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Biochemical composition and adenylate energy charge shifts in longfin yellowtail (*Seriola rivoliana*) embryos during development under different temperatures

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Abstract :

The longfin yellowtail Seriola rivoliana is an emerging species for aquaculture diversification worldwide and production relies on fertilized eggs from captive broodstock. Temperature is the main factor that influences the developmental process and success during fish ontogeny. However, the effects of temperature on the utilization of the main biochemical reserves and bioenergetics are scarcely investigated in fish, whereas protein, lipid and carbohydrate metabolism have critical roles in maintaining cellular energy homeostasis. In this context, we aimed to evaluate metabolic fuels (protein, lipids, triacylglicerides, carbohydrates), adenylic nucleotides and derivates (ATP, ADP, AMP, IMP), and the adenylate energy charge (AEC) during embryogenesis and in hatched larvae in S. rivoliana at different temperatures. For this purpose, fertilized eggs were incubated at six constant (20, 22, 24, 26, 28 and 30 °C) and two oscillating (21⇄29 °C) temperatures. Biochemical analyses were made at blastula, optic vesicles, neurula, prehatch and hatch periods. Results indicated that the developmental period had a major influence on the biochemical composition at any temperature regime tested during the incubation. Protein content decreased only at hatching mainly due to the loss of the chorion, total lipids tended to increase at the neurula period and variations in carbohydrates depended on the particular spawn analyzed. Triacylglicerides were a critical egg fuel during hatching. The high AEC during embryogenesis and even in hatched larvae suggested an optimal energy balance regulation. The lack of critical biochemical changes from different temperature regimes during embryo development confirmed that this species exhibits a high adaptive capacity in response to constant and fluctuating temperatures. However, the timing of hatching was the most critical period of development, where biochemical components and energy utilization significantly changed. The oscillating temperatures tested may have physiological advantages without detrimental energetic effects that will require further research on larval quality after hatching.

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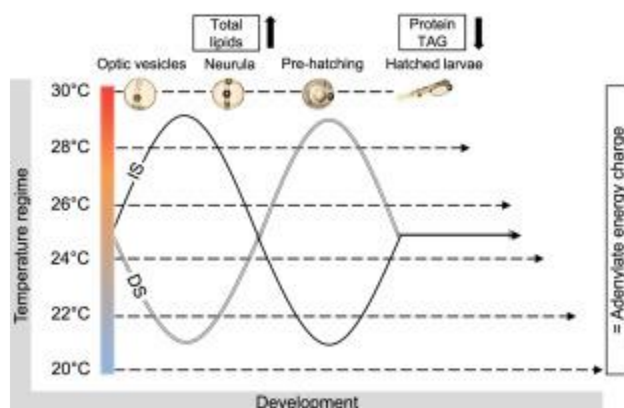
Graphical abstract



Highlights

► *S. rivoliana* embryonic development was analyzed at constant and oscillating temperatures. ► Development had a major influence on biochemical components, while temperature did not. ► The adenylate energy charge indicated an optimal embryo energy homeostasis. ► Protein content decreased at hatch associated with chorion breakdown and release. ► Triacylglicerides were the primarily metabolic fuel, mainly used during hatching.

Keywords : Seriola, embryogenesis, temperature, ATP, Adenylate energy charge

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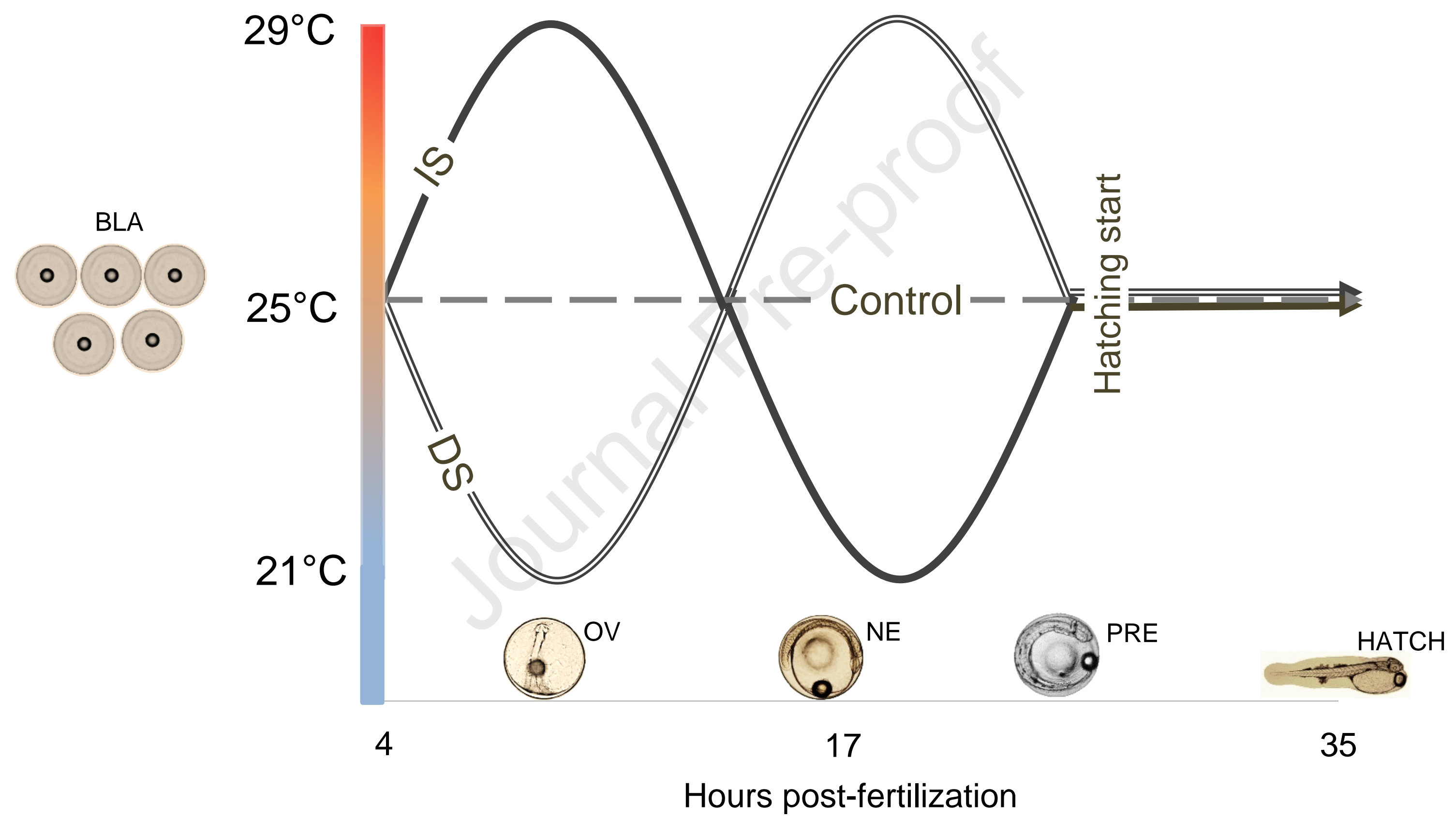

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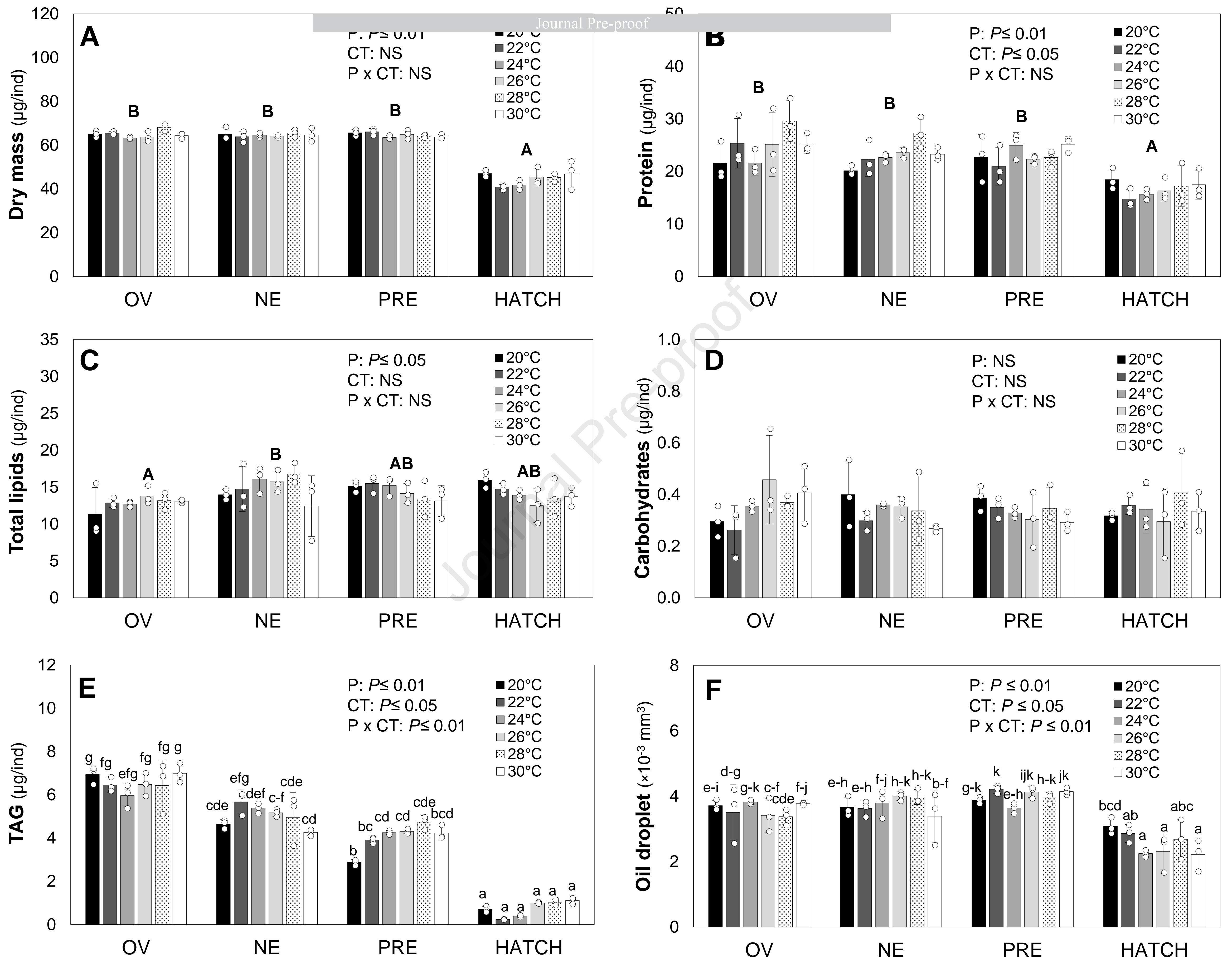

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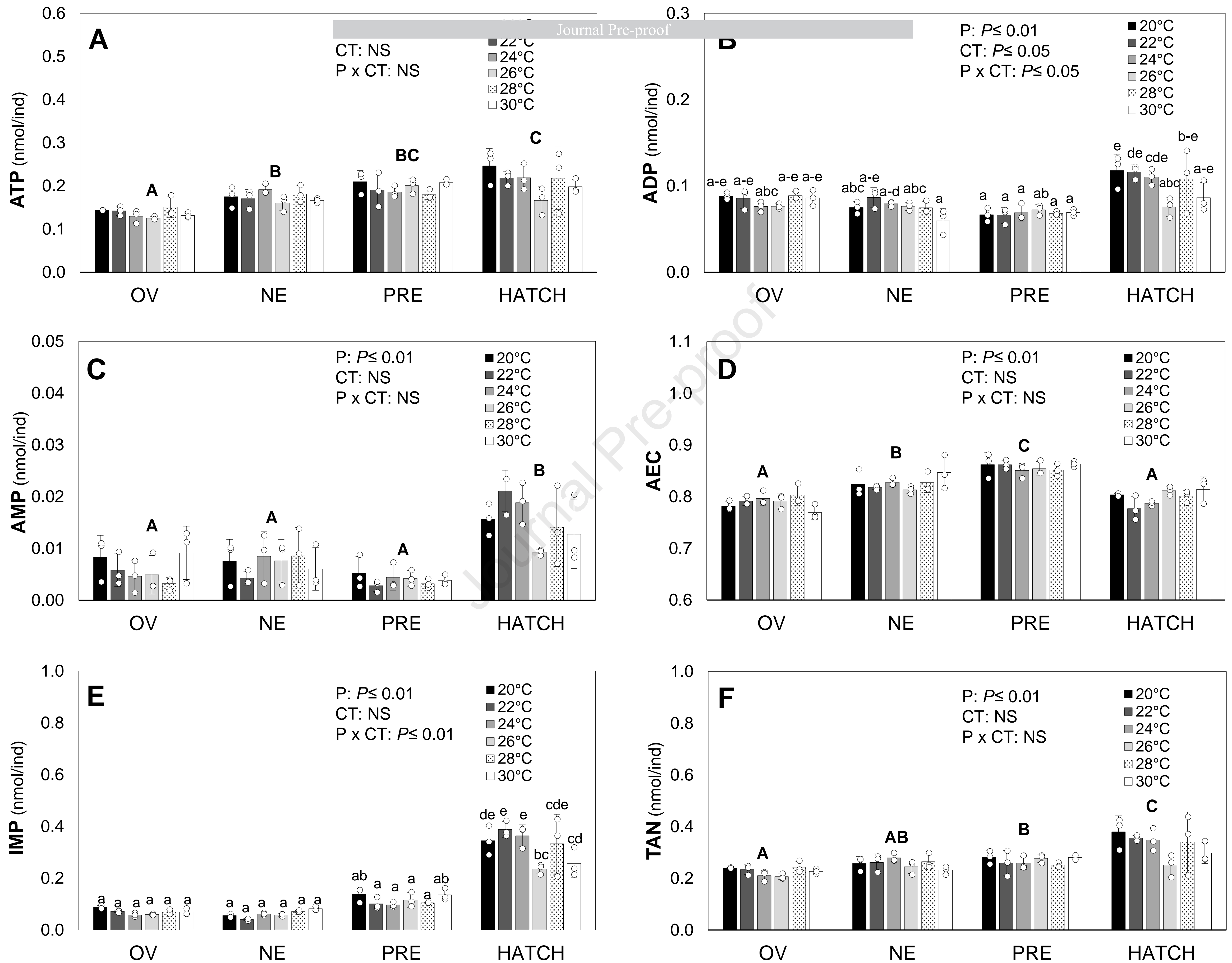

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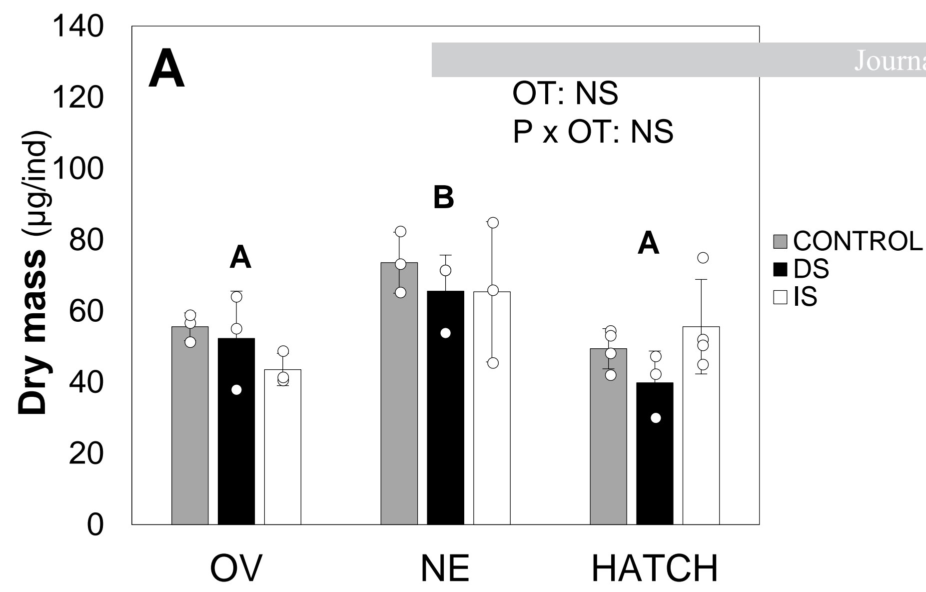

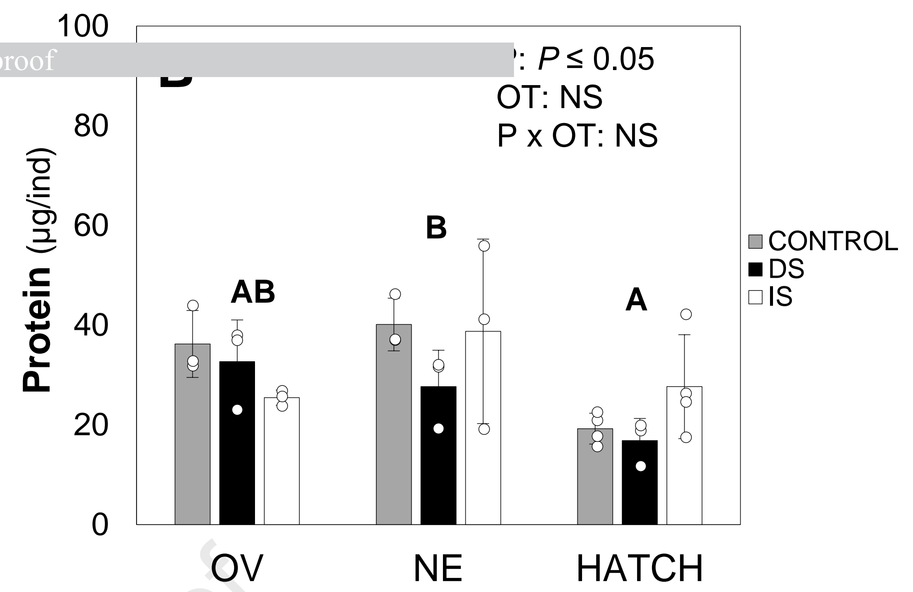

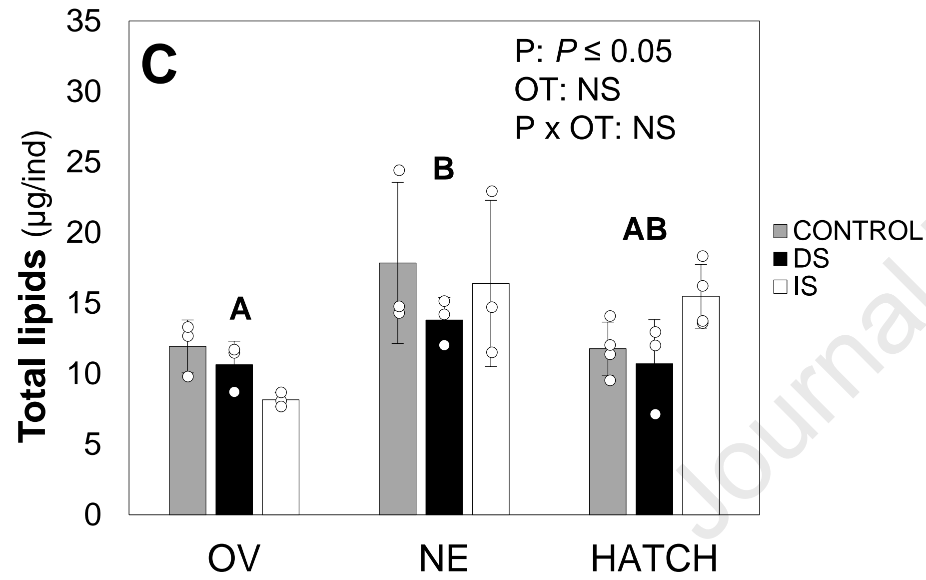

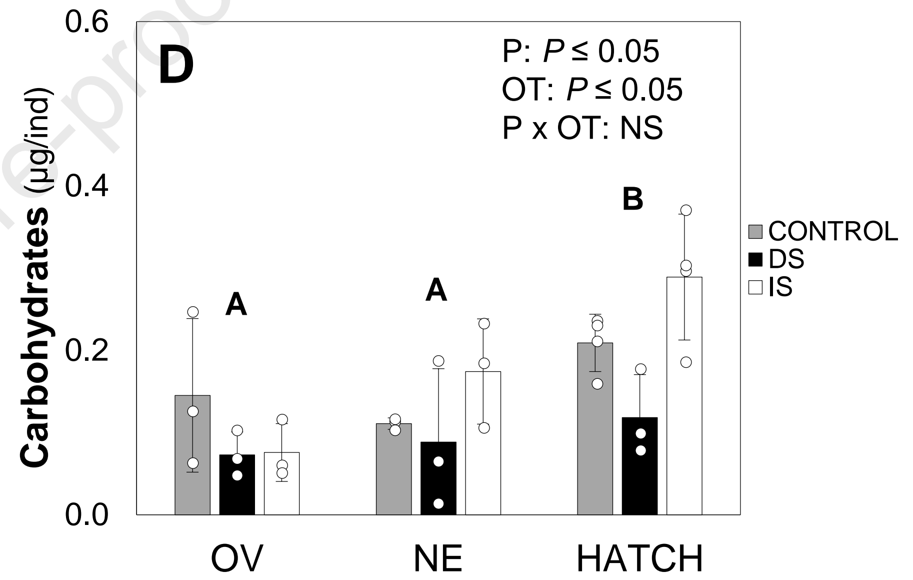

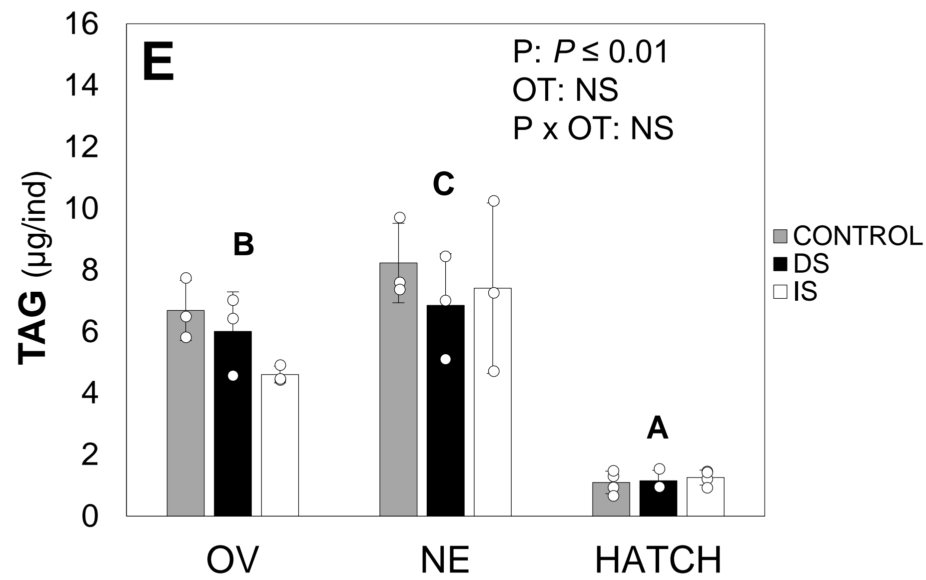

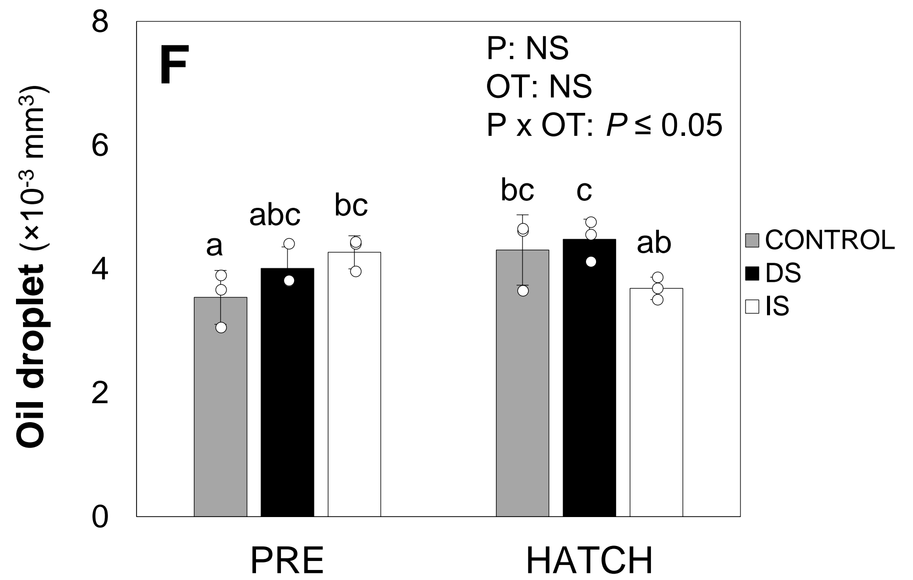

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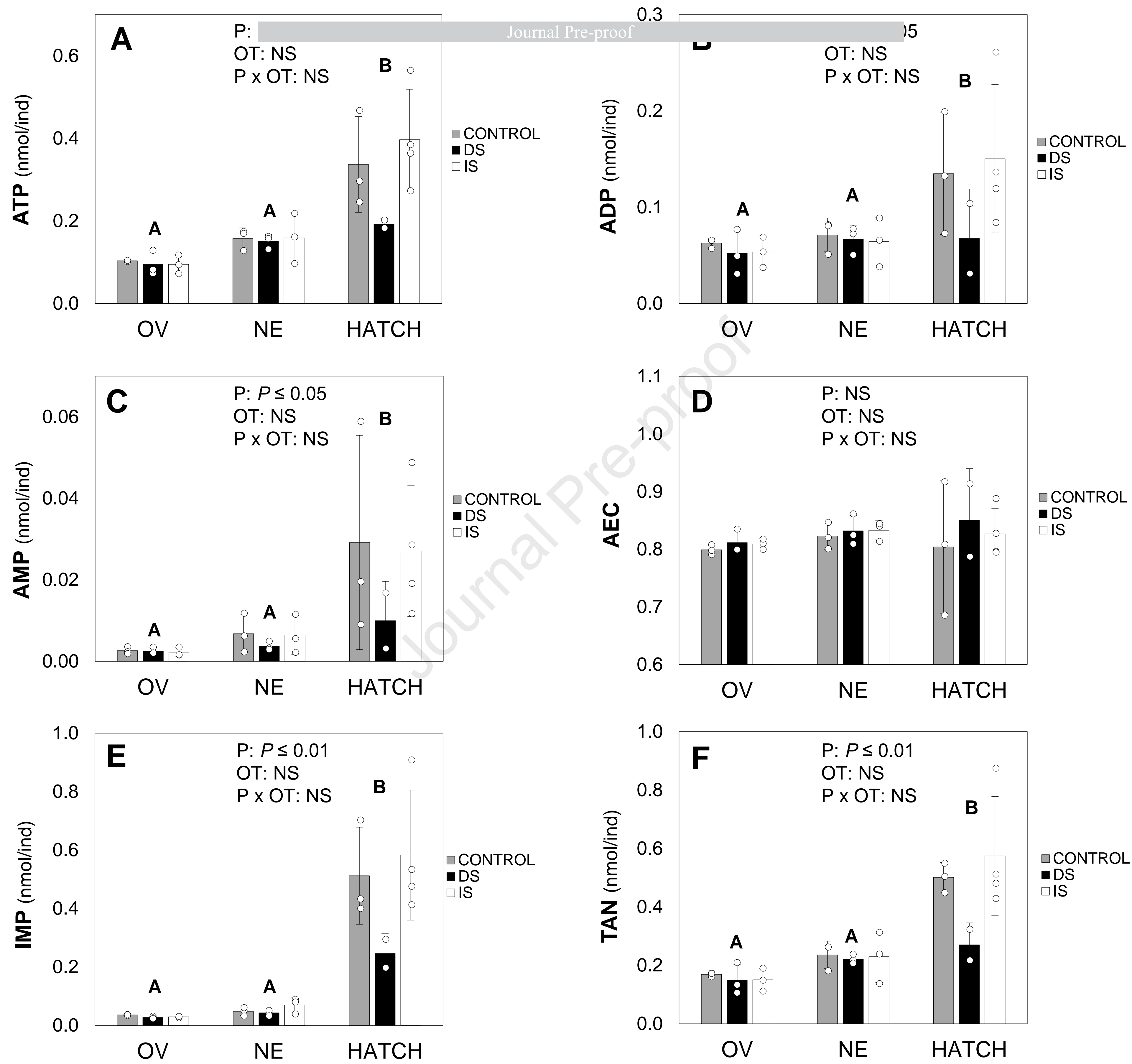

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52 **INTRODUCTION**

53 In marine ecosystems, temperature is one of the most important environmental factors
54 influencing the developmental process during the early fish ontogeny. Most studies analyze
55 the influence of constant temperatures on marine fish embryo development, namely on
56 performance (incubation time, hatching rate, survival and growth) as well as morphological
57 traits (larval length, yolk sac and oil droplet volumes) (Scoppettone et al., 1993; Moran et
58 al., 2007; Kupren et al., 2011; Puvanendran et al., 2015; Imsland et al., 2019; Viader-
59 Guerrero et al., 2021). Although this is a practical approach for artificial rearing conditions
60 to establish an optimal temperature range and predict the detrimental influence of global
61 warming scenarios, it does not necessarily reflect natural or anthropogenic induced thermal
62 fluctuations. Therefore, the evaluation of variable temperature regimes during the early
63 ontogeny of several fish, mainly cold to temperate species, has been gaining attention in
64 the last few years to further improve larval performance (Blanco-Vives et al., 2010;
65 Lahnsteiner et al., 2012; Mueller et al., 2015; Jeuthe et al., 2016; Eme et al., 2018).

66 The endogenous feeding phase in marine fish is a period in which maternally derived
67 biochemical reserves in the yolk and oil droplet constitute the main energy sources and
68 structural components for metabolism, development and growth (Finn et al., 1995a; 1996;
69 Rønnestad et al., 1998). However, the effects of constant or fluctuating temperatures on
70 biochemical reserves (protein, lipids and carbohydrates) during ontogeny have been
71 scarcely assessed (Wen et al., 2013; Mueller et al., 2017).

72 Another approach used to understand bioenergetics of early development facing
73 environmental challenges is the evaluation of the composition of adenylic nucleotides
74 (ATP, ADP, AMP) (Vetter et al., 1983). During the larval lecithotrophic phase, the

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75 metabolic transformations are energetically supported by the hydrolysis of adenosine
76 triphosphate (ATP), which is formed via the coupled oxidation of the reserves stored in the
77 yolk and oil globule (Finn et al., 1996). Hence, ATP must be tightly regulated, and a low
78 concentration of ATP indicates a loss of energy balance between energy production and
79 utilization. Rather than ATP alone, Atkinson (1968) proposed that cells regulate their
80 energy metabolism based on the relative levels of the three adenylic nucleotides (ATP,
81 ADP and AMP), experimentally expressed as the adenylate energy charge (AEC) that
82 ranges from 0.0 to 1.0, with normal values between 0.8 and 0.9 indicating enough cellular
83 energy availability. In eggs of marine and freshwater fish, few studies have evaluated ATP
84 and/or AEC levels during embryogenesis. A decrease in ATP and AEC was observed
85 during embryogenesis in carp *Ciprinus carpio* (Bouleckbache et al., 1989) suggesting that
86 the concomitant mobilization of reserves for oxidative phosphorylation of ADP to
87 synthetize ATP did not fully compensate for ATP hydrolysis. In accordance, Lahnsteiner
88 and Patarnello (2003) found a higher AEC in viable (floating) compared to non-viable
89 (sinking) seabream *Sparus aurata* eggs, probably due to inadequate levels of energy
90 sources (reserves) or to a low rate of catabolic pathways for ATP synthesis.

91 The cosmopolitan longfin yellowtail *Seriola rivoliana* Valenciennes is a fast-growing
92 species inhabiting subtropical oceans and has emerged for aquaculture diversification
93 around the world (Espinoza et al., 2009; Jerez, 2013). The commercial production of
94 juveniles depends on larviculture from eggs of captive broodstock, which naturally spawn
95 at an optimal temperature of 26 °C (Blacio, 2004). However, the quality of spawns is still
96 highly variable and inconsistent, and larval survival is usually low with only a few batches
97 successfully raised to juveniles (Pacheco-Carlón et al., 2021).

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98 In the context of improving larval survival of *S. rivoliana*, the aim of this research was to
99 provide insight into the energetic condition of longfin yellowtail *S. rivoliana* throughout
100 embryogenesis and in early hatched larvae in relation to constant and oscillating
101 temperatures. We performed biochemical and adenylic nucleotide analyses at specific
102 periods of development to identify critical energetic windows during the early larval
103 development in this species.

104

105 **MATERIAL AND METHODS**

106 **Ethics Statement**

107 Experimental protocols and procedures used for fish maintenance and sampling were
108 ethically reviewed and approved by the Aquaculture Program Animal Welfare Committee
109 of Centro de Investigaciones Biológicas del Noroeste S.C., La Paz, Baja California Sur,
110 Mexico (approval number CIBNOR-CEI-2022-01)

111

112 **Broodstock and embryo management**

113 The experimental conditions and procedures were the same as in a previous study
114 (Pacheco-Carlón et al., 2021). Briefly, wild broodstock of longfin yellowtail consisted of
115 20 females and 20 males, weighing around 20 kg each and held in two 40-ton tanks in an
116 experimental pilot hatchery facility at our research center in La Paz, Mexico (24°08′32″ N;
117 110°18′39″ W). Broodstock had been reproductively active for the last four years,
118 maintained at a constant temperature of ~26°C, salinity of 36-38 psu, and under artificial
119 manipulation of natural photoperiod for this species. Spontaneous spawning occurred twice

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120 to three times per week in each tank between 8 and 10 a.m. Buoyant eggs were obtained 4
121 h after fertilization at the early blastula period. The total volume of eggs was assessed after
122 density setting in a 10-mL graduated cylinder. Then, the egg count was calculated from a
123 previously established ratio of 755 ± 8 eggs/mL (Pacheco-Carlón et al., 2021). Buoyant
124 eggs were incubated with dissolved oxygen levels between 6.0 and 6.8 mg L^{-1} (>80 %
125 saturation), measured every hour, pH 7.8 – 8.0, measured twice a day, and constant light
126 (~800 lux). Each unit had air diffuser stones to maintain eggs and larvae gently in
127 suspension and homogeneously distributed and with a daily 30–50% water exchange.
128 Seawater temperature in the experimental units was measured every 10 min and maintained
129 (± 0.2°C) with one or three 250W-heaters with thermostats and air conditioning in the
130 laboratory. Eggs were then constantly monitored for embryo development and hatching
131 occurrence using a 4x stereomicroscope (Leica, amscope, USA).

132

133 **Experimental design and sampling**

134 *Description of Trial #1 at constant temperatures and Trial #2 at oscillating temperatures*.
135 The experimental design was the same as previously reported (Pacheco-Carlón et al.,
136 2021). In summary, eggs from a first spawning (Trial #1) were exposed to six constant
137 temperatures (20, 22, 24, 26, 28 and 30°C) decreasing or increasing it from 26°C at a rate
138 of 2.0°C h^{-1}) until hatching, using three 18-L containers inside a 150-L bath-container per
139 temperature, at a stocking density of 700 eggs L^{-1}. Eggs from a second spawning (Trial #2)
140 were subjected to two oscillating temperature treatments and one control treatment at a
141 constant temperature, using three 150-L bath-containers per treatment at a lower stocking
142 density of 200 egg L^{-1}, because of a smaller spawn volume available. The first oscillating

143 temperature treatment (decreasing-starting 'DS') started 4 h post-fertilization at 25°C,
144 decreased to 21°C at a rate of 0.5°C h^{-1} during 8 h, then increased to 29°C (1°C h^{-1} during
145 8 h), and then returned to 25°C (0.5°C h^{-1} during 8 h) until hatching. Conversely, the second
146 oscillating temperature treatment (increasing-starting 'IS') started 4 h post-fertilization at
147 25°C, increased to 29°C (0.5°C h^{-1} during 8 h), then decreased to 21°C (1°C h^{-1} during 8
148 h), and then returned to 25°C (0.5°C h^{-1} during 8 h), until hatching. The third treatment was
149 maintained at a constant temperature of 25°C (Control). In this way, gastrulation (*i.e.*,
150 blastula, optic vesicles, and neurula) and organogenesis (*i.e.*, pre-hatched larvae) had
151 different temperature regimes during development (Fig. 1), although the mean temperature
152 (25°C) was the same for all treatments.

153

154 *Embryonic developmental periods*. Samples of eggs, embryos and larvae (~0.5 g per
155 replicate; ~700 eggs/embryos or 1400 larvae) were obtained at different key developmental
156 periods (Kimmel et al., 1995) identified and monitored using a 4x stereomicroscope (Leica,
157 amscope, USA). Five periods of lecithotrophic development were sampled when 100% of
158 the specimens represented a particular morphology, whereas the hatch period was
159 considered when at least 50% of free larvae occurred: 1) eggs at the blastula period (BLA)
160 4 h post-fertilization, 2) the embryo showing optic vesicles (OV) at the early neurula
161 period, 3) the advanced neurula period (NE), when embryo had body myomeres and an
162 apple-shaped yolk sac, 4) pre-hatch period (PRE), when embryo showed head and tailbud
163 fully developed and 5) larvae immediately after hatching (HATCH), (Fig. 1) (see Pacheco-
164 Carlón et al., 2021 for embryo developmental periods). In both trials, we collected the eggs
165 from the broodstock tanks at the blastula period (BLA), and therefore, only one initial

166 biochemical reference datum was obtained (not shown). In trial #2, we skipped sampling
167 for biochemical analysis at the pre-hatch period (PRE) because of insufficient larvae,
168 prioritizing the hatching period (HATCH).

169

170 *Sample collection and storage*. Buoyant embryos and larvae were drained onto a 100 µm
171 mesh, the excess water was dried with absorbent paper, scooped, weighed, using an
172 analytical balance with a precision of 0.1 mg (Ohaus, A&D HR60, USA), immediately
173 plunged into liquid nitrogen, and finally stored at -80°C until analysis. To determine
174 biochemical and adenylic nucleotide contents per individual, we counted the eggs,
175 embryos, or larvae in a small parallel sub-sample (~30 mg) and then extrapolated to the
176 number of individuals inside the sample analyzed. Data were reported on a weight or mole
177 basis per individual, because it represents a more accurate way to describe quantitative
178 changes through development in which embryo/larval weight varies itself and generally
179 decreased according to development (Finn et al., 1995a; Finn et al., 1996; Rønnestad et al.,
180 1998; Podrabsky and Hand, 1999; Hilton et al., 2008).

181

182 *Oil droplet measurement.* Samples of eggs and larvae (~20 individuals per replicate) were
183 placed with a small drop of water onto a slide without cover glass and immediately
184 photographed using an Olympus camera attached to a compound light microscope CX-31
185 (Olympus, Japan). The oil drop diameters (mm) were directly measured in images
186 previously calibrated in the microscope using a stage micrometer slide (100 x 0.01 = 1 mm)
187 and analyzed using ImageJ software. The oil droplet volume (ODV, mm^3) was calculated
188 as $ODV=[(4/3\pi) * r^3]$ (Avila and Juario, 1987), where 'r' is the radius of the oil droplet. In

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189 Trial #1, the oil droplet volume was obtained at all developmental periods, whereas in Trial
190 #2 it was obtained only at blastula (BLA), pre-hatch (PRE), and hatching (HATCH)
191 periods.

192

193 *Biochemical analysis.* Frozen samples of eggs, embryos and larvae were lyophilized, then
194 pulverized using a homogenizer (MPI, Fast Prep-24, CA-USA), and then re-hydrated with
195 0.5 mL of distilled water per 10 mg of dry sample and homogenized again. For total protein
196 quantification, the crude homogenate was digested in NaOH 0.1N for 120 min, then reacted
197 with bicinchoninic acid at 60°C for 15 min (Fujimoto et al., 1985). The sample was
198 quantified using a protein-dye reagent and bovine serum albumin as a standard (Sigma-
199 Aldrich, Merck, St Louis, MO). Total lipids were determined by the sulphophosphovanillin
200 method (Barnes and Blackstock, 1973). Sulfuric acid was added to the homogenate and
201 heated at 90°C for 10 min, then reacted with phosphovanillin 0.2% in 80% H_2SO_4. For total
202 carbohydrates quantification, the proteins in the homogenate were precipitated using 20%
203 trichloroacetic acid (TCA) and the supernatant was reacted with 0.1% anthrone in 72%
204 H_2SO_4 (Roe et al. 1961). Triacylglicerides were determined from the enzymatic hydrolysis
205 with lipases according to Koditschek and Umbreit (1969), using an enzymatic colorimetric
206 test (kit de RANDOX, TR 1697). The dry mass of eggs, embryos, and larvae was obtained
207 by leaving samples at 60°C for 24 h in an oven (VWR, USA).

208

209 *Adenylic nucleotides.* Frozen samples of eggs, embryos and larvae were ground to a fine
210 powder with a ball mill mixer (MM400, Retsh, Germany) precooled with liquid nitrogen.
211 Nucleotides within grounded samples (100 mg) were extracted and processed according to

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212 the method of Moal et al. (1989), with modifications described by Robles-Romo et al.
213 (2016). Acidic extracts (500 µl) were neutralized with a mixture of dichloromethane and
214 trioctylamine (5:1 v/v), after which they were passed through a 0.2 µm filter and then
215 maintained at -80°C until further analysis. The nucleotides were separated by ion-pairing
216 reversed-phase HPLC (model 1100, Agilent Technologies, Palo Alto, CA) with a
217 Hyperclone ODS C18 column (150 × 4.6 mm, 3 µm particle size, Phenomenex, Torrance,
218 CA) connected to a C18 guard column (40 × 3 mm; Phenomenex, Torrence, CA).
219 Separation was carried out in a mobile phase consisting of 0.15 M sodium phosphate
220 monobasic (H_2NaO_4P), 3 mM tetrabutylammonium (Sigma-Aldrich, Merk, St. Louis, MO)
221 and 8% methanol at pH 6.0, which was adjusted with 5N NaOH. Nucleotide signals were
222 detected at 254 nm at 0.8 mL min^{-1} for 22 min. Nucleotide identification was performed
223 using a mixture of standards of adenosine triphosphate (ATP), adenosine diphosphate
224 (ADP), adenosine monophosphate (AMP) and inosine monophosphate (IMP) (Sigma-
225 Aldrich, Merck, St. Louis, MO) at known concentrations. The adenylate energy charge
226 (AEC) was estimated according to the method of Atkinson (1968) as follows:
227 [(ATP+0.5ADP)/(ATP+ADP+AMP)]. The total adenine nucleotide (TAN) was also
228 calculated as the sum of ATP, ADP and AMP.

229

230 *Statistical analysis.* Data were expressed as mean ± Standard Deviation (SD). All variables
231 were analyzed for normality (Bartlett test) and homoscedasticity (Levene test). Two-way
232 ANOVA was used to test the effects of period and temperature regime (constant and
233 oscillating) on biochemical and nucleotide composition. When a significant interaction was
234 observed, individual means within each factor were compared using Tukey´s test.

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235 Otherwise, global means within each factor (period and/or temperature) regardless of the
236 influence of the other factor were compared and indicated in figures with capital letters for
237 developmental periods or in the text for temperatures. All the statistics and graphics were
238 analyzed by Statistica version 8.0 (StatSoft, Tulsa, OK). In all cases, statistical significance
239 was accepted at $P < 0.05$.

240

241 **RESULTS**

242 *Effects of constant temperatures*

243 In Trial #1, the two-way ANOVA showed that developmental period had a much larger
244 influence on dry mass (DM) of embryos compared to the effects of temperature. The DM
245 of embryos showed a significant difference related to the developmental period only ($P <$
246 0.01), remaining stable during embryogenesis (OV, NE and PRE) with values of ~64
247 µg/ind, and then, there was a significant decrease to 44.6 µg/ind at the HATCH period (Fig.
248 2A). The protein content in embryos showed significant differences linked to the
249 developmental period ($P < 0.01$) and constant temperatures ($P < 0.05$). Comparisons of
250 global means by period showed that embryos maintained steady levels of proteins during
251 embryogenesis (~23 µg/ind), followed by a significant decrease to 16.7 ± 0.6 µg/ind at the
252 HATCH period (Fig. 2B). On the other hand, the global means of protein content in
253 embryos at 28°C (24.2 ± 5.7 µg/ind), followed by 30°C (22.8 ± 3.7 µg/ind) were
254 significantly higher than the other constant temperatures (20.7 ± 3.1, 20.9 ± 5.0, 21.2 ± 3.9
255 and 21.9 ± 4.4 µg/ind, at 20, 22, 24, and 26°C, respectively). The total lipidic content in
256 embryos was significantly influenced by the developmental period ($P < 0.05$), where global
257 means comparisons indicated a significant increase from the OV (12.8 ± 0.4 µg/ind) to the

258 NE (15.0 ± 0.6 μg/ind) periods, whereas the PRE and HATCH periods showed
259 intermediate values (Fig. 2C). The carbohydrate content in embryos during development
260 was negligible (~0.33 μg/ind) in comparison to lipids and proteins and without any main
261 effect or interaction between the developmental period and constant temperatures (Fig.
262 2D). The triacylglicerides (TAG) content had significant differences between
263 developmental periods ($P < 0.01$), with a progressive and significant decrease during
264 embryogenesis, leaving the hatched larvae with very low levels of TAG (Fig. 2E).
265 Additionally, there was a significant interaction between the developmental period and
266 constant temperatures ($P < 0.01$). However, this effect was not consistent for all
267 temperatures during the first three embryonic periods (OV, NE and PRE). There was a
268 significant decrease from the OV to the NE periods at 20, 28 and 30°C, and from the NE
269 to the PRE periods at 20 and 22°C (Fig. 2E). The oil droplet volume (ODV) was influenced
270 by the developmental period, constant temperatures and their interaction ($P < 0.01$). The
271 ODV significantly increased from the OV (3.6×10^{-3} mm^3) to the PRE (4×10^{-3} mm^3)
272 periods and then, it significantly decreased to 2.6×10^{-3} mm^3 at the HATCH period.
273 Changes before hatching were variable depending on temperature, whereas the decrease at
274 hatching was evident for all temperatures. Larvae at hatching had a higher ODV at 20°C
275 than at 24, 26 and 30°C, with intermediate values at 22 and 28°C (Fig. 2F).

276 The ATP content was significantly dependent on the developmental period ($P < 0.01$),
277 showing a progressive and significant increase from the OV (0.14 ± 0.0 nmol/ind) to the
278 HATCH (0.21± 0.01 nmol/ind) periods (Fig. 3A). The ADP content was also significantly
279 influenced by the developmental period ($P < 0.01$), with a significant decrease from the
280 OV (0.084 ± 0.002 nmol/ind) to the PRE (0.068± 0.001 nmol/ind) periods, followed by a

281 significant increase at the HATCH period (0.102 ± 0.005 nmol/ind) (Fig. 3B). Additionally,
282 there were significant effects of constant temperatures and the interaction between
283 development period and temperature (P < 0.05). Indeed, ADP content did not increase at
284 the same rate in hatched larvae at 26 and 30°C regimes compared to the other temperatures
285 (Fig. 3B). The developmental period had a significant effect on the AMP content (P <
286 0.01), showing a stable level content during the early embryogenesis, followed by a
287 significant increase from the PRE (0.004 ± 0.00 nmol/ind) to the HATCH (0.015 ± 0.001
288 nmol/ind) periods (Fig. 3C). The AEC was maintained at high values near 0.8 that
289 increased significantly from the OV (0.79 ± 0.0) to the PRE (0.86 ± 0.00) periods, and then
290 decreased at the HATCH period, reaching a similar value as in the OV period (Fig. 3D).
291 The IMP content was significantly influenced by the developmental period (P < 0.01), with
292 a first increase at the PRE period (0.12 ± 0.01 nmol/ind), and a further 2.6-fold increase at
293 the HATCH period (0.32 ± 0.02 nmol/ind) (Fig. 3E). Additionally, the interaction was also
294 significant (P < 0.05), as the increase at hatching was less pronounced at 26 and 30°C (Fig.
295 3E). The developmental period had a significant effect on total adenine nucleotide (TAN)
296 content only (P < 0.01), showing a progressive increase from the OV (0.23 ± 0.0 nmol/ind)
297 to the HATCH (0.33 ± 0.02 nmol/ind) periods (Fig. 3F).

298

299 *Effects of oscillating temperatures*

300 In Trial #2, as in Trial #1, the two-way ANOVA showed that developmental period had a
301 much larger influence on DM of embryos compared to the effects of temperature. The DM
302 and protein contents were significantly influenced by the developmental period (P < 0.05).
303 Global means indicated that embryos significantly gained weight at the NE period (68.2 ±

304 4.2 µg/ind), compared to the OV period (50.5 ± 3.0 µg/ind), and then significantly
305 decreased at the HATCH period (49.1 ± 3.3 µg/ind) (Fig. 4A). A similar trend was observed
306 for protein levels with a significant decrease at the HATCH period (21.7 ± 2.4 µg/ind),
307 although without a significant difference between the OV (31.5 ± 2.4 µg/ind) and the NE
308 (35.6 ± 4.0 µg/ind) periods (Fig. 4B). The developmental period showed a significant effect
309 on the total lipidic content ($P < 0.05$), with an increase from the OV (10.2 ± 0.7 µg/ind) to
310 the NE (16.0 ± 1.5 µg/ind) periods, followed by a non-significant decrease at the HATCH
311 period (12.8 ± 0.9 µg/ind) (Fig. 4C). The total carbohydrate content was also negligible
312 compared to lipids and proteins, but significantly influenced by the developmental period
313 and oscillating temperatures ($P < 0.05$). The embryos significantly increased their content
314 from the NE (0.12 ± 0.02 µg/ind) to the HATCH (0.21 ± 0.03 µg/ind) periods (Fig. 4D).
315 On the other hand, there was a significantly higher level of carbohydrates at the HATCH
316 period in the IS (0.19 ± 0.03 µg/ind) than in the DS (0.09 ± 0.02 µg/ind) oscillating
317 treatments, whereas the control group (at constant 25°C) had intermediate values (0.16 ±
318 0.02 µg/ind) (Fig. 4D). The TAG content showed a significant effect linked to
319 developmental period ($P < 0.01$), with an increase from the OV (5.8 ± 0.4 µg/ind) to the
320 NE (7.5 ± 0.6 µg/ind) periods, followed by a significant decrease at the HATCH period
321 (1.21 ± 0.1 µg/ind) (Fig. 4E). The ODV was influenced by a significant interaction ($P <$
322 0.01), with a significant increase from the PRE (3.5×10^{-3} mm^3) to the HATCH ($4.4 \times 10^{-}$
323 ^{3} mm^3) periods only in the control treatment, at a constant temperature of 25°C. In contrast,
324 there was a significant decrease of ODV in the IS oscillating regime from the PRE ($4.3 \times$
325 10^{-3} mm^3) to the HATCH (3.7×10^{-3} mm^3) periods. The DS oscillation regime maintained
326 similar values at these periods (Fig. 4F). All adenylic nucleotides (ATP, ADP, AMP), as

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327 well as IMP and TAN, showed a significant effect linked to developmental period ($P <$
328 0.05). The ATP content showed a progressive and significant increase from the OV (0.10
329 ± 0.01 nmol/ind) to the HATCH (0.33 ± 0.04 nmol/ind) periods (Fig. 5A). The ADP, AMP,
330 IMP and TAN contents maintained low levels during the OV and the NE periods (~0.060,
331 ~0.004, ~0.04 and ~0.20 nmol/ind, respectively) and then, all of them showed a significant
332 increase at the HATCH period (0.127 ± 0.023, 0.024 ± 0.006, 0.48 ± 0.07 and 0.48 ± 0.06
333 nmol/ind, respectively) (Fig. 5). Although not significant, there was a tendency in the DS
334 oscillating treatment to present lower levels of all nucleotides in hatched larvae compared
335 to the IS oscillating and the control treatments (Fig 5A, B, C). Finally, the AEC was
336 maintained at high values above 0.8 during embryogenesis and hatching without any
337 changes related to the developmental period, oscillatory temperature regimes (IS and DS
338 treatments), and the control at a constant temperature of 25°C (Fig. 5D).

339

340 **DISCUSSION**

341 *Biochemical changes during embryo development*

342 During embryonic development, the dry mass (DM) of the egg can decrease (Red sea
343 bream *Pagrus major,* Seoka et al., 1997), increase (Cobia *Rachycentron canadum,* Huang
344 et al., 2021), or be maintained relatively constant (Turbot *Scophthalmus maximus,* Finn et
345 al., 1996). The latter was the case for *Seriola rivoliana* eggs during embryo development,
346 with a 31% average decrease at hatching, calculated from PRE to HATCH periods
347 (Trial#1). Such decrease corresponded mainly to the loss of chorion (protein envelope) and
348 associated perivitelline colloids, although it was greater than the 17-19% obtained in
349 Turbot *Scophthalmus maximus* (Finn et al., 1996). Similarly, the magnitude of the isolated

350 chorion in bigger eggs like Cod *Gadus morhua* (Solberg and Tilseth, 1984) and Halibut
351 *Hippoglossus hippoglossus* (Finn et al., 1991) resulted in chorion weights equivalent to 17
352 and 11% of the egg DM, respectively. It seems likely that the drop of dry weight in hatched
353 larvae can be further explained by the loss of energy reserves due to such event, probably
354 associated with chorion breakdown (Garcia-Guerrero et al., 2003; Wen et al., 2013),
355 although in the trout it was concluded that both aerobic and anaerobic energetic cost of
356 chorion disposal was minimal (Ninness et al., 2006a; 2006b).

357 The rate of utilization of different energy substrates during embryonic and larval
358 development of fishes has been widely studied (Vetter, 1983; Finn et al., 1995a; 1995b;
359 1996; Rønnestad et al., 1998; Jaroszewska and Dabrowski, 2011). Embryos use
360 carbohydrates during blastomere divisions and then are mainly supported by free amino
361 acids, with secondary participation of phosphatidyl choline and wax esters. During and
362 after hatching, an initial use of wax esters, triacylglicerides, and remaining free amino acids
363 predominates, whereas protein catabolism gradually increases until first feeding (Finn et
364 al., l996). Our study showed that protein did not change from OV to PRE periods over
365 embryo development, whereas the drop in protein content at hatching would also be
366 associated with the loss of chorion, as isolated chorions of halibut *Hippoglossus*
367 *hippoglossus* eggs contained 84% of the DM as protein (Finn et al., 1991).

368 Total lipids slightly increased at the neurula period in both trials, as observed in eggs from
369 lake whitefish *Coreogonus clupeaformis* (Mueller et al., 2017) and cobia *Rachycentron*
370 *canadum* (Huang et al., 2021), indicating that specific egg lipid components were used as
371 energy sources. Neutral lipids, specifically wax esters and triacylglicerides (TAG), are
372 indeed the main fuels during embryogenesis and hatching (Finn et al., 1996; Rønnestad et

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373 al., 1998). TAG levels slightly decreased during embryogenesis and then abruptly
374 collapsed at hatching in both trials. Therefore, the increase in total lipids between OV and
375 NE corresponded more likely to an increase of phospholipids (PLs), as seen in wild
376 silverside *Chirostoma estor estor*, suggesting that PLs were synthesized from TAG fatty
377 acids (Palacios et al., 2007). Lipids in pelagic eggs could be present in two distinct forms:
378 the yolk lipids and lipids present in the oil droplet (Silversand et al., 1996). Despite their
379 importance, few studies present results of changes in the size or composition of the oil
380 droplet during embryogenesis (Finn et al., 1996; Santamaría-Miranda et al., 2021). In
381 general, the oil droplet is composed of non-polar lipids (triacylglicerides, cholesterol esters,
382 and wax esters) (Silversand et al., 1996; Wiegand, 1996; Finn et al., 1996). Although the
383 exact composition of the oil droplet was not measured in embryos of *Seriola rivoliana*, the
384 decrease in TAG levels between prehatching embryos and hatched larvae (83% in Trial #1)
385 did not fully match the decrease in oil droplet volume (35% in Trial #1). The above
386 suggested that TAG were preferentially used over other components of the oil droplet
387 because of the high energy demand required for hatching, whether it was related to the
388 chorion breakdown/disposal or not.

389 Content of individual adenylic nucleotides and the resulting AEC through embryo
390 development reflect the ability to adjust cellular energy derived from the oxidation of
391 different reserves to the energy consumed for different processes occurring during
392 development (energy homeostasis). Therefore, the overall constancy of AEC observed in
393 this work would reflect an optimal energy balance in embryos, except at hatching in Trial
394 #1, as discussed below. Variable results were obtained in previous studies with an increase
395 in AEC or ATP (the main determinant of AEC) in medaka *Oryzias latipes* (Pincetich et al.,

396 2005) and Atlantic cod *Gadus morhua* (Jung et al., 2012), constant values in gilthead
397 seabream *Sparus aurata* (Lahnsteiner and Patarnello, 2003) and a decrease in red drum
398 *Sciaenops ocellata* (Vetter et al., 1983). A possible explanation for such differences was
399 attributed to intraspecific differences in the duration of development (Lahnsteiner and
400 Patarnello, 2003; Jung et al., 2012), with a decrease in AEC and ATP in species exhibiting
401 a short incubation period (less than 24 h), such as the red drum (Vetter et al., 1983) and no
402 change or an increase in species with longer incubation periods (4.5 to 8 days), such as
403 medaka, cod, and seabream (Lahnsteiner and Patarnello, 2003; Pincetich et al., 2005; Jung
404 et al., 2012). Time to hatching in *Seriola rivoliana* eggs ranged from 22 to 52 hours,
405 depending on incubation temperatures (Pacheco-Carlón et al., 2021), and could be
406 considered more closely related to a short incubation period, with an overall constancy of
407 AEC. Another study suggested that different patterns of ATP and AEC variation in trout
408 *Salmo gairdneri* compared to loach *Misgurnus fossilis* could be related to different
409 developmental temperatures between species (Boulekbache 1981). However, we did not
410 observe such an effect at different temperature regimes in *Seriola rivoliana*, as discussed
411 below. The denominator used for biochemical concentrations could explain ATP or AEC
412 differences in embryos and larvae. Nucleotides are expressed on a molar basis per wet or
413 dry weight in all the previous studies cited. However, if the embryo´s weight decreases, it
414 may skew results reported on the basis of weight.

415 AEC slightly decreased at hatch from 0.86 to 0.80 in Trial #1, but not in Trial #2 (although
416 no data were obtained in pre-hatch). Nevertheless, such a decrease was mainly attributed
417 to increased ADP and AMP levels, indicating a higher energy requirement during hatching,
418 with higher use of ATP. An increase in IMP levels was observed at pre-hatch (Trial #1)

419 and, more importantly, after hatching (both trials). It is well known that IMP is the primary
420 metabolite of adenylic nucleotide degradation, occurring mainly postmortem (Tejada,
421 2009) but also during intense energy consumption (Wang et al., 1994; Robles-Romo et al.,
422 2016). In such conditions, ATP is restored from ADP via adenylate kinase with an
423 accumulation of AMP that is transformed to IMP by AMP deaminase, avoiding an
424 excessive accumulation of AMP that would disrupt cellular energy balance (Atkinson,
425 1977). Such IMP increase was also observed in fish embryogenesis with a concomitant
426 decrease in ATP and total adenylic nucleotides (TAN) (Vetter et al., 1983). In contrast, in
427 the present study, TAN increased in both trials, suggesting that even if IMP was involved
428 in such cellular adenylic balance, *de novo* synthesis of adenylic nucleotides was also
429 occurring. Indeed, IMP is also involved in purine synthesis (Zalkin and Dickson, 1992),
430 reflecting active DNA synthesis occurring during embryogenesis (Podrabsky and Hand,
431 1999). More particularly, purine synthesis is importantly involved in pigmentation and
432 ocular formation (Ng et al., 2009) that occurs prior to hatching in *Seriola rivoliana*
433 (Pacheco-Carlón et al., 2021).
434
435 *Effect of temperature*
436 The temperature range in which normal embryo development and hatching occur has been
437 established for several fish species (Scoppettone et al., 1993; Kupren et al., 2011;
438 Puvanendran et al., 2015; Imsland et al., 2019; Viader-Guerrero et al., 2021). From the
439 same spawns, we observed smaller hatched *S. rivoliana* larvae with increasing temperature
440 from 20 to 30°C without a clear pattern in yolk sac volume (Pacheco-Carlón et al., 2021).
441 In Trial #1, the biochemical and physiological data showed that only the oil droplet volume

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442 and TAG were affected by constant temperatures alone or by the interaction between
443 temperature and developmental period. For TAG, the quantitative decrease between NE to
444 PRE and then to HATCH periods was more accentuated at lower temperatures (20-24°C),
445 which could be related to a longer duration of development and larger larvae (Pacheco-
446 Carlón et al., 2021). In contrast to TAG, the oil droplet appears to be spared during hatching
447 at low temperatures (*e.g.*, 20°C), for which a bigger oil droplet in hatched larvae was
448 observed compared to upper temperatures (26-30°C). Taken together, these results suggest
449 that other neutral lipids of the oil droplet (*e.g.*, wax esters) were spared over TAG,
450 especially at the lowest temperatures. For *S. rivoliana*, we can expect bigger hatchlings
451 obtained at lower temperatures (22-24°C) (Pacheco-Carlón et al., 2021) and with higher
452 levels of reserves (total lipids and oil droplet) (this study) than those observed at warmer
453 temperatures (26-30°C). It seems that a delayed larval development at low temperatures
454 would benefit of a higher initial availability of lipids in the oil droplet after hatching in *S.*
455 *rivoliana*.

456 The effect of temperature on nucleotide levels was principally observed at hatching with a
457 higher accumulation of ADP, AMP, TAN, and IMP in the low-temperature range.
458 Conversely, the decline of AEC at this period was slightly more pronounced at these low
459 temperatures. Such results point to a higher absolute energy use linked to a longer duration
460 of embryogenesis with more tissue built (bigger hatched larvae) at low temperatures.
461 However, it did not seriously compromise the overall availability of energy at hatching
462 (ATP and AEC), even when TAG levels were almost depleted.

463 The influence of shifts in temperature during embryo incubation has been examined in
464 previous studies to assess critical windows for development and to reflect possible natural

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465 or anthropogenic thermal fluctuations (Eme et al., 2015; Mueller et al., 2015; Lim et al.,
466 2017; Eme et al., 2018). These studies revealed that fluctuating temperatures resemble
467 more closely constant warm temperatures than cold ones when considering growth, oxygen
468 consumption, and yolk sac absorption (Eme et al., 2018). For practical purposes (*e.g.*,
469 aquaculture), we can suggest that an oscillating regime could have the advantages of a
470 particular temperature on certain traits without its possible detrimental effects on other
471 characteristics. Such "ideal larvae" would also depend on the particular temperature shifts
472 during critical windows of development. In a previous associated study, a greater larval
473 size at hatching was observed in the increasing starting oscillation (IS) and a smaller one
474 in the decreasing starting oscillation (DS) (Pacheco-Carlon et al., 2021). We suggested that
475 the ultimate temperature shift that lasted ~8 h was a window of higher embryo growth,
476 which was boosted at low temperature in the IS oscillation treatment from the advanced
477 neurula to the hatch periods. In the present study, few effects were observed at biochemical
478 level related to oscillating temperature regimes. Carbohydrate content fitted the same
479 pattern as larval size (Pacheco-Carlon et al., 2021), with the highest content in the IS group
480 and the lowest one in DS, a difference more accentuated with advanced development (NE
481 and HATCH periods), suggesting that it reflects mainly polysaccharide compounds. The
482 lower oil droplet volume in the IS regime at hatching was in accordance with increasing
483 temperatures before hatching as observed with constant higher temperatures in Trial #1.
484 Because no statistical differences were observed for adenylic nucleotides and the resulting
485 AEC and IMP, it was not possible to attribute variation in energy efficiency or availability
486 at hatching to different oscillating regimes.
487

488 *Concluding remarks*

489 During embryogenesis in longfin yellowtail *S. rivoliana*, the increase in energy levels
490 (ATP) supported the high energy demand required by the embryo, where energetic reserves
491 were regulated in response to the thermal environment, resulting in high larval viability at
492 any temperature regime. The high adenylic energy charge (AEC) values during
493 embryogenesis suggested an adequate energy balance. However, hatching was the most
494 critical period of development, where energy metabolism significantly changed, with a
495 decrease in AEC, protein, lipids and body mass, indicating a higher energy requirement.
496 Triacylglicerides (TAG) were preferentially used over the other components, suggesting
497 their pivotal role as an energy source during hatching and phospholipid synthesis. The
498 general lack of critical biochemical changes under different temperature regimes confirms
499 that this species has a high adaptive capacity in response to constant and fluctuating
500 temperatures. Oscillating temperatures may have physiological advantages without
501 detrimental energetic effects that will require further research.

502

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743 **FIGURE CAPTIONS**

744

745 **FIGURE 1.** Oscillatory temperature treatments (Trial #2) during embryogenesis in longfin
746 yellowtail *Seriola rivoliana*. Decreasing-starting (DS, grey line), increasing-starting (IS,
747 black line) and control at 25°C. Periods of development and approximate time of sampling
748 are indicated: blastula (BLA); optic vesicles (OV); neurula (NE); pre-hatch (PRE) (not
749 sampled in Trial #2); larvae at hatching (HATCH). (Modified from Pacheco-Carlón et al.,
750 2021).

751

752 **FIGURE 2.** Biochemical components (µg/individual) and oil droplet volume ($\times 10^{-3}$ mm^3)
753 (mean ± SD) in longfin yellowtail *Seriola rivoliana* embryos exposed to constant
754 temperature (20-30°C) (Trial #1). (A) dry mass, (B) total protein, (C) total lipids, (D)
755 carbohydrates, (E) triacylglicerides and (F) oil droplet volume. The two-way ANOVA,
756 developmental period (P) × constant temperature (CT) results, are inserted in the figure.
757 Following Tukey's post hoc test mean comparisons, the different capital letters indicate
758 significant differences among developmental periods. Only when the interaction was
759 statistically significant, the different lowercase letters represent significant differences
760 between means for the different P × CT combinations. N=3 samples (approx. 150 embryos
761 or 200 larvae/sample) for each temperature-period combination, obtained from 3 different
762 replicated tanks for each temperature.

763

764 **FIGURE 3.** Adenylic nucleotide values (nmol/individual) (mean ± SD) in longfin
765 yellowtail *Seriola rivoliana* embryos exposed to constant temperatures (20-30°C) (Trial

766 #1). (A) adenosine triphosphate (ATP), (B) adenosine diphosphate (ADP), (C) adenosine
767 monophosphate (AMP), (D) adenylic energy charge (AEC), (E) inosine monophosphate
768 (IMP) and (F) total adenine nucleotide (TAN). See Fig. 2 for statistics.
769
770 **FIGURE 4.** Biochemical components (µg/individual) and oil droplet volume ($\times10^{-3}$ mm^3)
771 (mean ± SD) in longfin yellowtail *Seriola rivoliana* embryos exposed to constant 25 °C
772 (control) and oscillating temperature treatments during incubation (21 °C ⇄29 °C) (Trial
773 #2). (IS) increasing starting, (DS) decreasing starting (see Fig 1). (A) dry mass, (B) protein,
774 (C) total lipids, (D) carbohydrates, (E) triacylglicerides and (F) oil droplet volume. The
775 two-way ANOVA, developmental period (P) × oscillating temperature (OT) results, are
776 inserted in the figure. Following Tukey's post hoc mean comparisons, the different capital
777 letters indicate significant differences among developmental periods. Only when the
778 interaction was statistically significant, the different lowercase letters represent significant
779 differences between means for the different P × OT combinations. N=3 samples (aprox 150
780 embryo or 200 larvae/sample) for each temperature regime -period combination, obtained
781 from 3 different replicated tanks for each temperature regime.
782
783 **FIGURE 5.** Adenylic nucleotide values (nmol/individual) (mean ± SD) in longfin
784 yellowtail *Seriola rivoliana* embryos exposed to constant 25 °C (control) and oscillating
785 temperature treatments during incubation (21 °C ⇄29 °C) (Trial #2). (IS) increasing
786 starting, (DS) decreasing starting (see Fig 1). (A) adenosine triphosphate (ATP), (B)
787 adenosine diphosphate (ADP), (C) adenosine monophosphate (AMP), (D) adenylic energy
788 charge (AEC), (E) inosine monophosphate (IMP) and (F) total adenine nucleotide (TAN).
789 See Fig. 4 for statistics.

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Declaration of interests

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

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

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