1 **Toxicity of 2 pg ethynylestradiol in brown trout embryos (*Salmo trutta*)**

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10

11 **Abstract**

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13 Endocrine disrupting chemicals are a threat to natural fish populations in the aquatic
14 environment. Their toxicity is usually discussed relative to concentrations in the water the fish are
15 exposed to. In the case of the synthetic compound 17-alpha-ethynylestradiol (EE2), a common
16 and persistent estrogen, concentrations around 1 ng/L have repeatedly been found to induce toxic
17 effects in fish. Here, we used brown trout (*Salmo trutta*) from a natural population to study EE2
18 take up and how it affects early life-history. We collected adults during the spawning season,
19 produced 730 families *in vitro* (to control for potential maternal and paternal effects on embryo
20 stress tolerance), and singly raised 7,300 embryos (in a 2 mL static system) that were either
21 exposed to one dose of EE2 at 1 ng/L (i.e., 2 pg/embryo) or sham-treated. We found that EE2
22 concentration did not significantly change over a period of 3 months in control containers without
23 embryos. Embryos took up most of the 2 pg EE2 within about 4 weeks at 4.6°C. EE2 treated
24 embryos experienced higher mortality, delayed hatching of the survivors, and had reduced size at
25 hatching. Our findings suggest that the toxicity of EE2 is often underestimated when discussed at
26 the level of concentrations in water only.

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28 **Key words:** Estrogen toxicity, salmonidae, embryo uptake, embryo development, larval traits.

Introduction

Pollution by endocrine disrupting chemicals is a threat to wildlife (Wedekind, 2017). The synthetic estrogen 17α-ethynylestradiol (EE2) is a persistent and powerful endocrine disruptor (reviewed in Corcoran et al., 2010, Aris et al., 2014). This steroid is a compound of oral contraceptive pills and reaches the environment through household sewage (Ternes et al., 1999a, Chèvre, 2014). During the sewage treatment process, EE2 removal is expected to be on average 68%, i.e. its degradation is likely not complete (Johnson et al., 2013). Thus, rivers that carry treated sewage effluent also carry EE2. Modelling work suggests that EE2 is present at 10 pg/L or higher in 20% of the entire European river network (Johnson et al., 2013) and concentrations around 1 ng/L have been regularly measured in surface waters (Tiedeken et al., 2017).

In fish, EE2 exposure is believed to have two main types of effects. First, it may affect early life-history stages, when individuals are often more sensitive than at later stages, by altering traits such as embryo survival, developmental time and larval growth (reviewed in Aris et al., 2014). Secondly, as EE2 is a hormone and sex differentiation in fish can often be affected by environmental factors (Devlin & Nagahama, 2002), EE2 can disturb fish reproductive traits in juveniles and mature individuals (reviewed in Leet et al., 2011, Caldwell et al., 2012). Either type of effect has potential consequences for individual and population fitness (Cotton & Wedekind, 2009, Wedekind, 2017). Significant effects have been observed in concentrations around 1 ng/L (Caldwell et al., 2012, Aris et al., 2014). For example, in zebrafish (*Danio rerio*) an exposure to 1 ng/L EE2 increased vitellogenin body content (Van den Belt et al., 2003) and 2 ng/L were sufficient to reduce embryo survival (Santos et al., 2014) and growth (Xu et al., 2008). Moreover, juvenile medaka (*Oryzias latipes*) exposed to 10 ng/L of EE2 showed reduced survival and growth (Scholz & Gutzeit, 2000). Similar concentrations have been linked to the appearance of gonadal deformations in mature zebrafish males (Xu et al., 2008) and led to intersex medaka males (Metcalfe et al., 2001). The toxicity of EE2 has, however, rarely been studied in non-model fish. While model species offer good background for ecotoxicology tests, understanding the toxicity of compounds with wild non-model species is an important step in validating results in a more ecologically-relevant context (Wedekind et al., 2007). Moreover, fish ecotoxicology studies often focus only on the effects of constant concentrations of chemical compounds in the water (OECD, 1992, Leet et al., 2011, Aris et al., 2014), but see Bjerregaard et al. (2008) and Knudsen et al. (2011). However, determining both, exposure content and individual uptake, is necessary to better understand the toxicity of a substance (Quinnell et al., 2004, Skillman et al., 2006).

Species from the salmonidae family are typically of high economic, cultural and ecological relevance. Nonetheless, salmonids have been suffering from anthropogenic changes in the environment and several populations face demographic shifts (e.g. Wedekind et al., 2013) and population declines (e.g. Gustafson et al., 2007, Wedekind & Küng, 2010). Because many salmonid species spawn in rivers – many of which affected by effluent discharges – and embryos typically develop in an immobile stage over a period of several months, EE2 may be a particular threat for early life stages of these species. Moreover, EE2 is likely a stronger threat in rivers than in lakes because it typically reaches higher concentrations in smaller rivers that receive high effluent loads. When Brazzola et al. (2014) exposed two species of whitefish (*Coregonus palaea* and *C. albellus*) to low concentrations of EE2 (from 1 ng/L), they found EE2 to reduce embryo survival and larval growth and to delay hatching (Brazzola et al., 2014). However, whitefish are lake-spawning salmonids that in the natural environment may be less exposed to EE2 than their river-spawning counterparts. Schubert et al. (2014) investigated the effects of exposures to the natural estrogen 17β-estradiol (E2) on embryos of brown trout (*Salmo trutta*), a river-spawning

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76 salmonid. The authors found an E2 exposure of 3.8 and 38 ng/L to not significantly reduce
77 embryo survival, but to delay hatching time and reduce body length at hatching (Schubert et al.,
78 2014). However, the trout population used in Schubert et al. (2014) was captive. Because
79 salmonids display large maternal effects (Einum & Fleming, 1999, Clark et al., 2014) that include
80 environmentally-dependent compounds that females allocate to their eggs (e.g. Wilkins et al.,
81 2017, Wilkins et al. submitted manuscript), the toxicity of chemicals in an ecologically-relevant
82 context requires investigations on wild individuals.
83   The brown trout population of the Aare river system in Switzerland has been extensively
84 monitored and has shown a decline of about 50% over the past three decades (Burkhardt-Holm,
85 2007, Stelkens et al., 2012). The observed population decline is not yet sufficiently understood
86 but habitat fragmentation (Stelkens et al., 2012) and estrogenic pollution (Burkhardt-Holm et al.,
87 2008) have been investigated as candidates contributing to it. In this study, we used brown trout
88 from the Aare river system to investigate (i) whether and how much embryos take up EE2 from a
89 low and ecologically-relevant exposure and (ii) the effects of such uptake on early life-history
90 traits.
91
92 **Methods**
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94 *Experimental design and embryo rearing*
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96 Adult males (N= 142) and females (N=145) were sampled during their spawning season from the
97 Aare river system, in Switzerland. Their gametes were stripped and used for *in vitro* block wise
98 full-factorial fertilizations (Lynch & Walsh, 1998) for a study on variance components and genetic
99 correlations (Marques da Cunha et al., in preparation). Here, the block-wise breeding of gametes
100 of many males and females controls for potential parental effects on stress tolerance. The
101 experimental crosses were performed in 4 different days, once per week from mid-November to
102 mid-December. The breeding block design varied from 4 x 5 (i.e., 4 females crossed with 5 males
103 in all possible combinations to produce 4 x 5 sibgroups) to 6 x 5, depending on the availability of
104 individuals. In total, 29 breeding blocks yielding 730 sibgroups were produced. After hardening
105 for 2 hours, samples of freshly fertilized eggs of each sibgroup were brought to the laboratory
106 where 15 freshly fertilized eggs per sibgroup were used for another study (Marques da Cunha et
107 al., in preparation) and 10 per sibgroup (N = 7,300) were used for the present study. They were
108 washed as in von Siebenthal et al. (2009) and singly raised in polystyrene 24-well plates (Greiner
109 Bio-One, Austria) filled with 1.8 mL of autoclaved standardized water per well (OECD, 1992).
110 Plates were incubated in a climate chamber at 4.6°C.
111
112 *Treatment preparation, exposure and trait measurements*
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114 A spike solution of 10 ng/L of analytical 17α-ethynylestradiol (Sigma-Aldrich, USA) was prepared
115 through a 3-steps serial dilution. Because EE2 is poorly soluble in water, absolute ethanol (VWR
116 International, USA) was used for the first step of the dilutions. This led to a concentration of
117 0.004% of ethanol in the EE2 spike solution. Analogously, a control spike solution with the same
118 concentration of ethanol but without EE2 was prepared. All of the dilutions were prepared with
119 autoclaved standardized water (OECD, 1992). One day post fertilization, either 0.2 mL of the EE2
120 or 0.2 mL of the control spike solution was added to the wells for a final volume of 2 mL. The
121 nominal concentrations in the wells were 1 ng/L of EE2 and 0.0004% of ethanol for EE2-exposed

122 embryos (i.e. a total content per well of 2 pg EE2) or 0.0004% of ethanol for sham-treated
123 individuals.
124   At the day of hatching, embryos were singly transferred to 12-well plates (Greiner Bio-
125 One, Austria) filled with 3 mL of autoclaved standardized water (OECD, 1992), i.e. there was no
126 EE2 treatment at that stage. These 12-well plates were scanned for embryo body measurements
127 (Epson Perfection V37, Japan), i.e. hatchling length and yolk sac length and width at hatching.
128 After 24 days, the plates were again scanned for the same trait measurements. Larval growth was
129 calculated as larval length at 24 days post hatching minus length at hatching. Yolk sac volume at
130 hatching was calculated as in Jensen et al. (2008). All the trait measurements were performed with
131 ImageJ (http://rsb.info.nih.gov/ij/).
132
133 *Statistical analyses*
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135 Embryo survival was analyzed as a binomial response variable in generalized linear mixed
136 models (GLMM) and hatching time, hatchling length, yolk sac volume at hatching and larval
137 growth as continuous response variables in liner mixed models (LMM). Treatment was entered as
138 fixed effect and breeding block (which comprises the variation of family and fertilization date) as
139 random effect in the models. The significance of the model terms was obtained by comparing a
140 model including or lacking the term of interest to a reference model with likelihood ratio tests
141 (LRT) and Akaike information criterion (AIC). All the statistical analyses were performed in R
142 (R Development Core Team, 2015) and mixed models with the lme4 package (Bates et al., 2015).
143
144 *Determining EE2 concentrations in 24-well plates*
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146 A second experiment was performed to estimate embryo EE2 uptake and determine the
147 persistence of EE2 in the same model of polystyrene 24-well plates as used in the first
148 experiment. In total 4,080 brown trout embryos (from other parents of the same populations as in
149 the first experiment; seven 4 x 6 breeding blocks, i.e. 168 sibgroups of 28 females and 42 males)
150 were raised in 170 24-well plates (Greiner Bio-One, Austria) at the same conditions as in the first
151 experiment (i.e. same methods, temperature, treatment and concentrations). Furthermore, 170 24-
152 well plates without embryos were prepared and analogously treated with EE2 or control spike
153 solutions. Measuring EE2 in plates without embryos and comparing these measurements with
154 plates that contanined embryos allowed for estimations of embryo EE2 uptake. Water samples
155 were collected at 5 time points across embryonic development and stored at -20° C for later EE2
156 measurements. For each time point, the water of 12 entire plates was pooled per treatment (576
157 mL of water sample per treatment). The first time point was performed only in empty plates and
158 was collected 30 minutes after the spike. The following time points were performed in plates with
159 and without embryos and were collected 7, 28, 56 and 84 days after exposures (the latter was
160 collected a few days before embryo hatching started).
161   EE2 was quantified with liquid chromatography-tandem mass spectrometry (LC-MS/MS).
162 First, the water samples were thawed and filtered with glass fibre filters. Then, sample volume
163 and pH were set to 250 mL and 3, respectively. After that, 4 ng/L of 17α-ethynylestradiol D4
164 were added to control for recovery and matrix effects. Water samples were enriched on LiChrolut
165 EN / LiChrolut RP-C18 cartridges (previously conditioned with hexane, acetone, methanol and
166 water at a pH of 3 as in Escher et al. (2008)). Cartridges were dried with nitrogen and eluted with
167 acetone and methanol. Solvents were exchanged to hexane/acetone at a ratio of 65:35 and the
168 extracts were passed through Chromabond Silica columns (Ternes et al., 1999b). Finally, the

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169   volume of the extracts was set to 0.25 mL. LC-MS/MS was performed with an Agilent6495
170   Triple Quadrupole. The column used was an XBridge BEH C18 CP (2.5 µm, 2.1 mm X 75 mm).
171   A gradient of acetonitrile/water was used for the liquid chromatography followed with a post-
172   column addition of ammonium fluoride solution. Mass transitions that were monitored are listed
173   in the SI (Table S1). The LC-MS/MS method also covered estrone (E1), 17β-estradiol (E2) and
174   bisphenol A (BPA) – mass transitions described in Table S1 All three compounds were detected
175   in the 24 well-plates with BPA at significant concentrations (see SI).

176

177   **Results**

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179   *EE2 water quantifications and embryo uptake*

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181   The measured concentration of the EE2 and control spike solutions corresponded to the nominal
182   concentrations. The EE2 spike solution concentration was 10.1 ng/L (nominal concentration = 10
183   ng/L) and the control spike solution concentration was lower than the limit of quantification
184   (LOQ = 0.05 ng/L). The concentrations of EE2 in water samples from 24-well plates depended
185   on the presence of embryos. In EE2-spiked plates without embryos, the concentrations of EE2
186   remained near the expected nominal concentration (1 ng/L, i.e. a total content of around 2 pg per
187   well) throughout the observational period (Fig. 1). In EE2-spiked plates with embryos, the level
188   of EE2 gradually declined from near the nominal concentration to the limit of quantification (Fig.
189   1). Nearly all of this decline happened during the first month of embryogenesis (Fig. 1). Control-
190   spiked plates with or without embryos did not show EE2 concentrations above LOQ (which was
191   now at 0.1 ng/L EE2 for these measurements).

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193   *EE2 effects on brown trout early traits*

194

195   Exposure to EE2 reduced embryo survival by 0.7%, i.e. an increase in mortality by 34.9 % (Table
196   1a; Fig. 2a). Breeding blocks varied by their survival rates, however the differences among
197   breeding blocks were not dependent on the treatment (block x treatment interactions in Table 1a).
198   The exposure to EE2 led to delayed hatching time by about half a day (Table 1b, Fig. 2b) and to
199   reduced hatchling length by on average 0.25% (Table 1c; Fig. 2c). Again, breeding blocks
200   differed in their overall hatching time and hatchling length and these effects were not treatment-
201   specific (Tables 1b – c). Yolk sac volume at hatching and larval growth were not significantly
202   affected by the EE2 treatment (Tables 1d – e; Fig. 2d – e). Variation in these traits was again
203   strongly dependent on breeding blocks (Table 1d – e).

204

205   **Discussion**

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207   EE2 is a common and potent endocrine disrupting pollutant of surface waters (Ternes et al.,
208   1999a, Chèvre, 2014). However, its toxicology has mainly been studied on model fish that were
209   bred and raised under laboratory conditions. Studies on wild populations are rare but necessary in
210   this context, because of the many differences in stress tolerance within and among species and
211   between wild and captive populations (Wedekind et al., 2007, Nyman et al., 2014). For example,
212   maternal environmental effects on egg content and hence on embryo viability and stress tolerance
213   typically differ considerably between populations (Pompini et al., 2013, Sopinka et al., 2016,
214   Wilkins et al., submitted manuscript). Moreover, the toxicology of EE2 has mainly been studied
215   relative to concentrations that were typically kept constant in experimental setups, and little is

216   known about the uptake of EE2 in embryos especially of wild populations. We therefore sampled
217   gametes from wild-caught spawners to produce embryos that represent their counterparts in the
218   wild. We exposed them to 2 pg EE2, estimated EE2 uptake and determined various measures of
219   embryo viability in response to this pollutant.
220   EE2 concentrations in plates without embryos remained constant throughout the
221   observational period of three months. This confirms the high stability and low degradation of
222   EE2 as compared to natural estrogens (Aris et al., 2014). When Ternes et al. (1999a) investigated
223   the behavior and occurrence of estrogens in activated sludge from sewage treatment plants under
224   aerobic conditions, the authors found EE2 to be largely persistent over an 80 hour treatment,
225   while E2 and E1 were rapidly degraded. Moreover, in a natural situation (water from 3 rivers in
226   England), the half-life of EE2 was calculated to be on average 17 days against 1.2 days for E2
227   (Jürgens et al., 2002). The stable concentrations of EE2 in our experimental setup also reveal that
228   adsorption of EE2 to the polystyrene walls of the plate wells plays no role. Various types of
229   materials and plastics can retain chemical compounds dissolved in water through adsorption
230   (Walker & Watson, 2010). This can be a challenge in ecotoxicology studies if adsorption makes
231   the tested compounds biologically unavailable and produces misleading results (Lung et al.,
232   2000, Walker & Watson, 2010).
233   In our study, the high stability of EE2 concentrations in plates without embryos and its
234   rapid decrease in plates with embryos indicates a quick and continuous uptake of the 2 pg EE2 by
235   the embryo. When Bhandari et al. (2015) investigated EE2 uptake by embryonic stages of
236   medaka, they also found significant uptakes. However, the authors exposed individuals to a much
237   higher EE2 content of 50 pg per embryo, which is not likely in the wild (Bhandari et al., 2015).
238   With an exposure content of 2 pg/embryo, estimated uptake in our experiment was continuous at
239   around 0.5 pg per week and seemed to slow down after the first 4 weeks. This may be linked to
240   the lower content of EE2 available in the water after 4 weeks of uptake. However, because of
241   technical constraints (i.e. large water volumes are needed to determine low EE2 concentrations)
242   we have only a single measurement per time point and treatment which limits our understanding
243   on the measurement error. We can, nonetheless, estimate the measurement error based on the
244   variance on measurements in plates without embryos (i.e. it was around 0.3 pg). The uptake of
245   micropollutants may well be species dependent and vary with the chemical characteristics of the
246   compounds. Lipophilic substances such as EE2 are taken up by eggs and embryos that are rich in
247   lipids (Vermeirssen et al., unpublished data), such as salmonids eggs (Murzina et al., 2009).
248   A one-time exposure to 2 pg of EE2 was sufficient to reduce embryo survival by 0.7%.
249   Given the high overall survival rate of nearly 98% in controls, this low reduction in survival
250   represents a 35% increase in mortality. In our experimental setup embryos were singly raised
251   under conditions that are arguably close to optimal for their development (e.g. minimizing
252   pathogen growth and mechanical stress, etc.). In the wild, embryos are typically exposed to very
253   strong selection (Elliott, 1994) and have to cope with a combination of challenges such as
254   opportunistic microbes (Wilkins et al., 2015) and various types micropollutants (Chèvre, 2014,
255   Moschet et al., 2014). If EE2-induced mortality is amplified by further stress factors (Wedekind
256   et al., 2007, Segner et al., 2012, Segner et al., 2014), the results from our experimental treatments
257   are likely to underestimate the potential ecotoxicological relevance of EE2.
258   The increased mortality we observed supports studies in typical model species (Caldwell
259   et al., 2012, Aris et al., 2014) and the results of Brazzola et al. (2014), which found 1 ng/L of EE2

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260   to reduce whitefish survival until hatching. In brown trout, Schubert et al. (2014) was unable to
261   find a significant link between brown trout embryo mortality rates and E2 exposure. However,
262   despite the high concentrations tested by the authors, E2 is naturally present in brown trout eggs
263   and has a lower estrogenic potency than EE2 (Segner et al., 2003).
264   Embryos exposed to EE2 hatched slightly later (on average about 10 hours later) and at
265   smaller size, i.e. EE2 reduced development and growth. Although small in an experimental setup,
266   such differences in hatching time may create strong effects in the wild. Hatching time is fitness
267   relevant because fast developing, and hence early emerging, salmonid embryos are more likely to
268   establish and successfully defend a feeding territory than individuals that develop slower and
269   emerge later (Einum & Fleming, 2000, Skoglund et al., 2012). Moreover, while embryos exposed
270   to EE2 hatched at a smaller size, their yolk sac volume was not significantly larger than those of
271   controls. This suggests that, more than only a delay in development, exposure to EE2 may have
272   imposed an energetic cost on developing embryos. In accordance, EE2 has been described to
273   change various aspects of development in typical model species (Caldwell et al., 2012, Aris et al.,
274   2014) and to delay early development (Brazzola et al., 2014, Marques da Cunha et al., in prep.)
275   and sexual differentiation (Selmoni et al., 2017) in salmonids.
276   EE2 is known to reduce growth of larvae of model species (Aris et al., 2014) and other
277   salmonids. Brazzola et al. (2014) and Marques da Cunha et al. (in prep.), for example, have found
278   a concentration of 1 ng/L EE2 to reduce larval growth in whitefish and European grayling
279   (*Thymallus thymallus*), respectively. Here we found larval growth not to be significantly affected
280   by the one-time addition of EE2 during early embryogenesis. These seemingly contrasting results
281   may be caused by the different experimental protocols used, rather than revealing species-specific
282   susceptibilities. While Brazzola et al. (2014) and Marques da Cunha et al. (in prep.) raised
283   hatchlings under EE2 exposure until the last day of measurements, we transferred embryos to
284   plates containing only standard water at the day of hatching. Moreover, from our chemical
285   measurements, we conclude that there was nearly no EE2 left in the water at that time, i.e. our
286   protocol did not allow for any further uptake of EE2 during the larval stages. The reduction of
287   growth observed in Brazzola et al. (2014) and Marques da Cunha et al. (in prep.) may therefore
288   be caused by an additional EE2 uptake by the larvae rather than revealing long-term effects of
289   EE2 uptake during earlier embryonic stages.
290   In conclusion, our results show that embryos from a natural population of brown trout
291   take up EE2 from water even at low and ecologically relevant concentrations. A total uptake of
292   only 2 pg was sufficient to significantly affect embryo viability and development, despite the
293   high levels of natural estrogens E1 and E2 in the embryos (estimated based on the increasing
294   water content in Table S2). Ecotoxicology studies on EE2 and other micropollutants generally
295   focus on concentrations that are kept constant during the assessments (Caldwell et al., 2012, Aris
296   et al., 2014), i.e. they do not always consider exposure content and biological uptake. This can
297   potentially lead to an underestimation of toxicities, as suggested by Quinnell et al. (2004). We
298   found that an uptake of only 2 pg EE2 induces significant reduction in embryo viability in a
299   species that is often exposed continuously to EE2 in their surrounding water. Our results suggest
300   that toxicity of EE2 may be currently underestimated.

310 **Author contributions**
311
312 LMC, AU and CW designed the experiment on early trout viability, and LMC, AU, DN and CW
313 performed the fieldwork. LMC and AU performed the experiment. ES and DN analyzed larval
314 images. Data analysis was performed by LMC and CW. The experiment on EE2 measurements
315 was designed by LMC, EV and CW. LMC, AU, DN and CW performed the fieldwork. The EE2
316 exposures and water collection was performed by LMC, and all chemical analyses were
317 supervised by EV. LMC and CW wrote the first version of the manuscript that was then critically
318 revised by all authors.

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486   **Figure legends**
487
488   Fig. 1: The persistence of 17α-ethynylestradiol (EE2) in 24-well plates with and without embryos
489   across 5 time points. Triangles represent plates with embryos and circles without embryos. Sham-
490   treatment data points are not shown because they were always below LOQ (0.1 ng/L or a total
491   well content of 0.2 pg).
492
493   Fig. 2: The effects of 17α-ethynylestradiol (EE2) on embryo early phenotype: (A) embryo
494   survival, (B) hatching time, (C) hatchling length, (D) yolk sac volume at hatching, and (E) larval
495   growth. Bars represent means of family means and error bars are 95 % confidence intervals, ***
496   $= p < 0.001$, * $= p < 0.05$, and ns $= p > 0.05$. See Table 1 for statistics.

497 **Tables**

498

499 Table 1. The effects of treatment (exposure to EE2) and breeding block on (A) embryo survival,

500 (B) hatching time, (C) hatchling length, (D) yolk sac volume at hatching, and (E) larval growth.

501 Likelihood ratio tests on mixed model regressions were used to compare a reference model (in

502 bold) with models including or lacking the term of interest. Significant effects (p < 0.05) are

503 highlighted in bold.

504

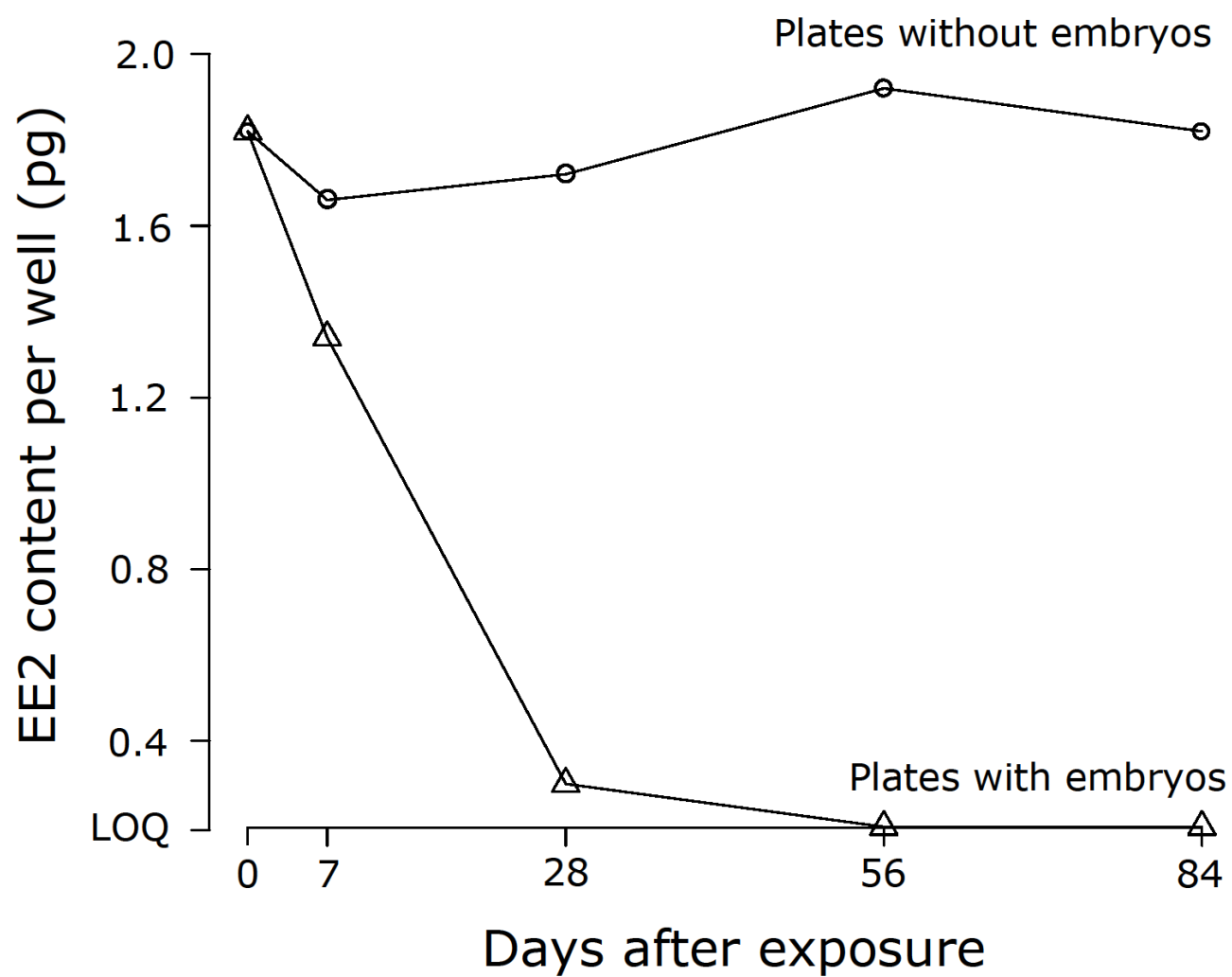| Model terms	Effect tested	AIC	d.f.	X^2	P
(A) Embryo survival					
t + block		1219	4		
block	t	1222	3	4.9	**0.03**
t	block	1360	3	142.9	**<0.001**
t + block + t x block	t x block	1223	6	0.2	0.89
(B) Hatching time					
t + block		29068	5		
block	t	29101	4	34.8	**<0.001**
t	block	38808	4	9742.0	**<0.001**
t + block + t x block	t x block	29069	7	3.2	0.20
(C) Hatchling length					
t + block		5485	5		
block	t	5488	4	4.1	**0.04**
t	block	8113	4	2629.0	**<0.001**
t + block + t x block	t x block	5490	7	0.0	0.99
(D) Yolk sac volume at hatching					
t + block		35225	5		
block	t	35226	4	2.7	0.10
t	block	38521	4	3297.2	**<0.001**
t + block + t x block	t x block	35229	7	0.8	0.66
(E) Larval growth					
t + block		5204	5		
block	t	5204	4	1.3	0.26
t	block	5724	4	522.1	**<0.001**
t + block + t x block	t x block	5208	7	0.5	0.77

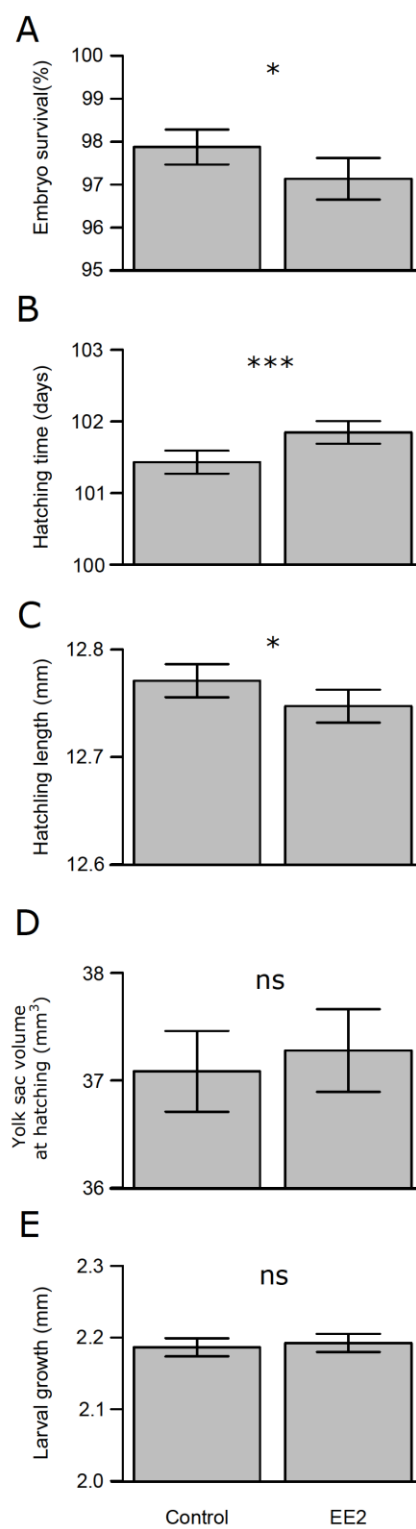505 Fixed effect: t, treatment. Random effect: breeding block.

506

507 **Figures**
508
509 Fig. 1.

510

511   Fig. 2.

512

513