



Acclimatizing laboratory-reared hatchling cod (*Gadus morhua*) to salinity conditions in the Baltic Sea

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ABSTRACT

Decades of overfishing and unsustainable management, together with habitat degradation and eutrophication, depleted the cod (*Gadus morhua*) stocks in the Baltic Sea. Accompanying severe oxygen deficiency and decreased salinity in their spawning grounds restricted successful spawning today to the Bornholm basin, resulting in decreased recruitment. In order for the species to recover, several different measures are required and proposed, among others restocking. We therefore investigated the possibility of producing laboratory-reared cod larvae acclimatized to the current environment in the Baltic Sea. For this, cod were reared from newly fertilized eggs to non-feeding yolk-sac larvae, testing the effect of different salinity reduction treatments during early development on mortality, hatching success, and neutral buoyancy. The results show that a sudden ambient salinity decrease after hatching has no strong effect on survival or hatching (around 60% and 95%, respectively), while it decreased neutral buoyancy of larvae from 18 to minimum 12.5 psu. Lowest buoyancy was reached in treatments with a salinity change in the early egg stage. Gradual salinity decrease starting early in the egg stage yielded to significantly increased mortality and reduced hatching success, but also lowest buoyancy of 12 psu. We showed that a decrease of ambient salinity enables the production of yolk-sac cod larvae with reduced buoyancy, which are potentially better acclimatized to survive in current environmental conditions in the Baltic Sea.

1. Introduction

Atlantic cod *Gadus morhua* (Linnaeus, 1758) is a commercially and ecologically important fish species in and around the North Atlantic Ocean, including the Baltic Sea. After decades of overfishing and unsustainable management, paired with an increase in natural mortality in recent years, the Baltic cod stocks today are depleted (ICES, 2019). While fishing for Eastern Baltic cod is banned since July 2019, the stock biomass and quality of fish remain critically low (Eero et al., 2020). Several factors are thought to influence the bad status of this cod stock, such as reduced spatial overlap between cod and its fish prey (Eero et al., 2012), as well as increased abundance of predators, i.e. grey seals *Halichoerus grypus* (Fabricius, 1791), which also negatively impacts cod through infections with parasites (Harding et al., 2007; Horbowy et al., 2016). However, mainly ecosystem changes are considered as drivers for the drastic change in Eastern Baltic cod productivity.

In the brackish waters of the Eastern Baltic Sea, cod is inhabiting an environment close to its limits regarding salinity and oxygen conditions for successful reproduction (Mackenzie et al., 2007; Hinrichsen et al.,

2011). The environmental conditions in historical spawning grounds of Baltic cod, i.e., the Bornholm, Gdansk and Gotland basins, are to a large extent controlled by so called major Baltic inflow events of saline, oxygen-rich water from the North Sea, as they are the only source of deep-water ventilation. Instead, the impact of increased eutrophication during the last century, leading to increased biomass production, oxygen deficiency, and sedimentation, is identified as a driving factor (Zillén et al., 2008; Conley et al., 2009). Nowadays, nutrient inputs are considerably reduced, but concentrations of nutrients in deeper waters and sediments remain high due to accumulation and can have effects over multiple decades (HELCOM, 2018).

As a consequence of severe oxygen deficiency and decreased salinity in the deep basins, successful spawning of Eastern Baltic cod today is restricted to the Bornholm basin. Requirements for successful fertilization and healthy development of eggs, meaning a salinity of ≥ 11 psu (Westin and Nissling, 1991) and oxygen concentrations of ≥ 2 mL L⁻¹ (Wieland et al., 1994), are currently only met in this basin. Additional reduced parental condition has negatively affected egg production, fertilization and hatching success, along with post-hatch survival

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(Røjbek et al., 2014; Hinrichsen et al., 2016). Accordingly, recruitment production declined, impeding stock recovery.

Measures for cod stock recovery are, among others, reducing bycatches, reducing predator populations, preserving recruitment habitats, seasonal fishing restrictions, and restocking (Bryhn et al., 2022). Several programs attempted to increase wild cod populations by hatching and releasing young fish already since the mid-1880s (Svåsand et al., 2000). The most extensive manipulation of fish recruitment worldwide has been carried out in Flødevigen, Norway (Smith, 2002). This stock enhancement program released over seven billion three to five days old cod larvae between the end of the 19th century and 1971 (Chan et al., 2003). Hatchery-release programs are of recurrent popularity, among others in the Baltic Sea. A previous investigation on the possibility to enhance cod stocks by releases of yolk-sac larvae has been conducted in the Eastern Baltic Sea from 2005 to 2007 (Støttrup et al., 2008) and a similar project (ReCod) is currently being launched in Sweden. Large-scale releases of first-feeding larvae could potentially enhance the spawning stock biomass of Eastern Baltic cod as a long-term solution towards stock recovery. However, it is yet unclear whether released cod larvae, bred in controlled laboratory conditions, have a potential to survive in the low salinity in the Baltic Sea and maintain buoyant or if they will sink into deeper water layers with unfavorable environmental conditions.

In this study, we investigate the effect of ambient salinity changes during egg and early larvae development of Baltic cod on survival, hatching success, and neutral buoyancy. Our aim is to evaluate in a laboratory experiment whether we can acclimatize laboratory-reared yolk-sac larvae to reach neutral buoyancy at salinity levels similar to the central Baltic Sea. Acclimatization of yolk-sac larvae to low salinities has implications for their survival in the Baltic Sea, i.e., their ability to avoid lethal oxygen conditions and reach sufficient feeding conditions. Ultimately, the results of this study will give insight into the possibility to produce and successfully use yolk-sac larvae for stock enhancement efforts of Baltic cod.

2. Material and methods

Animal Research Reporting in Vivo Experiment (ARRIVE) guidelines (Percie du Sert et al., 2020) were used throughout this study.

2.1. Field sampling and maintenance of broodstock fish

The parental cod were caught in February 2021 by trawling in ICES subdivision (SD) 25 (area between 55°41'19.4"N 14°18'11.8"E, 55°28'46.3"N 14°36'21.3"E and 55°46'37.6"N 16°03'41.8"E), corresponding to the Eastern Baltic cod stock. Fish were transported to the research station Ar, Gotland, Sweden, where they were kept in a 15 m³ tank with recirculating seawater (7 psu, 12 °C, DO: 12 mg/L, pH: 7.5) and a natural light cycle. The seawater in this closed system was filtered by a Recirculating-Aquaculture-System (RAS) with a turnover time of 1×/h and a daily water intake of 3%. The broodstock comprised 40 cod ranging in size and weight from 45 to 66 cm total length (mean: 54 ± 0.8 (SE) cm) and 1028–3078 g (mean: 2108 ± 87.6 (SE) g), respectively. As cod has no apparent sexual-dimorphism outside of the spawning, fish were characterized as gravid females when abdominal swelling could be observed during spawning. About 2/5 of the fish were accordingly characterized as spawning females. Fish were fed daily to satisfaction, alternating with Atlantic herring *Clupea harengus* (Linnaeus, 1758), European sprat *Sprattus sprattus* (Linnaeus, 1758) or northern prawn *Pandalus borealis* (Krøyer, 1838).

2.2. Induction of spawning

To induce spawning of the breeding cod, the photoperiod was manipulated, as light is the main factor influencing the spawning status (Hansen et al., 2001). For this, the annual light cycle was shortened from

twelve to nine months, starting in September 2021 with 12 h of light exposure. Hence, the shortest day with 6.5 h light exposure was two months later, in November, and the longest day with 18 h light exposure was after six months, in March 2022, when the cod started to spawn. The salinity in the breeding tanks was increased by adding synthetic sea salt (Hoss) one month prior to spawning in steps of 1 psu/day until 17 psu was reached. At this salinity fertilized eggs are positively buoyant and float whereas unfertilized or dead eggs sink. Other water quality parameters, e.g., temperature, pH and DO, were kept constant. Concurrently the diet of broodstock fish was changed to low-fat high-protein, i. e., solely northern prawn, as this is beneficial for reproductive performance (Gunasekera et al., 1996; Coldebella et al., 2011).

2.3. Sampling and maintenance of fish eggs and larvae

Fertilized eggs were collected daily from the water surface in the broodstock tank via a floating airlift egg collector (Ohs et al., 2019) and placed in a 5 L beaker filled with seawater (17 psu, 7 °C, DO: 12 mg/L). Ten eggs from the collected daily batch were observed under a stereo microscope to assess the batch quality and embryonic development stage according to Hall et al., 2004. Batches with minimum nine out of ten living eggs without deformities were used for the experiment. The average experimental batch contained eggs in stage 6.4 ± 0.7 (SE), corresponding to the 16-cell stage of the cleavage period, about 810 min post fertilization, of which not one had deformities.

2.4. Experimental set-up

Ten different treatments were tested to find the most suitable way to adapt yolk-sac cod larvae fertilized in 17 psu to 7 psu seawater (Table 1). In one-time acclimatization treatments a salinity of 7 psu was reached in the beaker with one water change, whereas for gradual acclimatization treatments the salinity in the beaker was lowered slowly in steps. One-time acclimatization treatments comprised treatments with a salinity change within 24 h after fertilization, 8, 10, 12, 14 and 16 DPF (Days-Post-Fertilization), respectively. Gradual adaptation treatments include starting the salinity decrease within 24 h after fertilization (AF), after gastrulation (AG), i.e., 3 DPF, close to estimated hatching 8 DPF, and after first hatching. The control group (C) did not undergo a salinity change and stayed in 17 psu seawater throughout the experiment.

From batches with a good quality 100 eggs each were incubated in 400 mL beakers filled with water of the respective salinity, prepared from filtered seawater (0.2 µm) and synthetic sea salt (Hoss) for a total of 16 days. Each treatment was replicated 9 times, i.e., once in each of nine daily batches collected between March and May. Dead eggs and larvae, as well as egg shells were siphoned from the bottom daily and their respective numbers noted. Further, 2/3 of the water was changed daily and temperature, salinity, and oxygen content were measured (WTW Multi 3420; temperature accuracy: ± 0.5 °C, conductivity accuracy: ± 1.0% of measured value, D.O. concentration accuracy: ± 0.03 mg/L). For one-time acclimatization treatment 16 DPF the final water change occurred 1 h before the end of the experiment.

Table 1

Treatments used for the salinity acclimatization experiment, organized according to treatment intensity.

One-time acclimatization (OTA)	Gradual acclimatization (GA)
AF: 17 psu – 7 psu after fertilization	1AF: 1 psu/day after fertilization
D08: 17 psu – 7 psu 8 DPF	1AG: 1 psu/day after gastrulation
D10: 17 psu – 7 psu 10 DPF	2D8: 2 psu/day after day 8
D12: 17 psu – 7 psu 12 DPF	2H: 2 psu/day after hatching
D14: 17 psu – 7 psu 14 DPF	
D16: 17 psu – 7 psu 16 DPF	

C: control 17 psu.

2.4.1. Survival and hatching

The number of living eggs was calculated for every day of the experiment by subtracting the number of dead eggs taken out from the number of surviving eggs from the previous day, and the number of hatched larvae was counted. Percentage of daily egg mortality was calculated relative to the number of eggs at the start of the experiment, whereas the percentage of hatching was calculated relative to the number of surviving eggs at the respective time point in the experiment.

2.4.2. Buoyancy

After the experiment, i.e., 16 DPF, ten larvae from each treatment were sedated with 50 mg/L benzocaine for 10 min and transferred to density gradient columns (height: 75 cm, volume: 1.6 L) with salinity ranging from 5 to 31 psu. After 10 min in the columns, the positions of the larvae were compared to the positions of nine density floats of known specific gravity (Coombs, 1981). The linearity between specific gravity of the density floats and their positions was high for all measurements ($R^2 \geq 0.95$).

2.4.3. Body length

Ten larvae from each treatment were observed under a stereo microscope after the experiment, photographed (IC Measure, version 2.0.0.286) and their total length was measured.

2.5. Statistical analysis

The Cox mixed-effects model was used to test for significant difference between risk of death of fish larvae in different treatments and batches. For this, every individual larva was treated as an individual observation. Plotted scaled Schoenfeld residuals showed homoscedasticity, i.e., no variation over time for either variable, indicating proportional hazards ratio over time. Random effects, i.e., unobserved covariates, were incorporated by including batch, as well as the interaction between treatment and batch to account for the fact of larvae from each batch per treatment being placed in the same beaker as an additional source of variability. A comparison of the model including the random effects with models excluding them revealed that both random effects have a significant effect on the model, and therefore need to be included (analysis of variance: $F_{(1)} = 27.93$, $p \leq 0.001$ and $F_{(1)} = 614.35$, $p \leq 0.001$, respectively). The AIC (Akaike Information Criterion) value is lower for the integrated log-likelihood model than for the penalized model (1710.44 vs 1891.17), while the BIC (Bayesian Information Criterion) is higher (1630.14 vs 1208.72). As we prioritize model fit over model simplicity, the integrated model was used in this study.

To compare overall hatching, i.e., first hatching, time when $<50\%$ and time when $<80\%$ of surviving larva hatched, and total hatching success 16 DPF, of different salinity treatments, the nonparametric Kruskal-Wallis test followed by a Dunn-Bonferroni post-hoc test was used, as Shapiro-Wilk's test revealed non-normal distribution of data ($p < 0.05$). The standard error is reported along with mean values of first hatching and hatching success.

For buoyancy and length data, Brown-Forsythe's test approved the null hypothesis of equal variances ($p = 0.2$ and $p = 0.71$, respectively) and normality was approved by the Shapiro-Wilk test for all groups ($p = 0.07$ and $p = 0.09$, respectively). One-way analysis of variance was therefore used as a parametric test for significant differences between the treatments, followed by a Dunn-Bonferroni post-hoc test to compare the groups in pairs to find out which was significantly different. The standard error is reported along with mean values of length. One-way analysis of variance was also used to test for significant differences in length between batches.

The relationship between larva neutral buoyancy and body length was tested with a linear regression followed by a mixed linear model with treatment and length as fixed effects and the interaction between length and treatment as random effects to investigate the effect of length

on buoyancy. A likelihood ratio test comparing the model including the random effect with a model excluding it revealed that the random effect has a significant effect on the model, and is therefore included ($X^2_{1,110} = 26.5$, $p \leq 0.001$).

3. Results

3.1. Survival

As expected, survival of Baltic cod eggs and larvae decreased over time in all treatments (Fig. 1). In the control group, a maximum of 78 (mean: 61.6 ± 3.6) % of eggs and larvae survived until 16 DPF. Treatments with a one-time salinity decrease at or after expected hatching, i.e., OTA-D10, OTA-D12, OTA-D14 and OTA-D16, followed a similar survival curve as the control group. Among all treatments, lowest overall survival with a maximum of 41 (mean: 27.9 ± 3.5) % was observed in treatment OTA-AF, which was exposed to a strong salinity decrease within 24 h after fertilization. After expected first hatching around 10 DPF, mortality increased in most treatments, while this trend was strongest in treatments with a salinity decrease before 10 DPF, i.e., from the egg stage on, namely GA-1AF, GA-1AG, GA-2D8 and OTA-D08. Survival is affected by treatment and differs between batches ($X^2_{12,11,500} = 1734.44$, $p = 0$). In summary, there was an overall significantly higher risk of death in treatments with a decrease in ambient salinity in the egg stage (Table 2).

3.2. Hatching

Larvae in the control group first hatched 10.2 ± 0.3 DPF and latest first hatching occurred in treatment OTA-AF at 11.6 ± 0.5 DPF. Differences in time of first hatching between treatments are however not statistically significant ($H_{10,110} = 15.1$, $p = 0.128$). At 12.69 ± 0.2 days after fertilization, $\geq 50\%$ of surviving larvae have hatched in the control group, which is similar to other treatments, with the exception of GA-1AF and OTA-AF ($p \leq 0.05$). The majority of the surviving larvae, i.e., $\geq 80\%$, have hatched in the control group 13.69 ± 0.2 days after fertilization, which is similar to the treatments OTA-D16, OTA-D14, OTA-D12, and GA-2H ($p = 1$). Other treatments didn't result in an average hatching of $\geq 80\%$. On average $97 \pm 1.7\%$ of surviving larvae have hatched in the control group 16 DPF, while lowest hatching success of $29.2 \pm 5.7\%$ was observed in treatment OTA-AF (Fig. 2). A pairwise post-hoc Dunn test with Bonferroni adjustments indicates that hatching success of the OTA-AF and GA-1AF treatment is significantly lower than in treatments resulting in on average $> 95\%$ hatching success, i.e., the control group, OTA-D14, OTA-D16, and GA-2H ($p \leq 0.003$). However, GA-1AF, which results in $61 \pm 5\%$ hatching success, is not significantly different from OTA-AF.

3.3. Buoyancy

The larvae 16 DPF from the control group were neutrally buoyant at a minimum of 14.8 (mean: 18.3 ± 0.3) psu, which was similar to larvae from the OTA-D16 treatment, 1 h after decreasing salinity (Fig. 3). The one-factorial ANOVA indicated a significant effect of treatment on buoyancy ($F_{10,110} = 52.58$, $p \leq 0.001$). Larvae that experienced either a gradual or a sudden salinity decrease early on in the egg stage or prior to expected hatching 10 DPF, i.e., treatments GA-1AF, GA-1AG, GA-2D8, OTA-D08, OTA-D10 and OTA-AF, yielded lower neutral buoyancy scores than larvae from the control group or treatment OTA-D16 ($p \leq 0.001$). While the treatments with early salinity decreases did not differ statistically regarding neutral buoyancy of larvae, GA-1AF resulted in lowest overall neutral buoyancy at a minimum of 9.8 (mean: 11.9 ± 0.2) psu. The treatments OTA-D14 and GA-2H don't show significant differences among each other and all result in intermediate buoyancy levels between on average 13.5 and 15.5 psu. However, these treatments are statistically different from treatments with a salinity decrease prior to

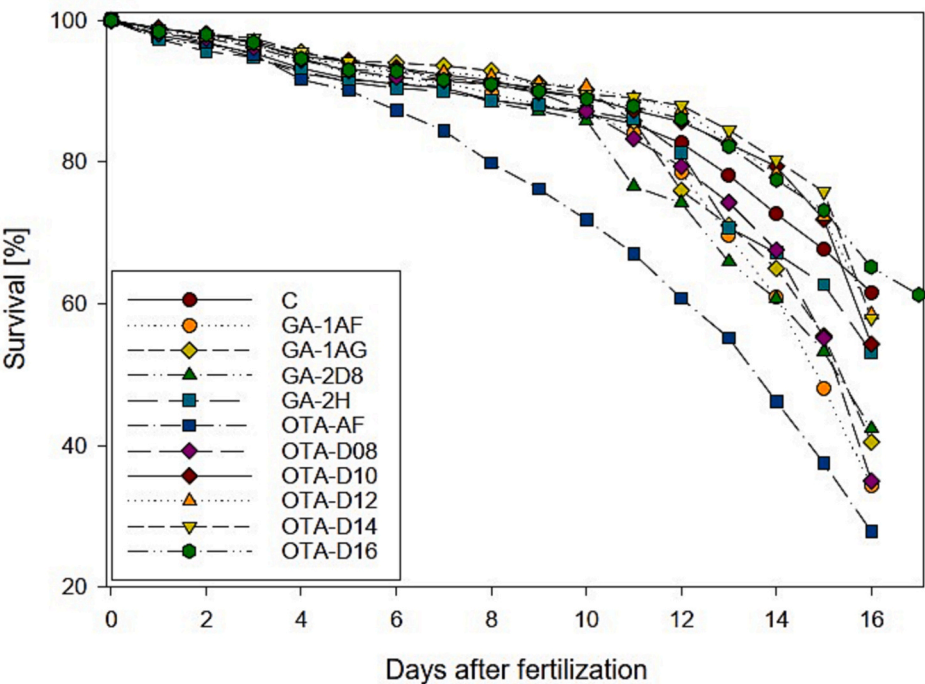


Fig. 1. Survival of Baltic cod eggs and larvae over time. Shown are mean values of different treatments.

Table 2
Cox mixed-effect model predicting event-free survival in cod larvae exposed to different ambient salinity reduction treatments ($n = 11,500$, number of events = 5956, iterations = 1790). Significance codes: 0 ‘****’ 0.001 ‘***’ 0.01 ‘**’.

Characteristic	Coefficient	Hazard ratio (\pm SE)	P value
<i>Treatment</i>			
Control	Reference		
GA-1AF	0.73	2.08 (0.17)	<0.001 ***
GA-1AG	0.60	1.80 (0.17)	<0.001 ***
GA-2D8	0.64	1.90 (0.16)	<0.001 ***
GA-2H	0.29	1.33 (0.17)	<0.001 ***
OTA-AF	1.16	3.20 (0.18)	<0.001 ***
OTA-D08	0.63	1.87 (0.17)	<0.001 ***
OTA-D10	0.13	1.13 (0.17)	0.47
OTA-D12	0.02	1.02 (0.18)	0.89
OTA-D14	-0.02	0.98 (0.18)	0.93
OTA-D16	-0.20	0.82 (0.18)	0.25

hatching, which result in lower neutral buoyancy, as well as from the control and OTA—D16, which had higher neutral buoyancy ($p \leq 0.001$).

3.4. Body length

Larvae of the control group were smaller than larvae that experienced a salinity decrease (Fig. 4). On average, the control larvae reached a total length of 4.4 ± 0 mm whereas larvae from treatments that include a decrease in salinity prior to 16 DPF reached between 4.7- and 4.9 mm. The one-factorial ANOVA indicated a significant effect of treatment on length ($F_{10, 110} = 12.72, p \leq 0.001$). Post hoc comparison using the Bonferroni test indicated that the control group was significantly smaller than all treatment groups ($p < 0.001$). The length of larvae exposed to the salinity reduction treatments did not differ significantly between the treatments. Additionally, no differences between batches were statistically significant ($F_{1, 110} = 3.47, p = 0.06$). A regression of larva body length and neutral buoyancy suggest a negative relationship ($y = 44.76 - 6.37 \cdot x, R^2 = 0.24$), i.e., larger larva having a lower neutral buoyancy (Fig. 5). However, in a mixed linear model with treatment and length as fixed effects, the effect of length on

buoyancy is not statistically significant, while the effect of the different treatments is ($p = 0.63$ vs $p \leq 0.001$, Table 3). This indicates a strong relationship between treatment and buoyancy, potentially masking an effect of body length.

4. Discussion

Restocking efforts by hatching and releasing of fish larvae have previously been introduced as a method to strengthen depleted cod stocks in the Baltic Sea (Støttrup et al., 2008). In this study, we investigated the effect of ambient salinity changes during egg and early larvae development of Baltic cod on survival, hatching success, and neutral buoyancy. Our results reveal the possibility to produce yolk-sac larvae in the laboratory, which are better acclimatized to salinity conditions in the Baltic Sea, and hence have potential for successful restocking efforts of Baltic cod. A sudden decrease in salinity from 17 to 7 psu 10 DPF, i.e., at first hatching, was shown to be the most promising approach.

4.1. Larval condition in hatchery

In this study, we found an average survival of 62% at 16 DPF in eggs and larvae incubated in 17 psu throughout the experiment, i.e., the control group. While mortality was particularly low in the egg stage, mortality increased with the onset of hatching, starting 10 DPF; > 50% hatched 13 DPF. Hatching success was on average 97%. A previous study on survival of Baltic cod eggs fertilized in 17 psu and transferred shortly after to a similar salinity of 15 psu until 15 DPF found a much lower survival of about 35% (Nissling and Westin, 1991). In contrast to our study, the authors also recorded a strong increase in mortality during the first three days of egg incubation, until gastrulation, of up to 40%. High mortality in the early egg stage has been reported for several cod populations (Laurence and Rogers, 1976; Iversen and Danielssen, 1984; Kjorsvik et al., 1984), including the Baltic cod (Grauman, 1973). However, survival during the egg stages in our experiment stayed at >90%. The higher early egg survivability, but also overall survivability of eggs and larvae in our study, is likely linked to endogenous factors, such as good parental condition prior to spawning. While exogenous factors, such as temperature, salinity, oxygen concentration and light,

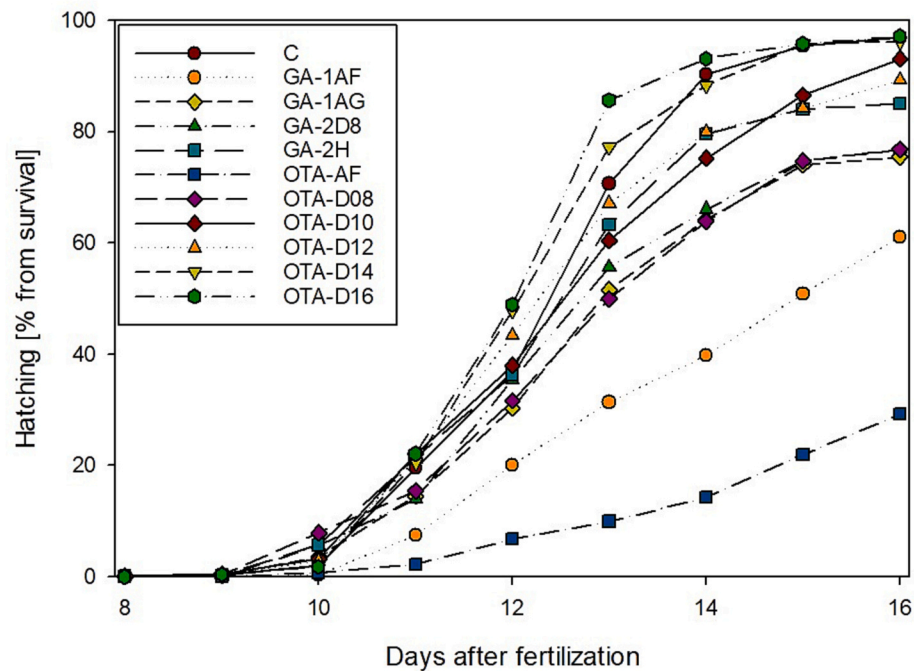


Fig. 2. Hatching of Baltic cod larvae over time. Shown are mean values of different treatments.

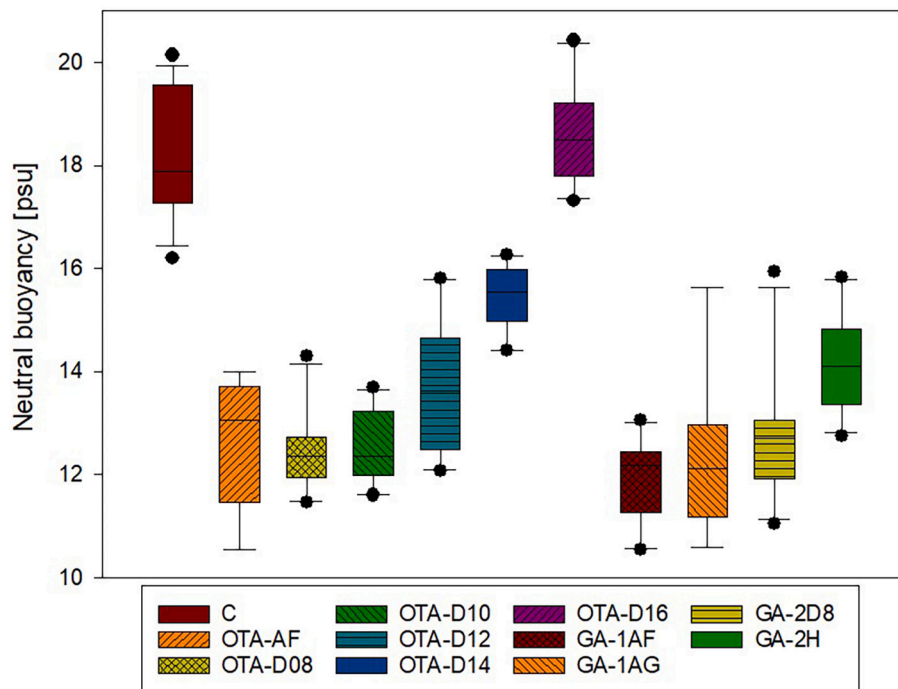


Fig. 3. Buoyancy of Baltic cod larvae 16 DPF in different treatments. The box represents the interquartile range with median, upper and lower whiskers represent scores outside the middle 50%, dots represent the 5th/95th percentile.

didn't differ in our experimental set-up and the one used by [Nissling and Westin \(1991\)](#), these authors obtained eggs via stripping, whereas parental fish used in our study were let spawning naturally. The act of handling fish, especially in combination with stripping, causes stress, which may negatively impact the eggs.

An increase in mortality around hatching ([Iversen and Danielssen, 1984](#); [Laurence and Rogers, 1976](#)), as well as generally high mortality in the larval stage ([Avery et al., 2009](#); [Stiasny et al., 2016](#)) have been previously reported and align with our results. The main reasons for

high mortality in this stage is believed to be the difficulty in successful feeding ([Llopiz et al., 2014](#)). Larvae in our experiment however still possessed their yolk-sac and were feeding mixed endogenously while approaching the onset of exogenous feeding. During the first days after hatching, total mortality of marine fish larvae has been observed to be as high as 43%, with 35% mortality occurring during the period of exclusively endogenous feeding, mainly affecting small larvae ([Garrido et al., 2015](#)). Potential reasons for this pattern could be metabolic differences during early embryonic development, meaning small size-at-hatch

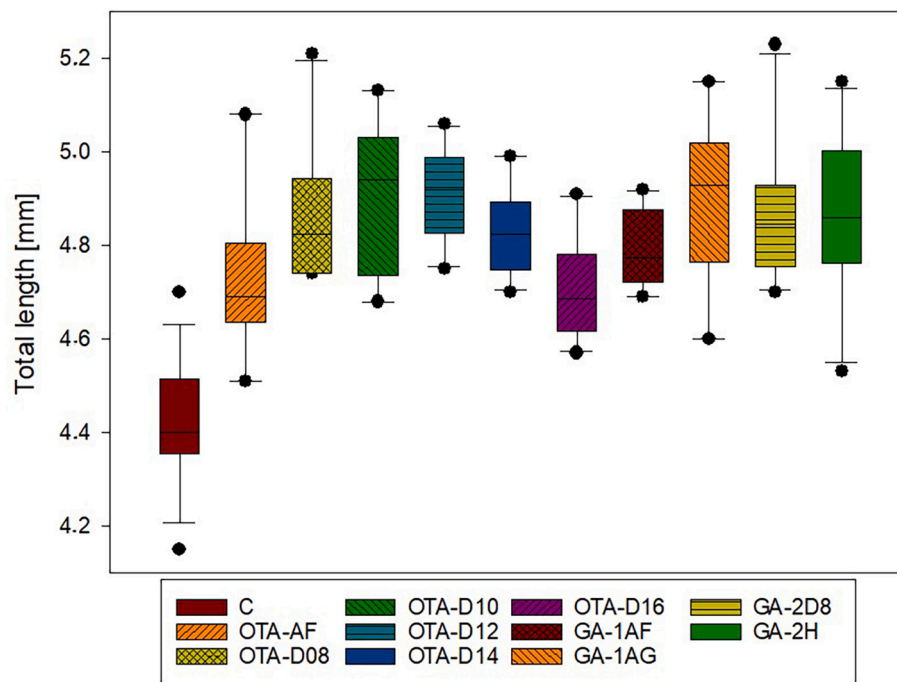


Fig. 4. Total length of Baltic cod larvae 16 DPF in different treatments. The box represents the interquartile range with median, upper and lower whiskers represent scores outside the middle 50%, dots represent the 5th/95th percentile.

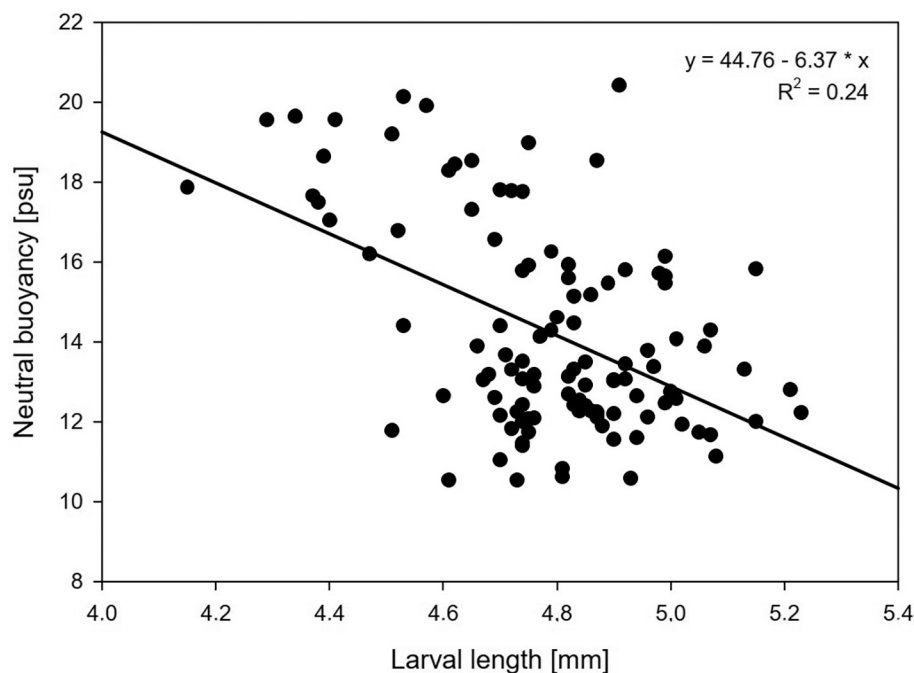


Fig. 5. Scatter plot and linear regression of cod larval length [mm] vs neutral buoyancy [psu].

leading to disadvantages in growth and survival due to metabolic limits and high development costs in young larvae, or small larvae being below an existing threshold of yolk reserves, hindering embryonic or early life development (Garrido et al., 2015).

Larvae raised in 17 psu were neutrally buoyant at 18 psu, while individuals ranged from 15 to 25 psu. Other studies working with Baltic cod eggs fertilized in 17 psu found them to be neutrally buoyant at a much lower salinity of 14.5 ± 1.2 psu (Nissling and Westin, 1991; Nissling et al., 1994a). These studies however obtained eggs via stripping cod used to 7 psu sea water, while the parental fish in our study were

acclimatized to 17 psu sea water prior to spawning. Since regulatory processes within the ovary of the parental fish affect egg buoyancy (Nissling et al., 2003), a neutral larval buoyancy similar to the maternal ambient salinity conditions is plausible. Egg density varies between batches from different females and between batches from the same female (Nissling and Westin, 1991), which explains the consequential high variation in larval density observed in this study. Since eggs for this experiment were collected from a naturally spawning broodstock kept in a shared tank, batches collected daily from the water surface will likely have originated from different females in different stages of spawning.

Table 3

Mixed linear model predicting neutral buoyancy in cod larvae with treatment and length as explanatory variables and the interaction of treatment*length as a random effect ($n = 110$). Significance codes: 0 '****' 0.001 '***' 0.01 '**'.

Variable	Estimate	Std. error	t value	Pr(> t)
<i>Treatment</i>				
Control	16.51	3.70	4.46	<0.001 ***
GA-1AF	-6.51	0.58	-11.23	<0.001 ***
GA-1AG	-6.10	0.64	-9.58	<0.001 ***
GA-2D8	-5.71	0.61	-9.30	<0.001 ***
GA-2H	-4.31	0.61	-7.05	<0.001 ***
OTA-AF	-5.57	0.56	-9.94	<0.001 ***
OTA-D08	-5.99	0.63	-9.57	<0.001 ***
OTA-D10	-5.92	0.62	-9.49	<0.001 ***
OTA-D12	-4.82	0.64	-7.56	<0.001 ***
OTA-D14	-2.99	0.60	-5.00	<0.001 ***
OTA-D16	0.23	0.55	0.42	0.67
<i>Length</i>	0.40	0.83	0.48	0.63

The total length of larvae was on average 4.4 mm, showing high within-group variation ranging from 3.8 to 5 mm. This range corresponds with the expectable length of cod larvae between hatching and the first-feeding stage (Kane, 1984; Marteinsdottir and Steinarsson, 1998). Differences in individual length are likely due to different age of individual larvae. Hatching mainly occurred between 10 and 14 DPf, resulting in larvae being anywhere between 2 and 6 days old by the end of the experiment.

4.2. Effects of different salinity reduction treatments on key developmental parameters

The different treatments resulted in survival patterns similar to the control group, showing a strong increase in mortality with the onset of hatching. While survival during the egg stage did not differ between treatments, the increase in mortality with hatching was strongest in treatments with a salinity decrease before hatching (10 DPf), from the egg stage on. A decrease in ambient salinity at or after hatching however resulted in survival ($\geq 53\%$) and hatching success ($\geq 85\%$) similar to the control group. Treatment OTA-AF, which comprised of a strong salinity decrease within 24 h after fertilization, formed the exception. The number of surviving eggs and larvae decreased from the start of the experiment, resulting in lowest overall survival of 28%. This treatment also showed latest first hatching at 12 DPf, going along with lowest hatching success of 29% of surviving eggs. In a comparable treatment of a salinity reduction experiment on Baltic cod eggs 15 DPf, Nissling and Westin (1991) observed an increase in number of deformities in the late egg stage, resulting in low hatching success of about 5%, along with low survival of about 7%.

Studies on the salinity tolerance of eggs from other cod populations, i.e., Atlantic and Belt Sea cod, found that eggs transferred to lower salinities shortly after fertilization died before gastrulation or closure of the blastopore (von Westernhagen, 1970; Kjorsvik et al., 1984). However, if eggs were transferred after gastrulation, they were able to develop and hatch (von Westernhagen, 1970). While this pattern is not reflected regarding survival in our study, hatching success was clearly higher in treatments with a salinity decrease after gastrulation. Water exchange with the environment is minimized after fertilization, as water permeability of the vitelline membrane surrounding the yolk is low (Riis-Vestergaard, 1984; Mangor-Jensen, 1987). After gastrulation, when the epiboly is completed, the permeability of the vitelline membrane increases, and functioning chloride cells are present in the embryo, which enable the ingestion of ambient water and excretion of salt (Alderice, 1971; Riis-Vestergaard, 1984; Mangor-Jensen, 1987). This mechanism leads to a decrease in specific gravity until just prior to hatching (Nissling and Vallin, 1996) and coincides with the timing of increasing tolerance towards low salinities observed in this and previous studies. Observed differences between batches are likely due to high

variation in mortality and quality of eggs from different individual females (Nissling et al., 1994b).

Treatments with a salinity decrease early on in the egg stage or prior to hatching, 10 DPf, yielded to lower neutral buoyancy than larvae from the control group or treatments with salinity decreases in the early larval stage. This study clearly shows that acclimatization to new environmental conditions takes time. One hour after being exposed to a lowered salinity, no difference in neutral buoyancy of cod larvae could be observed. Two, four, and six days after the ambient salinity decrease, neutral buoyancy of 18 psu decreased by 3-, 5-, and 6 psu, respectively. Lowest neutral buoyancy of 12 psu was observed in treatments with a salinity decrease in the early egg stage or prior to hatching. Nissling and Vallin (1996) previously showed that specific gravity of newly hatched cod larvae differs due to incubation salinity, with gravity being lowest in lowest incubation salinity. Incubation of cod eggs at salinities $\geq 10.6 \pm 0.8$ psu [yolk osmolality of unfertilized Baltic cod eggs (Westin and Nissling, 1991)], e.g., 17 psu as in this study, is equivalent to hyper-osmotic conditions, leading to water loss, and consequently higher buoyancy (Nissling and Vallin, 1996). Hence, lower incubation salinity such as 7 psu leads to an inflow of water and a decrease in neutral buoyancy.

The total length of larvae was higher than in the control group for all treatments. Nissling and Vallin (1996) observed different height of cod larvae depending on incubation salinity, suggesting differences in subdermal spaces, i.e., different dilute fluids contents, which are known to affect buoyancy of pelagic fish larvae (Shelbourne, 1956). While the larval length didn't differ in their study, larval height increased significantly with decreasing ambient salinity. These observations combined with our results of larva body length being negatively correlated with buoyancy, indicate an effect of larval size on neutral buoyancy of cod larvae. However, larval size only explained about 24% of variance in buoyancy, meaning that other factors related to the different treatments influence neutral buoyancy as well.

4.3. Implications for stock enhancement

Nowadays, there are only a few places in the Baltic Sea with the required oxygen and salinity levels for cod to succeed in their reproduction, which make it difficult for an overfished and weakened stock to recover. Generally, mortality of cod eggs in the wild is high, and even small variations in mortality rate can have a big impact on year-class strength. In the Baltic Sea, early estimations on cod egg survival were correlated with hydrographical conditions and ranged from 1 to 21% (Grauman, 1973, 1974). In today's last remaining spawning ground, the Bornholm basin, daily egg mortality is estimated to be around 27%, resulting in an overall mortality of about 99.9% (Wieland, 1988).

Investigations in the Western Baltic Sea found a mortality of ~50% from successively released eggs in early life stages of Eastern Baltic cod (Hüssy et al., 2016), which was caused almost exclusively by bottom contact due to low environmental salinity and high egg buoyancy (Petereit et al., 2014). Mainly an increase in sedimentation and exposure to detrimental oxygen concentrations close to the seafloor are expected to result in reduced egg and early larval survival of Baltic cod (Hinrichsen et al., 2012; Petereit et al., 2014). Thus, the ability to maintain buoyancy is crucial for yolk-sac cod larvae that are to be released to the Baltic Sea in order to successfully strengthen the local cod stock.

We showed that a decrease of ambient salinity in the hatchery enables the production of yolk-sac cod larvae with reduced neutral buoyancy approaching environmental conditions in the Baltic Sea, while not affecting survival or hatching success negatively. As the vertical distribution of marine fish eggs and early larvae is known to be mainly influenced by ambient water density, turbulent mixing via wind, and individual buoyancy (Sundby, 1991), being able to manipulate the latter is the only promising approach to acclimatize laboratory-reared fish larvae to the wild. Body structures that can influence the larvae's specific location in the water column, i.e., the swim bladder, fins and

muscles, are fully functional at about 10 days post hatch in cod (Hunt von Herbing et al., 1996; Nissling and Vallin, 1996), which is 4 days after the end of our experiment and after previously mentioned release efforts. Thus, early yolk-sac larvae are relying strongly on their neutral buoyancy to be similar to the destined water depth in order to stay buoyant and avoid sinking. Maintaining the position in the water column during the mixed feeding period, i.e., from 4 to 8 days after hatching in Baltic cod (GrønkJaer and Wieland, 1997), is further highly energetically costly in times when the swim bladder not yet fully developed. Energy conservation is essential during this early time, as marine fish larvae have very low initial feeding rates (Yin and Blaxter, 1987), while being fully relying on exogenous feeding after completion of the yolk-sac. With ongoing larval development, yolk-sac consumption and formation of body structures, the larval specific gravity depends increasingly on their own body density, which is influenced by the environment rather than maternal or egg characteristics (Saborido-Rey et al., 2003). From this stage onwards, fish larvae are able to maintain buoyant in their respective environment and adjust to it naturally.

When testing different salinity reduction treatments to find the most suitable approach to produce cod larvae for release efforts, a sudden decrease in salinity from 17 to 7 psu 10 DPf, i.e., at first hatching, yielded the most promising results. While reducing ambient salinity in the egg stage leads to a strong increase in mortality and reduced hatching success, reducing ambient salinity just a few days after hatching had limited effects on larval neural buoyancy. Hence, we propose a reduction of ambient salinity at first hatching as a way to produce cod larvae for hatch and release efforts, which are potentially better acclimatized to survive in current environmental conditions in the Baltic Sea and avoid lethal oxygen conditions. Ultimately, large-scale releases of first-feeding larvae acclimatized to the Baltic Sea can have a higher chance of success than previous trials and could potentially enhance the spawning stock biomass of Eastern Baltic cod as a long-term solution towards stock recovery.

Future studies should investigate the effect of a lower ambient salinity for the broodstock on key larval parameters, such as survival and buoyancy. Egg density, i.e., buoyancy, is regulated through the process of oocyte hydration within the ovary of the parental fish (Nissling et al., 2003) in order to adjust the egg buoyancy to ambient salinity conditions experienced by the spawning adults (Goarant et al., 2007). Consequently, keeping the broodstock in a salinity <17 psu but ≥11 psu, that is still sufficient for fertilization and healthy development of eggs (Westin and Nissling, 1991), can potentially lower the mortality of the eggs and larvae during salinity acclimatization and increase the success of acclimatization treatments.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Neele Schmidt reports financial support was provided by BalticWaters.

Data availability

Data will be made available on request.

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