

Water salinity effects on embryogenesis of the lesser sandeel, *Ammodytes tobianus* (Linnaeus, 1758)

Research Article

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Abstract: Effects of water salinity (3.0-9.0 PSU) on the duration and outcome of embryogenesis in the lesser sandeel (*Ammodytes tobianus* (L.)) as well as on survival, size, and malformation rate of the newly hatched larvae were followed, based on observations involving equipment consisting of a microscope and still and digital cameras. The images obtained and measurements of egg and larval dimensions were analyzed using the NIS elements Br. computer software package. The lowest salinity tested (3.0 PSU), similar to that in the inshore Baltic Sea waters native for the species studied, produced a lower fertilization rate and resulted in extended embryogenesis, reduced embryo survival, changes in the hatch size, and increased larval malformation rate. The high-end salinity (7.5-9.0 PSU), higher than that prevailing in the species's spawning grounds in the Baltic Sea, resulted – similarly to the low-end salinity – in disturbed development and growth of the embryos.

Keywords: Salinity • Fertilization • Eggs • *Ammodytes tobianus*

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1. Introduction

The lesser sandeel (*Ammodytes tobianus* (L.)), a representative of the family Ammodytidae, is one of the 26 marine fish species occurring in the Baltic Sea. The species inhabits coastal areas of European seas, from the Bay of Biscay to the Barents Sea; it is found off the British Isles, Iceland, Norway, and in the Baltic Sea. The lesser sandeel are small, spear-shaped, and very motile; they form schools [1]. They live down to the depth of 30 m, close to or at the sandy bottom. The fish stay buried in the sand during the day and emerge at night. Depending on the location, the lesser sandeel spawn in autumn-winter (the English Channel) to spring-summer (the North

Sea). In the Baltic Sea the spawning of the lesser sandeel takes place in the summer and the late autumn [1,2]. The lesser sandeel spawn in the nearshore zone; the female lays 4-22 thou. eggs, in small clumps of a few egg each, on the sandy bottom. The eggs measure 0.8-1.0 mm in diameter [3]. The larvae are pelagic; the newly hatched larvae are, on the average, 4.0-8.0 mm long [1].

As shown by earlier studies on, e.g., herring (*Clupea harengus* L.), lump sucker (*Cyclopterus lumpus* L.), cod (*Gadus morhua*), gilthead seabream (*Sparus aurata* L.) [7]; white halibut (*Hippoglossus hippoglossus* L.), obscure puffer (*Takifugu obscurus*), and Brazilian flounder (*Paralichthys orbignyanus*) (Valenciennes, 1839), differences in the concentration of minerals

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in water, including salinity, are important for how the embryogenesis proceeds and for the size of newly hatched larvae [4–10].

The Baltic Sea is a salt-water Mediterranean sea, therefore some fish species occurring there are migratory species from the North Sea. Most of the Baltic Sea shows the salinity ranging within 5.0–8.0 PSU, for which reason the Baltic water is too freshened for the truly marine fish fauna and too salty for the freshwater fish. The salinity range of 5.0–8.0 PSU is frequently referred to as the “minimum species richness zone”, as the lowest species richness is encountered in a region where the salinity grades from fresh to fully marine.

Over millennia, the typically marine fish species living in the Baltic have gradually adapted to the reduced salinity. The adaptation involved size reduction: the local races of a species are smaller than their conspecifics inhabiting more saline waters.

The Baltic Sea is a sea where the influx of salty ocean water is hindered and the water supply from rivers and precipitation waters is considerable, therefore its average salinity amounts to only ca. 7.0 PSU. The further away from the Danish Straits, the more the salinity level decreases. In the Bay of Puck the salinity drops to 6.2 PSU, and in the Vistula Lagoon it equals only 1.0 – 3.0 PSU, whereas in the Gulf of Finland and the Gulf of Bothnia it amounts to mere 2.0 PSU.

The North Sea feeds the Baltic Sea with salty water in a very irregular manner. Sometimes the feeds take place every several months and sometimes every several years or several dozen years, which may result in the freshening of the Baltic Sea.

The lesser sand eel is very common along the Baltic Sea coast, where its spawning grounds are located. It lays its eggs on the sea bottom, up to 30 m deep, where the salinity level is lower than in the deeper areas. Because there are differences in salinity levels in various parts of the Baltic Sea along the Polish coast, the breeding of the discussed fish species must take place in waters characterized by various salinities. Therefore, in light of the above facts it was decided to check whether freshened waters originating from a considerable influx of river waters into the Baltic Sea (and the lowering of the degree of water mineralization), and the presence of waters characterized by salinity levels exceeding 7.0 PSU (as found in the Danish Straits) constituted factors restricting the breeding of the lesser sandeel.

2. Experimental Procedures

The study was carried out in May 2011. The lesser sandeel eggs and sperm were extracted from adult

spawners caught in the Pomeranian Bay (53°58'5.83"N; 14°30'11.81"E). The eggs were collected from 4 females (mean length 14.25 cm), whereas the sperm was provided by 6 males (mean length 12.18 cm).

The eggs and sperm were transported, in separate containers placed in thermoses, to the isothermal laboratory of the Department of Hydrobiology, Ichthyology and Biotechnology of Reproduction, where the eggs were fertilized using the “dry method” - this method induces the female and male fish to release their eggs and sperms, respectively, through ‘hand-stripping’ under dry conditions (which is how it got its name).

After insemination, eggs were placed in the water originating from the location where spawners were collected (water from the Pomeranian Bay) – control, K, salinity 6.5 PSU and in salt solutions mimicking salinities of 3.0 (treatment A); 6.0 (B); 7.5 (C); and 9.0 PSU (D).

The salt solutions were prepared with sea salt (hw-Meersalz Professional) and deionised (ultra-clean) water. Salt dissolution was facilitated by using a magnetic stirrer (MS HS, Vigo). The concentration of minerals in the water used in the experiment was assayed (Table 1) using a Dione ICS 3000 ionic chromatograph at the Department of Hydrochemistry and Water Protection.

The fertilized eggs were incubated in 0.5 dm³ glass beakers filled with treatment water (number of eggs incubated - Table 1). To ensure appropriate dissolved oxygen content in the water, aerators were placed in the beakers. The water was changed as the embryogenesis progressed; the water temperature was kept at 11.0±0.2°C.

Individual stages of embryogenesis were observed under a Nikon ECLIPSE TE-2000S stereomicroscope, and were recorded with an image recording system (a Nikon Coolpix 5400 digital still camera, SONY video recorders, a computer) coupled with the microscope. The microscope was equipped with a stage cooling system ensuring an optimal, constant temperature during observations. The recorded images were analyzed using a NIS Elements Br image analysis system which made it possible to, *e.g.*, measure the eggs, their internal structures, and the newly hatched larvae.

The eggs were measured (two egg diameters and two yolk sphere diameters) once they ceased to absorb water (30 min.). The values obtained were averaged and the egg and yolk sphere volumes were calculated as (equation 1.1):

$$V=4/3\cdot p\cdot r^3\text{ mm}^3 \text{ (1.1)}$$

where: p (π) $\approx 3,14$;

Treatment	Salinity [PSU]				
	6.5 K	3.0 A	6.0 B	7.5 C	9.0 D
pH	8.06	8.22	8.36	8.99	8.52
Lithium [mg Li ⁺ ·dm ⁻³]	0.032	0.001	0.002	0.002	0.002
Sodium [mg Na ⁺ ·dm ⁻³]	2022.9	801.8	1799.8	2317.1	2722.8
Magnesium [mg Mg ²⁺ ·dm ⁻³]	176.0	68.2	121.5	141.2	150.5
Calcium [mg Ca ²⁺ ·dm ⁻³]	111.9	26.9	61.2	77.2	88.9
Potassium [mg K ⁺ ·dm ⁻³]	70.7	29.8	61.4	74.2	79.6
Bromine [mg Br·dm ⁻³]	13.9	3.5	12.6	13.4	13.3
Fluorine [mg F·dm ⁻³]	2.3	7.8	0.9	1.8	0.4
Chlorine [mg Cl·dm ⁻³]	3936.0	1535.6	3399.9	4387.5	5142.8
Number of eggs	912	550	667	853	741

Table 1. Treatment water pH, ion concentrations and number of eggs incubated.

*Differences between means in rows denoted with identical symbols are not significant ($p \geq 0.05$) (Duncan's multiple range test)

The newly hatched larvae were measured within a few hours following their leaving the eggs. A total of 30 individuals were measured for the total body length (*longitudo totalis*, l.t.); should fewer larvae be obtained, as in treatment A with 14 larvae, all were measured. The yolk sacs were measured as well and their volumes were calculated as in [11] (equation 1.2):

$$Vs = (\pi/6) \cdot l \cdot h^2 \text{ mm}^3 \quad (1.2)$$

where: l-yolk sac length (mm); h-yolk sac height (mm)

The magnitude of the perivitelline space was expressed as the percentage of the total egg volume.

The survival rate, determined as the percentage of hatched larvae, was calculated on termination of hatching from 100 fertilized eggs (selected at the blastopore closure stage).

The egg fertilization rate (%) was calculated from 100 eggs observed at the blastopore closure stage.

Larval malformation rate was determined as a percentage of malformed larvae among all the hatch in a treatment.

The data were processed statistically with the Statistica 9.0 PL software (StatSoft Poland) by running the one-way analysis of variance (ANOVA, $p < 0.01$) and Duncan's multiple range test ($p < 0.05$) to test for significance of between-treatments differences in the size of eggs, egg cells, perivitelline space, body length and yolk sac volumes of the larvae, and percentage of fertilization, percentage of survival, percentage of deformed larvae.

3. Results

Results of the conducted measurements of eggs of the lesser sandeel shown in Table 2 (diameters and egg volumes calculated on the basis of those diameters) reveal statistically significant differences between the sizes of eggs incubated in waters varying with respect to salinity levels.

The lowest egg diameter was observed in treatment A (salinity of 3.0 PSU), the highest diameter being recorded in treatment B (6.0 PSU). The differences in egg diameter were translated into differences in egg volume, with the lowest (0.24 mm³) and the highest (0.29 mm³) mean volumes being shown by eggs in treatments A and B, respectively (Table 2). There were no significant between-treatments differences in egg yolk sphere dimensions ($\varnothing = 0.45 \text{ mm}$; $v = 0.05 \text{ mm}^3$) (Table 2).

The perivitelline space, i.e., a milieu in which the embryo develops, was found to take from 79.32% (treatment A) to 82.77% (treatment B) of the whole egg volume. There were no significant differences in the perivitelline space between treatments K, C, and D (Table 2).

The highest percentage of fertilized eggs (65.0%) was recorded in the control treatment (K); lower percentages (in excess of 50.0%) were found in treatments B, C, and D. In contrast, as few as 22.0% of the eggs were fertilized in treatment A (Table 2).

Despite salinity differences, eggs in all the treatments showed the embryos to develop. There were no significant between-treatments differences in the rate of development during early embryogenesis.

Dimension	Treatment				
	K	A	B	C	D
Egg diameter [mm]	0.79 ^b ± 0.02	0.76 ^a ± 0.04	0.81 ^c ± 0.01	0.79 ^b ± 0.02	0.78 ^b ± 0.02
Yolk sphere diameter [mm]	0.45 ^a ± 0.02	0.45 ^a ± 0.02	0.45 ^a ± 0.02	0.45 ^a ± 0.02	0.45 ^a ± 0.01
Egg volume [mm ³]	0.25 ^b ± 0.02	0.24 ^a ± 0.04	0.29 ^c ± 0.03	0.26 ^c ± 0.03	0.26 ^b ± 0.02
Yolk sphere volume [mm ³]	0.05 ^a ± 0.005	0.05 ^a ± 0.005	0.05 ^a ± 0.008	0.05 ^a ± 0.005	0.05 ^a ± 0.004
Perivitelline space - PVS [%]	80.89 ^b ± 1.72	79.32 ^a ± 2.31	82.77 ^c ± 3.89	81.14 ^b ± 2.93	81.18 ^b ± 1.61
Fertilization success rate [%]	65.0 ^c	22.0 ^a	55.0 ^b	53.0 ^b	52.0 ^b

Table 2. Egg size and fertilization rate in the treatments used.

**Differences between means in rows denoted with identical symbols are not significant ($p \geq 0.05$) (Duncan's multiple range test)*

At 9.0 DD (degree-days – the product of the number of days of embryogenesis and the mean daily temperature), 60% of eggs in all the treatments showed the small-cell morula (Figure 1). At 19.0 DD, the yolk sphere epiboly (blastopore closure) was observed in all the treatments, the embryo's body outline being then visible.

On day 5 of the embryonic development, at about 50.0 DD, the embryonic heart in eggs of treatments K, B., C, and D was observed to begin slow contractions, while – at the same time – the treatment A embryos, less well developed and retarded in their embryonic development, started to die out (Figure 2).

At 75.0 DD, the embryos in all the treatments except A were observed to move vigorously in the eggs.

Retardation of embryogenesis in the lowest-salinity treatment (A) was very pronounced at the “eyeing” stage, *i.e.*, when the pigment appears in the embryo's eyes. In treatments K, B, C, and D, the well-visible pigment appeared on day 10 (about 108 DD), the pigment being visible as late as on day 13 (140 DD) in treatment A (Figure 3).

As shown by this study, differences in salinity did affect the timing of hatching. The earliest hatching occurred in treatments C and D (7.5–9.0 PSU) (Table 2), followed by the control (K, 6.5 PSU) and treatment B (6.0 PSU). On the other hand, the eggs incubated in the lowest salinity (treatment A, 3.0 PSU) hatched as late as in 173.0 DD (Table 3).

The highest hatching success rate was recorded in the control (treatment K) as well as in treatments C and D (92.0–96.0%), while as few as 16.5% of the eggs hatched in treatment A (Table 3).

The lesser sandeel larvae showed significant between-treatments differences in their mean total body length (Figure 4). The longest individuals hatched in treatment K (4.67 ± 0.266 mm) and B (4.70 ± 0.260 mm), the two treatments using water of a similar salinity (6.0–6.5 PSU). The shortest individuals (4.01 ± 0.281 mm)

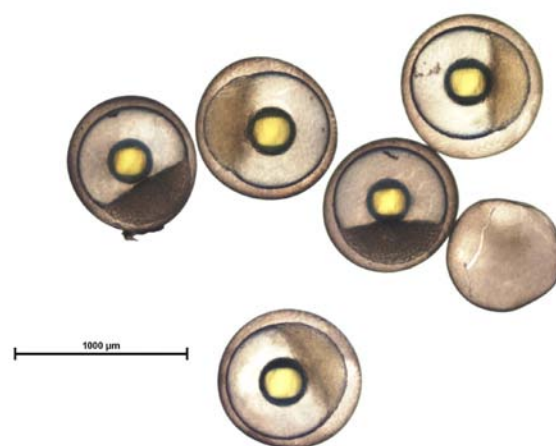


Figure 1. Eggs of the lesser sandeel on 9.0 DD, at the stage of a small-molecule morula (treatment B) – a lipidal droplet is visible in the central part of an egg.

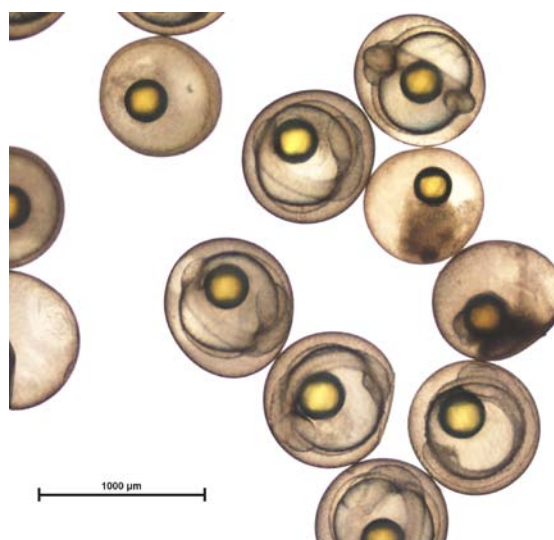


Figure 2. Eggs of the lesser sandeel on 50.0 DD (treatment A) – an embryo with eye cups visible in the cephalic area and with distinctive myomeres in the thoracic area.

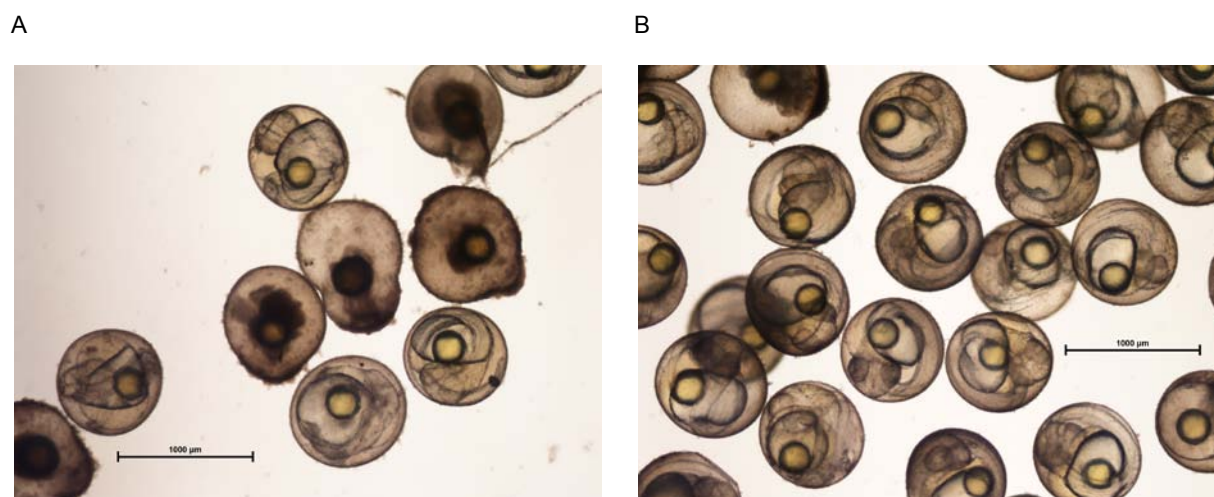


Figure 3. Eggs of the lesser sandeel on 108.0 DD of their development – brain vesicles are visible in the cephalic area of the embryo, situated behind the eye cups. The length of the embryo exceeds the perimeter of the yolk sphere. Lens can be seen in the eyeballs; in treatment A there is no pigment, while in treatment C the pigment is clearly visible.

Embryogenesis stage	Treatment				
	K	A	B	C	D
Start of hatching	162.0	173.0	162.0	141.0	141.0
50% eggs hatched	201.0	184.0	201.0	184.0	172.0
End of hatching	228.0	228.0	228.0	217.0	217.0
Incubation duration (days)	14-20	15-20	14-20	12-19	12-19
Survival (%)	96.0 ^c	16.5 ^a	85.0 ^b	92.0 ^c	95.0 ^c
Malformed larvae (%)	8.0 ^a	35.0 ^b	9.0 ^a	9.0 ^a	10.0 ^a

Table 3. Duration of embryogenesis (DD), hatching, and survival of the lesser sandeel embryos and malformation of their bodies.

hatched in the low-salinity (3.0 PSU) treatment A (Figure 4).

As a consequence of varying larval body length in the treatments, the yolk sac volumes differed significantly between the treatments as well (Figure 5).

The largest yolk sacs (mean $v = 0.034 \text{ mm}^3$) were typical of the larvae hatched from eggs incubated in the lowest salinity (treatment A), smaller yolk sacs being observed in the larvae from treatments C and D (0.023 and 0.022 mm^3 , respectively) (Figure 5). The larvae from the control (K; 0.013 mm^3) and from treatment B (0.015 mm^3) (Figure 5) showed the smallest yolk sacs.

Among the newly hatched larvae, there were – in addition to healthy individuals – some showing various malformations of the body. The highest malformation rate occurred among the larvae hatched from eggs kept under the lowest salinity (treatment A). The remaining treatments produced 9.0-10.0% of malformed larvae (Table 3; Figure 6).

The most frequent malformations included axial and lateral curvature of the spine, the larvae thus affected are referred to as the “C-shaped larvae” (Figure 6b). Deformed yolk sacs were observed as well, primarily in treatment A.

4. Discussion

Our study on salinity effects on the lesser sandeel embryogenesis showed the water minerals content to have a potential of affecting first the gametes (sperm and eggs), then fertilization, and eventually individual stages of the embryogenesis, and thus the percentage of healthy hatch.

Similar studies carried in the 1960s and targeting the embryogenesis of herring (*Clupea harengus*) and flounder (*Pleuronectes platessa*) showed the two species, which occur in the Baltic Sea, to have

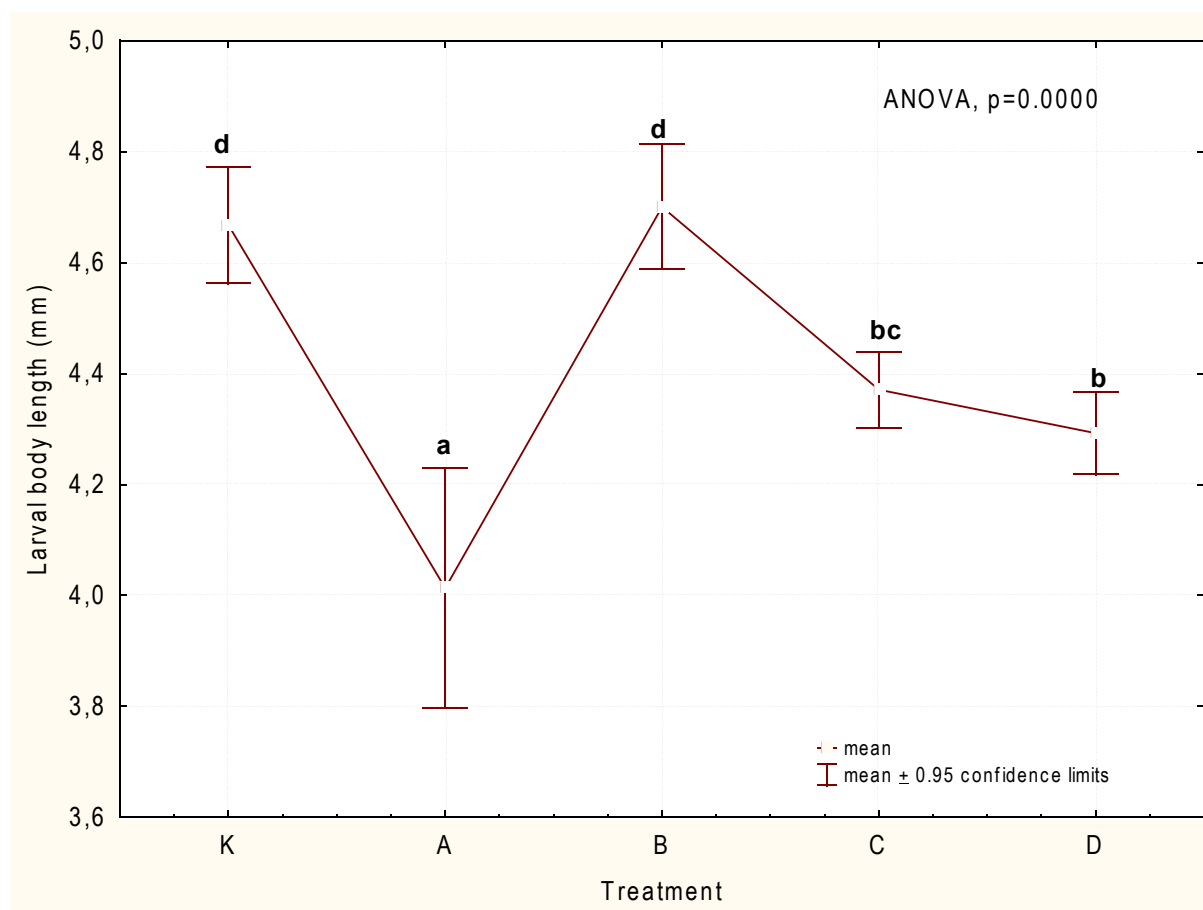


Figure 4. Mean total body length of the lesser sandeel larvae in different treatments *means denoted with identical letters (low case fonts) were not significantly different at $p \geq 0.05$ (Duncan's multiple range)

a high tolerance to salinity changes [4,12]. The eggs of herring and flounder were successfully fertilized at the salinity ranging within 10.0-60.0 and 30.0-60.0 PSU, respectively; however, the gametes were exposed to those salinities for 1-2 min. only [4,12]. The salinities used in this study (not exceeding 10.0 PSU) affected, in a continuous manner, first the gametes alone and then the embryos developing in eggs.

In addition, the study showed that the lesser sandeel eggs and sperm placed in both the low and elevated water salinity (different from the optimal level) tolerated various salinity levels, as all the eggs were fertilized, albeit with a different success (Table 2). The fertilization rate in the lowest salinity (treatment A, 3.0 PSU), equal to about one-third of that in the control, was most likely a result of a too low salinity; the salinity much lower than the optimum may adversely affect sperm motility, as reported from the Baltic cod (*G. morhua*) [6]. The authors cited followed effects of salinity changes from 11.5 PSU to 35.0 PSU on sperm motility and found the sperm to be most active in 15.5-26.0 PSU. At

lower salinities (11.5-12.0 PSU), the spermatozoa were virtually motionless or showed vibrating movements only. A high fertilization success rate was obtained for the eggs incubated in water of 12.5-30.0 PSU salinity, the lower salinity treatments (10.0-12.0 PSU) reducing the fertilization rate [6].

It has to be borne in mind that fertilization should be understood as a cycle of consecutive and strongly related processes which may be disturbed by a change in salinity in the water surrounding the egg. When the egg cell fuses with a spermatozoon, the membranes of cortical alveoli in the oocyte cytoplasm fuse with the egg cell membrane; as a result, hydrophilous colloids penetrate the gap between the egg membrane and that of the yolk, a process known as the cortical reaction [13-18]. Subsequently, a high osmotic pressure liquid formed in the perivitelline pit induces sucking of the water into the egg, whereby the egg membrane expands and the egg achieves the required turgor [13,14,18-24]. As the egg membrane requires external calcium ions (Ca^{2+}) to harden [25,26], it may be presumed that a reduced

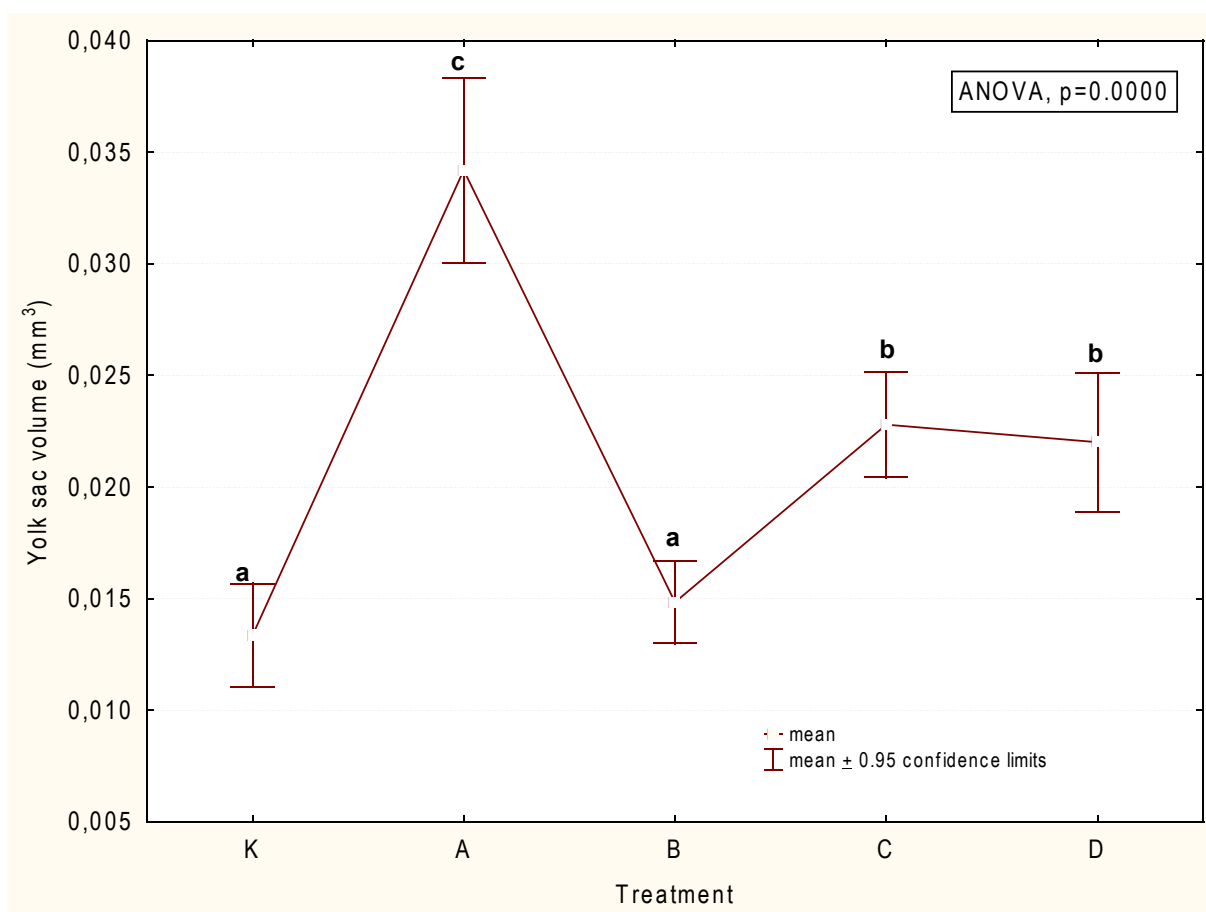


Figure 5. The lesser sandeel larval yolk sac volumes in different treatments * means denoted with identical letters (low case fonts) were not significantly different at $p \geq 0.05$ (Duncan's multiple range test).

a



b



Figure 6. Larvae of the lesser sandeel: a) a healthy, well-developed larva (treatment C); b) a deformed larva with the body bent in the shape of the letter "c" (treatment A).

or elevated calcium ion concentration in water is likely to produce various adverse effects. Differences in the calcium ion concentrations between the treatments applied in this study were in fact revealed by the chemical assays (Table 1). The seawater (treatment K) showed the calcium content more than four times that in the lowest salinity treatment (A) (Table 1). In addition, although the calcium content increased with the salinity, the highest salinity water (treatment D, 9.0 PSU) showed the calcium content lower by $23.33 \text{ mgCa}^{2+}\text{dm}^{-3}$ than that in the seawater (treatment K) (Table 1).

It may be presumed that the differences in ionic concentrations induce disturbances which prevent the egg from attaining a sufficient turgor. An indirect effect can be seen as differences in the perivitelline space size in the eggs in different treatments.

It can thus be concluded that:

- firstly, an insufficient calcium ion concentration could disturb egg membrane hardening, whereby the egg will not attain the required turgor. The egg volume and the size of the perivitelline space will be smaller than those in eggs undergoing embryonic development in more saline water, which was the case in this study (Table 2);
- secondly, the differences in the size of the perivitelline space, observed in this study, could have been an effect of differences in ion concentrations on egg membrane permeability. Eddy and Talbot [27] reported that higher concentrations of some ions, including those of sodium (Na^+), could be involved in slowing down the perivitelline space formation in the eggs of salmon (*Salmo salar* L.). As revealed by the chemical assays, treatment C and D showed concentrations of the sodium cation to be somewhat higher (about the value $300\text{--}700 \text{ mg dm}^{-3}$) than that in treatment B (Table 1) in which the eggs were the largest (Table 2);
- thirdly, the ionic composition of the water differed between the treatments; as the water, along with ions and respiratory gases, easily penetrates the egg through its membranes during swelling [28–30], the differences in ionic composition could have also affected formation of the perivitelline space which supports the developing embryo. As shown by this study, the embryos developing in eggs incubated in treatments A, C, and D had a reduced space to develop in (reduced PVS); the composition of the perivitelline fluid filling this space was altered as well, which resulted in disturbed development and growth of the embryo.

The disturbances resulted in: different (elongated in treatment A and reduced in treatments C and D) duration of the embryogenesis, elevated percentage of malformed hatch, and reduced survival (treatment A) as well as altered dimensions of the newly hatched larvae (reduced body length and therefore larger egg yolk) (treatments A, C, D).

In addition, results reported by Luberda *et al.* [31] confirm that the proteolytic activity of the hatching enzyme (a metalloenzyme) responsible for digestion of the egg membrane and regular hatching [32,33] is controlled by metal ions contained in the perivitelline fluid. Consequently, delay or acceleration of hatching of eggs kept in water in different mineral regimes may be due to effects of metal ions, e.g., Na^+ , Mg^{2+} , K^+ present at various concentrations in the water. According to the research, in the water with the lowest salinity (treatment A), the content of the above mentioned ions was over 2.5 times lower than in sea water (treatment K – water from the Pomerania Bay). Furthermore, the concentration of chlorine ions was over 2.5 times lower in sample A than in sample K, and in the case of bromine, the concentration was over 4 times lower. As for lithium, its content in the water with the lowest salinity level was 30 times lower than in sea water (Table 1).

The results obtained in this study show water salinity, both lower and higher than the species-specific optimal level, to induce disturbances in the embryogenesis, resulting in reduced fertilization success and embryo survival rates, and in changes in the size and condition of the newly hatched larvae. Those findings are similar to effects reported by numerous workers studying other marine fish species [4,8–10,12,34].

The inferences and facts presented above demonstrate that, for the lesser sandeel embryogenesis in the Baltic Sea to proceed unhampered, it needs, *i.a.*, a suitable mineral composition of the water in which embryonic development is taking place. Various populations of a species (the lesser sandeel in this study) living in waters of different salinities (e.g., the more saline North Sea) may differ in their tolerance limits. As shown by this study, the lesser sandeel is highly plastic. This plasticity, an evolution-derived trait, is very advantageous in the face of, *i.a.*, progressing environmental changes (including changes in water salinity), and one that enables the species to adapt to altered habitat conditions.

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