Early and abrupt salinity reduction impacts European eel larval culture

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6

7 **Abstract**

8 Reducing water salinity towards isosmotic conditions is a common practice applied in euryhaline fish farming

9 to limit osmoregulation costs and enhance growth. In this respect, the present study investigated the timing

10 of salinity reduction in an abrupt manner during European eel (Anguilla anguilla) larval culture by examining

11 associated impacts on morphological and molecular levels. Larvae from 3 different parental combinations

12 (families) were reared at constant 36 psu for 6 days (control) or subjected to a direct reduction to 18 psu on

13 1-, 2-, or 3-days post-hatch. Overall, salinity reduction enhanced growth and survival, resulting from more

14 efficient energy resource utilization. In the control group, expression of growth-related igf2 remained

15 constant, demonstrating a steady growth progression, while igf1 expression increased over time only for the

16 salinity reduced treatments, potentially qualifying as a useful biomarker for growth performance. Even

17 though each parental combination seems to have a different capacity to cope with salinity alterations, as

18 observed by family-driven water-transport-related aquaporin (aqp1, aqp3) gene expression, it could be

19 inferred that the abrupt salinity change is generally not stressful, based on non-upregulated heat shock

20 proteins (hsp70, hsp90). However, the applied salinity reduction (irrespective of timing) induced the

21 development of pericardial edema. As such, we conclude that despite the positive effect of salinity reduction

22 on early growth and survival, the long-term benefit for eel larval culture lies in establishing a protocol for

23 salinity reduction, at a precise developmental time point, without causing pericardial malformations.

24 **Keywords**

26

Introduction

To facilitate growth during fish larval development in culture, the high energy demand of osmoregulation can be reduced by adjusting the culture environment towards salinity conditions, which facilitate energy resources to be utilized with greater efficiency (reviewed in Bœuf and Payan 2001). Intermediate salinity regimes have been frequently employed, with positive results concerning larval survival and growth for several fsh species (Gaumet et al. 1995; Tandler et al. 1995; Imsland et al. 2001; Rahmah et al. 2020). However, the mechanisms involved in the processes of decreasing osmoregulatory energy expenditure and acting benefcially can be multidimensional. Along these lines, better feed intake (Bœuf and Payan 2001) and feed conversion efciency rates (Gaumet et al. 1995) have previously been reported. Overall, an aquatic environment with osmotic pressure matching the osmolality of larvae (iso-osmotic) is suggested to be less stressful (Deane et al. 2002), thus allowing marine fish larvae in culture to thrive at lowered salinity due to reduced energy costs for maintenance of homeostasis. European eel (Anguilla anguilla) is a catadromous fish with a migration pattern connecting distant oceanic spawning habitats with continental juvenile on-growing habitats (Schmidt 1923; Righton and Metcalfe 2011). In these corresponding native environments, eels have adapted to different salinity regimes, developing elaborate osmoregulatory mechanisms (Cutler and Cramb 2001; McCormick 2001; Bone and Moore 2008). During early life history stages, eels are encountered exclusively in the ocean (Schmidt 1923; Tsukamoto 1992), where they experience hyperosmotic conditions (Lee et al. 2013). It is therefore anticipated that eel larvae must possess genetically pre-programmed mechanisms for osmoregulation, allowing to cope with osmotic and ionic gradients between their body fluid osmolality and the surroundings (Varsamos et al. 2005). Even though osmoregulatory organs are absent during the early developmental stages, eel larvae exhibit numerous ion-transporting ionocytes on their body surface already at hatch (Sasai et al. 1998, 2007; Seo et al. 2015; Kuroki et al. 2016). These mitochondrial-rich cells require a significant proportion of metabolic energy to be allocated for osmoregulation (Morgan and Iwama 1991; Bœuf and Payan 2001; Bone and Moore 2008). In aquaculture, eel offspring are commonly reared in seawater at 36 psu (Sørensen et al. 2016a), but applying a reduction in salinity to half-strength seawater during larval rearing has proven beneficial (Okamura et al. 2009; Kurokawa et al. 2013; Kuroki et al.

53   2016; Politis et al. 2018a). However, the application techniques and timing of such a salinity reduction have

54   received little attention. In European eel, it was shown that the earlier a salinity reduction is applied, the

55   better survival and growth rates are achieved (Politis et al. 2018a). For the closely related Japanese eel, a

56   stable full-strength salinity regime is suggested until at least 4 days post-hatch (dph), based on the

57   emergence of morphological deformities after early exposure of pre-leptocephali to low-salinity conditions

58   (Kurokawa et al. 2013). More specifically, malformations, such as pericardial edema and open lower jaw may

59   occur, with high mortalities during early ontogeny often being attributed to such abnormal phenotypes

60   (Okamura et al. 2007, 2011, 2016, 2018; Okamoto et al. 2009). Regarding the aforementioned

61   considerations, on the one hand, the timing of application should accommodate early life development, while

62   on the other, salinity change should be in line with culture efficiency. Politis et al. (2021) showed that a

63   stepwise salinity reduction close to iso-osmotic levels (18 psu) improves larval growth and survival. However,

64   such an approach challenges technical aspects of recirculating aquaculture system (RAS) technology, by the

65   need of either multiple RAS units stabilized to different fixed salinities or by dynamically altering salinity in

66   each operating RAS. The latter scenario substantially compromises RAS biofilter stability (Kinyage et al. 2019),

67   where particularly in saltwater systems, abrupt lowered salinity negatively affects biofilter function and

68   reduces vital nitrification processes (Lyssenko and Wheaton 2006). Considering these aspects, the above

69   approaches do not serve as sustainable solutions, calling for more understanding regarding physiological

70   tolerances during larviculture. Interestingly though, eel larvae are able to tolerate a drastic change of salinity,

71   performed by moving the larval rearing tanks from one stable RAS unit at 36 psu to another stable RAS unit

72   at 18 psu (Politis et al. 2021), which represents a cost-efficient solution. Nonetheless, the biologically most

73   suited timing for applying this salinity reduction is yet to be elucidated. The present study aimed to

74   investigate the application timing of such a salinity decrease and the biological significance for eel larvae

75   from a morphometric (survival, biometrics, and deformity) and molecular (gene expression) perspective. For

76   this, European eel larvae from three parental combinations (families) were reared either at constant 36 psu

77   for 6 days (control) or subjected to a direct salinity reduction to 18 psu on 1, 2, or 3 dph.

78   **Materials and methods**

79   *Broodstock management and gamete production*

80   Female broodstock were collected from nature (Saltbæk Vig, 55°44'48.8"N 11°09'09.5"E, Denmark; and Lake

81   Vandet, 57°00'50.1"N 8°33'16.5"E, Denmark), while male broodstock originated from glass eels raised at a

82   commercial Danish eel farm (Royal Danish Fish, Hanstholm, Denmark). Males and females were transported

83   to the EEL-HATCH facility in Hirtshals (Denmark), where they were reared in closed recirculation systems with

84   a flow rate of 10–15 L min−1 and acclimated to 36 psu and 20 °C over the course of 2 weeks. Stimulation of

85   spermatogenesis and milt collection were performed according to previously described assisted reproduction

86   protocols (Tomkiewicz et al. 2011; Butts et al. 2014; Sørensen et al. 2016b), while stimulation of

87   vitellogenesis and ovulation was performed using salmon pituitary extract and dihydroprogesterone,

88   respectively (Tomkiewicz et al. 2019; Kottmann et al. 2020). The reproductively mature males (n=14) had a

89   mean standard length of 39.1±3.8 cm and a body weight of 132.0±23.6 g, while females (n=3) had a mean

90   standard length of 70.3±5.7 cm and a body weight of 695.3±221.3 g, respectively. Egg fertilization and

91   incubation The eggs of each female (n=3) were strip spawned, mixed with milt originating from a combination

92   of 3–5 males (Butts et al. 2014; Benini et al. 2018) and fertilized at an effective salinity of 36 psu and 20 °C

93   (Sørensen et al. 2016a), to create the 3 family crosses used in this study. The buoyant eggs/embryos were

94   transferred to 60-L conical flow-through incubators, as described in Sørensen et al. (2016b) and Politis et al.

95   (2018b). Light was kept dimmed (Politis et al. 2014), temperature set at ~ 18 °C (Politis et al. 2017), and

96   gentle aeration was applied until ~ 52 h post fertilization (hpf), while bottom purging was regularly applied

97   for removal of dead eggs. Air diffusers were removed prior to hatching, which occurred at ~ 56 hpf.

98   *Experimental design and conditions*

99   The experimental setup included two identical RAS units (Fig. 1), each consisting of a~1-m3 reservoir, 0.2-

100   m3 header tank, 0.2-µm cartridge flter for particle removal (Cuno/3 M, 10-in. PDA 0.2-µm cartridge, Silhorko-

101   Eurowater A/S, Skanderborg, Denmark), and UV treatment (UltraAqua, Aalborg, Denmark). The systems did

102   not contain a bioflter and did not receive returning water from the 2-L acrylic experimental rearing jars. One

103   system was kept at 36±0.3 psu, while the other was adjusted to 18±1.8 psu using municipal tap water (non-

104 chlorinated groundwater). Temperature in both systems was maintained at 18.8±0.4 °C (Politis et al. 2017).

105 The experiment was repeated 3 times, each time using offspring from a different family (4 salinity

106 treatments×3 families×3 replicates=36 experimental units). For each family, newly hatched larvae (day 0)

107 were randomly distributed (~800 individuals per replicate) into 12 acrylic 2-L fow-through jars (drz400sm

108 hank, JugDesk Type, Taipei, Taiwan) with custom-designed bottom inflow and top outflow. All larval rearing

109 tanks were initially connected to the 36 psu system. Thereafter, 3 jars were connected to the 18 psu system

110 on day 1 (treatment 1), another 3 jars on day 2 (treatment 2), and finally another 3 jars on day 3 (treatment

111 3). The last 3 jars (control) remained connected to the 36-psu system the entire period (Fig. 2). Water

112 exchange rate was adjusted to~0.1 L min−1 for all jars. Eel larvae were reared under these experimental

113 regimes for 6 days under low light intensity (Politis et al. 2014).
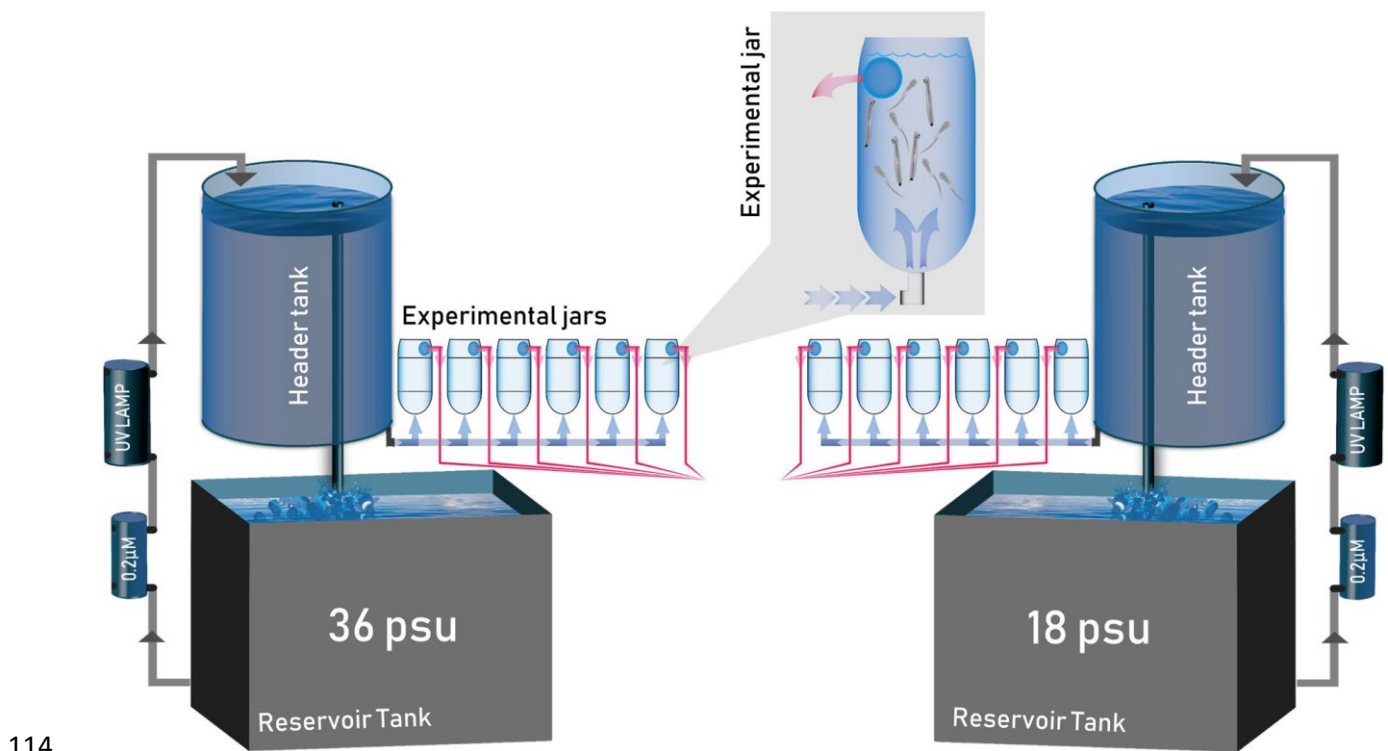
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115 *Figure 1. Schematic representation of the experimental setup. European eel (Anguilla anguilla) larvae were*

116 *reared either at a constant salinity of 36 psu (control) over 6 days post-hatch (dph) or experienced an abrupt*

117 *salinity reduction on 1, 2, or 3 dph by moving larval rearing tanks from the 36-psu system to*

118 *the 18-psu system.*

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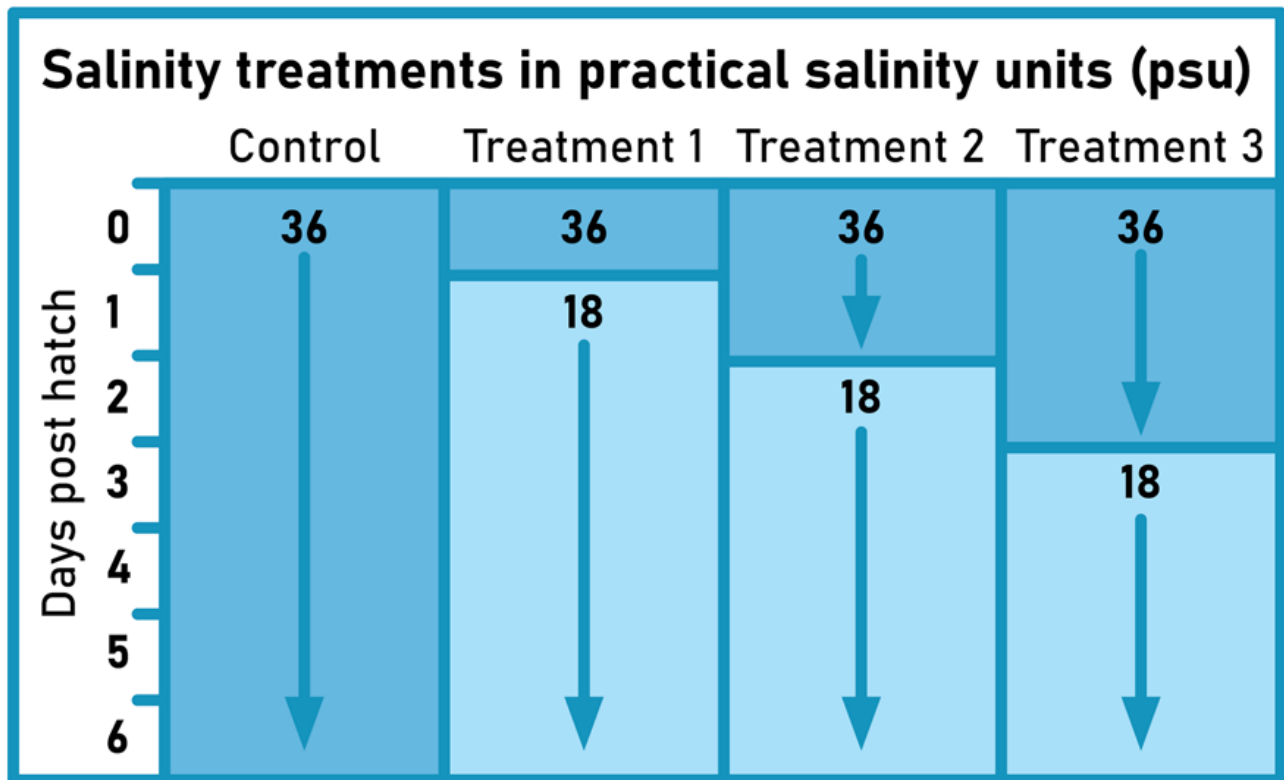
Figure 2. *Schematic representation of the experimental treatments, where European eel (Anguilla anguilla)*

larvae were reared over 6 days post-hatch (dph) under constant salinity of 36 psu (control) or experiencing a

salinity reduction to 18 psu on 1 (treatment 1), 2 (treatment 2), or 3 (treatment 3) dph

Data collection

Mortality and biometry

Larval sampling was conducted on 0, 1, 2, 3 and 6 dph. Here, ~10 larvae from each replicate (n=3), family

(n=3), and treatment (n=4) were randomly sampled, anesthetized using ethyl 3-aminobenzoate

methanesulfonate (MS-222, Sigma-Aldrich Chemie, Steinheim, Germany), and photographed using a zoom

stereomicroscope (SMZ1270i fitted DS-Fi2 Camera Head, Nikon Corporation, Tokyo, Japan). Subsequently,

larval images were analyzed for total body area, oil-drop area, and pericardial edema using the NIS-Elements-

D analysis software (Nikon Corporation, Tokyo, Japan). Based on the derived measurements, the following

estimates were calculated:

Growth rate = [(final body area – initial body area) ∕number of days]

133 Oil droplet utilization = [(initial oil droplet area − final oil droplet area) ∕number of days]

134 Growth efficiency = (% growth rate∕% oil droplet utilization)

135 To study the severity of heart malformation, the ratio of edema height relative to neurocranium height was

136 discriminated and categorized as either minor (0.5–0.8) or severe (>0.8) according to Kurokawa et al. (2008)

137 and Okamoto et al. (2009). Dead larvae were counted and removed from all experimental units on a daily

138 basis to minimize microbial interference (Sørensen et al. 2014). At the completion of the experiment, all

139 larvae were enumerated and survival was estimated taking into account all the sampled larvae throughout

140 the experimental process. Larval cumulative mortality was calculated as a percentage from hatch until 6 dph.

141 *Gene expression*

142 For molecular analysis, a pool of~20 larvae from each replicate (n=3), family (n=3), and treatment (n=4) were

143 randomly sampled on 0, 1, 2, 3, and 6 dph. These larvae were recorded, euthanized using MS-222, preserved

144 in RNAlater Stabilization Reagent, and kept at−20 °C following the procedures suggested by the supplier

145 (Qiagen, Germany). RNA was extracted using the NucleoSpin® RNA Kit (Macherey–Nagel, Germany) following

146 the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry using

147 Nanodrop® ND-1000 (Peqlab, Germany) and then reverse-transcribed using the qScript™ cDNA Synthesis Kit

148 (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipe out

149 step (PerfeCta® DNase I Kit (Quantabio, Germany)). The expression levels of target genes were determined

150 by quantitative real-time PCR (RT-qPCR), using specific primers (Table 1). Primers were designed to target all

151 known potential isoforms with an amplification size ranging from 75 to 200 nucleotides and optimal Tm of

152 60 °C, using primer 3 software (http://frodo.wi.mit.edu/primer3/) based on cDNA sequences available in

153 GenBank databases. Expressions of genes in each larval sample from 2 randomly selected replicates, from

154 each family (n=3), treatment (n=4), and larval age (0, 1, 2, 3, and 6 dph) were analyzed in two technical

155 replicates using the qPCR Biomark™ HD technology (Fluidigm, USA) based on dynamic arrays (GE chips). The

156 chip was run according to the Fluidigm protocol with a Tm of 60 °C. The relative quantity of target gene

157 transcripts was normalized (ΔCT) to the geometric mean of the 2 (reference) genes (ef1a and npr3), after

158 qBase+ software revealed that these mRNA levels were stable throughout analyzed samples (Hellemans et al

159 2007). Coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of

160 gene expression was carried out according to the 2−ΔΔCt method (Livak and Schmittgen 2001).

161 *Table 1* *European eel, Anguilla anguilla, primers used for amplification of genes by qRT-PCR. Designed based*

162 *on sequences available on GenBank databases*

Function	Gene name	Abbreviation	5´forward 3´	5´reverse 3´	Accession Nr
Housekeeping	Atrial natriuretic peptide receptor 3	*npr3*	AACCCTCCACGTGTA GACTG	TGACCAGAATTGCTC CCTCTT	XM_035380325.1
	Elongation factor 1α	*ef1α*	CTGAAGCCTGGTATG GTGGT	CATGGTGCATTTCCA CAGAC	EU407824
Stress-repair	Heat shock protein 70	*hsp70*	TCAACCCAGATGAAG CAGTG	GCAGCAGATCCTGAA CATTG	AZBK01685255
	Heat shock protein 90	*hsp90*	ACCATTGCCAAGTCA GGAAC	ACTGCTCATCGTCAT TGTGC	AZBK01838994
Growth	Insulin-like growth factor 1	*igf1*	TTCCTCTTAGCTGGG CTTTG	AGCACCAGAGAG AGGGTGTG	EU018410.1
	Insulin-like growth factor 2	*igf2*	AAAGCTTTGGGACAG CTTCA	CGCAGCTGTGTACGT GAAAT	AZBK01622663
Water transport	Aquaporin 1 like	*aqp1*	GAATTCCTGGCAACC TTTCA	CAAGATGACCCAGAC CCACT	AJ564421
	Aquaporin 3 like	*aqp3*	GCTCTCATGGCTTGT TCCTC	AAGGTCACAGTG GGGTTCAG	AJ319533

163

Statistical analysis

165 All data were analyzed using SAS software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were evaluated

166 for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted valves) to ensure they

167 met model assumptions. Data were log10 or arcsine square root transformed, when necessary. Alpha was

168 set at 0.05 for main effects and interactions. Treatment means were contrasted using Tukey's honest

169 significance test. Cumulative mortality data were analyzed using a mixed model repeated measures ANOVA,

170 where the main model variables were salinity treatment (fixed factor), age (fixed factor), and the salinity

171 treatment × age interaction (fixed factor). Body area, oil droplet area, growth rate, oil droplet utilization,

172 growth efficiency, and pericardial edema ratio were analyzed using a series of mixed model factorial ANOVAs.

173 Additionally, a series of mixed effects models were run to investigate how salinity treatment impacts gene

174 expression at each age and how gene expression changes throughout early larval ontogeny for each salinity

175 regime. In these above models, family and the family × salinity treatment interaction were considered

176 random factors. Variance components (VC) for random effects were generated using the restricted maximum

177 likelihood (REML) estimation method and expressed as a percentage.

178 **Results**

179 *Mortality and biometry*

180 The earlier the salinity reduction was induced, the smaller the larval size in terms of (final) body area reached

181 on 6 dph, while larvae raised in full strength salinity water (Control) developed the significantly smallest body

182 area compared to any of the salinity reduction treatments (p = 0.021; Fig. 3A). Accordingly, larval growth rate

183 was lower for the control group, with no significant differences observed among the rest of the treatments

184 (p = 0.021; Fig. 3B). Significantly less energy reserves, in terms of (final) oil droplet area on 6 dph, were

185 observed in larvae experiencing the abrupt salinity reduction on 3 dph (treatment 3) compared to treatments

186 1 and 2, while the lowest energy reserves were observed in larvae reared in full-strength seawater (control)(p

187 = 0.001; Fig. 3C). Furthermore, significantly lower oil droplet utilization was observed when salinity reduction

188 was implemented on 1 and 2 dph (p = 0.001; Fig. 3D). Consequently, this coincides with the growth efficiency

189 values, which were highest for larvae experiencing a salinity reduction on 1 and 2 dph (p < 0.001; Fig. 3E),

190 while the stable salinity regime of 36 psu (control) displayed the lowest growth efficiency among all

191 treatments. Concerning the condition of pericardial edema, it was exclusively observed in larvae from the

192 three salinity reduced treatments (pericardial edema severity ratio > 0.8). Nevertheless, the developmental

193 stage at which salinity reduction was introduced had no significant implications on the magnitude of the

194 specific malformation (p = 0.005; Fig. 3F). The cumulative mortality (Fig. 4) was significantly (p < 0.05) higher

195 for larvae reared in full-strength seawater (control), while no differences were detected among the salinity

196 reduction treatments. Moreover, no significant salinity treatment × age interaction was observed.

197

198 *Gene expression*

199

200 As revealed by the VC analysis (Table 2), the expression patterns of genes related to water transport (aqp1,

201 aqp3) were significantly ($p < 0.05$) family driven on 1, 2, and 3 dph, while they were driven by a significant

202 genome (family). environment (salinity treatment) interaction on 6 dph (aqp1: $p = 0.037$; aqp3: $p = 0.015$).

203 The family effect was also significant ($p < 0.05$) for stress-related genes (hsp70, hsp 90) on 2 and 3 dph, where

204 it explained up to 47.1% of the observed variability. Similarly, expression patterns of growth-related genes

205 were found to be predominantly influenced by family on 2 dph for igf1 and on 1 and 2 dph for igf2. The

206 expression levels of neither aquaporin nor heat-shock protein groups were affected ($p > 0.05$) by larval age

207 and remained steady throughout ontogeny. Likewise, no significant fluctuations were noted for the

208 expression of igf2 among developmental stages. Still, igf1 expression was affected by developmental age (Fig.

209 5), where expression increased over time in all reduction treatments (treatment 1: $p = 0.002$; treatment 2: $p

210 = 0.046$; treatment 3: $p < 0.0001$), while it remained stable throughout ontogeny for larvae reared at constant

211 36 psu.

212

213 **Discussion**

214 Today, production of European eel offspring is deemed essential to support sustainable aquaculture

215 development and alleviate pressure from the depleted wild eel stocks (ICES 2020). Despite the notion that

216 farming should imitate the conditions corresponding to the species- and stage-specific natural habitat, the

217 employment of rather abnormal rearing environments may in fact facilitate production. The present study

218 investigated the application timing of unnatural and abrupt salinity changes from 36 to 18 psu during eel

219 larviculture, to elucidate the biological significance for eel larvae from a morphometric and molecular point

220 of view. At the end of the experiment (6 dph), growth in body area was significantly increased by salinity

221 reduction towards iso-osmotic conditions. As such, the assumption of energy saving due to lower

222 osmoregulation costs was here confirmed by the higher availability of internal energy reserves (in terms of

223 yolk and oil-droplet) in larvae experiencing a salinity reduction, which were more efficiently utilized and

224 invested into development instead of maintaining ionic and water balance. However, larvae experiencing this

225 salinity change already on day 1 did not grow as big as larvae switching to 18 psu later in development. This

226 indicates that newly hatched larvae might have restricted ability to cope with abrupt salinity changes, which

227 could be compromising their growth potential. Similar to growth, survival was lowest for the control group,

228 where larvae were reared in full strength salinity. Interestingly, mortality increased the longer the larvae

229 remained in high-salinity conditions, confirming previous observations by Politis et al. (2021), where applying

230 such a drastic salinity change on 6 dph did not improve eel larval survival compared to larvae constantly

231 reared in full-strength seawater. As such, the present study confirms that the early establishment of iso-

232 osmotic conditions generally accommodates the survival of weak larvae that do not survive in full-strength

233 salinity. However, it needs to be mentioned that in the current study, all salinity reduction treatments,

234 irrespective of the timing the salinity reduction was induced, caused deformities in terms of pericardial

235 edema. This pathology seems to be directly provoked by low salinity since almost all individuals reared in

236 reduced salinity were diagnosed with severe heart edema on 6 dph. There is no evidence that pericardial

237 edema is directly lethal, at least until yolk absorption (Kurokawa et al. 2008), but negative consequences,

238 especially during the first-feeding stages, are most likely. In order to prevent this deformity in Japanese eel

239 culture, it is suggested to maintain full-strength seawater during the early yolk sac stages (Kurokawa et al.

240 2013) and shift to low salinity conditions on 6 dph (Okamura et al., 2016), enabling feeding procedures on

241 the bottom of so-called Kreiseltanks, while positive effects on survival are sustained in the long term.

242 However, it is worth mentioning that Japanese eel larvae are reared at higher temperatures than European

243 eel larvae; and thus, the same age (6 dph) corresponds to a later developmental stage (first-feeding). In this

244 regard, it still remains to be clarified whether salinity preferences and tolerance limits, as well as respective

245 physiological responses to environmental alterations, are developmental stage (reviewed in Varsamos et al.

246 2005) and/or species-dependent. Moreover, in this study, we investigated the mechanism of cellular protein

247 repair, which is mainly driven by heat shock proteins (HSP) and activated when cellular protein damage

248 occurs under osmotic and/or heat stress (Deane et al. 2002). As such, a hyper- or hypo-osmotic shock is

249 expected to upregulate the expression of *hsps* compared to iso-osmotic conditions (Deane et al. 2002).

250 However, in this study, the expression patterns of genes (*hsp70*, *hsp90*) relating to this mechanism were not

251 directly affected by salinity, supporting the assumption that either the mechanism has not gained full

252 functionality yet to respond to such extreme physiological changes, or that the salinity regimes applied in the

253 current study are within the physiological tolerance threshold limits of this species. Additionally, the

254   expression patterns of *hsp70* and *hsp90* were not affected by the age or stage of eel larvae, as they were

255   constantly expressed throughout development, but differed among batches on 2 and 3 dph, indicating that

256   each family might have a different capacity to handle environmental (salinity) changes. In this regard, the

257   sensitivity of the relevant molecular mechanism could be inheritable, rendering eel larvae genetically pre-

258   programmed to respond according to the "good genes" hypothesis (Neff and Pitcher 2005). We also targeted

259   processes such as growth, metabolism, and development, which are regulated by underlying molecular

260   mechanisms involving the somatotropic axis, interlinking insulin-like growth factors (IGF) and growth

261   hormones (GH) (Reinecke et al 2005). Here, we attempted to molecularly trace growth, associated to salinity

262   treatments, by following expression patterns of two genes (*igf1* and *igf2*), which are an integral part of the

263   abovementioned mechanism. So far, it has been reported that isoosmotic conditions enhance growth and

264   trigger the *igf1* transcription (Deane et al. 2002). However, we did not detect differences in expression of

265   neither *igf1* nor *igf2* between the salinity treatments. This insinuates that it is either developmentally too

266   early to clearly decipher the sensitivity of this mechanism to environmental parameters (such as salinity), or

267   that growth is mediated post-translationally (Scott et al. 2004). With respect to the latter, similar results have

268   been obtained for stunted coho salmon, *Oncorhynchus kisutch*, when osmoregulation was out of control and

269   *igf1* production was hampered despite the high GH plasma levels (reviewed in Boeuf and Payan 2001). In this

270   regard, further research possibly examining the larval dry weight and protein levels under different salinity

271   scenarios and/or over a longer period may shed more light on the above theory.

272   Moreover, similar to *hsps*, growth-related expression of *igf1* and *igf2* was influenced by family (on 1 and 2

273   dph), indicating the importance of maternally supplied yolk and the oil droplet, as growth of larvae in the

274   present study was exclusively dependent on internal energy reserves. These internal sources are commonly

275   utilized throughout the endogenous feeding phase, until larvae become dependent on external dietary

276   factors throughout the following exogenous feeding stage (which was not explored in this study). Therefore,

277   it was presumable to experience a sustained expression of *igf2* over time, demonstrating a steady growth

278   progression. On the contrary, expression of *igf1* increased over time, at least for the salinity reduction

279   treatments, indicating that *igf1* might be a useful future biomarker to molecularly trace growth throughout

280   development. Furthermore, we molecularly explored the mechanism of water transport, mainly regulated

281   by aquaporins (AQP). These membrane proteins function as water channels, which control body fluid

282   homeostasis (Borgnia et al. 1999). Different homologues have been described depending on their selectivity.

283   Among them, *aqp1* is exclusively permeable to water, whereas *aqp3* is additionally permeable to glycerol

284   and urea (Borgnia et al. 1999). In adult European eel, their expression varies depending on the tissue, with

285   renal *aqp1* being downregulated (Martinez et al. 2005a) and intestinal *aqp1* being upregulated (Martinez et

286   al. 2005b) during seawater acclimation. On the other hand, gill *aqp3* levels have been shown to decrease

287   under similar conditions (Tse et al. 2006). In this study of eel larvae, deciphering tissue specific expression

288   was not possible due to size limitations. Thus, whole larvae were used instead, where *aqp1* and *aqp3* were

289   both expressed at constant levels within the ontogenetic window investigated (not affected by age/stage),

290   potentially revealing to be of basal importance throughout eel early larval ontogeny. Moreover, expression

291   of both *aqp1* and *aqp3* was family driven during the earliest stages, but on 6 dph, expression patterns were

292   controlled by the genome (family) x environment (salinity) interaction instead.

293   As such, these results reveal a family-specific phenotypic plasticity translating into a variable sensitivity

294   of each batch to salinity, which should be taken into consideration in future larviculture of this species.

295

296   **Conclusion**

297

298   The present study showed that applying drastic salinity changes in European eel larviculture, where larvae

299   are directly transferred from full strength seawater (36 psu) to 18 psu, can be realized, since this technique

300   requires only two stable RAS units "balanced" at the desired salinity levels. Even though, the capacity of eel

301   larvae to adapt to salinity changes was found to be batch-specific, the abrupt salinity changes applied in this

302   study were within the threshold tolerance limits of eel larvae, indicating no physiological injury by the

303   osmotic stress applied and supported by the even improved growth and survival. However, larvae suffer from

304   pericardial edema, with no observed negative consequence during the endogenous pre-feeding period, but

305   with potential sub-chronical implications during the later exogenous feeding stage. As such, we conclude that

306   despite the positive effect of salinity reduction on early survival (probably by saving weak individuals), the

307   long-term benefit for eel larviculture lies in the production of healthy offspring. Thus, the challenge ahead is

308 finding the balance between this trade-off process and establishing a protocol for salinity reduction, at a

309 developmental time point, without causing heart edema, while still benefiting feeding procedures and

310 survival throughout the feeding culture.

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Author contributions

JT, SP, SS, and IB provided funding, while JT, SP, and SS designed the study. JT established the assisted broodstock protocols providing gametes for the experiment. SS constructed and tailored the larval systems and rearing tanks. ES, SP, and EB conducted the experiment and collected samples. SP and EB carried out the gene expression analysis. IB performed the statistical analysis. SP and SS made illustrations. ES wrote original manuscript draft. All the authors contributed to data interpretation and manuscript revision.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest

The authors declare no competing interests.

Ethics approval

All fish were handled according to the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). The experimental protocol for the present study was approved by the Danish

347   Ministry of Food, Agriculture and Fisheries (permit number: 2015–15- 0201–00696). In particular, broodstook

348   used were anesthetized with ethyl p-aminobenzoate (benzocaine) prior to any handling, while efforts were

349   made to minimize animal handling stress.

350   **Consent for publication**

351   All the authors approved the submitted version of this manuscript.

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353   **Reference**

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