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

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Abstract :

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

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

Introduction

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

The duration of egg receptivity of marine and freshwater fishes is species specific and is closely related to different water flow regimes on the spawning ground (Mann 1996; Merz, Setka, Pasternack & Weathon 2004; Probst, Stoll, Hofmann, Fisher & Eckmann 2009). Sperm longevity, the velocity of sperm, and the duration of egg receptivity impact the success of a fertilization event (see Trippel & Morgan 1994; Butts, Trippel & Litvak 2009 among others). For instance, if sperm are viable for longer periods of time in the activation medium, then the potential of contacting and fertilizing an egg increases (Butts *et al.* 2009). On the other hand, when longevity of sperm is very short, than egg receptivity may be necessary to increase fertility, since longer periods of egg receptivity are predicted to increase the probability of a successful fertilization event (Trippel 2003). Therefore, duration of egg receptivity provides

78 valuable insights on reproductive behavior for any fish species (Butts, Roustaian & Litvak
79 2012).

80 The activation process for fish eggs represents several complex changes including the
81 release of the developmental block of meiosis at metaphase (II), consecutive breakdown of
82 cortical granules, and formation of the perivitelline space (Pavlov, Emel'yanova & Novikov
83 2009). Egg activation is induced by the fusion of sperm in the majority of marine fishes, while
84 in freshwater fishes and salmonids it is induced by contact with water or mechanical
85 stimulation (Dettlaff 1962; Ginzburg 1972; Pavlov et al. 2009). In general, the influx of
86 intracellular free Ca^{2+} in eggs mediates the cortical alveoli to initiate exocytosis (Finn 2007;
87 Vasilev, Chun, Gragnaniello, Garante & Santella 2012). Following this Ca^{2+} wave, cortical
88 glycoproteins are then broken into smaller units by proteolysis and form the osmotic gradient
89 that facilitates uptake of ambient seawater across the egg membrane (Lønning & Davenport
90 1980; Govoni & Forward 2008). The perivitelline space between the oocyte plasma
91 membrane and egg envelopes fills with perivitelline fluid, which is formed by imbibed water
92 and the substances released from the cortical granules (Siddique *et al.* 2014). Until now, there
93 is a knowledge gap on activation mechanisms and formation of perivitelline space of sea bass
94 eggs.

95 The European sea bass *Dicentrarchus labrax* (L.) is a leading species for aquaculture
96 in the Mediterranean due to its emerging economic importance in the Mediterranean and
97 North East Atlantic regions (Vandeputte, Dupont-Nivet, Haffray, Chavanne, Cenadelli, Parati,
98 Vidal, Vergnet & Chatain 2009; Colléter, Penman, Lallement, Fauvel, Hanebrekke, Osvik,
99 Eilertsen, D'Cotta, Chatain & Peruzzi 2014). Adults usually exhibit demersal behavior and
100 inhabit coastal waters down to 100 m depth but are more common in the littoral zone on
101 various kinds of bottoms in estuaries, lagoons and occasionally in rivers. In the
102 Mediterranean, first sexual maturity occurs at 2 to 4 years and fish spawn once a year in

groups (Froese & Pauly 2015). The egg envelope of sea bass consists of three distinct layers with a funnel shaped micropylar canal (Fausto, Carcupino, Scapigliati, Taddei & Mazzini 1994; Scapigliati *et al.* 1994). Sea bass eggs are pelagic, small in size (1.1 to 1.5 mm diameter), freely floating in seawater, and fertilized externally (Froese & Pauly 2015). In teleost fishes, eggs can be activated by contact with water or by the penetration of sperm (Pavlov *et al.* 2009). In case of sea bass, eggs can be activated in seawater and after a few seconds of activation, a thin perivitelline space is formed beneath the egg membrane. Currently, there is lack of knowledge about how long sea bass eggs can remain viable after being activated in seawater within the natural environment.

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

Materials and methods

Broodstock husbandry and gamete collection

Sea bass broodstock (aged 4 to 6 years and weighted 2 to 5 kg) were kept at the Ifremer Experimental Aquaculture Station (Palavas-les-Flots, France). Males and females were kept separately in recirculation systems (8 m^3 volume). Mature males were recognized

by gentle abdominal pressure and females were selected by assessing the maturation stage with ovarian biopsies. Maturation stages were determined based on oocyte diameter and migration of the germinal vesicle using a light microscope (4× magnification). Females at "stage B" of development (when the germinal vesicle started its migration to the animal pole) were selected for hormonal induction (Fauvel & Suquet 1988). Each female received a single dose (10 $\mu g.kg^{-1}$ body weight mixed with physiological solution) of Luteinizing Hormone Releasing Hormone analogue (LHRHa, Sigma, France) in order to induce final maturation and ovulation (Fauvel & Suquet 1988). The treated females were isolated in individual tanks (1.5 m^3, 17 $L.h^{-1}$ water renewal, and low air flow) at 13°C water temperature. Ovulated oocytes were collected from females after 72 h of hormonal stimulation by abdominal pressure. Sperm were collected from the male's genital papilla by applying pressure to the abdominal region. Samples were drawn into 5 ml syringes. Before collection, the genital papilla was wiped dry and extra care was taken to avoid contamination with urine. Sperm were then held at 4°C until use. The males and females both were ~~fished~~ treated without anesthetics and immediately wrapped in a dark wet towel to limit stress and fish movement during sperm and oocyte collection.

Quantification of sperm density and motility

Sperm concentration was measured before pooling using a Thoma hemocytometer (depth 0.1 mm × length 0.05 mm) after dilution of sperm by 1:2000 in distilled water. After allowing 10 min for sedimentation of the sperm, three frames of 24 squares were recorded randomly through a video camera (Axiolab, Zeiss + SSC-D50AP video camera, Sony). The cells were then automatically counted using image analysis software (Image J, NIH, USA).

Sperm motility was measured according to the procedure by Fauvel, Boryshpolets, Cosson, Leedy, Labbe, Haffray & Suquet (2012).

We determined the effects of pre-incubation time for fresh and overripe sea bass eggs in seawater as well as the duration of egg receptivity. For this experiment we used proportionally pooled sperm from 4 males. The mean (±SD) sperm density of pooled sperm was $5.3 \pm 0.03 \times 10^9$ sperm mL^{-1}, while sperm motility was 60 ± 8.16%, and mean sperm longevity was 42 ± 6 s.

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

Pooled eggs from 4 females were used for this experiment. The difference between egg collection from the first female to fourth female was 20 min. Freshly stripped eggs (10 mL) were first placed in 18 × 250 mL plastic dishes. Then 5 mL of seawater (pH 8.22, salinity 37.4 psu) was added to each dish. The eggs were pre-incubated in seawater for 0 min (no incubation to serve as the experimental control), 0.5 min, 1 min, 3 min, 10 min, and 30 min before sperm was added for fertilization. There were 3 plastic dishes for each allotted pre-incubation period for replication. Immediately following pre-incubation, 5 mL of sperm were added to the eggs using a micropipette and the plastic dishes were ~~shaken~~ rotated by hand for 30 s to facilitate fertilization. For the experimental control, with no pre-incubation, the seawater and sperm were added simultaneously.

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

177 At 3 h post-fertilization, cleavage was observed. After removing water from the plastic
178 dishes, eggs were mixed properly with a plastic spoon and 300 eggs were randomly chosen
179 for observation. Only eggs that had developed to 2-4 cells after 3 h incubation were used for
180 analysis. Additionally, we took pictures for the unfertilized eggs under light microscope at 0,
181 60, 100, 220, 300, 450 and 500 s post activation by water to observe the swelling process and
182 formation of perivitelline space.
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185 **Statistical analyses**
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187 All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute
188 Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test) and
189 homogeneity of variance (plot of residuals vs. predicted values). If data violated ANOVA
190 assumptions fertilization success was arcsin square-root transformed. Alpha was set at 0.05
191 for main effects and interactions. A-posteriori analyses were performed using Tukey's
192 multiple comparisons procedure.
193 Fertilization success was analyzed using a two-way repeated measures ANOVA
194 model containing the pre-incubation time (0 min, 0.5 min, 1 min, 3 min, 10 min, and 30 min;
195 fixed repeated factor) and egg status (fresh and overripe; fixed factor) main effects as well as
196 the pre-incubation time × egg status interaction term. When a significant pre-incubation time
197 × egg status interaction was detected the saturated model was decomposed into a series of
198 lower-order statistical models following Keppel (1991). Here, the decomposed ANOVA
199 models were run to (i) determine the effect of pre-incubation time for each egg status category
200 using a series of one-way repeated measures ANOVA models, and (ii) determine the effect of
201 egg status category for each pre-incubation time using a series of t-tests. These reduced

models involved only pre-planned comparisons, so alpha-level corrections for *a posteriori* comparisons were not necessary.

Results

The saturated two-way repeated measures ANOVA model revealed a significant pre-incubation time × egg status interaction term ($P < 0.05$); therefore, the model was decomposed into a series of lower-order statistical models. When the saturated model was decomposed to determine the effect of pre-incubation time for each egg status category a significant pre-incubation time effect was detected for both the fresh ($P < 0.01$; Fig. 1A) and overripe eggs ($P < 0.01$; Fig. 1B), such that egg fertilization success significantly declined for both these treatments after 3 min of pre-incubation. Together, this means that sea bass eggs have the capability to be fertilized within 3 min post-activation. After that window of receptivity the eggs lose their ability to be fertilized. Moreover, when the saturated model was decomposed to determine the effect of egg status for each pre-incubation time a significant egg status effect was detected at the 0.5 min ($P < 0.01$; Fig. 1D), 1 min ($P < 0.05$; Fig. 1E), 3 min ($P < 0.05$; Fig. 1F), and 30 min pre-incubation times ($P < 0.05$; Fig. 1H); here, the fresh eggs had a higher fertilization success than overripe eggs. This means that fertilization ability is decreased in overripe eggs; therefore, short term storage of sea bass eggs is not feasible due to their fast over ripening process. On the contrary, no significant effect was detected between the fresh and overripe eggs at the 0 min ($P > 0.05$; Fig. 1C) and 10 min pre-incubation times ($P > 0.05$; Fig 1G).

Sea bass eggs showed a very rapid swelling process after releasing into seawater (Fig 2A-B). During activation of eggs in seawater, a small perivitelline space was observed in several eggs across the females. After 60 s of activation, the perivitelline space became

clearly visible (Fig 2C) and there were no substantial changes in perivitelline space after 100 s (Fig 2D) and 220 s (Fig 2E) of activation. Then, the perivitelline space became large after 300 s (Fig 2F) to 450 and 500 s (Fig 2G & 2H) of activation.

Discussion

Egg activation is a key process in early embryonic development of fish, but not fully understood (Webb & Miller 2013). Fish eggs are activated upon contact with water and this activation mechanism is initiated by the release of intracellular stored Ca^{2+} in the egg cytosol (Coward, Bromage, Hibbitt & Parrington 2002; Finn 2007), which resulted in formation of the fertilization membrane (Minin & Ozerova 2008). As seen in our study, sea bass eggs swell very rapidly (within 10-20 s) upon activation by contact with seawater. However, when in contact with seawater the egg only takes a few seconds to achieve flexible size and structure, and to acquire fertilization ability. In this study, mean fertilization rate of fresh eggs slightly increased for 30 s and 1 min of pre-incubation in seawater, but it was not statistically significant. In our decomposed statistical models, a significant pre-incubation time effect was detected for both the fresh and overripe eggs, where a significant decline of egg fertilization rate was observed for both these treatments after 3 min of pre-incubation. This means that sea bass eggs have the capability to be fertilized within 3 min after activation in seawater; after that period the eggs lose their fertilization ability. Moreover, in the decomposed model, fertilization success of the fresh eggs was significantly higher than overripe eggs at the 0.5 min, 1 min, 3 min, and 30 min pre-incubation times. Thus, our results reveal that the fertilization ability of overripe eggs are less than the fresh eggs, but they are still capable of being fertilized for up to 3 min after being released into seawater.

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

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

sperm entry site. This is the mechanism of polyspermy block for acipenserids (Linhart & kudo 1997; Siddique *et al.* 2014).

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

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

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446 **Figure legend**

447

448 Figure 1. Effects of pre-incubation time (0 min, 0.5 min, 1 min, 3 min, 10 min and 30 min)
449 on fertilization rate of (A) fresh eggs and (B) overripe eggs of sea bass *Dicentrarchus labrax*;
450 and effects of egg status on fertilization rate for (C) 0 min, (D) 0.5 min, (E) 1 min, (F) 3 min,
451 (G) 10 min and (H) 30 min pre-incubation time.

452

453 Figure 2. Micrograph of sea bass *Dicentrarchus labrax* eggs; ovulated, non-activated eggs
454 (A); unfertilized eggs 20 s after activation in seawater (B); unfertilized eggs developing
455 perivitelline space at 60 s (C), 100 s (D), 220 s (E), 300 s (F), 450 s (G), and 500 s (H) after
456 activation in seawater. PS - perivitelline space, LD – lipid droplet, EM – egg membrane.
457 Micrographs were taken at 4× magnification.

Table 1: Duration of egg receptivity and spawning habitat of marine and freshwater fishes.

Species	Duration of egg receptivity	Natural spawning habitat [a]	Reference
Silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844)	30-40 s	Rivers and tributaries	Mikodina & Makeyeva (1980)
Sockeye salmon *Oncorhynchus nerka* (Walbaum, 1792)	40 s	Stream	Hoysak & Liley (2001)
Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)	40 s	Lake and streams	Liley *et al.* (2002)
Crucian carp *Carassius carassius* (Linnaeus, 1758)	1 min	Shallow pond, lake and rivers	Żarski *et al.* (2014)
Goldfish *Carassius auratus* (Linnaeus, 1758)	<1 min	Shallow water, river, and lakes	Hamano (1951)
European perch *Perca fluviatilis* Linnaeus, 1758	2.5 min	Lake and rivers	Żarski *et al.* (2012)
Sea bass *Dicentrarchus labrax* (Linnaeus, 1758)	3 min	Sea	Present study
Vendace *Coregonus albula* (Linnaeus, 1758)	4 min	Lakes and shallow waters	Lindroth (1947)
Japanese rice fish *Oryzias latipes* (Temminck & Schlegel, 1846)	4 min	Pond, marsh, paddy field, and small streams	Yamamoto (1944)
Pond loach *Misgurnus anguillicaudatus* (Cantor, 1842)	5 min	Stream and pond	Gamo, Yamauchi & Suzuki (1960)
European weatherfish *Misgurnus fossilis* (Linnaeus, 1758)	10 min	Open water, lake, and streams	Minin & Ozerova (2008)

Mummichog *Fundulus heteroclitus* (Linnaeus, 1766)	10-30 min	Salt marsh and tidal creeks	Kagan (1935)
Chum salmon *Oncorhynchus keta* (Walbaaum, 1792)	15-30 min	River	Yamamoto (1951)
Winter flounder *Pseudopleuronectes americanus* (Walbaum, 1792)	32 min	Sea	Butts *et al.* (2012)
Atlantic cod *Gadus morhua* Linnaeus, 1758	2 h	Offshore water	Davenport *et al.* (1981)
Pontic shad *Alosa immaculate* Bennett, 1835	>2 h	Large river	Kryzhanovskii (1956)
Atlantic herring *Cluoea harengus* Linnaeus, 1758	4 h	Shallow coastal areas or offshore banks	Kryzhanovskii (1956)
Russian sturgeon *Acipenser gueldenstaedtii* Brandt and Ratzeburg, 1833	6 h	River	Ginzburg (1972)

Note: [a] Data are retrieved from FishBase (Froese & Pauly 2015)

Fig 1

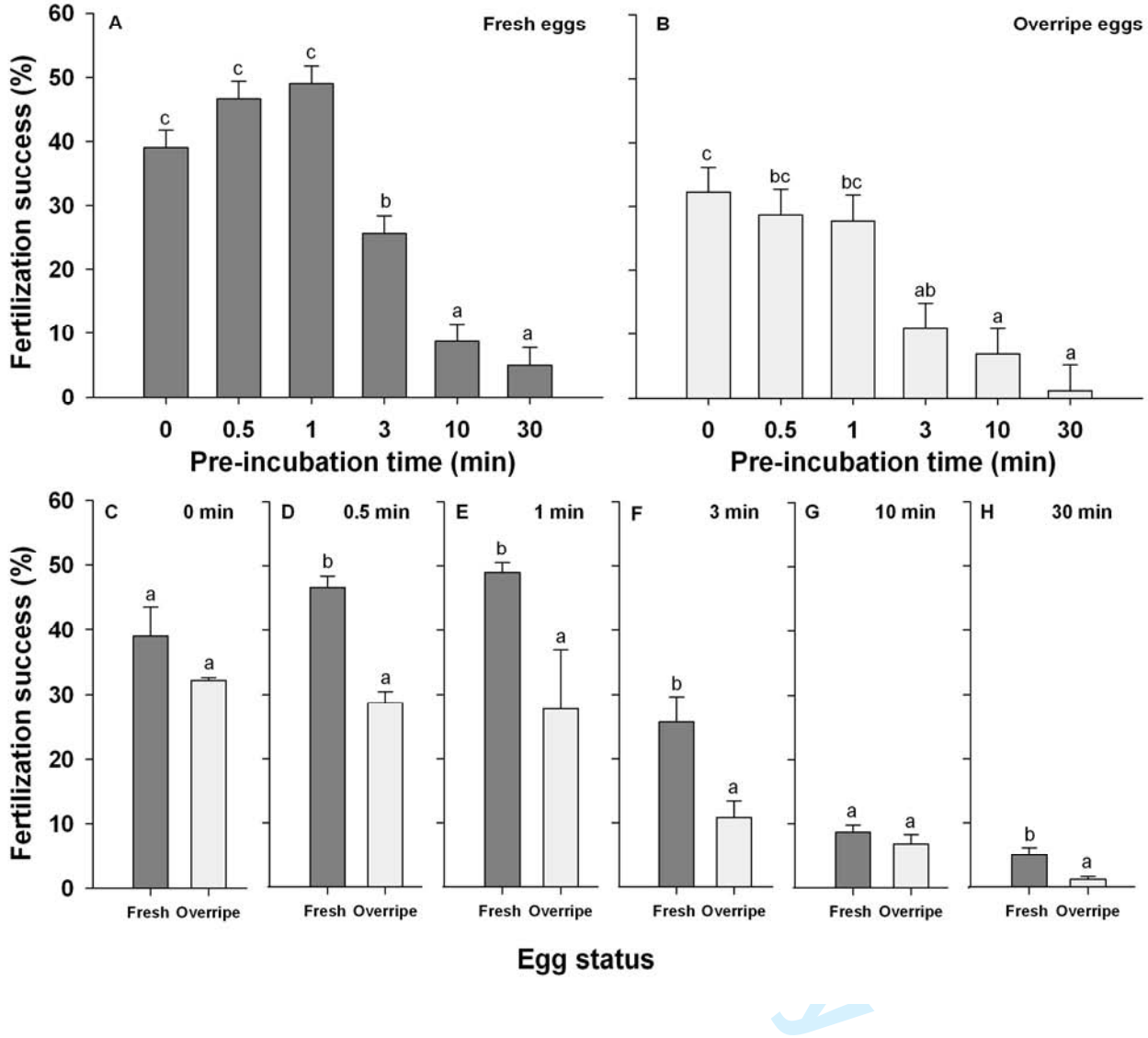
A
Fresh eggs
B
Overripe eggs
Fertilization success (%)
Pre-incubation time (min)
0
0.5
1
3
10
30
C 0 min
D 0.5 min
E 1 min
F 3 min
G 10 min
H 30 min
Fresh
Overripe
Egg status

Fig 2