Effect of a low water concentration in chloride, sodium and potassium on oocyte maturation, oocyte hydration, ovulation and egg quality in rainbow trout

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1 # Effect of a low water concentration in chloride, sodium and
2 # potassium on oocyte maturation, oocyte hydration, ovulation
3 # and egg quality in rainbow trout.

4

5 Emilien Segret[1,2*], Emilie Cardona[1,3], Sandrine Skiba-Cassy[3], Frédéric Cachelou[2], Julien Bobe[1*].

6 [1] INRAE, LPGP, Fish Physiology and Genomics, F-35000 Rennes, France.

7 [2] Viviers de Sarrance, 64490 Sarrance, France.

8 [3] INRAE, Univ. Pau & Pays Adour, E2S UPPA, NUMEA, 64310, Saint-Pée-sur-Nivelle, France.

9 *corresponding authors: Emilien Segret : emilien.segret@inrae.fr / +33633782979

10 Julien Bobe : julien.bobe@inrae.fr

11 Key words: ultrasound staging; non-invasive phenotyping; egg viability; VisEgg; ion and water

12 transport.

13 Highlights:

14 - **Low water salinity impairs final oocyte maturation and egg quality in rainbow trout**
15 - **Low water salinity induces delayed ovulation and impaired oocyte hydration**
16 - **Low water salinity induces a dysregulation of several key ovulatory genes**
17 - **Monitoring of final oocyte maturation can be performed using ultrasound staging**

18

19

Abstract

Water salinity is an important environmental factor known to have detrimental effects on salmonid reproduction, mostly when migrating female broodfish are held in sea water. In contrast, data obtained in freshwater are scarce and the impact of low water salinity, and more specifically of low water concentrations in sodium, chloride and potassium, during reproduction in freshwater is currently unknown. For this reason, and because ion and water fluxes are critical for the final steps of the female gamete formation, including oocyte hydration and ovulation, the aim of the present study was to investigate the impact of low salinity water on final oocyte maturation, ovulation and, ultimately, on egg quality, using rainbow trout as a physiological model and relevant aquaculture species.

Fish from the same commercial strain were raised either in a site characterized by low concentrations of Na+, K+, and Cl- ions in the water or in a closely located control site exhibiting higher concentration in these elements. Egg quality and duration of final oocyte maturation were investigated using innovative phenotyping tools such as automatic assessment of egg viability using the VisEgg system and non-invasive echograph-based monitoring of final oocyte maturation duration, respectively. Oocyte hydration during final oocyte maturation and after ovulation was also investigated. Finally, molecular phenotyping was performed using real-time PCR-based monitoring of several key players of final oocyte maturation and ovulation associated with ion and water transport, inflammation, proteolytic activity, and coagulation. Oocyte hydration and gene expression data were analyzed in the light of the duration of final oocyte maturation.

Here we show that low water salinity (i.e., low water concentration in chloride, sodium and potassium) negatively influences final oocyte maturation, ovulation and, ultimately, egg quality. Low water salinity triggered delayed ovulation and lower oocyte viability. When investigating the impact of low water salinity on final oocyte maturation duration, individuals presenting the most severe phenotypes

44 exhibited impaired oocyte hydration and abnormally reduced gene expression levels of several key
45 players of the ovulatory process. While the under expression of water (i.e., aquaporins) and ion (i.e.,
46 solute carriers) transporters is consistent with impaired oocyte hydration, our observations also
47 indicate that the overall ovulatory gene expression program is disrupted. Our results raise the question
48 of the mechanisms underlying the negative influence of low salinity water on the dynamics of the
49 preovulatory phase, on the control of the oocyte homeostasis, including hydration, and on the overall
50 success of the maturation-ovulation process.

51

52 # 1 Introduction

53 Fish egg quality is defined as the egg ability to be fertilized and to subsequently develop into a normal
54 embryo (Bobe and Labbé, 2010). Egg quality can be highly variable in fish under both natural and
55 aquaculture conditions. In most species, a lack of knowledge of mechanisms influencing egg quality is
56 a limiting factor for the mass production of fry (Migaud et al. 2013). Many external factors such as
57 broodstock nutrition, temperature and photoperiod, to name just a few, are known to have a major
58 impact on egg quality in various fish species (Aegerter and Jalabert, 2004; Bonnet et al., 2007; Carrillo
59 et al., 1989; Izquierdo et al., 2001; Pankhurst et al., 1996). In contrast, other factors such as
60 composition and physico-chemical parameters of the water have received far less attention (Brooks et
61 al., 1997; Bobe and Labbe, 2010). Ionic composition of the water is however likely to influence the final
62 steps of oogenesis (i.e., final oocyte maturation - FOM) given the importance of water exchange during
63 this critical period (Dolomatov, 2012). Among mineral elements, chloride, sodium and potassium are
64 of specific interest because (i) they are the most abundant electrolytes in the body of living organisms
65 and (ii) they serve a vital function in controlling osmotic pressure and acid-base equilibrium (Lall, 2003).
66 It is commonly accepted that deficiencies in chloride, sodium and potassium are difficult to trigger
67 because fish derive these mineral elements from surrounding water (Lall, 2003). To our knowledge,
68 the impact of a low ionic composition in these three key mineral elements on female reproduction was
69 never investigated.

70 Several concomitant, yet distinct, events take place in the full-grown ovarian follicle during FOM. The
71 follicle-enclosed oocyte progressively acquires the ability to resume meiosis whereas the follicle is
72 preparing the release of oocyte from surrounding somatic layers at ovulation (Grier et al., 2007;
73 Lubzens et al., 2010). In fish, as in other vertebrates, the mechanisms involved in the ovulatory process
74 are described as inflammatory-like (Espey, 1994; Thibault and Levasseur, 1988). In contrast to meiosis
75 resumption and ovulation that are common to all vertebrates, FOM in fish is associated with a
76 significant hydration of the oocyte. This phenomenon results in a dramatic increase of oocyte volume
77 in marine species and is triggered by water entry due to the increase in free amino acid content

78 resulting from yolk protein processing (Finn et al., 2002). In the saltmarsh species, *Fundulus*
79 *heteroclitus*, the influx in potassium and sodium is a major cause of the uptake of osmotically obligated
80 water and subsequent volume increase experienced by maturing oocytes (Wallace et al., 1992). In
81 fresh water species, a more limited, yet significant, increase in water content is observed during FOM
82 that could contribute mechanically to the ovulatory process, ultimately leading to oocyte release from
83 surrounding somatic follicular layers (Craik and Harvey, 1984; Milla et al., 2006).

84 Rainbow trout (*Oncorhynchus mykiss*) is a major aquaculture species that produces eggs every year
85 starting at age 2 (Bromage et al., 1992). In this species oocyte maturation, ovulation (Cerdà et al., 2007;
86 Grier et al., 2007; Jalabert and Fostier, 1984 ; Lubzens et al., 2010), and egg quality (Bobe, 2015; Bobe
87 and Labbé, 2010; Bromage and Jones, 1992; Brooks et al., 1997) have been extensively studied,
88 including at the molecular level (Bobe et al., 2004, 2006, 2009; Yamashita et al., 2000). As previously
89 documented, exposition to stress factors (e.g., temperature) during FOM can alter the quality of
90 unfertilized eggs released from the ovary at ovulation (Aegerter and Jalabert, 2004; Bobe and Labbé,
91 2010; Colson et al., 2019).

92 For these reasons, and because water and ion fluxes are important for several key steps of oocyte
93 development during FOM, the aim of the present study was to investigate the impact of water ionic
94 composition, and more specifically low concentrations of sodium, chloride and potassium on FOM,
95 ovulation and, ultimately, on egg quality, using rainbow trout as a physiological model and relevant
96 aquaculture species. To achieve this objective, we used two closely located production sites in the
97 French Pyrenees where the stream water exhibits dramatically different concentrations of chloride,
98 sodium and potassium all year round, including during the reproductive period.

99 2 Material and methods

100 2.1 Ethical statement

101 Investigations were conducted in compliance with EU Directive 2010/63/EU for animal experiments.
102 The experimental protocol registered under the reference #201904021747602 was specifically

approved by INRAE-LPGP Animal Care and Use Committee. For oocyte sampling, echograph-based monitoring and ovulation detection, fish were anaesthetized in tricaine metanesulfonate (MS222) at a concentration of 130 mg/liter. For gonad sampling, fish were euthanized in MS222 at a concentration of 300mg/liter, until 10 minutes after cessation of opercular movement.

2.2 Experimental sites and water characterization

Two experimental sites located in Rébénacq [43.155948,-0.405067] and Sarrance [43.049999,-0.6014075] (French Pyrenees), and presenting similar water temperature [9°-10°C] and oxygen conditions were used in the present study. The two sites are close (<20 km) but located in different valleys (i.e., hydrogeologic systems) differing mostly by ionic abundance in Sodium (Na^+), Chloride (Cl^-) and Potassium (K^+) in the water (Fig 1.A , Supplemental datafiles 1 and 2). The site presenting significantly lower abundance of these 3 mineral elements will be referred to hereafter as “Low Salinity Water (LSW)”, whereas the other site, for which water ionic composition was significantly higher, will be referred to as “Control”.

For both sites, water composition analyses were performed monthly throughout the year, from the water flowing into the experimental raceways using a dedicated water sampling flask to avoid any contamination. Samples were analyzed by a specialized analytical laboratory (Ultra Traces Analyses Aquitaine, Pau, France) that provided the sampling flasks. Ionic abundance analysis was performed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) technique for Na^+ and K^+ and by ionic chromatography for Cl^-. Data obtained were not normally-distributed (Shapiro-Wilk test, $p > 0.005$). Ionic concentration in the 2 sites was compared using non parametric U-tests. A comprehensive water analysis of both sites is available in supplemental data files 1 and 2.

2.3 Fish rearing

Three years old female rainbow trout (*Oncorhynchus mykiss*) from the same commercial strain (Viviers SA Company strain, France) were used in this study. After rearing in a nursery site, fish were transferred to either Rébénacq (control site) or Sarrance (LSW site) locations at 6 months post-fertilization. On

128 both sites, fish were reared under natural photoperiod in 125m^3 raceways at a 60kg.m^{-3} density, with
129 a water renewal of 75% per hour until the beginning of the experiment. Stream water was used in an
130 open circulation system without any recirculation. Similar feeding protocols, including feeding rate and
131 diet composition, were applied for both sites throughout their lifecycle. Fish were fed at an identical
132 daily rate (0.6% of the live weight) with the same commercial diet (46% crude protein, 19% crude fat,
133 0.9% phosphorus, 11.5% ash, 19 MJ/kg gross energy, Omega-3 4.8%, Omega-6 1.25%) (Le Gouessant,
134 Lamballe, France). Following a first reproduction at age 2, fish were held under natural photoperiod
135 during their second reproductive cycle. Spawning naturally occurred during November-December.

136 2.4 Experimental design

137 Approximately two weeks before expected ovulation (estimated based on previous ovulation dates),
138 fish were transferred into a 8 m^3 raceway to facilitate manipulations. Experimental raceways on both
139 sites were of similar dimensions (8 m in length, 1 m in width and 1 m in depth) with a water renewal
140 of 200% per hour originating directly from the stream water. Ovulation was monitored three time a
141 week. Feeding was stopped when first ovulations were detected. On the control site, a total of 32 fish
142 were used including 18 for egg quality assessment using the VisEgg system (Cardona et al. 2020) (4 of
143 them being also used for the oocyte hydration study and 11 of them being also used for blood
144 sampling) and 14 for the molecular analysis. On the LSW site, a total of 46 fish were used including 30
145 for egg quality assessment using the VisEgg system (4 of them being also used for the oocyte hydration
146 study and 9 of them being also used for blood sampling) and 16 for the molecular analysis.

147 2.5 Egg quality assessment using VisEgg

148 In both groups, specific fish (control, n=18; LSW, n=30) were individually monitored until ovulation.
149 Ovulations were checked every 2-3 days, under anesthesia, by gentle pressure on the abdomen.
150 Oocyte viability evaluation was systematically performed using the VisEgg protocol when ovulation
151 occurred. This recently developed tool allows a robust assessment of different egg features including
152 egg viability (Cardona et al., 2020). The process uses a standardized image-based batch analysis

153 method of 24h-hydrated unfertilized eggs and an image processing algorithm. Outputs are the
154 presence and percentage of whitening (i.e., non-viable) eggs within the batch. These features can be
155 used to reliably assess egg integrity and viability. Analyze of presence of white eggs depending on
156 rearing sites or the maturation duration yielded contingency tables analyzed using Khi-2 tests. Data
157 obtained from VisEgg were not normally distributed (Shapiro-Wilk test, $p < 0.001$). Comparison of two
158 groups were performed using non parametric U-tests.

159 2.6 Oocyte development staging during FOM

160 A non-invasive to the female ultrasound-based staging was used to estimate progress into FOM. This
161 approach, previously described for assessing reproductive stages in wild salmons (Nevoux et al., 2019),
162 was used here for the first time to assess progression into FOM in rainbow trout. An ultrasound scanner
163 M-Turbo (Sonosite) with a 5-10MHz linear transducer (multiple frequency) was used. Anesthetized fish
164 were placed in a plastic tray for a short duration (<30 sec) and sagittal images of the abdominal cavity
165 were obtained by aligning the transducer with the lateral line of the fish. Late vitellogenic oocytes
166 appeared white on the screen, while a dark area progressively appeared at the center of the oocyte as
167 the oocytes progressed into FOM (Fig 3.A).

168 In order to validate echograph-based staging, direct brightfield observation was performed. In rainbow
169 trout, late-vitellogenic (LV) oocytes observed with the naked eye appear opaque and no noticeable
170 changes in yolk structure or any sign of meiosis resumption can be observed. Post-vitellogenesis (PV)
171 oocytes exhibit a central yolk clarification as meiosis progressively resumes. Germinal vesicle is still
172 visible and lipid droplets remain relatively small. During oocyte maturation (Mat), germinal vesicle
173 breakdown (GVBD) progressively occurs and oocytes become totally clear with well-defined lipid
174 droplets (Grier et al., 2007; Jalabert and Fostier, 1984; Milla et al., 2006; Patiño and Sullivan, 2002;
175 Hurk and Peute, 1979; Wallace and Selman, 1990). The above description of phenotypic changes
176 occurring in the rainbow trout oocyte during FOM was used to validate the echograph-based staging.
177 In 100% of the cases (n= 57 fish) the direct observation confirmed the echograph-based staging of the

oocyte during FOM. Echograph-based oocyte staging was preferred due to its non-invasiveness nature and was thus subsequently used to measure the duration of the oocyte maturation phase (i.e., between fully transparent oocyte (Mat) stage and detected ovulation) in the present study. The duration of the maturation phase was temperature-normalized using the actual temperature recorded on both sites during experiments and expressed in hours at 10°C in both experimental groups. Comparisons of the two groups were performed using non-parametric U-tests.

2.7 Plasma sampling and analysis

Blood was sampled during oocyte maturation on both sites (control, n=9; LSW, n=11). Blood samples were centrifuged (1000 g, 10 min, 4°C), and plasma were sampled and stored at −20 °C until further analysis. Plasma samples were analyzed by a specialized analytical laboratory (Ultra Traces Analyses Aquitaine, Pau, France). Ionic abundance analysis was performed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) technique for Na^+ and K^+ and by ionic chromatography for Cl^-. Comparison of two groups were performed using non parametric U-tests.

2.8 Hydration measurement

For analytical purposes, individual fish originating from the LSW site were separated in two groups, based on the duration of the maturation phase [Mat<64H at 10°C (n=10), Mat⩾64H at 10°C (n=21)]. Oocyte hydration was measured (n=4 individuals of each group) and monitored from late vitellogenesis stage until ovulation. The method used has previously been described (Milla et al., 2006). Briefly, gentle manual striping was performed under anesthesia to collect oocytes, from late vitellogenesis stage until ovulation. As previously described (Finet et al., 1988), non-ovulated oocytes were expelled from the follicle layers using two fine tip tweezers. Oocyte development staging was performed under a binocular microscope. A total of 60 to 80 oocytes per female were obtained and rapidly weighted to obtain wet mass (WM). After heating these oocytes 24 h at 105°C in a drying oven (Memmert, Buechenbach, Germany), dry mass (DM) was measured. The water content (WC) was measured from subtracting DM from WM. For each individual, WC was measured (n=4 individuals of each group) and

monitored from late vitellogenesis stage until ovulation. Analysis of the increase in water content (WC) in the two experimental groups (Mat <64H ; Mat ⩾ 64H) was performed using non parametric U-test.

2.9 Molecular analysis

2.9.1 Tissue collection

Ovaries from control (n=4 late-vitellogenesis stage and n=10 oocyte maturation stage) and LSW (n=5 late-vitellogenesis stage and n=11 oocyte maturation stage) groups were sampled for RNA extraction. After euthanasia, ovaries were dissected out of the abdominal cavity. Oocyte staging was performed using ultrasound images prior to euthanasia followed by macroscopic validation of oocyte stage under microscope after dissection. Ovarian follicles were deyolked as previously described (Bobe and Goetz, 2000) by pressing the entire tissue between two stainless steel screens while continuously applying ice-cold Cortland medium with a squirt bottle, then immediately frozen in liquid Nitrogen and stored at -80°C until RNA extraction.

2.9.2 Total RNA extraction and reverse transcription

Ovarian tissue was homogenized in Trizol reagent (Molecular Research Center, Cincinnati, USA) at a ratio of 1ml per 100mg of tissue. Tissue was grinded using bead beating technology (Precellys Evolution, Bertin Technologies, Saint-Quentin en Yvelines, France). Total RNA extraction was performed following the manufacturer’s instructions. Briefly, 2 µg of total RNA were reverse transcribed using Maxima First Strand cDNA Synthesis Kit (Maxima Reverse Transcriptase enzyme, derived from Moloney murine Leukemia virus enzyme). Reverse transcription was performed by 10 minutes of incubation at 25°C followed by a 15 minutes step at 60°C and a 5-minute step at 75°C. Control reactions were run without Maxima reverse transcriptase and used as negative controls in the real-time polymerase chain reaction study.

2.9.3 Real-time PCR analysis

Real-time PCR was performed using a LightCycler480 (Roche, Switzerland). Reverse transcription products were diluted to 1/30. Technical replicates of each biological sample were run. Runs were

228 performed in 5µL reactions containing 2µL of RT product, 250 nM of each primer and 1X PowerUp
229 SYBR Green Master Mix (Applied Biosystems). The program used was: 50°C for 2 minutes, then 95°C
230 for 2 minutes for initial denaturation, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. After
231 amplification, a fusion curve was obtained by 15-second step followed by a 0.5°C increase, repeated
232 70 times, and starting at 60°C

233 ### 2.9.4 Gene expression analysis

234 Several concomitant events occur in the ovary during FOM that involve distinct biological processes
235 including ovulation, an inflammatory-like process characterized by major tissue rearrangements, blood
236 coagulation, detachment of somatic follicular cells from the oocyte, and oocyte hydration which
237 involves specific water and ion transports. Genes used for the molecular analysis were thus selected
238 for their putative role in inflammation, proteolytic activity, or water and ion transport and were
239 previously reported to be markedly differentially up-regulated during FOM in rainbow trout (Bobe et
240 al., 2006). Primers used in this prior study (Bobe et al., 2006) were used here to specifically monitor
241 the expression of the following genes. *aquaporin 4* (*aqp4,* GenBank accession # BX885214), *vasotocin-*
242 *neurophysin VT 1* (*vt1,* GenBank accession # CA375992) and *solute carrier family 26* (*slc26a4,* GenBank
243 accession # BX873066) are involved in ion and water transport processes whereas *A Disintegrin And*
244 *Metalloproteinases 22* (*adam22*, GenBank accession # CA363158), *CXC chemokine L14* (*cxcl14,*
245 GenBank accession # BX868653) and *coagulation factor V* (*f5*, GenBank accession # BX879767) are
246 inflammation-related genes. 18S RNA level was measured in each sample and used for messenger RNA
247 abundance normalization. Expression levels at the late-vitellogenic (LV) stage are not presented but
248 were not significantly different between groups and were arbitrarily set to 1 for each group. Expression
249 data at the oocyte maturation stage were expressed as a percentage of the transcript abundance at
250 the LV stage. Difference between LSW and control groups at oocyte maturation stage were analyzed
251 using non-parametric U-tests.

252

253 # 3 Results and discussion

254 ## 3.1 Low water salinity results in lower egg viability

255 The automatic phenotyping of egg viability using the VisEgg system (Fig 2.A) revealed that 60% of the
256 females exhibited non-viable eggs at the time of ovulation in the LSW group in sharp contrast with the
257 control group in which only 18% of the females exhibited at least one non-viable egg at ovulation.
258 More specifically, the percentage of non-viable eggs was dramatically increased (+378%) in the LSW
259 group in comparison to the control group (Fig 2.B). Salinity is known to have an impact on reproduction
260 in fish, including in salmonids. In Atlantic salmon (*Salmo salar*) and Coho salmon (*Oncorhynchus*
261 *kisutch*), whole-life breeding in freshwater can result in the production of poor quality gametes
262 including poor semen production for males and presence of atretic follicles in females (Valdebenito et
263 al., 2015). Similarly, other studies reported that rearing of Atlantic salmon in sea water induces delays
264 and variability in the timing of ovulation (Haffray, 1995) and poor gamete quality (Maisse et al., 1998).
265 In Arctic charr (*Salvelinus alpinus*), gamete quality is improved for broodstock transferred in saltwater
266 during their gametogenesis (Atse et al., 2002). Together, these studies indicate that water salinity is a
267 key factor for the reproduction success of salmonids that all reproduce in freshwater even though a
268 part of their life cycle occurs in sea water in some species. Most studies have however focused on the
269 impact of high water salinity during final oocyte maturation. In contrast, data on low salinity water
270 remain scarce. In addition, most studies have focused on migrating species that return to freshwater
271 to reproduce. In the present study, we confirm that water salinity is a key factor of reproductive
272 success and show that, similarly to high salinity, low salinity can also have a detrimental effect on
273 reproductive success.

274 ## 3.2 Low water salinity triggers delayed ovulation

275 Together, our results indicate that low water concentration in chloride, sodium and potassium triggers
276 a significant decrease in egg viability at ovulation. In order to shed light on underlying mechanisms, we
277 aimed at investigating the preovulatory period that is known to be critical for egg quality. Given the

278 favorable temperature range (i.e., 9-10°C) and similar feeding regime throughout the reproductive
279 cycle, we reasoned that LSW could perturbate FOM and investigated the duration of the maturation
280 phase from meiosis resumption to ovulation. Using non-invasive echograph-based analysis we could
281 measure the duration of the oocyte maturation phase. After monitoring the first signs of intra-oocyte
282 clearing (i.e., post-vitellogenic stage) (Fig 3.A and B), oocytes became fully transparent (i.e., maturation
283 stage) until they were released from the ovary at ovulation. The duration of the maturation phase was
284 calculated in each group after normalization by the daily temperature on site. We observed (Fig 3.C)
285 that the maturation phase was significantly longer in the LSW group in comparison to the control
286 group. The duration of oocyte maturation was on average 53 hours (at 10°C) in the LSW group while it
287 was 70 hours (at 10°C) in the control group, which corresponds to a 32% increase in FOM duration in
288 the LSW group.

289 Several studies described the temporal aspects of final oocyte maturation in rainbow trout. The *in vivo*
290 duration of FOM, including progressive central clearing of the oocyte, ranged from 48 to 72 hours at
291 13°C (Bry, 1981). The detachment of the mature oocyte from surrounding somatic follicular layers was
292 reported to occur between 72 and 96 hours after the first observable signs of oocyte maturation (i.e.,
293 central clarification) at 10°C (Jalabert, 1978; Jalabert and Szöllösi, 1975). Other authors showed that
294 ovulation occurs generally 48-96 hours at 14°C after the peak concentration of maturation-inducing
295 steroid (Fostier, 1981). Another study shown a duration of 48-72 hours at 12°C between maturation
296 and detected ovulation for rainbow trout (Breton et al., 1998).

297 The duration of oocyte maturation, from full oocyte cytoplasm clearing to ovulation, reported here for
298 the control group, is therefore fully consistent with the duration of FOM reported in these prior studies
299 that usually included the post-vitellogenic phase (i.e., germinal vesicle migration towards the periphery
300 of the oocyte). Our observations however suggest that the duration of FOM in the LSW group is higher
301 or in the upper range of what is classically observed in rainbow trout at 10°C. In addition, the LSW
302 group exhibits a strong interindividual variability with individuals that ovulated 97 hours (2 individuals)
303 and 130 hours (1 individual) after detected oocyte maturation (Fig 3.C). These individuals presented

304 the usual phenotypic traits of maturation (i.e., color pattern and body shape), but ovulation occurred
305 drastically later. Heterogeneity in FOM duration and, in some cases, absence of ovulation has often
306 been reported in trout and other fish. This heterogeneity has however systematically always been
307 associated with premature induction of FOM and ovulation, typically when FOM was triggered in
308 maturationally incompetent (i.e., vitellogenic) follicles *in vivo* or *in vitro*. This is for instance the case
309 for hormonally injected fish (Bry, 1981; Goetz and Bergman, 1978; Jalabert, 1978). Using non-invasive
310 ultrasound-based FOM monitoring, we were able to demonstrate here that oocyte maturation (i.e.,
311 oocyte clearing) occurred but that ovulation was delayed. Previous studies have demonstrated that
312 oocyte maturation and ovulation, while being interconnected, were two independent processes. In
313 rainbow trout it is possible to trigger ovulation without oocyte maturation (Jalabert, 1972). Together,
314 our results show that low salinity (i.e., low water concentration in chloride, sodium and potassium)
315 during FOM triggers delayed ovulation, sometimes by several days. Our observations also indicate that,
316 while oocyte maturation occurs normally, the ovulatory process appears impaired or inhibited,
317 ultimately leading to abnormally long FOM.

318 3.3 Reduced egg viability in low salinity water is linked with delayed ovulation

319 Together our results show that low water concentration in chloride, sodium and potassium triggered
320 reduced egg viability and delayed ovulation. We also noticed a high inter-individual variability in both
321 egg viability and maturation phase duration in the LSW group (Figs 2.B & 3.C). We reasoned that
322 delayed ovulation could be at the origin of lower egg viability in the LSW group and investigated further
323 any link between egg viability and maturation phase duration. For data analysis, LSW fish were
324 separated in two groups, based on the duration of the maturation phase. We observed a dramatic and
325 highly significant difference in egg viability in the LSW group depending on the duration of the
326 maturation phase (Fig 4). A very high proportion (85%) of the fish exhibiting a maturation phase
327 duration of 64 hours or more exhibited non-viable eggs at ovulation, while only 10% of the fish that
328 underwent oocyte maturation in less than 64 hours exhibited this feature (Fig 4.A). In addition, the

329 percentage of non-viable eggs was dramatically increased in the $\geqslant$64h group in comparison to the
330 <64h group (Fig 4.B).

331 These results strongly support our hypothesis of a significant loss of egg quality after delayed ovulation.
332 A possible explanation for the higher rate of non-viable eggs would be the ageing of the oocytes. This
333 well-known phenomenon has been extensively described (Aegerter and Jalabert, 2004; Billard, 1981;
334 Craik and Harvey, 1984; Escaffre, 1976; Lahnsteiner, 2000; Samarin et al., 2015, 2008; Springate et al.,
335 1984) and is known to trigger major egg quality defects. All existing studies on oocyte ageing prior to
336 fertilization were however conducted after post-ovulatory ageing. To our knowledge, oocyte ageing
337 prior to ovulation (i.e., within the ovarian follicle) is reported here for the first time in a fish species. In
338 salmonids, in contrast to other fish species, post-ovulatory ageing does not induce a rapid decrease of
339 egg quality. In rainbow trout, ovulated eggs can be held in the body cavity for 5 days at 12°C without
340 any significant decrease of their developmental success (Aegerter et al., 2005). It is therefore unlikely
341 that the modest intra follicular post-meiotic ageing observed here in the LSW group, in which ovulation
342 is delayed, on average, by 17 hours, is sufficient to trigger alone the drop of egg quality observed in
343 LSW. The osmotic properties of the rainbow trout egg have been thoroughly investigated in rainbow
344 trout (Gray, 1932) and another explanation for the increase in whitening egg occurrence could be a
345 loss of the oocyte homeostasis in LSW ultimately leading to non-viable eggs. In brook trout, a
346 diminution of ionic concentration (Cl-) in surrounding medium has as direct negative effect of
347 membrane resistance (Marshall et al., 1985). Disruption of the oocyte homeostasis could therefore
348 originate from a fragile oocyte vitelline membrane. In addition, the loss in oocyte homeostasis could
349 originate from difficulties (i.e., high energetic cost) in maintaining the osmotic difference across oocyte
350 membrane in a hypotonic external medium.

351 Together, our results show that the reduced egg viability observed in LSW is linked to delayed
352 ovulation. This reduced egg viability can be due, at least in part, to intra-follicular oocyte ageing. We
353 cannot, however, rule out the possibility that LSW also affects egg viability through other mechanisms
354 that could disrupt the maintenance of oocyte homeostasis and ultimately decrease egg viability.

Further investigations are needed to unravel the mechanisms triggering decreased egg viability when FOM is occurring in LSW.

3.4 Low water concentration in chloride, sodium and potassium results in reduced oocyte hydration during FOM

The release of the oocyte from surrounding follicular layers at the time of ovulation results from the ovulatory process that has originally been described in mammals as an inflammatory-like reaction. In fish, in which the oocyte is directly in contact with surrounding follicular layers, a prerequisite is the separation of the oocyte from surrounding granulosa layers (Jalabert, 1976; Lubzens et al., 2010). This phenomenon has been thoroughly described in rainbow trout in which suppression of tight junctions between granulosa cells and the oocyte results in the apparition of a thin periplasmic space separating the oocyte from surrounding somatic layers (Lubzens et al., 2010). Many observations, including direct brightfield *in vitro* follicle observation, have shown that preovulatory rainbow trout oocyte exhibits a significant mechanical pression onto surrounding somatic layers (Lubzens et al., 2010). Somatic follicular layers appear to be stretched around the oocyte at that stage. Because of the angiotensin-like nature of the ovulatory process and the contractile actions of theca layers during ovulation (Hajnik and Goetz, 1998; Hsu et al., 1992), postovulatory follicles appear shrinked after ovulation. It was hypothesized that the modest (i.e., 20%), yet significant, increase in water content observed during rainbow trout FOM would contribute to the increasing pressure applied by the oocyte onto surrounding somatic layers and would ultimately mechanically facilitate the exit of the oocyte through a hole in somatic layers (Milla et al., 2006). Mechanic pressure applied by the oocyte swelling is one cause of follicle rupture and the completeness of hydration facilitates the complete liberation of unfertilized eggs into the abdominal cavity. We reasoned that a lower hydration would decrease the efficiency of ovulation and investigated hydration in the two above defined sub-groups exhibiting respectively <64h and ⩾64h maturation phase duration. Oocyte water content (WC) was monitored throughout FOM in both groups. In the <64h group, WC progressively increased throughout FOM in

380 comparison to the late vitellogenic (LV) stage, with a 15% increase during oocyte maturation and a
381 27% increase at ovulation to reach 36% during the post-ovulatory period (Fig 5, light blue). In contrast,
382 a much more limited increase in water content was observed in the ⩾64h group. In comparison to the
383 LV stage, a 2% increase in water content was observed during oocyte maturation while a 13% increase
384 was monitored at ovulation that did not further increase during the post-ovulatory period.
385 Observations in the <64h group are fully consistent with existing data in rainbow trout that were
386 obtained using the same methodology (Milla et al., 2006). In contrast the hydration in the ⩾64h
387 appears to be drastically reduced. At all stages, the water increase was significantly lower in the ⩾64h
388 group in comparison to the <64h group. Together these results indicate that oocyte hydration is
389 impaired during FOM occurring in low salinity water.

390 Oocyte hydration has been described for several species (Finn et al., 2002; LaFleur and Thomas, 1991;
391 Milla et al., 2006; Watanabe and Kuo, 1986). Liberation of free amino acids and an uptake of inorganic
392 ions occur during maturation. These events create a change in osmotic equilibrium, resulting in the
393 passive transport of water molecule into the oocyte. This transport mechanism uses several proteins
394 activity such as solute carriers, Na+/K+ ATPases, Aquaporins, and demonstration was made that
395 protein activity and hydration were closely dependent on K+ concentration in the medium (Ecker and
396 Dennis Smith, 1971; LaFleur and Thomas, 1991). K+ and Na+ accumulation in the oocyte during
397 maturation plays an important role in the oocyte hydration process. For *Fundulus heteroclitus*, oocytes
398 Na+ and K+ uptakes by gap junctions (Lubzens et al., 2010) are main responsible of osmolality increase
399 (Greeley et al., 1991; Wallace et al., 1992). For Atlantic halibut *Hippoglossus hippoglossus*, Cl- uptakes
400 also contributes to the increase of oocyte osmolality (Finn et al., 2002). Together these studies
401 demonstrate the importance of Na+, K+ and Cl- ions for the oocyte hydration process during FOM. In
402 contrast, the impact of low water salinity was never investigated, mostly because these experiments
403 were often conducted in marine species. We hypothesize that FOM in low salinity water is associated
404 in reduced ion~~s~~ uptake during maturation ultimately leading to incomplete oocyte hydration and

405 thereby causing a large variability in the timing of ovulation. Further investigations are needed to
406 elucidate underlying mechanisms.

407 3.5 Low water concentration in chloride, sodium and potassium induces
408 dysregulated expression of ovulation genes

409 The ovulatory process involves the activation of a specific gene expression program that has been
410 extensively documented in mammals (Richards et al., 2002) and in fish (Bobe et al., 2009, 2006; Cerdà
411 et al., 2007; Fabra et al, 2005; Hajnik and Goetz, 1998; Knoll-Gellida, 2006). The inflammatory-like
412 nature of the ovulatory process has been well characterized and the expression of many inflammation-
413 related genes have been reported in various fish species (Hagiwara, 2020; Ohnishi, 2005; Takahashi,
414 2019) including in rainbow trout (Bobe et al., 2006). A subset of these previously identified genes
415 related to inflammation, proteolytic activity, or ion and water transport was monitored to characterize
416 the ovulatory process in low salinity water.

417 *Solute transporters and hydration-related genes are under-expressed in low salinity water*

418 Aquaporin are molecular channels responsible of passive water transport across membranes. The role
419 of aquaporins during fish oocyte hydration has been described by several studies. The up-regulation
420 of *aquaporin 4* (*aqp4*) was reported during rainbow trout follicular maturation (Bobe et al., 2006),
421 suggesting that oocyte hydration of fresh water fish is aquaporin mediated, in consistency with the
422 demonstrated role of *aquaporin1-like* in oocyte hydration of saltwater fish (Cerdà, 2009; Fabra, 2005).
423 The *solute carrier family 26 member 4* gene (*slc26a4*), previously known as *pendrin*, codes for a
424 transmembrane anion exchanger and is involved in osmoregulation mechanisms (Nakada et al., 2005).
425 Identified as dramatically up-regulated in the rainbow trout ovary during maturation, *slc26a4* is
426 believed to play an important role in the osmotic regulation, causing water influx into oocyte during
427 meiotic maturation. In the present study, a marked under expression of *slc26a4* and *aqp4* is observed
428 in the ovary when FOM occurs in LSW (Fig 6). Given the role of both genes in ion and water transport,

429 this under expression is fully consistent with the impaired oocyte hydration observed in LSW, in
430 comparison to the control group.

431 Vasotocin-neurophysin VT 1 (*vt1* gene) has two key roles in FOM-ovulation in catfish (Joy and Chaube,
432 2015). It is an important link in the gonadotropin cascade controlling FOM and ovulation. By a common
433 action with maturation inductions hormones, VT 1 is a regulator of germinal vesicle breakdown and
434 meiosis resumption. VT 1 also plays a key role in follicular hydration by regulating the aquaporin action
435 (Balment et al., 2006). Here we show a marked under expression of *vt1* observed in the LSW group in
436 comparison to the control group (Fig 6). This profile is fully consistent with both the observed impaired
437 ovulatory process and the under expression of *aquaporin 4*. In addition, the position of *vt1* in the
438 gonadotropin-dependent regulatory cascade controlling oocyte maturation and ovulation suggests
439 that it could be a relay in mediating the inhibitory effect of LSW.

440 *Inflammation-related genes are under-expressed in low salinity water*

441 Ovulation is accompanied by a broad-spectrum proteolysis within follicular layers that involves a large
442 number of proteases. Members of A Disintegrin and Metalloproteinase gene family are dramatically
443 up-regulated during trout FOM-ovulation phases (Berndtson and Goetz, 1988; 1990; Bobe et al., 2006).
444 Adam22 plays an important role in cell-to-cell and cell-to-matrix interaction regulation (White, 2003).
445 By regulating or processing the deposit of the collagen fibrils, ADAMs proteases cause the extra-cellular
446 matrix proteolysis (Ohnishi et al., 2005).

447 CXC chemokines stimulate recruitment of leukocytes and play a role of pro-inflammatory mediators.
448 Cxcl14 implication in immune–related functions has been described in fish (Baoprasertkul et al., 2005).
449 The over-expression of *cxcl14* during FOM-ovulation phases is fully in line with the inflammatory-like
450 nature of the ovulatory process.

451 Coagulation factor V (*f5* gene) is involved in synthesis of blood coagulant factors and has been
452 characterized in mammals and fish (Hanumanthaiah et al., 2002; Rothberger, 1984). It was speculated

453 that synthesis of coagulation factor V prevents bleeding caused by rupture of follicle layers during
454 ovulation.

455 Here we show that *cxcl14*, *adam22*, and *f5* are dramatically under expressed during oocyte maturation
456 in the LSW group in comparison to the control group (Fig 6). These expression profiles are consistent
457 with the delayed ovulation observed in LSW and suggest that the inflammatory nature of ovulation is
458 affected. This is especially true for *cxcl14* for which a 5-fold decrease in gene expression is observed in
459 the LSW group in comparison to the control group. Together, these results show that several important
460 components of the ovulatory process are toned down when FOM occurs in low salinity water. It is
461 noteworthy that most, if not all, aspects of the ovulatory process including inflammation, coagulation,
462 swelling, water and ion transport as well and proteolysis are under activated. Even if previous studies
463 have shown gene expression changes when fish adapt to water salinity change (e.g. migration,
464 precipitation) for salmonids (Morro, 2020; Norman et al., 2014) and other species (Fiol, 2006; Pujante,
465 2018), evidence of such an impact of low water salinity on the overall ovulatory process were, to our
466 knowledge, previously unreported. This raises the question of how low water salinity can inhibit the
467 overall ovulatory process and not only osmotic exchanges.

468 3.6 Lower blood plasma levels of sodium, chloride and potassium are observed in the 469 LSW site

470 In order to assess the impact of water concentration in chloride, sodium and potassium on fish
471 homeostasis, we measured blood plasma concentration in these three elements in both control and
472 LSW sites. We observed a significantly lower plasmatic concentration of these three mineral elements
473 in fish of the LSW site in comparison to the control site (Fig 1.B). This indicates that the water
474 composition can lead to significant differences in plasma concentration in these elements. This also
475 further supports our hypothesis that reproduction in fresh water exhibiting low sodium, chloride and
476 potassium concentration perturbates the fish oocyte osmolarity and the hydration process during final
477 oocyte maturation and ovulation.

478 ## 3.7 Role of other water components

479 While marked and highly significant differences in water concentration of chloride, sodium and
480 potassium are found between the two experimental sites, other differences exist in water composition
481 that could also contribute, at least in part, to the impaired FOM and ovulation and reduced egg quality
482 phenotype observed in the LSW site. A lower concentration in Iron (Fe) and Manganese (Mn) is
483 observed in the LSW site in comparison to the control site. For Iron, the difference is however not
484 significant when only the reproductive period is considered (Supplemental datafile 2). Manganese
485 (Mn) is known to inhibit ovulation in rainbow trout (Jalabert and Szollosi, 1975) and inhibit meiotic
486 maturation in terrestrial animals (Bilodeau-Goeseels, 2001). It is therefore difficult to link low Mn
487 concentration with impaired FOM and ovulation. In addition, it is commonly accepted that the diet is
488 considered to be the major source of Manganese and Iron (Lall, 2002). It is therefore extremely unlikely
489 that the lower water concentration in Mn and Fe found in the LSW site is responsible for the impaired
490 reproductive phenotype observed in the LSW site. Similarly, a lower nitrate concentration was
491 observed in the LSW site. While we cannot rule out that this could contribute to the abnormal
492 reproductive phenotype observed in the LSW site, this seems however, to our knowledge and in the
493 light of previous studies led on salmonids and others fish species (Dolomatov, 2011; Tripathi and
494 Krishna, 2008), extremely unlikely.

495 # 4 Conclusion

496 Here we show that low water concentration in chloride, sodium and potassium negatively influences
497 final oocyte maturation, ovulation and, ultimately, egg quality. Low salinity water triggers delayed
498 ovulation and lower oocyte viability. When investigating the impact of LSW on FOM duration,
499 individuals presenting the most severe phenotypes exhibited impaired oocyte hydration and
500 abnormally reduced gene expression levels of several key players of the ovulatory process. While the
501 under expression of water (i.e., aquaporins) and ion (i.e., solute carriers) transporters is consistent with
502 impaired oocyte hydration, our observations also indicate that the entire ovulatory gene expression

sequence is disrupted. Our results raise the question of the mechanisms underlying the negative influence of low water concentration in chloride, sodium and potassium on the dynamics of the preovulatory phase, on the control of the oocyte homeostasis, including hydration, and on the overall success of the maturation-ovulatory process. A global analysis of differentially regulated genes in LSW during final oocyte maturation is needed to unravel these mechanisms.

5 Declarations

5.1 Competing interests

The authors declare no competing interest.

5.2 Authors' contributions

ES conceived the study, performed experiments and data analysis and drafted the manuscript. EC participated in experiments. SS-K and FC participated in the design of the study. JB conceived the study and participated in manuscript writing and data analysis.

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526 # 6 Figure legends

527 *Color should be used for all figures in print.*

528 **Figure 1: Water and plasma composition on both experimental sites**

529 A: Distribution of Potassium, Chloride and Sodium concentration (mg/L) in Control (in grey, n=12) and
530 Low Salinity Water (in blue, n=12) sites.

531 B: Distribution of Potassium, Chloride and Sodium concentration (g/L) in fish blood plasma reared in
532 Control (in grey, n=11) and Low Salinity Water (in blue, n=9) sites.

533 **Figure 2: Egg quality in the two experimental sites**

534 A: Occurrence of non-viable eggs in batches evaluated using the VisEgg system in Control (n=18) and
535 LSW (n=30) groups. Khi-2 test (p = 0.007).

536 B: Rate (percent) of non-viable eggs evaluated using the VisEgg system in Control (n=18) and LSW
537 (n=30) groups.

538 **Figure 3: Ultrasound staging for measure of maturation duration**

539 A: Echograph-based observation of oocyte changes during final oocyte maturation. Changes in oocyte
540 aspect from LV to MAT is illustrated by a progressive central darkening, with brightening of peripheral
541 layer before ovulation.

542 B: Pictures of direct observation of oocytes classified by stage. Oocyte were obtained by stripping and
543 defolliculation (Finet et al., 1988). Yolk clarification and lipid droplet formation are observable.

544 C: Distribution of maturation duration evaluated by ultrasound in Control (n=18) and LSW (n=30) from
545 central oocyte clarification and ovulation.

546 **Figure 4: Egg quality depending on the duration of oocyte maturation**

A: Percentage of females exhibiting non-viable eggs in low salinity water, evaluated using the VisEgg system. Individuals are separated in two groups depending on the duration of oocyte maturation (Mat<64H, n=10; Mat⩾64H, n=20). Khi-2 test (p = 1.34E-04).

B: Percentage of non-viable eggs depending on the duration of oocyte maturation (Mat<64H, n=10; Mat⩾64H, n=20).

Figure 5: Hydration of oocytes during final oocyte maturation

Increase of oocyte water content (WC), in comparison to late vitellogenic stage, for oocytes sampled at four ovarian stages: Late-Vitellogenesis (LV), oocyte maturation (Mat), ovulation (Ov) and 3 days post-ovulation (Post-Ov). Individuals are separated in two groups based on the duration of oocyte maturation (Mat<64H, n=4; Mat⩾64H, n=4).

Figure 6: Ovarian expression profiles of genes involved in FOM and ovulation

Ovarian expression profiles of *aqp4*, *slc26*, *cf5*, *adam22*, *cxcl14* and *vt5* during oocyte maturation in control (n = 10) and low salinity water (LSW, n = 11) groups. Messenger RNA abundance was measured using real-time PCR and normalized using the abundance of 18S. Abundance was arbitrarily set to 1 in the LV stage in each group.

7 Additional Files

	CONTROL		LSW		
	Mean	SD	Mean	SD	
Conductivity (µS/cm)	264,00	41,64	248,92	34,07	
Major ions, in milligrams per liter					
Bicarbonate	145,50	17,77	140,00	17,04	
Calcium	44,79	8,57	43,60	5,78	
Chloride	2,29	0,84	1,24	0,39	*
Magnesium	4,29	0,51	5,16	2,41	
Potassium	0,47	0,13	0,21	0,09	*
Sodium	1,83	0,28	1,09	0,27	*
Sulfur	3,03	0,79	5,08	3,32	*
Sulfate	8,33	2,06	13,05	9,54	
Nutrients, in milligrams per liter					
Nitrate	2,92	1,28	1,03	0,27	*
Trace elements, in micrograms per liter					
Copper	0,22	0,09	0,14	0,07	
Iron	14,99	14,12	3,00	4,58	*
Manganese	1,47	1,28	0,15	0,18	*
Selenium	0,13	0,02	0,15	0,05	
Iode	1,35	0,53	1,04	0,18	
Zinc	1,37	0,70	1,35	0,83	

Supplemental data file 1: Water composition in control (Rébénacq) and LSW (Sarrance) sites.

Mean (N= 12) yearly (2020-2021) values and standard deviation are shown. Difference between LSW and control water were analyzed using non-parametric U-tests.

* denotes significant differences.

	CONTROL		LSW		
	Mean	SD	Mean	SD	
Conductivity (µS/cm)	282.00	20.785	242.67	29.48	
Major ions, in milligrams per liter					
Bicarbonate	154.50	25.05	143.67	10.97	
Calcium	48.97	5.11	42.43	4.66	
Chloride	2.98	0.45	1.47	0.06	*
Magnesium	4.59	0.32	3.51	2.66	

Potassium	**0.60**	0.19	**0.18**	0.04	*
Sodium	**2.02**	0.17	**0.99**	0.18	*
Sulfur	**3.60**	0.09	**3.10**	2.82	
Sulfate	**9.70**	0.65	**9.5**	8.37	
Nutrients, in milligrams per liter					
Nitrate	**4.09**	1.79	**1.11**	0.09	*
Trace elements, in micrograms per liter					
Copper	**0.26**	0.15	**0.16**	0.13	
Iron	**8.07**	6.72	**2.00**	0.44	
Manganese	**0.70**	0.33	**0.12**	0.07	*
Selenium	**0.13**	0.01	**0.17**	-	
Iode	**1.45**	0.50	**1.15**	0.08	
Zinc	**1.26**	0.56	**1.44**	0.54	

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579 **Supplemental data file 2: Water composition in control (Rébénacq) and LSW**

580 **(Sarrance) site during the reproductive period (October-December).**

581 Mean (N= 3) values (from 2020-10-15 to 2020-12-15) and standard deviation are shown. Difference

582 between LSW and control water were analyzed using non-parametric U-tests.

583 * denotes significant differences.

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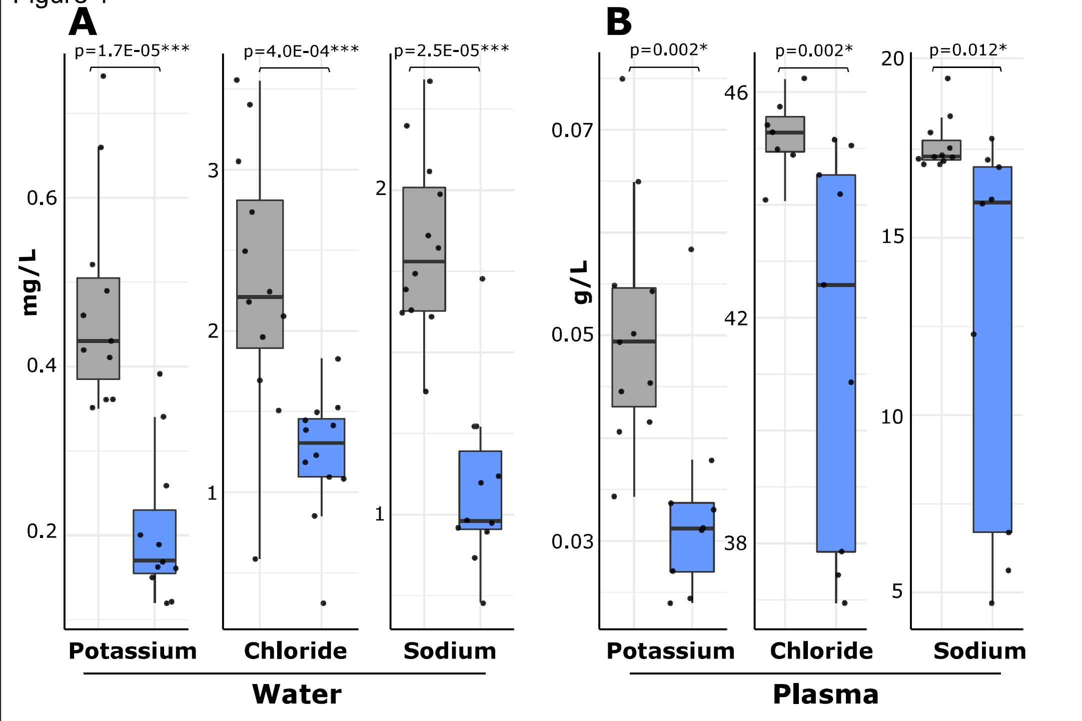


Figure 1
A
B
p=1.7E-05***
p=4.0E-04***
p=2.5E-05***
p=0.002*
p=0.002*
p=0.012*
mg/L
g/L
0.6
0.4
0.2
3
2
1
2
1
0.07
0.05
0.03
46
42
38
20
15
10
5
Potassium
Chloride
Sodium
Potassium
Chloride
Sodium
Water
Plasma

Figure 2

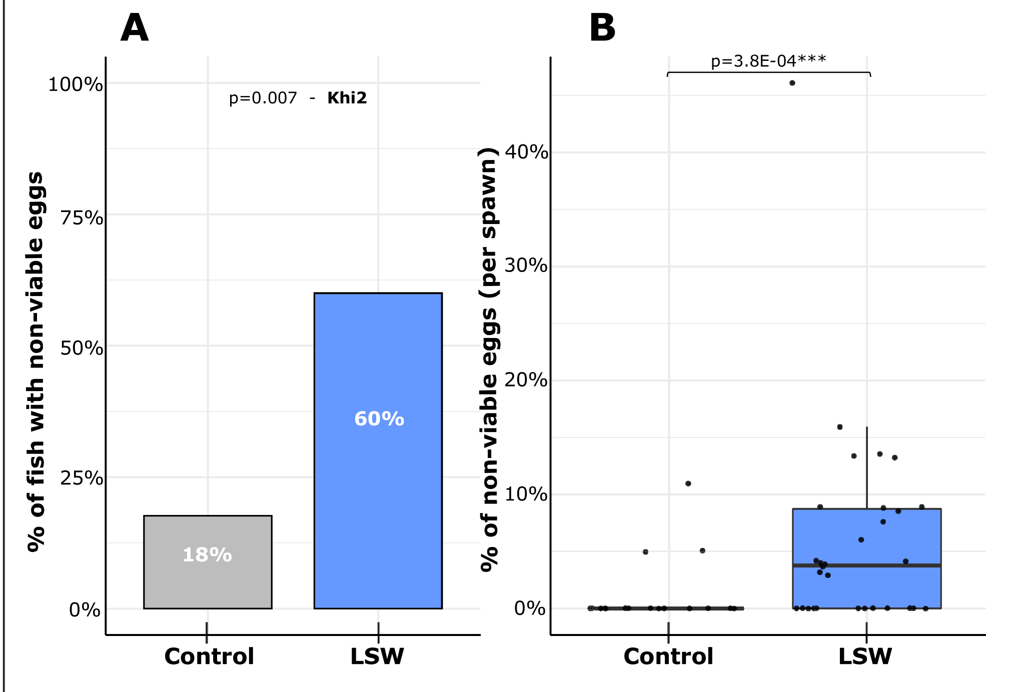
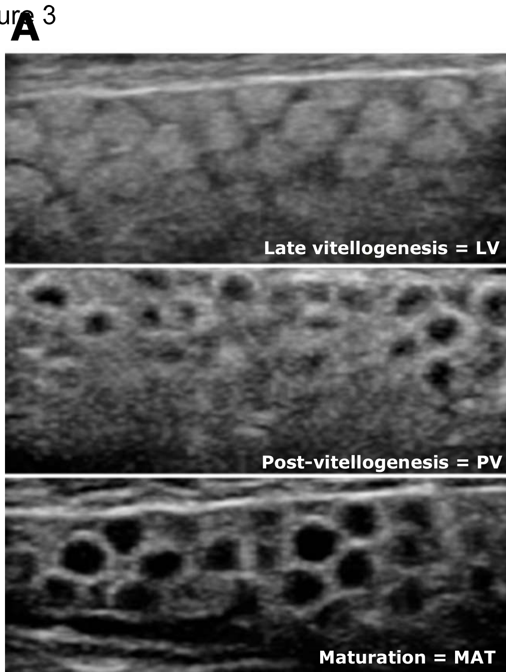
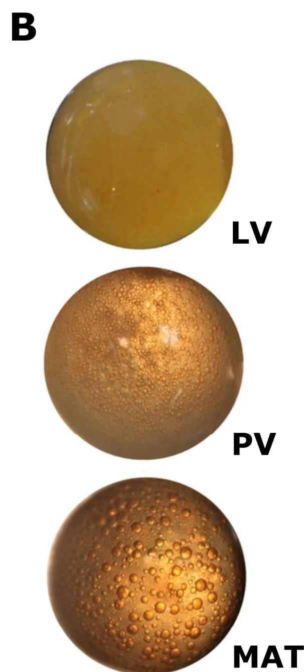
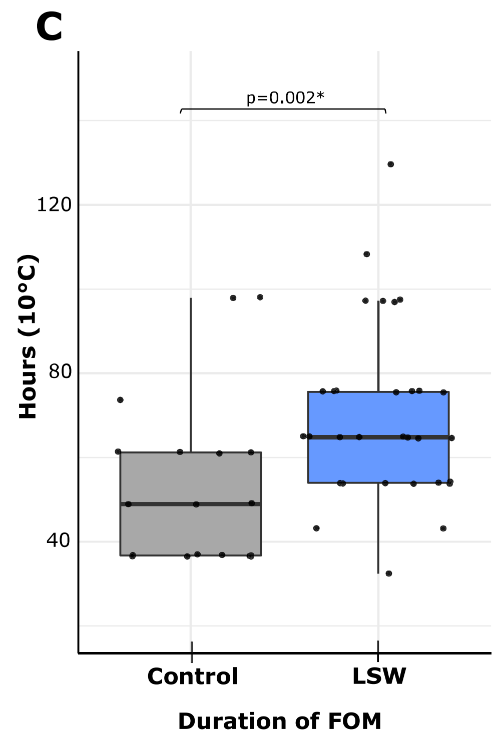


Figure 3







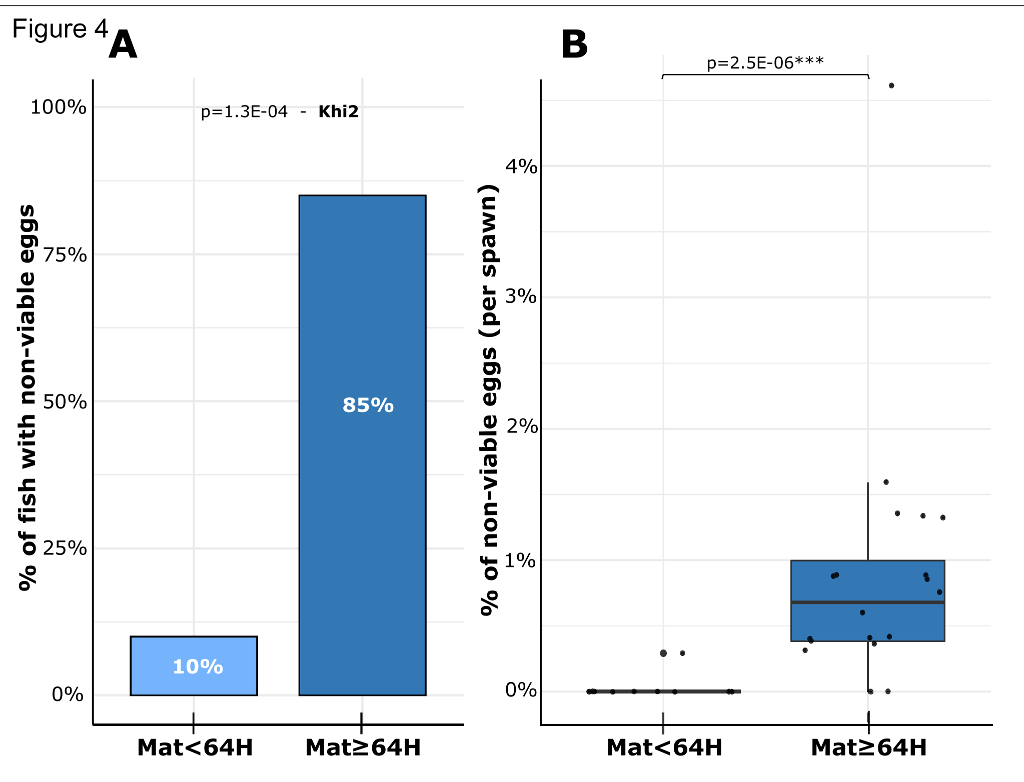


Figure 4
A
p=1.3E-04 - Khi2
100%
75%
50%
25%
0%
% of fish with non-viable eggs
10%
85%
Mat<64H
Mat≥64H
B
p=2.5E-06***
4%
3%
2%
1%
0%
% of non-viable eggs (per spawn)
Mat<64H
Mat≥64H

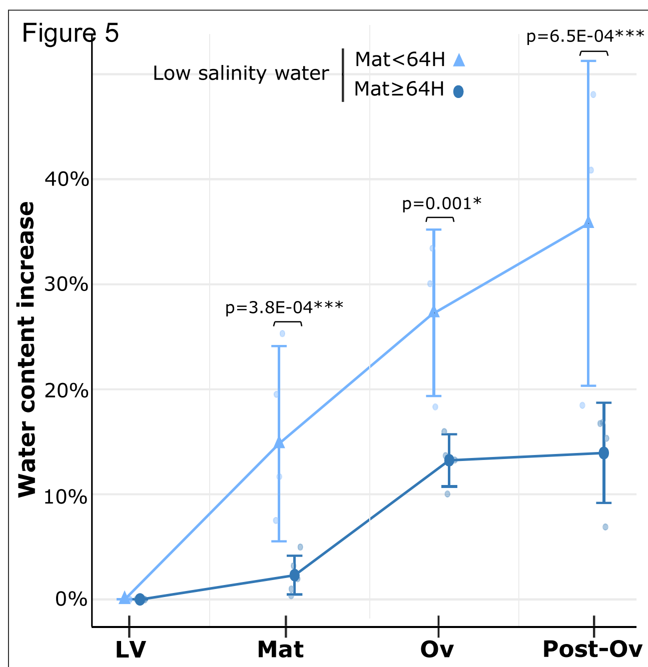


Figure 5
Low salinity water
Mat<64H
Mat≥64H
p=6.5E-04***
p=0.001*
p=3.8E-04***
Water content increase
40%
30%
20%
10%
0%
LV
Mat
Ov
Post-Ov

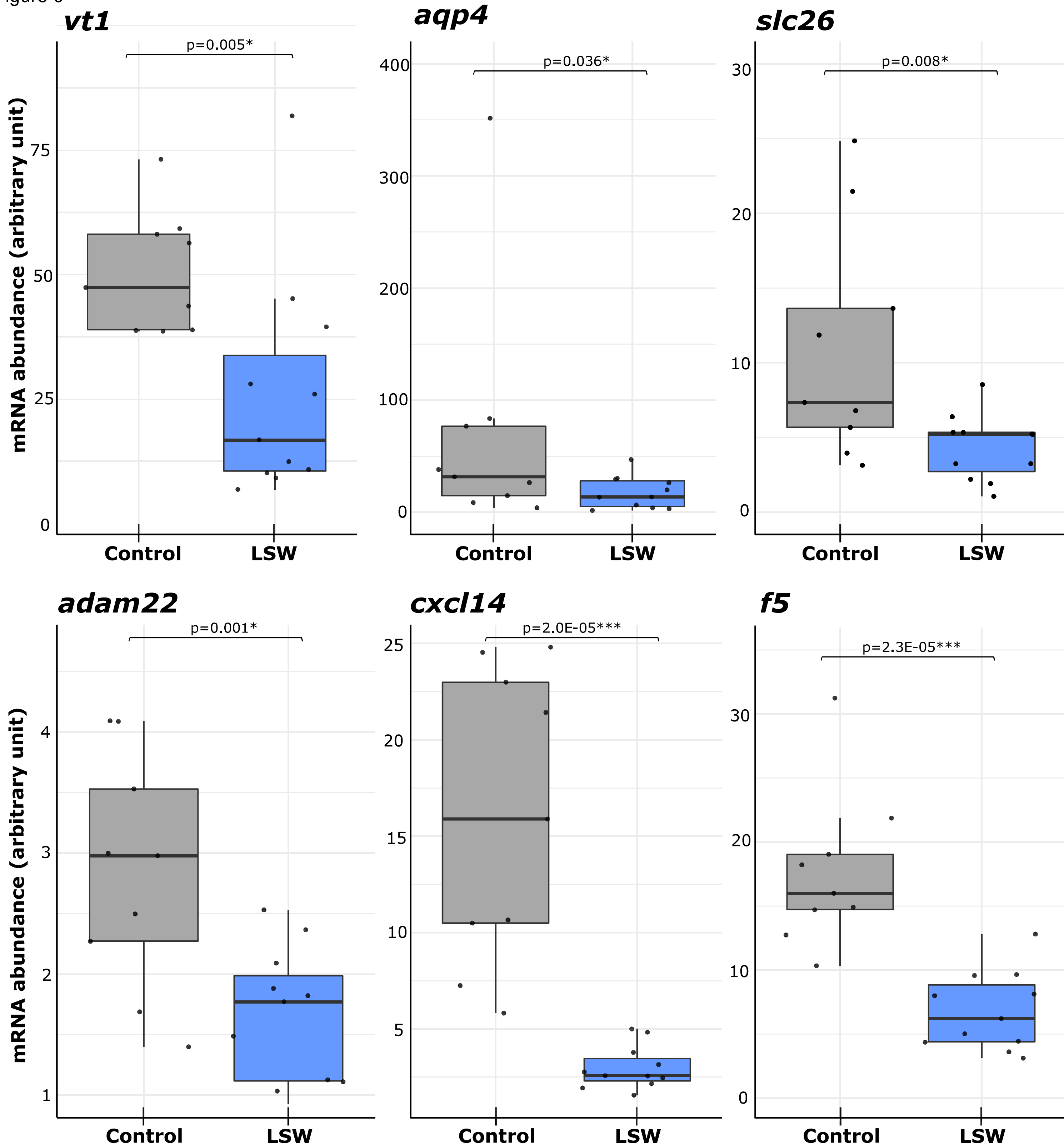


Figure 6
vt1
p=0.005*
aqp4
p=0.036*
slc26
p=0.008*
adam22
p=0.001*
cxcl14
p=2.0E-05***
f5
p=2.3E-05***
mRNA abundance (arbitrary unit)
Control
LSW

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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

Authors statement

Emilien Segret: Conceptualization, formal analysis, investigation, data curation, writing – original draft.

Emilie Cardona: Investigation, formal analysis.

Sandrine Skiba-Cassy: Conceptualization, supervision, project administration

Frederic Cachelou: Conceptualization, supervision, project administration, funding acquisition.

Julien Bobe: Conceptualization, methodology, validation, visualization, writing – review and editing, supervision, funding acquisition.