamn 7th Aug.2014

Rosenthal, H., Klumpp, D., Willführ, J., 1988. Influence of sperm density and contact time on herring egg fertilization. J. Appl. Ichthyol. 4, 79–86.

Rousseau, K., 2009. Silvering: Metamorphosis or Puberty? 39–63.

Salvesen, I., Øie, G., Vadstein, O., 1997. Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. Aquac. Int. 5, 249–258.

Salvesen, I., Vadstein, O., 1995. Surface disinfection of eggs from marine fish: evaluation of four chemicals. Aquac. Int. 3, 155–171.

Schmidt, J., 1912. The Reproduction and Spawning-Places of the Fresh-Water Eel (*Anguilla Vulgaris*). Nature 89, 633–636.

Schmidt, J., 1923. The Breeding Places of the Eel. Philos. Trans. R. Soc. Lond. Ser. B Contain. Pap. Biol. Character 211, 179–208.

Shibata, Y., Iwamatsu, T., Oba, Y., Kobayashi, D., Tanaka, M., Nagahama, Y., Suzuki, N., Yoshikuni, M., 2000. Identification and cDNA Cloning of Alveolin, an Extracellular Metalloproteinase, Which Induces Chorion Hardening of Medaka (*Oryzias latipes*) Eggs upon Fertilization. J. Biol. Chem. 275, 8349–8354.

Skjermo, J., Salvesen, I., Øie, G., Olsen, Y., Vadstein, O., 1997. Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquac. Int. 5, 13–28.

Skjermo, J., Vadstein, O., 1999. Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177, 333–343.

Suantika, G., Dhert, P., Sweetman, E., O'Brien, E., Sorgeloos, P., 2003. Technical and economical feasibility of a rotifer recirculation system. Aquaculture 227, 173–189.

Suzuki, Y., Otake, T., 2000. Skin lectin and the lymphoid tissues in the leptocephalus larvae of the Japanese eel *Anguilla japonica*. Fish. Sci. 66, 636–643.

Swain, P., Nayak, S.K., 2009. Role of maternally derived immunity in fish. Fish Shellfish Immunol. 27, 89–99.

Tinh, N., Dierckens, K., Sorgeloos, P., Bossier, P., 2008. A Review of the Functionality of Probiotics in the Larviculture Food Chain. Mar. Biotechnol. 10, 1–12.

Tomkiewicz, J., 2012. Reproduction of European eel in aquaculture (REEL), Consolidation and new production methods (DTU Aqua Report No. 249-2012), DTU Aqua Report. DTU Aqua.

Tomkiewicz, J., Jarlbæk, H., 2008. Kunstig reproduktion af ål: ROE II og IIB., DTU Aqua rapport nr. 180-08. DTU Aqua.

Trippel, E., 2003. Estimation of Male Reproductive Success of Marine Fishes. J. Northwest Atl. Fish. Sci., Journal of Northwest Atlantic Fisheries Science 33, 81–113.

Tsukamoto, K., 2009. Oceanic migration and spawning of anguillid eels. J. Fish Biol. 74, 1833–1852.

Tsukamoto, K., Chow, S., Otake, T., Kurogi, H., Mochioka, N., Miller, M.J., Aoyama, J., Kimura, S., Watanabe, S., Yoshinaga, T., Shinoda, A., Kuroki, M., Oya, M., Watanabe, T., Hata, K., Ijiri, S., Kazeto, Y., Nomura, K., Tanaka, H., 2011. Oceanic spawning ecology of freshwater eels in the western North Pacific. Nat. Commun. 2, 179.

Unuma, T., Kondo, S., Tanaka, H., Kagawa, H., Nomura, K., Ohta, H., 2005. Relationship between egg specific gravity and egg quality in the Japanese eel, *Anguilla japonica*. Aquaculture 246, 493–500.

Vadstein, O., Bergh, Ø., Gatesoupe, F.-J., Galindo-Villegas, J., Mulero, V., Picchietti, S., Scapigliati, G., Makridis, P., Olsen, Y., Dierckens, K., 2012. Microbiology and immunology of fish larvae. Rev. Aquac.

Van Ginneken, V., 2007. Does a 5500-km swim trial stimulate early sexual maturation in the European eel (*Anguilla anguilla* L.)? Comp. Biochem. Physiol. -Mol. Integr. Physiol. 147, 1095–1103.

Vidal, B., 2004. Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: A neuroendocrine lock for the onset of puberty. Biol. Reprod. 71, 1491–1500.

Vine, N.G., Leukes, W.D., Kaiser, H., 2006. Probiotics in marine larviculture. FEMS Microbiol. Rev. 30, 404–427.

Wedekind, C., Gessner, M.O., Vazquez, F., Maerki, M., Steiner, D., 2010. Elevated resource availability sufficient to turn opportunistic into virulent fish pathogens. Ecology 91, 1251–1256.

Yoshinaga, T., Miller, M.J., Yokouchi, K., Otake, T., Kimura, S., Aoyama, J., Watanabe, S., Shinoda, A., Oya, M., Miyazaki, S., Zenimoto, K., Sudo, R., Takahashi, T., Ahn, H., Manabe, R., Hagihara, S., Morioka, H., Itakura, H., Machida, M., Ban, K., Shiozaki, M., Ai, B., Tsukamoto, K., 2011. Genetic identification and morphology of naturally spawned eggs of the Japanese eel *Anguilla japonica* collected in the western North Pacific. Fish. Sci. 77, 983–992.

Paper 1:

Evaluation of Methods to Determine Sperm Density for the European eel, *Anguilla anguilla*

Evaluation of Methods to Determine Sperm Density for the European eel, *Anguilla anguilla*

SR Sørensen[1], V Gallego[2], L Pérez[2], IAE Butts[1], J Tomkiewicz[1] and JF Asturiano[2]

[1]*National Institute of Aquatic Resources, Technical University of Denmark, Charlottenlund, Denmark;* [2]*Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Valencia, Spain*

Contents

European eel, *Anguilla anguilla*, is a target species for future captive breeding, yet best methodology to estimate sperm density for application in *in vitro* fertilization is not established. Thus, our objectives were to evaluate methods to estimate European eel sperm density including spermatocrit, computer-assisted sperm analysis (CASA) and flow cytometry (FCM), using Neubauer Improved haemocytometer as benchmark. Initially, relationships between spermatocrit, haemocytometer counts and sperm motility were analysed, as well as the effect of sperm dilution on haemocytometer counts. Furthermore, accuracy and precision of spermatocrit, applying a range of G-forces, were tested and the best G-force used in method comparisons. We found no effect of dilution on haemocytometer sperm density estimates, whereas motility associated positively with haemocytometer counts, but not with spermatocrit. Results from all techniques, spermatocrit, CASA and FCM, showed significant positive correlations with haemocytometer counts. The best correlation between spermatocrit and haemocytometer counts was obtained at $6000 \times g$ ($r = 0.68$). Of two CASA variants, one or three photographic fields (CASA-1 and CASA-2), CASA-2 showed a very high accuracy to haemocytometer counts ($r = 0.93$), but low precision (CV: CASA-2 = 28.4%). FCM was tested with and without microfluorospheres (FCM-1 and FCM-2), and relationships to haemocytometer counts were highly accurate (FCM-1: $r = 0.94$; FCM-2: $r = 0.88$) and precise (CV: FCM-1 = 2.5; FCM-2 = 2.7%). Overall, CASA-2 and FCM-1 feature reliable methods for quantification of European eel sperm, but FCM-1 has a clear advantage featuring highest precision and accuracy. Together, these results provide a useful basis for gamete management in fertilization protocols.

Introduction

European eel, *Anguilla anguilla,* is a well-known species in aquaculture with a commercial value in 2010 of approximately 8.3 € per kg and production approaching 7000 tons (FIGIS 2012). Still, the eel farming industry relies solely on wild-caught juveniles for production, as protocols for commercial production of glass eels are not available. Since 2006, new integrated methods have expanded this research field for European eel, thus enabling researchers to produce multiple batches of competent gametes, embryos and yolk-sac larvae (PRO-EEL 2013; Tomkiewicz 2012).

For several species of marine finfish, it is challenging to produce high-quality gametes for fertilization (Bobe and Labbé 2010). As such, research has focused on how to optimize fertilization strategies for a given species (Butts et al. 2009, 2012). Standardizing the sperm to egg ratio that has been used to improve fertilization rates (Suquet et al. 1995; Bart and Dunham 1996; Christopher et al. 2010). Generally, lowering the sperm density reduces the fertilization percentage, but any excess sperm sticking to the egg chorion serves as a substrate for microbial activity, which is known to impair embryonic development (Oppenheimer 1955; Bergh et al. 1992). Determining the optimal sperm to egg ratio (among other methods) is therefore important for successful *in vitro* fertilization, thus implying the need for accurate and precise methods for quantification of sperm concentration and density.

Sperm quality is commonly assessed using density and motility/velocity. In the literature, sperm density and motility have been correlated with quality (Rideout et al., 2004). Quantifying spermatozoa density is routinely carried out by counting the number of spermatozoa in a specific volume of ejaculate (Alavi et al. 2008). The most common counting method is performed using a haemocytometer, which is classified by the World Health Organization as the 'gold standard' for sperm quantification in humans (WHO 1999). This method, however, is time consuming (Suquet et al. 1992), and precision relies on skilled personnel. As such, studies have been conducted to discover faster and more automated counting methods (reviewed in Fauvel et al. 2010).

Spermatocrit, defined as the ratio of packed sperm to the total volume of milt $\times$ 100, is a fast and easy method to estimate spermatozoa concentration. Positive significant correlations between spermatocrit and sperm density estimates, using a haemocytometer, have been reported for several species (Ciereszko and Dabrowski 1993; Rideout et al., 2004; Hatef et al. 2007; Agarwal and Raghuvanshi 2009). However, it is important to note that sperm sedimentation is a reported feature in marine fish species (Fauvel et al. 2010), potentially compromising the accuracy of spermatocrit estimates. In addition, fluctuations in spermatozoa size during the spawning season potentially bias and influence spermatocrit values; for instance, spermatozoa head size changes in marine fish during a spawning season, such as in Atlantic cod (Butts et al. 2011).

Computer-assisted sperm analysis (CASA) automates sperm quality assessment, which in turn provides quick, precise and objective results (Fauvel et al. 2010; López

Rodríguez et al. 2011). The strength of CASA lies in quantification of motility, velocity and behavioural trajectories (i.e. linearity, amplitude of lateral head movement). CASA is furthermore capable of quantifying density of sperm as shown by Ehlers et al. (2011) together making it a versatile descriptor of sperm quality. Flow cytometry (FCM) is another automated technique that is able to measure the amount of one or more fluorescent stains in a cell. It features high precision, sensitivity, accuracy and speed (Cordelli et al. 2005) and due to this deemed a potentially valuable method for assessing male germ cell quality (Cordelli et al. 2005). Within this context, there is a need to assess the applicability of these automated counting methods for the European eel.

Spermatogenesis in eels applied in captive reproduction experiments is induced using human chorionic gonadotropin (hCG) (Pérez et al. 2000; Tomkiewicz et al. 2011). Spermiation in European eel starts approximately week 5 using 1.5–2.0 IU hCG/g fish in weekly treatment (Pérez et al. 2000; Asturiano et al. 2006) with sperm volume increasing until week 8–12 of treatment after which it stabilizes (Asturiano et al. 2006; Tomkiewicz et al. 2011). At this stage, spermatozoa densities are in the range of $5–18 \times 10^9$ cells/ml (Pérez et al. 2000; Gallego et al. 2012). During spermatozoa maturation, spermatozoa size changes in European eel (Asturiano et al. 2006; Marco-Jiménez et al. 2006). This includes an increase in spermatozoa head length from the 5th to 7th week and head thickening continuing until the 8th week of hormonal treatment (Asturiano et al. 2006; Marco-Jiménez et al. 2006). After the 8th week, only minor changes in spermatozoa/sperm cells head size occur, followed by a decrease in head length from the 12th week and onwards (Marco Jiménez et al. 2006; Pérez et al. 2009; Peñaranda et al. 2010). Within the last decade, European eel sperm have been analysed using CASA techniques to describe motility parameters (Pérez et al. 2009; Peñaranda et al. 2010; Gallego et al. 2013), ratio of viable spermatozoa (Asturiano et al. 2004, 2005) and their morphology (Marco-Jiménez et al. 2006). Furthermore, spermatocrit (12 000 × g) has been used to standardize sperm: egg ratios in European eel fertilization experiments (Tomkiewicz 2012). However, no studies have been conducted to quantify eel sperm density using CASA or FCM nor has the accuracy and precision of different methods to quantify sperm density been evaluated.

The purpose of this study was to provide fast and reliable tools to measure sperm density for European eel. More specifically, our objectives were to (i) test the relationship between spermatocrit and Neubauer Improved haemocytometer counts; (ii) test whether spermatocrit and haemocytometer counts correlates with sperm motility class; (iii) assess the effect of sperm dilution on haemocytometer counts; (iv) test the accuracy of spermatocrit for sperm quantification and identify the G-force for best correlation between spermatocrit and haemocytometer counts; (v) evaluate accuracy and precision of spermatocrit, CASA, FCM using haemocytometer counts as benchmark; and (vi) discuss these results in context of applicability for use in hatchery production of the European eel.

Materials and Methods

Data collection

Fish and hormonal treatment

Male European eels (n = 43; mean standard length and body weight ± SD: 40 ± 2.6 cm and 124 ± 21 g, respectively) were obtained from a commercial eel farm, Stensgård Eel Farm A/S in Jutland, Denmark (55.655461N: 9.20051E). Age of the fish ranged from 2 to 6 years. The fish were transported to a research facility (55.407444N: 9.403414E) of the Technical University of Denmark (DTU) in September 2011, and acclimatized to saltwater over a 10-day period. While at DTU, the eels were kept in 300-l tanks equipped with a closed recirculation system. The salinity and temperature of the system ranged from 36.7 to 37.3 ppt and 19.5–20.5°C, respectively. Saltwater was made artificially using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany). Fish were maintained under a 12 l light photoperiod at approximately 20 lux and 12 h dark with a 30-min gradual transition. No feed was provided during the experiment to mimic nature, as eels cease feeding in the silvering stage (Dollerup and Graver 1985).

Hormonal treatment was initiated on 22 September 2011. Prior to onset of hormonal treatment, all males were anesthetized using ethyl p-aminobenzoate at 20 mg/l (benzocaine; Sigma-Aldrich Chemie, Steinheim, Germany). Each fish was tagged with a passive integrated transponder (PIT tag) in the dorsal muscle tissue. Each week, fish were weighed and received dorsal injections of recombinant human chorionic gonadotropin at 1.5 IU/g fish (rhCG; Ovitrelle, Madrid, Spain) following Gallego et al. (2012).

Sperm sampling

Milt was collected after the 8th (trail 1) and 9th (trials 2 + 3) hormonal treatment, coinciding with the recommended time to strip sperm for high-quality gametes (Asturiano et al. 2006). Sperm samples were obtained 24 h after injection of rhCG to optimize sperm quality (Pérez et al. 2000). Prior to harvest, males were anesthetized using benzocaine, as earlier. The urogenital pore was thoroughly cleaned using Milli-Q water and dried prior to sperm collection. The first ejaculate of milt was omitted to avoid urine and faeces contamination. Ejaculated milt was kept in sterilized 50-ml Falcon tubes, covered using Parafilm®M, and stored at 4°C until motility estimation (max. 30 min). Following motility estimation, sperm was refrigerated at 4°C until further assessment (within 5 h).

Sperm dilution

Dilutions used for haemocytometer counting, CASA and FCM were 1 : 1000 or 1 : 2000 (see below). Haemocytometer counts were performed on fresh sperm, while the other treatments were conducted on preserved sperm samples. Sperm dilutions were carried out immediately after milt collection in P1 medium (Peñaranda et al. 2010) containing glutaraldehyde 2.5% (v/v) (Sigma-Aldrich Chemie, Steinheim, Germany) to avoid movement of sperm. Dilutions were carried out

using a two-step procedure by first diluting sperm 1 : 20 and subsequently, 1 : 50 or 1 : 100 to obtain final dilutions of 1 : 1000 or 1 : 2000, respectively.

Sperm motility determination

Immediately after milt collection, sperm motility was assessed by mixing 2 µl of milt with 200 µl of 37 ppt artificial seawater (Aqua Medic Sea salt, GmbH, Bissendorf, Germany), with 2% w/v Bovine Serum Albumin (Sigma-Aldrich, Chemie, Steinheim, Germany), adjusted to 8.2 pH (Peñaranda et al. 2010). After activation, 2 µl of sperm was assessed in a SpermTrack-10® chamber (Proiser R+D, S.L.; Paterna, Spain) and observed between 15 and 30 s after activation using a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan), fitted with a Nikon DS-Fi1 camera head, and 100× magnification (10 × CFI Plan Flour). All the samples were performed in triplicate and analysed by the same trained observer to avoid subjective differences in motility evaluation. Motility of each replicate was characterized to the nearest 10% increment, averaged and then categorized into an arbitrary scale where 0: represents no motile sperm; while I: <25%; II: 25–50%; III: 50–75%; IV: 75–90%; and V: 90–100% represent per cent of motile spermatozoa (Pérez et al. 2009).

Spermatocrit

Spermatocrit, defined as the ratio of packed sperm to the total volume of milt × 100, was used to estimate sperm concentration. Fresh milt from each male was drawn into three Vitrex™ microhaematocrit tubes, 75 mm long, with a 1.1–1.2 mm opening and sealed using Vitrex™ Sigillum wax. Tubes were centrifuged (Haematokrit 210, Andreas Hettich GmbH & Co.KG, Tuttlingen Germany) for 10 min at specific G-forces ranging from 500 to 14 000 × g (see below for further details). The mean of three measurements per male was used for statistical analyses. Spermatocrit was determined using a digital calliper (±0.05 mm).

Haemocytometer counting

A Neubauer Improved haemocytometer was used for counting sperm cell density diluted at 1 : 1000 or 1 : 2000 (see section Sperm dilution). Sperm counts were carried out in triplicate and results expressed as spermatozoa × 10^9/ml.

CASA counting

Milt samples preserved and diluted at 1 : 2000 in P1 medium (see section Sperm dilution) were used for CASA counting. Sperm (2.5 µl) were added to the SpermTrack-10® chamber (Proiser R+D, S.L.; Paterna, Spain) and density was assessed by the concentration module of the Integrated Semen Analysis System (ISAS; Proiser R+D, S.L.; Paterna, Spain). Images for CASA analyses were captured using a Nikon Eclipse E-400 microscope (Nikon Corporation, Tokyo, Japan) equipped with a 10× negative phase objective lens. The image captured represented approximately 90% of the whole microscope field.

The mean number of cells per field varied between 15 and 45 sperm, depending on sperm density. All analyses were performed in triplicate and two different methods were used: CASA-1 = capturing one microscope field per replicate and CASA-2 = capturing three microscope fields per replicate.

Flow cytometer counting

Milt samples used for flow cytometer analyses (Cytomics FC500; Beckman Coulter, USA) were diluted at 1 : 2000 in P1 medium (see section Sperm dilution). Two different methods were applied to calculate sperm density: FCM-1 = at least 5000 events (spermatozoa detected, after discarding debris) were analysed by a medium flow rate (30 µl/min) with time as the measured factor in each sample; and FCM-2 = a known concentration of fluorospheres (Flow-Check™ Fluorospheres, Beckman Coulter) were diluted in each sperm sample and at least 5000 events (spermatozoa and fluorospheres detected, after discarding debris) were analysed by a medium flow rate. Here, the ratio of sperm cells/fluorospheres was the registered factor in each sample. In both methods, sperm density was determined by the number of spermatozoa per volume analysed for each sample. All spermatozoa were stained using 0.1 µM SYBR-14 for 10 min, making sperm distinguishable from the remaining particles. We used a 20-mW air-cooled Argon ion laser with excitation wavelength of 488 nm and measured emission light using the FL1 photodetector channel to read the green light (525 nm).

Experimental design

Trial 1: Relationships between spermatocrit, sperm density and motility

Males (n = 43) were stripped and spermatocrit was measured in triplicate for individual males by centrifuging at 12 000 × g for 10 min. Sperm samples were counted using a haemocytometer with a dilution of 1 : 1000. Sperm motility was assessed for each male.

Trial 2: Effect of sperm dilution

In total, 14 randomly chosen males were stripped and sperm from six of these individuals were selected to have a good dispersion of motility values and avoid bias (10–45%). For haemocytometer counts, sperm samples from the same males were diluted at 1 : 1000 and 1 : 2000 in P1 medium.

Trial 3: Identification of the optimal G-force

Initially, milt from 35 mature males was collected. From these fish, sperm from 10 males were selected covering the range from low to high (27–95%) spermatozoa motility. Spermatocrit was measured using 500; 2000; 4000; 6000; 8000; 10 000; 12 000; and 14 000 × g at a centrifugal time of 10 min. For each G-force, new aliquot samples of sperm were used. For each male, haemocytometer counts were obtained using samples diluted at 1 : 2000 (see section Haemocytometer counting).

Trail 4: Test accuracy of automated methods (CASA, FCM) with haemocytometer counts

Data were collected using the same 10 sperm samples as in Trial 3. Automated counting was performed using CASA (CASA-1 and CASA-2) and FCM (FCM-1 and FCM-2). In addition, sperm were counted using a haemocytometer. Measurements were taken in triplicate.

Statistical analyses

Data were analysed using Sigmaplot v. 11 (Systat Software Inc, Hounslow, UK), and R (R Core Team, 2012, Vienna, Austria). Shapiro–Wilk and Levene's test were used to check for normality and homoscedasticity assumptions, respectively. Data were expressed as mean ± SD. Alpha was set at 0.05 for main effects and interactions.

Trial 1: Relationships between spermatocrit, sperm density and motility

To compare spermatocrit and haemocytometer counts, Model II linear regression was used [ordinary least products regression as described by (Ludbrook 2010)] due to possible variation on both x- and y-axis. Model II regression was run for all males and also for a subset of males exhibiting motility values >80%. Furthermore, one-way ANOVAS were run to test whether spermatocrit and haemocytometer counts were independent of sperm motility class. Haemocytometer data violated ANOVA assumptions. As such, a Kruskal–Wallis test was used for further analyses.

Trial 2: Effect of sperm dilution on sperm density

A student's t-test was used to compare sperm density estimates in samples diluted in the ratios 1 : 1000 and 1 : 2000, respectively.

Trial 3: Identification of the optimal G-force

Model II linear regression was used to compare haemocytometer counts and spermatocrit for each G-force.

Trial 4: Test accuracy of automated methods (CASA, FCM) with haemocytometer counts

Model II linear regression was used to compare CASA-1, CASA-2, FCM-1, FCM-2, spermatocrit with haemocytometer counts. Next, coefficient of variation (CV) was used for each counting technique to assess between subject variability; spermatocrit values for this analysis were obtained from Trial 3.

Results

Trial 1: Relationships between spermatocrit, sperm density and motility

Spermatocrit at 12 000 × g ranged from 12.3 to 100%, and haemocytometer counts ranged from 1.4 to 21.4 × 10^9 sperm/ml (Fig. 1). For these 43 males, there was a significant positive relationship between spermatocrit and haemocytometer counts ($r = 0.53$,
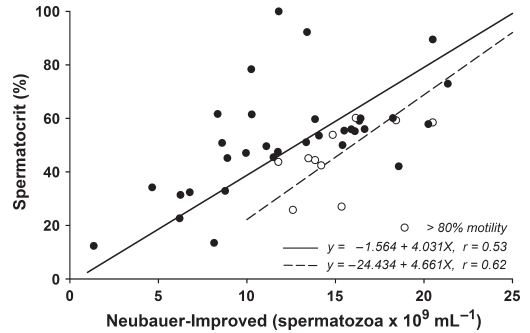

Fig. 1. Relationships between spermatocrit and hemocytometer counts in the European eel, *Anguilla anguilla*. Model II linear regression was used [ordinary least products regression as described by (Ludbrook 2010)] due to possible error in both x- and y-axes. Regression analyses were run for all males ($n = 43$) and this is represented by a solid line; those males with motility >80% ($n = 10$) are represented by open circles and a dashed line

$F_{1,42} = 15.60$, $p < 0.001$, $y = -1.564 + 4.031 \times$). However, a high degree of scatter was observed in the spermatocrit values; that is, spermatocrit values for haemocytometer counts approximately 8 × 10^9/ml ranged from 15 to 60%. The haemocytometer counts for males showing motility >80% ($n = 10$) were generally higher, resulting in a different relationship between spermatocrit and haemocytometer counts ($r = 0.62$, $F_{1,9} = 5.02$, $p = 0.030$, $y = -24.434 + 4.661 \times$).

Haemocytometer counts were associated with motility class, such that sperm counts were significantly higher in motility class V (approaching 100%) than in class 0 with lowest motility ($F_{5,37} = 2.73$, $p = 0.034$; Fig. 2). On the contrary, spermatocrit values did not vary among sperm motility classes ($H = 4.789$, $p = 0.442$; Fig. 2); class 0 showed high variability as it was composed of two individuals.

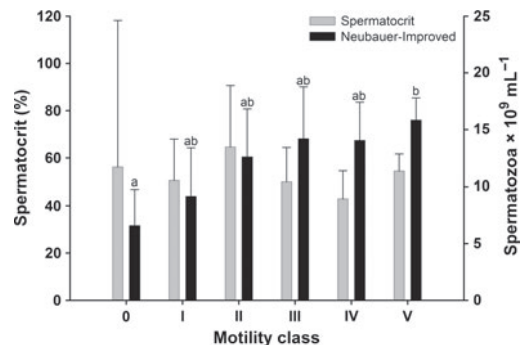Fig. 2. Spermatocrit (gray bars on primary y-axis) and hemocytometer counts (black bars on secondary $y$axis) for five sperm motility classes in the European eel, *Anguilla anguilla*. Data are expressed as mean ± SD. Values with common letters were not significantly different via one-way ANOVA. 0 = 0% motility; I: 1–25% motility; II: 25–50% motility; III: 50–75% motility; IV: 75–90% motility; V: 90–100% motility

Trial 2: Effect of sperm dilution on sperm density

The effect of dilution (1 : 1000 vs 1 : 2000) on haemocytometer estimates of sperm density was non-significant ($t_{10} = 0.048$, p = 0.963; Fig. 3); only the variation among replicates tended to be higher at lower dilution.

Trial 3: Identification of optimal G-force

Sperm from Male 3 and Male 8 showed a rapid decrease in spermatocrit over the G-force gradient (Fig. 4). There were significant positive relationships between spermatocrit and haemocytometer counts at 500; 4000; 6000; 12 000; and 14 000 × g (r values ranged from 0.33 to 0.68, p ≤ 0.049; Fig. 5.). The best relationship was found between spermatocrit and haemocytometer counts at 6000 × g (r = 0.68, p = 0.016; Fig. 5), as such these G-force data were used for further comparisons.

Trail 4: Test accuracy of automated methods (CASA, FCM) with haemocytometer counts

Computer-assisted sperm analysis (CASA)-1 (r = 0.70, $F_{1,9}$ = 7.61, p = 0.012) and CASA-2 (r = 0.93, $F_{1,9}$ = 51.16, p < 0.001; Fig. 6) density estimates were positively related to haemocytometer counts. Furthermore, there were significant positive relationships between FCM-1 (r = 0.94, $F_{1,9}$ = 62.921, p < 0.001) and FCM-2 (r = 0.88, $F_{1,9}$ = 26.84, p < 0.001) and haemocytometer counts.

The CVs for CASA-1 (17.9%) and CASA-2 (28.4%) were in the order of 7.5 times greater compared to the other counting techniques (CV ranges from 2.5 to 5.9%; Table 1).

Discussion

In this study, we report several key findings: (i) haemocytometer counts were positively associated sperm motility; (ii) haemocytometer counts were not affected by milt dilution ratio; (iii) optimizing G-force for centrifuging milt improved the relationship between
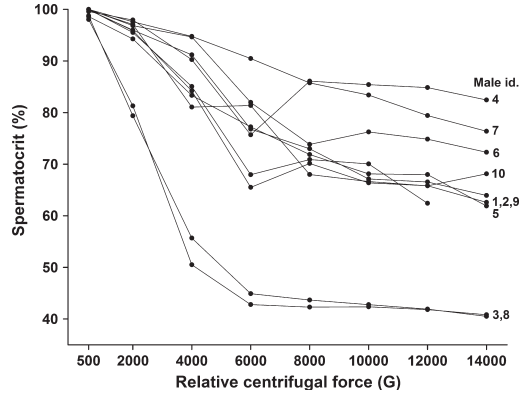
Fig. 4. Values of spermatocrit for 10 males over a G-force gradient (500–14 000 × g) in the European eel, *Anguilla anguilla*. Male Id is shown on the right (1–10)

spermatocrit and haemocytometer counts; (iv) spermatocrit, CASA and FCM were all positively related to haemocytometer counts with CASA-2 and FCM-1 having the strongest relationship to haemocytometer counts.

Spermatocrit has been used to estimate sperm concentration for several species of fish (Rakitin et al. 1999; Rideout et al. 2004). such as yellow perch, *Perca flavescens* (Cierezsko and Dabrowski 1993), haddock, *Melanogrammus aeglefinus* (Rideout et al., 2004), Atlantic halibut, *Hippoglossus hippoglossus* (Tvedt et al. 2001), snow trout, *Schizothorax richardsonii* (Agarwal and Raghuvanshi 2009), brown trout, *Salmo trutta* (Poole and Dillane 1998), Atlantic salmon, *Salmo salar* (Aas et al. 1991), rainbow trout, *Oncorhynchus mykiss* (Cierezsko and Dabrowski 1993) and lake whitefish, *Coregonus clupeaformis* (Cierezsko and Dabrowski 1993). Together these studies found spermatocrit as a quick and easy technique for estimating sperm concentration (Alavi et al. 2008). In the present study, we evaluated the relationship between spermatocrit and haemocytometer counts for the European eel and showed a significant positive relationship between these two quantitative sperm metrics. However, its relationship with haemocytometer counts showed considerable scatter and appeared inferior to the automated counting methods. Furthermore, the tests of different centrifugal G-forces revealed that r-values varied between 0.33 and 0.68 and the best relationship between spermatocrit and haemocytometer counts was obtained at 6000 × g. Higher centrifugal forces tended to result in low correlation coefficients, as a result of changes in cell packing within the microhaematocrit tube.

A non-significant relationship between spermatocrit and haemocytometer counts was found in Atlantic cod, *Gadus morhua* (Rakitin et al. 1999). The authors suggested this might be an artefact of small volumes of milt being diluted in immobilizing media before sperm density was quantified using a haemocytometer. This study by Rakitin et al. (1999) used a one-step 500-fold dilution and their reported variability was high
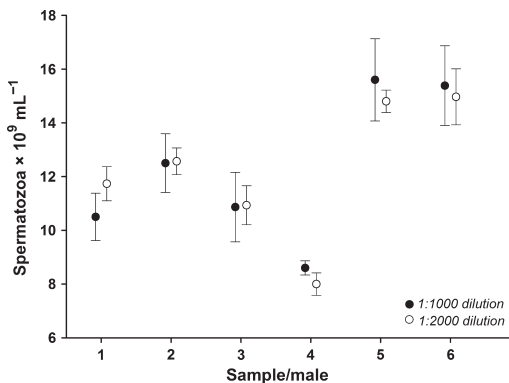
Fig. 3. Hemocytometer counts for six males using two different milt dilutions in the European eel, *Anguilla anguilla*. Solid symbols = 1 : 1000; open symbols = 1 : 2000 dilution
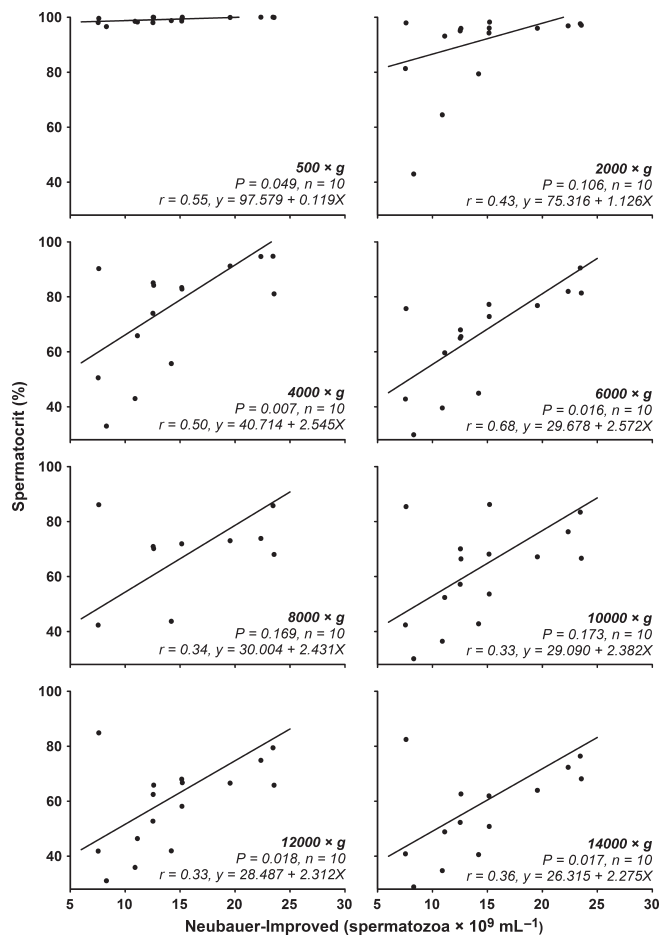
Fig. 5. Relationships between spermatocrit and hemocytometer counts over a G-force gradient (500–14000 × *g*) in the European eel, *Anguilla anguilla*. Model II linear regression was used [ordinary least products regression as described by (Ludbrook 2010)] due to possible error in both *x* and *y*-axes. For each plot the p-value, sample size, correlation coefficient, and equation of line are shown

Fig. 6. Relationships between computer-assisted sperm analysis (CASA)-1, CASA-2, flow cytometry (FCM)-1, FCM-2 and hemocytometer for the European eel, *Anguilla anguilla*. Model II linear regression was used [ordinary least products regression as described by (Ludbrook 2010)] due to possible error in both *x* and *y*-axes. For each plot the p-value, sample size, correlation coefficient, and equation of line are shown
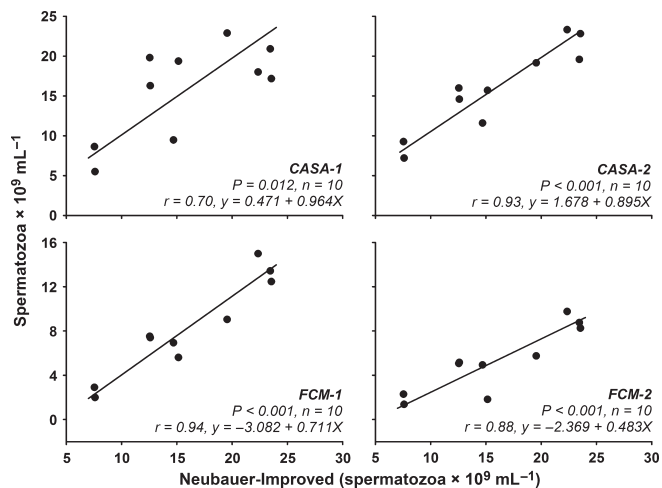
Table 1. Coefficients of variation for haemocytometer, spermatocrit at $6000 \times g$, computer-assisted sperm analysis (CASA-1 and CASA-2) and flow cytometry (FCM-1 and FCM-2) for the European eel, *Anguilla anguilla*. Mean values are shown for each counting method. Measurements were performed in triplicate for 10 males

Male number	Neubauer improved	Spermatocrit	CASA-1	CASA-2	FCM-1	FCM-2
1	6.1	6.5	36.5	34.7	5.5	3.6
2	11.2	3.0	11.7	31.5	1.7	2.5
3	8.4	2.1	13.8	27.4	1.8	3.5
4	0.0	10.1	6.9	36.0	2.1	1.5
5	5.1	9.6	30.6	27.3	1.7	1.4
6	4.7	6.8	12.0	21.0	2.5	3.9
7	8.7	6.8	1.8	16.0	3.8	4.2
8	6.0	4.0	29.0	21.5	2.0	3.2
9	7.6	4.2	32.4	26.4	2.8	3.0
10	0.9	3.1	3.8	42.2	3.6	0.5
Mean	5.9	5.6	17.9	28.4	2.5	2.7

(CV = 27.7%). We found negligible effect of milt dilution ratio on haemocytometer counts as well as a low coefficient of variation (CV = 5.9%). The precision and accuracy of haemocytometer counts have been addressed in the literature (see Alavi et al. 2008 and Fauvel et al. 2010 for review) and errors due to pipetting, dilution ratio, sperm settling times and operator biases are emphasized (Rakitin et al. 1999). Therefore, there is a need for species-specific guidelines for fishes as set by the WHO for humans (WHO 1999).

Sperm motility and spermatocrit values were independent, while haemocytometer density estimates increased with motility class, such that the low motility class 0 (no motility) had significantly lower sperm density than the high motility class V (90–100%

motility). The latter concurs with final hydration of spermatozoa coinciding with final maturation and increase in motility (Gallego et al. 2012). Useful future research should relate these quantitative sperm metrics to other estimates of quality, such as sperm velocity and fertilization success.

In our study, CASA-2 and FCM-1 show strong predictive relationships with haemocytometer counts ($r = 0.93$ and 0.94, respectively). FCM-1 gave the strongest relationship. FCM has an advantage over CASA in that it has a 10-fold lower coefficient of variation. Similarly, sperm counts measured by haemocytometer and flow cytometer were also highly correlated ($r^2 = 0.85$) in the razorback sucker, *Xyrauchen texanus* (Jenkins et al. 2011). CASA, although not commonly used for quantification of fish sperm density, gave us promising result. This indicates that CASA is a universal tool for sperm quality/quantity assessment and further complements flow cytometry which, besides quantification, can describe the physiology of milt parameters (i.e. membrane potential, cell integrity; Cordelli et al. 2005; Fauvel et al. 2010). CASA software is commonly used throughout the field of sperm biology (Marco-Jiménez et al. 2006; Pérez et al. 2009; Peñaranda et al. 2008, 2010). as open-source systems have immerged, resulting in inexpensive alternatives for sperm quality assessment (Komori et al. 2006; Wilson-Leedy and Ingermann 2007). We recommend these automated systems for studying reproductive physiology and for routine assessment of sperm density for the European eel. Additionally, spectrophotometry methods should be examined (Fauvel et al. 1999).

When deciding which method to use for quantification of sperm, both economic feasibility and accuracy/precision of specific device(s) need to be considered. In

Table 2. Resource requirements, advantages and disadvantages for the different quantitative methods used to determine sperm density for the European eel, *Anguilla anguilla*

Quantification method	Requirements	Advantages	Disadvantages
Neubauer Improved haemocytometer	Microscope required Neubauer Improved haemocytometer Trained personnel	Cheap Precise – low CV Described in literature	Time consuming
Spermatocrit	Centrifuge required Microhaematocrit tubes Tube sealant Haematocrit tube reader	Fast Precise – low CV Low level of training	Inaccurate – low r Sperm sedimentation
CASA-1	CASA software Software calibration Computer and microscope with frame grabber Training	Fast Additional measures of sperm quality obtained	Low precision – high CV Inaccurate – low r Trained personnel
CASA-2	CASA software Software calibration needed Computer and microscope with frame grabber Training	Fast Accurate – high r Additional measures of sperm quality easy obtainable	Low precision – high CV Trained personnel
FCM-1	Flow cytometer required Training	Precise – low CV Accurate – high r	Trained personnel Need to extrapolate by equation
FCM-2	Flow cytometer and fluorospheres required Training	Precise – low CV Accurate – high r	Fluorospheres making it more expensive than FCM-1 Lower accuracy than FCM-1 Need to extrapolate by equation

Table 2, we provide an overview of resource requirements, advantages and disadvantages for the different quantitative methods investigated. In summary, the haemocytometer features low operational costs, precise measurements, but is time consuming and precision relies on skilled personnel. Spermatocrit measurements require a centrifuge, low level of operator training, are fast, but are not as accurate as other methods. CASA-1 requires special software and a microscope with video frame grabber. Additionally, CASA-1 gives fast results, but has relatively low accuracy and precision. CASA-2, like the aforementioned, needs software, requires a microscope and video frame grabber. Furthermore, CASA-2 gives an accurate result, but at low precision. FCM-1 requires expensive equipment, gives both accurate and precise results, while FCM-2 features the same characteristics, although slightly more expensive and less accurate. Both the haemocytometer and automated counting techniques differ from spermatocrit by giving counts rather than concentration, and therefore are likely less subjective to bias from changes in spermatozoa head morphology (Marco-Jiménez et al. 2006).

In conclusion, we found highly predictive relationships between CASA-2 and FCM-1 and haemocytometer counts, which can be considered as accurate methods for quantification of European eel sperm. These methods appear the most efficient for developing standardized fertilization protocols, enabling optimized sperm to egg ratios. We also found a lower, but significant correlation between spermatocrit and haemocytometer counts, although not as clear as reported in some other fish species.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

SRS, JT and JFA conceived the experiment. SRS, VG, LP and JFA performed the experimental design and experiment execution. SRS, VG, IEAB, JT and JFA performed data analyses and interpretation. JT and JFA supervised the study design, execution, analysis and approved the final version. All authors read and approved the manuscript.

References

Aas GH, Refstie T, Gjerde B, 1991: Evaluation of milt quality of Atlantic salmon. Aquaculture 95, 125–132.

Agarwal NK, Raghuvanshi SK, 2009: Spermatocrit and sperm density in snowtrout (Schizothorax richardsonii): correlation and variation during the breeding season. Aquaculture 291, 61–64.

Alavi SMH, Linhart O, Coward K, Rodina M, 2008: Fish spermatology: implications for aquaculture management. In: Fish spermatology. Alavi SMH, Cosson JJ, Coward K, Rafiee G (eds), Alpha Science Ltd, Oxford, UK, pp. 397–460.

Asturiano JF, Pérez L, Garzón DL, Marco-Jiménez F, Peñaranda DS, Vicente JS, Jover M, 2004: Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiol Biochem 30, 283–293.

Asturiano JF, Pérez L, Garzón DL, Peñaranda DS, Marco-Jiménez F, Martínez-Llorens S, Tomás A, Jover M, 2005: Effect of different methods for the induction of spermiation on semen quality in European eel. Aquac Res 36, 1480–1487.

Asturiano JF, Marco-Jiménez F, Pérez L, Balasch S, Garzón DL, Peñaranda DS, Vicente JS, Viudes-de-Castro MP, Jover M, 2006: Effects of hCG as spermiation inducer on European eel semen quality. Theriogenology 66, 1012–1020.

Bart AN, Dunham RA, 1996: Effects of sperm concentration and egg number on fertilization efficiency with channel catfish (Ictalurus punctatus) eggs and blue catfish (I. furcatus) spermatozoa. Theriogenology 45, 673–682.

Bergh Ø, Hansen GH, Taxt RE, 1992: Experimental infection of eggs and yolk sac larvae of halibut, Hippoglossus hippoglossus L. J Fish Dis 15, 379–391.

Bobe J, Labbé C, 2010: Egg and sperm quality in fish. Gen Comp Endocrinol 165, 535–548.

Butts IAE, Trippel EA, Litvak MK, 2009: The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod Gadus morhua L. Aquaculture 286, 89–94.

Butts IAE, Trippel EA, Ciereszko A, Soler C, Sowińska M, Alavi SMH, Litvak MK, Babiak I, 2011: Seminal plasma biochemistry and spermatozoa characteristics of Atlantic cod (Gadus morhua L.) of wild and cultivated origin. Comp Biochem Physiol A: Mol Integr Physiol 159, 16–24.

Butts IAE, Roustaian P, Litvak MK, 2012: Fertilization strategies for winter flounder: Effects of spermatozoa density and the duration of gamete receptivity. Aquat Biol 16, 115–124.

Christopher JG, Ag M, Sukumaran N, 2010: Optimization of artificial fertilization in the stinging catfish Heteropneustes fossilis (Bloch). Zygote 19, 63–66.

Ciereszko A, Dabrowski K, 1993: Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. Aquaculture 109, 367–373.

Cordelli E, Eleuteri P, Leter G, Rescia M, Spanò M, 2005: Flow cytometry applications in the evaluation of sperm quality: Semen analysis, sperm function and DNA integrity. Contraception 72, 273–279.

Dollerup J, Graver C, 1985: Repeated induction of testicular maturation and spermiation, alternating with periods of feeding and growth in silver eels, Anguilla anguilla (L.). Dana 1985, 19–39.

Ehlers J, Behr M, Bollwein H, Beyerbach M, Waberski D, 2011: Standardization of computer-assisted semen analysis using an e-learning application. Theriogenology 76, 448–454.

Fauvel C, Savoye O, Dreanno C, Cosson J, Suquet M, 1999: Characteristics of sperm of captive seabass in relation to its fertilization potential. J Fish Biol 54, 356–369.

Fauvel C, Suquet M, Cosson J, 2010: Evaluation of fish sperm quality. J Appl Ichthyol 26, 636–643.

FIGIS, 2012: FAO Fisheries & Aquaculture - Aquatic species. Available: http://www.fao.org/fishery/species/2203/en (accessed 2 January 2013).

Gallego V, Mazzeo I, Vîlchez MC, Peñaranda DS, Carneiro PCF, Pérez L, Asturiano JF, 2012: Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (Anguilla anguilla) during induced sexual maturation. Aquaculture 354–355, 7–16.

Gallego V, Carneriro PCF, Mazzeo I, Vílchez MC, Peñaranda DS, Soler C, Pérez L, Asturiano JF, 2013: Standardization of European eel (*Anguilla anguilla*) sperm motility evaluation by CASA software. Theriogenology **79**, 1034–1040.

Hatef A, Niksirat H, Amiri BM, Alavi SMH, Karami M, 2007: Sperm density, seminal plasma composition and their physiological relationship in the endangered Caspian brown trout (*Salmo trutta caspius*). Aquac Res **38**, 1175–1181.

Jenkins JA, Eilts BE, Guitreau AM, Figiel CR, Draugelis-Dale RO, Tiersch TR, 2011: Sperm quality assessments for endangered razorback suckers *Xyrauchen texanus*. Reproduction **141**, 55–65.

Komori K, Tsujimura A, Ishijima S, Tanjapatkul P, Fujita K, Matsuoka Y, Takao T, Miyagawa Y, Takada S, Okuyama A, 2006: Comparative study of sperm motility analysis system and conventional microscopic semen analysis. Reprod Med Biol **5**, 195–200.

López Rodríguez A, Rijsselaere T, Bijttebier J, Vyt P, Van Soom A, Maes D, 2011: Effectiveness of the sperm quality analyzer (SQA-Vp) for porcine semen analysis. Theriogenology **75**, 972–977.

Ludbrook J, 2010: Linear regression analysis for comparing two measurers or methods of measurement: but which regression?, Linear regression analysis for comparing two measurers or methods of measurement: but which regression? Clin Exp Pharmacol Physiol **37**, 692–699.

Marco-Jiménez F, Pérez L, de Castro MPV, Garzón DL, Peñaranda DS, Vicente JS, Jover M, Asturiano JF, 2006: Morphometry characterisation of European eel spermatozoa with computer-assisted spermatozoa analysis and scanning electron microscopy. Theriogenology **65**, 1302–1310.

Oppenheimer CH, 1955: The effect of marine bacteria on the development and hatching of pelagic fish eggs, and the control of such bacteria by antibiotics. Copeia **1955**, 43–49.

Peñaranda DS, Pérez L, Marco-Jiménez F, Jover M, Asturiano JF, 2008: Advances in techniques for the control of European eel reproduction: spermiation induction, sperm quality evaluation and cryopreservation. Cybium **32**, 323.

Peñaranda DS, Pérez L, Gallego V, Barrera R, Jover M, Asturiano JF, 2010: European eel sperm diluent for short-term storage. Reprod Domest Anim **45**, 407–415.

Pérez L, Aturiano JF, Tomás A, Zegrari S, Barrera R, Espinós FJ, Navarro JC, Jover M, 2000: Induction of maturation and spermiation in the male European eel: assessment of sperm quality throughout treatment. J Fish Biol **57**, 1488–1504.

Pérez L, Peñaranda D, Gallego V, Asturiano J, 2009: Testis development, sperm quality evaluation and cryopreservation in the European eel. In: Thillart G, Dufour S, Rankin JC (eds), Spawning Migration of the European eel, Fish and Fisheries Series. Springer Netherlands, Dordrecht, pp. 333–362.

Poole WR, Dillane MG, 1998: Estimation of sperm concentration of wild and reconditioned brown trout, *Salmo trutta* L. Aquac Res **29**, 439–445.

PRO-EEL, 2013: PRO-EEL. Available: http://www.pro-eel.eu/ (accessed 20 February 2013).

Rakitin A, Ferguson MM, Trippel EA, 1999: Spermatocrit and spermatozoa density in Atlantic cod (*Gadus morhua*): correlation and variation during the spawning season. Aquaculture **170**, 349–358.

Rideout RM, Trippel EA, Litvak MK, 2004: Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. J Fish Biol **65**, 319–332.

Suquet M, Omnes MH, Normant Y, Fauvel C, 1992: Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*). Aquaculture **101**, 177–185.

Suquet M, Billard R, Cosson J, Normant Y, Fauvel C, 1995: Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. Aquaculture **133**, 83–90.

Tomkiewicz J (ed.), 2012: Reproduction of European Eel in Aquaculture (REEL): Consolidation and New Production Methods. DTU Aqua Report No 249-2012. National Institute of Aquatic Resources, Technical University of Denmark, 1–47.

Tomkiewicz J, Kofoed TMN, Pedersen JS, 2011: Assessment of testis development during induced spermatogenesis in the European eel *Anguilla anguilla*. Mar Coast Fish **3**, 106–118.

Tvedt HB, Benfey TJ, Martin-Robichaud DJ, Power J, 2001: The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture **194**, 191–200.

WHO, 1999: World Health Organisation. Laboratory Manual for the Examination of Human Semen and Semen Cervical Mucus Interaction. Cambridge University Press, Cambridge, UK.

Wilson-Leedy JG, Ingermann RL, 2007: Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology **67**, 661–672.

Author's address (for correspondence): SR Sørensen, National Institute of Aquatic Resources, Technical University of Denmark, Kavalergården 6, 2920 Charlottenlund, Denmark. E-mail: srs@aqua.dtu.dk

Paper 2:

Standardization of fertilization protocols for the European eel, *Anguilla anguilla*

Standardization of fertilization protocols for the European eel, *Anguilla anguilla*

Ian A.E. Butts [a,*], Sune R. Sørensen [a,1], Sebastian N. Politis [a,1], Trevor E. Pitcher [b,c], Jonna Tomkiewicz [a]

[a] DTU Aqua-National Institute of Aquatic Resources, Section for Marine Ecology, Technical University of Denmark, Charlottenlund Slot, Jægersborg Allé 1, 2920 Charlottenlund, Denmark
[b] Department of Biology, University of Windsor, 401 Sunset Ave, Windsor, Ontario N9B 3P4, Canada
[c] Great Lakes Institute for Environmental Research, University of Windsor, 401 Sunset Ave, Windsor, Ontario N9B 3P4, Canada

ARTICLE INFO

ABSTRACT

Standardization of artificial fertilization protocols for the European eel, *Anguilla anguilla*, is a prerequisite for optimizing the use of available gametes in hatchery facilities and for conserving sperm from high quality males, which is either cryopreserved or in living gene banks. The objectives of this research were to provide a rapid, accurate and precise method to quantify sperm density by examining the relationship between sperm density and absorbance by use of a spectrophotometer, determine the optimal number of sperm required to fertilize eggs in a controlled setting, and explore how long eggs are receptive to fertilization post-stripping. Mean sperm density and absorbance at 350 nm were $1.54e + 10 \pm 4.95e + 9$ sperm/mL and 1.91 ± 0.22 nm, respectively. Regression analysis demonstrated a highly significant positive relationship between sperm density and absorbance using a spectrophotometer at 350 nm ($R^2 = 0.94$, $p < 0.001$, $y = 2.273e + 10x - 2.805e + 10$); significant but slightly weaker relationships were also detected at 400, 500, and 600 nm ($R^2 \geq 0.93$, $p < 0.001$). Fertilization success using sperm to egg ratios ranging from $1.3e + 3$ to $1.0e + 6$ sperm per egg increased from 37.5 to 68.1%, respectively. Sperm to egg ratio had a significant effect on fertilization success ($p < 0.0001$), where fertilization success increased from $1.3e + 3$ to $2.5e + 4$ sperm per egg; adding greater than $2.5e + 4$ sperm per egg had no significant effect. Furthermore, the duration of time post-stripping had a significant effect on egg fertilization success ($p < 0.0001$), such that between 0 and 10 min post-stripping 57.4 to 78.2% of the eggs were fertilized while at 15 min post-stripping a significant decrease in fertilization success was detected (47.5%). For all statistical models, the female variance component was significant for fertilization success ($p < 0.0001$) and explained $\geq 84\%$ of the models variance. In conclusion, European eel eggs should be fertilized within 10 min post-stripping using $2.5e + 4$ sperm per egg. Together, these findings will contribute to the development of European eel breeding technology and further our understanding on sperm biology and reproductive biology in fishes.

1. Introduction

European eel, *Anguilla anguilla*, has long been a highly valued species targeted for aquaculture production (Gousset, 1990; Ottolenghi et al., 2004). Nevertheless, the eel farming industry relies exclusively on wild-caught juveniles, as rearing protocols for commercial production of glass eels (an intermediary stage in the eel's life history) are not yet available. Recently, pioneering research has raised eel breeding from a state of reproductive failure to a stable production of yolk-sac larvae (reviewed in Tomkiewicz et al., 2012). Although basic procedures for artificial fertilization have been described (Mordenti et al., 2013; Tomkiewicz et al., 2012), as of yet no empirical research has been directed towards standardizing fertilization protocols. This is especially critical for species whose gametes are hand-stripped, such as the European eel, as it may increase fertilization success and embryonic survival.

Before standardizing fertilization protocols, for any given species, there is a need for accurate and precise methods to quantify sperm density. Currently, several methods are available to quantify sperm density for the European eel (Sørensen et al., 2013). In brief, haemocytometer counting featured low operational costs, high precision and accuracy, but was a tedious and time-consuming technique. Spermatocrit measurements were cost-effective and rapid, but not as accurate as the other methods, while computer-assisted sperm analysis and flow cytometry offered fast and unbiased estimation, but required expensive software/equipment and skilled personnel (Sørensen et al., 2013). Determination of sperm density by use of a spectrophotometer however, has yet to be explored for this species. This method is known to be reliable, rapid, simple, and inexpensive, thus would allow for more efficient use of time and prevent stripped gametes from standing too long before fertilization (Alavi et al., 2008; Dong et al., 2005).

---

* Corresponding author. Tel.: +45 23 10 02 86; fax: +45 35 88 33 33.
 E-mail addresses: ianb@aqua.dtu.dk, iana.e.butts@gmail.com (I.A.E. Butts).
[1] Both authors equally contributed to this work.

Fertilization success is predicted to increase when more sperm are incorporated into an artificial and/or natural spawning event (Butts et al., 2009; Hatef et al., 2009). However, the benefit of sperm number should decrease as the total number of cells approaches the number required to fertilize an entire egg batch (Ball and Parker, 1996; Casselman et al., 2006). Excess sperm sticking to the chorion of adhesive eggs can also serve as a substrate for microbial activity, which is known to impair embryonic development until hatching (Bergh et al., 1992). Additionally, two or more sperm entering the egg (termed polyspermy) may lead to abnormal embryonic development and ultimately to death of an embryo, even though mechanisms exist to minimize and/or block polyspermy in vertebrates and invertebrates (Psenicka et al., 2010; Snook et al., 2011). Therefore, determining the "optimal" sperm to egg ratio is a critical step towards establishing successful in vitro fertilization protocols.

Gamete receptivity, defined as the length of time gametes are receptive to be fertilized, also impacts reproductive yields and is another factor which should be considered when spawning fishes in captivity (Butts et al., 2012a). For instance, when European eel sperm are activated they lose their fertilization capability within 1–2 min due to limited amounts of energy reserves available for motility, whereas non-activated cells can be stored in a refrigerator for extended periods and still maintain a high capacity to fertilize eggs upon activation (~80% motility at 7 days of storage; Peñaranda et al., 2010). To the best of our knowledge, it is unclear how long European eel eggs are receptive to fertilization post-stripping. In fishes, eggs of high quality, are usually viable for only a restricted time post-stripping, but may be prolonged at appropriate temperatures (Ciereszko et al., 2000). Therefore, defining the trajectory of this decline is important to optimize spawning collection procedures for aquaculture and/or research activities (Johnston et al., 2008).

Therefore, the main goal of this study was to standardize artificial fertilization protocols for the European eel. More specifically, the objectives were to (i) develop a relationship between sperm density and absorbance by use of a spectrophotometer, (ii) use this newly developed relationship to determine the optimal number of sperm required to fertilize eggs in a controlled setting, and (iii) explore how long eggs are receptive to fertilization post-stripping. Overall, these findings will contribute to the development of European eel aquaculture and further our understanding on sperm biology and reproductive biology in fishes.

2. Materials and methods

2.1. Broodstock collection and management

Female broodstock (n = 42; mean standard length and body weight ± SEM were 68.8 ± 1.4 cm and 669.0 ± 47.7 g, respectively) were caught in the silvering stage from a freshwater lake (Vandet Sø) in northern Jutland, Denmark. Eels were transported, using an aerated transportation tank, to a research facility (55.407444 N: 9.403414E) of the Technical University of Denmark (DTU). While at DTU, eels were injected with a passive integrated transponder (PIT tag) in the dorsal muscle and housed in 300 L tanks equipped with a closed re-circulation system. Acclimatization to saltwater took place over a 14-day period. Salinity was adjusted artificially using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany). During maturation, eels were kept at a density of ≤30 kg per m³, salinity of ~36‰, and temperature of ~19 to 21 °C. Eels were maintained under dimmed light conditions at ~20 lx and a natural daily photoperiod with gradual transition taking 30 min. No feed was provided during experimentation as eels in silvering stage cease feeding (Dollerup and Graver, 1985).

To induce vitellogenesis females received weekly (10 to 20 weeks) injections of salmon pituitary extract (SPE; 18.75 mg/kg body weight, Argent Chemical Laboratories, Washington, USA) (Kagawa et al., 2005). Biopsies were routinely taken for evaluation of oocyte development (Palstra et al., 2005). Based on body weight increase and oocyte developmental stage indices, females received another injection of SPE as a priming dose. To stimulate final maturation and induce ovulation females were later injected, ~24 h after receiving the priming dose, with the maturation-inducing steroid (MIS; 17α,20ß-dihydroxy-4-pregnen-3-one; 2 mg per/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) (Ohta et al., 1996). Within 12 to 14 h of receiving MIS, eggs were stripped by applying pressure to the abdomen of the fish. Expelled eggs were collected into dry weight boats (7 cm × 7 cm with volume of 80 mL).

Male broodstock were reared in a commercial eel farm (Stensgård Eel Farm A/S) in Jutland, Denmark (55.655461 N: 9.20051E) on a standard diet (DAN-EX 2848, BioMar A/S, Brande, Denmark) (for composition see Støttrup et al., 2013). Males (n = 60; mean standard length and body weight were ± SEM 38.1 ± 0.3 cm and 110.0 ± 2.0 g, respectively) were transported to DTU's research facility (husbandry conditions as above). Male eels received weekly injections of recombinant human chorionic gonadotropin at 1.5 IU g⁻¹ fish (Ovitrelle, Madrid, Spain) (Gallego et al., 2012). Milt was collected after 7 weeks of hormonal treatment (~12 h after administration of hormone). For milt collection, the genital pore was wiped dry using deionized water. The first ejaculate of milt was omitted to avoid urine and faeces contamination. Samples were then collected into dry weight boats (3.6 cm × 3.6 cm with volume of 7.5 mL), by applying slight pressure along the abdominal region. Within 10 s, 500 μL of milt from each male was pipetted into 20 mL of immobilizing medium (Peñaranda et al., 2010).

2.2. Sperm motility assessment

Sperm motility was assessed according to Sørensen et al. (2013). In brief, within 30 to 40 min post-stripping, the percentage of motile sperm per male was estimated by adding 0.2 μL of milt onto the centre of a microscope slide, situated on the stage of a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan) maintained at room temperature. Sperm were activated by adding 200 μL of ~20 °C seawater obtained from the North Sea and adjusted to 36 ppt with artificial Red Sea salt (Red Sea Europe, Verneuil sur Avre, France). No coverslip was added during sperm activation. Sperm motility was assessed at 400× magnification within 10 to 15 s after the addition of seawater. Motility was characterized using an arbitrary scale where 0: represents no motile sperm; while I: <25%; II: 25–50%; III: 50–75%; IV: 75–90%; and V: 90–100% represent percentage of motile spermatozoa (Pérez et al., 2009; Sørensen et al., 2013). All samples were performed in triplicate and analyzed by the same trained observer to avoid subjective differences in motility assessment. Activation motility scores were all ≥75% (characterized as stage IV or V); no significant difference in sperm motility was detected between the sperm pools (p < 0.05).

2.3. Experiment 1: Determination of sperm density by use of a spectrophotometer

Milt (50 μL) was collected from 18 males and each sample was diluted into disposable glass test tubes (16 mm × 100 mm, Fisher Scientific, Loughborough, UK) containing 3000 μL of immobilizing media (P1 media as described in Peñaranda et al., 2010). The resulting sperm suspensions were homogenized using a vortex mixer for 10 s (Minishaker MS2; IKA, Staufen, Germany). Homogenized samples were immediately transferred to plastic cuvettes, the outside of each cuvette was wiped with Kimwipes®, and then placed into a spectrophotometer (DR 2800; Hach-Lange Aps, Brønshøj, Denmark). Absorbance values were randomly generated across four different wavelengths spanning the visible spectrum: 350, 400, 500 and 600 nm. The mean of three replicates per male was used for statistical analyses.

Sperm density was counted under a Zeiss Axiostar compound microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) at 400× magnification using an improved Neubauer haemocytometer (see

Butts et al., 2012b for details). Homogenized milt (200 μL) from the plastic cuvettes was further diluted in 3200 μL of immobilizing media and mixed using a vortex mixer for 10 s. A sample of this sperm suspension (10 μL) was then micropipetted onto a haemocytometer that had been pre-covered with a coverslip. The number of sperm in each of the five larger squares on the haemocytometer was counted. There are 25 of these large squares on the haemocytometer and each of these large squares has 16 smaller squares within it. Sperm were counted in the four large corner squares and the large centre square. The mean number of sperm per large square count (i.e. mean of the five counts) was multiplied by 25 (to obtain the mean per 5 × 5 large-square grid), by 10 (the depth of the chamber in mm), and then by the initial volume of the sample to estimate the sperm density. Sperm densities are expressed as the total number of sperm per mL of a male's ejaculate. The mean of three replicates per male was used for statistical analyses.

2.4. Experiment 2: Determination of the optimal sperm to egg ratio

Eggs were collected from six females and milt was stripped from 24 haphazardly selected males. Milt (500 μL) from four males was diluted into 20 mL of immobilizing media to create six unique sperm pools (500 μL of milt × 4 new males = 2000 μL of diluted milt into each sperm pool). Eggs from each female were then "crossed" (see below) with a new sperm pool from the four males.

Using a micropipette, a known volume of sperm from each pool (adjusted according to the calculated sperm density using results outlined for Experiment 1 in Section 3.1) was added to 2 mL microcentrifuge tubes (Sarstedt AG & Co., Nümbrecht, Germany) creating 12 experimental sperm to egg ratios for each sperm pool (1.3e + 3:1, 2.5e + 3:1, 5.0e + 3:1, 1.0e + 4:1, 2.5e + 4:1, 5.0e + 4:1, 7.5e + 4:1, 1.0e + 5:1, 2.5e + 5:1, 5.0e + 5:1, 7.5e + 5:1, 1.0e + 6:1 sperm to egg). Three replicate microcentrifuge tubes were used for each sperm to egg ratio. Each microcentrifuge tube was filled to 1000 μL with immobilizing media. Approximately 500 eggs (mean ± SEM = 506 ± 10 eggs) were placed into dry weight boats (7 cm × 7 cm) using a 1.0 mL syringe. The tip of each syringe was cut off to prevent the eggs from being compressed or damaged. Sperm in each microcentrifuge tube was then added to the eggs in the weight boats. Activation media (20 mL of 37 ppt seawater at ~20 °C) was immediately added to the eggs. After 5 min gamete contact time, eggs were transferred into 250 mL plastic tri-corner beakers containing 200 mL of 36 ppt seawater for incubation. The embryos were incubated at 20 °C until being examined for fertilization success. Fertilization success was determined 3 to 5 h post-fertilization, by examining a mean (± SEM) of 70 ± 2 eggs per replicate. Embryos were observed and images captured using a compound microscope equipped with a digital camera (as above). Fertilization success was calculated as the percent fertilized eggs. Fertilized eggs were identified by the presence of blastomere cleavage (4 to 64 stages), and those not showing >4-cell cleavages were considered unfertilized.

2.5. Experiment 3: Effect of time post-stripping

Eggs from three females were "crossed" with a sperm pool from four males (in total 12 males were used) at 0, 5, 10, 15, 20, 30, 40, 60 min (± 3 min) post-egg stripping (see Section 2.4 for further details on fertilization procedures). For each female, three replicate crosses were performed at each time post-egg stripping. Throughout storage, the eggs were held in dry weight boats (without extender media), sealed in plastic bags, and stored in a cooler at 20 °C. Based on results outlined in Experiment 2 (see Section 3.2), crosses were conducted using a 2.5e + 4:1 sperm to egg ratio.

2.6. Statistical analyses

Data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) and RMA software for reduced major axis regression (v. 1.17; http://www.bio.sdsu.edu/pub/andy/rma.html) (Bohonak, 2004). Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Fertilization success was arcsin square-root transformed (Zar, 1996). Alpha was set at 0.05.

2.6.1. Experiment 1: Determination of sperm density by use of a spectrophotometer

Linear regression analysis was used to examine the relationship between sperm density and absorbance by use of a spectrophotometer. Separate regressions were run at each absorbance. One of the underlying assumptions of standard ordinary least squares regression is that the independent variable, or X-axis, is measured with no error (Zar, 1996). Because absorbance was measured with "possible error", data were analyzed using RMA software (Model II regression, Ludbrook, 2010).

2.6.2. Experiments 2 and 3: Determination of the optimal sperm to egg ratio, and effects of time post-stripping

Fertilization success was analyzed using a mixed-model ANOVA (PROC MIXED; SAS Institute, 2003) where sperm to egg ratio was considered a fixed factor and female was considered a random blocking factor. A-posteriori analyses were not performed on random female effects. Instead, variance components were constructed using the restricted maximum likelihood (REML) estimation method in SAS PROC Mixed, and expressed as a percentage. To test for significance among variance components (VC) greater than zero, likelihood ratio statistics were generated (SAS Institute, 2003). Denominator degrees of freedom for all F-tests were approximated using the Kenward-Roger method (Spilke et al., 2005). A-posteriori analyses performed on fixed effects were constructed using Tukey's multiple comparisons procedure.

3. Results

3.1. Experiment 1: Determination of sperm density by use of a spectrophotometer

The overall mean (± SD) sperm density for the 18 males was 1.54e + 10 ± 4.95e + 9 sperm/mL with male mean values ranging from 7.13e + 9 to 2.32e + 10 sperm/mL. Regression analysis demonstrated that the most variance was explained between sperm density and absorbance using a spectrophotometer at 350 nm (R^2 = 0.94, $F_{1,17}$ = 235.63, p < 0.001, y = 2.273e + 10x − 2.805e + 10; Fig. 1). Additionally, significant positive relationships were detected at 400 (R^2 = 0.93, $F_{1,17}$ = 231.31, p < 0.001, y = 2.358e + 10x −
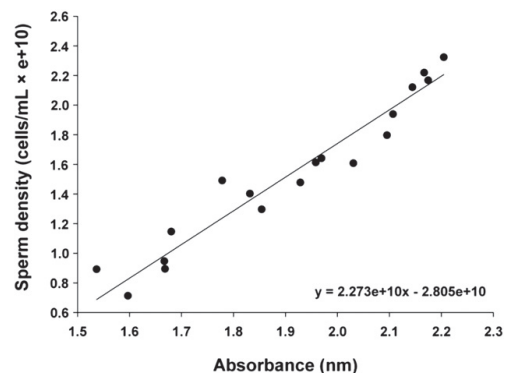

Fig. 1. Relationship between sperm density and absorbance for the European eel, *Anguilla anguilla*. Density was determined using a haemocytometer and absorbance was determined at 350 nm by use of a spectrophotometer.

2.745e + 10), 500 (R^2 = 0.93, $F_{1,17}$ = 227.36, p < 0.001, y = 2.476e + 10x − 2.628 + 10), and 600 nm (R^2 = 0.93, $F_{1,17}$ = 220.50, p < 0.001, y = 2.472e + 10x − 2.408e + 10).

3.2. Experiment 2: Determination of the optimal sperm to egg ratio

Mean fertilization success, using sperm to egg ratios ranging from 1.3e + 3 to 1.0e + 6 sperm per egg increased from 37.5 to 68.1%, respectively (Fig. 2). Sperm to egg ratio had a significant effect on fertilization success ($F_{11,180}$ = 12.24; p < 0.0001; Fig. 2). Fertilization success increased from 1.3e + 3 to 2.5e + 4 sperm per egg, while adding greater than 2.5e + 4 sperm per egg had no significant effect on fertilization success (Fig. 2). The female VC was significant for fertilization success (p < 0.0001) and explained 86.4% of the models variance, while the residual error explained only 13.6%.

3.3. Experiment 3: Effect of time post-stripping

Duration of time post-stripping had a significant effect on fertilization success ($F_{7,50.1}$ = 9.94; p < 0.0001; Fig. 3). Between 0 and 10 min post-stripping 57.4 to 78.2% of the eggs were fertilized on average (Fig. 3). At 15 min post-stripping, a significant decrease in fertilization success was detected; i.e. decreased to 35.2% at 60 min post-stripping. The female VC was significant for fertilization success (p < 0.0001) and explained 84.3% of the models variance while the residual error explained 15.7%.

4. Discussion

In this study, we report several key findings: (i) haemocytometer counts for quantification of sperm density were positively related to absorbance by use of a spectrophotometer, (ii) sperm to egg ratio had a significant effect on fertilization success, where adding greater than 2.5e + 4 sperm per egg had no significant effect, (iii) duration of time post-stripping had a significant effect on egg fertilization success, such that at 15 min post-stripping a significant decrease in fertilization success was detected, and (iv) the female VC was significant for fertilization success (for both Experiments) and explained ≥ 84% of the models variance.

Here, we showed that European eel sperm could be rapidly and accurately (94% of the variance was explained) quantified by use of a spectrophotometer. Similar relationships have also been established for small-bodied biomedical fishes (Tan et al., 2010), as well as economically important marine (Rouxel et al., 2008), and freshwater species (Cierezsko and Dabrowski, 1993). To establish our method, we tested

Fig. 2. The effect of sperm to egg ratio on fertilization success in the European eel, *Anguilla anguilla*. Sperm to egg ratios with different letters are significantly different (p < 0.05, least square means, ANOVA). Error bars represent least square means standard error.
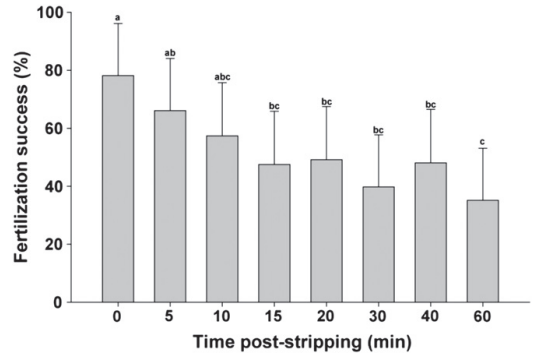

Fig. 3. The effect of time post-stripping of eggs on fertilization success in the European eel, *Anguilla anguilla*. Times with different letters are significantly different (p < 0.05, least square means, ANOVA). Error bars represent least square means standard error.

four different wavelengths spanning the visible spectrum. Our results showed highly significant relationships among readings at 350 to 600 nm, indicating that all tested wavelengths can be used for quantifying sperm density for this species. Using small-bodied biomedical model fishes, Tan et al. (2010) observed a similar phenomenon; i.e. no single maximal absorbance peak was detected across the visible spectrum (380 to 750 nm). This likely occurs because of variability in milt composition, through an assortment of lipids and proteins (among others), which essentially makes it difficult to find a discrete peak in the absorbance spectra via spectrophotometry (Dong et al., 2005). Nevertheless, the 350 nm wavelength appeared to be the most suitable for sperm quantification based on having a slightly higher coefficient of determination (R^2 = 0.94 vs. 0.93). Ultimately, this methodology can now be incorporated into routine hatchery and experimental protocols to avoid potential confounding factors relating to male-to-male variation in sperm density. Additionally, it should increase the effectiveness of cryopreservation and fertilization protocols by standardizing the number of cells in each cryogenic sperm straw and optimizing sperm to egg ratios (Alavi et al., 2008; Butts et al., 2011; Cierezsko et al., 2000), respectively.

Based on a literature review by Butts et al. (2012a), the number of sperm required to fertilize a fish egg is relatively high and species dependent (mean ± SEM for 15 species was ~550,000 ± 480,000 sperm per egg). To our knowledge, this is the first study that assesses how sperm number influences egg fertilization for the European eel. An increase in fertilization success was observed up to 2.5e + 4 sperm per egg; adding additional sperm had no significant effect on the dependent variable. Consequently, this gamete ratio is now recommended for studies dealing with European eel fertility, as it secured maximal fertilization rates, while at the same time used limited sperm cells. However, when using this ratio we still have to be cognizant of the fertilization environment, as varying the sperm extender, adding additional activation media, "crossing" gametes in containers with different dimensions, and modifying gamete contact times may all influence the final outcome of a fertilization event, thus altering the optimal sperm to egg ratio (Alavi et al., 2008; Casselman et al., 2006; Cierezsko et al., 2000). Thus, these variables (among other factors) should coincide with those used in our experimentation, especially if our optimized sperm to egg ratio is to be used for fertilization. Ultimately, this will enable the fertilizing capability of individual males to be measured consistently and each male's sperm, whether stored frozen or in living gene banks, to be used wisely.

We found that at 15 min post-stripping of the eggs, a significant decrease in fertilization success was detected. This suggests that European eel eggs are much more sensitive to storage time post-stripping relative to that of salmonids, such as Chinook- (*Oncorhynchus tshawytscha*) and Atlantic salmon (*Salmo salar*), where a delay in activation did not affect fertilization for these species until 1 h post-stripping (Munkittrick et al.,
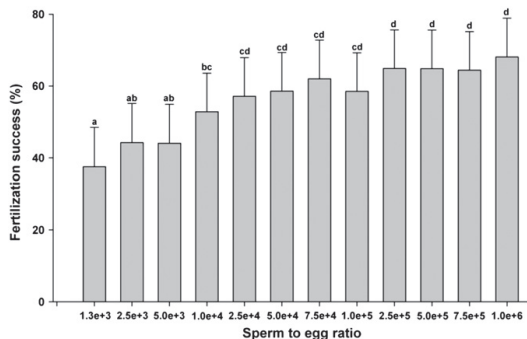
1992). In the Japanese eel (*Anguilla japonica*), Ohta et al. (1996) found that time post-ovulation, within the ovary, to in vitro egg activation is an important factor for fertilization with a clear negative effect occurring at 6 to 7 h post-ovulation. This suggests that we should mimic this ovarian environment in situ to decrease this rapid rate of egg decay as observed in our study; perhaps through extenders that mimic the ovarian fluid of European eel under a strict thermal and/or oxygenated regime.

Fertilization success was strongly influenced by female identity. In our studies, eggs were obtained from wild-caught females that experienced different conditions prior to capture and size/age varied, although they were housed under similar conditions during the reproductive season (e.g. photoperiod, temperature) and received similar hormonal therapy. Ultimately, these factors may have contributed to this variability in female responsiveness to treatment and egg quality. Eggs from each female were fertilized using a new sperm pool from multiple males (having no difference in sperm motility), thus masking any potential paternal effects that may have been active during and immediately after embryogenesis (Rideout et al., 2004). Nevertheless, there is still a suite of other genetic and environmental factors, as well as the associated genetic × environmental interaction, which modulate nuclear-genetic and extra-nuclear non-genetic constituents (e.g. yolk, lipids, immunoglobulin, mRNAs, hormones, and stage of oocyte maturation) that foster the development, quality, and size of an egg, in turn influencing female fertility (reviewed in Babin et al., 2007). Future studies should explore these maternal processes, to ultimately decipher the direct mechanisms leading to enhanced egg quality and higher fertilizations success for this species.

In conclusion, we have now standardized artificial fertilization protocols for the European eel under controlled experimental conditions. Based on our findings, we conclude that spectrophotometry is a valuable tool for estimating sperm density for this species. Furthermore, we suggest that European eel eggs should be fertilized using 2.5e + 4 sperm per egg within the first 10 min post-stripping to maintain a high capacity to be fertilized. Overall, these findings will contribute to the development of European eel breeding technology and further our understanding on sperm biology, cryobiology, and reproductive biology in fishes.

Acknowledgements

References

Alavi, S.M.H., Linhart, O., Coward, K., Rodina, M., 2008. Fish spermatology: implications for aquaculture management. In: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Ltd., Oxford, pp. 397–461.

Babin, P.J., Cerdà, J., Lubzens, E., 2007. The fish oocyte: from basic studies to biotechnological applications. Springer, New York.

Ball, M.A., Parker, G.A., 1996. Sperm competition games: external fertilization and "adaptive" infertility. J. Theor. Biol. 180 (2), 141–150.

Bergh, Ø., Hansen, G.H., Taxt, R.E., 1992. Experimental infection of eggs and yolk sac larvae of halibut, *Hippoglossus hippoglossus* L. J. Fish Dis. 15 (5), 379–391.

Bohonak, A.J., 2004. RMA: software for reduced major axis regression, version 1.17. San Diego State University, San Diego.

Butts, I.A.E., Trippel, E.A., Litvak, M.K., 2009. The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod *Gadus morhua* L. Aquaculture 286 (1–2), 89–94.

Butts, I.A.E., Babiak, I., Ciereszko, A., Litvak, M.K., Słowińska, M., Soler, C., Trippel, E.A., 2011. Semen characteristics and their ability to predict sperm cryopreservation potential of Atlantic cod, *Gadus morhua* L. Theriogenology 75 (7), 1290–1300.

Butts, I.A.E., Roustaian, P., Litvak, M.K., 2012a. Fertilization strategies for winter flounder: effects of spermatozoa density and the duration of gamete receptivity. Aquat. Biol. 16 (2), 115–124.

Butts, I.A.E., Love, O.P., Farwell, M., Pitcher, T.E., 2012b. Primary and secondary sexual characters in alternative reproductive tactics of Chinook salmon: associations with androgens and the maturation-inducing steroid. Gen. Comp. Endocrinol. 175 (3), 449–456.

Casselman, S.J., Schulte-Hostedde, A.I., Montgomerie, R., 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can. J. Fish. Aquat. Sci. 63 (9), 2119–2125.

Ciereszko, A., Dabrowski, K., 1993. Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. Aquaculture 109 (3–4), 367–373.

Ciereszko, A., Glogowski, J., Dabrowski, K., 2000. Fertilization in landlocked sea lamprey: storage of gametes, optimal sperm:egg ratio, and methods of assessing fertilization success. J. Fish Biol. 56 (3), 495–505.

Dollerup, J., Graver, C.M., 1985. Repeated induction of testicular maturation and spermiation, alternating with periods of feeding and growth in silver eels, *Anguilla anguilla* (L.). Dana J. Fish. Mar. Res. 4, 19–39.

Dong, Q.X., Eudeline, B., Huang, C., Tiersch, T.R., 2005. Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg). Aquac. Res. 36 (1), 86–93.

Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L., Asturiano, J.F., 2012. Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354, 7–16.

Gousset, B., 1990. European eel (*Anguilla anguilla* L.) farming technologies in Europe and in Japan: application of a comparative analysis. Aquaculture 87 (3–4), 209–235.

Hatef, A., Niksirat, H., Alavi, S.M.H., 2009. Composition of ovarian fluid in endangered Caspian brown trout, *Salmo trutta caspius*, and its effects on spermatozoa motility and fertilizing ability compared to freshwater and a saline medium. Fish Physiol. Biochem. 35 (4), 695–700.

Johnston, T.A., Kaufman, S.D., Moles, M.D., Wiegand, M.D., Leggett, W.C., 2008. Effects of delayed fertilization on embryo viability in walleye *Sander vitreus* (Mitchill): the role of maternal effects. J. Fish Biol. 72 (10), 2634–2644.

Kagawa, H., Tanaka, H., Ohta, H., Unuma, T., Nomura, K., 2005. The first success of glass eel production in the world: basic biology on fish reproduction advances new applied technology in aquaculture. Fish Physiol. Biochem. 31 (2–3), 193–199.

Ludbrook, J., 2010. Linear regression analysis for comparing two measurers or methods of measurement: but which regression? Clin. Exp. Pharmacol. Physiol. 37 (7), 692–699.

Mordenti, O., Di Biase, A., Bastone, G., Sirri, R., Zaccaroni, A., Parmeggiani, A., 2013. Controlled reproduction in the wild European eel (*Anguilla anguilla*): two populations compared. Aquac. Int. 21 (5), 1045–1063.

Munkittrick, K.R., McGeachy, S.M., Burke, M.G., Flett, P.A., 1992. Effects of delay in water addition or rinsing on fertilization rates of Chinook salmon, coho salmon, Atlantic salmon, and rainbow trout eggs. Progress. Fish Cult. 54 (1), 14–20.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K., 1996. Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica*. Aquaculture 139 (3–4), 291–301.

Ottolenghi, F., Silvestri, C., Giordano, P., Lovatelli, A., New, M.B., 2004. Eels. Capture-based aquaculture — the fattening of eels, groupers, tunas and yellowtails. FAO, Rome, Italy.

Palstra, A.P., Cohen, E.G.H., Niemantsverdriet, P.R.W., van Ginneken, V.J.T., van den Thillart, G.E.E.J.M., 2005. Artificial maturation and reproduction of European silver eel: development of oocytes during final maturation. Aquaculture 249 (1–4), 533–547.

Peñaranda, D.S., Pérez, L., Gallego, V., Barrera, R., Jover, M., Asturiano, J.F., 2010. European eel sperm diluent for short-term storage. Reprod. Domest. Anim. 45 (3), 407–415.

Pérez, L., Peñaranda, D., Gallego, V., Asturiano, J., 2009. Testis development, sperm quality evaluation and cryopreservation in the European eel. In: Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning Migration of the European Eel, vol. 30. Springer, Dordrecht, pp. 333–362 (Netherlands).

Psenicka, M., Rodina, M., Linhart, O., 2010. Ultrastructural study on the fertilisation process in sturgeon (*Acipenser*), function of acrosome and prevention of polyspermy. Anim. Reprod. Sci. 117 (1–2), 147–154.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. Paternal effects on haddock early life history traits. J. Fish Biol. 64 (3), 695–701.

Rouxel, C., Suquet, M., Cosson, J., Severe, A., Quemener, L., Fauvel, C., 2008. Changes in Atlantic cod (*Gadus morhua* L.) sperm quality during the spawning season. Aquac. Res. 39 (4), 434–440.

SAS Institute Inc., 2003. SAS System, version 9.1. SAS Institute Inc., Cary, NC, USA.

Snook, R.R., Hosken, D.J., Karr, T.L., 2011. The biology and evolution of polyspermy: insights from cellular and functional studies of sperm and centrosomal behavior in the fertilized egg. Reproduction 142 (6), 779–792.

Sørensen, S.R., Gallego, V., Pérez, L., Butts, I.A.E., Tomkiewicz, J., Asturiano, J.F., 2013. Evaluation of methods to determine sperm density for the European eel, *Anguilla anguilla*. Reprod. Domest. Anim. 48 (6), 936–944.

Spilke, J., Piepho, H.P., Hu, X., 2005. Analysis of unbalanced data by mixed linear models using the mixed procedure of the SAS system. J. Agron. Crop Sci. 191 (1), 47–54.

Støttrup, J.G., Jacobsen, C., Tomkiewicz, J., Jarlbaek, H., 2013. Modification of essential fatty acid composition in broodstock of cultured European eel *Anguilla anguilla* L. Aquac. Nutr. 19 (2), 172–185.

Tan, E., Yang, H., Tiersch, T., 2010. Determination of sperm concentration for small-bodied biomedical fishes by use of microspectrophotometry. Zebrafish 7 (2), 233–240.

Tomkiewicz, J., Tybjerg, L., Støttrup, J., McEvoy, F., Ravn, P., Sørensen, S.R., Lauesen, P., Graver, C., Munk, P., Holst, L.K., Vestbö, B., Svalastoga, E., Jacobsen, C., Holst, B., Steenfeldt, S.J., Buelund, L., Hornum, T., Kofoed, T., 2012. Reproduction of European Eel in Aquaculture (REEL). DTU Aqua Report Series (47 pp.).

Zar, J.H., 1996. Biostatistical analysis, Third edition. Prentice-Hall, New Jersey.

Paper 3:

Microbial interference and potential control in culture of European eel (*Anguilla anguilla*) embryos and larvae
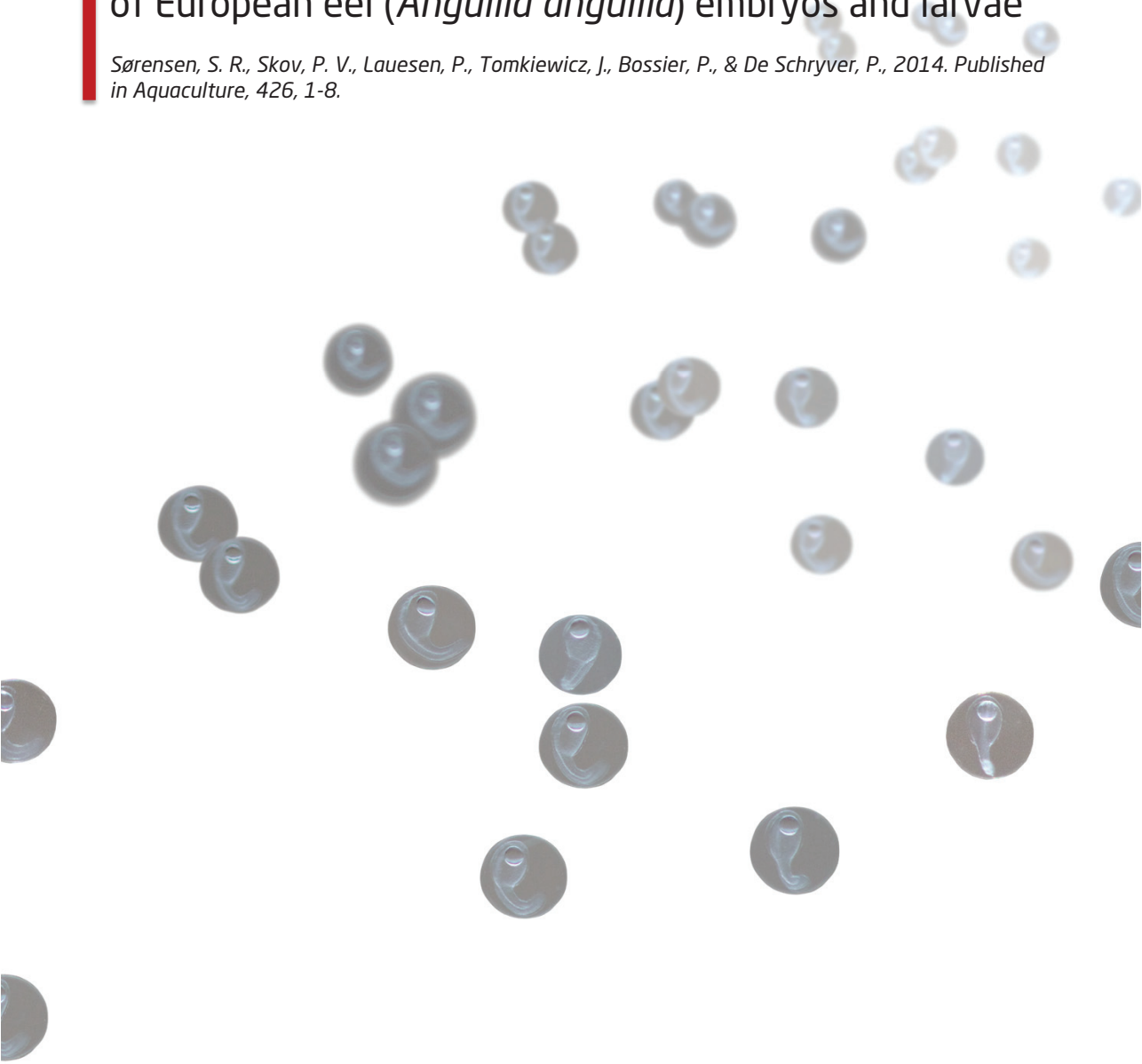
Microbial interference and potential control in culture of European eel (*Anguilla anguilla*) embryos and larvae

Sune Riis Sørensen [a],[*], Peter V. Skov [b], Peter Lauesen [c], Jonna Tomkiewicz [a], Peter Bossier [d], Peter De Schryver [d]

[a] *National Institute of Aquatic Resources, Technical University of Denmark, Jægersborg Allé 1, Charlottenlund 2920, Denmark*
[b] *National Institute of Aquatic Resources, North Sea Science Park, Technical University of Denmark, 9850 Hirtshals, Denmark*
[c] *Billund Aquaculture Service Aps., Kløvermarken 27, Billund 7190, Denmark*
[d] *Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium*

ARTICLE INFO

ABSTRACT

Recent experimental research applying hormonally induced maturation in European eel has resulted in production of viable eggs and yolk-sac larvae. However, present incubation and larval rearing conditions are suboptimal and few larvae survive until onset of first feeding. The aim of this work was to investigate if high mortality during egg incubation and larval culture resulted from microbial interference. By suppressing microbial coverage and activity on fertilised eel eggs using antibiotic and disinfection treatment, egg hatching success and larval longevity were significantly improved. A new approach based on scanning electron microscopy was developed to quantify microbial coverage of eggs. Measurements of microbial coverage in combination with growth curves of egg-associated bacteria indicated that microbial activity rather than physical coverage led to reduced hatch success. In addition, an inverse relationship between microbial coverage of eggs and larval survival indicated that attachment of micro-organisms on the egg surface during the last 24 h of incubation affected later larval survival. These results suggest that microbial control through application of egg surface disinfection in combination with microbial management will be fundamental for improved post-hatch larval survival.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

For marine fish in aquaculture, the interactions between micro-organisms and mucosal surfaces of eggs and larvae have been associated with a reduction in egg hatch success and post-hatch survival of larvae (Hansen and Olafsen, 1999). Microbial colonisation may damage the *zona pelucida* by bacterial secretion of proteolytic enzymes exposing the underlying *zona radita* (Hansen et al., 1992; Pavlov and Moksness, 1993). Reduced hatch success and premature or delayed hatching have been attributed to toxins secreted by certain bacterial colonies (Olafsen, 2001). Similarly, lethal effects may arise from the physical prevention of chorion gas exchange and intra-oocyte oxygen supply as reviewed by Hansen and Olafsen (1999). The requirements for oxygen during embryonic development is greatest during the late incubation period (Hempel, 1979), which often coincides with the densest bacterial coverage (Hansen and Olafsen, 1989; Pavlov and Moksness, 1993). Such microbial colonisation may be prominent in aquaculture, especially in egg incubators that often contain high egg densities and ample substrate supporting growth of micro-organisms (Olafsen, 2001). Under natural circumstances, the number of bacteria associated with eggs will generally be lower and colonisation effects on hatch success are likely to be limited as reported for sardine (Míguez et al., 2004).

European eel (*Anguilla anguilla*) is a well-known species in aquaculture, but captive breeding has not yet been established partly due to complex natural hormonal control mechanisms of eel reproduction (Dufour et al., 2003) and difficulties in eel larval rearing and ongrowing (Okamura et al., 2009). Recent experimental research applying hormonally induced maturation has resulted in production of leptocephalus larvae and glass eels of Japanese eels (*Anguilla japonica*) (Ijiri et al., 2011) as well as production of viable eggs and yolk-sac larvae of European eel (Tomkiewicz, 2012). For European eel, present incubation and larval rearing conditions are suboptimal, resulting in high mortality in the embryonic and yolk-sac stages, thus few larvae survive until first feeding. Mortality and disease during egg and larval culture of marine species in aquaculture are often associated with an uncontrolled microbial community (Olafsen, 2001). Microbial interference is likely a challenge for the embryonic and early post-hatch development of European eel. In nature, early life stages of European eel prevail in the Sargasso Sea with eggs assumed to be neutrally buoyant in the depth range between 50 and 150 m (Castonguay and McCleave, 1987; Riemann et al., 2010). Water sample analyses from this aquatic layer suggest that this is an oligotrophic environment, low in phytoplankton and zooplankton (Rowe et al., 2012) and bacterial density (app. 4.6–8.8×10^5 colony forming units (CFU) mL^{-1}) (Rowe et al., 2012).

Sensitivity of European eel eggs and larvae towards microbes is presently unknown. A previous study on sardines in their native environment examined bacteria loads on eggs by methods of washing

* Corresponding author. Tel.: +45 21314983; fax: +45 35883333.
E-mail address: srs@aqua.dtu.dk (S.R. Sørensen).

and detaching adhering microbes prior to quantitative analysis (Míguez et al., 2004). Here bacteria were found in low numbers (1×10^2 CFU) and attachment to eggs had no effect on hatch and survival. In aquaculture, however, bacteria numbers can be higher (Hansen and Olafsen, 1999) and bacteria composition is different from natural habitats (Bergh et al., 1992). As reported for several marine fish species, microbial interference can be severe (e.g. Bergh et al., 1992) and microbial interference may be of significance for European eel embryos and larvae in captive breeding. Assessing the importance of microbial interference will therefore be useful for optimising culture of early life stages for this species.

In this context, the aim of this study was to investigate if high mortality during egg incubation and larval culture of European eel results from microbial interference. Microbial presence and activity on fertilised eggs were manipulated using antibiotic and disinfection treatments to investigate effects on hatching success and subsequent larval mortality. An analytical method based on scanning electron microscopy (SEM) was developed for quantification of microbial coverage of the egg surface. The estimated microbial coverage of eggs was related to hatching success and larval survival to assess the impact on European eel larviculture and to make recommendations for future culture methods.

2. Materials and methods

2.1. Broodstock and gamete production

Female silver eels were obtained from a freshwater lake, Lake Vandet, in northern Jutland, Denmark, while males originated from a commercial eel farm (Stensgård Eel Farm, Denmark). The broodstock was transported to a research facility of the Technical University of Denmark (DTU) and transferred to 300 L tanks in a recirculation system and acclimatised to artificial seawater adjusted by Tropic Marin® Sea Salt (Dr. Biener Aquarientechnik, Germany) to ~36 ppt and ~20 °C (Tomkiewicz, 2012). Broodstock was maintained in a natural daylight regime i.e. a 12 h photoperiod with a gradual 30 minute shift to dark conditions. Prior to experiments, fish were anaesthetized (ethyl p-aminobenzoate, 20 mg L^{-1}; Sigma-Aldrich Chemie, Steinheim, Germany) and tagged with a passive integrated transponder (PIT tag).

Females were thereafter matured by weekly injections of salmon pituitary extract (SPE 18.75 mg kg^{-1} body weight) and males similarly by weekly injections of human chorionic gonadotropin (hCG 1.0 mg kg^{-1} body weight). Female ovulation was induced using the maturation inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (Tomkiewicz, 2012).

2.2. Water quality in fertilisation, incubation and larval culture

Natural North Sea seawater (32.5 ppt) was filtered using a drop-in housing cartridge filter (0.8 μm, CUNO 3M®, St. Paul, MN, USA) and salinity adjusted to full-strength seawater (FSW; 36 ppt) using Tropic Marin® Sea Salt. Salinity was verified using a conductivity metre (WTW Multi 3410, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). FSW was used in egg activation, fertilisation, incubation, and larval cultures. Water was kept at 20 °C and aerated using an airstone during the experiments. FSW used in Experiments 1 and 2, on microbial interference, was autoclaved prior to use, i.e. filtered autoclaved sea water (FASW).

2.3. Fertilisation and incubation

Milt was collected 2 h prior to fertilisation from three to four males by applying gentle abdominal pressure. Sperm motility was characterised within 30 s of activation using an arbitrary scale where 0 represents no motile sperm, I represents <25%, II represents 25–50%, III represents 50–75%, IV represents 75–90% and V represents

90–100% of motile spermatozoa (Sørensen et al., 2013). The percentage of motile cells was determined using a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan), equipped with a Nikon $400\times$ magnification ($40\times$ CFI Plan Flour). Sperm with a motility lower than 50% was not used. Milt was diluted in an artificial seminal plasma medium, hereafter P1 media (Asturiano et al., 2004). The milt dilution ratio was adjusted (Ohta et al., 1996) to 1:99. Diluted sperm was kept in sterile culture flasks at 4 °C prior to mixing with eggs (Sørensen et al., 2013). Eggs were stripped into dry and sterilised containers and 1 ml of diluted sperm was added per 2 g of eggs, and then activated by adding FSW. After activation, gametes were moved to 15 L of FSW for 1 h. Buoyant eggs were skimmed into 15 L of fresh FSW to remove excess sperm and negatively buoyant (dead) eggs.

Two hours later, eggs were moved to a 60 L incubator holding FSW for ~300 g buoyant eggs at 20 °CC ± 0.5. Eggs were kept in suspension by gentle aeration through an air diffuser. Dead eggs were removed regularly by purging from a bottom valve to prevent build-up of bacteria (Keskin et al., 1994). Fifteen hours post fertilization (HPF), incubator FSW was renewed. Each batch of eggs was quality screened and only egg batches showing normal embryonic development were applied in Experiments 1 and 2. All incubation equipment was cleaned using Virkon-S 2% solution (Virkon-S®, DuPont, USA) between batches.

2.4. Experiment 1: effect of antibiotic treatment on egg hatching and larval survival

In Experiment 1, ~10,000 eggs were collected from the incubator at 23 HPF, rinsed gently twice using a 100 μm sieve and 1 L FASW, and transferred to a glass beaker containing 1 L FASW. From the rinsed eggs, 4000 eggs were evenly distributed to ten beakers with FASW. Beaker one was kept as control and beaker two to ten received different antibiotic treatments as described in Table 1. The final volume in each beaker was 900 mL.

For assessment of hatching success, 3×48 eggs from each treatment beaker were transferred to multiwell plates (48-well, Nunc® Non-treated, Thermo Scientific) with each well containing 1 egg and 1 mL of treatment water (Table 1). Subsequently, multiwell plates were covered with lids and incubated in a temperature controlled environment at 20 ± 0.5 °C (MIR-154 Incubator, Panasonic Europe B.V.) at a light intensity <5 lx. Hatching success was assessed by counting the number of hatched larvae in each plate at 55 HPF.

For assessment of larval survival, the ten treatment beakers with remaining eggs were incubated in the same temperature controlled environment as the multiwell plates. After hatching at 55 HPF, 4×30 hatched larvae were chosen at random from each treatment and transferred to 4 sterile media flasks (Nunc® 75 cm^2 Flasks, Non-treated with Ventilated Caps, Thermo Scientific) containing 200 mL of new FASW. Each flask contained newly prepared water with the same antibiotic mixture as the beaker from which larvae originated, and was incubated as described above. Survival was determined daily by counting the larvae, until 350 HPF, which coincides with the expected time of first feeding (Tomkiewicz, 2012) avoiding starvation effects. All counting procedures were performed under low intensity red light to avoid stressing the larvae.

In order to assess the antimicrobial activity of applied antibiotics, five eggs were transferred at 40 HPF from the FASW beaker (control) to a petri dish with marine agar (BD Difco™, BD Diagnostic Systems) for culturing egg-associated microbes. The plate was incubated at 20 ± 0.5 °C for 24 h. A mixture of the grown bacteria was sampled and further grown in a marine broth (BD Difco™, BD Diagnostic Systems) for 24 h at 28 °C on an orbital shaker. The mixed culture was then washed with FASW and diluted using FASW to an optical density of 1 at 600 nm. Antibiotic solutions was then prepared according to Table 1 and inoculated with freshly grown culture at 1 vol.% (resulting in ~10^6 CFU mL^{-1}) along with a negative control (FASW). From inoculated cultures 3×200 μL aliquots were

Table 1

Overview of antibiotic treatments used in Experiment 1 and disinfection treatments used in Experiment 2 for incubation of eggs and larvicultures of European eel (*Anguilla anguilla*).

Experiment 1: antibiotics[a]	Treatment	Antibiotic mixtures and concentrations	
	FASW	Filtered autoclaved seawater — control	
	PS^{low}	Penicillin (20 ppm), streptomycin (30 ppm)	
	PS^{med}	Penicillin (45 ppm), streptomycin (65 ppm)	
	PS^{high}	Penicillin (60 ppm), streptomycin (100 ppm)	
	AR^{low}	Ampicillin (10 ppm), rifampicin (10 ppm)	
	AR^{med}	Ampicillin (50 ppm), rifampicin (50 ppm)	
	AR^{high}	Ampicillin (100 ppm), rifampicin (100 ppm)	
	$ARKT^{low}$	Ampicillin (10 ppm), rifampicin (10 ppm), kanamycin (10 ppm) trimethoprim (10 ppm)	
	$ARKT^{med}$	Ampicillin (50 ppm), rifampicin (50 ppm), kanamycin (50 ppm), trimethoprim (50 ppm)	
	$ARKT^{high}$	Ampicillin (100 ppm), rifampicin (100 ppm), kanamycin (100 ppm), trimethoprim (100 ppm)	
Experiment 2: disinfection [b]	Treatment	Disinfection compound	Disinfection treatment
	FASW	No treatment	Filtered autoclaved seawater[c]
	GLUT	Glutaraldehyde	100 ppm, 2.5 min
	S HYP 100	Sodium hypochlorite	100 ppm, 10 min
	S HYP 50	Sodium hypochlorite	50 ppm, 5 min
	H_2O_2 0.2%	Hydrogen peroxide	2000 ppm, 15 min
	H_2O_2 0.6%	Hydrogen peroxide	6000 ppm, 5 min

[a] All antibiotics were purchased from Sigma-Aldrich. Penicillin G sodium salt, streptomycin sulphate salt, ampicillin sodium salt and kanamycin sulphate salt (from *Streptomyces kanamyceticus*) were dissolved in FASW. Rifampicin and trimethoprim were dissolved in methanol prior to addition in FASW. Salt-content of antibiotics was considered and not included in concentrations.

[b] Glutaraldehyde (25% Grade II), sodium hypochlorite, and hydrogen peroxide (30% active) were purchased from Sigma-Aldrich.

[c] Mimicking handling associated with disinfection treatment.

transferred to a 96-well plate (Multiwell, Nunc® Non-treated, Thermo Scientific). Optical density at 600 nm (OD_{600nm}) was measured every hour for 24 h (Tecan Infinite M200, Tecan Group Ltd. Männedorf, Switzerland). Cultures that did not show an increase in OD_{600nm} after 24 h were serial dilution plated on marine agar to determine the bacterial density.

2.5. Experiment 2: effect of egg surface disinfection on hatching success and larval survival

Approximately 10,000 eggs from a different egg batch were collected at 28 HPF. Eggs were rinsed twice using a 100 µm sieve and 1 L FASW and transferred to a glass beaker containing 1 L FASW. From the rinsed eggs, 4000 eggs were evenly distributed into six beakers each subjected to a disinfection treatment as described in Table 1, using the following procedure: eggs were concentrated on a 100 µm sieve and submerged into disinfection solution while gently swirling the sieve. Subsequently, eggs were washed two times on the sieve in 800 mL FASW. Each batch of eggs was finally incubated in a beaker containing 900 mL FASW with AR^{low} antibiotic mixture.

For determination of hatching success, 3 × 48 eggs were transferred from the beakers to 48-well plates as in Experiment 1. Similarly, larval survival was determined as described in Experiment 1, with the exception that all media flasks contained FASW with AR^{low} antibiotic mixture.

Eggs were sampled for assessment of egg chorion microbial coverage. Immediately after disinfection and washing, 5 eggs from each treatment were sampled and fixed in 2.5% glutaraldehyde (Grade I, Sigma-Aldrich, Missouri, USA) with 0.1 M phosphate buffered saline (PBS), pH 7.4. Preserved eggs were dehydrated according to a modified procedure of Laforsch and Tollrian (2000). In brief, eggs were rinsed in 0.1 M PBS and dehydrated in a graded series of ethanol (70%, 95%, 3 × 100% for 30 min each). After the last dehydration step, eggs were transferred to a 50:50 solution of pure ethanol and hexamethyldisilazane (HMDS) (Sigma-Aldrich, USA) for 30 min, and then to pure HMDS for 30 min. After this step, excess HMDS was removed leaving only enough to cover the sample, which was then transferred to a desiccator with

evacuation. Once eggs were chemically dried, they were mounted on aluminium stubs onto double-sided carbon tape by aid of a dissection microscope, and sputter coated with gold (E5000, Polaron).

2.6. Assessment of microbial egg chorion coverage using SEM

Samples were viewed on an XL FEG 30 scanning electron microscope (Philips, The Netherlands) with an acceleration voltage of 2 kV and a working distance of 5 mm. Quantification of microbial egg surface coverage was done as follows: the exposed hemisphere of each egg was photographed at five surface regions, in the centre of the egg and in each quadrant (Fig. 1, Step 1). A region of interest (ROI) was selected at a low magnification (×20) that precluded any visual details of egg surface and bacterial coverage to assure an unbiased selection of images. Magnification was subsequently increased to ×5.000 for image acquisition (Fig. 1, Step 2). If initial ROI provides an image unsuited for quantification, such as a folded or slanted surface area, the SEM stage was moved towards the right until a frame with an image suited for acquisition appeared. From each of the five surface images, three replicate conversions to binary images were done following adjustment of contrast and threshold to distinctly outline colonies from chorionic surface (Fig. 1, Step 3). Subsequently, the three binary images were analysed using the particle analyser function in the software ImageJ (Rasband, 1997–2012) to measure surface coverage (Fig. 1, Step 4). For each binary image, an initial measurement of the smallest and largest bacterial colony was made to set the range of colonies to measure. Finally, the average values of the 15 binary surface images yielded the average egg surface coverage.

2.7. Statistical analysis

Statistical analyses for survival data in Experiments 1 and 2 were performed using SAS software (v.9.1; SAS Institute Inc., Cary, NC, USA; SAS Institute Inc., 2003). Data for hatching and microbial coverage was analysed using Sigmaplot v. 11 (Systat Software Inc., Hounslow, UK). Data were square root transformed when necessary to meet normality and homoscedasticity assumptions. Differences in treatment means
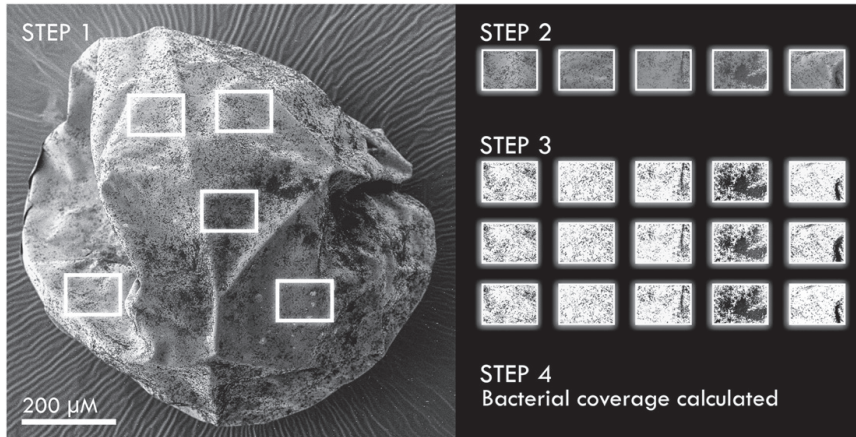
Fig. 1. Procedure to obtain average percent of microbial surface coverage on European eel, *Anguilla anguilla*, eggs based on scanning electron microscopy (SEM) images. Step 1: choose photo frame at random sites within five regions on the egg hemisphere; central region and in each quadrant. Step 2: capture suitable images. Step 3: convert to 5×3 binary images. Step 4: calculate average coverage.

were detected using the Tukey's least squares means method. All data are presented as mean $\pm$ standard deviation (SD). Significance was set at α of 0.05 for main effects and interactions.

Larval survival data were analysed using repeated measures ANOVAs using the following selected time points for Experiment 1: 100, 200, 300, and 350 HPF, and for Experiment 2: 100, 200, and 250 HPF (50 HPF was time of loading newly hatched larvae i.e. 100% survival). Data were square root transformed when necessary to meet normality and homoscedasticity assumptions. When interactions were detected (time × treatment), reduced one-way ANOVA models were run at each sampling time to determine treatment effects. Within the reduced models only pre-planned comparisons were performed with no repeated use of the same data. Therefore α-level corrections for comparisons were not necessary. Hatching success data for Experiments 1 and 2 were analysed using a one-way ANOVA.

The microbial coverage of egg chorion in Experiment 2 was tested using a one-way ANOVA on ranks (Kruskal–Wallis) and pairwise multiple comparison tests (Dunn's method) due to violation of the homoscedasticity assumption.

3. Results

3.1. Experiment 1: effect of antibiotic treatment on hatching success

The hatching success was significantly improved by the application of any of the antibiotic treatments (one-way ANOVA; $P < 0.001$) with about a doubling of the number of hatched larvae as compared to the FASW control treatment without antibiotics. The mean hatching success in FASW was 37.8%, while the antibiotic treatments ranged from 67.7 to 81.9% (Fig. 2). No significant differences were detected in hatching success between the antibiotic treatments.

3.2. Experiment 1: effect of antibiotic treatment on larval survival

Larval survival decreased over the experimental period but the antibiotic treatments showed capable of improving larval survival (Fig. 3). The repeated measures ANOVA showed a significant interaction between time and antibiotic treatment (repeated measures ANOVA; $P < 0.001$), therefore separate one-way ANOVA tests were run at 100, 200, 300, and 350 HPF. At each of these time points, significant treatment effects were detected (one-way ANOVA's; $P < 0.001$). In summary, the AR[med], ARKT[med], and ARKT[low] showed the highest survivals

throughout the experimental period. Within the specific time point at 100 HPF, the AR and ARKT treatments had the highest survival as compared to the PS treatments, while the FASW showed the lowest survival. At 200 HPF, no survival was detected in the PS and FASW treatments, while AR[med], ARKT[med] and ARKT[low] showed the highest survival. At 300 HPF, AR[med], ARKT[med], and ARKT[low] showed a higher survival as compared to AR[low], AR[high], and ARKT[high]. Finally, at 350 HPF the ARKT[low] and ARKT[med] showed the highest larval survival.

3.3. Experiment 1: effect of antibiotic treatments on growth of egg-associated microbiota

Optical density (OD_{600nm}) curves, representing growth of egg-associated bacteria over time in antibiotic solutions, are shown in Fig. 4. FASW showed the highest indicator level of bacterial growth, with an OD_{600nm} that increased from 0.06 to 0.6 over the 24 h period. The PS[low] and PS[med] treatments also showed growth, although a bacteriostatic effect was evident as compared to the FASW control. The remaining cultures showed no growth and resembled the negative control treatment. Dilution plating of cultures with no growth after 24 h
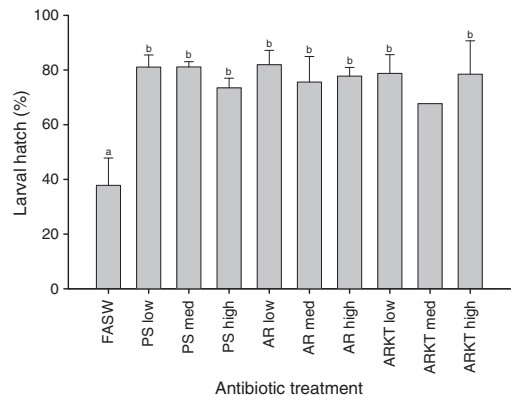
Fig. 2. Larval hatch of European eel, *Anguilla anguilla* (in %) treated with the different combinations of antibiotics indicated in Table 1. Bars show means $\pm$ SD. Bars with different superscripts are significantly different ($\alpha = 0.05$; one-way ANOVA analysis). Standard deviation (SD) is not shown for ARKT[med] due to loss of one of three replicates.
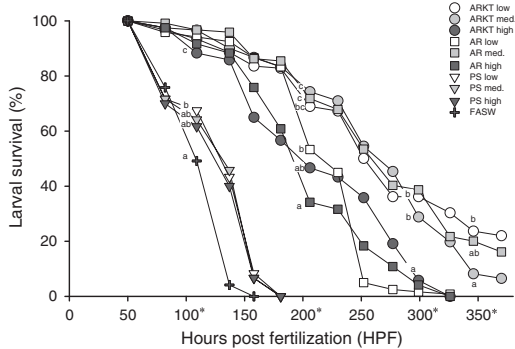
Fig. 3. Mean cumulative % survival of European eel, *Anguilla anguilla*, larvae hatched (48 HPF) from eggs that were incubated in FASW and different combinations of antibiotics (see Table 1). Asterisks denote the times at which treatments were tested for significant differences (Tukey's test). Different superscripts indicate significant differences between treatments ($\alpha = 0.05$).

indicated that the PS[high] culture acted bacteriostatic, resulting in a constant density, while the remaining treatments acted bactericidal, as there was a decrease in cell density from ~1×10^6 to ~1×10^3 or less (Table 2).

3.4. Experiment 2: effect of disinfection on hatching success

Hatching success following disinfection treatment is illustrated in Fig. 5. Egg disinfection with GLUT and H_2O_2 was not found significantly different regarding hatching success compared to non-disinfected eggs (FASW) (one-way ANOVA; $P = 0.403$). In contrast, treatments with sodium hypochlorite proved fatal for the eggs, as these immediately turned opaque white and died.
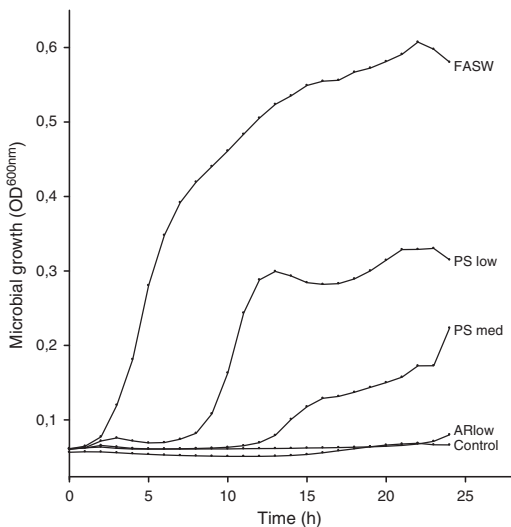
Fig. 4. Microbial growth of European eel, *Anguilla anguilla*, egg-associated bacteria (OD_{600}) subjected to different antibiotic treatments (see Table 1). Only treatments showing growth are presented (FASW, PS[low], PS[med] and AR[low]).

3.5. Experiment 2: effect of disinfection on larval survival

Larval survival decreased over the experimental period (Fig. 6). Repeated measures ANOVA showed a significant interaction between time and antibiotic treatments (repeated measures ANOVA; $P < 0.001$) as such separate one-way ANOVA tests were run at 100, 200, and 250 HPF. At each time point significant treatment effects were detected (one-way ANOVA; $P < 0.001$). In summary, the 0.6% H_2O_2 treatment showed the highest survival throughout the experimental period. Within the specific time point at 100 HPF, the disinfection treatment with 0.6% H_2O_2 had the highest survival and the control treatment (FASW) had the lowest survival. At 200 HPF, this pattern was retained with larval survival being highest in the 0.6% H_2O_2 followed by 0.2% H_2O_2, GLUT and then FASW. At 250 HPF, the pattern was similar with the exception of GLUT now being similar to FASW survival. The control treatment (FASW) in this Experiment 2 (as well as the other treatments) contained the AR[low] antibiotic mixture during the incubation phase similar to the AR[low] treatment in Experiment 1 (Table 1), but larval survival was lower than seen in Experiment 1 in this treatment (Fig. 3).

3.6. Experiment 2: assessment of microbial egg chorion coverage

A clear visual effect of disinfection treatment on microbial egg chorion coverage was observed on eggs fixed after disinfection (Fig. 7A). The average coverage of each treatment is exemplified with the selected pictures illustrated in Fig. 7B. One-way ANOVA on the numerical data supports this observed effect (one-way ANOVA; $P < 0.001$). The box plot of microbial surface area coverage in Fig. 8 shows the 0.6% H_2O_2 treatment to efficiently remove microbial surface coverage and being significantly different from the other treatments (Dunn's; $P < 0.05$). A wide scatter in coverage was observed for the FASW treatment mainly due to one particular egg with an average coverage of only 3.5%, while the remaining 4 eggs had an average of $30.6 \pm 12.5\%$. The FASW treatment was not significantly different from the GLUT or 0.2% H_2O_2 treatment (Dunn's; $P > 0.05$) but differed from the 0.6% H_2O_2 treatment (Dunn's; $P < 0.05$). Regression analysis showed a significant linear relationship between microbial egg surface coverage and larval survival at both 100 HPF ($R^2 = 0.45$, $P = 0.002$), 150 HPF ($R^2 = 0.43$, $P = 0.004$); 200 HPF ($R^2 = 0.48$, $P = 0.002$); and 250 HPF ($R^2 = 0.42$, $P = 0.005$).

4. Discussion

Microbial interference is known to be an important factor affecting egg and larviculture for several marine fish species (Hansen et al., 1992; Oppenheimer, 1955; Shelbourne, 1963), and severity can be species-specific (Hansen and Olafsen, 1989). Studies on the closely related Japanese eel indicate that microbial interference may be an issue in eel larviculture (Ohta et al., 1997; Okamura et al., 2009; Unuma et al., 2004) as a high concentration of streptomycin (100 ppm) and penicillin (60 ppm) has been used for egg incubation and larvae cultures. Our study is the first to investigate the sensitivity to and effects of microbial interference on early life stages of European eel. The findings suggest that microbial control will be required for successful hatchery culture of this species.

Antibiotics serve as an effective tool in microbial management (Alderman and Hastings, 1998; Cabello, 2006) and led in our study to a 100% increase in egg hatch success as compared to untreated controls. The antibiotic treatments of the eggs were applied in the last half of the incubation period; considering that European eel embryonic development lasts ~48 h at 20 °C. The improvement of larval hatching success using antibiotic treatment implies that activity of egg-associated microbiota in the last half of the incubation period significantly influence embryonic survival. The application of egg surface disinfection prior to incubation in antibiotics (AR[low]) did not significantly improve egg

Table 2

Cell density before and after incubation of European eel (*Anguilla anguilla*) egg-associated bacteria in different antibiotic treatments for 24 h.

Antibiotics	PS^{high}	AR^{low}	AR^{med}	AR^{high}	ARKT^{low}	ARKT^{med}	ARKT^{high}
Initial density (CFU mL^{-1})	4.1×10^6	4.1×10^6	4.1×10^6	4.1×10^6	4.1×10^6	4.1×10^6	4.1×10^6
Density after 24 h (CFU mL^{-1})	2.9×10^6	6.2×10^3	2.6×10^3	2.0×10^3	3.4×10^3	$<1 \times 10^3$	$<1 \times 10^3$
Δ density (CFU mL^{-1})	1.90×10^6	-9.94×10^5	-9.97×10^5	-9.98×10^5	-9.97×10^5	-9.99×10^5	-9.99×10^5

hatching success. The novel SEM based approach to quantify microbial egg surface coverage showed that the different disinfection treatments significantly altered the degree of microbial presence on the egg surface. It can thus be concluded that physical coverage by bacteria was not the sole determinant for good eel egg hatch success.

Microbial colonisation of the egg surface may interfere with oxygen supply, which is an important cue for larval hatching, operating both as a trigger or inhibitor of hatching depending on the species under consideration (Korwin-Kossakowski, 2012; Martin et al., 2011). In the case of hypoxia triggering hatching, it is the short-term low oxygen level that stimulates increased movement of the embryo, mixing of perivitelline fluids within the egg and increased distribution of released chorionase enzymes (hatching enzymes) secreted from embryonic glands (Czerkies et al., 2001; Martin et al., 2011). The demand for oxygen is generally low in fish eggs during the early embryonic stages but increases gradually during the later stages (Czerkies et al., 2001; Hempel, 1979). Consequently, an effect of chorion blockage on embryonic development is most critical at the later stages. During these stages, tail formation occurs in the eel embryo and vital functions like heartbeat, motion and finally hatching set in (Pedersen, 2004). A limitation of oxygen supply through longer periods of the incubation will presumably lead to detrimental effects on embryonic development simply due to oxygen transfer blockage as has been shown for clay particle blockage in salmon eggs (Greig et al., 2005). Increased microbial activity or coverage on the egg surface may limit oxygen availability and thus may have impaired embryo development.

The similarity in hatching success for different disinfection treatments despite the observed differences in egg chorion coverage renders it unlikely that physical coverage by bacterial colonisation is a determining factor for hatching success. On the eel eggs, however, the microbial coverage was less complex and thinner than observed for Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Bergh et al., 1992; Hansen and Olafsen, 1989, 1999) which may relate to the shorter egg incubation time for eels (48 h at 20 °C). Aerobic bacterial activity, however, may have scavenged oxygen from the egg surface thereby limiting oxygen diffusion and creating internal hypoxic conditions (Hansen and Olafsen, 1999; Czerkies et al., 2001). While

the activity of egg surface bacteria has been shown to disrupt the fish egg chorion by exoproteolytic enzyme production (Hansen and Olafsen, 1989; Hansen et al., 1992; Pavlov and Moksness, 1993), none of our SEM images showed indications of abnormal changes to the egg chorion. The mechanism that reduced hatch success in our studies cannot be deduced from our data, however the fact that all treatments with antibiotics had higher success than the control and even the ones only acting mildly bacteriostatic (e.g. PS^{low}), suggests that microbial activity is influencing embryonic survival.

In addition to affecting egg hatch success, microbial activity during egg incubation also clearly affected larval quality. The experiment using antibiotics demonstrated that survival of larvae was positively related to the degree of microbial control. The survival curves distinguished two groups, one group surviving no longer than ~180 HPF (FASW, PS^{low}, PS^{med} and PS^{high}) and another surviving longer than ~300 HPF (AR^{low} to ^{high} and ARKT^{low} to ^{high}). This grouping corresponded to whether the antibiotics acted bacteriostatic (PS^{low}, PS^{med}, and PS^{high}) or bactericidal (AR^{low} to ^{high} and ARKT^{low} to ^{high}) according to the microbial growth curves and subsequent plating cultures. Treatments that acted bactericidal gave highest larval longevity and in particular the low and medium concentrations seemed to increase survival. Studies on the toxicity of ampicillin and rifampicin towards fish embryos and larvae are limited, but our data suggest an upper limit for mixed concentrations between 50 ppm and 100 ppm. Decreasing microbial coverage by disinfection also increased the survival of hatched larvae and an inverse relationship between the microbial surface coverage on egg and the larval survival was observed.

Microbial interference during early stages of yolk-sac larvae is known to induce mortality (Vadstein et al., 2007). The bacterial growth curves from the PS treatments (PS^{high}, PS^{med}, and PS^{low}) illustrate that the merely bacteriostatic effect of PS treatments may have allowed microbial activity during culture of larvae and thus may have contributed to larval mortality in the PS mixture masking potential effect of treatment during egg incubation. No studies are available on immune
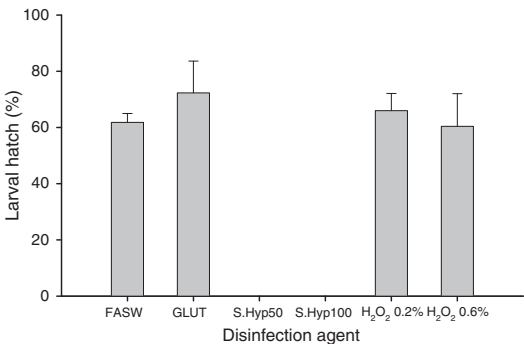
Fig. 5. Larval hatch of European eel, *Anguilla anguilla* (in %) shown as means ± SD after different disinfection treatments (see Table 1) followed by incubation in FASW with the antibiotic mixture AR^{low}. Treatments are not significantly different (α = 0.05; one-way ANOVA analysis).
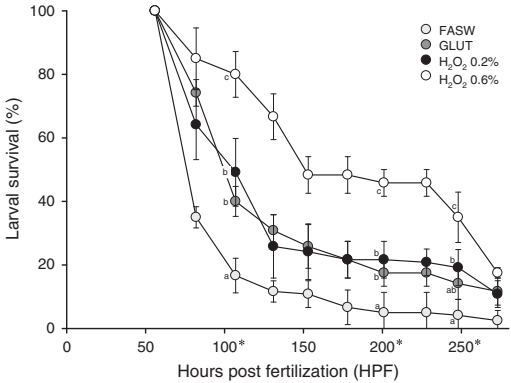
Fig. 6. Survival of European eel, *Anguilla anguilla*, larvae (in %) hatched from eggs treated with different types of surface disinfection (see Table 1) and incubated in FASW with the antibiotic mixture AR^{low}. Egg hatch occurred at ~48 HPF. Data are expressed as means ± SD. Asterisks denote the times at which treatments were tested for significant differences (Tukey's test). Different superscripts indicate significant differences between treatments (α = 0.05).
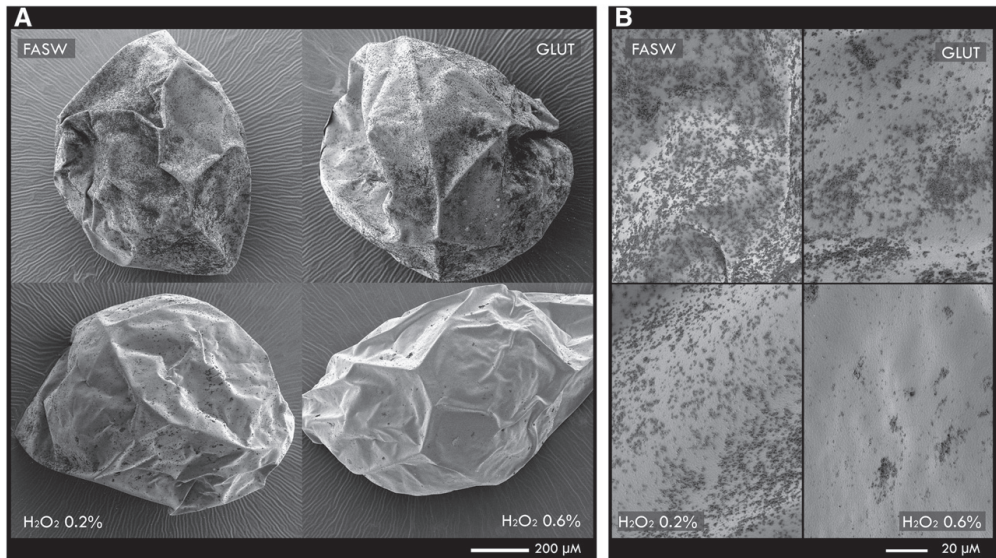
Fig. 7. (A) Scanning electron microscopy (SEM) images representing average microbial surface coverage of eggs from European eel, *Anguilla anguilla* after disinfection. Eggs were fixed immediately after disinfection (treatments described in Table 1). Sodium hypochlorite treated eggs are not included. (B) Selected images of 5000× magnification with an average microbial surface area coverage equal to the respective treatment average. Average microbial surface coverage percentages are as follows: FASW (25.1% coverage); GLUT (22.2% coverage); 0.2% H_2O_2 (17.8% coverage); 0.6% H_2O_2 (3.8% coverage).

response for European eel larvae, but Japanese eel larvae have been shown to possess a weak innate immune system in the yolk-sac stage with only few blood cells and hardly any lymphocytes (Suzuki and Otake, 2000). This indicates a low tolerance to host–microbe interactions in the early preleptocephali stage and suggests that eel larvae are highly sensitive to microbial interference. As proposed by Suzuki and Otake (2000) this may relate to life in the oligotrophic ocean depths. Japanese eel larvae do, however, show exceptionally high concentrations of lectins in the skin mucus. Lectins are a part of the non-specific immune system, transferred maternally (Dong et al., 2004; Zhang et al., 2013), capable of binding to the carbohydrate surface of pathogens facilitating neutralisation of these (Magnadottir et al., 2005; Nielsen and Esteve-Gassent, 2006; Swain and Nayak, 2009; Watanabe

et al., 2013). Lectins are, however, likely highly pathogen specific (Watanabe et al., 2013), and thus may not ensure protection against the pathogens in hatcheries.

Overall, the findings from the present experiments illustrate that microbial control during the egg and larval stages is a contributing factor of importance for larviculture of European eel. As prophylactic use of antibiotics is not a sustainable way to prevent negative microbial interactions, the application of environment friendly alternatives is a necessity. The application of egg surface disinfection with hydrogen peroxide at 24 HPF appears adequate for the removal of egg associated micro-organisms and is not associated with health hazards as is the case for glutaraldehyde (Jara et al., 2013). Re-colonisation of the egg surface by fast-growing opportunistic micro-organisms should however be prevented. Future studies implementing the principle of r/K-selection in the environmental microbial community (Andrews and Harris, 1986; Skjermo et al., 1997; Vadstein et al., 1993) implying incubation of disinfected eggs in water containing bacteria with a high half saturation constant (Ks) and low growth rate, the so-called K-strategists, is suggested. Colonisation by such bacteria could prevent colonisation of the egg chorion by fast growing opportunists (r-strategists) and may be a useful strategy (Hansen and Olafsen, 1999). Recirculation systems implying a selection for a microbial community composed of mainly K-strategists have been proven to sustain high larval survival (Attramadal et al., 2012a, 2012b) and are probably the best culture systems for future eel egg incubation and larviculture. Microbial conditions in such systems may mimic the oligotrophic environment of the Sargasso Sea, dominated by K-strategists due to low nutrient availability (Rowe et al., 2012).

Acknowledgements
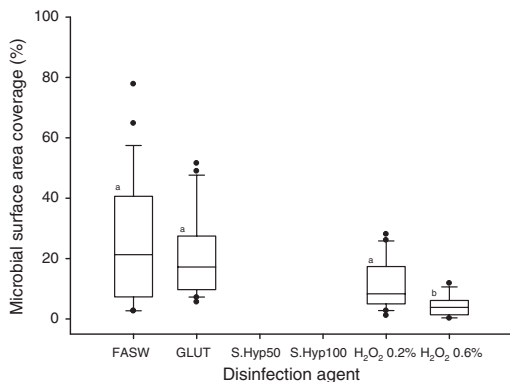
Fig. 8. Box-plot showing microbial surface area coverage for European eel, *Anguilla anguilla*, eggs ~2 h after disinfection treatment (see Table 1). Error bars indicate 95% confidence limits and a Dunn's multiple comparison test for treatment effect was run and superscripts show significant differences (α = 0.05).

References

Alderman, D.J., Hastings, T.S., 1998. Antibiotic use in aquaculture, development of antibiotic resistance — potential for consumer health risks. Int. J. Food Sci. Technol. 33, 139–155.

Andrews, J.H., Harris, R.F., 1986. r- and K-selection and microbial ecology. Adv. Microb. Ecol. 9, 99–147.

Asturiano, J.F., Pérez, L., Garzón, D.L., Marco-Jiménez, F., Peñaranda, D.S., Vicente, J.S., Jover, M., 2004. Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiol. Biochem. 30, 283–293.

Attramadal, K.J.K., Øie, G., Størseth, T.R., Alver, M.O., Vadstein, O., Olsen, Y., 2012a. The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. Aquaculture 330–333, 121–129.

Attramadal, K.J.K., Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2012b. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac. Eng. 46, 27–39.

Bergh, Ø., Hansen, G.H., Taxt, R.E., 1992. Experimental infection of eggs and yolk sac larvae of halibut, Hippoglossus hippoglossus L. J. Fish Dis. 15, 379–391.

Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 8, 1137–1144.

Castonguay, M., McCleave, J.D., 1987. Vertical distributions, diel and ontogenetic vertical migrations and net avoidance of leptocephali of Anguilla and other common species in the Sargasso Sea. J. Plankton Res. 9, 195–214.

Czerkies, P., Brzuzan, P., Kordalski, K., Luczynski, M., 2001. Critical partial pressures of oxygen causing precocious hatching in Coregonus lavaretus and C. albula embryos. Aquaculture 196, 151–158.

Dong, C.-H., Yang, S.-T., Yang, Z.-A., Zhang, L., Gui, J.-F., 2004. A C-type lectin associated and translocated with cortical granules during oocyte maturation and egg fertilization in fish. Dev. Biol. 265, 341–354.

Dufour, S., Burzawa, Gerard E., Belle, N.L., Sbaihi, M., Vidal, B., 2003. Reproductive endocrinology of the European eel, Anguilla anguilla. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer, Japan, pp. 373–383.

Greig, S.M., Sear, D.A., Smallman, D., Carling, P.A., 2005. Impact of clay particles on the cutaneous exchange of oxygen across the chorion of Atlantic salmon eggs. J. Fish Biol. 66, 1681–1691.

Hansen, G.H., Olafsen, J.A., 1989. Bacterial colonization of cod (Gadus morhua) L. and halibut (Hippoglossus hippoglossus) eggs in marine aquaculture. Appl. Environ. Microbiol. 55, 1435–1446.

Hansen, G.H., Olafsen, J.A., 1999. Bacterial interactions in early life stages of marine cold water fish. Microb. Ecol. 38, 1–26.

Hansen, G.H., Bergh, Ø., Michaelsen, J., Knappskog, D., 1992. Flexibacter ovolyticus sp. nov., a pathogen of eggs and larvae of Atlantic halibut, Hippoglossus hippoglossus L. Int. J. Syst. Bacteriol. 42, 451–458.

Hempel, G., 1979. Early life history of marine fish: the egg stage. Washington Sea Grant Publication. University of Washington Press.

Ijiri, S., Tsukamoto, K., Chow, S., Kurogi, H., Adachi, S., Tanaka, H., 2011. Controlled reproduction in the Japanese eel (Anguilla japonica): past and present. Aquac. Europe 36, 13–17.

Jara, M.A.G., Hidalgo, A.M., Gulin, J.C.A., Albiach, M.L., Ortiz, L.M., Monserrat, P.T., Ollé, X.E., 2013. Exposure of health workers in primary health care to glutaraldehyde. J. Occup. Med. Toxicol. 8, 31.

Keskin, M., Keskin, M., Rosenthal, H., 1994. Pathways of bacterial contamination during egg incubation and larval rearing of turbot, Scophthalmus maximus. J. Appl. Ichthyol. 10, 1–9.

Korwin-Kossakowski, M., 2012. Fish hatching strategies: a review. Rev. Fish Biol. Fish. 22, 225–240.

Laforsch, C., Tollrian, R., 2000. A new preparation technique of daphnids for scanning electron microscopy using hexamethyldisilazane. Arch. Hydrobiol. 149, 587–596.

Magnadottir, B., Lange, S., Gudmundsdottir, S., Bøgwald, J., Dalmo, R.A., 2005. Ontogeny of humoral immune parameters in fish. Fish Shellfish Immunol. 19, 429–439.

Martin, K., Bailey, K., Moravek, C., Carlson, K., 2011. Taking the plunge, California grunion embryos emerge rapidly with environmentally cued hatching. Integr. Comp. Biol. 51, 26–37.

Míguez, B., Combarro, M.P., Guisande, C., Vergara, A.R., Riveiro, I., 2004. Effect of bacterial epiflora on egg hatching of the Atlantic sardine (Sardina pilchardus). FEMS Microbiol. Ecol. 50, 111–115.

Nielsen, M.E., Esteve-Gassent, M.D., 2006. The eel immune system: present knowledge and the need for research. J. Fish Dis. 29, 65–78.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K., 1996. Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4-pregnen-3-one in the Japanese eel, Anguilla japonica. Aquaculture 139, 291–301.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Iinuma, N., Hirose, K., 1997. Artificial induction of maturation and fertilization in the Japanese eel, Anguilla japonica. Fish Physiol. Biochem. 17, 163–169.

Okamura, A., Yamada, Y., Horita, T., Horie, N., Mikawa, N., Utoh, T., Tanaka, S., Tsukamoto, K., 2009. Rearing eel leptocephali (Anguilla japonica Temminck & Schlegel) in a planktonkreisel. Aquac. Res. 40, 509–512.

Olafsen, J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200, 223–247.

Oppenheimer, C.H., 1955. The effect of marine bacteria on the development and hatching of pelagic fish eggs, and the control of such bacteria by antibiotics. Copeia 43–49.

Pavlov, D.A., Moksness, E., 1993. Bacterial destruction of the egg shell of common wolffish during incubation. Aquac. Int. 1, 178–186.

Pedersen, B.H., 2004. Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel Anguilla anguilla. Aquaculture 237, 461–473.

Rasband, W.S., 1997–2012. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA. http://imagej.nih.gov/ij/.

Riemann, L., Alfredsson, H., Hansen, M.M., Als, T.D., Nielsen, T.G., Munk, P., Aarestrup, K., Maes, G.E., Sparholt, H., Petersen, M.I., Bachler, M., Castonguay, M., 2010. Qualitative assessment of the diet of European eel larvae in the Sargasso Sea resolved by DNA barcoding. Biol. Lett. 6, 819–822.

Rowe, J.M., DeBruyn, J.M., Poorvin, L., LeCleir, G.R., Johnson, Z.I., Zinser, E.R., Wilhelm, S.W., 2012. Viral and bacterial abundance and production in the Western Pacific Ocean and the relation to other oceanic realms. FEMS Microbiol. Ecol. 79, 359–370.

Shelbourne, J.E., 1963. A marine fish-rearing experiment using antibiotics. Nature 198, 74–75.

Skjermo, J., Salvesen, I., Øie, G., Olsen, Y., Vadstein, O., 1997. Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquac. Int. 5, 13–28.

Sørensen, S.R., Gallego, V., Pérez, L., Butts, I.A.E., Tomkiewicz, J., Asturiano, J.F., 2013. Evaluation of methods to determine sperm density for the European eel, Anguilla anguilla. Reprod. Domest. Anim. 48, 936–944.

Suzuki, Y., Otake, T., 2000. Skin lectin and the lymphoid tissues in the leptocephalus larvae of the Japanese eel Anguilla japonica. Fish. Sci. 66, 636–643.

Swain, P., Nayak, S.K., 2009. Role of maternally derived immunity in fish. Fish Shellfish Immunol. 27, 89–99.

Tomkiewicz, J., 2012. Reproduction of European Eel in Aquaculture REEL: Consolidation and New Production Methods. DTU Aqua Report No. 249–2012 (DTU Aqua Report. DTU Aqua).

Unuma, T., Kondo, S., Tanaka, H., Kagawa, H., Nomura, K., Ohta, H., 2004. Determination of the rates of fertilization, hatching and larval survival in the Japanese eel, Anguilla japonica, using tissue culture microplates. Aquaculture 241, 345–356.

Vadstein, O., Øie, G., Olsen, Y., Salvesen, I., Skjermo, J., Skjåk-Bræk, G., 1993. A strategy to obtain microbial control during larval development of marine fish. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnereim, K. (Eds.), Fish Farming Technology. A.A. Balkema Publishers, Rotterdam, pp. 69–75.

Vadstein, O., Mo, T.A., Bergh, Ø., 2007. Microbial interactions, prophylaxis and diseases. In: Moksness, E., Kjørsvik, E., Olsen, Y. (Eds.), Culture of Cold-Water Marine Fish. Blackwell Publishing Ltd., pp. 28–72.

Watanabe, Y., Naganuma, T., Ogawa, T., Muramoto, K., 2013. Lectins of marine origin and their clinical applications. In: Fang, E.F., Ng, T.B. (Eds.), Antitumor Potential and Other Emerging Medicinal Properties of Natural Compounds. Springer, Netherlands, pp. 33–54.

Zhang, S., Wang, Z., Wang, H., 2013. Maternal immunity in fish. Dev. Comp. Immunol. 39, 72–78.

Paper 4:

Effects of salinity and sea salt type on egg activation, fertilisation and early embryology of European eel, *Anguilla anguilla*
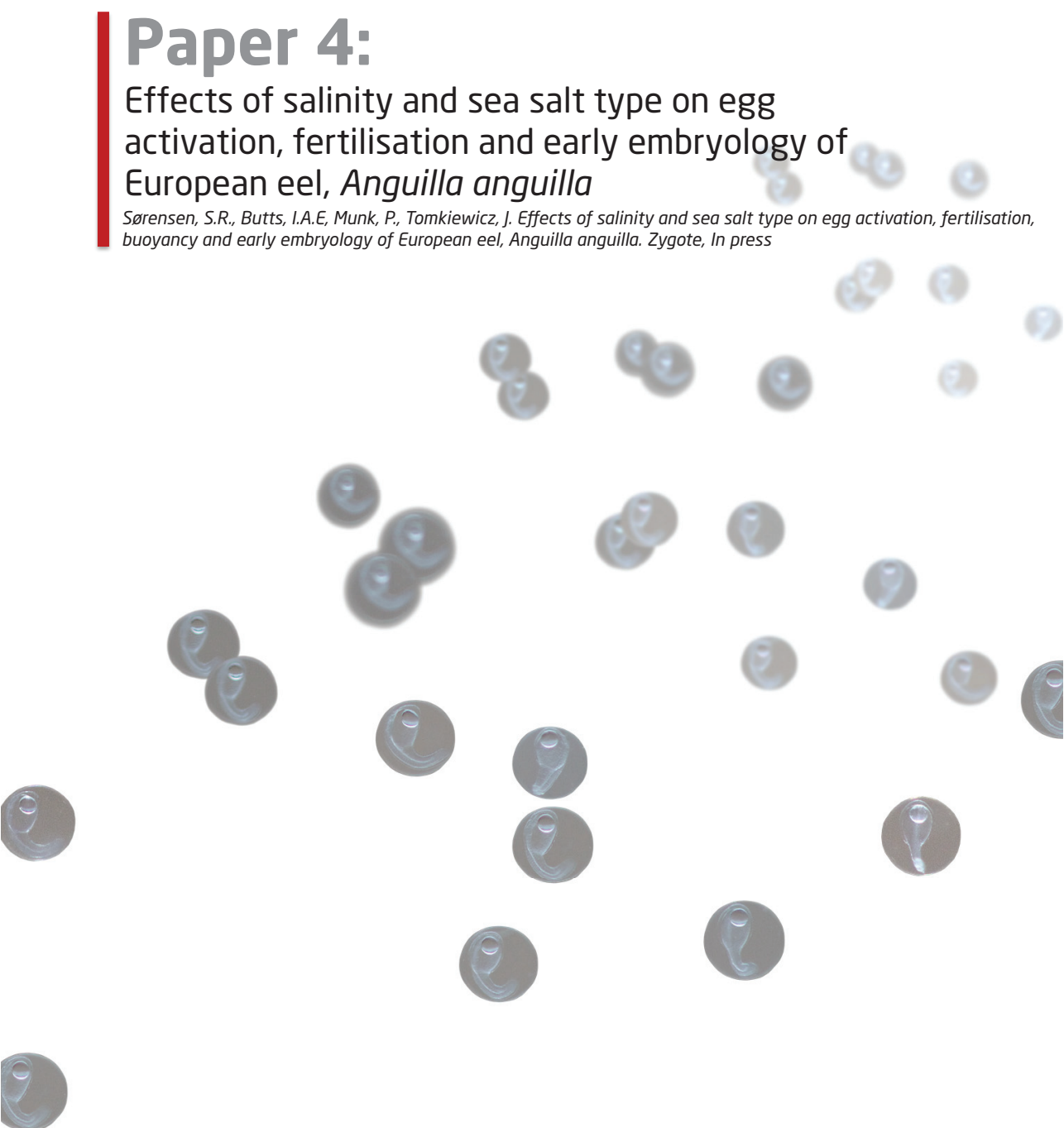
Effects of salinity and sea salt type on egg activation, fertilization, buoyancy, and early embryology of European eel, *Anguilla anguilla*

Sune Riis Sørensen[1], Ian Anthony Ernest Butts[2], Peter Munk[2], and Jonna Tomkiewicz[2]

Running title: Activation of European eel eggs

[1]All correspondence to: Sune Riis Sørensen. Section for Marine Ecology and Oceanography, National Institute of Aquatic Resources, Technical University of Denmark, Jægersborg Allé 1, DK-2920 Charlottenlund, Denmark. Tel: +45 21 31 49 83. Fax: +45 35 88 33 33. E-mail: srs@aqua.dtu.dk.

[2]Section for Marine Ecology and Oceanography, National Institute of Aquatic Resources, Technical University of Denmark, Jægersborg Allé 1, DK-2920 Charlottenlund, Denmark.

Summary

Improper activation and swelling of *in vitro* produced eggs of European eel, *Anguilla anguilla*, has been shown to negatively affect embryonic development and hatching. We investigated this phenomenon by examining the effects of salinity and sea salt type on egg dimensions, cell cleavage patterns, and egg buoyancy. Egg diameter after activation, using natural seawater adjusted to different salinities, varied among female eels, but no consistent pattern emerged. Activation salinities between 30 to 40 psu produced higher quality eggs and generally larger egg diameters. Chorion diameters reached maximal values of 1642 ± 8 µm at 35 psu. A positive relationship was found between egg neutral buoyancy and activation salinity. Nine salt types were investigated as activation and incubation media. Five of these induced a substantial perivitelline space (PVS), leading to large egg sizes, while the remaining four salt types resulted in smaller eggs. All salt types except NaCl treatments led to high fertilization rates and had no effect on fertilization success as well as egg neutral buoyancies at 7 h post-fertilization. The study points to the importance of considering ionic composition of the media when rearing fish eggs, and further studies are encouraged.

Keywords: Artificial fertilization; Egg quality; Egg size; Perivitelline space; Buoyancy, Cortical reaction

1. Introduction

Salinity has a major influence on the distribution of marine fishes in the oceanic environment (Holliday, 1969). Adults, juveniles, and most larvae are well adapted to changing environments, (Holliday and Blaxter, 1960), while egg and embryonic stages tend to be more sensitive to ambient conditions. Salinity optima and tolerance limits for fertilization and early embryonic development are apparent for marine fish species; even for those inhabiting variable environments, such as plaice, *Pleuronectes platessa* (Holliday and Blaxter, 1960). Based on catches of young larvae the spawning area for European eel, *Anguilla anguilla* is delimited to the southern Sargasso Sea, however, spawning adults or eggs have still not been encountered (Tsukamoto *et al.*, 2011). Captive reproduction of eel is challenged by an endocrinological inhibition of maturation during the silvering stage, which in nature precedes spawning migration to the Sargasso Sea (Dufour *et al.*, 2003). Eel broodstock are therefore matured via hormone therapy (Dufour *et al.*, 2003), and recent advancements in reproduction methods of European eel have advanced to a state where large quantities of viable gametes, eggs and yolk-sac larvae can be regularly obtained and cultured using standardized methods (Tomkiewicz, 2012, Butts *et al.*, 2014; Sørensen *et al.*, 2014). This now allows us to conduct studies on basic eco-physiological factors that are not known in the natural environment for European eel, such as salt and salinity effects on eggs/embryos, which are important for gamete activation and fertilization.

Egg activation is a key process in early embryonic development of fishes, triggered by the contact between eggs, sperm, and an aqueous medium (i.e. seawater or freshwater). The process of egg activation, although not fully understood (Webb and Miller, 2013), follows a general mechanism initiated by a release of intra-cellular stored Ca^{2+} in between the chorion (egg envelope) and the yolk membrane (Coward *et al.*, 2002; Alavi *et al.*, 2008). Ca^{2+} release mediates the cortical alveoli, embedded in the plasma membrane around the yolk to exocytose cortical proteins (Laale, 1980; Cerdà *et al.*, 2007). Cortical proteins are then broken into smaller units by proteolysis and form the osmotic gradient that facilitate uptake of seawater from the environment across the chorion (Lønning and Davenport, 1980; Govoni and Forward, 2008). This process forms and expands the perivitelline space (PVS) and is facilitated by the flexible structure of the chorion. The release of intra-cellular stored Ca^{2+} is relatively high during this period, and besides triggering the cortical reaction, it is assumed to counteract polyspermy, early cell cleavage

patterns, and embryonic development (Coward *et al.*, 2002; Claw and Swanson, 2012). Size of the PVS determines final egg size and differs between species (Davenport *et al.*, 1981). The PVS serves as an important "cushioning" incubation medium for the embryo and provides an essential buffer and sink for nitrogenous wastes during embryogenesis (Finn, 2007).

Activated eggs of European eel and Japanese eel, *A. japonica* have, as seen for other primitive species, a remarkably large PVS (Govoni and Forward, 2008) and a prominent oil droplet that is part of the egg yolk (Heinsbroek *et al.*, 2013). Natural egg size of European eel remains unknown, but the diameter of Japanese eel eggs in nature range from 1490 to 1710 μm in contrast to the somewhat smaller eggs from captivity-bred broodstock, i.e. 1300 to 1600 μm (Tsukamoto *et al.*, 2011). In early reproduction experiments of European eel, unfertilized egg sizes ranged from 930 to 1400 μm (Fontaine *et al.*, 1964), 1000 to 1100 μm (Villani and Lumare, 1975; Boëtius and Boëtius, 1980) and up to 1200 to 1600 μm (Kokhnenko *et al.*, 1977). Pedersen, 2003 obtained fertilized eggs of ~1000 μm and recorded unfertilized eggs from a spontaneous spawning female to be 1400 μm. Additionally, Palstra (2005) obtained a few developing embryos and presents an illustration with an egg of unspecified size (Palstra *et al.*, 2005), although this appears large, presumably ~1500 μm. Such variation among eggs together with the apparent larger size of wild-caught Japanese eel eggs, points to a suboptimal activation process in the assisted reproduction procedures. Insights into factors affecting egg swelling are therefore seen as crucial for successful *in vitro* production of eel offspring (Yoshinaga *et al.*, 2011).

Besides the protective PVS, marine pelagic fish eggs depend on a hydrostatic lift to facilitate embryonic development in their environment (Govoni and Forward, 2008) and lack of sufficient buoyancy is a known defect of eel eggs produced in captivity (Seoka *et al.*, 2003). Egg buoyancy is determined during oocyte maturation in the ovary and pelagic species exhibit a particularly high proteolysis of yolk proteins into free amino acids (FAA) (Cerdà *et al.*, 2007; Thorsen and Fyhn, 1996). FAA increases the osmotic potential and cause an aquaporin mediated water uptake of maternal isosmotic water during the hydration process. In turn, this causes the oocyte to swell (Fabra *et al.*, 2005; Cerdà *et al.*, 2007; Govoni and Forward, 2008). The difference between egg osmolality and that of the seawater environment promotes final egg buoyancy for which 80% is due to maternal fluids absorbed by the oocyte, and the remaining ~20% is facilitated by oil droplets and yolk lipids (Cerdà *et al.*, 2007; Govoni and Forward, 2008).

Preliminary studies on the European eel revealed that use of natural seawater was a prerequisite for obtaining viable eggs (Tomkiewicz and Jarlbæk, 2008). Use of natural seawater in experiments is however problematic, while collection of water is influenced by the natural variability. A selection of commercial available marine salt types, varying in composition (Atkinson and Bingman, 1997), enables the possibility to test whether such artificial seawater could be a more stable media for *in vitro* fertilization and experiments. Investigation of the applicability of artificial seawater would therefore be of value for understanding the eco-physiological response of oocytes in different media, and could lead to an improvement of *in vitro* fertilization conditions.

In this study, activation and fertilization processes were explored using a range of salinities, seawater and salt types in order to reveal their effects on: 1) magnitude and size of the PVS, 2) resulting egg size, 3) fertilization success and early cleavage patterns, and 4) neutral buoyancy of the eggs.

2. Material and methods

2.1 Broodstock and gamete management

Female silver eels were obtained from a freshwater lake (Lake Vandet, Denmark). Eels were transported, to a research facility of the Technical University of Denmark (DTU) and stocked in 300 L tanks in a recirculation system at a density ≤ 30 kg per m^3. Broodstock was acclimatized to artificial seawater adjusted by Tropic Marin$^®$ Sea Salt (Dr. Biener Aquarientechnik, Germany) to ~35 psu and maintained at ~20°C under dimmed light conditions at ~20 lux imitating natural daylight, dusk/dawn; local diurnal periodicity. Prior to experiments, fish were anaesthetized (ethyl p-aminobenzoate, 20 mg L^{-1}; Sigma–Aldrich Chemie, Steinheim, Germany) and tagged with a passive integrated transponder (PIT tag). No feed was provided during experiments, as maturing eels cease feeding (Pankhurst and Sorensen, 1984).

To induce vitellogenesis females received weekly injections of salmon pituitary extract (SPE; 18.75 mg kg^{-1} body weight, Argent Chemical Laboratories, Washington, USA). Females were weighed weekly and at 10% increase, biopsies were routinely made for evaluation of oocyte

development. At ~oocyte stage 3 (Palstra *et al.*, 2005), females received another injection of SPE as a priming dose to stimulate final maturation. Females were injected, ~24 h after receiving the SPE priming, with an injection of maturation-inducing steroid (MIS; 17α,20ß-dihydroxy-4-pregnen-3-one; Sigma-Aldrich Denmark A/S,) (Ohta *et al.*, 1996). In the timespan of 12 to14 h after receiving MIS, eggs were stripped into dry and sterilized trays by applying slight pressure to the abdomen of the fish and stripped eggs were weighed (± 1 g).

Male broodstock were reared at Stensgård Eel Farm in Jutland, Denmark. Males (mean ± SD standard length and body weight were 37.6 ± 2.1 cm and 110.2 ± 13.6 g, respectively) were transported to DTU facility (husbandry as above) and received weekly injections of human chorionic gonadotropin (Sigma Aldrich Denmark A/S) at 1.5 IU g^{-1} fish. Milt was collected after 7 to 8 weeks of hormonal treatment (~12 h after administration of hormone) by applying slight pressure along the abdominal region and for each fertilization event a pool of milt from 3 to 4 males was utilized. Prior to milt collection, the urogenital pore was wiped dry using deionized water and the initial ejaculate was omitted to avoid contamination. Milt was collected into sterile beakers.

Sperm motility was characterized, within 30 s of activation, using a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan), equipped with a Nikon 400 × mag. objective (40 × CFI Plan Flour) and motilities < 50% were discarded. Sperm concentration was measured using spermatocrit (Haematokrit 210, Andreas Hettich GmbH & Co.KG, Tuttlingen Germany), obtained from spinning milt for 10 min at 6000 × g (Sørensen *et al.*, 2013). After motility analyses, milt was diluted in P1 medium (Asturiano *et al.*, 2004; Butts *et al.*, 2014) at a ratio of 1:99. Diluted milt was kept in sterile culture flasks at 20˚C, prior to fertilization (within 2 h post-stripping).

2.2 Experiment 1: Activation salinity

Eggs from each female (n=5, Table 1) extruded as described above were fertilized with sperm from an independent pool of 3-4 males, using a ratio of 1 mL of diluted milt per 2 g of newly stripped eggs. Activation seawater from the North Sea, kept in a 2000 L aerated tank, was adjusted to targeted salinities (± 0.1 psu) using an electronic conductivity meter (WTW Multi 3410 + TetraCon325, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany);

taking into consideration the salinity of P1 milt diluent being 10.3 psu. The seawater was adjusted to the desired salinities using Tropic Marin Sea Salt. pH was measured using pH-indicator strips (pH 6.5 to 10.0, Merck Millipore, KGaA, Darmstadt, Germany) and adjusted to pH 8.2 using HCl.

Following mixing of gametes, samples of 3.5 g eggs (~7000 eggs) were distributed into triplicate weigh boats (100 mL, 7 cm × 7 cm) for each of the treatments: 30, 35, 40, 45, and 50 psu. Each replicate was immediately activated, after mixing of gametes, using 11.7 mL of activation media from pre-filled disposable 20 mL syringes. Loading and gamete activation were performed randomly across treatments. After activation, gametes were gently poured (10 min post-activation) into 250 mL glass beakers containing 200 mL of the corresponding salinity seawater, supplemented with penicillin G at 45 ppm and streptomycin sulphate at 65 ppm (Sigma-Aldrich, St. Louis, Missouri). Egg/embryos were incubated in light below 50 lux at 20 ± 0.2 °C. After 3.5 to 5 h of incubation, eggs were randomly sampled from each replicate beaker by gently mixing eggs to obtain a homogenous sample (see Section 2.4). Digital images of sampled egg were then taken (see Section 2.5.1).

2.3 Experiment 2: Activation salt type

Following stripping, samples of 3.5 g of eggs from each of females (n=5, Table 1) were randomly distributed into dry weigh boats (100 mL, 7 cm × 7 cm). There were triplicate samples for each activation treatment, which consisted of commercial salts types and natural seawater (Table 2). pH was measured using an electronic pH meter (model 827, Metrohm Inc. Riverview, Florida, USA), measuring three replicate 35 psu seawater mixtures 1 h after mixing ($\pm$ 0.01 pH). Treatments were mixed with MilliQ water prior to use and adjusted to 35 ± 0.1 psu, taking into consideration the salinity of P1 milt diluent being 10.3 psu. Activation salinity was confirmed using an electronic conductivity meter. Each replicate was activated as described in Exp. 1 and sampling for imaging was performed similar to Exp. 1.

2.4 Egg evaluation

2.4.1 Egg sizes

Four digital images, from each replicate (4 × ~20 eggs), formed the basis for analysis of egg sizes as well as egg categories (see Section 2.4.2). Egg sizes were measured using NIS Elements image software (v. 3.32, Nikon Corporation, Tokyo, Japan). Except for the egg categorization (see Fig. 1), all egg measurements were based solely on fertilized eggs. Image software was used to calculate egg diameter by marking five reference points around each egg membrane circumference thereby deriving a diameter equivalent to a circle with sphericity of 1. The chorion and the yolk (vitelline membrane) circumference hence formed egg chorion diameter and yolk diameter, respectively. Sizes were measured within 3.5 to 5 h post fertilization and obtained in association to egg categories described in Section 2.4.2. In Exp. 1 and 2, ≥ 15 eggs were measured per replicate.

To quantify the rate of eggs having egg chorion diameters >1600 μm a dataset was formed based on egg measurements from treatments of salt and salt type from both Exp. 1 and Exp. 2. Treatments of salt and salt type providing significantly smaller egg sizes were not included in this dataset.

2.4.2 Egg categorization and fertilization percent

Four digital images, from each replicate (4 × ~20 eggs) were acquired at 3.5 to 5 h post fertilization and a categorization scheme was established (Fig. 1) that distinguished fertilized, unfertilized, and dead eggs, outlining specific characteristics within each of five egg types. Fertilized eggs were categorized as Type 1 (T-1), having >4 visible cells (pathogenic first cleavage occurs) and represent the proportion of fertilized eggs. To evaluate the regularity of cell cleavage patterns, fertilized eggs were subdivided into three subcategories according to early cell cleavage symmetry: T-1a) regular cleaved cells showing cleavage symmetry and well-defined cell margins with full adhesion between cells; T-1ab) cells with an overall even appearance and margin but minor cleavage irregularity; and T-1b) irregular cleavage planes with uneven cell sizes and low or no adhesion between cells (Fig. 1). Unfertilized eggs were divided into four categories: T-2) unfertilized but activated, showing a PVS, buoyant with mean diameter (± SD) of 1163 μm ± 181 (N = 234), and variable oil droplets; T-3) eggs failing to activate and form a PVS, buoyant with a mean diameter of 917 μm ± 39 (N = 219), and variable oil droplets; T-4)

unripe, non-ovulated eggs with visible nucleus, non-buoyant with mean diameter of 872 μm ± 43 (N = 378), and variable oil droplets; and T-5) white or opaque eggs cf. dead eggs, non-buoyant with mean diameter of 1029 μm ± 75 (N = 481).

2.4.3 Egg size difference between T-1a, T-1ab, and T-1b

To test for overall size effects between each subcategory of fertilized eggs (T-1a, T-1ab, and T-1b) data obtained from Exp. 1 and 2 were used. In specific, the 30, 35, 40, and 45 psu treatments were used from Exp. 1, while the Std, RS, RS^{pro}, RC, and TM treatments were used from Exp. 2.

2.4.4 Egg activation with and without milt

Following stripping of female *f5* (Table 1), 3.5 g of eggs were randomly distributed into three replicate dry weigh boats per treatment (100 mL, 7 cm × 7 cm) and two activation treatments were tested, differing in presence and absence of milt. Eggs were activated and incubated as described for Exp. 1 and 2 using 35 psu seawater. Digital images was obtained and analyzed as described in 2.4 above.

2.5 Measurement of egg neutral buoyancy

Eggs from two females were used for buoyancy measurements; one used in Exp. 1 and one in Exp 2. Neutral buoyancy of eggs, following treatments and incubation, were conducted at 7 and 30 h post-fertilization. A glass tank (thermo glass preventing temperature gradients) 100 × 10 × 10 cm was filled from the bottom using methods modified from Coombs (1981). A peristaltic pump (Economy Console fitted Easy-Load® 3 pumphead, Masterflex, Cole-Parmer, Vernon Hills, Illinois, USA) provided steady addition of high saline water (58 psu, 2.5 to 2.8 mL × min^{-1}) into a closed stirred mixing beaker containing low saline water (9 psu). A gradual increasing salinity gradient formed from the bottom of the glass tank within ~6 h. Water was premixed using demineralised water and Tropic Marine sea salt. Four density glass floats covering the range of 5.5 to 38 psu (Martin Instruments Co. Welwyn Garden City, Hertz, England) were inserted in the salinity gradient and positions used to establish the logarithmic function connection positions and salinity within the gradient. A correlation coefficient lower than r < 0.98 was chosen as the lower limit for a usable gradient. All salinity-density conversions were temperature-compensated by measurements in the gradient (± 0.1˚C) (Chapman, 2006).

From each replicate, 5 to 10 eggs (~20 eggs per treatment) were gently inserted into the gradient using a disposable inoculation needle (1.0 mm), avoiding addition of water to the salinity gradient, which can disturb the gradient. Position of the eggs and reference floats were read on a metric scale (± 1 mm) 30 min after loading. Positioning was aided by a self-levelling laser on a tripod in darkness (SuperCross 2, Laserliner[®], Frauenfeld, Switzerland) giving a thin-lit cross section in the gradient to ensure an unbiased reading of position in the gradient. A new salinity gradient was established between each time point. In the reading at 7 HPF in Exp. 1, eggs were left for 210 min to estimate their sinking rate. Sinking rate was measured as egg density change in psu min^{-1}.

2.6 Statistical analyses
Data were analyzed using SAS (v.9.1; SAS Institute Inc., Cary, NC, USA) and Sigmaplot (v.11; Systat Software Inc, Hounslow, UK). Residuals were tested for normality (Shapiro-Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). When necessary, data were transformed to meet the assumptions of normality and homoscedasticity. Treatment means were contrasted using a Tukey's test. All data are presented as mean ± SD. Significance was set at α of 0.05.

Relationships between female body size (independent variable, i.e. length and weight), egg chorion diameter, yolk diameter, and overall fertilization percentage (depended variables) were examined using linear regression.

Effects of salinity and salt type on fertilization success and egg traits were analyzed using a series mixed-model ANOVAs (PROC MIXED), where salinity and salt type were considered fixed factors and the Female and Female × Salinity and/or Salt type interaction were considered random factors. *A-posteriori* analyses were not performed on random effects. Instead, variance components (VC) were constructed using the restricted maximum likelihood estimation method in SAS PROC Mixed, and expressed as a percentage. To test for significant variability among variance components that were > 0, likelihood ratio statistics were generated (PROC MIXED). To further explore within female effects, salinity and salt type were analyzed using a series of one-way ANOVA models.

T-tests were used to compare egg and yolk size between the treatments activated with and without milt, while egg buoyancy data were analyzed using one-way ANOVA models. Relationships between salinity difference (activation salinity subtracted the measured buoyancy) (dependent variable), and the activation salinity (independent variable) were analyzed using linear regression.

3. Results

3.1 Experiment 1: Activation with different salinities

3.1.1 Egg size

No relationship was detected between female body size and egg characteristics ($R^2 < 0.11$). Average egg chorion diameter for the five females ranged from 1207 ± 212 to 1338 ± 203 μm across the salinity gradient (Fig. 2A). For the mixed-model ANOVA including all females, salinity had no significant effect on egg chorion diameter (Fig. 2A). For the model's random effects, the Female × Salinity interaction was significant and explained the majority of the models variance (VC = 99.7%), while the Female VC was not significant (VC = 0.28%). For within female effects, salinity had a significant effect on egg chorion diameter for all females (Fig. 2B to F). Egg chorion diameter was largest for Female *f1* and *f2* at 30 and 35 psu (Fig. 2BC), *f3* at 40 to 50 psu (Fig. 2D), *f4* at 35 to 50 psu (Fig. 2E), and *f5* at 35 to 50 psu (Fig. 2F).

Yolk diameter ranged from 947 ± 16 to 953 ± 9 μm (Fig. 2A). The mixed-model ANOVA showed that salinity had no effect on yolk diameter (Fig 2A), while for the models random effects, both the Female (VC = 74.2%) and Female × Salinity VC were significant (VC = 10.8%). For within female effects, salinity had no effect on yolk diameter for *f1* (Fig. 2B), *f2* (Fig. 2C), and *f3* (Fig. 2D), but significant effects were detected for both *f4* (Fig. 2E) and *f5* (Fig. 2F).

3.1.2 Fertilization rate and zygote development

No relationship was detected between female body size and fertilization percent ($R^2 < 0.05$). Overall fertilization success ranged from 22.1 ± 18.6 to 60.9 ± 16.9 % across the salinity gradient (Fig. 3A). When considering all fertilized eggs (egg category = T-1), salinity had a significant effect on fertilization percent, such that the 30 to 40 psu treatments had the highest fertilization rates (Fig. 3A). For the model's random effects, both the Female (VC = 58.4%) and Female ×

Salinity interaction were significant (VC = 23.1%). When considering within female effects, salinity had a significant effect on fertilization success for females $f1$, $f2$, $f3$, $f4$, (Fig. 3B to E) although not for $f5$ (Fig. 3F). The highest fertilization rate was achieved for $f1$ (Fig. 3B), $f2$ (Fig. 3C), and $f4$ (Fig. 3E) at 30 to 40 psu and $f3$ at 30 to 35 psu (Fig. 3D). Typically, the lowest fertilization percentage was detected at 45 to 50 psu (Fig. 3).

The overall rate of regular cleaved fertilized eggs (egg subcategory = T-1a), ranged from 0.65 ± 0.98 to 14.71 ± 21.57% (Fig. 3A). The mixed-model ANOVA showed that salinity had no effect on the proportion of regular cleaved fertilized eggs (Fig 3A). The Female VC was significant and explained 58.7% of the models variance. The Female × Salinity VC was also significant and explained 35.0% of the variance. For within female effects, $f2$ (Fig 3C) and $f4$ (Fig 3E) were significant, where the percentage of regular cleaved fertilized eggs declined across the salinity gradient.

For the fertilized eggs that showed mostly regular cell cleavages (egg subcategory = T-1ab), salinity had an impact (Fig 3A), where the percentage of fertilized eggs declined at the 50 psu treatment. For the models random effects, both the Female VC (VC = 52.2%), and Female × Salinity interaction were significant (VC = 17.0%). For within female effects, salinity had a significant effect on the distribution of T-1ab fertilized eggs for all females (Fig. 3) except for $f3$ (Fig. 3D).

Salinity had no effect on the proportions of fertilized eggs with irregular cell cleavages, (egg subcategory = T-1b) (Fig. 3A). On the contrary, both the Female (VC = 72.9%) and Female × Salinity (VC = 19.4%) random VCs were significant. When comparing the rate of irregular cleaved with overall fertilization percent, the proportion of irregular cleaved eggs increased with increasing salinity (Fig. 3A). For within female effects, salinity had a significant effect on the distribution of T-1b fertilized eggs for $f1$, $f2$, $f3$, $f4$ (Fig. 3B to E), but not for $f5$ (Fig. 3F).

Egg chorion diameter did not differ between eggs activated with and without the presence of milt, however yolk diameter was larger in the activated unfertilized eggs (Fig. 4A). Eggs activated without the presence of sperm also formed a PVS (Fig. 4B), i.e. T-2 eggs in the

categorization scheme (Fig. 1), and occasionally show disk-like structure (Fig. 4B egg 1) and first cell cleavage (Fig. 4B egg 2 and 3).

3.1.3. Egg neutral buoyancy

Activation salinity had a significant effect on egg buoyancy at 7 h post fertilization (HPF) (Fig. 5A), such that fertilized eggs in the 50 psu treatments had the highest buoyancy, while the 30 and 35 psu treatments eggs showed lower and similar buoyancy. At 30 HPF, salinity continued to significantly affect buoyancy, showing a similar trend (Fig. 5B). Sinking speed of eggs recorded over a period of 210 minutes, from 7 to 10.5 HPF, in the salinity gradient column from the 35 psu treatment were on average 0.04 ± 0.0009 mm $\times$ min^{-1} corresponding to 0.0025 ± 0.001 psu $\times$ min^{-1} (N = 13).

The deviation between measured buoyancies and salinity in activation and incubation, in relation to treatments, is shown in Figure 5C for 7 HPF and Figure 5D for 30 HPF. The deviation was significantly related to the activation salinity, with negative values i.e. buoyancy lower than activation salinity at high values at activation salinities and vice versa ($R^2 = 0.99$, y= 0.825X - 27.87). At 30 HPF, data violated statistical assumptions as buoyancy of the 50 psu treatment increased more than the remaining treatments; therefore, the 50 psu treatment was omitted and the model was rerun. After rerunning the model, a significant relationship between the salinity deviation (activation salinity subtracted the measured buoyancy) was also detected at 30 HPF ($R^2 = 0.93$, y= 0.655X - 22.073).

3.2 Experiment 2: Activation of eggs with different salt types

3.2.1 Egg size

Average egg chorion diameter ranged from 1055 ± 59 to 1562 ± 75 μm across the nine salt types (Fig. 6A). The mixed-model ANOVA showed that salt type had a significant effect on chorion diameter, such that the RS, RC, RS^{pro}, and Std treatments had the largest egg chorion diameter (Fig. 6A). The Female VC was non-significant (VC = 8.5%), while the Female $\times$ Salt Type interaction was significant and explained the majority of the models variance (VC = 83.5%). For within female effects, salt type significantly influenced egg chorion diameter for all females (Fig. 6). For instance, the RC salt type evoked the largest chorion diameter for *f6* (Fig. 6B), while the RS salt type caused the largest egg chorion diameter for females *f9* (Fig. 7E). For *f7, f8, f10* (Fig.

6C,D,F), the largest egg chorion diameter was observed across a wide-range of salt types. For all females, the IO, TE, NC^{RS}, and NC salt types typically resulted in small egg chorion diameters compared to the other treatments (Fig. 6).

Salt type had a significant effect on yolk diameter (Fig 6A). The Female VC was significant and explained 46.1% of the variance, while the Female × Salt Type VC was also significant and explained 31.8% of the variance. For within female effects, salt type had no effect on yolk diameter for female *f8* (Fig. 6D) and *f10* (Fig. 6F), while salt type had a significant effect for the other females.

3.2.2 Fertilization rate and zygote development
Considering all fertilized eggs, (egg category = T-1), fertilization percentage for the five females ranged from 0.3 ± 0.7 to 85.7 ± 9.0% across the nine salt types (Fig. 7A). The mixed-model ANOVA showed a significant salt type effect in the fertilization percent. Within this regard, RS, RC, RS^{pro}, Std, TM, IO and TE had higher fertilization success than the NC and NC^{RS} treatments (Fig. 7A). For the models random effects, the Female VC was significant (VC = 27.4%). The Female × Salt Type interaction was also significant and explained 33.7% of the models variance. Salt type had a significant effect on fertilization for all females, where the NC treatment always had the lowest fertilization while the RS, RC, TM, and IO treatments had the highest fertilization (Fig. 7B to F).

The proportion of fertilized eggs with regular cell cleavage (egg subcategory = T-1a), differed significantly depending on salt type (Fig 7A), with low proportions in NC and NC^{RS} treatments compared to other salt types. The Female VC was significant (VC = 62.1%), and similarly the Female × Salt Type interaction, explaining 30.5% of the model's variance. For each female, the effect of salt type on the proportion of eggs with regular cell cleavage was also significant (Fig. 7B to F).

With respect to the fertilized eggs that showed mostly regular cell cleavage, (egg subcategory = T-1ab), salt type had a significant impact on its distribution (Fig. 7A). Furthermore, for the model's random effects the Female VC was significant (VC = 41.7%), and the Female × Salt Type interaction was also significant (VC = 39.0%). Salt type had a significant effect on the

percentage of mostly regular cleaved eggs for the females *f5*, *f7*, *f8*, *f9* (P ≤ 0.002; Fig. 7), while the effect of salt type was non-significant for *f10* (Fig. 7F). The NC and NC^{RS} salt type treatment resulted in the lowest percentage of T-1ab for the five females, while for the remaining salt types showed no general pattern (Fig. 7B to F).

Salt type had a significant effect on the proportion of eggs with irregular cell cleavages (egg subcategory = T-1b) (Fig. 7A), where the NC differed from the NC^{RS} treatment (Fig. 7A). The Female VC was significant (VC = 42.9%) and the Female × Salt Type VC was significant and explained 49.5% of the model. Salt type effects were significant for all females with respect to proportion of T-1b eggs (Fig. 7B to F).

3.2.3 Egg neutral buoyancy

Mean neutral buoyancy of eggs from the different salt type treatments ranged from 32.4 ± 0.5 to 37.1 ±1.9 psu at 7 HPF (Fig. 8A) and 32.8 ± 0.5 to 33.8 ± 0.2 psu at 30 HPF (Fig. 8B). At 7 HPF, activation and incubation salt type had a significant effect on egg buoyancy, with no significant difference between Std, RS, RS^{pro}, RC TM, and TE salt types, which all had the lowest egg buoyancy. The NC and NC^{RS} salt types had the highest buoyancy (Fig. 8A). Additionally, RS was also significantly lighter than IO. At 30 HPF, the buoyancy of eggs was also significantly affected by activation and incubation salt type (Fig. 8B) with NC and NC^{RS} treatments no longer buoyant and RS, RS^{pro} and TM having lower neutral buoyancy than IO and TE.

3.3 Size difference among fertilized egg types

Selection of treatments from Exp. 1 (30 to 45 psu) and Exp. 2 (Std, RS, RS^{pro}, RC, and TM) formed a dataset of 896 eggs with regular cell cleavages (T-1a); 753 eggs having mostly regular cell cleavages (T-1ab), and 1535 eggs with irregular cell cleavages (T-1b) (Table 3). The fertilized egg types significantly differed with respect to egg diameter, yolk diameter, egg volume, yolk volume, and the size of the perivitelline space. For all egg metrics, the three fertilized egg types differed significantly from each other (Table 3).

4. Discussion

Egg sizes of marine fish show both inter- and intra-species variation (Kennedy *et al.*, 2007). Maternal effects on egg diameter have been studied in many fish species and are generally found significant, depending on diet, broodstock condition, and timing in relation to the reproductive season (Kjørsvik *et al.*, 1990; Laale, 1980; Morley *et al.*, 1999). Similarly, paternal effects are important, impacting processes related to fertilization (Evans and Geffen, 1998; Kroll *et al.*, 2013). In our study, wild-caught females were crossed with farmed males, and we found a clear female effect that accounted for a large proportion of the models variance, e.g. yolk size had a Female VC of 74.2%. However, no relationship was found between female size and egg chorion diameter, yolk diameter, or overall fertilization percentage.

Currently, no information is available regarding the size of European eel eggs in nature, and we are limited to compare our observations with wild-captured eggs from Japanese eel. These wild-captured eggs are reported larger than eggs from their captivity-bred counterparts (Tsukamoto *et al.*, 2011; Yoshinaga *et al.*, 2011). In captive breeding of Japanese eel, egg sizes are in the range of 1300 to 1600 μm, while eggs captured in nature range from 1490 to 1710 μm with a mean of 1610 μm ± 70 μm (Tsukamoto *et al.*, 2011; Yoshinaga *et al.*, 2011). Overall, we found 13% of 3442 fertilized eggs to be in that range (1600 μm to 1785 μm), thus comparable to wild-caught Japanese eel. Our study points to effects of salinity and salt composition on egg size. Within females, we found that activation and incubation salinities had an effect on egg size and typically the largest eggs were obtained within the 30 to 40 psu interval. The variation among females regarding salinity tolerance and optima did however preclude a clear trend. Salinity induced swelling is described in the long rough dab, *Hippoglossoides platessoides* where PVS is accounting for up to 85% of the egg volume (Lønning and Davenport, 1980), which is close to the average of 74% PVS in regular cleaved eggs that we reported. The mechanisms during activation of a fish egg are still unknown (Webb and Miller, 2013) and specifically research on the initial swelling following activation has been urged in relation to improving captive rearing in eels (Yoshinaga *et al.*, 2011).

As seen in our study, regardless of sperm being present, eel eggs swell upon activation by contact with seawater. Activation is known to be triggered by release of intracellular stored Ca^{2+}

in between the yolk and chorion membrane (Finn, 2007). This stimulates the cortical alveoli in the yolk membrane, to exocytose glycoprotein into the perivitelline space leaving the broken cortical alveoli to fuse with the plasma membrane and form the new protective and impermeable vitelline membrane surrounding the yolk (Hart and Yu, 1980). The glycoproteins are cleaved into smaller colloid proteins (Gallo and Costantini, 2012) and form a colloid osmotic gradient between the exterior and the PVS across the chorion (Shephard, 1989). This osmotic gradient has a charge potential, called the perivitelline potential (PVP), and the more negative the PVP, the stronger the force of water uptake from the environment (Peterson and Martin-Robichaud, 1986). This force is possibly imposed by diffusion through the chorion by cations like sodium, Na^+ rather than chloride, Cl^- (Peterson and Martin-Robichaud, 1986) however the mechanism of water influx through the chorion in fish is not fully known (Yoshinaga *et al.*, 2011; Webb and Miller, 2013). In freshwater fishes, the ionic effects on swelling shows that polyvalent ions like Al^{3+} and Zn^{2+} inhibit PVS formation with increasing concentration (Eddy, 1983; Shephard, 1989; Li *et al.*, 1989). Marine fish are fundamentally different as they face a hypertonic environment but are likewise sensible to ion composition, as shown in herring by cadmium Cd^{2+} affecting the osmotic gradient plus water uptake and egg size, especially at lower salinities (Alderdice *et al.*, 1979a, 1979b). The increase in egg diameter we observed could hardly be due to the same effects, as increasing salinity would increase inhibiting ions and thus decrease water uptake. A more likely explanation seems to be related to a selective influx of Na^{2+}, as suggested by Peterson and Martin-Robichaud (1986). This process would enforce a more negative PVP and create a stronger force of water uptake. Alone or playing in concert, osmotic shock exerted by the activation salinity may also contribute by triggering activation; similar to the mechanical touch of the chorion found to onset the activation process in Medeka, *Oryzias latipes* (Webb and Miller, 2013).

The diameter of egg yolk showed no consistent size change in relation to salinity supporting the impermeable nature of the vitelline membrane; precluding further yolk size increases due to osmotic pressure as described by Hart and Yu (1980). This finding is in line with other studies, such as those on Lumpsucker, *Cyclopterus lumpus* (Kjørsvik *et al.*, 1984) or long rough dab (Lønning and Davenport, 1980) where even strong hyperosmotic pressure had a low impact on yolk osmolality and size. The long rough dab, *Hippoglossoides plantessoides limandoides*, which has an expandable chorion, shows egg properties similar to the eel with a large expansion of the

PVS and no size change in yolk plasma diameter (Lønning and Davenport, 1980). Studies have shown the cortical reaction and formation of vitelline membrane, is faster once initiated at the egg animal pole, i.e. when sperm penetrates (Iwamatsu and Ito 1986) as compared to when activated merely by contact with seawater. Consequently, a delayed formation of this membrane is expected once activated without sperm cells. This may validate why we found a slightly larger yolk diameter once activated without sperm cells, since the slower activation process may cause extended membrane exposure to hypertonic medium increasing the yolk membrane diameter by osmotic pressure.

In two out of five females, the proportion of regular cleaved eggs (T-1a) showed significant peaks around 30 and 35 psu. In spite that the proportion of regular cleaved eggs was low for the remaining three females, a general decrease in higher quality blastulas, T-1a as well as T-1ab, was observed with increasing salinity. Coincidental at high salinity the irregular cleaved, T-1b, accounted for a high proportion of overall fertilized eggs, along with an increase in unactivated and dead eggs, T3 and T-5 respectively. Early cell cleavage symmetry, evenness, and mutual adhesion at the 8 to 128 cell stage are known to correlate with larval survival in several species (Shields *et al.*, 1997; Kjørsvik *et al.*, 2003). Activation and incubation salinity are likely to impact the success of *in vitro* fertilization in eel and the optimum is presumably close to the salinity of the proposed spawning area, i.e. ~36.5 psu (Munk *et al.*, 2010). Mechanisms of salinity effects on the zygote are scarcely known but they are overall related to external and internal supplies of Ca^{2+}. The initial wave of calcium upon sperm entry is released mainly from internal stores, while external supplies found in the seawater environment act to renew these stores and facilitate complex mechanisms; e.g. inositol triphosphate-mediated calcium release (IP_3) (Stricker 1999). The different levels of action of this calcium wave may be an underlying explanation for variation in fertilization quality patterns we observed at different salinities. A study on Japanese eel embryos illustrate the importance of salinity changes as eggs activated at 33 psu, followed by incubation treatments from 24 to 45 psu show a discrete effect on hatching but reduced larval survival above 36 psu, and increased deformities below 30 psu (Okamoto *et al.*, 2009).

Egg buoyancy characteristics are fundamental to European eel egg development both in nature and hatcheries as after spawning the PVS fills with seawater, equaling the surrounding salinity

(Davenport *et al.*, 1981). Neutral buoyancy is therefore determined by a balance between the seawater salinity and the yolk osmolality and lipids sealed by the vitelline membrane. Neutral buoyancy of eggs were correlated to the activation salinity. By using a range of salinities for activation and incubation it was possible to deduce a regression intercept at 33.8 psu, which indicates neutral buoyancy regardless of activation salinity. A similar pattern was confirmed at 30 HPF, although 50 psu had damaging effects. The measurement of neutral buoyancy in a salinity gradient can however potentially lead to overestimation of egg density because pelagic eggs with time tend to equilibrate across the chorion membrane with the surrounding water (Coombs *et al.*, 2004; Govoni and Forward, 2008). As defined by Stokes Law, sinking speed is related to egg size (Coombs *et al.*, 2004) and we observed a rapid sinking speed until the point of neutral buoyancy. Sardine eggs were shown to continue sinking from point of neutral buoyancy in a salinity gradient, challenging estimation of neutral buoyancy (Coombs, 2004). In our study however, we found eel eggs remaining at the point of neutral buoyancy and sink only marginally from 7 to 10.5 HPF. This means that from the time of the fully distended chorion and likely during period of chorion hardening, the exchange of water across the chorion is very limited which validates our neutral buoyancies measures.

Commercial synthetic salt products are often branded as being similar in composition to seawater and thus potentially useful for standardizing water conditions for fish culture. Our results showed that several salt types were able to activate eggs at the same level as natural seawater (Std), but do so more consistently reducing variability. Similarly, some artificial sea salt brands as Red Sea Salt (RS); Red sea salt pro, (RS[pro];) Reef Crystal, (RC) and Tropic Marin, (TM) provided egg sizes as large as, or larger than, Natural seawater (Std). In contrast, Instant Ocean (IO) and Tetra Marine (TE), consistently resulted in eggs with a small PVS which also pertained to pure NaCl, (NC) and NaCl +10% RS, (NC[RS]). In summary RS and RC seemed consistent in forming large eggs. Hardly any eggs could be fertilized in NC, however adding only 10% RS proved, in several cases, to enable fertilization and improve PVS formation and thereby adding important input to the discussion of external calcium during zygote formation. NC lacks ions needed for activation and fertilization like i.e. K^+ and Ca^{2+} but indeed also impacts eel sperm functionality that further needs K^+, Na^+, and HCO_3^- (Ohta *et al.*, 2001; Gallego *et al.*, 2014).

The mineral compositions for the RS, RC, TM, and IO brands are published (Atkinson and Bingman, 1997; Hovanec and Coshland, 2004; Arnold *et al.*, 2007) however variation between studies and composition of minor elements between reported brands is considerable (Atkinson and Bingman, 1997; Hovanec and Coshland, 2004; Arnold *et al.*, 2007). Minor elements differing make up more than twenty components in the salt types, including several of the metals believed to affect egg activation. A reported difference between brands relates to the alkalinity and pH (Atkinson and Bingman, 1997). IO and RC differed in their ability to induce a large PVS, resembled each other in composition (Atkinson and Bingman, 1997; Hovanec and Coshland, 2004), but showed a difference in pH of 0.6. Such pH variation is however also found between the salt types leading to consistently large PVS, thus why pH within the range observed cannot determine PVS formation. Variability in alkalinity, not determined in this study on the other hand, could have an impact on the sperm via HCO_3^- which has been seen to increase motility in Japanese eel sperm (Ohta *et al.*, 2001).

Artificial salt types contains many different ions, and several are known to block the cortical reaction e.g. Al^{3+} in Atlantic salmon (Finn, 2007), or inhibit like Zn^{2+}, Mg^{2+}, and SO_4^{2-} (Eddy, 1983). A study on *Mytilus edulis*, found salt type, IO to contain a higher amount of Cu ions from industrial processing (Arnold *et al.*, 2007). Until recently, aquaporin was only described as acting in tissue of fish and in the fish oocyte during the maturation process (Cerdà and Finn, 2010). Amaroli *et al.*, (2013) showed for sea urchins *Paracentrotus lividus*, that water uptake during activation was highly dependent on aquaporin channels in the chorion (Amaroli, 2013) and that PVS formation and fertilization was highly inhibited by ions like Cu, Hg and Ni. Assuming aquaporins are present in fish egg chorion, then variability in the concentrations of metal ions in marine salt types likely impacts the formation of PVS.

Egg neutral buoyancy at 7 HPF did not show differences among salt types reflecting the remarkable deviation in egg chorion diameter observed in the eggs from IO and TE. However, at the stage of embryo formation (30 HPF) the small eggs obtained using IO and TE became heavier and this likely indicates the first signs of insufficient size of PVS. This influences metabolism and wastes from the embryo and causes suboptimal incubation conditions (Eddy, 1983). This scenario resembles previous reported observations where artificial seawater invoked small eggs that were unable to hatch (Tomkiewicz and Jarlbæk, 2008). The neutral buoyancy found from the

salts providing large eggs was around 33.8 psu, which is similar to buoyancy estimates of Japanese eel eggs (Tsukamoto, 2009).

In summary, salinity and sea salt type significantly influenced egg activation, fertilization, buoyancy, and early embryonic development, although with substantial variation in optima and tolerance between females. Fertilization success was highest in the salinity range of 30 to 40 psu. Salt type significantly affected the egg chorion diameter, and RS and RC lead to consistently large eggs while IO, TE, NC and NC[RS] generated eggs with limited PVS. Yolk diameters showed low variability across treatments supporting the impermeability of the vitelline membrane during early embryonic development. Activation and incubation salinity affected neutral buoyancy, and only within a salinity range of 30 to 40 psu could egg neutral buoyancy be sustained during incubation to embryo formation. Salt types were found to induce only small egg chorion diameters and failed to sustain buoyancy. In conclusion, salt concentration and elemental composition strongly affected processes during egg activation and embryonic development. The effects would often play in concert and mechanisms are not fully understood. Successful activation and development of the eggs of European eel are crucial for the progress in the artificial propagation of the species and further studies on these quality aspects of the activation media are encouraged.

Acknowledgements

Conflict of interest

None

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Furthermore we assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

References

Alavi, S.M.H., Linhart, O., Coward, K., Rodina, M. (2008). Fish spermatology: implications for aquaculture management. In: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Ltd., Oxford, pp. 397–461.

Alderdice, D.F., Rao, T.R., Rosenthal, H. (1979a). Osmotic responses of eggs and larvae of the Pacific herring to salinity and cadmium. Helgoländer Wiss. Meeresunters. **32**, 508-538.

Alderdice, D.F., Rosenthal, H., Velsen, F.P. (1979b). Influence of salinity and cadmium on the volume of Pacific herring eggs. Helgol. Wiss. Meeresunters. **32**, 163–178.

Amaroli, A. (2013). Identification of aquaporins in eggs and early embryogenesis of the sea urchin *Paracentrotus lividus*. Acta Histochem. **115**, 257–263.

Arnold, W.R., Cotsifas, J.S., Winter, A.R., Klinck, J.S., Smith, D.S. & Playle, R.C. (2007). Effects of using synthetic sea salts when measuring and modelling copper toxicity in saltwater toxicity tests. Environ. Toxicol. Chem. **26**, 935–943.

Asturiano, J.F., Pérez, L., Garzón, D.L., Marco-Jiménez, F., Peñaranda, D.S., Vicente, J.S. & Jover, M. (2004). Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiol. Biochem. **30**, 283–293.

Atkinson, M.J. & Bingman, C. (1997). Elemental composition of commercial seasalts. J. Aquaric. Aquat. Sci. VIII, 39-43.

Boëtius, I. & Boëtius, J. (1980). Experimental maturation of female silver eels, *Anguilla anguilla*. Estimates of fecundity and energy reserves for migration and spawning. Dana **1**, 1-28.

Butts, I.A.E., Sørensen, S.R., Politis, S.N & Tomkiewicz, J. (2014). Standardization of fertilization protocols for the European eel. Aquaculture **426-427**, 9-13.

Cerdà, D.J., Fabra, M., Raldúa, D. (2007). Physiological and molecular basis of fish oocyte hydration. In: Babin, P.J., Cerdà, D.J., Lubzens, E. (Eds.), The Fish Oocyte. Springer Netherlands, pp. 349–396.

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

Chapman, R., 2006. A sea water equation of state calculator. APL Ocean Remote Sensing. [Online]. John Hopkins University Applied Physics Laboratory. Accessed online March 10, 2013 at:< http://fermi.jhuapl.edu/denscalc.html >.

Claw, K.G. & Swanson, W.J. (2012). Evolution of the Egg: New Findings and Challenges. Annu. Rev. Genomics Hum. Genet. **13**, 109-125.

Coombs, S.H. (1981). A density-gradient column for determining the specific gravity of fish eggs, with particular reference to eggs of the mackerel *Scomber scombrus*. Mar. Biol. **63**, 101–106.

Coombs, S.H., Boyra, G., Rueda, L.D., Uriarte, A., Santos, M., Conway, D.V.P. & Halliday, N.C. (2004). Buoyancy measurements and vertical distribution of eggs of sardine (*Sardina pilchardus*) and anchovy (*Engraulis encrasicolus*). Mar. Biol. **145**, 959–970.

Coward, K., Bromage, N.R., Hibbitt, O. & Parrington, J. (2002). Gamete physiology, fertilization and egg activation in teleost fish. Rev. Fish Biol. Fish. **12**, 33–58.

Davenport, J., Lønning, S. & Kjørsvik, E. (1981). Osmotic and structural changes during early development of eggs and larvae of the cod, *Gadus morhua* L. J. Fish Biol. **19**, 317–331.

Dufour, S., Burzawa-Gerard, E., Belle, N.L., Sbaihi, M. & Vidal, B. (2003). Reproductive Endocrinology of the European Eel, *Anguilla anguilla*. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer Japan, pp. 373–383.

Eddy, F.B. (1983). Formation of the perivitelline fluid in Atlantic salmon eggs (*Salmo-salar*) in fresh-water and in solutions of metal-ions. Comp. Biochem. Physiol. Pharmacol. Toxicol. Endocrinol. **75**, 1–4.

Evans, J.P. & Geffen, A.J. (1998). Male characteristics, sperm traits, and reproductive success in winter-spawning Celtic Sea Atlantic herring, *Clupea harengus*. Mar. Biol. **132**, 179–186.

Fabra, M., Raldúa, D., Power, D.M., Deen, P.M.T. & Cerdà, J. (2005). Marine fish egg hydration is aquaporin-mediated. Science **307**, 545–545.

Finn, R.N. (2007). The physiology and toxicology of salmonid eggs and larvae in relation to water quality criteria. Aquat. Toxicol. **81**, 337–354.

Fontaine, M., Bertrand, E., Lopez, E. & Callamand, O. (1964). Sur la maturation des organes génitaux de l'anguille femelle (*Anguilla anguilla* L.) et l'émission spontanée des oeufs en aquarium. CR Acad Sci Paris **259**, 907–2.

Gallego, V., Martínez-Pastor, F., Mazzeo, I., Peñaranda, D.S., Herráez, M.P., Asturiano, J.F. & Pérez, L. (2014). Intracellular changes in Ca^{2+}, K^+ and pH after sperm motility activation in the European eel (*Anguilla anguilla*): Preliminary results. Aquaculture **418–419**, 155–158.

Gallo, A. & Costantini, M. (2012). Glycobiology of reproductive processes in marine animals: The state of the art. Mar. Drugs **10**, 2861-2892.

Govoni, J.J. & Forward, R.B.J. (2008). Buoyancy. In: R.N. Finn and B.G. Kapoor (Eds.), Fish Larval Physiology. Science Publishers, Enfield, NH, USA, pp. 495–521.

Hart, N.H. & Yu, S.-F. (1980). Cortical granule exocytosis and cell surface reorganization in eggs of *Brachydanio-rerio*. J. Exp. Zool. **213**, 137–159.

Heinsbroek, L.T.N., Støttrup, J.G., Jacobsen, C., Corraze, G., Kraiem, M.M., Holst, L.K., Tomkiewicz, J. & Kaushik, S.J. (2013). A review on broodstock nutrition of marine pelagic spawners: the curious case of the freshwater eels (*Anguilla spp.*). Aquac. Nutr. **19**, 1–24.

Holliday, F.G.T. (1969). The effects of salinity on the eggs and larvae of teleosts. In: Hoar, W.S., & Randall, D.J., (Eds.) Fish Physiology, Excretion, Ionic Regulation and Metabolism. Academic Press Inc. New York, USA, pp 293-309.

Holliday, F.G.T. & Blaxter, J.H.S. (1960). The effects of salinity on the developing eggs and larvae of the herring. J. Mar. Biol. Assoc. U. K. **39**, 591-603.

Hovanec, T.A. & Coshland, J.L. (2004). A chemical analysis of select trace elements in synthetic sea salts and natural seawater. Sea Scope Aquar. Syst. **21**(3).

Iwamatsu T., Ito, S. (1986). Effects of microinjected cations on the early event of fertilization in the Medeka egg. Develop. Growth and Differ. **28** 303-310

Kennedy, J., Geffen, A.J. & Nash, R.D.M. (2007). Maternal influences on egg and larval characteristics of plaice (*Pleuronectes platessa* L.). J. Sea Res. **58**, 65-77.

Kjørsvik, E., Davenport, J. & Lönning, S. (1984). Osmotic changes during the development of eggs and larvae of the lumpsucker, *Cyclopterus lumpus* L. J. Fish Biol. **24**, 311–321.

Kjørsvik, E., Hoehne-Reitan, K. & Reitan, K.I. (2003). Egg and larval quality criteria as predictive measures for juvenile production in turbot (*Scophthalmus maximus* L.). Aquaculture **227**, 9–20.

Kjørsvik, E., Mangor-Jensen, A. & Holmefjord, I. (1990). Egg quality in fishes. In: J.H.S. Blaxter and A.J. Southward (Eds.), Advances in Marine Biology **26**, 71–113.

Kokhnenko, S.V., Bezdenezhnykh, V.A. & Gorovaya, S.L. (1977). Maturation of the European eel (*Anguilla anguilla*) when artificially reared. J. Ichthyol. **17**, 878–883.

Kroll, M.-M., Peck, M.A., Butts, I.A.E. & Trippel, E.A. (2013). Paternal effects on early life history traits in Northwest Atlantic cod, *Gadus morhua*. J. Appl. Ichthyol. **29**, 623–629.

Laale, H.W. (1980). The perivitelline space and egg envelopes of bony fishes: A review. Copeia **1980**, 210–226.

Li, X., Jenssen, E., & Fyhn, H.J. (1989). Effects of salinity on egg swelling in Atlantic salmon (*Salmo salar*). Aquaculture **76**, 317–334.

Lønning, S., & Davenport, J. (1980). The swelling egg of the long rough dab, *Hippoglossoides platessoides limandoides* (Bloch). J. Fish Biol. **17**, 359–378.

Morley, S.A., Batty, R.S., Geffen, A.J. & Tytler, P. (1999). Egg size manipulation: a technique for investigating maternal effects on the hatching characteristics of herring. J. Fish Biol. **55**, 233–238.

Munk, P., Hansen, M.M., Maes, G.E., Nielsen, T.G., Castonguay, M., Riemann, L., Sparholt, H., Als, T.D., Aarestrup, K., Andersen, N.G. & Bachler, M. (2010). Oceanic fronts in the Sargasso Sea control the early life and drift of Atlantic eels. Proc. R. Soc. B Biol. Sci. **277**, 3593–3599.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K. & Hirose, K. (1996). Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica*. Aquaculture **139**, 291–301.

Ohta, H., Kagawa, H., Tanaka, H. & Unuma, T. (2001). Control by the environmental concentration of ions of the potential for motility in Japanese eel spermatozoa. Aquaculture **198**, 339–351.

Okamoto, T., Kurokawa, T., Gen, K., Murashita, K., Nomura, K., Kim, S.-K., Matsubara, H., Ohta, H. & Tanaka, H. (2009). Influence of salinity on morphological deformities in cultured

larvae of Japanese eel, *Anguilla japonica*, at completion of yolk resorption. Aquaculture **293**, 113–118.

Palstra, A.P., Cohen, E.G.H., Niemantsverdriet, P.R.W., van Ginneken, V.J.T., Van den Thillart, G.E.E.J.M. (2005). Artificial maturation and reproduction of European silver eel: Development of oocytes during final maturation. Aquaculture **249**, 533–547.

Pankhurst, N.W., & Sorensen, P.W. (1984). Degeneration of the alimentary tract in sexually maturing European *Anguilla anguilla* (L.) and American eels Anguilla rostrata (LeSueur). Can. J. Zool., **62**, 1143–1149.

Pedersen, B.H. (2003). Induced sexual maturation of the European eel *Anguilla anguilla* and fertilization of the eggs. Aquaculture **224**, 323–338.

Peterson, R.H. & Martin-Robichaud, D.J. (1986). Perivitelline and vitelline potentials in teleost eggs as influenced by ambient ionic-strength, natal salinity, and electrode electrolyte - and the influence of these potentials on cadmium dynamics within the egg. Can. J. Fish. Aquat. Sci. **43**, 1445–1450.

Seoka, M., Yamada, S., Iwata, Y., Yanagisawa, T., Nakagawa, T. & Kumai, H. (2003). Differences in the biochemical content of buoyant and non-buoyant eggs of the Japanese eel, *Anguilla japonica*. Aquaculture **216**, 355–362.

Shephard, K.L. (1989). An analysis of the ion-exchange characteristics of fish-egg chorions. Fish Physiol. Biochem. **6**, 395–401.

Shields, R.J., Brown, N.P. & Bromage, N.R. (1997). Blastomere morphology as a predictive measure of fish egg viability. Aquaculture **155**, 1–12.

Sørensen, S.R., Skov, P.V., Lauesen, P., Tomkiewicz, J., Bossier, P., & De Schryver, P. (2014). Microbial interference and potential control in culture of European eel (*Anguilla anguilla*) embryos and larvae. Aquaculture **426**, 1–8.

Sørensen, S.R., Gallego, V., Pérez, L., Butts, I.A.E., Tomkiewicz, J., & Asturiano, J.F. (2013). Evaluation of methods to determine sperm density for the European eel, Anguilla anguilla. Reproduction in Domestic Animals **48**, 936-944.

Stricker, S.A. (1999). Comparative biology of calcium signalling during fertilization and egg activation in animals. Developmental Biology **211**, 157-176

Thorsen, A. & Fyhn, H.J. (1996). Final oocyte maturation in vivo and in vitro in marine fishes with pelagic eggs; yolk protein hydrolysis and free amino acid content. J. Fish Biol. **48**, 1195–1209.

Tomkiewicz, J. (2012). Reproduction of European eel in aquaculture (REEL), Consolidation and new production methods. In, DTU Aqua Research Report No. 249-2012. Technical University of Denmark.

Tomkiewicz, J. & Jarlbæk, H. (2008). Kunstig reproduktion af ål: ROE II og IIB. In, DTU Aqua Research Report No. 180-08 (Danish). Technical University of Denmark.

Tsukamoto, K. (2009). Positive buoyancy in eel leptocephali: an adaptation for life in the ocean surface layer. Mar. Biol. **156**, 835–846.

Tsukamoto, K., Chow, S., Otake, T., Kurogi, H., Mochioka, N., Miller, M.J., Aoyama, J., Kimura, S., Watanabe, S., Yoshinaga, T., Shinoda, A., Kuroki, M., Oya, M., Watanabe, T., Hata, K., Ijiri, S., Kazeto, Y., Nomura, K. & Tanaka, H. (2011). Oceanic spawning ecology of freshwater eels in the western North Pacific. Nat. Commun. **2**, 179.

Villani, P. & Lumare, F. (1975). Nota Sull'accrescimento ovarico indotto in *Anguilla anguilla* L. Investig. Pesq. **39**.

Webb, S.E. & Miller, A.L. (2013). Ca^{2+} signaling during activation and fertilization in the eggs of teleost fish. Cell Calcium **53**, 24–31.

Yoshinaga, T., Miller, M.J., Yokouchi, K., Otake, T., Kimura, S., Aoyama, J., Watanabe, S., Shinoda, A., Oya, M., Miyazaki, S., Zenimoto, K., Sudo, R., Takahashi, T., Ahn, H., Manabe, R., Hagihara, S., Morioka, H., Itakura, H., Machida, M., Ban, K., Shiozaki, M., Ai, B. & Tsukamoto, K. (2011). Genetic identification and morphology of naturally spawned eggs of the Japanese eel *Anguilla japonica* collected in the western North Pacific. Fish. Sci. **77**, 983–992.

Table 1: Length (cm) and weight (g) for wild-caught female European eels *Anguilla anguilla*, used in Experiment 1 and 2.

	Female ID	Lengths cm	Weight g
Experiment 1	*f1*	62	432
	f2	71	612
	f3	78	955
	f4	60	492
	f5	77	890
	f6	71	750
	mean ±SD	69.8 ±7.5	676.2 ± 212.4
Experiment 2	*f5*	77	890
	f7	69	599
	f8	80	832
	f9	75	781
	f10	69	551
	mean ±SD	74.0 ± 4.9	730.6 ± 148.2

Table 2: Specification of salt type treatments used in Experiment 2 for activation and incubation of eggs of European eel, *Anguilla anguilla*.

Treatment	Salt type	Components	Salinity ±0.1 ppt	pH
Std	Natural sea water	Filtered 0.8 μm, salinity adjusted by Tropic Marin[d]	35.0	8.2 ± 0.02
RS	Artificial sea water	Red Sea[a] + MilliQ water	35.0	8.6 ± 0.06
RS[pro]	Artificial sea water	Red Sea Pro[b] + MilliQ water	35.0	8.4 ± 0.03
RC	Artificial sea water	Reef Crystal[c] + MilliQ water	35.0	9.1 ± 0.01
TM	Artificial sea water	Tropic Marine[d] + MilliQ water	35.0	8.5 ± 0.03
IO	Artificial sea water	Instant Ocean[e] + MilliQ water	35.0	8.5 ± 0.01
TE	Artificial sea water	Tetra Marin[f] + MilliQ water	35.0	8.8 ± 0.04
NC	Artificial sea water	NaCl[g] + MilliQ water	35.0	7.4 ± 0.04
NC[RS]	Artificial sea water	NaCl[g] + 10% Red Sea[a] (v/w) + MilliQ water	35.0	7.5 ± 0.05

Sea salt brands: [a]Red Sea, RedSea International, Eilat, Israel; [b]Red Sea Coral Pro salt, RedSea International, Eilat, Israel; [c]Reef Crystal, Aquarium system, Sarrebourg, France; [d]Tropic Marin® Sea Salt, Dr. Biener GmbH, Wartenberg, Germany; [e]Instant Ocean, Aquarium system, Sarrebourg, France; [f]Tetra Marine, TetraEurope, Paris, France; [g]NaCl, Sigma-Aldrich, 0.2 μm filtered

Table 3: Average eggs metrics of *in vitro* fertilised eggs from European eel, *Anguilla anguilla* according to egg categories T-1a, T-1ab, and T-1b defined by cleavage symmetry 3.5-5 h after fertilisation. Within each egg measure sub-letters indicate significant differences ($\alpha = 0.05$).

| Egg category | N | Diameter μm ± SD | | Volume mm^3 ± SD | | |
		Egg	Yolk	Egg	Yolk	Perivitelline Space
T-1a: Regular cleavage	896	1453 ± 162^a	934 ± 31^a	1.666 ± 0.52^a	$0.427 \pm 0.04a$	1.239 ± 0.52^a
T-1ab: Mostly regular cleavage	753	1422 ± 172^b	940 ± 27^b	1.571 ± 0.54^b	$0.436 \pm 0.38b$	1.135 ± 0.53^b
T-1b: Irregular cleavage	1535	1374 ± 174^c	946 ± 31^c	1.421 ± 0.52^c	$0.445 \pm 0.04c$	0.976 ± 0.50^c

Figures
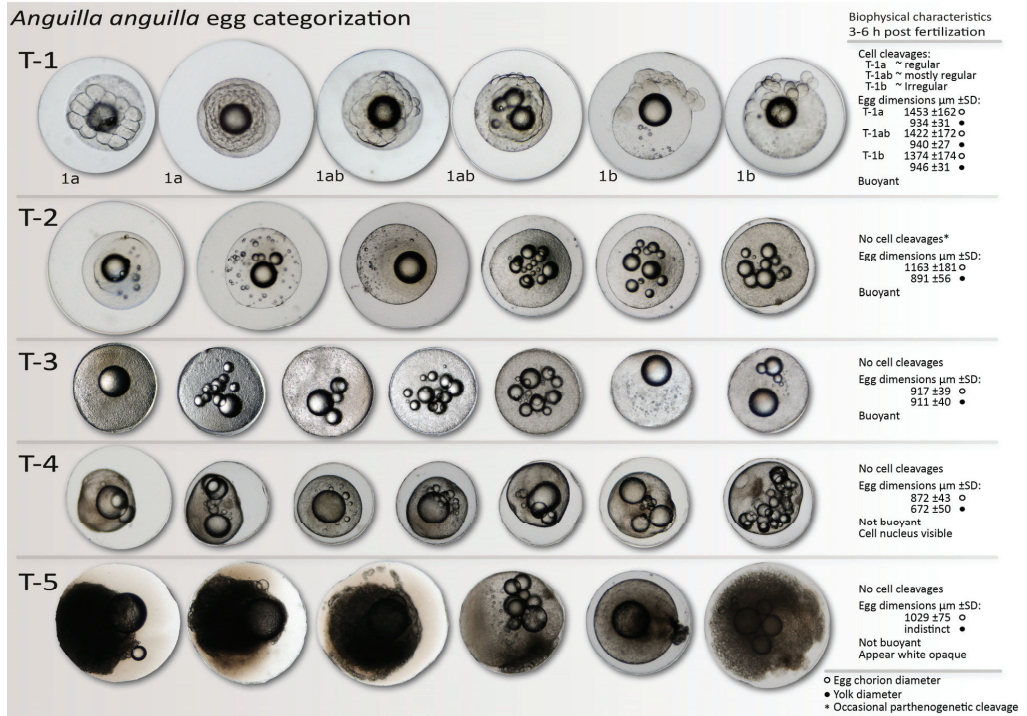
Fig. 1. Characteristics of egg types used to categorize eggs of European eel, *Anguilla anguilla,* after *in vitro* activation and fertilisation. Five main types of eggs are displayed based on morphology, size and buoyancy at 35 psu, 3 to 6 h post fertilisation and incubation at 20 ± 0.5˚C. T-1 includes fertilised eggs and is subdivided into three sub-categories based on cell cleavage pattern: T-1a: regular and symmetric; T-1ab: mostly regular with minor irregularities; T-1b: irregular.
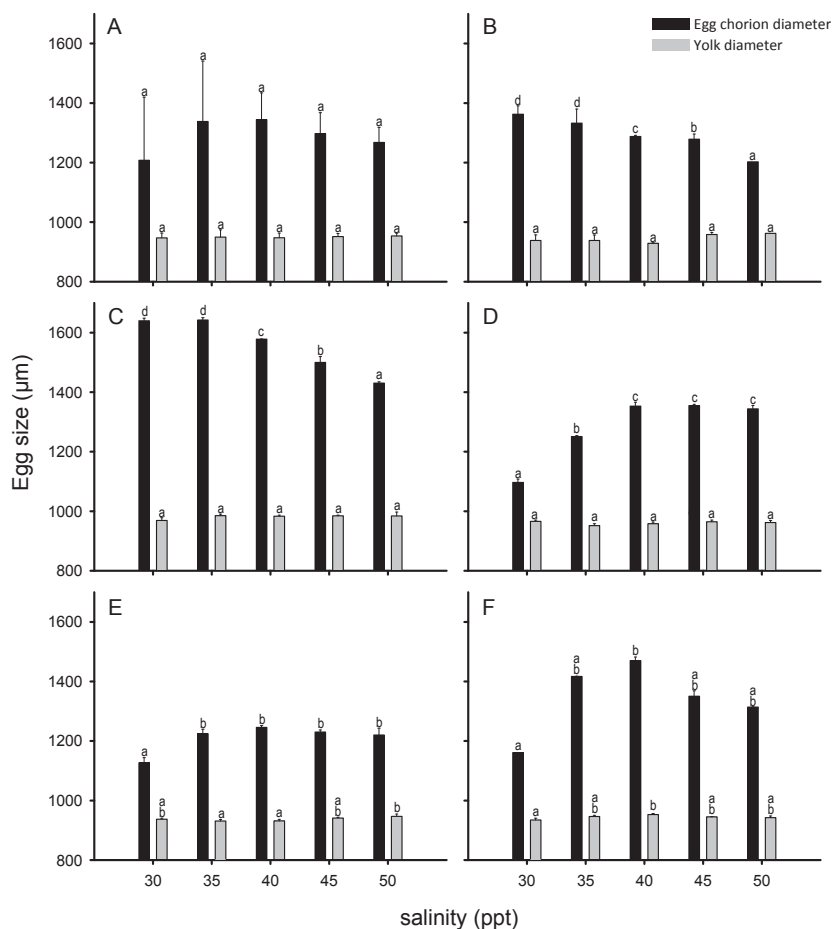
Fig. 2. Egg size of European eel, *Anguilla anguilla*, in relation to activation salinity (psu). Measurements include chorion and yolk diameter (μm ± SD) of fertilised eggs 3.5 to 5 h post in vitro fertilisation and incubated at 20 ± 0.5˚C. (A) Average egg sizes all females. (B to F) egg sizes are for female f1 to f5. Letters indicate significant differences (α = 0.05) among salinities within groups.
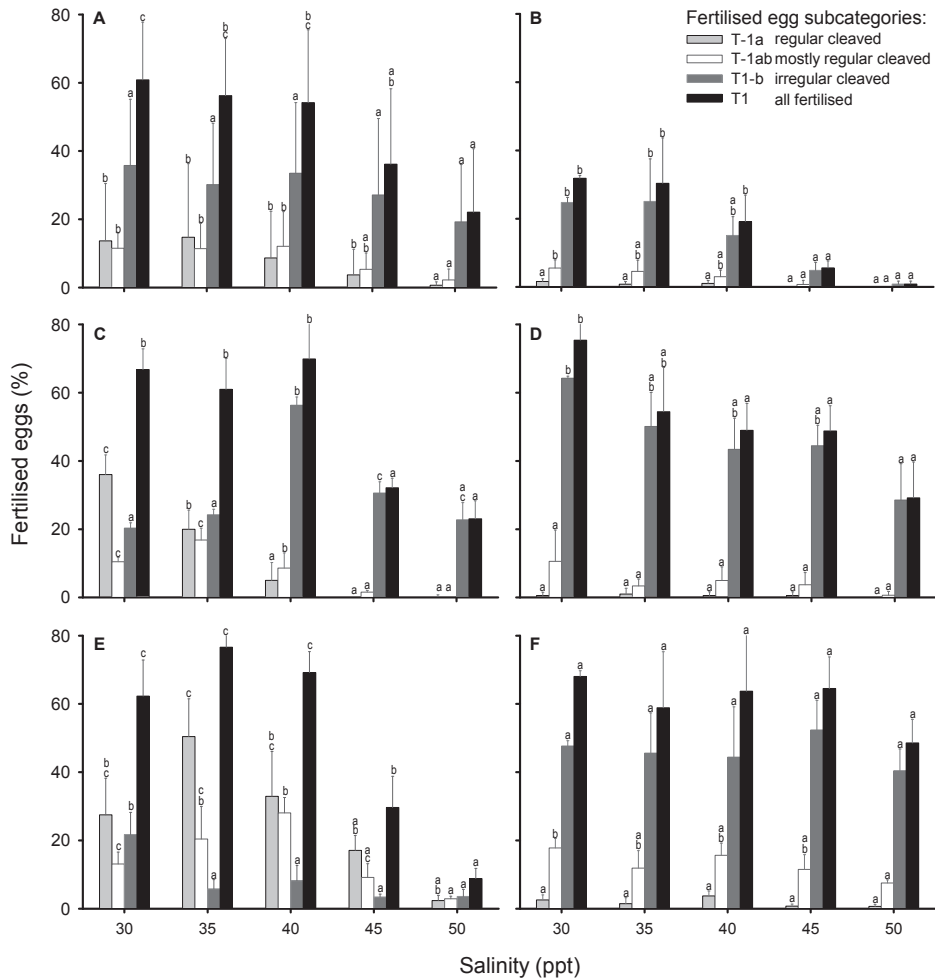
Fig. 3. Fertilisation success and cleavage pattern of *in vitro* fertilised eggs of European eel, *Anguilla anguilla*, in relation to activation and incubation salinity. Fertilisation percentage and distribution on sub-categories T-1a, T1ab, and T-1b were assessed 3.5-5 h post fertilisation and incubation at $20 \pm 0.5°$ C. Bars show percentage + SD. (A) Average egg sizes all females. (B-F) egg sizes for female *f1-f5*. Letters indicate significant differences ($\alpha = 0.05$) among salinities within groups.
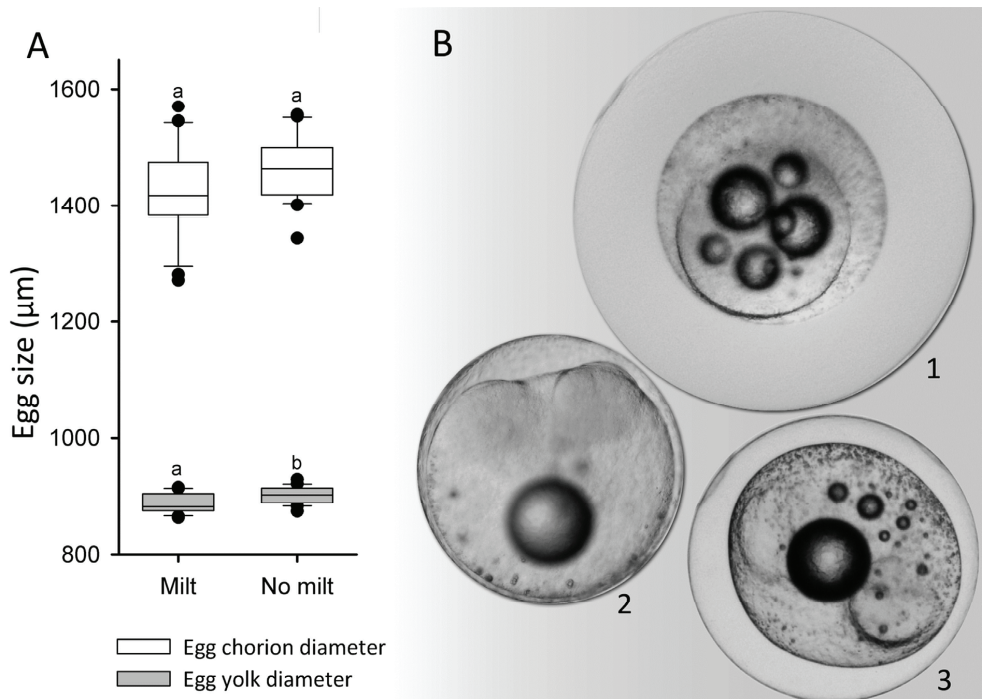
Fig. 4. Eggs of European eel, *Anguilla anguilla*, activated with and without the presence of sperm. (A) Boxplot of egg chorion and yolk diameter at 3.5 h post fertilisation. (B) Images of unfertilised eggs 1.5 to 4 h post fertilisation with signs of cell cleavage. Characteristics of egg 1: large perivitelline space (PVS) and a single disk; egg 2 and egg 3: Negligible PVS and parthenogenic cleavage.
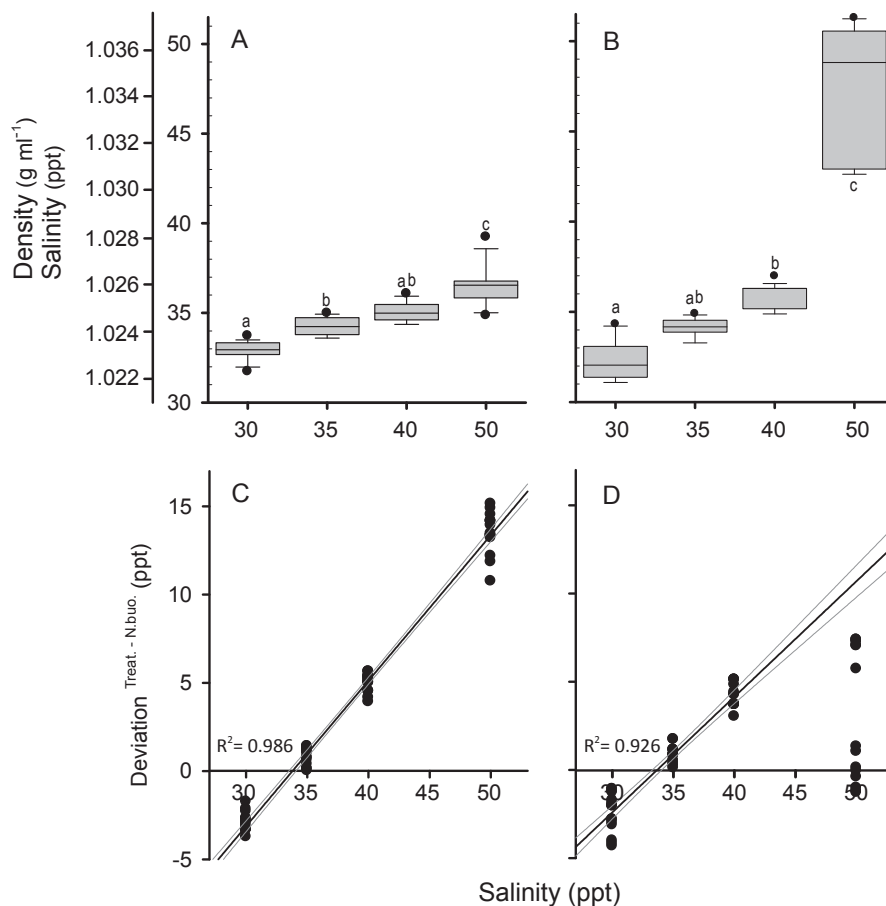
Fig. 5. Buoyancy characteristics of eggs from European eel, *Anguilla anguilla*, activated and fertilised at different salinities and incubated at 20 ± 0.5˚C. (A+B) Neutral buoyancy of eggs (N ≥ 15) from each treatment after: (A) 7 HPF, (B) 30 HPF. Letters indicate significant differences (α = 0.05) among groups. (C+D) Deviation between activation salinity and salinity at neutral buoyancy in relation to activation salinity at (C) 7 HPF (linear regression ± 95% CL, R2 = 0.99, y=0.825X - 27.87), and (D) 30 HPF (linear regression excl. 50 psu ± 95% CL, R2=0.93, y=0.655X – 22.073).
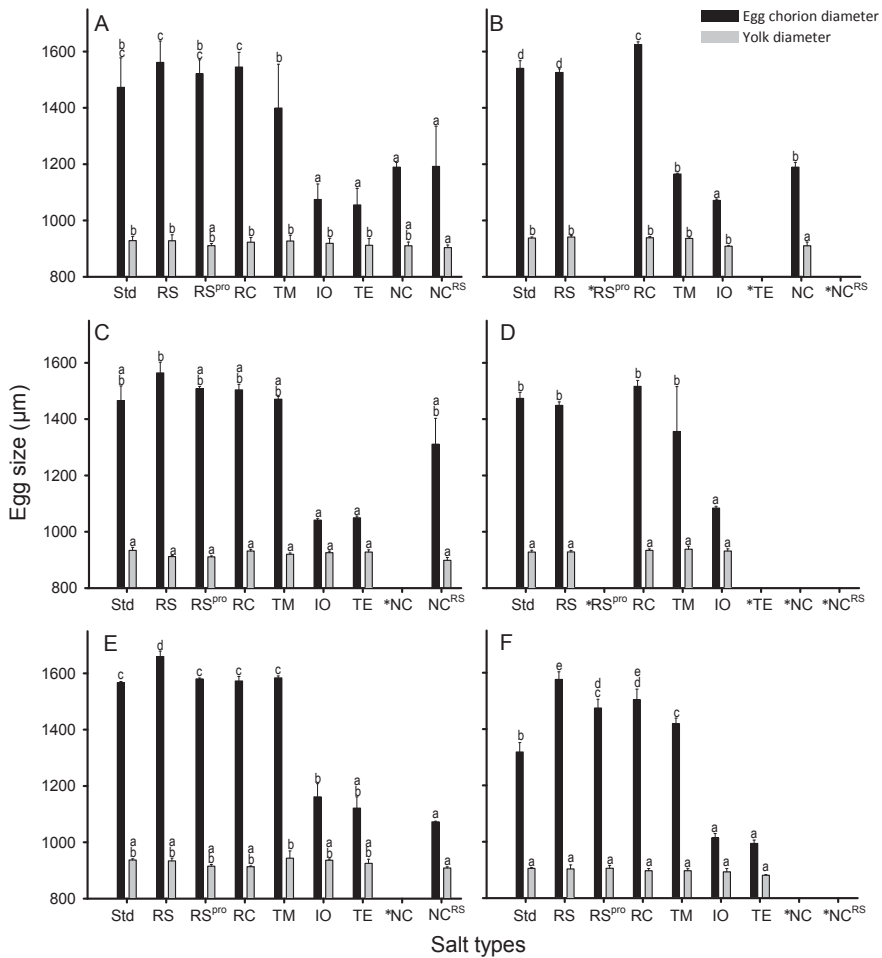
Fig. 6. Egg size of European eel, *Anguilla anguilla*, in relation to salt type treatment adjusted to 35.0 ± 0.1 psu. Measurements include chorion and yolk diameter (µm ±SD) of fertilised eggs 3.5 to 5 h post in vitro fertilisation and incubated at 20 ± 0.5˚C. Salt type treatment, Std: Natural seawater; RS: Red Sea salt; RSpro: Red Sea Pro; RC: Reef Crystal; TM: Tropic Marine; IO: Instant Ocean; TE: Tetra Marine; NC: NaCl; NCRS: NaCl + 10% Red Sea salt. (A) average of all females. (B to F) are for female f6 to f10. Letters indicate significant differences (α = 0.05) among groups. * mark treatments without fertilised eggs or not tested for a particular female
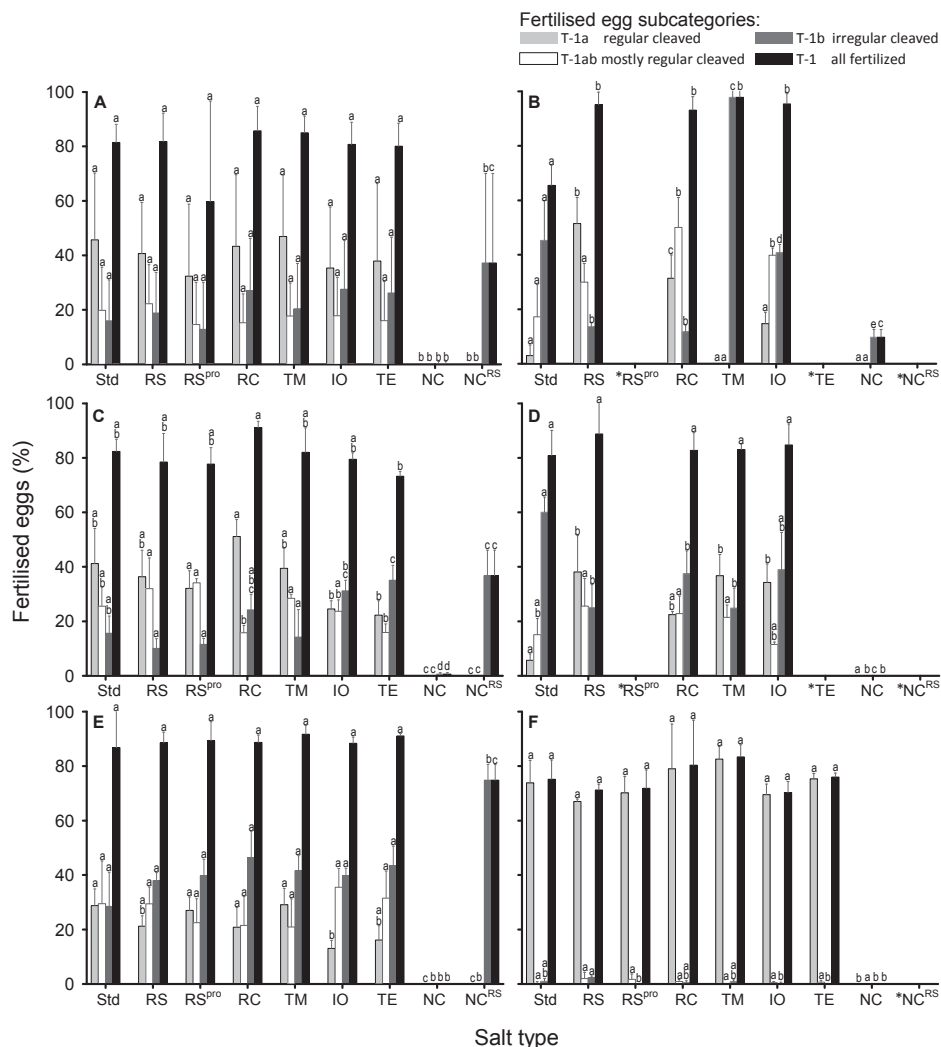
Fig. 7.

Fig. 7. Fertilisation success and cleavage pattern of in vitro fertilised eggs of European eel, *Anguilla anguilla*, in relation to salt type treatment adjusted to 35.0 ± 0.1 psu. Fertilisation percentage and distribution of sub-categories T-1a, T1ab, and T-1b were assessed 3.5 to 5 h post fertilisation and incubation at 20 ± 0.5°C. (A) average of all females. (B to F) are for female f6 to f10. Letters indicate significant differences (α = 0.05) among groups. Bars show percentage + SD. Salt type treatment, Std: Natural seawater; RS: Red Sea salt; RSpro: Red Sea Pro; RC: Reef Crystal; TM: Tropic Marine; IO:

Instant Ocean; TE: Tetra Marine; NC: NaCl; NCRS: NaCl + 10% Red Sea salt. * mark treatments without fertilised eggs or not tested for a particular female.
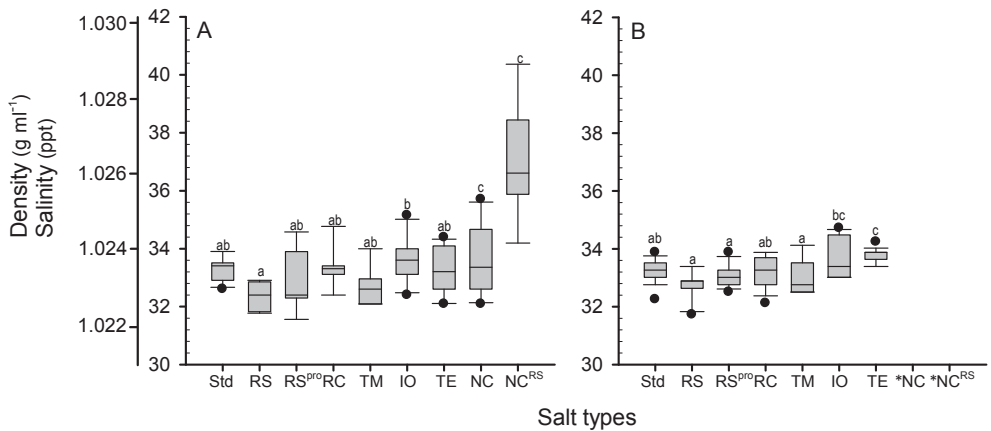
Fig. 8

Fig. 8. Neutral buoyancy of eggs from European eel, *Anguilla anguilla*, in vitro activated and fertilised, in relation to salt type treatment adjusted to 35.0 ± 0.1 psu and incubated at $20 \pm 0.5°C$. Box-plot including outliers show density and salinity at neutral buoyancy of (A) eggs (N $\geq$ 15) at 7 HPF, (B) eggs (N $\geq$ 15) at 30 HPF. Letters indicate significant differences ($\alpha = 0.05$) among salt type treatments and * mark treatments with non-buoyant eggs

Paper 5:

Ichthyodinium infection in eggs of European eel (*Anguilla anguilla*) spawned in captivity

Ichthyodinium identified in the eggs of European eel (*Anguilla anguilla*) spawned in captivity

S.R. Sørensen [a,*], J. Tomkiewicz [a], A. Skovgaard [b]

[a] National Institute of Aquatic Resources, Section for Marine Ecology, Technical University of Denmark, Jægersborg Allé 1, 2920 Charlottenlund, Denmark
[b] Department of Veterinary Disease Biology, Laboratory of Aquatic Pathobiology, University of Copenhagen, Stigbøjlen 7, 1870 Frederiksberg C, Denmark

ABSTRACT

A presumed parasitic protozoan was found in the eggs of European eel obtained from an experiment on captive breeding of eel, *Anguilla anguilla*, based on silver eels from a freshwater lake in the northern part of Denmark. Gross morphology of the organism was comparable to that of early stages of *Ichthyodinium*, a syndinian dinoflagellate parasite found in pelagic eggs of various marine fish species. Sequences of genes coding for small subunit ribosomal RNA confirmed that the organism was an *Ichthyodinium* species, and molecular phylogenetic analysis demonstrated the presence of two *Ichthyodinium* genotypes: one occurring in the Atlantic Ocean and adjacent coastal waters and one in the Pacific Ocean area. The inclusion of several GenBank-derived environmental gene sequences, from the Caribbean Sea, revealed to represent *Ichthyodinium*, suggesting that this parasite genus is ubiquitous in the World's oceans.

© 2014 Elsevier B.V. All right reserved.

1. Introduction

Ichthyodinium chabelardi is a parasitic dinoflagellate that infects pelagic eggs of several marine fish species (Hollande and Cachon, 1952, 1953; Stratoudakis et al., 2000; Yuasa et al., 2007). It has the life cycle of a parasitoid, infecting and devouring the egg yolk sac, eventually causing the death of the unhatched fish embryo or newly hatched larva. *Ichthyodinium* infections have currently been reported from eggs of a dozen of marine fish species and several studies have reported on rDNA sequences from the parasite and partial life cycle descriptions (Table 1). However, a lethal parasitoid behaviour has so far only been observed in fish species from tropical, subtropical, and warm temperate waters, such as European pilchard *Sardina pilchardus* (Hollande and Cachon, 1952), Atlantic mackerel *Scomber scombrus* (Meneses et al., 2003) and yellowfin tuna *Thunnus albacares* (Yuasa et al., 2007). In fishes of cold temperate waters, on the other hand, infections with *Ichthyodinium* have never been observed to develop to a degree at which the fish embryos die due to infection (Pedersen et al., 1993; Skovgaard et al., 2010). An exception to this may be the eggs of Atlantic mackerel, which are presumed to be killed by the parasite and have been observed to host *Ichthyodinium* at low prevalence also at higher latitudes (Meneses et al., 2003). The reason for this discrepancy is not fully understood, but it has been suggested that *Ichthyodinium* depends on high salinity and water temperature to fulfil its life cycle, or

alternatively, that some fish species such as Atlantic cod *Gadus morhua* and turbot *Scophthalmus maximus* are not suitable hosts for the parasite and that infection in the eggs of these species is a 'dead end' resulting in the disintegration of parasite cells and potential survival of the embryo (Skovgaard et al., 2010).

Ichthyodinium was originally described as a syndinid dinoflagellate by Hollande and Cachon (1952). This systematic position has been confirmed by recent molecular phylogenetic studies, demonstrating that *Ichthyodinium* is genetically affiliated with the syndinid dinoflagellates, which constitute parasitic sister groups to the typical dinoflagellates. More specifically, *Ichthyodinium* belongs to the so-called Marine Alveolate Group I (López-García et al., 2001; Skovgaard et al., 2009). Only few species are known from this group, but a large number of environmental SSU rRNA gene sequences fall into the group in molecular phylogenetic analysis, suggesting that microorganisms related to *Ichthyodinium* are abundant in the oceans (Guillou et al., 2008). SSU rDNA sequences of *Ichthyodinium* are remarkably similar across host species and only limited genetic variation has been found between *Ichthyodinium* from Northern Europe and Southeast Asia (Mori et al., 2007; Skovgaard et al., 2009). So far, only a single species of the genus has been formally described.

This study presents data on the occurrence of *Ichthyodinium* in European eel, *Anguilla anguilla*, in an experimental eel reproduction facility and summarizes available SSU rDNA sequence information. Reproduction of European eel in captivity has been attempted for several decades (Bruun et al., 1949; Pedersen, 2004) with very limited success in hatching larvae (Bezdenezhnykh et al., 1983). The mechanisms of

Table 1

Fish species reported as host of the fish egg parasite *Ichthyodinium*. Absence of 'x' denotes that infection has been mentioned in text of published papers only.

Fish species	Data on morphology available	SSU rDNA sequences available	Lethal infection observed	Reference
Sardina pilchardus	x	x	x	Borges et al. (1996), Gestal et al. (2006), Hollande and Cachon (1952, 1953), Marinaro (1971), Meneses and Ré (1992), Skovgaard et al. (2009), Silva and Miranda (1992), Stratoudakis et al. (2000)
Maurolicus muelleri				Hollande and Cachon (1953)
'Gadoids'				Hollande and Cachon (1953)
Sparus aurata	?			Marinaro (1971)
Maurolicus pennanti				Marinaro (1971)
'Other sparids'				Marinaro (1971)
Gadus morhua	x	x	–[a]	Pedersen and Køie (1994), Pedersen et al. (1993), Skovgaard et al. (2010), Bloch et al. (1997)
Scophthalmus maximus	x		–[a]	Pedersen (1993), Pedersen and Køie (1994)
Trachurus trachurus				Farinha in Stratoudakis et al. (2000)
Engraulis encrasicolus				Farinha in Stratoudakis et al. (2000)
Micromesistius poutassou				Farinha in Stratoudakis et al. (2000)
Thunnus albacares	x	x	x	Mori et al. (2007)
Plectropomus leopardus	x	x	x	Yuasa et al. (2007)
Scomber scombrus	x		x	Meneses et al. (2003)
Boops boops	x	x	x	Skovgaard et al. (2009)
'Fish eggs'	x	x	x	Shadrin et al. (2002), Shadrin et al. (2010)
Thunnus orientalis	x	x	x	Ishimaru et al. (2012)
Anguilla anguilla	x	x	–[a]	Present study
Environmental sequences		x		Edgcomb et al. (2011)

[a] No mortality has been documented.

hormonal control in eel maturation are complex and impede reproduction in captivity (Dufour et al., 2003). Recent research applying hormonally induced maturation has, however, led to the production of viable eggs and yolk sac larvae of European eel, enabling detailed study of morphology and structure and development of embryos and larvae (Tomkiewicz, 2012). This work led to the discovery of a spherical foreign object within the developing embryo, resembling the morphology of the parasitic protozoan *Ichthyodinium*, and our aim in the present work was to isolate and identify this organism that has not previously been reported from eggs of European eel.

2. Methods and materials

2.1. Production and sampling of fertilized eel eggs

Gametes were obtained from artificially matured female silver eels, originating from a freshwater lake, Lake Vandet, in northern Jutland, Denmark, and farmed male eels from the commercial eel farm, Stensgård Eel Farm (central Jutland, Denmark) (Tomkiewicz, 2012). Female and male broodstocks were held separately in recirculation systems with artificial seawater adjusted to ~36 ppt using Tropic Marin® Sea Salt (Dr. Biener Aquarientechnik, Germany) and at ~20 °C (Tomkiewicz, 2012).

Natural sea seawater obtained from the North Sea was used in egg activation, fertilization, incubation, and larval culture. Prior to use, the water was adjusted from native salinity (32.5 ppt) to 36 ppt using Tropic Marin® Sea Salt and kept at 20 °C in an aerated reservoir prior to use (hereafter referred to as hatchery seawater, HSW). Salinity was measured using an electronic conductivity meter (WTW multi3410, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). All incubation equipment was cleaned using Virkon-S 2% solution (Virkon-S®, DuPont, USA) between egg batches.

Sperm was obtained shortly before fertilization from four males and diluted in artificial seminal plasma (Asturiano et al., 2004) at a volume ratio of 1:99 in sterile culture flasks. Eggs were stripped into sterilized and dried containers and 1 mL of diluted sperm was added per 2 g of eggs followed by activation adding HSW. Prior to stripping, fish were anaesthetized with ethyl p-aminobenzoate (20 mg L^{-1}; Sigma-Aldrich Chemie, Steinheim, Germany). After activation, gametes were transferred to 15 L of HSW. After 1 h, all buoyant eggs were skimmed into

15 L fresh HSW to remove excess sperm and negatively buoyant (dead) eggs.

Two hours post-fertilization (HPF) eggs were moved to a 60 L incubator holding HSW at 20 °C $\pm$ 0.5 for incubation. At regular intervals, dead eggs were flushing from a bottom valve to avoid build-up of bacteria. Fifteen HPF, HSW was renewed in the incubator.

During the experiment conducted in June 2012, many eggs appeared to be infected with a protozoan parasite. Infected eggs (n = 30) were isolated 15 and 30–35 HPF from 6 selected females and single eggs were placed in sterile Eppendorf tubes and kept at −20 °C until processing. Each egg sample was labelled individually and photographed using a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan), fitted with a Nikon DS-Fi1 camera head, using 40, 100, and 400× magnification (CFI Plan Flour objectives). Larvae were photographed with 20 and 40× magnifications and during photography held in physiological saline solution of 0.90% w/v of NaCl to prevent osmotic stress and anaesthetized using MS222. In addition, samples consisting of 250 mL water, were obtained from an incubator containing many newly hatched larvae, originating from an egg batch with widely infected eel eggs. The water samples were filtered through a sterile 0.2 μm pore size filters (Sartorius Minisart® NML hydrophilic syringe filters) to retain particles present in the sampled water. A total of 16 samples were prepared for PCR and sequence work.

2.2. Infection pathway

In addition to collection of infected eggs, a test of infection pathway was made. The exterior body parts of a female eel (69 cm, 789 g) and male eels (n = 4; mean standard length $\pm$ SD: 40 $\pm$ 2.0 cm and body weight 121 $\pm$ 11 g) were carefully cleaned exterior body parts before stripping to exclude transfer of parasites from the ambient environment during stripping. The used bloodstock fish was anaesthetized and before stripping rinsed on the entire abdominal region using sterile MilliQ water and thereafter wiped around gat area using alcohol (96%).

Eggs were stripped into four replicate sterile trays and activated as described above using sterile filtered seawater (0.2 μm, Sartorius Minisart® NML hydrophilic syringe filters). Following 10 min of activation and fertilization, samples of ~200 eggs from each replicate were inserted in media flasks (Nunc® 75 cm² flasks, non-treated with ventilated caps, Thermo Scientific) containing 200 mL of fresh 0.2 μm filtered

seawater. As a control, 4 subsequent trays of eggs were activated in a similar way, except that activating and incubation followed the standard procedure, i.e. using HSW and the general clean but non-sterile equipment used at the hatchery (such as containers and incubators). Samples of ~200 eggs were then transferred to four 250 mL beakers holding each 200 mL HSW.

After 20 h of incubation, 20 eggs were picked randomly from each replicate flask or beaker and checked for infection under a dissection microscope (Nikon Eclipse 55i microscope, Nikon Corporation, Tokyo, Japan), fitted with a Nikon DS-Fi1 camera head, and 20× magnification (2 × CFI Plan Flour). The number of spheres was quantified in each egg and if above 10 spheres, a category of >10 was used.

2.3. DNA extraction, PCR and sequencing

Prior to PCR, DNA was extracted by proteinase K digestion of samples in 20 μL of lysis buffer (Skovgaard et al., 2011). DNA was PCR-amplified in 25 μL reactions, using 5 μL of the resulting crude DNA extract and 1.0 μM of each primer of the pair Icht1F and Icht4R, which amplifies approx. 1000 bp of the *Ichthyodinium* SSU rRNA gene (Skovgaard et al., 2010). The 25 μL PCR mixture further contained 200 μM of each dNTP, 3.0 mM $MgCl_2$, 1 unit of Taq polymerase, and the PCR buffer supplied with the enzyme (Bioline no. BIO21040). The PCR was run in an automated thermocycler (Biometra T3 Thermocycler) under the following conditions: an initial denaturing step at 94 °C for 5 min followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 6 min. Positive controls were omitted throughout DNA amplification in order to eliminate this as a source of potential contamination of samples. Negative 'controls' were plentiful, since most samples did not result in products suitable for sequencing. No PCR products from the first PCR were visible on an ethidium bromide-stained agarose gel, and PCR products were therefore re-amplified. Second rounds of PCR were run both as re-amplification using the initial primers and as semi-nested PCR under the same conditions as the initial PCR. One microliter of the products from the initial PCR served as a template for the second PCR, and the primer combinations used for semi-nested PCR were Icht1F/18S-EUK1134-R (Bower et al., 2004) and ND4F/Icht4R (Ekelund et al., 2004). PCR products were sequenced bidirectionally with an ABI3730xl sequencer (Macrogen, Korea) using the same primers as used for PCR and semi-nested PCR. Sequence reads were aligned and assembled using the software ChromasPro 1.75 (Technelysium, Australia), and their similarities to other known sequences were established by BLAST searches (Altschul et al., 1997).

2.4. Phylogenetic analysis

BLAST search revealed that the obtained sequence (GenBank accession number) was similar to several *Ichthyodinium* sequences. An alignment was, therefore, made based on the new sequence and *Ichthyodinium* SSU rDNA sequences available in GenBank plus a number of 'environmental' sequences with highest similarity to *Ichthyodinium* as detected through the BLAST search. The alignment further included the syndinian dinoflagellate *Euduboscquella cachoni* with highest similarity to *Ichthyodinium* and two dinophycean dinoflagellates *Karenia mikimotoi* and *Pentapharsodinium tyrrhenicum* for rooting of the phylogenetic tree. The resulting 21 sequences were aligned by the use of ClustalX2.1 (Larkin et al., 2007). A phylogenetic analysis was then conducted in MEGA5.21 (Tamura et al., 2011), using the Maximum Likelihood method based on the Tamura 2-parameter model (K2 + I, as suggested by MEGA5.21). Calculation of bootstrap values was based on 1000 replicates. Positions containing gaps and missing data were eliminated, resulting in a final dataset covering a total of 568 positions. Similarities of sequences (including gaps) were calculated by pairwise comparison using BioEdit 7.1.11 (Hall, 1999).

3. Results

### 3.1. Infection and morphology

The presumed parasite was visible in all eggs and the smallest stages of the parasite appeared to be unicellular spheres with a diameter of approximately 10 μm (Figs. 1–2). These spheres appeared around 15 h post-fertilization (HPF), i.e. when germ ring appeared (Fig. 1A). Larger, presumably multicellular life-cycle stages, up till approximately 100 μm (Fig. 2B) in diameter, were visible from around 30 HPF (Figs. 1 and 2). In some cases, these appeared to have been dividing in a bud-like manner as viewed from depressions in the larger stages with size and shape corresponding to that of the minor stages (Fig. 2A). These two stages were exclusively found in the yolk sac of embryos and newly hatched larvae, but in addition, intermediate sized 'amoeboid' (or amorphous) stages (or cell types) were at some occasions seen in embryos outside the yolk sac (Figs. 3 and 4). No flagella or signs of motility were observed in any cell types.

Degenerating embryos, containing many parasites of different stages were observed (Fig. 1), however specific causes of mortality could not be ascertained as other factors also challenge these pioneering experiments. No flagellated parasite stages were observed and no parasite cells were ever seen outside eel eggs or embryos. It appeared that there was an initial proliferation of parasite cells until the stage when
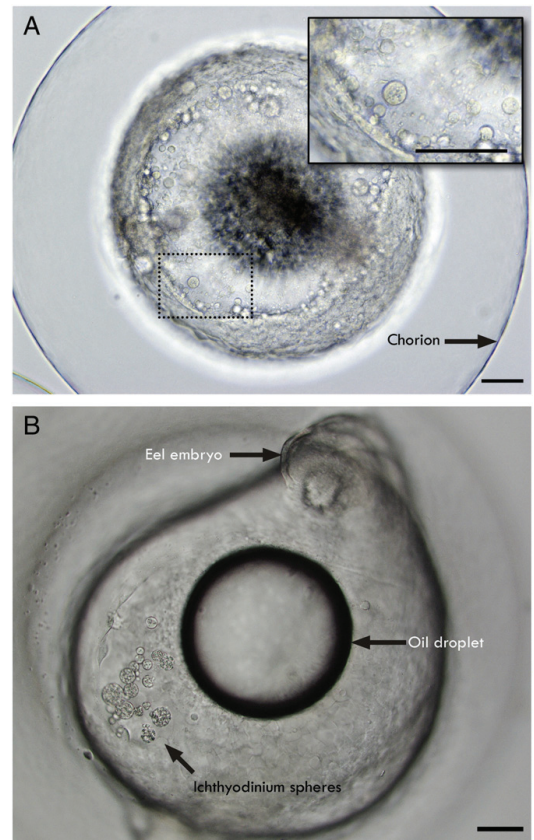
Fig. 1. Infection with *Ichthyodinium*-like spheres in European eel (*Anguilla anguilla*) egg and embryos. A: Fertilized egg 15 HPF showing large variety of spheres. B: Embryo 30 HPF showing spheres positioned on or in yolk of eel embryo. Scale bar = 100 μm.
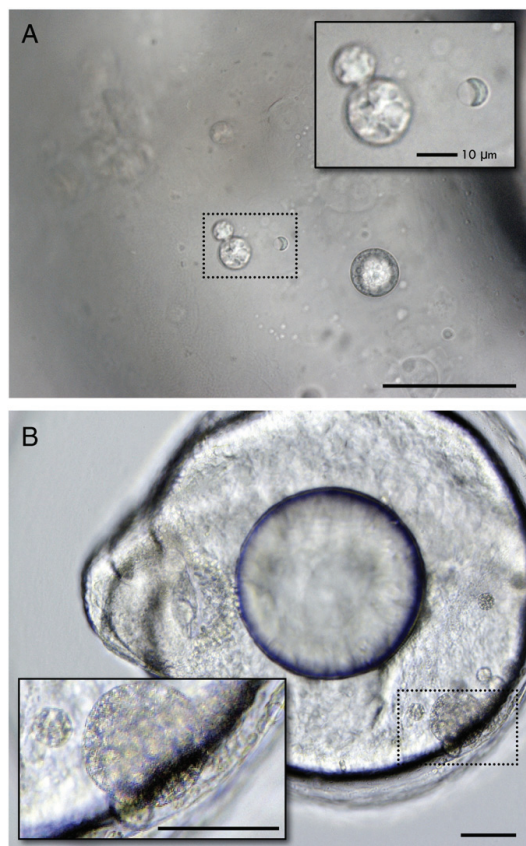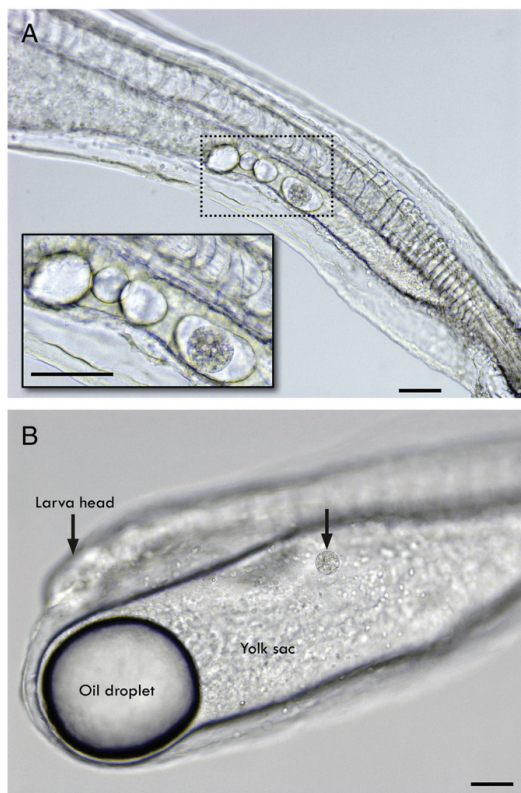
Fig. 2. Egg and embryo of European eel (*Anguilla anguilla*) infected with *Ichthyodinium*-like parasite. A: 400× magnification of yolk surface of embryo showing budding of a small cell from a large multicellular sphere. B: Embryo 25 HPF showing one of the largest spheres found positioned on or in yolk of eel embryo. Scale bar = 100 µm.


Fig. 3. Infection with *Ichthyodinium*-like spheres in hatched larvae of European eel (*Anguilla anguilla*). Although rarely seen, infections were observed in few larvae always associated with yolk sac of these. A: Larvae at day 1 after hatch and B: larvae on day of hatch (0 DPH). Scale bar = 100 µm.

these became noticeable inside the eggs (15 HPF). However, after this point there was no obvious growth in parasite numbers and all infected egg batches resulted in hatching percentages of 1 to 5% of all stripped eggs and longevity of 7 to 9 days post-hatch, which was in the normal range for reproduction experiments during that experimental period. Egg infection was common also in egg batches that resulted in normal hatching rates and initial survival of larvae. The transition from egg to larvae coincided with a clear drop in visual detection of the parasite and observations of infected larvae were rare. However, the filtrates of water from rearing containers obtained after the first 24 h after hatch revealed no detectable DNA from *Ichthyodinium*. Fig. 4A shows an exceptional case of yolk sac infection found only in this particular larva, while Fig. 4B represents typical infections when observed in larvae.

3.2. Sequence data

A 972 bp long partial SSU rDNA sequence was achieved on the basis of several PCRs performed on DNA extract of a single sample (2BFE). This sample contained several infected *A. anguilla* eggs from a batch with some of the highest infection levels observed during the study. Eggs from this batch were observed infected at 15 HPF and all infected embryos died before hatch. The sequence obtained was identical to SSU rDNA of *I. chabelardi* from *S. pilchardus* and *Boops boops* and to

that of a 553 bp long partial sequence of *Ichthyodinium* sp. from *G. morhua*. Similarities to other *Ichthyodinium* sequences, originating from the Southeast Asian hosts *T. albacares*, *Plectropomus leopardus*, and *Thunnus orientalis*, were 98.3%. It would have been desirable also to obtain sequences of more variable genes or regions than the SSU rDNA, such as the ITS region, which is already available for *I. chabelardi* from *S. pilchardus* and *B. boops*. However, attempts were unsuccessful, which may be due to insufficient amounts of *Ichthyodinium* DNA in the samples or, perhaps, a suboptimal match between target DNA and primers.

3.3. Molecular phylogeny

In the phylogenetic analyses (Fig. 5) sequences from *I. chabelardi* from fish of European waters and hatcheries (*S. pilchardus*, *B. boops*, *G. morhua*, and *A. anguilla*) grouped together with sequences from environmental samples from the Cariaco Basin, Caribbean Sea. These sequences formed a clade with *Ichthyodinium* sp. sequences from the Pacific area (waters and hatcheries in Japan, Indonesia and Vietnam) branching out as a subclade.

Thus, two distinct groups of *Ichthyodinium* ribotypes could be identified and these were geographically separated with one group originating from the Atlantic region and the other group from the Pacific region. The similarity between representatives of these two groups (represented by *I. chabelardi* ex *S. pilchardus* versus *Ichthyodinium* sp. ex *P. leopardus*) was
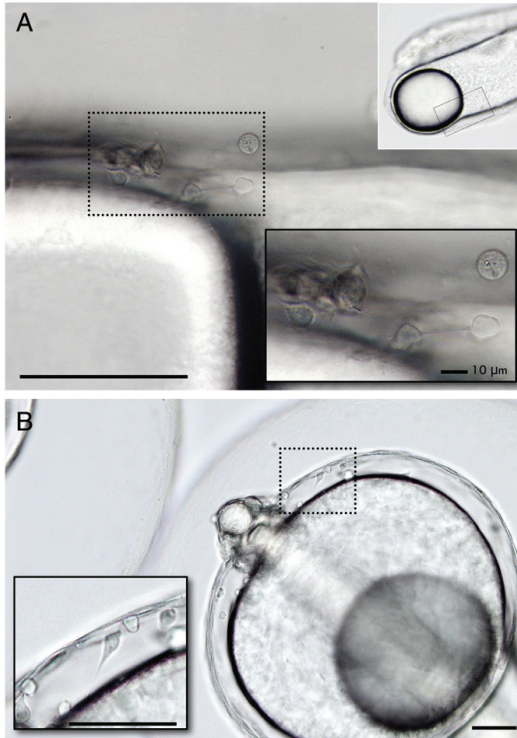
Fig. 4. Different morphotypes of *Ichthyodinium*-like spheres in the larvae of European eel (*Anguilla anguilla*). A: Larvae at day 1 after hatch with thumbnail in upper right, showing position of high magnification image. Possible formation of swarmer cells is seen and in B: embryo ~30 h post-fertilization similar cell morphotypes are visible. Scale bar = 100 μm.

97.9% similar over the nearly entire SSU rRNA gene (1746 bp), corresponding to 37 indels.

The three environmental sequences from the Caribbean Sea (retrieved form GenBank) clustered with the Atlantic *Ichthyodinium* clade with high support (99.6% sequence similarity). Apart from these, the most similar sequences obtained through BLAST search were various environmental sequences that branched out basal to all *Ichthyodinium* sequences (Fig. 5). A single one of these environmental sequences (clone SSRPB64 originating from the Sargasso Sea, Not et al., 2007) branched out basally within the *Ichthyodinium* clade (with 97.3% similarity with *I. chabelardi* ex *S. pilchardus*).

3.4. Infection pathway

The test of infection pathway showed spheres present in eggs fertilized in filtered water and under sterilized conditions with mean prevalence $\pm$ SD of 80 $\pm$ 10.8% compared to standard hatchery produced eggs at 90 $\pm$ 7.1%. No difference was found between prevalence in the two groups (t-test, $t = -1.55$, $P = 0.17$), but a significant higher number of spheres per egg (i.e. infection intensity) was found for the controls 3.5 $\pm$ 0.6 (mean $\pm$ SD) compared to filtered conditions having 4.5 $\pm$ 0.5 (t-test, $t = -2.50$, $P = 0.047$). The number of eggs with more than 10 spheres was equal in both treatments.

4. Discussion

Light microscopy revealed clear morphological resemblances between the presumed parasitic protozoan in *A. anguilla* eggs and the parasitic dinoflagellate *I. chabelardi*: size and shape of the small unicellular spheres and the larger multicellular stages in *A. anguilla* eggs were similar to some of the stages of *I. chabelardi* primordial schizonts reported from *S. pilchardus* and *S. scombrus* (Hollande et al., 1953; Meneses et al., 2003). These morphological similarities were supported by the identity of SSU rRNA genes of the presumed parasite in *A. anguilla* eggs and *I. chabelardi* sequences from the confirmed *Ichthyodinium* hosts *S. pilchardus*, *B. boops*, and *G. morhua* (Gestal et al., 2006; Skovgaard et al., 2009, 2010).

The more advanced life cycle stages such as secondary and tertiary schizonts (Meneses et al., 2003) and swarmer cells (Skovgaard et al.,
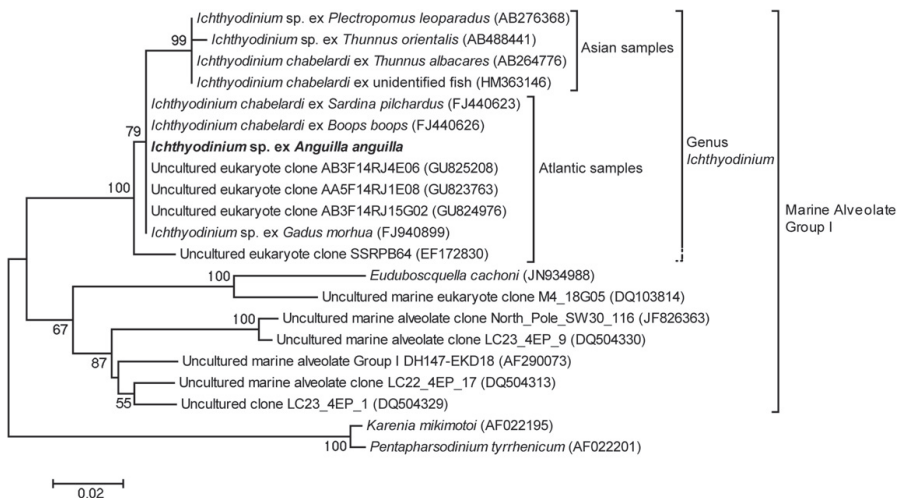
Fig. 5. Phylogenetic analysis based on the Maximum Likelihood method conducted in MEGA5.21. The datasets consisted of a 568-bp alignment comprising 21 SSU rDNA sequences of members of Alveolata with high similarity to *Ichthyodinium*. The tree is rooted with two sequences from the members of Dinokaryota. Numbers at internal branches are bootstrap values (only values above 50 are shown). Numbers in brackets are GenBank accession numbers. Asian samples refer to fish eggs collected in aquaculture and coastal waters in Southeast Asia. Atlantic samples were collected in the Atlantic Ocean and adjacent coastal waters, including European aquaculture.

2009) of *Ichthyodinium* appeared to be lacking in *A. anguilla* eggs and embryos. The *A. anguilla* eggs were incubated in water with a salinity of 36 ppt and temperature of 20 °C. According to previous reports (Mori et al., 2007; Skovgaard et al., 2009), such conditions should be favourable for growth of the parasite. Although a clear drop in detection of parasite spheres was observed once eggs hatched to larvae, no proliferation of swarmer cells was observed or detected in filtrate of hatching water. In full strength tropical seawater, lethal effects of the infection are the results of proliferation of flagellated swarmer cells that rupture the yolk sac, which is described as clearly visible (Hollande and Cachon, 1952, 1953; Meneses et al., 2003; Mori et al., 2007; Stratoudakis et al., 2000; Yuasa et al., 2007). The infection and low amount of spheres in the eel embryos were not comparable to such severe cases and we did not observe changes in sphere morphology into the amorphous shapes shown by Yuasa et al. (2007). The numbers and morphology of the parasite resemble more that shown for cod (Skovgaard et al., 2010) in which mortality and swarmers did not occur. The effect of infection in eel larvae and the influence on survival therefore suggest to be minor, however, a dedicated study of larvae survival versus infection has yet to be conducted. Such study would call for a special setup, since larvae are sensitive to handling, and the minute size of *Ichthyodinium* spheres necessitates microscopic examination to reveal infections, hence compromising larvae survival.

The phylogenetic analysis in this study confirmed a previous observation that at least two distinct and geographically separated ribotypes of *Ichthyodinium* exist (Skovgaard et al., 2009). Whether these ribotypes represent separate species cannot presently be determined, given that no morphological differences have been recognized. No host-specificity has been detected in *Ichthyodinium*; genetically identical parasites have been found in the eggs of different fish species (Skovgaard et al., 2009). Very little genetic variation is present between parasites from different host species in the same geographic area (Fig. 5). In addition, parasites from different parts of the world (i.e. Atlantic region versus Pacific region) are strikingly similar: SSU rRNA gene sequences from the Atlantic *Ichthyodinium* and the (Pacific) parasite found in *T. albacares* are 98% similar. Interestingly, three Caribbean Sea environmental sequences (Edgcomb et al., 2011) branched out as members of the Atlantic *Ichthyodinium*-clade in the phylogenetic analysis (Fig. 5). The exact match of these sequences leaves no doubt that they must be of *Ichthyodinium* origin, providing additional evidence of a worldwide occurrence of this parasite genus.

Irrespective of the number of *Ichthyodinium* species existing, this parasite is bound to have large ecological effects through its exploitation of pelagic fish eggs as food source. *Ichthyodinium* appears to be abundant in coastal waters all over the world (Meneses et al., 2003; Shadrin et al., 2010; Stratoudakis et al., 2000) and it infects eggs of a large number of marine fish species (Table 1). Thus, even though not all pelagic fish eggs are necessarily suitable hosts for the parasite (Skovgaard et al., 2010), those that are may suffer high egg mortality rates due to infections with *Ichthyodinium* (Meneses et al., 2003).

Infection with *Ichthyodinium* may also pose challenges for the production of marine fish larvae in hatcheries. However, infection has been found controllable through good husbandry, avoiding horizontal infection from the environment to newly spawned eggs (Mori et al., 2007; Yuasa et al., 2007). Mori et al. (2007) were able to avoid disease outbreak in *P. leopardus* eggs by rearing broodstock and incubating fertilized eggs in oxidant-treated seawater. Likewise, Yuasa et al. (2007) did not observe an infection in *T. albacares* eggs, when spawned eggs were immediately transferred to sterilized seawater. In the present study, however, results were contradictory in the way that no reduction in prevalence was observed, if eggs were transferred to the sterile filtered seawater. The infection pathway in our tests thus appeared to be vertical. However, several studies have shown evidence for a horizontal transfer of this parasite (Mori et al., 2007). This questions why we observe such high prevalence following fertilization in sterile filtered seawater. The catadromous life-strategy of eels implies that glass eels

returning from the Sargasso Sea live in freshwater for 12 to more than 20 years before the onset of their spawning migration to the seawater (Tesch, 2003). Broodstock females used in our study were caught at the onset of autumn migration, while still in a freshwater habitat and male counterparts originated from domestic stocks kept in freshwater tanks for 2–3 years prior to induction of maturation. This implies that the marine *Ichthyodinium* schizonts, if present inside the broodstock gonads should have survived for several years, while the broodstock lived in freshwater. Alternatively, the parasite schizonts might have infected broodstock eels while kept in seawater recirculation system during experimentation. In Anguillids, the ovaries open into the body cavity, i.e. the gymnovarian type, and the eggs are conveyed through an open funnel to the oviduct. The gymnovarian condition may provide parasites an easier access to ovaries and eggs, than in most teleosts, having cystovarian condition (Barton, 2007). Sequencing ovarian tissue to identify *Ichthyodinium* DNA would prove useful, answering such questions, however such analyses are challenged by the ratio of maternal to parasite DNA. Although *Ichthyodinium* infection did not appear to induce mortality in eel larvae in the present study, studies to clarify the infection pathway are important to ensure the future healthy production of offspring from captive breed European eels.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Asturiano, J.F., Pérez, L., Garzón, D.L., Marco-Jiménez, F., Peñaranda, D.S., Vicente, J.S., Jover, M., 2004. Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiol. Biochem. 30, 283–293.

Barton, M., 2007. Bond's Biology of Fishes, 3rd edition. Thomson Brook/Cole, Belmont, California (912 pp.).

Bezdeznhykh, V., Prokhorcik, G., Petrikov, A., Petukov, V., Plyuta, M., 1983. Obtaining the larvae of European eel *Anguilla anguilla* L. (Pisces, Anguillidae), under experimental conditions. Dokl. Akad. Nauk SSSR 268, 1264–1266 (in Russian).

Bloch, B., Pedersen, B.H., Jensen, P.V., 1997. An enigmatic and possibly parasitic organisms in the tissue of embryonated eggs of Baltic cod *Gadus morhua*. Dis. Aquat. Org. 30, 121–137.

Borges, R., Ré, P., Azevedo, C., 1996. *Ichthyodinium chabelardi* (Hollande e Cachon 1952), dinoflagelado parasita dos ovos de sardinha. Ciência biológica. Ecology and Systematics, 16.

Bower, S.M., Carnegie, R.B., Goh, B., Jones, S.R.M., Lowe, G.J., Mak, M.W.S., 2004. Preferential PCR amplification of parasitic protistan small subunit rDNA from metazoan tissues. J. Eukaryot. Microbiol. 51, 325–332.

Bruun, A., Hemmingsen, A., Møller-Christensen, E., 1949. Attempts to induce experimentally maturation of the gonads of the European eel, *Anguilla anguilla* L. Acta Endocrinol. 2, 212–226.

Dufour, S., Burzawa-Gerard, E., Belle, N., Sbaihi, M., Vidal, B., 2003. Reproductive endocrinology of the European Eel, *Anguilla anguilla*. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer, Japan, pp. 373–383.

Edgcomb, V., Orsi, W., Bunge, J., Jeon, S., Christen, R., Leslin, C., Holder, M., Taylor, G.T., Suarez, P., Varela, R., Epstein, S., 2011. Protistan microbial observatory in the Cariaco Basin, Caribbean. I. Pyrosequencing vs Sanger insights into species richness. ISME J. 5, 1344–1356.

Ekelund, F., Daugbjerg, N., Fredslund, L., 2004. Phylogeny of *Heteromita, Cercomonas* and *Thaumatomonas* based on SSU rDNA sequences, including the description of *Neocercomonas jutlandica* sp. nov., gen. nov. Eur. J. Protistol. 40, 119–135.

Gestal, C., Novoa, B., Posada, D., Figueras, A., Azevedo, C., 2006. *Perkinsoide chabelardi* n. gen., a protozoan parasite with an intermediate evolutionary position: possible cause of the decrease of sardine fisheries? Environ. Microbiol. 8, 1105–1114.

Guillou, L., Viprey, M., Chambouvet, A., Welsh, R.M., Kirkham, A.R., Massana, R., Scanlan, D.J., Worden, A.Z., 2008. Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). Environ. Microbiol. 10, 3349–3365.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hollande, A., Cachon, J., 1952. Un parasite des oeufs de sardine: l'*Ichthyodinium chabelardi*, nov. gen., nov. sp. (Péridinien parasite). C.R. Hebd. Seances Acad. Sci. 235, 976–977.

Hollande, A., Cachon, J., 1953. Morphologie et évolution d'un Péridinien parasite des oeufs de sardine (*Ichthyodinium chabelardi*). Bulletin des Travaux publiés par la Station d'Aquiculture et de Pêche de Castiglione, 4, pp. 321–331.

Hollande, A., Enjumet, M., Manciet, J., 1953. Les péridiniens parasites des Phæodariés et le problème de la sporogénèse chez ces Radiolaires. C. R. Acad. Sci. 236, 1607–1609.

Ishimaru, K., Iida, N., Okada, T., Miyashita, S., 2012. *Ichthyodinium* infection in the embryos and yolk sac larvae of Pacific bluefin tuna *Thunnus orientalis*. Fish Pathol. 47, 143–146.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

López-García, P., Rodríguez-Valera, F., Pedrós-Alió, C., Moreira, D., 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409, 603–607.

Marinaro, J.Y., 1971. Contribution à l'étude des oeufs et larves pélagiques de poissons Mediterraneans. V. Oeufs pélagiques de la Baie d'Alger. Pelagos 3, 1–118.

Meneses, I., Ré, P., 1992. Infection of sardine eggs by a parasitic dinoflagellate (*Ichthyodinium chabelardi* Hollande and Cachon) off the Portuguese coast. Bol. Inst. Esp. Oceanogr. 8, 201.

Meneses, I., Vendrell, C., Stratoudakis, Y., 2003. Mackerel (*Scomber scombrus*) eggs parasitized by *Ichthyodinium chabelardi* in the north-east Atlantic: an overlooked source of mortality. J. Plankton Res. 25, 1177–1181.

Mori, K.I., Yamamoto, K., Teruya, K., Shiozawa, S., Yoseda, K., Sugaya, T., Shirakashi, S., Itoh, N., Ogawa, K., 2007. Endoparasitic dinoflagellate of the genus *Ichthyodinium* infecting fertilized eggs and hatched larvae observed in the seed production of leopard coral grouper *Plectropomus leopardus*. Fish Pathol. 42, 49–57.

Not, F., Gausling, R., Azam, F., Heidelberg, J.F., Worden, A.Z., 2007. Vertical distribution of picoeukaryotic diversity in the Sargasso Sea. Environ. Microbiol. 9, 1233–1252.

Pedersen, B.H., 1993. Embryos and yolk-sac larvae of turbot *Scophthalmus maximus* are infested with an endoparasite from the gastrula stage onwards. Dis. Aquat. Org. 17, 57–59.

Pedersen, B.H., 2004. Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. Aquaculture 237, 461–473.

Pedersen, B.H., Køie, M., 1994. A protistan endoparasite in embryos and yolk-sac larvae of cod *Gadus morhua* and turbot *Scophthalmus maximus*. Dis. Aquat. Org. 19, 39–46.

Pedersen, B.H., Buchmann, K., Køie, M., 1993. Baltic larval cod *Gadus morhua* are infested with a protistan endoparasite in the yolk-sac. Dis. Aquat. Org. 16, 29–33.

Shadrin, A.M., Pavlov, D.S., Novikov, G.G., 2002. The phenomenon of endoparasitism at early stages of fish development. Dokl. Biol. Sci. 385, 361–363.

Shadrin, A.M., Pavlov, D.S., Kholodova, M.V., 2010. Long-term dynamics of infection of fish eggs and larvae with the endoparasite *Ichthyodinium* sp. (Dinoflagellata) in Nha Trang Bay, Vietnam. Fish Pathol. 45, 103–108.

Silva, A., Miranda, A., 1992. Laboratory rearing of sardine larvae *Sardina pilchardus* (Walb.) and early effects of starvation: a preliminary experiment. Bol. Inst. Esp. Oceanogr. 8, 163–174.

Skovgaard, A., Meneses, I., Angélico, M.M., 2009. Identifying the lethal fish egg parasite *Ichthyodinium chabelardi* as a member of Marine Alveolate Group I. Environ. Microbiol. 11, 2030–2041.

Skovgaard, A., Meyer, S., Overton, J.L., Støttrup, J., Buchmann, K., 2010. Ribosomal RNA gene sequences confirm that protistan endoparasite of larval cod *Gadus morhua* is *Ichthyodinium* sp. Dis. Aquat. Org. 88, 161–167.

Skovgaard, A., Bahlool, Q.Z.M., Munk, P., Berge, T., Buchmann, K., 2011. Infection of North Sea cod, *Gadus morhua* L., larvae with the parasitic nematode *Hysterothylacium aduncum* Rudolphi. J. Plankton Res. 33, 1311–1316.

Stratoudakis, Y., Barbosa, A., Meneses, I., 2000. Infection of sardine eggs by the protistan endoparasite *Ichthyodinium chabelardi* off Portugal. J. Fish Biol. 57, 476–482.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Tesch, F.W., 2003. The Eel, Fifth edition. Blackwell Science, Oxford 408.

Tomkiewicz, J., 2012. Reproduction of European Eel in Aquaculture (REEL): consolidation and new production methods. DTU Aqua Report No 249–2012. Technical University of Denmark, National Institute of Aquatic Resources.

Yuasa, K., Kamaishi, T., Mori, K.I., Hutapea, J.H., Permana, G.N., Nakazawa, A., 2007. Infection by a protozoan endoparasite of the genus *Ichthyodinium* in embryos and yolk-sac larvae of yellowfin tuna *Thunnus albacares*. Fish Pathol. 42, 59–66.

DTU Aqua – National Institute of Aquatic Resources – is an institute at the Technical University of Denmark. DTU Aqua's mission is to conduct research, provide advice, educate at university level and contribute to innovation in sustainable exploitation and management of aquatic resources. We investigate the biology and population ecology of aquatic organism, aquatic physics and chemical processes, ecosystem structure and dynamics, taking account of all relevant natural and anthropogenic drivers.