

# Infections may select for filial cannibalism by impacting egg survival in interactions with water salinity and egg density

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**Abstract** In aquatic environments, externally developing eggs are in constant contact with the surrounding water, highlighting the significance of water parameters and pathogens for egg survival. In this study we tested the impact of water salinity, egg density and infection potential of the environment on egg viability in the sand goby (*Pomatoschistus minutus*), a small fish that exhibits paternal egg care and has a marine origin, but which in the Baltic Sea lives in brackish water. To manipulate the infection potential of the environment, we added either a *Saprolegnia* infection vector into UV-filtered water or a fungicide into natural Baltic Sea water. *Saprolegnia* are widely spread water moulds that are a key cause of egg mortality in aquatic organisms in fresh- and brackish water. We found that increased water salinity indeed decreased the egg infection rate and had a positive effect on egg viability, while high egg density tended to have the opposite effect. However, the different factors influenced egg viability interactively, with a higher egg density having negative effects at low, but not in high, salinity. Thus, the challenges facing marine organisms adapting to lower salinity levels can be amplified by *Saprolegnia* infections that reduce egg

survival in interaction with other environmental factors. Our results support the hypothesis that suppressing egg infections is an important aspect of parental care that can select for filial cannibalism, a common but poorly understood behaviour, especially in fish with parental care.

**Keywords** Adaptation · Egg density · Filial cannibalism · Salinity · *Saprolegnia* infection

## Introduction

The effects of salinity on reproduction are particularly topical in aquatic environments in a flux, such as the Baltic Sea. The present-day brackish water phase of the Baltic Sea, which was preceded by a more marine phase, is only 4,500 years old (Westman and Sohlenius 1999), meaning that, from an evolutionary perspective, the brackish phase is relatively young. The markedly lower than oceanic salinity levels in the Baltic Sea are therefore likely to have both direct and indirect effects on natural and sexual selection regimes experienced by its inhabitants. Indeed, fish living in the Baltic Sea display numerous adaptations to salinity which affect their growth (Marchinko and Schluter 2007; Defaveri and Merilä 2013) and egg development (Thorsen et al. 1996; Karås and Klingsheim 1997; Nissling et al. 2002; Nissling and Dahlman 2010). For example, eggs of the Atlantic cod (*Gadus morhua*) reach neutral buoyancy at very different water salinities, depending on whether the fish originate from marine conditions (Atlantic coast) or the Baltic Sea (Thorsen et al. 1996).

Because the surface of externally developing aquatic eggs is in constant contact with the surrounding water, water parameters are also likely to have an important role in egg infection rate. In this context, lowering the infection

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risk in the offspring's immediate environment is an important component of parental care in both aquatic and terrestrial habitats. Some bird species, for example, are known to incorporate antiparasitic fungal or vegetative material into their nests (Wimberger 1984; Clark and Mason 1985). Females of the European earwig (*Forficula auricularia*), in turn, attend to their egg clutches in burrows in the soil, spending an increasing amount of time on grooming as a response to increasing exposure to mould (Boos et al. 2014). Although relatively little is known about the interplay between parental care, microbial infections and egg viability in fish and other aquatic organisms (Knouft et al. 2003), the role of parental care in the avoidance of infections is potentially important in these species also. In species that normally provide care, unguarded eggs are known to become heavily invaded by water moulds or other microbes (Winn 1958; Brown and Clotfelter 2012). Parental activities that may reduce the rate of microbial infections on fish eggs include stirring of the water by rhythmic movements (e.g. fanning; Côté and Gross 1993; St Mary et al. 2004), body excretions (e.g. mucus or foam; Knouft et al. 2003; Giacomello et al. 2008; Little et al. 2008; Brown and Clotfelter 2012) and selective removal or eating of infected eggs (Winn 1958; Sargent 1992). Thus, pathogen control may be an important driver of filial cannibalism (i.e. parents eating some of their own young), a relatively common but poorly understood behaviour in fish with parental care (Rohwer 1978; Sargent 1992; Kraak 1996).

One particularly wide-spread group of pathogens that impacts the viability of eggs of fish and other aquatic organism is the oomycetes (water moulds) of the genus *Saprolegnia*. *Saprolegnia* water moulds affect both wild and farmed populations of aquatic organisms (Kitancharoen et al. 1997; Lategan et al. 2004; van West 2006; Fernández-Benéitez et al. 2011), being globally second only to bacterial diseases in terms of inflicting losses to aquaculture (Hussein and Hatai 2002; van West 2006). *Saprolegnia* water moulds are most commonly encountered in freshwater, although they can also be found in estuaries and brackish bodies of water (Padgett 1978; van West 2006). They are known to be sensitive to high salinity levels, to such an extent that sodium chloride baths are often used as an antifungal measure in egg hatcheries and tanks of fish hobbyists (Taylor and Bailey 1979; Edgell et al. 1993; Marking et al. 1994; Schreier et al. 1996; Racha et al. 2004). Indeed, the addition of sodium chloride has been found to increase hatching success of eggs of salmonids and other aquatic organisms reproducing in brackish or freshwater (Edgell et al. 1993; Marking et al. 1994; Schreier et al. 1996; St Mary et al. 2004). However, the relationship between salinity and egg survival is not straightforward, with lower doses of or shorter exposure times to sodium chloride sometimes being ineffective in

controlling the growth of *Saprolegnia* and other microbes (Edgell et al. 1993; Celada et al. 2004), while high salt concentrations can damage eggs that normally develop in lower salinities (Chatto 1979; Gerking and Lee 1980; Karås and Klingsheim 1997; Rasowo et al. 2007).

In this study, we assessed the effects of salinity on eggs of a Baltic population of the sand goby (*Pomatoschistus minutus*), a small marine fish with paternal egg care. The sand goby is a particularly apt model species for our study for several reasons. First, the background information on its reproduction is extensive and continuously increasing (reviewed in Forsgren 1999; Lehtonen 2012). Second, the species is widespread and occurs in a range of environments and salinity levels, with northern parts of the Baltic Sea representing the low end of its salinity range (Miller 1986; details on its occurrence in the Mediterranean Sea: Bouchereau and Guelorget 1997; Atlantic Ocean: Fonds 1973; Hesthagen 1977; Baltic Sea: Jansson et al. 1985; Zander 1990; Sundell 1994). Interestingly, the results of an earlier study suggest that although eggs of gobies from a marine environment develop normally in a relatively wide range of salinities, they encounter difficulties in salinities that correspond to the conditions of the Northern Baltic Sea (Fonds and Van Buurt 1974). Third, even close to the edge of its wide distribution range, the sand goby is the most numerous littoral fish species in many parts of the Baltic Sea (Jansson et al. 1985; Sundell 1994). Finally, the lower salinity levels in the Northern Baltic Sea affect the reproduction of sand gobies in multiple ways. For example, many of the marine egg predators of sand gobies do not live in the areas of low salinity, thereby allowing sand gobies to use habitats that they do not use for reproduction elsewhere, presumably due to the removal of pressure from egg predators (Lehtonen and Lindström 2004; Järvi-Laturi et al. 2008). In contrast, the lower salinity levels in the Baltic Sea are likely to increase the risk of microbial egg infection (especially *Saprolegnia*). Furthermore, the availability of mussel shells, important nesting resources of sand gobies, is low in the Northern Baltic Sea, probably due to the low salinity levels (Forsgren et al. 1996; Lehtonen and Lindström 2004; Singer et al. 2006; Wong et al. 2008). Nesting resources are therefore typically smaller and less numerous in the Baltic Sea (Forsgren et al. 1996), potentially resulting in denser egg masses, which, in turn, may benefit egg pathogens even further. Indeed, in addition to the physiological effects of low salinity, *Saprolegnia* and other microbes can potentially play a large role in reproduction of sand gobies and other species with parental care in the Baltic Sea and other brackish waters, such as estuaries. However, to date the interactions between low salinity levels, pathogen infections and egg success are underexplored, particularly in species that exhibit parental care. The aim of our study was to address this gap in current knowledge.

## Materials and methods

The study was carried out during the 2013 breeding season of the sand goby at the Tvärminne Zoological Station on the Finnish coast of the Baltic Sea (59°50.7'N, 23°15.0'E). Sand gobies were collected using a hand trawl near the field station and maintained in separate-sex aquariums (approx. 100 l) containing natural brackish water (salinity 5.5–6 ppt) that was pumped from the sea through the aquariums using a flow-through system. For the experiments, a male was placed in a flow-through tank measuring 70 × 25 cm (bottom area), with the water depth set to approximately 25 cm. Each tank had a 4-cm layer of fine sand and half a clay flowerpot (diameter 6.5 cm) as a nesting resource. The inner surface of the pot was lined with a piece of transparent plastic film that had been cut to fit the shape of the pot. Male sand gobies build nests by first piling sand on top of a nesting resource and then excavating under it, leaving a single narrow opening (see Japoshvili et al. 2012). After the male had completed nest-building, we added a female ripe with eggs to the tank. The female was removed from the tank after she had laid her eggs in a mono-layer on the film in the nest.

We carried out two experiments in which we adopted slightly different approaches to assess the impact of multiple factors on egg viability (see section “[Response variables](#)”). In both experiments, we controlled for potential effects of male care effort by rearing the eggs artificially. To this end, 12–24 h after the female had laid her eggs, we removed the transparent film with the egg batch from the tank. Using scissors which had been cleaned with ethanol and then wiped dry, we then cut the film into 12 strips, with each strip containing approximately the same number of eggs. Eggs on each piece of film were then photographed. Six of the 12 strips were used in experiment 1 and the other six in experiment 2. The strips with eggs were placed on petri dishes (diameter 90 mm) in an incubator for 7 days at a constant temperature of 15 °C. This time frame was chosen because it was long enough for eye-spots to develop in all eggs that had been alive up to a late stage of development, while also short enough not to result in eggs hatching before the end of the experiment (a few hatched following handling at the end of the experiment) (Fonds and Van Buurt 1974; Kvarnemo 1994; Lehtonen 2012; personal observations in 2013 and 2014).

### Experiment 1: *Saprolegnia* exposure and water salinity treatments

In experiment 1, we manipulated exposure to *Saprolegnia* water moulds to test egg success in relation to infections, water salinity and egg density. For egg incubation we used

natural water that, after having been pumped from a nearby bay, was sterilised with UV irradiation (ASTRA UV-C 11 W; AstraZeneca, London, UK) using a very slow (approx. 1 l/min) water flow to maximise the effect of the irradiation. UV irradiation does not change water parameters or produce harmful by-products, while it has been found to be efficient in reducing microbial counts in water (Heikkinen et al. 2013). We had two categorical treatment classes: ‘*Saprolegnia* infection’ with two treatment levels (infected and control) and ‘salinity’ with three treatment levels (low, medium and high, see below for values), resulting in a 2 × 3 design. One strip from each egg batch (see above) was used in each of the six (2 × 3) categorical treatments. In addition, we assessed the effect of egg density (see below).

*Saprolegnia* used for manipulating the level of infection had first been collected from naturally infected sand goby eggs, and the inocula were then cultured in petri plates containing half-strength Emerson YpSs agar with yeast extract, at 25 °C. Boiled hemp seeds were placed on the culture plates for about 3 days before being used as vectors in the infection treatment. Two of the infected seeds were then placed in a petri dish with goby eggs for the first 24 h, after which the seeds were removed. In the control treatment, two non-infected hemp seeds were similarly added to each petri dish (Sagvik et al. 2008).

In the low salinity treatment, goby eggs were incubated in an unmanipulated salinity of approximately 6 ppt (see below). Medium salinity level was created by adding a commercially available sea salt mix for aquarium use (‘Instant Ocean’; Spectrum Brands Inc., Middleton, WI) at 6 g/l of UV-irradiated seawater. In the high salinity treatment, 12 g salt mix/l UV-irradiated seawater was added. It should be noted that the salt mix was likely to contain some moisture (i.e. water) due to its high absorbing capacity. The amount of water in each petri dish was set at a depth of 2 cm and changed daily, without renewing the initial infection manipulations. Salinity of the water in the petri dishes was monitored at six haphazardly chosen occasions during the course of the experiment. Right after the water on a petri dish had been changed, the three salinity levels [mean ± standard error (SE)] were 5.77 ± 0.06 ppt (low treatment level), 11.2 ± 0.1 (medium treatment level) and 16.2 ± 0.1 ppt (high treatment level),  $N = 6$ ; due to evaporation, salinity levels right before a water change were higher for each treatment level (8.02 ± 0.24 ppt, 15.9 ± 0.4 ppt and 22.5 ± 0.7 ppt, respectively).

After the 7-day incubation period, eggs were photographed a second time and inspected visually. Egg density was later assessed from these photographs using the Sigma Scan Pro 5.0 software (Systat Software Inc., San Jose, CA). The diameters of ten haphazardly chosen eggs per each piece of film were first measured

(two measurements per egg, with an angle of 90° between the two diameters), and the average of these measurements was used as a scaling unit for egg density on that particular piece of film. Hence, an egg density value of 1 means that the eggs were packed as tightly as possible in a monolayer, while lower values indicate lower densities.

The experiment was repeated with 16 batches of sand goby eggs, providing  $16 \times 6 = 96$  replicates for the analyses.

#### Experiment 2: fungicide and water salinity treatments

In experiment 2 we inhibited growth of *Saprolegnia* and other microbial egg pathogens to investigate the relationship between water salinity, egg density, pathogen infections and egg viability. The  $2 \times 3$  design of experiment 2 was the same as that of experiment 1, with identical three levels of salinity. However, in contrast to experiment 1, eggs were not experimentally infected with *Saprolegnia* and water was not UV-irradiated. Rather, the experiment involved a fungicide treatment at two levels: a control and a fungicide addition. The fungicide used, Protozin (Waterlife Research Ind. Ltd, Longford, UK), is a widely available product and was used following the manufacturer's recommended dose. This fungicide was developed especially for home aquarium use and is commonly applied as a precautionary measure to avoid microbial infections on both adult fish and their eggs in fresh- and brackish water (personal observations; see also Rushbrook and Barber 2008). Experiment 2 was replicated using subsets of the same 16 egg batches as used in experiment 1, providing  $16 \times 6 = 96$  replicates.

#### Response variables

In both experiments we had the following three response variables: (A) the proportion of eggs that developed up to the 'eye-spot' stage; (B) the proportion of eggs that appeared to be healthy, i.e. were not damaged and/or infected; (C) the binomial outcome of whether or not *Saprolegnia* hyphae (filaments) were found growing on any of the eggs in a replicate. For response variable (A), we counted the total number of eggs from the photograph that was taken at the end of each trial. The same photograph was also used for counting the number of eggs that had developed up to the 'eye-spot' stage, and the starting phase egg photograph acted only as a back-up. Here, the presence of two visible 'eye-spots' at the end of the experiment was used as an indicator of the egg having successfully developed up to an advanced stage. To estimate response variable (B), we again used the counts of the total number of eggs in each replicate. At the end of the replicate, we also visually estimated the percentage of eggs that appeared to

be infected or otherwise had an unhealthy appearance, presumably due to pathogen infection, oxygen shortage, carbon dioxide overdose and/or osmotic stress. While these percentages are used in the figures, for the analyses, we used the combination of the total number of eggs and our percentage estimate to calculate (approximately) the number of eggs that appeared to be healthy (versus infected/damaged) in each replicate. Note that it was possible for an egg that was categorised as 'infected' at the end of the experiment to have developed up to the eye-spot stage. Lastly, at the final visual assessment, we also checked for response variable (C)—the presence of *Saprolegnia* hyphae growing on the eggs. If any *Saprolegnia* hyphae were seen, the replicate was given the value '1' and if no *Saprolegnia* filaments were seen, the value '0'.

#### Statistical analyses

In both experiments, our analyses involved the above-described three response variables (A, B, C), that were analysed separately. For the proportional variables, (A) and (B), the numbers of eggs with the two different states [eye-spots vs. no eye-spots in (A), and healthy vs. infected in (B)] were combined into a single independent variable for these analyses. In experiment 1, we applied a generalised mixed model (GMM) for each response variable, with the *Saprolegnia* infection treatment (control vs. infected), salinity level (three levels) and egg density as fixed effects and—to account for any quality variation among the egg batches—egg batch ID as a random effect. We initially used a binomial distribution with Logit Link function, appropriate for both proportional (A, B) and binomial (C) response variables (Zuur et al. 2013). However, for response variables A and B, our initial models were found to be overdispersed (see, for example, Zuur et al. 2013), and we therefore translated these to log-normal-Poisson models by including an individual-level random variable into each of these models (Elston et al. 2001). In all cases, we assessed, using  $\chi^2$ -tests (with  $p > 0.05$  as the cutting point), whether a particular model could be simplified by refitting it without its least significant, highest order interaction term. For clarity, any significant interaction effects were then further investigated by using simple statistics (rank correlations and Mann–Whitney  $U$  tests). Here, as well as in our visual presentations, the numbers of eggs in the two 'state categories' (see above) were reduced into simple values of proportion.

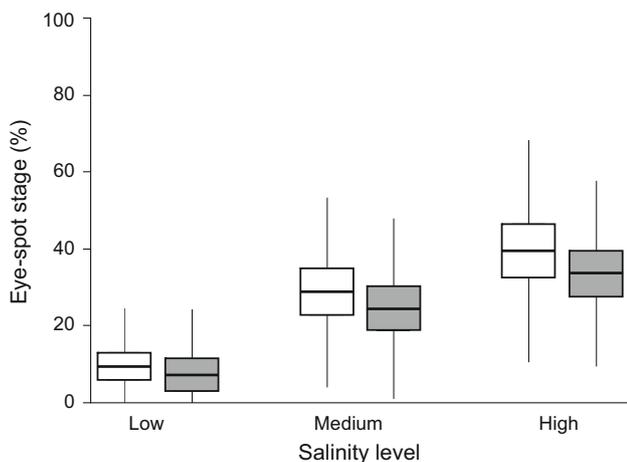
The analyses in experiment 2 were similar to those just described except that in the place of the *Saprolegnia* infection treatment we had the fungicide treatment (control vs. treated). We used R 3.0.2 software (R Development Core Team 2013) for the analyses and 'lme4' statistical package for running the GMMs.

**Results**

**Experiment 1**

The mean survival of eggs up to the eye-spot stage varied between 7.0 % for infected eggs in the low salinity water condition and 39 % for non-infected (i.e. control) eggs in the high salinity water condition (Fig. 1). When a GMM was applied to assess egg development up to the eye-spot stage, a stepwise simplification procedure revealed that all interaction terms were non-significant ( $p > 0.05$ ); these were consequently removed from the model. The effect of egg density was non-significant ( $\chi^2 = 2.784$ ,  $df = 1$ ,  $p = 0.095$ ) and it too was removed from the model. The final model showed that the presence of the infection vector (two *Saprolegnia*-infected hemp seeds) had a significant negative effect on egg survival up to the eye-spot stage ( $z = 2.128$ ,  $p = 0.033$ ), whereas higher water salinity levels had a positive effect (low vs. medium level:  $z = 7.175$ ,  $p < 0.001$ ; medium vs. high:  $z = 2.918$ ,  $p = 0.004$ ) (Fig. 1).

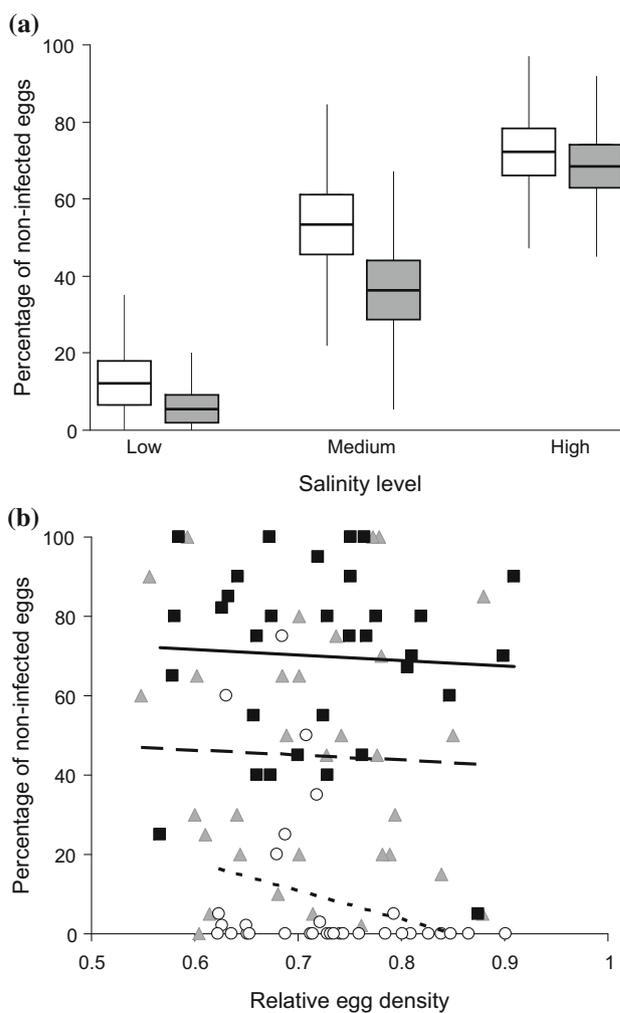
For the proportion of eggs that appeared ‘healthy’ (i.e. not visibly infected or damaged), we found a significant interaction between salinity and egg density ( $\chi^2 = 11.01$ ,  $df = 2$ ,  $p = 0.004$ ) and a negative effect of infection treatment on the proportion of healthy eggs ( $z = 2.092$ ,  $p = 0.036$ ) (Fig. 2a). The significant salinity  $\times$  density interaction was due to the fact that increasing density had a negative effect on the proportion of healthy-looking eggs in the low-level salinity treatment (Spearman’s rank correlation,  $\rho = -0.447$ ,  $n = 32$ ,  $p = 0.010$ ) but not in the



**Fig. 1** The percentage of eggs which developed up to the eye-spot stage at the end of experiment 1 ( $n = 16$  for each box). White boxes control treatment, grey boxes *Saprolegnia* infection treatment. Low, medium, and high refer to the three salinity treatments. Horizontal line within box the mean, horizontal box margins standard errors (SE), whiskers standard deviations (SD)

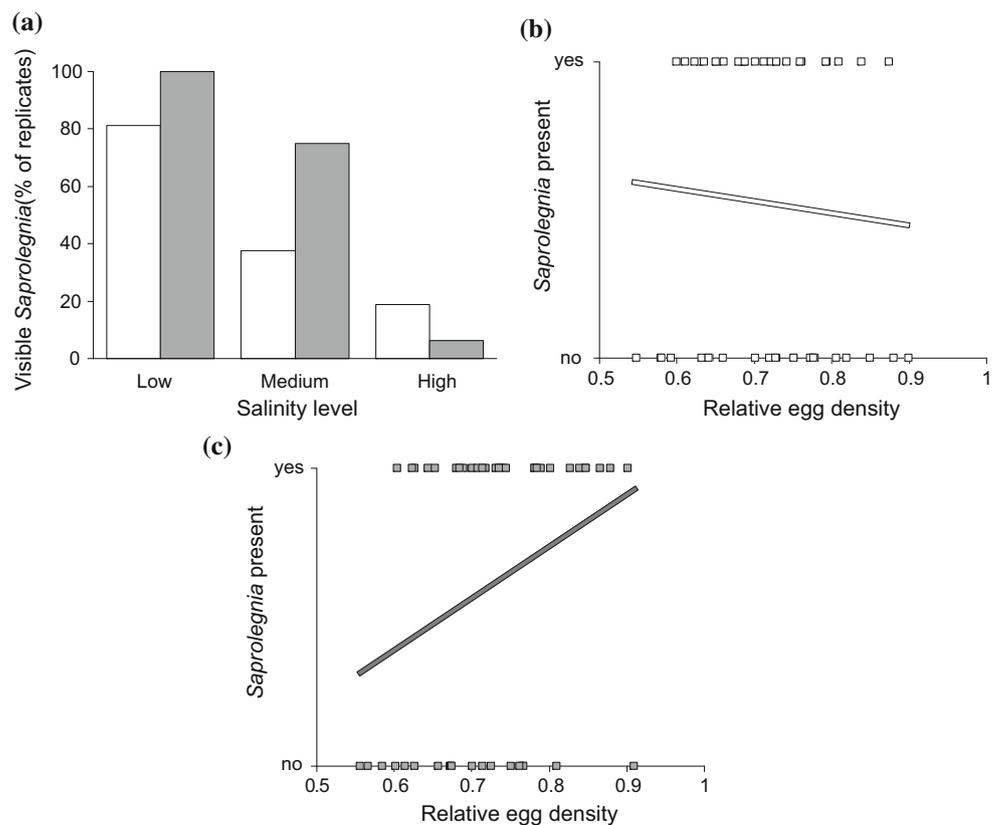
medium-level salinity condition (Spearman’s rank correlation,  $\rho = -0.047$ ,  $n = 32$ ,  $p = 0.80$ ) or the high-level salinity condition (Spearman’s rank correlation,  $\rho = -0.036$ ,  $n = 32$ ,  $p = 0.84$ ). Hence, the negative effect of low salinity on the proportion of healthy-looking eggs was even more pronounced at high egg density than at low egg density (Fig. 2b).

Finally, when we assessed the presence of visible *Saprolegnia* filaments, we found significant interactions both between infection treatment and salinity ( $\chi^2 = 10.44$ ,  $df = 2$ ,  $p = 0.005$ ), and between infection treatment and egg density ( $\chi^2 = 5.046$ ,  $df = 1$ ,  $p = 0.025$ ). In particular, the infection treatment



**Fig. 2** The percentage of eggs that in a visual inspection seemed not to be infected or damaged in experiment 1. **a** White boxes the control treatment, grey boxes the *Saprolegnia* infection treatment ( $n = 16$  for each box). Horizontal line within box the mean, horizontal margins of box SE, whiskers SD. **b** White circles/dotted trend line low salinity treatment, grey triangles/dashed trendline medium salinity treatment, black squares/solid trendline high salinity treatment ( $n = 32$  for each salinity level)

**Fig. 3** Replicates with and without visible signs of *Saprolegnia* infection in experiment 1. **a** Percentage of replicates in which *Saprolegnia* was recorded. *White bars* control treatment, *grey bars* *Saprolegnia* infection treatment ( $n = 16$  for each bar). **b** Each replicate of the *Saprolegnia* infection treatment in relation to egg density ( $n = 48$ ). **c** Replicates of the control treatment ( $n = 48$ )



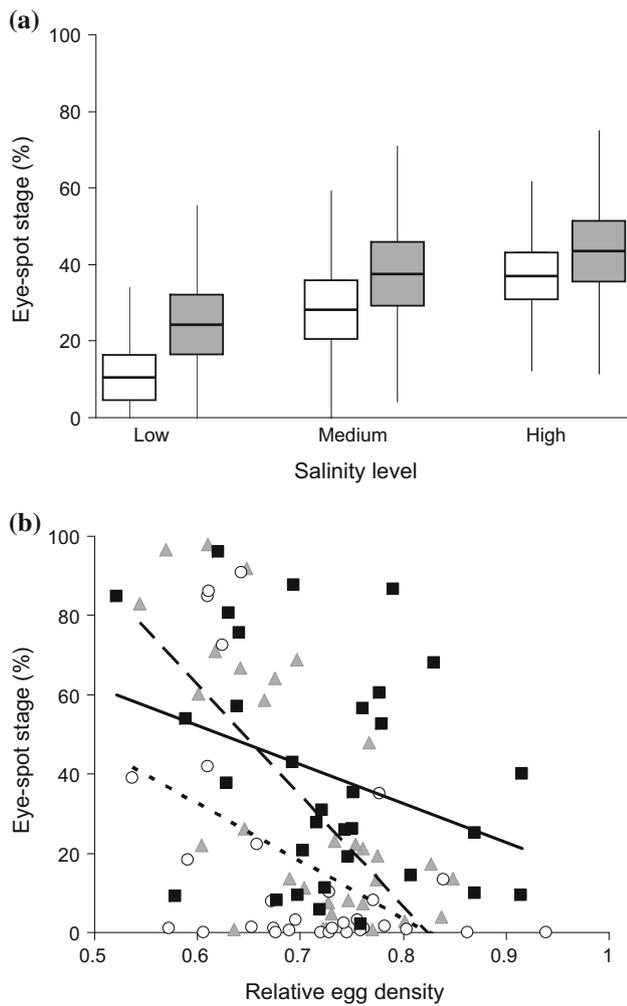
significantly increased the frequency of replicates with visible signs of *Saprolegnia* hyphae in the medium-level salinity treatment ( $z = 2.078$ ,  $p = 0.038$ ) but not in the two other salinity levels (low:  $z = 0.23$ ,  $p = 0.82$ ; high:  $z = 0$ ,  $p = 1.0$ ) (Fig. 3a). Furthermore, egg density increased the frequency of replicates with visible signs of *Saprolegnia* hyphae in the infection treatment ( $z = 2.082$ ,  $p = 0.037$ ) (Fig. 3b) but not in the control treatment ( $z = 0.050$ ,  $p = 0.96$ ) (Fig. 3c).

## Experiment 2

In this experiment, the average proportion of eggs that developed up to the eye-spot stage varied between 10 % in the control treatment at the low-salinity condition and 43 % in the fungicide treatment at the high-salinity condition (Fig. 4a). Here, we found a significant interaction between salinity level and egg density ( $\chi^2 = 7.475$ ,  $df = 2$ ,  $p = 0.024$ ). Furthermore, the fungicide supplement increased the proportion of eggs that reached the eye-spot stage ( $z = 4.197$ ,  $p < 0.001$ ) (Fig. 4a). The significant salinity level  $\times$  egg density interaction was driven by density having a significant negative effect on egg development up to the eye-spot stage at low (Spearman's rank correlation,  $\rho = -0.4015$ ,  $n = 32$ ,  $p = 0.023$ ) and

medium (Spearman's rank correlation,  $\rho = -0.5740$ ,  $n = 32$ ,  $p < 0.001$ ) salinities, but not at the high salinity treatment (Spearman's rank correlation,  $\rho = -0.217$ ,  $n = 32$ ;  $p = 0.23$ ). In other words, the effect of egg density on egg survival until the eye-spot stage depended on the salinity level (Fig. 4b).

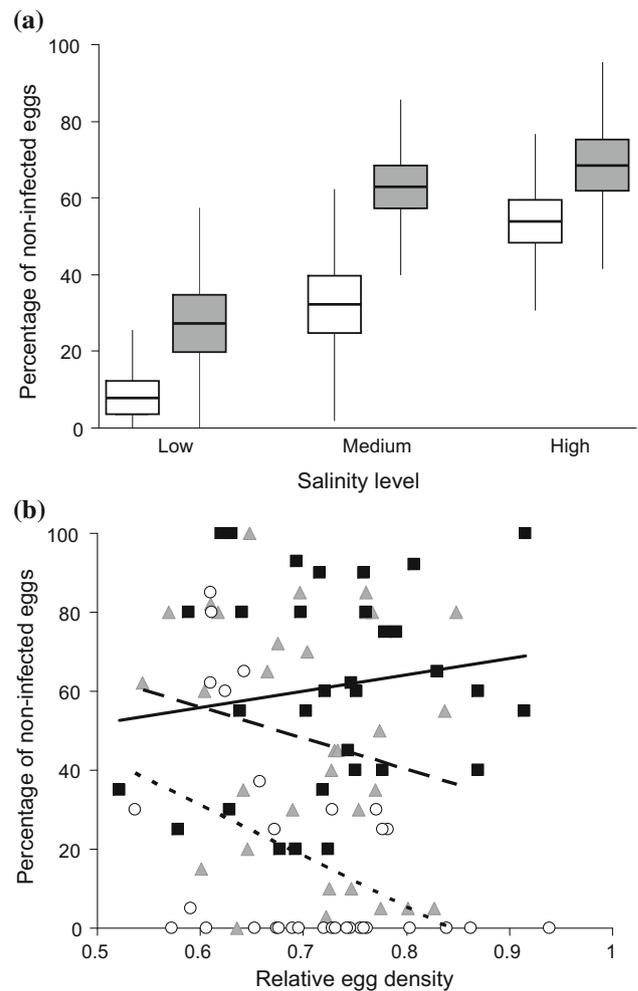
When assessing the proportion of eggs that looked healthy, we found significant interactions between salinity level and fungicide treatment ( $\chi^2 = 6.057$ ,  $df = 2$ ,  $p = 0.048$ ), and between salinity level and density ( $\chi^2 = 19.18$ ,  $df = 2$ ,  $p < 0.001$ ). Further analyses revealed that the fungicide increased the proportion of healthy-looking eggs at low (Mann-Whitney,  $W = 175$ ,  $n_1 = n_2 = 16$ ,  $p = 0.049$ ) and medium (Mann-Whitney,  $W = 202$ ,  $n_1 = n_2 = 16$ ,  $p = 0.006$ ) salinities, but not significantly in the high salinity treatment (Mann-Whitney,  $W = 171$ ,  $n_1 = n_2 = 16$ ,  $p = 0.11$ ) (Fig. 5a). Furthermore, increasing egg density was associated with a decreasing proportion of healthy eggs in the low salinity treatment (Spearman's rank correlation,  $\rho = -0.429$ ,  $n = 32$ ;  $p = 0.014$ ), whereas there was no significant relationship between egg density and the proportion of healthy eggs at medium (Spearman's rank correlation,  $\rho = -0.171$ ,  $n = 32$ ;  $p = 0.35$ ) or high salinities (Spearman's rank correlation,  $\rho = 0.154$ ,  $n = 32$ ;  $p = 0.40$ ).



**Fig. 4** The percentage of eggs that had developed up to the eye-spot stage at the end of experiment 2. **a** White boxes control treatment, grey boxes fungicide treatment ( $n = 16$  for each box). Horizontal lines within box the mean, margins of box SE, whiskers SD. **b** White circles/dotted trendline low salinity treatment, grey triangles/dashed trendline medium salinity treatment, black squares/solid trendline high salinity treatment ( $n = 32$  for each salinity level)

This pattern also means that the low salinity level had a more clearly distinguishable negative effect on the proportion of healthy eggs when egg density was high than when it was low (Fig. 5b).

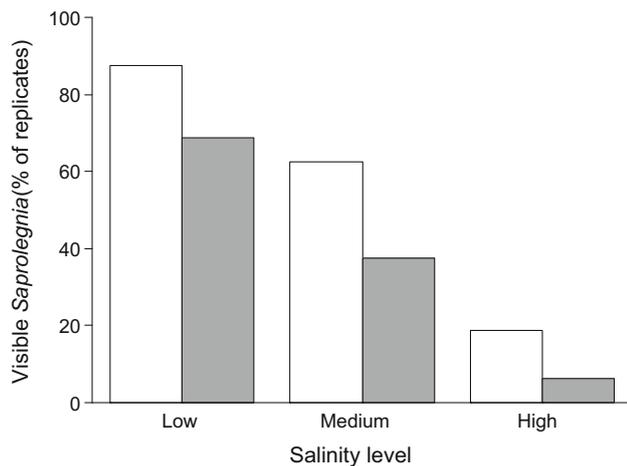
The analysis of the presence of *Saprolegnia* hyphae showed a significant interaction between salinity level and egg density ( $\chi^2 = 6.758$ ,  $df = 2$ ,  $p = 0.034$ ) and a significant negative effect of fungicide on *Saprolegnia* ( $z = -2.572$ ,  $p = 0.010$ ) (Fig. 6). Further analyses indicated that the significant interaction was driven by egg density having a significant positive effect on *Saprolegnia* occurrence at the low salinity level ( $z = 2.234$ ,  $p = 0.026$ ), but not at the medium ( $z = 0.458$ ,  $p = 0.65$ ) and high ( $z = 0.090$ ,  $p = 0.93$ ) salinity levels. Overall, *Saprolegnia* was more common the lower the level of salinity (Fig. 6).



**Fig. 5** The percentage of eggs in experiment 2 that in a visual inspection appeared not to be infected or damaged. **a** White boxes control treatment, grey boxes fungicide treatment ( $n = 16$  for each box). **b** White circles/dotted trendline low salinity treatment, grey triangles/dashed trendline medium salinity treatment, black squares/solid trendline high salinity treatment ( $n = 32$  for each salinity level)

### Discussion

We found that water salinity, egg density and exposure to *Saprolegnia* infection together impacted the infection rate and general viability of sand goby eggs. In most of our analyses, increased salinity had a strong, positive effect on egg viability (Figs. 1, 2, 3a, 4a, 5a, 6). A higher egg density, in turn, had a negative effect on egg success (Figs. 2b, 3c, 4b, 5b), but only at the low and/or medium salinity levels and not at a high salinity (Figs. 2b, 4b, 5b). Furthermore, our manipulations of the infection potential of the environment—i.e. the presence of a direct *Saprolegnia* infection vector in experiment 1 and an infection inhibitor (fungicide) in experiment 2—not only influenced the observed *Saprolegnia* prevalence but also affected the



**Fig. 6** The percentage of replicates in experiment 2 with visible signs of *Saprolegnia* infection. White bars control treatment, grey bars fungicide treatment ( $n = 16$  for each bar). Low, medium, and high indicate the three salinity treatments

proportion of eggs that successfully developed up to the eye-spot stage. In particular, treatments with a higher level of *Saprolegnia* exposure resulted in a higher percentage of unhealthy ('infected') eggs and a lower egg survival (Figs. 1, 2a, 3a, 4a, 5a, 6), with the magnitude of this effect being in some cases subject to the level of salinity (Figs. 3a, 5a). The exact pattern of these co-effects depended on the response variable, highlighting the complexity of the processes that define the egg infection rate and survival.

Despite the interplay between the different factors, our results suggest that growth of *Saprolegnia* (and other pathogens) played an important role in the observed density- and salinity-dependent variation in egg survival up to the eye-spot stage. Conditions that were less favourable for growth of *Saprolegnia* (and other infections) resulted in a higher survival. Indeed, sodium chloride's inhibitory effect on *Saprolegnia* growth provides a functional explanation for the improved egg survival up to the eye-spot stage with increased salinity. Interestingly, we also found that a high egg density was associated with both a high prevalence of *Saprolegnia* and a low percentage of egg survival in the low but not high salinity (with intermediate effects in the medium salinity). This finding is in line with those of previous studies which have demonstrated that *Saprolegnia* hyphae can suffocate and kill healthy eggs, but only when spreading from adjacent infected eggs (Smith et al. 1985; Celada et al. 2004; Rasowo et al. 2007; Thoen et al. 2011). Our result could also explain an apparent contradiction between two earlier studies that did (Klug et al. 2006) and did not (Norevik Andrén and Kvarnemo 2014) find an increase in egg survival at lower egg densities. The former study was conducted under low salinity conditions of the Northern Baltic Sea and the latter on the west coast of

Sweden, where salinity is even higher than it was in the high salinity treatment of our study.

The findings of our study have a direct bearing on parental egg-care of fish in the Baltic Sea and other brackish bodies of water. Earlier studies on a pupfish species, the flagfish (*Jordanella floridae*), indicated a higher need for egg-care in lower salinities (St Mary et al. 2001, 2004) and that nest-directed behaviours in general may have a relatively larger importance in such conditions (Hale 2008). Klug et al. (2006), in turn, suggested that their result of an increase in egg survival—and a decrease in filial cannibalism—with an experimental reduction of egg density was due to an improved oxygen environment for the less densely spaced eggs. However, similar to Lissåker et al. (2003), Klug et al. (2006) did not find an effect of experimentally reduced oxygen on the rate of egg survival or filial cannibalism. Indeed, our results provide an alternative (or additional) explanation to the lower egg survival and higher rate of filial cannibalism of dense egg batches, namely, *Saprolegnia*. In particular, conditions that reduce the growth of *Saprolegnia* also enhance egg survival, while the primary spreading mechanism of *Saprolegnia* to healthy eggs is hyphae growth from surrounding dead or damaged eggs (Smith et al. 1985; Celada et al. 2004; Rasowo et al. 2007; Thoen et al. 2011). Hence, a reduced density of eggs, either due to manipulation or cannibalism by the egg-tending male, should indeed inhibit the spread of *Saprolegnia* and other microbes, resulting in an increase in egg survival. In accordance with this interpretation, the overall rate of filial cannibalism in sand gobies seems to be lower on the Swedish west coast than on the Finnish coast of the Baltic Sea, with lower salinity conditions in the latter providing a more favourable environment for egg pathogens [Swedish west coast (Lindström 1998; Lissåker et al. 2003; Lissåker and Svensson 2008) vs. Northern Baltic Sea (Pampoulie et al. 2004; Klug et al. 2006; Lehtonen and Lindström 2007; Järvi-Laturi et al. 2008; Klug and Lindström 2008; Chin-Baarstad et al. 2009; Lehtonen 2012). Hence, our results support the suggestion by Sargent (1992) and Kraak (1996) that an important role of filial cannibalism is to control infections. However, more research on the rate of filial cannibalism in relation to, for example, egg density, *Saprolegnia* infections and infection inhibitors, such as water salinity, are needed to further investigate this underexplored hypothesis.

The stronger density-dependence of egg viability in low rather than high salinities may also influence patterns of egg-laying and hence sexual selection. For instance, a lower survival cost of high egg densities in more saline environments may increase the potential for male mating biases if selection against females to lay eggs in nests that already have relatively high egg densities is stronger in low salinity environments. Such salinity-dependent differences

in the potential for mate monopolisation may therefore help to explain differences in sexual selection regimes across populations (see, for example, Forsgren et al. 1996). Note, however, that the patterns of egg-laying are also likely to be influenced by the salinity-dependent size distribution and availability of nesting resources (Forsgren et al. 1996; Lehtonen and Lindström 2004). Furthermore, when given a choice between two nests for egg-laying, even sand goby females from the relatively high salinity environment of the Swedish west coast preferred the one with the smaller egg clutch (Norevik Andrén and Kvarnemo 2014).

In our study, we assessed the influence of variation in egg density on egg success without direct density manipulations. Consequently, egg density in each replicate was determined by at least the following three factors: (1) the female's way of depositing her eggs onto the substratum, (2) the pattern of any filial cannibalism by the male before the eggs were removed from the nest and (3) the way we cut the egg batches into smaller subsets and then (haphazardly) distributed the subsets among the different treatments. Therefore, we cannot rule out the possibility that males could have had time to selectively cannibalise eggs, possibly resulting in a lower proportion of eggs with low viability in replicates that we found to have a low egg density. However, even though this possibility would indicate that egg density per se might not be as important as our results suggest, it would still support the importance of filial cannibalism in reducing egg infections.

It also remains possible that the higher egg survival in water of increased salinities was due to not only lower rates of *Saprolegnia* infections in those conditions but also to the marine origin of sand gobies. In particular, sand goby eggs may not be optimally adapted to develop in the lower salinities of the geologically young Baltic Sea. Even in that case, our results imply that, in addition to any physiological challenges, *Saprolegnia* infections are an important driver of selection pressure on marine fish (and other organisms) adapting to lower salinity levels, such as those found in the Baltic Sea. Similarly, eggs of freshwater fishes living in brackish waters are likely to experience different infection challenges than those in their original lake or riverine environments. These considerations are timely because salinity levels in the Baltic Sea have been decreasing during recent decades (reviewed in Nissling et al. 2006; Neumann 2010; Wikner and Andersson 2012) and are predicted to decrease even further in the future (Meier 2006; Neumann 2010). Such changes are likely to have a greater negative impact on fish than on pathogens, such as *Saprolegnia*, which not only benefit from decreased salinity levels but also have a high capacity to adapt to novel conditions, sometimes even opportunistically switching their feeding substrates (Thoen et al. 2011). It is therefore important to consider

microbial egg infections when trying to predict the effects of a salinity drop on the fauna of the Baltic Sea. Our results suggest that increased rates of *Saprolegnia* infections can cause major difficulties to species of marine origin in reduced salinities.

**Author contribution statement** CK conceived the experiments with TKL providing input on design details and response variables. TKL performed the experiments and analysed the data. TKL wrote the manuscript with CK providing edits and advice.

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**Ethical standard** All applicable national guidelines for the care and use of animals were followed. The experimental procedures were approved by ELLA—the National Animal Experiment Board of Finland.

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