Aquaculture 426–427 (2014) 1–8

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

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

Article history:
Received 17 September 2013
Received in revised form 15 January 2014
Accepted 17 January 2014
Available online 25 January 2014

Keywords:
Bacteria
Biofilm
Chorion
Larval rearing
Egg incubation leptocephalus

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.

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 $\times 10^5$ 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).

0044-8486/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaculture.2014.01.011

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× magnification (40× 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

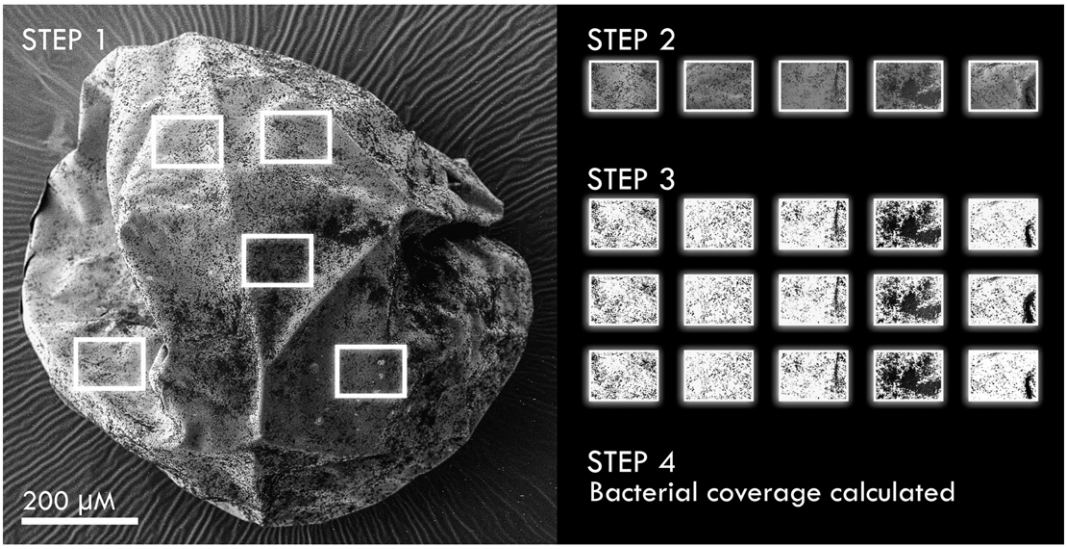

Fig. 1. Procedure to obtain average percent of microbial surface coverage on European eel, *Anguilla anguilla*, eggs based on scanning electron microscopy (SEM) images. Step 1: choose photo frame at random sites within five regions on the egg hemisphere; central region and in each quadrant. Step 2: capture 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 ± 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

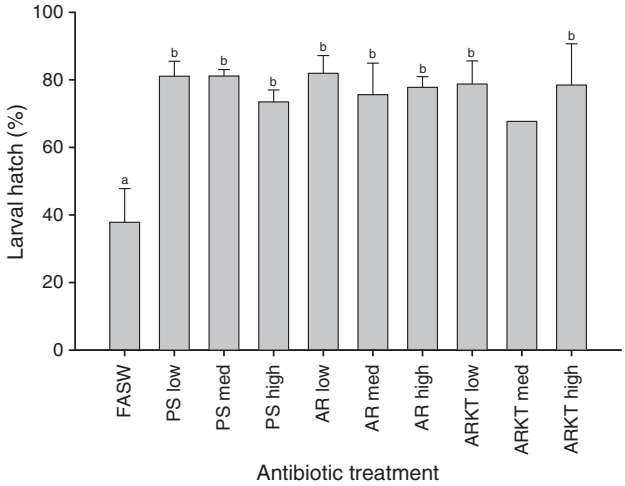

Fig. 2. Larval hatch of European eel, *Anguilla anguilla* (in %) treated with the different combinations of antibiotics indicated in Table 1. Bars show means ± SD. Bars with different superscripts are significantly different (α = 0.05; one-way ANOVA analysis). Standard deviation (SD) is not shown for $ARKT^{med}$ due to loss of one of three replicates.

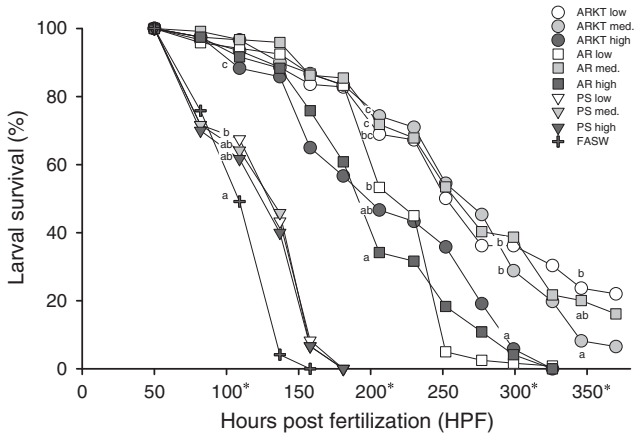

Fig. 3. Mean cumulative % survival of European eel, *Anguilla anguilla*, larvae hatched (48 HPF) from eggs that were incubated in FASW and different combinations of antibiotics (see Table 1). Asterisks denote the times at which treatments were tested for significant differences (Tukey's test). Different 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.

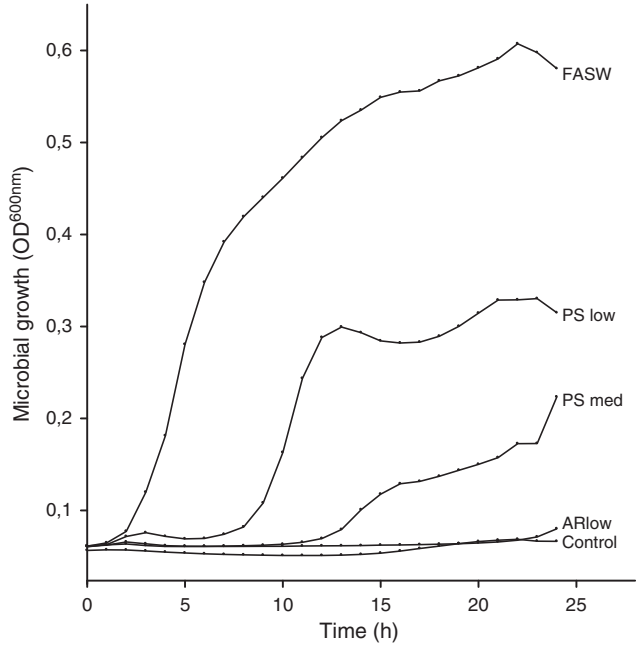

Fig. 4. Microbial growth of European eel, *Anguilla anguilla*, egg-associated bacteria (OD_{600}) subjected to different antibiotic treatments (see Table 1). Only treatments showing growth are presented (FASW, PS^{low}, PS^{med} and AR^{low}).

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 ± 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 of 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

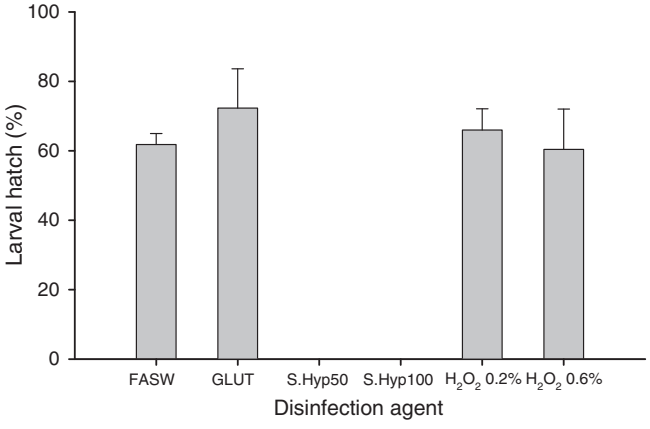

Fig. 5. Larval hatch of European eel, *Anguilla anguilla* (in %) shown as means ± SD after different disinfection treatments (see Table 1) followed by incubation in FASW with the antibiotic mixture AR^{low}. Treatments are not significantly different (α = 0.05; one-way ANOVA analysis).

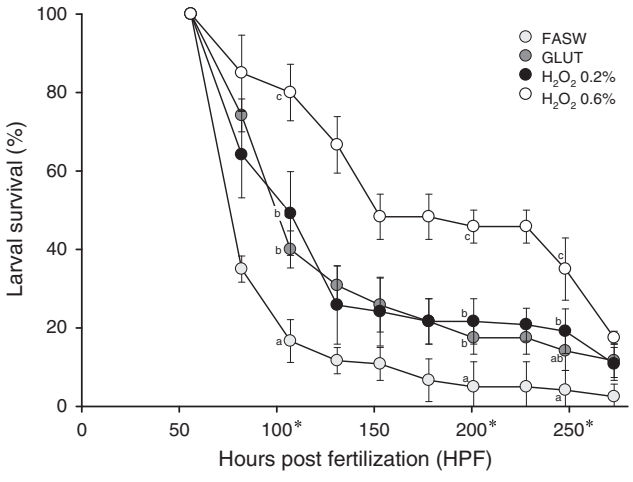

Fig. 6. Survival of European eel, *Anguilla anguilla*, larvae (in %) hatched from eggs treated with different types of surface disinfection (see Table 1) and incubated in FASW with the antibiotic mixture AR^{low}. Egg hatch occurred at ~48 HPF. Data are expressed as means ± SD. Asterisks denote the times at which treatments were tested for significant differences (Tukey's test). Different superscripts indicate significant differences between treatments (α = 0.05).

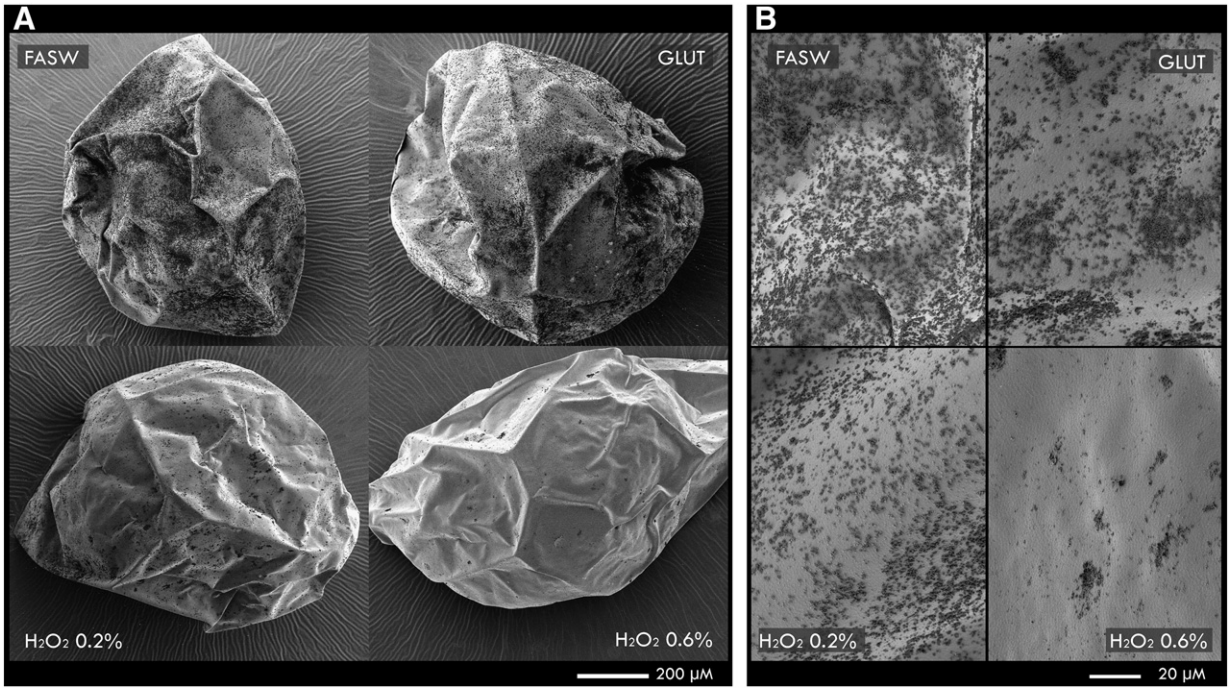

Fig. 7. (A) Scanning electron microscopy (SEM) images representing average microbial surface coverage of eggs from European eel, *Anguilla anguilla* after disinfection. Eggs were fixed immediately after disinfection (treatments described in Table 1). Sodium hypochlorite treated eggs are not included. (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.

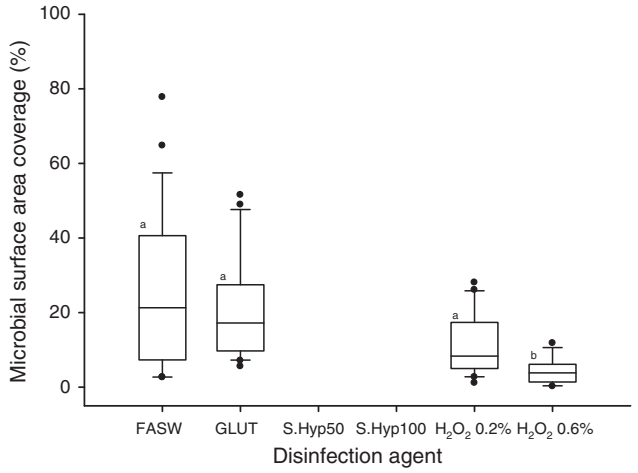

Fig. 8. Box-plot showing microbial surface area coverage for European eel, *Anguilla anguilla*, eggs ~2 h after disinfection treatment (see Table 1). Error bars indicate 95% confidence limits and a Dunn's multiple comparison test for treatment effect was run and superscripts show significant differences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

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

This work was funded by the European Commission's 7th Framework Programme through the collaborative research project "PRO-EEL: Reproduction of European Eel — Towards a Self-sustained Aquaculture" (Grant Agreement No.: 245527). Peter De Schryver was supported as a post-

doctoral research fellow by the Fund for Scientific Research (FWO) in Flanders (Belgium). The authors thank Elin Kjørsvik and Bart van Delsen for contributions to preliminary tests prior to this study, and thank Maria Krüger-Johnsen and Christian Graver for assistance in the experiments and Ian A.E. Butts for assistance in statistical analyses.

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.