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1 Sex steroid dynamics and mRNA transcript profiles of growth- and development-related genes
2 during embryogenesis following induced follicular maturation in European eel

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22 **Abstract**

23 Hormones and mRNA transcripts of maternal origin deposited in the egg may affect early
24 embryonic development in oviparous species. These hormones include steroids, such as estradiol-17β
25 (E2), testosterone (T), 11-ketotestosterone (11-kt), 17α,20ß-dihydroxy-4-pregnen-3-one (DHP), and
26 cortisol, which also play an important role in fish reproduction. In European eel, *Anguilla anguilla*,
27 which does not reproduce naturally in captivity, vitellogenesis in female broodstock is commonly
28 induced by administration of salmon or carp pituitary extract (PE) as an exogenous source of
29 gonadotropins, while follicular maturation is stimulated by a priming dose of PE followed by
30 provision of DHP as a maturation inducing hormone. In this regard, the main purpose of the present
31 study was to evaluate effects of induced follicular maturation on reproductive success in European
32 eel, focusing on maternal transfer and dynamics of steroids and mRNA transcripts of growth- and
33 development-related genes throughout embryogenesis. The results showed that maternal blood
34 plasma concentrations of E2, T and DHP were reflected in the unfertilized eggs. Moreover, a negative
35 relationship between concentrations of E2 and DHP in eggs and embryos and quality parameters
36 measured as fertilization success, cleavage abnormalities, embryonic survival, and hatch success was
37 found. Concomitant mRNA transcript abundance analysis including genes involved in stress response
38 (*hsp70, hsp90*), somatotropic axis (*gh*, *igf1*, *igf2a*, *igf2b*), lipid (*cpt1a*, *cpt1b*, *pigf5*) and thyroid
39 metabolism (*dio1*, *dio2*, *dio3*, *thrαb*, *thrβa*, *thrβb*) varied among unfertilized egg batches. For the
40 majority of genes, mRNA abundance increased during the maternal-to-zygotic transition in
41 connection to activation of the transcription of the embryos own genome. mRNA abundance of *dio1*,
42 *cpt1a* and *cpt1b* throughout embryogenesis was related to embryonic developmental competence.
43 Notably, mRNA abundance of *dio3* was positively associated with E2 concentrations, while the
44 mRNA abundance of *thrαb* was negatively related to T concentrations in the unfertilized eggs, which
45 may suggest an interaction between the thyroid and steroid hormone systems. Altogether, maternal

plasma concentrations of E2 and DHP were reflected in the eggs, with high concentrations of these steroids in the eggs being negatively associated with embryonic developmental competence. Additionally, high transcript levels of two of the investigated genes (*dio1*, *cpt1b*) were positively associated with embryonic developmental competence. This study reveals maternal transfer of steroids and mRNA transcripts to the eggs, which may be significant contributors to the variability in embryonic survival observed in European eel captive reproduction.

Keywords

Assisted reproduction; radioimmunoassay; gene expression; qPCR; hormone receptor

1. Introduction

In oviparous vertebrates, maternally derived constituents regulate early embryonic development (Groothuis et al., 2019; Lubzens et al., 2010; Paitz and Bowden, 2011). Besides nutritional components such as proteins and lipids, this includes lipophilic hormones and maternal mRNA that are incorporated into the egg cytoplasm during vitellogenesis (Brooks et al., 1997; Groothuis and Schwabl, 2008; Radder, 2007; Tokarz et al., 2015). The maternal influence on the endocrine environment during early offspring development has been evidenced for an array of oviparous species including birds (Groothuis et al., 2019; Groothuis and Schwabl, 2008), reptiles (Radder, 2007; Roush and Rhen, 2018) and teleosts (Tokarz et al., 2015). The endocrine state during embryonic development is expected to reflect maternally derived steroids, as the activation of the hypothalamus–pituitary–interrenal (HPI) axis and *de novo* steroid synthesis is likely not initiated until after hatch (Nesan and Vijayan, 2013). Overall, steroid hormones are involved in numerous physiological processes, such as metabolism, immune response, reproduction, embryonic development, and sex determination and differentiation (Tokarz et al., 2015).

In teleosts, the presence of different types of steroids, e.g. estradiol-17β (E2), testosterone (T), 11-ketotestosterone (11-kt), 17α,20ß-dihydroxy-4-pregnen-3-one (DHP), and cortisol, in unfertilized eggs and their temporal patterns throughout embryogenesis have been investigated in several species, e.g. zebrafish, *Danio rerio* (Alsop and Vijayan, 2008; Busby et al., 2010; Nesan and Vijayan, 2012; Pikulkaew et al., 2010), coho Salmon, *Oncorhynchus kisutch* (Feist et al., 1990), Arctic charr, *Salvelinus alpinus* (Khan et al., 1997), three-spined stickleback, *Gasterosteus aculeatus* (Paitz et al., 2015), medaka, *Oryzias latipes* (Iwamatsu et al., 2006), tilapia (Rothbard et al., 1987), Eurasian perch, *Perca fluviatilis* (Rougeot et al., 2007), white sturgeon, *Acipenser transmontanus* (Simontacchi et al., 2009), and Japanese flounder, *Paralichthys olivaceus* (de Jesus et al., 1991). Overall, these studies have shown that steroids are present in the unfertilized eggs, followed by a steady decline from fertilization towards hatch. This temporal pattern indicates an active role of the embryos in regulation of their early endocrine environment, suggested by their ability to metabolize them into free and conjugated steroids metabolites (Khan et al., 1997; Paitz et al., 2015; Paitz and Bowden, 2011, 2010). Recently, Paitz et al. (2016) demonstrated another mechanism for three-spined stickleback embryos, where eggs soon after fertilization can clear cortisol via ABC transporters without prior metabolization. In coho salmon, levels of E2 and DHP were higher in non-viable compared to viable eggs suggesting a negative effect of excessive concentrations (Feist et al., 1990). Notably, maternal exposure to cortisol in experiments on Atlantic salmon, *Salmo salar*, led to detrimental effects on the offspring, such as increased mortality and malformations and decelerated yolk sac utilization (Eriksen et al., 2006; 2007). Once endogenous production commences (often around hatch), steroids have been shown to play an important role in sex-differentiation (Iwamatsu et al., 2006; Rothbard et al., 1987; Rougeot et al., 2007; Simontacchi et al., 2009).

Also, maternally derived mRNA in the egg, deposited during oocyte development, is important for the development of early embryonic stages (Lubzens et al., 2017; Sirard, 2012; Winata

94 and Korzh, 2018). Specific maternal mRNA transcripts have profound impacts on egg quality and
95 early developmental competence, as shown in teleost species such as rainbow trout, *Oncorhynchus*
96 *mykiss*, Atlantic cod, *Gadus morhua*, Atlantic halibut, *Hippoglossus hippoglossus*, and European eel
97 (Aegerter et al., 2004; Lanes et al., 2013; Mommens et al., 2010; Rozenfeld et al., 2016). The embryo
98 then takes over control by its own genome activation during the maternal-to-zygotic transition (MZT)
99 (Newport and Kirschner, 1982). This takes place during the mid-blastula transition and includes both,
100 the clearance of maternal mRNA and activation of zygotic transcription representing a crucial step
101 during embryonic development (Giraldez et al., 2006; Lee et al., 2014).

102 In teleosts, oocyte development, including vitellogenesis and follicular maturation, is
103 regulated by the hypothalamus–pituitary–gonadal axis (HPG), where the gonadotropin-releasing
104 hormone (GnRH) regulates the synthesis and release of the two pituitary gonadotropic hormones,
105 follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Levavi-Sivan et al., 2010;
106 Nagahama and Yamashita, 2008; Patiño et al., 2003; Patiño and Sullivan, 2002). These hormones act
107 on the gonads to control gamete development and the production of sex steroids. However, in various
108 species due to endocrine impediments assisted reproduction needs to be applied to induce
109 gametogenesis or gamete maturation in captivity (Mylonas et al., 2010). While product type and
110 treatment schemes may vary, this hormone replacement therapy may lead to abnormal steroid levels
111 potentially affecting embryo quality.

112 The European eel, *Anguilla anguilla*, life cycle includes a prepubertal stage in which a strong
113 dopaminergic inhibition prevents sexual maturation in their continental habitats (Vidal et al., 2004).
114 Consequently, reproductive development does not occur in captivity unless hormonal treatments are
115 applied, which for female eels includes the administration of exogenous gonadotropins (Dufour et al.,
116 1983) followed by maturation-inducing steroid (MIS). During vitellogenesis, the importance of E2,
117 11-kt, and DHP was shown in Japanese eel, *A. japonica* (Ijiri et al., 1995; Kazeto et al., 2011;

118 Matsubara et al., 2005). While E2 and 11-kt play a primary role during previtellogenic and
119 vitellogenic growth of oocytes, DHP is important for the induction of follicular maturation. Also in
120 European eel, the steroid profile during different phases of the ovarian development and their
121 importance for successful follicular maturation has been described (Burgerhout et al., 2016; da Silva
122 et al., 2016). Here, levels of E2, T and 11-kt increase throughout the ovarian developmental process.
123 While E2 shows a constant increase from previtellogenic stage until germinal vesicle breakdown,
124 levels of T and 11-kt increase throughout vitellogenesis but show a decrease towards follicular
125 maturation (da Silva et al., 2016).

126 Pressured by a strong decline of natural stocks, efforts to establish a sustainable aquaculture
127 of European eel have increased and led to a stable production of viable eggs and first-feeding larvae
128 (Butts et al., 2016; Parmeggiani et al., 2020; Politis et al., 2018c; Tomkiewicz et al., 2019).
129 Nonetheless, high variability in egg quality and offspring performance call for further research to
130 enhance egg and larval quality aiming for a closed-cycle production. The expression profiles of
131 growth- and development-related genes involved in stress/repair, somatotropic axis, as well as thyroid
132 and lipid metabolism have been shown for European eel larvae (Politis et al., 2017; 2018a; 2018c).
133 Also, there are indications that maternal mRNA affects embryogenesis in *A. anguilla* (Kottmann et
134 al., 2020a; Rozenfeld et al., 2016) and *A. japonica* (Izumi et al., 2019, 2016). In *A. anguilla*, the
135 gonadotropin type used to induce vitellogenesis affected the mRNA transcript abundance of genes
136 involved in cell adhesion, MZT activation and immune response in the eggs and embryos influencing
137 their developmental potential. Moreover, gene specific temporal patterns were found for mRNA
138 transcripts with either increasing or decreasing abundance after the MZT (Kottmann et al., 2020a).
139 The MZT, which in European eel occurs ~ 10 hours post fertilization (hpf) at 18°C corresponding to
140 ~ 8 hpf at 20°C (Sørensen et al., 2016b), represents a major bottleneck in early life history (Kottmann
141 et al., 2020b). In the current study, transcriptional expression profiles during embryonic development

142 were obtained for genes involved in stress response (*hsp70, hsp90*), the somatotropic axis (*gh*, *igf1*,
143 *igf2a*, *igf2b*), as well as lipid (*cpt1a*, *cpt1b*, *pigf5*) and thyroid metabolism (*dio1*, *dio2*, *dio3*, *thrαb*,
144 *thrβa*, *thrβb*). These genes have been shown to be important for European eel larval ontogeny (Politis
145 et al., 2017; 2018a; 2018c) as well as embryos of teleost species such as Atlantic cod (Lanes et al.,
146 2013, 2012), rainbow trout (Aegerter et al., 2005, 2004; Li et al., 2007), or zebrafish (Campinho et
147 al., 2010; Vergauwen et al., 2018; Walpita et al., 2010).

148 The objectives of the current study were to examine maternal transfer of sex steroids and
149 mRNA transcripts as well as to investigate their temporal changes during embryogenesis in European
150 eel. Moreover, the effect of steroid and mRNA levels on egg quality and embryonic development was
151 explored by categorizing offspring into High, Medium, and Low quality groups.

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153
2. Materials and Methods
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2.1. Ethics statement

156 All fish were handled in accordance with the European Union regulations concerning the
157 protection of experimental animals (Dir 2010/63/EU). Eel experimental protocols were approved by
158 the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries
159 (permit number: 2015-15-0201-00696). Individual fish were anesthetized before tagging, biopsy, and
160 stripping of gametes. Females were euthanized after stripping and males at the end of the experiment
161 by submergence in an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg L^{-1}, Sigma
162 Aldrich, Germany).

163

164
2.2. Broodstock management and gamete production

165 Data were collected throughout two consecutive trials in 2016 and 2017 with female silver eels
166 caught during down-stream migration. In 2016, the study included 13 female eels (length = 76 ± 5.97
167 cm; weight = 897 ± 223.38 g) caught at Lower Bann, Toomebridge, Northern Ireland, and in 2017,
168 11 female eels (length = 63.55 ± 6.77 cm; weight = 518.36 ± 165.18 g) caught during downstream
169 migration from Lake Vandet, Denmark. The eels were transported to the EEL-HATCH experimental
170 facility at DTU in Hirtshals, Denmark, using an aerated freshwater tank. Here, fish were randomly
171 distributed into replicated 1150 L tanks, connected to a Recirculating Aquaculture System (RAS).

172 In both trials, male eels originated from Stensgård Eel Farm A/S, Randbøl, Denmark, where
173 they were raised from glass eels on a formulated diet (DAN-EX 2848, BioMar A/S, Denmark) at a
174 temperature of ~23°C. In 2016, experiments comprised 60 male eels (length = 38.2 ± 2.1 cm, weight
175 = 105.5 ± 15.3 g), and in 2017, 88 males (length = 38.5 ± 2.1 cm, weight = 114.7 ± 15.8 g). After
176 transport to the facility, males were randomly distributed in four tanks (485 L) connected to a RAS
177 unit at a density of ~15-22 eels per tank.

178 For acclimatization, salinity was gradually increased from 10 to 36 PSU over 14 days using
179 Blue Treasure Aquaculture Salt (Qingdao Sea-Salt Aquarium Technology Co. Ltd. Qingdao, China).
180 Subsequently, each individual was tagged with a passive integrated transponder (PIT tag) in the dorsal
181 muscle, while initial length and weight were recorded. At the facility, male and female broodstock
182 were reared at ~20°C and ~36 PSU under 12 h - 12 h light regime, with a 30 min twilight in the
183 morning and evening to resemble the Sargasso Sea photoperiod. The light source included eight
184 ceiling lamps each with two × 150 cm 58 w fluorescent bulbs (Osram LUMILUX Cool White, 4000K
185 - CRI: ≥77, Osram GmbH, München, Bayern, Germany) dimmed to 10 %. Mean surface light
186 intensity at ~5 cm above water surface was 0.02 μmol m^{-2} s^{-1}.

187 Vitellogenesis in the female broodstock was induced by weekly intramuscular injections of
188 salmon pituitary extract (SPE) at 18.75 mg kg^{-1} initial body weight (BW) for 11-21 weeks
189 (Tomkiewicz, 2012). The females were weighed weekly and once females reached a BW increase of
190 approx. 5-15 % of the initial weight, biopsies were taken to monitor oocyte developmental stage
191 followed by a priming injection of SPE (da Silva et al., 2018a). 12-24 hours after the primer, the
192 female received an injection of a MIS, 17α,20ß-dihydroxy-4-pregnen-3-one (DHP) (Sigma-Aldrich,
193 St. Louis, MO, USA), at 2 mg kg^{-1} current BW to stimulate final maturation and ovulation (Ohta et
194 al., 1996).

195 Male eels received weekly injections of human chorionic gonadotropin (Sigma-Aldrich,
196 Missouri, USA) at 150 IU/fish (Tomkiewicz, 2012). Prior to spawning, milt from 3-5 males was
197 collected, sperm concentration standardized (Sørensen et al., 2013), and the dilution kept in an
198 immobilizing medium (Peñaranda et al., 2010a). Sperm motility of the pooled milt was assessed
199 according to Sørensen et al. (2013). In brief, 2 µl of milt were added on a microscope slide using a
200 Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan) and activated by adding 200 µl of
201 20 °C seawater. Motility was categorized into: 0: <25 % ; I: 25-50 %; II: 50-75 %; and III: 75-100 %.
202 For all fertilized egg batches in the current study, sperm motility was classified as category III.

203 Eggs were strip-spawned and fertilized using a standardized sperm to egg ratio (Butts et al.,
204 2014; Sørensen et al., 2016a). After five min, a subsample of eggs was transferred to a 100 mL
205 graduated cylinder glass to estimate the percentage of floating eggs after 30 min. The remaining eggs
206 were transferred to 20 L buckets filled with ~15 L reverse osmosis water salted to ~36 PSU with Blue
207 Treasure (Qingdao Sea-Salt Aquarium Technology Co., Ltd., Qingdao, China) at ~19°C. After 60
208 min, the floating layer of eggs was further transferred to a second bucket (as above) and kept for 60
209 min. As anguillid eels are marine pelagophils that produce buoyant eggs with a high water content

210 and small oil globules (Cerdà et al., 2007), this procedure allows an initial crude separation of
211 fertilized, viable eggs from unfertilized eggs and debris from the spawning and milt dilution.

212 Subsequently, eggs/embryos for the experiment were taken from the floating layer of the
213 separation bucket and incubated in 10 × one L glass beakers (~5000 eggs/embryos per L), each filled
214 with filtered UV-treated seawater (FUV seawater; filter size: 10, 5, 1 µm) and supplemented with
215 rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA). Subsequent rearing
216 occurred in a temperature incubator at 18°C (Politis et al., 2018a) and 36 PSU. Additionally, 6 × 200
217 mL sterile tissue culture flasks filled with FUV seawater and supplemented with rifampicin and
218 ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) were stocked with eggs/embryos and
219 incubated as above. Three flasks stocked with ~2500 eggs/embryos were used to follow embryonic
220 development and three flasks stocked with ~600 eggs/embryos were used to assess hatch success.

221

222 **2.3. Sample and data collection**

223 Immediately after spawning, blood plasma was taken from the caudal vessel of the
224 anaesthetized female and centrifuged (10 minutes, 4360 RPM, 4 RCF). Plasma was distributed into
225 Eppendorf vials and stored at -20°C. The female was subsequently euthanized and ovarian tissue
226 sampled in Eppendorf vials filled with RNAlater (Sigma Aldrich, Germany). The samples were kept
227 in the fridge at 4°C for 24 hours and subsequently stored at -20°C. Unfertilized eggs (3 × 0.1 mL)
228 were kept in the oven at 60˚C for 24 h and weighed for dry-weight analyses.

229 Samples of unfertilized eggs were obtained for steroid analyses (4 × 0.25 ml, frozen at -20°C)
230 and gene expression (2 × 0.25 ml, kept in RNAlater in the fridge at 4°C for 24 hours and subsequently
231 stored at -20°C). Embryos were sampled at 2, 4, 6, 8, 24, 32 and 48 hours post fertilization (hpf).

232 Here, samples for steroid analyses (4 × 0.25 ml) were kept frozen at -20°C, while samples for gene
233 expression stored as described above.

234 For egg diameter (µm) at time of DHP administration, quantification of fertilization success,
235 egg size (mm^2) and occurrence at cleavage abnormalities (4 hpf), as well as embryonic survival (48
236 hpf), digital images were taken using a Nikon Eclipse 55i microscope equipped with a Nikon digital
237 sight DS-Fi1 Camera. Eggs were categorized as fertilized when >4 blastomeres could be observed
238 and fertilization success was calculated as the percentage of fertilized eggs divided by the total
239 number of floating eggs. Cleavage abnormalities at 4 hpf were determined by counting the number
240 of eggs with regular and irregular cell cleavages obtained from floating eggs. Embryonic survival at
241 48 hpf was measured by counting the number of dead and alive eggs and expressed as a percentage
242 (obtained from floating eggs). Hatch success was expressed as the number of hatched larvae divided
243 by the total number of fertilized eggs.
244

245 **2.4. Steroid analyses**

246 Concentrations of DHP, E2, T, 11-kt and cortisol were measured in female plasma, unfertilized
247 eggs, and embryos by means of radioimmunoassay (RIA), as described by Schulz (1985). Assay
248 characteristics and cross-reactivities of E2 and T antisera have previously been examined by Frantzen
249 et al. (2004) and validated for eel plasma by Mazzeo et al. (2014). For DHP, the procedure has been
250 described by Tveiten et al. (2010b) and validated for eel plasma by Peñaranda et al. (2010b). The
251 cross-reactivities of a new 11-kt antiserum have been described by Johnsen et al. (2013) and validated
252 for eel plasma by Baeza et al. (2015), while for cortisol, previous descriptions can be found in Tveiten
253 et al. (2010a). In short, free (i.e. non conjugated) steroids were extracted from the sample (200 µL
254 blood plasma or 360 mg eggs and embryos) with 4 mL diethyl ether under vigorous shaking for four
255 minutes. Subsequently, the aqueous phase was frozen in liquid nitrogen and separated from the

256 organic phase, which was then transferred to a new glass tube kept in a water bath at 45°C until all
257 ether was evaporated. The steroids were reconstituted by adding 900 µL of RIA-buffer and then
258 assayed for each sex steroid.

259 To validate the recovery of each steroid, triplicates of embryo samples were spiked with a
260 known amount of radiolabeled steroids (20-30 000 cpm (counts per minute)) of each steroid and
261 subsequently underwent the extraction procedure, as described above and assayed for the respective
262 steroid. The extraction efficiencies were 87.18±1.67 % for DHP, 77.91±1.31 % for T, 71.15±1.09 %
263 for E2, 68.70±2.15 % for 11-kt, and 75.48±2.04 % for cortisol. The extraction efficiency factor was
264 included in the scaling factor, when calculating concentrations of the different steroids. Moreover, a
265 dilution curve at 9 different dilutions was made and found to be parallel to the standard assay curve.

266

267 **2.5. Gene expression**

268 RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Germany) according to
269 manufacturer's instructions. RNA concentration and purity was analyzed by spectrophotometry using
270 Nanodrop One (Thermo Fisher Scientific, USA). 1 µg RNA was reverse transcribed using qScript
271 cDNA Synthesis Kit (Quantabio, Germany), following the manufacturer's instructions including a
272 DNase step using PerfeCta DNase I (Quantabio, Germany). Primers of 16 genes were retrieved from
273 previous studies (Table1). Primer of one gene was designed on the basis of the coding sequences of
274 the closely related species *A. japonica,* publicly available in Genebank, National Center for
275 Biotechnology Information (NCBI). Primers for the remaining two genes were designed based on the
276 assembly and annotation of the European Eel genome (Henkel et al., 2012) available at
277 https://dataverse.no/dataset.xhtml?persistentId=doi:10.18710/L7GO8T. Here, gene prediction and
278 exon coordinates were determined with Augustus v 2.4 (Stanke et al., 2008), and annotation was

279 performed using Blast2GO v 2.4.8 (Götz et al., 2008). The coding sequences used for the analyses
280 were then submitted to Genebank, NCBI and the related accession numbers are given in Table 1.
281 Primers were designed using primer 3 software v 0.4.01. All primers and predicted amplicons were
282 tested *in silico* for specificity using blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, the
283 expression of 19 genes (Table1) from all samples was analyzed with two technical replicates using
284 the qPCR BiomarkTM HD system (Fluidigm) based on 96.96 dynamic arrays (GE plates), as
285 previously described (Miest et al., 2016). In brief, a pre-amplification step was conducted with a 500
286 nM pool of all primers in PreAmp Master Mix (Fluidigm) and 1.25 μL cDNA per sample run in a
287 thermocycler for 2 min at 95°C; 10 cycles: 15 s each at 95°C and 4 min at 60°C. Obtained PCR
288 products were diluted 1:5 with low EDTA-TE buffer. The preamplified product was loaded onto the
289 chip with SsoFast-EvaGreen Supermix Low Rox (Bio-Rad) and DNA-Binding Dye Sample Loading
290 Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 μM in Assay Loading
291 Reagent (Fluidigm) and low EDTA-TE Buffer. The chip was run according to the Fluidigm 96.96
292 PCR protocol with a Tm of 60°C. qBase + software verified stability of housekeeping gene expression
293 throughout analyzed samples ($M < 0.4$; according to Hellemans et al. (2008)). Gene expression was
294 normalized (ΔCt) to the geometric mean of the four most stable housekeeping genes (*ccna2*, *cei*, *thaa*,
295 *igfr-1b*). Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method, in
296 relation to a random unfertilized egg sample, according to (Livak and Schmittgen, 2001).

297 **Table 1.**

298 Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR.

Full name	Abbreviation	Function	Primer Sequence (5' 3') (F: Forward; R: Reverse)	Reference/ Accession
Cyclin A2	*ccna2*	Reference	F: ATGGAGATAAAATGCAGGCCT R: AGCTTGCCTCTCAGAACAGA	AB061443.1
Cellular island	*cei*	Reference	F: CCTCAAACACCCCAACATCC R: AGCTCCTCCATGTACGTTGC	MT531390
Thyroid hormone receptor alpha a	*thaa*	Reference	F: GCAGTTCAACCTGGACGACT	(Politis et al., 2018b)

			R: CCTGGCACTTCTCGATCTTC	
Insulin like growth factor receptor 1b	*igfr-1b*	Reference	F: ATGGGAATCTTCAGCTCTTTAGA R: TCAAACTCCTCCTCCAAGCT	MT531391
Heat Shock Protein 70	*hsp70*	Stress response	F: TCAACCCAGATGAAGCAGTG R: GCAGCAGATCCTGAACATTG	(Politis et al., 2018a)
Heat Shock Protein 90	hsp90	Stress response	F: ACCATTGCCAAGTCAGGAAC R: ACTGCTCATCGTCATTGTGC	(Politis et al., 2018a)
Growth hormone	*gh*	Somtaotropic axis	F: TGAACAAGGGCATCAATGAA R: CGGAGCTTTCTCACATCCTC	(Politis et al., 2017)
Insulin like growth factor 1	*igf-1*	Somtaotropic axis	F: TTCCTCTTAGCTGGGCTTTG R: AGCACCAGAGAGAGGGTGTG	(Politis et al., 2018c)
Insulin like growth factor 2a	*igf-2a*	Somtaotropic axis	F: AGCCCAGAGGCTGAGGAG R: GATCAGATGTCGGTGGGATT	(Rozenfeld et al., 2016)
Insulin like growth factor 2b	*igf-2b*	Somtaotropic axis	F: CGGTCACAGAAGGGAATTGT R: GACGTCTCTCTCCGACTTGG	Rozenfeld et al., 2016)
carnitine O-palmitoyltransferase liver isoform-like 1a	*cpt1a*	Lipid metabolism	F: CCAGGCTGTGGATGAATCTT R: GCAAAGAGGACTGGAAGCTG	(Rozenfeld et al., 2016)
carnitine O-palmitoyltransferase liver isoform-like 1b	*cpt1b*	Lipid metabolism	F: TCTACGCTGGCTACGGAGTT R: ATAATGGGACTTCGCCCTCT	(Rozenfeld et al., 2016)
phosphatidylinositol glycan biosynthesis class F protein 5	*pigf5*	Lipid metabolism	F: ACAAGGTGTCCAAGGTCGTC R: GAAGGAGGACAGCAGGACAG	(Rozenfeld et al., 2016)
Deiodinase 1	*dio1*	Thyroid metabolism	F: AGCTTTGCCAGAACGACTGT R: TTCCAGAACTCTTCGCACCT	(Politis et al., 2018b)
Deiodinase 2	*dio2*	Thyroid metabolism	F: GAAGAGGAGGATCGCCTACC R: GCACTCTACCTCCGTCCAAA	(Politis et al., 2018b)
Deiodinase 3	*dio3*	Thyroid metabolism	F: TACGGGGCGTATTTTGAGAG R: GCTATAACCCTCCGGACCTC	(Politis et al., 2018b)
Thyroid Hormone Receptor alpha b	*thrαb*	Thyroid metabolism	F: GAAGCCTTCAGCGAGTTCAC R: ACAGCCTTTCAGGAGGATGA	(Politis et al., 2018b)
Thyroid Hormone Receptor beta a	*thrβa*	Thyroid metabolism	F: AGGAACCAATGCCAAGAATG R: GCCTGTTCTCCTCAATCAGC	(Politis et al., 2018b)
Thyroid Hormone Receptor beta b	*thrβb*	Thyroid metabolism	F: GAAGACTGAGCCCTGAGGTG R: AGGTAATGCAGCGGTAATGG	(Politis et al., 2018b)

299 Full name, abbreviation, function, and accession numbers or references for primers retrieved from
300 previous studies are listed.

301

302 **2.6. Statistical Analyses**

303 Data were analyzed using SAS Statistical Software (version 9.4; SAS Institute Inc., Cary,
304 North Carolina). Prior to analysis, residuals were tested for normality (Shapiro–Wilk test) and
305 homogeneity of variances (plot of residuals vs. fitted values). Data deviating from normality or
306 homoscedasticity were log_{10} transformed. Alpha was set at 0.05. Tukey's analysis was used to
307 compare least-squares means between groups. Akaike's (AIC) and Bayesian (BIC) information
308 criteria were used to assess which covariance structure was fitting the data most appropriately (Littell
309 et al., 1996). The division into three quality groups (High, Medium, Low) was based on fertilization
310 success, the occurrence of cleavage abnormalities, survival at 48 hpf, and hatch success.

311 For female morphometrics and offspring quality parameters, i.e. fertilization success,
312 cleavage abnormalities, embryonic survival, and hatch success, ANOVA models were run testing the
313 effect of broodstock series (2016, 2017), quality groups and their interaction. Here, no significant
314 interactions were found and therefore removed. While initial length ($p = 0.001$) and weight ($p = 0.002$)
315 differed between female broodstock from the two locations, quality parameters were similar among
316 female broodstock, including fertilization success ($p = 0.320$), cleavage abnormalities ($p = 0.560$),
317 embryonic survival at 48 hpf ($p = 0.095$), and hatch success ($p = 0.280$). Therefore, data from the two
318 broodstock series were pooled. Moreover, one-way ANOVA models were run testing the effect of
319 quality groups on oocyte diameter at the time of DHP administration, dry-weight of the unfertilized
320 eggs, as well as egg size at 4 hpf. Additionally, the relationship between egg size/mass and steroid
321 concentrations were tested for linear, quadratic and cubic relationship. Here, no significant
322 differences between quality groups and no significant relationships with steroids were found and thus,
323 an effect of egg size/mass on the egg quality and steroid concentrations was excluded.

324 Comparison of maternal plasma steroid concentrations in females among the three offspring
325 quality groups was analyzed using a series of one-way ANOVA models. DHP and E2 concentrations
326 in the unfertilized eggs and embryos were analyzed using a repeated measures two-way ANOVA
327 testing the main effects of quality group (High, Medium, Low) and age (unfertilized egg, 2, 4, 6, 8,
328 24, 48 hpf) as well as their interaction. Female ID (individual females and their offspring) was
329 considered random in all models. No significant interactions were detected for any of the tested
330 dependent variables and all models were re-run with the interaction effects removed, analyzing main
331 effects separately (Yossa and Verdegem, 2015). Comparison of T, 11-kt and cortisol concentrations
332 of the different quality groups in the unfertilized eggs and at 2 hpf were analyzed separately using a
333 series of one-way ANOVA models.

334 mRNA abundance data were analyzed using a repeated measures two-way ANOVA testing the
335 effect of quality group (High, Medium, Low) and age (unfertilized egg, 2, 4, 6, 8, 24, 32, 48 hpf) as
336 well as their interaction. A significant interaction was only found for *cpt1a*, where the model was
337 decomposed into a series of one-way ANOVA models testing the effect of quality at each age.
338 Moreover, relationships between steroid concentrations in the female blood plasma and unfertilized
339 eggs, between mRNA abundance in the female ovarian tissue and the unfertilized eggs as well as
340 between steroid concentrations and mRNA abundance levels were tested for linear, quadratic and
341 cubic relationship. In case more than one regression function was significant, F-statistics were used
342 to evaluate best fit.

343

344
3. Results

345 Overall, 16 out of 24 females successfully developed, ovulated and produced floating eggs
346 and were included in the analysis. An overview over all females and their reproductive success is
347 summarized in Table 2. Females were categorized into High, Medium and Low quality groups

348 depending on developmental competence of their eggs. Here, the High quality group was
349 characterized by low cleavage abnormalities (< 10 %) and high survival and hatch. The Low quality
350 group showed high occurrence of cleavage abnormalities (> 35 %) and low survival (< 20 %) as well
351 as hatch success (<15 %), while the Medium quality group showed intermediate developmental
352 potential. The used parameters have been verified as reliable egg quality and embryonic
353 developmental potential indicator for European eel (Kottmann et al., 2020a, 2020b).

354 **Table 2.**

355 Data on quality parameters for reproductive success of female European eel, *Anguilla anguilla* and
356 categorization into High, Medium and Low quality depending on developmental competence.

Female	Fertilization success (%)	Cleavage abnormalities (%)	Survival 48 hpf (% floating eggs)	Hatch success (% fertilized eggs)	Quality group
1	3.63	n.d.	0.00	0.0	Low
2	12.50	39.15	3.84	13.7	Low
3	14.78	67.32	2.45	0.0	Low
4	37.65	38.79	17.04	2.6	Low
5	44.35	55.56	14.58	n.d.	Low
6	26.73	44.52	21.80	69.4	Medium
7	37.37	16.20	21.88	46.8	Medium
8	54.57	25.93	40.60	62.9	Medium
9	58.10	27.78	57.32	69.2	Medium
10	61.68	5.19	56.68	85.2	High
11	61.97	4.43	64.40	81.8	High
12	72.94	1.04	70.78	72.7	High
13	76.66	7.84	61.30	48.7	High
14	81.10	7.08	n.d.	90.2	High
15	89.41	2.24	81.75	87.7	High
16	91.49	6.57	80.82	78.5	High

357 n.d.: no data available.

358

359 **3.1. Steroid concentrations**

360 DHP concentrations in female plasma after stripping did not differ significantly between
361 quality groups, although concentrations in the Low category tended to be higher ($p = 0.057$; Fig. 1A).
362 In unfertilized eggs and embryos, DHP concentrations decreased over time with lowest

363 concentrations in embryos close to hatch ($p < 0.0001$; Fig. 1D). Here, concentrations of DHP were
364 higher in the Medium and Low quality groups ($p < 0.0001$; Fig. 1B, C).

365 Female post-stripping E2 plasma concentrations did not differ between quality groups (Fig.
366 1E). On the other hand, E2 concentrations were found to be higher in the eggs and embryos from the
367 Low quality group ($p = 0.0003$), while the High and Medium groups did not differ (Fig 1G). Again,
368 concentrations decreased over time with highest levels in the unfertilized eggs and decreasing levels
369 towards hatch ($p < 0.0001$; Fig. 1F, H).

370 Concentrations of T, 11-kt, and cortisol are summarized in Table 3. Here, female post-
371 stripping T plasma concentrations did not differ among quality groups ($p = 0.381$), although a trend
372 towards higher concentrations in the Low category was observed. While differences between groups
373 were non-significant for unfertilized eggs ($p = 0.196$), T concentrations in embryos at 2 hpf differed
374 ($p = 0.027$) with concentrations in the Low quality group being significantly higher than in the
375 Medium quality group, while the concentrations in the High quality group did not differ. Overall,
376 concentrations at 2 hpf were close to the assay detection limits and no further measurements
377 throughout embryonic development were made.

378 **Table 3.**

379 Steroid concentrations in European eel, *Anguilla anguilla*.

Steroid	Female plasma (ng/ml)			Unfertilized eggs (ng/g)			Embryos at 2 hpf (ng/g)		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
T	57.28 ±11.85	58.37 ±15.67	82.05 ±14.02	1.19 ±0.13	1.52 ±0.18	1.53 ±0.15	0.53 ±0.04[ab]	0.44 ±0.06[a]	0.68 ±0.05[b]
11-kt	8.56 ±2.17	11.59 ±2.88	7.75 ±2.57	0.47 ±0.09	0.46 ±0.12	0.59 ±0.11	0.17 ±0.08	0.17 ±0.10	0.22 ±0.09
cortisol	24.57 ±6.03	14.84 ±7.97	24.07 ±7.13	0.48 ±0.10	0.58 ±0.13	0.59 ±0.12	n.d.	n.d.	n.d.

380 T: testosterone; 11-kt: 11-ketotestosterone; hpf: hours post fertilization; n.d.: no data available;
381 different lower-case letters represent a statistical difference ($p < 0.05$).

382

383 Female post-stripping 11-kt plasma concentrations also did not differ between quality groups 384 ($p = 0.594$). Furthermore, concentrations in the unfertilized eggs were similar among quality groups 385 and overall quite low ($p = 0.668$). Again, concentrations at 2 hpf did not differ between quality groups 386 ($p = 0.893$). Here, E2 concentrations were close to assay detection levels and no further analyses were 387 made.

388 Female post-stripping cortisol plasma concentrations did not differ between quality groups (p 389 $= 0.597$). Moreover, concentrations in the unfertilized eggs were overall low and also did not differ 390 among groups ($p = 0.733$). At 2 hpf, cortisol was below the assay detection levels in most of the 391 embryos, therefore no statistical analyses and no further analyses were performed.

392 Female post-stripping plasma steroid concentrations were associated to concentrations in the 393 unfertilized egg and best described by a positive linear regression for DHP (Fig 2A), E2 (Fig 2B), 394 and T (Fig 2C). No significant association was found for 11-kt (Fig 2D) and cortisol (Fig 2E).

395

396 **3.2. mRNA transcript abundance and gene expression patterns**

397 Overall, different mRNA abundance patterns over time were observed for gene groups, with 398 most of the genes showing increasing mRNA abundance after the MZT. Genes involved in 399 stress/repair mechanisms showed relatively low mRNA abundance during early development and 400 peaked towards hatch, i.e. 32 hpf for *hsp90* and 48 hpf for *hsp70* (Fig. 3A). Similar patterns were 401 observed for genes of the somatotropic axis (*gh*, *igf1*, *igf2a*, *igf2b*), which displayed low mRNA 402 abundance during the first eight hours and rapid increases after the MZT at 24 hpf (Fig. 3B). Genes 403 involved in lipid metabolism had low mRNA abundance during early development (Fig. 3C). Here, 404 *cpt1a* increased rapidly at 48 hpf, while *cpt1b* already increased at 24 and 32 hpf. Also, *pigf5*

405 increased towards hatch, however to a lower extent. Generally, genes involved in thyroid metabolism
406 (*dio1*, *dio2*, *dio3*, *thrαb*, *thrβa*, *thrβb*) also showed relatively low mRNA abundance during early
407 development, with the exception of *dio1*. The most rapid increases were found for *dio2* and *dio3* at
408 32 and 48 hpf. mRNA abundance of *thrαb*, *thrβa*, *thrβb* only showed slight increases towards hatch
409 (Fig. 3D).

410 The mRNA level in the female ovary was associated with the mRNA abundance in the
411 unfertilized eggs for eight genes (*hsp70*, *hsp90*, *cpt1a*, *cpt1b*, *pigf5,* *dio1*, *thrαb*, *thrβa*). Here,
412 associations were best described by linear regressions for *hsp70* ($Y = 0.34 + 0.65x$; $R^2 = 0.979$; $p <$
413 0.0001), *hsp90* ($Y = 0.09 + 0.42x$; $R^2 = 0.345$; $p = 0.021$), *dio1* ($Y = 70.76 + 0.26x$; Fig 4C), *thrαb*
414 ($Y = 0.82 + 0.19x$; $R^2 = 0.277$; $p = 0.044$), *thrβa* ($Y = 0.16 + 0.91x$; $R^2 = 0.617$; $p = 0.0005$), *cpt1a*
415 ($Y = 0.01 + 1.0x$; Fig 4H), *cpt1b* ($Y = 0.23 + 0.15x$; Fig. 4F), and *pigf5* ($Y = 0.33 + 0.43x$; $R^2 = 0.577$;
416 $p = 0.001$). The mRNA abundance of three genes differed between quality groups (Fig. 4). Here, *dio1*
417 mRNA levels were relatively stable throughout embryonic development but were significantly lower
418 in the Low quality group compared to the Medium and High quality group (Fig. 4A, B). The mRNA
419 abundance of *dio1* in the ovary was associated to the abundance in the unfertilized egg (Fig. 4C). The
420 mRNA levels of *cpt1b* were highest in the High and Medium quality group being significantly lower
421 in the Low quality group (Fig. 4D). mRNA abundance of this gene was relatively low in the
422 unfertilized egg and stayed stable until 8 hpf. Subsequently, a rapid increase was observed at 24 hpf
423 increasing further towards hatch. The mRNA abundance in the ovary was associated to the abundance
424 in the unfertilized egg. The mRNA abundance of *cpt1a* showed a significant interaction between
425 quality groups and hpf ($p = 0.001$) and was therefore analyzed separately at each sampling point.
426 Here, mRNA abundance until 24 hpf remained relatively stable and did not differ between quality
427 groups (Fig 4G). Hereafter, levels increased at 32 and 48 hpf. Moreover, at 32 hpf higher levels in
428 the Medium quality group were found compared to the High quality group, while the Low quality

429 group was intermediate. Again, mRNA abundance of *cpt1a* in the female ovary was positively
430 associated to levels in the unfertilized eggs (Fig. 4H).

431

432 **3.3. Steroid concentrations – mRNA transcript abundance**

433 The mRNA abundance of each analyzed gene was tested for association with each steroid
434 concentration in the unfertilized eggs. Here, an association between thyroid hormone receptors and
435 steroid levels in the unfertilized eggs was found. E2 concentrations were positively associated with
436 the relative mRNA abundance of *dio3* (Fig. 5A). Moreover, the T concentrations were negatively
437 associated with the relative mRNA abundance of *thrαb* (Fig. 5 B, C).

438

439 # 4. Discussion

440

441 **4.1. Steroids**

442 In female European eel, the importance of sex steroids and their dynamics has been studied
443 showing increasing concentrations of E2, T, and 11-kt throughout ovarian development (Burgerhout
444 et al., 2016; da Silva et al., 2016). Moreover, DHP is known to be the most effective MIS in many
445 teleost species (Nagahama, 1983; Nagahama and Yamashita, 2008). In anguillid species, the injection
446 of DHP is used to induce follicular maturation once oocytes reach the migratory nucleus stage (da
447 Silva et al., 2018b; Ohta et al., 1997; Palstra et al., 2005). The present study showed maternal transfer
448 of DHP as well as E2, T and 11-kt to the unfertilized eggs of European eel. More so, the
449 concentrations of DHP, E2 and T in the unfertilized eggs were associated with the concentrations in
450 female post-stripping plasma, confirming maternal transfer of these steroids to the developing
451 oocytes. Furthermore, high concentrations of DHP and E2 were negatively associated with embryonic
452 developmental competence. On this basis, assisted reproduction methods that are commonly used in

453 aquaculture to induce gametogenesis and maturation or synchronize spawning can be suspected to
454 have adverse effects on the egg quality. High maternal E2 concentrations may relate to the PE
455 injection provided prior to follicular maturation as a primer to sustain follicular maturation. FSH in
456 the PE stimulates E2 synthesis and E2 concentrations have been shown to increase following SPE as
457 well as DHP injections in European eel (H. Tveiten, unpubl. data). In the current study, this may have
458 led to high E2 concentrations in the ovary and thus leading to enhanced transfer of E2. Here, future
459 studies are needed to examine the exact influence of PE and DHP doses on transfer levels. Moreover,
460 elevated E2 might reflect that oocyte maturation may have been induced or boosted at a too early
461 stage of follicular development. This might also explain the association between high egg DHP
462 concentrations and poor egg quality that indicates possible premature recruitment into follicular
463 maturation. Unpublished in vitro studies show that European eel ovarian follicles are able to
464 metabolize DHP into inactive DHP-sulphate (H. Tveiten, pers. comm.) which may be a mechanism
465 to protect the oocyte from DHP overexposure and a premature entry into follicular maturation. This
466 metabolization, or inactivation mechanism, of MIS during follicular maturation is also found in other
467 marine teleosts (Scott et al., 1997; Scott and Sorensen, 1994; Tveiten et al., 2010b, 2000). The high
468 (150-200 ng/ml) DHP plasma concentrations associated with DHP induced follicular maturation in
469 eel, might supersaturate this inactivation system resulting in increased DHP accumulation in the
470 oocyte/egg. Thus, it can be speculated that eggs with high DHP concentrations and of low quality,
471 may have been subjected to MIS at a too early stage of development, negatively influencing their
472 further development (i.e. fertilization success, occurrence of cleavage abnormalities, embryonic
473 survival). To date, ideal timing to induce follicular maturation in order to allow optimal development
474 of the oocytes in eel is a widely discussed issue and criteria as well as oocyte stage classification are
475 constantly optimized (da Silva et al., 2018a; Tomkiewicz et al., 2019; Unuma et al., 2011). Results
476 from this study suggest further research focusing on optimal timing of the induction of follicular

477 maturation and ovulation to avoid potential negative effects due to untimely induction procedures.
478 Here, it is important to consider that evidence of eels being batch spawners with asynchronous or
479 group synchronous oocyte development is increasing (da Silva et al., 2018a; Palstra et al., 2020;
480 Tomkiewicz et al., 2019). This would underline the presence of oocytes in different stages during
481 induced follicular maturation, contributing to the variability in egg and embryonic quality. In addition
482 to optimal timing, the high dose of DHP given as MIS may play an important role in the individual
483 variability in the response and egg quality, an aspect that deserves further study.

484 Recently, studies on estrogen receptor expression have elucidated their important role during
485 follicular maturation in European eel (da Silva et al., 2018b; Lafont et al., 2016). In da Silva et al.
486 (2018b), the nuclear receptors *esr1* and *esr2a* were expressed at the time of SPE priming and DHP
487 injection but hardly in the ovulated eggs. While mRNA transcripts of the membrane receptor *gpera*
488 were present in the unfertilized eggs, levels of *gperb* were below the detection threshold. However,
489 at the time of DHP injection, a higher expression of *gperb* was found in females producing low quality
490 eggs. This observation may support the finding that high concentrations (signaling) of E2 were
491 negatively associated to egg quality in the present study. Further investigations are needed to reveal
492 if there is a direct relationship between receptor abundance and E2 with egg quality. Androgen
493 receptor (*ara*, *arb*) levels increase throughout ovarian development in both European eel (Peñaranda
494 et al., 2014) and Japanese eel (Tosaka et al., 2010). However, in the latter, mRNA transcript levels in
495 the ovulated eggs were very low (*ara*) or undetectable (*arb*) indicating only limited maternal transfer.

496 Concentrations of E2, T and 11-kt in eggs and embryos have been studied in several species.
497 For instance, a maternal transfer with presence of E2, T and 11-kt in the unfertilized eggs has been
498 suggested in Eurasian perch, *Perca fluviatilis* (Rougeot et al., 2007), coho salmon (Feist et al., 1990),
499 tilapia (T and E2) (Rothbard et al., 1987), white sturgeon, *Acipenser transmontanus* (T, E2 and
500 cortisol) (Simontacchi et al., 2009), medaka, *Oryzias latipes* (T and E2) (Iwamatsu et al., 2006), and

501 three-spined stickleback (T and E2) (Paitz et al., 2015). Also, the presence of maternally derived DHP
502 has been shown in eggs of coho salmon (Feist et al., 1990), Arctic charr (Khan et al., 1997), and three-
503 spined stickleback (Paitz et al., 2015). Similar to our results, a strong decline in concentration
504 following fertilization was found for all three species indicating that steroids may be removed. In the
505 present study, concentrations of T and 11-kt were almost non-detectable in fertilized eggs and there
506 were no apparent relationships with egg quality. However, there appeared to be a negative association
507 between E2 and DHP concentrations and embryonic developmental competence. These results are
508 similar to findings in coho salmon, where concentrations of E2 and DHP were higher in non-viable
509 eggs compared to viable eggs (Feist et al., 1990). However, the exact role and fate of steroids during
510 these early stages of embryonic development remains to be clarified. Some studies suggest the
511 metabolization by the embryo, e.g. in Arctic charr, it was suggested that at least two enzyme systems
512 were present, cytochrome P450 C_{21} side chain cleavage converting progesterone (P_4) to 17-
513 hydroxyprogesterone (17OHP) and further to androstenedione (A_4) and secondly, 11βhydroxylase
514 that convert A_4 to 11-oxyandrogens (Khan et al., 1997). This may be of advantage for the embryos,
515 as these products have a potentially lower biological activity than steroids such as E2 and T (Khan et
516 al., 1997). Other studies have provided evidence of the removal of steroids through ABC transporters
517 without prior metabolization, allowing the developing embryo to adapt to maternally derived steroid
518 concentrations, e.g. cortisol (Paitz et al., 2016). Future studies may investigate the present
519 mechanisms in European eel to examine whether the embryos are actively responding to these
520 maternal constituents.

521 Maternal stress may lead to increased deposition of cortisol into the egg with possible
522 implications on the embryonic developmental competence (Nesan and Vijayan, 2012). In this study,
523 cortisol was found in the blood plasma of the female in the 15-25 ng/ml range but only low
524 concentrations (and no association with plasma concentrations) were found in the unfertilized eggs.

525 Cortisol concentrations reached detection levels already at 2 hpf and no relationship with egg quality
526 was observed. In other fish species, protective measures to prevent excess cortisol entering the eggs
527 may be related to upregulation of cortisol inactivating enzymes, such as 11β-hydroxysteroid
528 dehydrogenase type 2 (11βHSD2) (Faught et al., 2016). Whether a similar mechanism may be present
529 in European eel ovaries remains unexplored.

530 **4.2. mRNA transcript abundance**

531 Overall, this study investigated the mRNA transcript profiles of genes assumed to be
532 important for embryonic development in European eel. The genes selected are known to be involved
533 in stress/repair responses, growth and development, as well as thyroid and lipid metabolism. In our
534 study, most of these genes showed low mRNA abundance before MZT and an increase in expression
535 upon activation of the embryos own genome.

536 **4.3. Genes related to cellular stress**

537 Heat shock proteins, such as *hsp70* and *hsp90* function as chaperones and are recognized to
538 be upregulated in response to cellular stress (Roberts et al., 2010). In teleost embryos, it has been
539 shown that *hsp* levels are both affected by developmental age (Blechinger et al., 2002; Lanes et al.,
540 2012) and cellular stress (Hallare et al., 2005; Sales et al., 2019; Uchimura et al., 2019; Yeh and Hsu,
541 2002). In the present study, *hsp90* peaked at 32 hpf with a subsequent decline towards hatch indicating
542 a role in embryonic development and possible stress response during organogenesis, while *hsp70*
543 showed a slight increase towards hatch indicating a possible role for hatched larvae. In European eel
544 larvae, both genes are affected by environmental parameters, such as temperature and salinity (Politis
545 et al., 2017; 2018a), which remains to be investigated for embryos. *Hsp70* is required to prevent
546 stress-induced cell death (Mosser et al., 2000) and *hps90* is essential for cell viability and normal
547 embryonic development in zebrafish embryos allowing intracellular signaling and proliferation

548 and/or differentiation (Lele et al., 1999). However, in the current study, no difference in
549 concentrations was observed for embryos between quality groups.

550 ### 4.4. Genes related to the somatotropic axis

551 Genes involved in the somatotropic axis take place in numerous processes including
552 reproduction and growth during embryonic development (Reinecke et al., 2005; Reinecke and Collet,
553 1998). In the present study, all genes involved in growth and development showed a similar pattern
554 with strong increases after the MZT indicating that the somatotropic axis is functional and may play
555 a role already during embryonic development in European eel. Additionally, no differences between
556 quality groups were observed, which is in agreement with a previous study on eel with no differences
557 in the expression of *igf2a and igf2b* between high and low hatch groups (Rozenfeld et al., 2016). This,
558 however, appears to be species specific, as *igf1*, *igf2*, and *igfr1b* have been positively associated with
559 embryonic survival in rainbow trout (Aegerter et al., 2004; 2005). The expression patterns of *igf* and
560 *gh* during embryogenesis have been shown for various species, such as zebrafish (Li et al., 2014; Zou
561 et al., 2009), maraena whitefish, *Coregonus maraena* (Nipkow et al., 2018), gilthead seabream,
562 *Sparus* aurata (Perrot et al., 1999), and seabass, *Dicentrarchus labrax* (Besseau et al., 2013).
563 Similarly, these patterns appear to be species specific and for some species expression has only been
564 observed after hatch, as in the closely related Japanese eel (Ozaki et al., 2006). Moreover, *gh* and *igf*
565 may show differential expression patterns indicating that the *igf* expression is not *gh*-dependent, yet,
566 at that stage (Li et al., 2006). Nonetheless, in the current study patterns for all growth- and
567 development-related genes were similar. Moreover, environmental parameters such as temperature
568 influence these genes in embryos (Li et al., 2006; Nipkow et al., 2018) as well as larvae (Politis et al.,
569 2017). Thus, these genes can be used as indicators to optimize rearing protocols for European eel
570 embryos.

571 **4.5. Genes related to lipid metabolism**

572 The great importance of fatty acids for reproductive success and high egg quality is widely
573 accepted and extensively studied (Sargent et al., 1999; Tocher, 2003), including in European eel
574 (Kottmann et al., 2020b; Støttrup et al., 2016). However, knowledge on the importance of maternal
575 mRNA and the expression patterns of fatty acid metabolic genes throughout early development is
576 scarce. In the present study, we investigated the expression dynamics of *cpt1a*, *cpt1b*, and *pigf5*,
577 which are involved in β-oxidation. Though being maternally derived, we observed overall low mRNA
578 levels of these genes during early development with strong increases after commencement of the
579 embryos own transcription. Moreover, we found higher expression of cpt1b in the High and Medium
580 quality group compared to the Low quality group. This is in agreement with a previous study on
581 European eel, where a higher relative abundance of all three genes was found for the hatch group
582 compared to the non-hatch group, but only during later embryonic development (Rozenfeld et al.,
583 2016). Similarly, in Atlantic cod higher expressions of these genes were found in embryos originating
584 from wild broodstock (high quality) compared to embryos obtained from farmed broodstock (low
585 quality) (Lanes et al., 2013). In zebrafish, the knockdown of *cpt1a* lead to impaired lymphatic
586 development demonstrating its importance for early development in fish (Zecchin et al., 2018). The
587 expression pattern found in the current study may indicate a functional role in lipid metabolism for
588 late embryonic development and possibly early larval development. In the orange-spotted grouper,
589 *Epinephelus coioides*, *cpt1* expression was also initially low with a rapid increase during main organ
590 formation processes, however, decreasing again towards hatch (Tang et al., 2013). Interestingly, the
591 expression of *cpt1* did not change over time in embryos and early larval stages of turbot,
592 *Scophthalmus maximus* (Cunha et al., 2013), indicating the function and dynamics during early life
593 history may be species specific.

594 **4.6. Genes related to thyroid metabolism and signaling**

595 Thyroid hormones (TH) play essential roles in growth, maturation, development and
596 metabolism and have been extensively studied in humans, mammals and birds (Power et al., 2001).
597 Knowledge on their function in early development in fish is still incomplete but THs are likely to be
598 maternally deposited into the oocyte, regulating early development until the offspring are capable of
599 endogenous hormone production (Brown et al., 2014). TH bind to nuclear thyroid hormone receptors
600 (THR), which mediate their actions (Power et al., 2001). In European eel, four different subtypes of
601 THR (t*hrαa*, *thrαb*, *thrβa*, *thrβb*) and three different subtypes of deiodinases (*dio1*, *dio2*, *dio3*) have
602 been characterized (Politis et al., 2018b). In the present study, most of the genes (except *dio1*)
603 involved in thyroid metabolism and signaling showed relatively low initial mRNA levels but
604 increased after the MZT and the embryos own genome activation. Here, *thrαb*, *thrβa*, *thrβb* showed
605 only slight increases towards hatch. A different pattern was found for *dio1* that appeared to have
606 higher maternally derived levels during the early embryonic stages and more stable levels throughout
607 embryogenesis, but with a slight decrease after MZT. Moreover, mRNA transcript levels in embryos
608 of High and Medium quality were higher compared to embryos with low developmental potential
609 indicating that *dio1* may be of particular importance during early embryogenesis. In teleost, *dio1* and
610 *dio2* have similar functions being capable to convert T4 (thyroxine) to T3 (3,5,30-triiodothyronine),
611 while *dio3* is a purely inactivating enzyme (Orozco and Valverde-R, 2005). In zebrafish, knockdown
612 of *dio1* and *dio2* had severe impacts on embryonic development (Walpita et al., 2010). Contradictory
613 to our results, where *dio2* was hardly expressed during early stages, *dio2* was found to be of higher
614 importance compared to *dio1* in zebrafish, indicating that functional roles may be stage specific and
615 vary among species. In the present study, the expression of *dio2* and *dio3* showed a pronounced peak
616 towards hatch, which is in line with results from European eel larvae, where elevated levels of these
617 two genes were observed in larvae at hatch (Politis et al., 2018b). Moreover, the present study
618 observed an association between E2 concentrations and *dio3* expression in the unfertilized egg as

well as between T and *thrab* indicating a possible interplay between the two hormone systems. Thyroid hormone receptors belong to the steroid-thyroid super family that also contains receptors for ligands, such as steroids, retinoids and vitamins (Power et al., 2001; Tsai and O'Malley, 1994) indicating cross-talk between the hormone systems through receptor binding (Duarte-Guterman et al., 2014). However, little is known about the extent of this in teleosts. In goldfish, injections with E2, T or 11-kt did not affect the expression of *thrαa*, *thrαb*, *thrβa* and *thrβb* in adult tissues (Nelson and Habibi, 2009). Nonetheless, in human cells a tissue specific positive effect of E2 on the expression of *dio3* was found (Kester et al., 2006). Overall, similar expression patterns of genes involved in thyroid metabolism throughout embryogenesis with increasing expression levels after MZT have been found for zebrafish (Campinho et al., 2010; Vergauwen et al., 2018), rainbow trout (Li et al., 2007), Atlantic salmon (Jones et al., 2002), sea bass (Nowell et al., 2001; Walpita et al., 2007) and fathead minnow, *Pimephales promelas* (Vergauwen et al., 2018). Previous results have shown the importance of thyroid metabolism on larval stages of Japanese (Kawakami et al., 2013) and European eel (Politis et al., 2018b, 2018c). Together, these findings may indicate that the thyroid hormone system is functional already during early stages of eel embryogenesis.

Altogether, results from this study substantiate knowledge on the maternal transfer of steroid hormones and mRNA transcripts to eggs and their temporal changes throughout embryonic development in teleosts, using European eel as a model. Steroid concentrations in the eggs/embryos showed a drastic decline at fertilization. Nonetheless, high levels of maternally derived DHP and E2 were negatively associated with embryonic developmental competence. Future studies investigating the mechanisms behind the steroid removal and whether the embryos actively modulate their exposure to maternal constituents are of interest. Furthermore, the present study revealed pronounced changes in mRNA transcripts of genes related to growth, development, and metabolism during early

642 ontogeny of the European eel. These results provide novel information on the transfer of maternal
643 steroids and mRNA transcripts, which may affect a wide array of oviparous species.

644

645 ## Acknowledgements

646 Wild-caught female silver eels in 2016 were donated by the Lough Neagh Fishermen's Co-
647 operative Society, Northern Ireland. This work was financially supported by Innovation Fund
648 Denmark (grant numbers 5184-00093B and 7076-00125B). Dr. Joanna J. Miest was supported by
649 internal grants from the University of Greenwich. Maria K. Johnsen, Elisa Benini, Dr. Sebastian N.
650 Politis (Technical University of Denmark), and Dr. Sune Riis Sørensen (Billund Aquaculture A/S)
651 took part in the experimental work. Dr. Dhivya Thiyagarajan (Uit The Arctic University of Norway)
652 assisted with steroid analyses and Adrian Loh (University of Greenwich) assisted with gene
653 expression analyses. Dr. Ian A.E. Butts (Auburn University) assisted with the statistical analyses.

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Figure captions:

Fig 1. Steroid concentrations in European eel, *Anguilla anguilla*. (A) Post-stripping plasma DHP concentrations in female eels producing High, Medium and Low quality eggs, (B) DHP concentrations in eggs and embryos from High, Medium and Low quality groups over sampling time (C) DHP concentrations in different quality groups (main effect quality) and (D) E2 concentrations over sampling time (main effect age). (E) Post-stripping plasma E2 concentrations in female eels producing High, Medium and Low quality eggs, (F) E2 concentrations in eggs and embryos from High, Medium and Low quality groups over sampling time (G) E2 concentrations in different quality groups (main effect quality) and (H) E2 concentrations over sampling time (main effect age). Values represent means (± SEM) among females at each sampling time and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

Fig 2. Association between female post-stripping steroid plasma concentrations and concentrations in the unfertilized eggs of European eel, *Anguilla anguilla* for (A) DHP, (B) E2, (C) T, (D) 11-kt, and (E) Cortisol. Circles mark outlier that have been removed from the regression analyses.

Fig 3. mRNA transcript abundance throughout embryonic development in European eel, *Anguilla anguilla*. Conceptual overview – mRNA abundance ($2^{-\Delta\Delta Ct}$) was calculated in relation to the average abundance in the unfertilized eggs of each gene. Relative mRNA abundance of (A) *hsp70, hsp90,* (B) *gh, igf1, igf2a, igf2b,* (C) *cpt1a, cpt1b, pigf5, and (D) dio1*, *dio2*, *dio3*, *thrαb*, *thrβa*, *thrβb*. Bar represents timeframe of maternal-to-zygotic transition (MZT).

Fig 4. mRNA transcript abundance in European eel, *Anguilla anguilla*. Relative mRNA abundance of *dio1* (A) in different quality groups (B) throughout embryonic development and (C) association between *dio1* between mRNA abundance in female ovary and unfertilized egg, and relative mRNA abundance of *cpt1b* (D) in different quality group (E) throughout embryonic development and (F) association between *cpt1b* between mRNA abundance in female ovary and unfertilized egg, and (G) interaction of relative mRNA abundance of *cpt1a* between quality groups throughout development and (H) association between *cpt1a* between mRNA abundance in female ovary and unfertilized egg. Values represent means (± SEM) among females at each sampling time and treatment. Different lower-case letters represent a significant statistical difference ($p < 0.05$).

Fig 5. Relationship between steroid concentrations and mRNA abundance in European eel, *Anguilla anguilla*. Relationship in the unfertilized eggs between (A) E2 concentration and *dio3* mRNA abundance and (B) T concentration and *thrαb* mRNA abundance.

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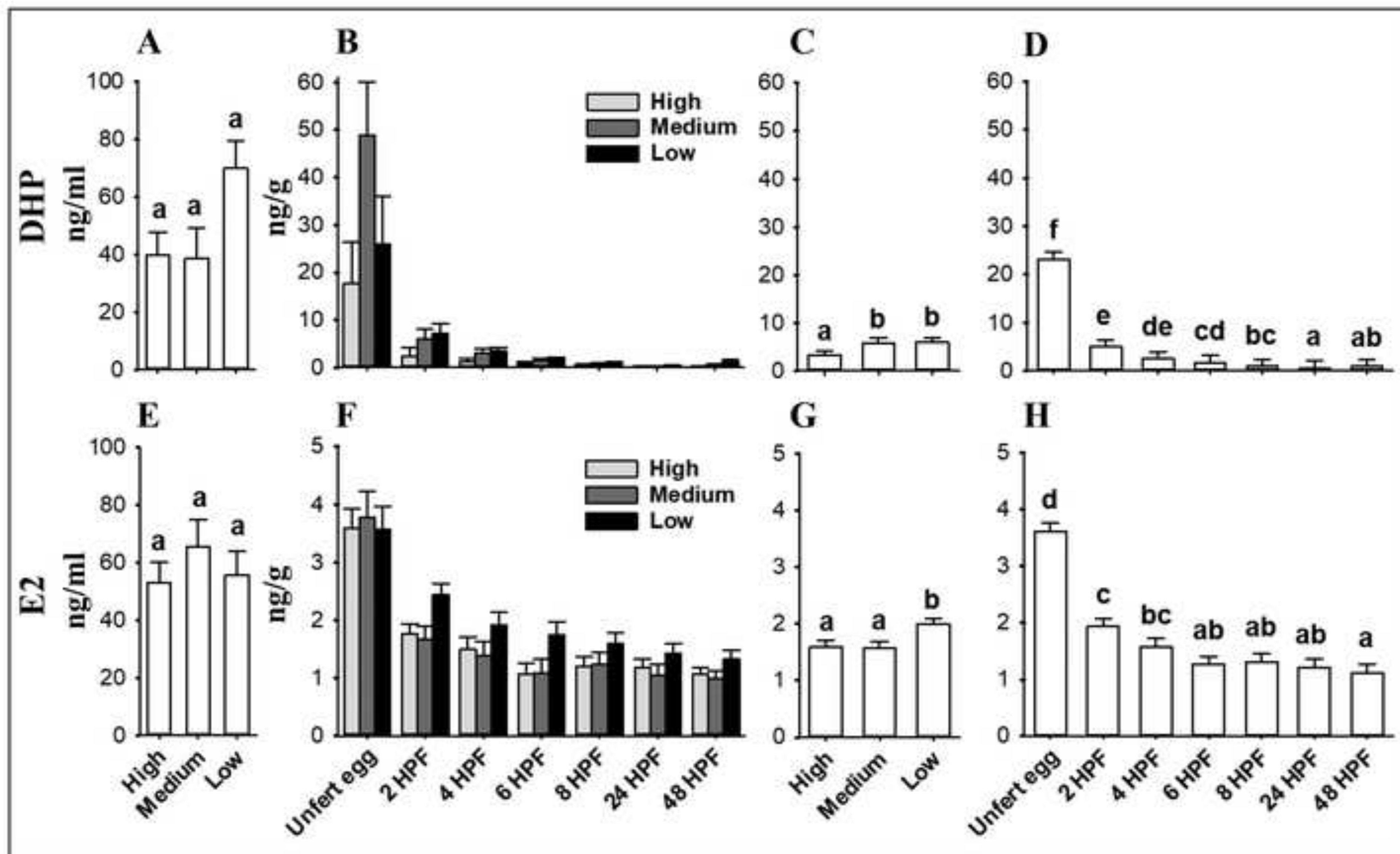

Figure 2

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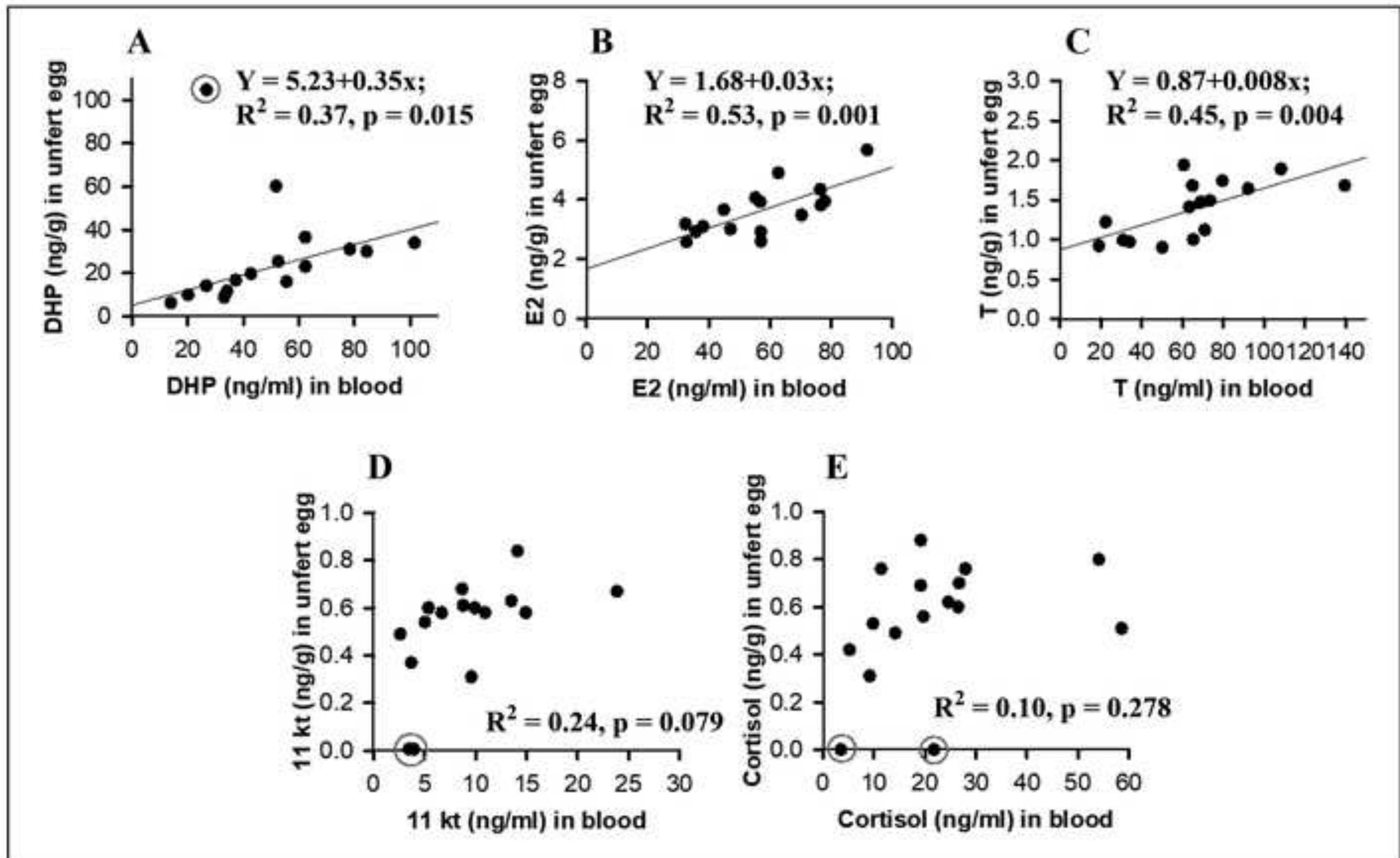

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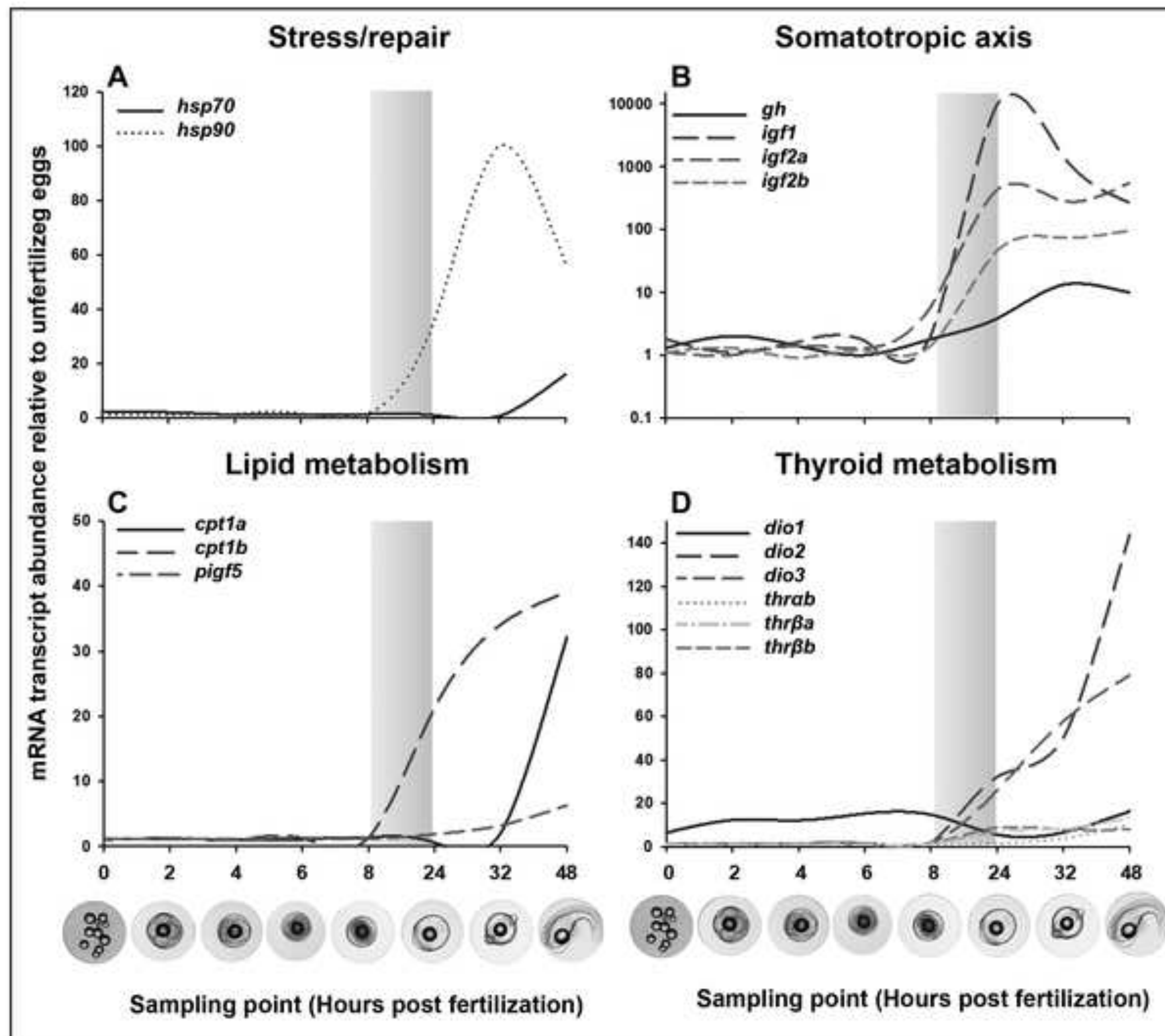

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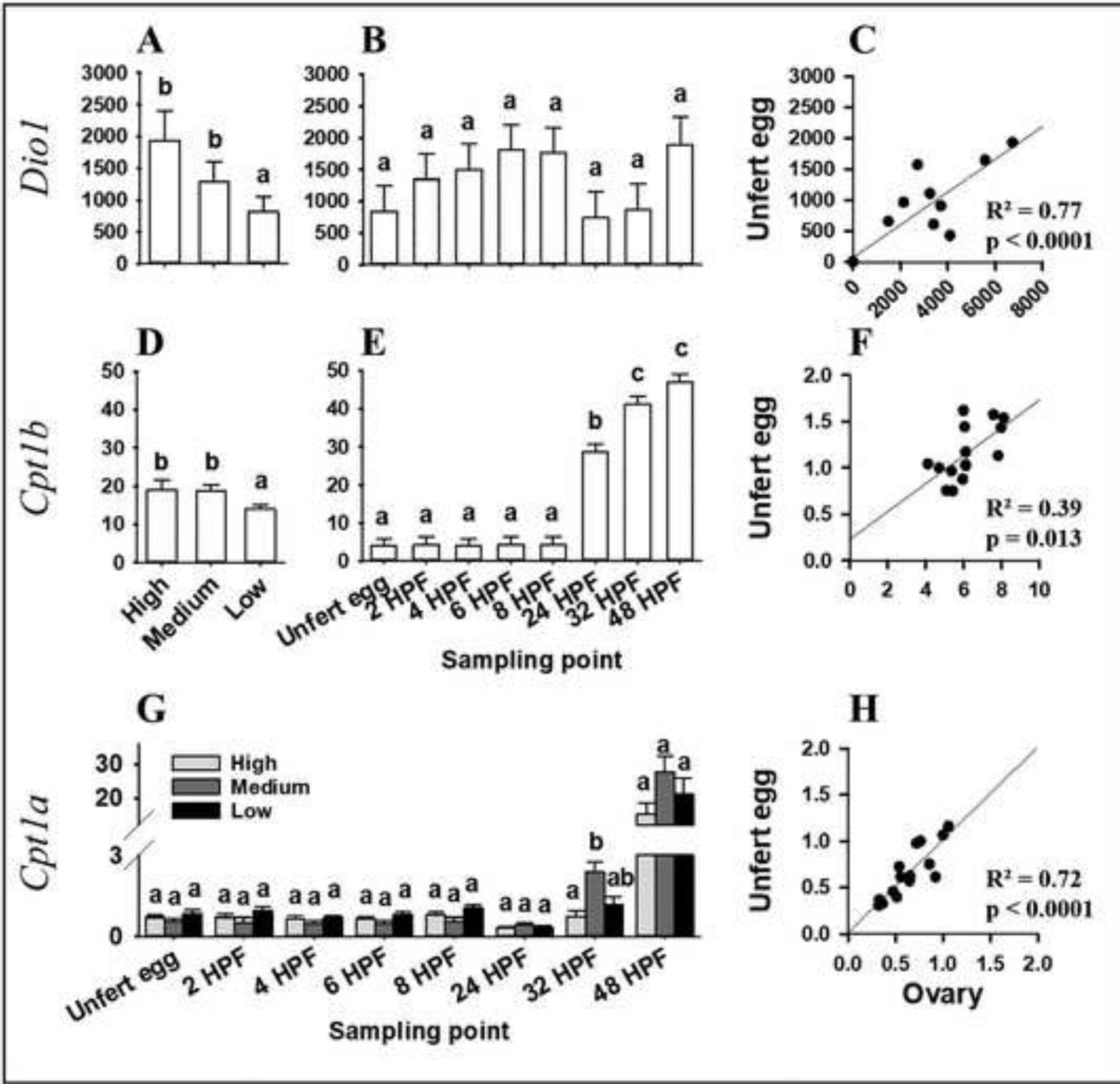

Figure 5

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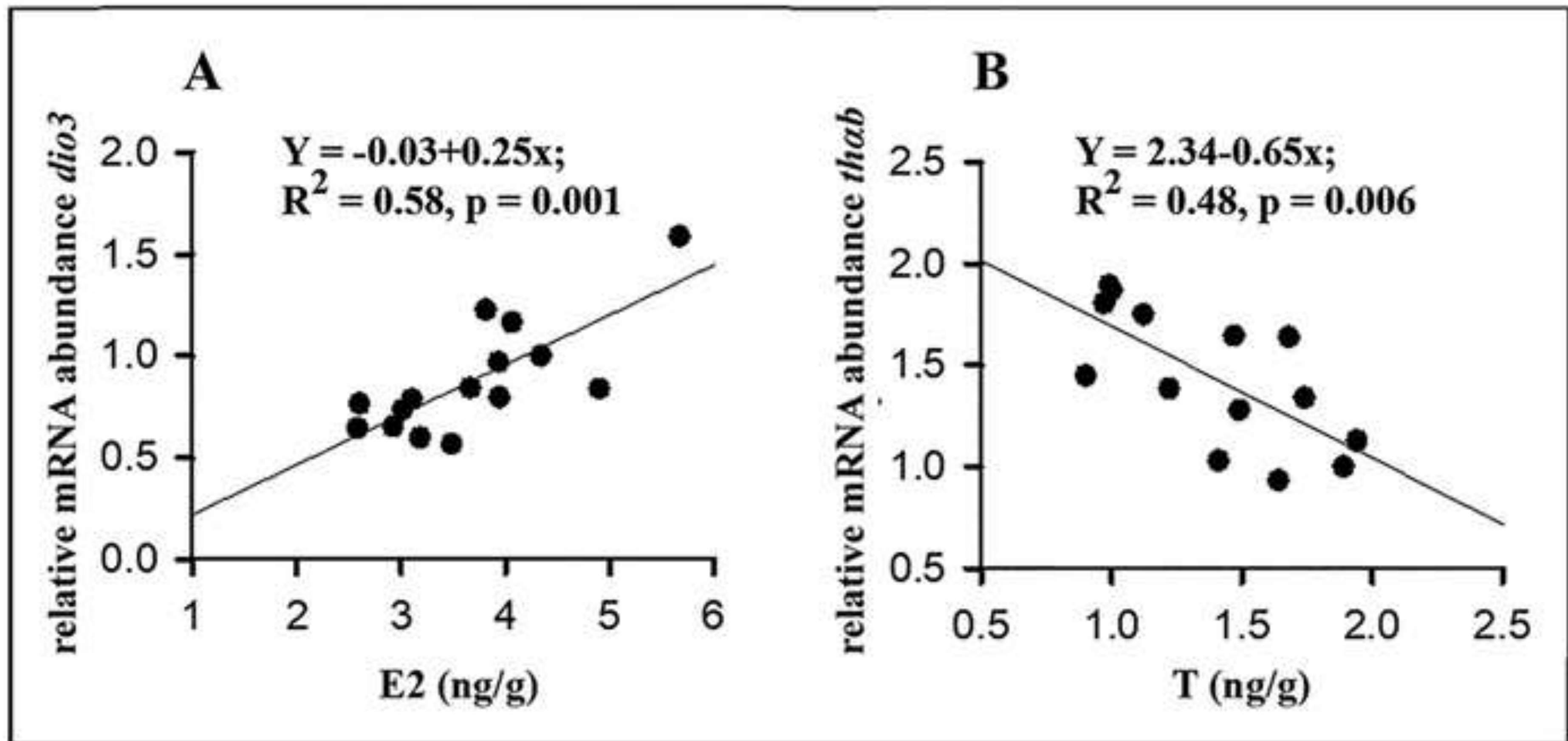

Author statement

Johanna Kottmann: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft preparation, Writing - review & editing

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