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THE INFLUENCE OF SEX, PARASITISM, AND ONTOGENY ON THE PHYSIOLOGICAL RESPONSE OF EUROPEAN EEL (*Anguilla anguilla*) TO AN ABIOTIC STRESSOR

Short title: *Interaction of biotic and abiotic factors on stress response of eels*

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What is already known:

The consequences of different biotic factors and their interaction on the physiological stress response of eels to abiotic stressors have long been assumed. Yet, very few studies have explored these relationships using empirical research. Such information is crucial to develop effective management practices needed to assist with the recovery of the European eel, currently classified as an Endangered species.

What this study adds:

This study revealed the importance of considering the role of biotic factors (in this case: sex, parasitism and ontogeny) acting together to influence the stress response of the European eel to abiotic stressors. To our knowledge this is the first physiological study that simultaneously examines these different biotic factors. Furthermore, this study is highly relevant as there is a paucity of information on the influence of biotic factors on the physiological response of the European eel and other fish species to different abiotic stressors.

ABSTRACT

Migration of adult European eel (*Anguilla anguilla*) from freshwater feeding grounds to oceanic spawning grounds is an energetically demanding process and is accompanied by dramatic physiological and behavioural changes. Humans have altered the aquatic environment (e.g. dams) and made an inherently challenging migration even more difficult; human activity is regarded as the primary driver of the collapse in eel populations. The neuroendocrine stress response is central in coping with these challenging conditions, yet, little is known about how various biotic factors such as sex, parasites, and ontogeny influence (singly and via interactions) the stress response of eel. In this study, mixed effect models and linear models were used to quantify the influence of sex, parasitism (*Anguillicola crassus*), life-stage (yellow and silver eels), and silvering stage on the stress response of eels when exposed to a standardized handling stressor. The physiological response of eels to a standardized abiotic stressor (netting confinement in air) was quantified through measurements of blood glucose and plasma cortisol. The relationships between biotic factors and the activity of gill Na^+/K^+ - ATPase was also examined. Analyses revealed that in some instances a biotic factor acted alone while in other cases several factors interacted to influence the stress response. Blood glucose concentrations increased following exposure to the standardized stressor and remained elevated after 4 hours. Variation in plasma cortisol concentrations following exposure to the stressor were found to be time-dependent, which was exacerbated by the life-stage and parasitism condition. Males and non-parasitized silver eels had the highest Na^+/K^+-ATPase activity. Silvering stage was strongly positively correlated with Na^+/K^+-ATPase activity in female eels. Collectively, these findings confirm that the factors mediating stress responsiveness in fish are complicated and aspects of inherent biotic variation cannot be ignored.

61 **Keywords:** silver eel, yellow eel, stress response, *Anguillicola crassus,* cortisol, glucose,

62 Na^+/K^+-ATPase activity

63 **INTRODUCTION**

64 In freshwater and marine ecosystems fish are often exposed to natural and anthropogenic
65 stressors (Arthington et al. 2016). To compensate for the challenge imposed by a stressor, fish
66 undergo a series of biochemical and physiological changes (i.e., the stress response; Wendelaar
67 Bonga 1997; Gorissen, and Flik 2017). The glucocorticoid stress response is an essential
68 mediator of allostasis that maintains stability (homeostasis) or facilitates adaptation to changing
69 conditions (McEwen and Wingfield 2003; Angelier 2013), therefore promoting the survival and
70 recovery of individuals (Sapolsky et al. 1999). The stress response is characterized by the
71 production and release of glucocorticoid steroid hormones (i.e., cortisol in fish) shortly after
72 the perception of the stressor (Axelrod and Reisine 1984). In the short term, this stress response
73 is adaptive, providing the fuel (i.e., glucose) needed to respond to a stressor (Mommsen et al.
74 1999; Barton 2002). However, if the stressor persists, the action of glucocorticoids can occur at
75 the expense of other life-history components through a reduction in the amount of energy
76 available for essential functions (Korte et al. 2005). In fish, stress can negatively affect growth,
77 health (immunocompetence), reproduction, and welfare, and ultimately result in mortality
78 (Schreck 1981, 2000; Barton 2002; Fuzzen et al. 2011).

79 For diadromous fish species, the transition from life in freshwater (FW) to seawater (SW)
80 is a very important and a challenging period usually characterized by high levels of mortality
81 (Bruijs et al. 2009; Piper et al. 2015). The European eel (*Anguilla anguilla*), a catadromous
82 species, undertakes an outward migration of ~5000–6000 km to spawning grounds in the
83 Sargasso Sea (van Ginneken et al. 2005; Aarestrup et al. 2009), which is known as the longest
84 spawning migration among all the species of eels (Aoyama 2009) and is performed without
85 feeding (Righton et al. 2012). Before migrating to SW eel's life is spent feeding in freshwater
86 (for up to 25 years) to store enough fat (>20% of the body mass; Tesch 2003) (yellow eel stage)
87 to fuel migration that may take many months (Righton et al. 2016), as well as, to provide

88 sufficient energy to produce offspring. After attaining an adequate lipid reserve, eels start lipid
89 mobilization (EELREP 2005; Trischitta et al. 2013) and sexual maturation, metamorphosing
90 into "silver eels". During this stage, eels stop feeding, and begin the long migration back to the
91 Sargasso Sea for spawning (Righton et al. 2012). Males (on average 40 cm) usually start their
92 migration in August while females (on average bigger than 40 cm) leave later, during October
93 and December (Tesch 2003).
94 Spawning migration of eels is a complex and energetically demanding process during
95 which eels are very vulnerable to natural and anthropogenic challenges that can impair their
96 migratory capacity as they transition from freshwater to saline water (Gollock et al. 2005,
97 Iversen et al. 2013, Trischitta et al. 2013, Wilson 2013). Durif et al. (2005) described five
98 different stages of the silvering process in female eels according to their physiological changes
99 as they prepare for their spawning migration: a growth phase (I and II) a pre-migration phase
100 (II) and two migration phases (IV and V). In part due to their catadromous lifestyle, European
101 eel populations have seen marked declines throughout their natural range in the past few
102 decades and are currently classified as Critically Endangered (Jacoby and Gollock 2014) and
103 listed under Appendix I-III of the Convention on International Trade in Endangered Species of
104 Wild Fauna and Flora (CITES 2013). Several factors are thought to have contributed to these
105 declines including barriers to migration, habitat loss, parasites (e.g. *Anguillicola crassus*),
106 disease, climate change, bioaccumulation of toxins, predation, changes in ocean currents and
107 overfishing (Dekker 2003; Knights 2003; Van Ginneken et al. 2005; Belpaire et al. 2009;
108 Geeraerts and Belpaire 2010; Durif et al. 2011; Kettle et al. 2011; Wahlberg et al. 2014). The
109 drastic decline of European eel populations has hastened the implementation of management
110 measures aimed at restoring stocks by preventing mortality during migration (European Union
111 implemented the Eel Recovery Plan 2007- Council Regulation No. 1100/2007/EC and the
112 International Council for the Exploitation of the Sea -ICES 2014).

113 Despite the extensive body of literature that has explored the stress response of fish in
114 general (reviewed in Schreck 2010; Pankhurst 2011), to our knowledge no studies have
115 specifically explored how biotic characteristics acting in concert may influence the stress
116 response and recovery in European eel, as analysed in this study. The main goal of this study
117 was to analyze how individual factors such as sex, parasitic load (non-parasitized and
118 parasitized with *A. crassus*), and ontogenetic phase (yellow, silver, and different silvering
119 stages) interact to influence the physiological response to a standardized handling and air
120 exposure stressor. To determine which biotic characteristics are associated with the stress
121 response, we used mixed effect and linear models to quantify the physiological responses of
122 eels. We measured blood parameters (i.e., plasma cortisol and body glucose) immediately
123 (baseline), 1 hour (stress response) and 4 hours (recovery period) post-exposure to the stressor.
124 We also tested for relationships between biotic factors and the activity of gill Na^+/K^+ - ATPase
125 given the important role of this gill enzyme in diadromous species. Moreover, plasma cortisol
126 is also associated with branchial Na^+/K^+-ATPase activity, which plays a central role in whole-
127 body osmoregulation (Towle 1981; Sancho et al 1997) such that stress has the potential to also
128 influence osmoregulatory processes.
129

130 **MATERIAL AND METHODS**

131 *Animals and experimental design*

132 European eels were caught during downstream migration between October and November
133 of 2014 in a trap located in River Gudenå at Vestbirk hydropower station, at a downstream trap
134 in Flade Sø and by electrofishing at Bygholm Å and Lake Stigsholm, Denmark. The eels (N=72,
135 mean total length (L_t) ± S.D. = 51.9 ± 8.3 cm, mean total weight (W_t) ± S.D. = 249.7 ± 127.3
136 g) were transported and held in three 8000L holding tanks (water temperature 12-15ºC) at the
137 National Institute of Aquatic Resources, Technical University of Denmark, in Silkeborg,

138 Denmark, until the experiments were carried out (holding time of between 5 and 9 days). To
139 minimize stress during holding and facilitate recovery from capture, transportation and
140 handling, shelter was provided for the eels. This shelter was comprised of 3.0 and 4.5 cm
141 diameter by 70 cm long PVC pipes that were placed in the holding tanks. These pipes also
142 limited the influence of removal of an individual for treatment on the remaining eels in the
143 holding tank since a single pipe could be removed without disturbing the other eels. Overall,
144 57 females eels (mean L_t ± S.D. = 54.9 ± 6.1 cm, mean W_t ± S.D. = 289.7 ± 111.9 g) and 15
145 males (mean L_t ± S.D. = 40.5 ± 3.4 cm, mean W_t ± S.D. = 105.13 ± 27.2 g), were tested. Each
146 eel received the same experimental treatment. First, an eel was removed from the holding tank
147 by netting a PVC pipe on either end and lifting it from the tank, with minimal disturbance. A
148 blood sample was then collected within 3-min of capture to act as baseline sample of plasma
149 cortisol and blood glucose (as per Lawrence et al. 2018). Next, the eel was exposed to a
150 standardized stressor in the form of a 10-min air exposure, before being moved into an
151 individual 80-L holding tank with 20-L of water. To measure the magnitude of the stress
152 response in each eel, blood samples were collected again at one and four hours after their
153 baseline sample. Eels were not anaesthetised during this procedure because it has been shown
154 to influence gill Na^+/K^+-ATPase activity (Toni et al. 2014) – another parameter measured in
155 this study (details provided in the *Plasma and Gill sample analysis* section) and would have
156 confounded our ability to measure the stress response. Anesthesia can influence the stress
157 response in a number of ways – both muting it and also serving as a stressor itself (there is a
158 significant metabolic demand associated with clearing anesthetics; Neiffer and Stamper 2009).
159 We acknowledge that the blood sampling at the 1 hr time point would have served as a stressor
160 that had the potential to influence the stress levels measured at the 4 hr time point but all fish
161 were handled similarly and this occurred during a period when the stress response was already
162 at its peak. Stress associated with sampling during the first blood sampling period was simply

163 part of the standardized stressor while stress associated with sampling during the final time
164 point was irrelevant given that no further sampling would occur. Blood sampling without
165 anaesthesia is relatively common in the study of stress physiology in wild fish (e.g., Cooke et
166 al., 2005) including studies that involve repeated sampling of individuals (e.g., Cook et al.
167 2012). To minimise disturbance of fish during blood sampling, this procedure was always
168 conducted by the same operator. Fish were euthanized via decapitation using a sharp knife. All
169 applicable international, national, and/or institutional guidelines for the care and use of animals
170 were followed. Animal care approval for this study falls under the Danish Animal Experiment
171 Inspectorate (licence number: 2013-15-2934-00808).

172

173 *Individual condition*

174 At the end of each experiment eels were sacrificed and measured for body mass, total length,
175 body width at maximum body depth, body height at maximum body depth, pectoral fin length
176 and horizontal and vertical eye diameters. These measurements were used to distinguish males
177 from females and to calculate three morphometric indices: eye index, fin index and Fulton's
178 condition factor (Durif et al. 2005; Bolger and Connolly 1989). These indices were used
179 together with the external morphological characteristics of silver-phase eel (presence of black
180 corpuscles in the lateral line; dark dorsal part of the body and lighter "silver" ventral region;
181 and snout shape and dark coloration of the extremities of the pectoral fins and tail), as selective
182 criteria to distinguish between the yellow and silver phases, as well as to determine the different
183 silvering stage (stage I to V; Pankhurst 1982; Durif et al. 2005). The swimbladder of each eel
184 was also removed and any *A. crassus* present in the swimbladder lumen were removed and
185 enumerated.

186

187 *Plasma and Gill sample analysis*

188 Blood samples were obtained by puncture of the caudal vasculature using pre-heparinised
189 (10 000 USP units/ml heparin sodium: Sandoz, Canada), needles (25 G 1/2”) and 1 ml syringes
190 (BD Plastipak, 1ml) and the blood was stored briefly in ice. The total sampling time never
191 exceeded 3 min. The volume of blood removed for each sample was approximately 0.2 ml.
192 After each blood sample was obtained, sub-samples were removed for immediate determination
193 of blood glucose concentrations using a glucose meter (Accuchek, Roche Diagnostics; Stoot et
194 al. 2014) and the remainder of the sample was centrifuged for 10 min at 4,000 RPM to separate
195 plasma from the blood cells. The aliquoted plasma was immediately frozen in liquid N_2 and
196 then stored frozen at –80°C for later analysis. Individual plasma cortisol concentrations (ng/mL)
197 were determined according to the radioimmunoassay procedure described in Pottinger and
198 Carrick (2001) with two minor adjustments. The antibody used in this study was IgG-F-2 rabbit
199 anti-cortisol (IgG Corp; Nashville, TN, USA) and tracer ([1,2,6,7]^{3}H-cortisol, 2.59 TBq/mmol;
200 Perkin-Elmer, U.K.) was added in a 25 μL aliquot of buffer at the same time as the antibody
201 was dispensed.

202 Measurement of gill Na^+/K^+-ATPase activity followed procedures outlined by McCormick
203 (1993). Gill filaments from the second right gill arch were removed from each eel, placed in a
204 tube containing ice-cold SEI buffer (300 mM sucrose, 20 mM Na_2EDTA, 50 mM imidazole,
205 pH 7.3) frozen in N_2 and stored at –80°C until analysed. Gill homogenates were centrifuged at
206 1000 g for 1 min and the supernatant was assayed for ATPase activity in the presence and
207 absence of 0.5 mM ouabain. Each assay was run in triplicate. Protein content was measured by
208 the Lowry (1951) method modified for a plate reader. The difference between the two
209 determined activities (with and without ouabain) was calculated as the Na^+/K^+-ATPase activity.
210

211 *Statistical Analysis*

212 Data were analysed for normality using the Shapiro-Wilcoxon test. To meet the normality
213 requirements of parametric analysis, cortisol and glucose data were log(x) transformed (log-
214 cortisol (logC) and log-glucose (logG), respectively.

215 Response variables logC and logG were fitted with linear mixed effects models (LME)
216 with individual fish as a random factor and time (baseline, 1 hour and 4 hours), sex (male or
217 female), life-stage (yellow or silver) and parasite condition (non-parasitized vs parasitized with
218 *A. crassus*) as fixed effects. Silvering stages (I to V) could not be compared independently due
219 to the small number of individual females in each stage; therefore, individuals were grouped in
220 three groups according to their similarities of development (after Durif et al. 2005). Group 1
221 included all the individuals belonging to the silvering stage I and II, group 2 had individuals in
222 stage III and group 3 had individuals in stage IV and V. To understand the effects of silvering
223 stage on logC and logG, a new LME model was run with silvering condition included as a fixed
224 effect and sex and stage (redundant factor) removed as possible predictors. Only females
225 (N=57: silver N=35; yellow N=22) were used in this analysis as the number of silver males was
226 very low for a statistical analysis (N=15: silver N=6; yellow N=9)

227 Linear models (LM) were used to assess the effect of sex, life-stage, parasite condition and
228 silvering stages on gill Na^+/K^+-ATPase activity. Data were analysed using the *nlme* function
229 implemented in the R statistical environment (package version 3.1-117, R core team ; Pinheiro
230 et al, 2017). To compare model fits objectively, and determine which was the most appropriate,
231 an information theoretic approach was performed to compare models using Akaike's
232 information criterion (AIC; Akaike 1974; Burnham and Anderson, 2002). Models were
233 validated by examining histograms of the normalized residuals, plotting the normalized
234 residuals against fitted values. The final models were refitted using maximum likelihood (ML).
235 Mean values are reported together with standard error (mean ± S.E) and results were considered
236 significant for $\alpha<0.05$.

237

238 **RESULTS**

239 *Parasitism*

240 Overall, 20 eels were parasitized with *A. crassus* . The number of parasites in the eels varied

241 between 1 and 11 individuals per specimen, and it was different according to the : sex (females:

242 N= 19, males N=1), life-stage (yellow eels N= 7, silver eels N=13) and silvering condition

243 (life stage I: N= 7, life stage II, N= 8, Life stage III: N= 4). The different number of parasites

244 in each silvering stage resulted in different levels of Glucose, Cortisol and Gill Na+/K+-ATPase

245 activity (Table 1).

246

247 *Glucose*

248 The final model for blood glucose (logG) contained time and parasite condition as the main

249 explanatory factors (logG~time*parasitism; AIC : -222.05, dF: 8). Blood glucose varied

250 significantly with time (Table 2), and increased from 4.5 ± 0.3 mmol/L (mean ± S.E) in

251 unstressed eels at 0 h to 7.6 ± 0.4 mmol/L at 1 hour after the stressor and to 10.2 ± 0.5 mmol/L

252 4 hours following the stressor (Fig. 1). Temporal variation of glucose was similar between

253 parasitized and non-parasitized eels, with parasitized eels exhibiting slightly higher overall

254 glucose levels (7.5 ± 0.4 mmol/L) than non-parasitized eels (7.1 ± 0.3 mmol/L; Fig. 1), although

255 this difference was not significant.

256 Variation of plasma glucose in female eels was best explained by a model that included

257 both time and the interaction of parasite condition with silvering (logG~

258 time+parasitism*silvering; AIC: -145.53, dF: 8). Plasma glucose levels at 1h (7.7 ± 0.5

259 mmol/L) and 4h (10.2 ± 0.6 mmol/L) after the stressor were significantly different from the

260 values in unstressed eels (4.5 ± 0.4 mmol/L) (Table 2). Parasitism and life-stage were also

261 important covariates in explaining the variation of plasma glucose in female eels, improving

262 the statistical model, nevertheless their effects were not statistically significant (Table 2).
263 Indeed, similar number of parasites in each silvering stage led to approximately the same values
264 on plasma glucose on eels. Although, minor, still, blood glucose increased the number of *A.*
265 *crassus* existent in each eel, in particularly in eels parasitized with more than 4 individuals.
266 (Table 1). Overall, mean glucose levels in non-parasitized female eels were lower (6.8 ± 0.4
267 mmol/L) than those in parasitized female eels (7.9 ± 0.4 mmol/L).
268

269 *Plasma cortisol*

270 Plasma cortisol levels varied significantly with time (Table 2) and were also dependent on
271 parasite condition and life stage of eels (logC~time*parasitism + parasitism*life-stage; AIC:
272 29.42, dF: 10). Mean plasma cortisol levels significantly increased in the first hour after the
273 stressor from 29.19 ± 4.0 ng/mL to 57.84 ± 3.48 ng/mL after which they decreased to levels
274 slightly higher than those in unstressed eels (37.73 ± 3.6 ng/mL) (Table 2). Although non-
275 parasitized eels exhibited higher levels of cortisol overall (48.6 ± 3.8 ng/mL) than parasitized
276 eels (33.1 ±2.4 ng/mL) (Table 2), net changes in variation was larger in parasitized eels (Fig.
277 2a). This was particularly evident in the first hour where mean plasma cortisol concentrations
278 rose significantly from baseline levels of 16.9 ± 2.0 ng/mL to 54.2 ± 3.2 ng/mL (Table 2, Fig.
279 2a). Overall, non-parasitized silver eels had higher plasma cortisol levels (58.6 ± 6.7 ng/mL)
280 when compared to non-parasitized yellow eels (39.15 ± 3.5 ng/mL). Nonetheless, parasitism
281 strongly influenced cortisol response in silver eels, which had the lowest levels of cortisol found
282 (30.7 ± 2.5 ng/mL) (Fig. 2b, Table 2).

283 In female eels, plasma cortisol concentrations were found to vary with silvering stage and
284 the interaction between time and parasitism (logC~silvering+time*parasitism; AIC : 36.06, dF:
285 10). Female eels belonging to the maximum silvering stage group exhibited higher levels of
286 plasma cortisol (58.02 ± 6.3 ng/mL), when compared to eels of the second (27.9 ± 2.3 ng/mL)

287 and first group (35.5 ± 3.3 ng/mL) (Table 2). Parasitized female eels exhibited the lowest levels
288 of plasma cortisol (32.3 ± 2.8 ng/mL) when compared to non-parasitized eels (49.1 ± 5.0
289 ng/mL). The variation of plasma cortisol in female parasitized eels was found to increase with
290 the number of *A. crassus* (Table 1). When parasitized with more than 4 individuals eels had an
291 increased on plasma cortisol levels. Nevertheless when in the last silvering stage, even a small
292 number *A. crassus* appears to elicit a strong increase of plasma cortisol.Variation in plasma
293 cortisol was also time dependent (Table 2); plasma cortisol significantly increased from 27.9 ±
294 5.0 ng/mL to 56.5 ± 4.1 ng/mL in the first hour following the stressor, decreasing to values
295 close to the baseline levels after 3h (38.6 ± 4.5 ng/mL) (Table 2). This temporal variation was
296 found to be related to the parasitism status of the individual (Table 2). After exposure to a
297 stressor, parasitized eels exhibited a stronger increase in cortisol levels when compared to non-
298 parasitized eels (Table 2). This variation was clearly evident in the first hour following
299 disturbance (non- parasitized eels: 61.9 ± 7.1 ng/mL, parasitized eels: 53.9 ± 3.5 ng/mL) (Table
300 2). However, by the 4h time point, plasma cortisol levels had recovered to near the levels seen
301 in non-parasitized eels (4h: 44.4 ± 8.0 ng/mL, baseline: 39.9 ± 9.7 ng/mL), but not in parasitized
302 eels (4h: 26.2 ± 3.0 ng/mL, baseline: 14.7 ± 2.2 ng/mL) (Table 2).
303
304 *Gill Na^+/K^+-ATPase activity*
305 Gill Na^+/K^+-ATPase activity varied between individuals of different sexes, life stages and
306 parasitisim levels (Na^+/K^+-ATPase activity~ sex + life-stage*parasitism; AIC: 293.97, dF: 6).
307 Males exhibited higher levels of Na^+/K^+-ATPase (8.71 ± 0.8 μmol ADP/mg protein/h) than
308 females (6.02 ± 0.5 μmol ADP/mg protein/h) (Table 2). Na^+/K^+-ATPase levels were found to
309 vary with the life-stage of eels, with the highest values found in silver eels (7.97 ± 0.6 μmol
310 ADP/mg protein/h) when compared to yellow eels (4.74 ± 0.5 μmol ADP/mg protein/h) (Fig.
311 3, Table 2). Within life stages the variation of Na^+/K^+-ATPase activity was conditioned by the

312 parasitism level, particularly in silver eels where non-parasitized individuals exhibited
313 sginificantly higher Na^+/K^+-ATPase activity (10.26 ± 0.8 µmol ADP/mg protein/h) than
314 parasitized silver eels (7.22 ± 0.5 µmol ADP/mg protein/h) (Fig. 3, Table 2).

315 In female eels, gill Na^+/K^+-ATPase activity increased through silvering stage (Table 2).
316 The highest values of Na^+/K^+-ATPase activity were found in the third silvering group (7.74 ±
317 0.8 µmol ADP/mg protein/h), decreasing in the second group (5.79 ± 0.6 µmol ADP/mg
318 protein/h) and were lowest in the first goup (4.02 ± 0.5 µmol ADP/mg protein/h). Gill Na^+/K^+-
319 ATPase activity in female parasitized eels increased with the number of *A.crassus,* in particular
320 in eels parasitized with more than 4 *A. crassus* (Table 1).

321

322 **DISCUSSION**

323 In this study we examined the effect of sex, parasite burden, and ontogeny, alone and in
324 combination, on the stress response and Na^+/K^+-ATPase activity of European eels when
325 exposed to a standardized handling stressor. To our knowledge, this is the first study to examine
326 the impact of the interaction of different biotic factors on the physiological response of eels.
327 The results of this study revealed a physiological response to our experimental handling stressor
328 with the extent of the response modulated by biotic factors. Interestingly, in some instances
329 biotic factors acted alone while in other cases several factors interacted to influence the
330 physiological response.

331 Eels subjected to the stressor exhibited significantly higher concentrations of glucose
332 throughout the 4 h duration of the study, with the most significant increase observed during the
333 first hour after disturbance. The prolonged elevation of glucose reflects a mobilisation of energy
334 to provide short-term support for immediate coping activities to promote survival. Parasitized
335 and non-parasitized eels showed similar levels of glucose, a result consistent with Gollock et
336 al. (2004) and their study on parasite-mediated stress responses to handling stressors in

337 European eel. Moreover, we observed that the number of *A. crassus* in female parasitized eels
338 led to slightly higher concentrations of blood glucose, but this was not significantly different
339 between the three silvering stages.

340 As expected, eels also exhibited a strong cortisol response to stress. Cortisol significantly
341 increased in the first hour after exposure to the stressor followed by a decrease in the next 3
342 hours. This is noteworthy given that we repeatedly sampled fish such that there would have
343 been some level of stress associated with blood sampling at the 1 hour time point. Despite that,
344 cortisol recovery was still evident at the 4 hour time point. When considering both males and
345 females, the temporal variation in cortisol was similar in both parasitized and non-parasitized
346 eels; parasitized eels exhibited a stronger response in terms of increment of cortisol when
347 compared to non-parasitized eels. This finding suggests that parasitic state plays an important
348 role in the stress response of eels. The similarity on the variation and levels of cortisol between
349 this study and Gollock et al. (2004), as well as, the fact that eels used in this study were also
350 wild and may have been infected by *A. crassus* for a long period of time, support the argument
351 of Gollock et al. (2004) that the results obtained can reflect an adaptation to the effects of
352 chronic parasitism. Moreover, Sures et al. (2001) found that there is a strong stress response of
353 eels to the larval and young adult stages of *A. crassus*, but no chronic response to older adults.
354 Although we have not analyzed the life stage of *A. crassus* infecting the tested specimens, it is
355 possible that the tested eels could have been in an early onset of infection. The environmental
356 characteristics of the system where eels lived (water temperature, water salinity) also played an
357 important role on the results obtained as it is known that the spread, extent and intensity of
358 infestation by *A. crassus* is dependent on water salinity and the age and size of the fish (Sures
359 et al. 2001; Lefebvre and Crivelli 2012). Differences in plasma cortisol levels between non-
360 parasitized and parasitized specimens was strongly evident on female silver eels, even if overall
361 there were no significant differences were found between the two life-stages. This evidence that

362 there is a synergistic influence of multiple stressors on the stress response. Female eels
363 categorized as being in the last stage of silvering (III) exhibited the highest levels of plasma
364 cortisol which may have some implications for reproductive function. Moreover, eels on the
365 third silvering stage were found to be more susceptible to the presence of parasites, as the
366 highest levels of plasma cortisol were found even when the number of parasites was low (<4
367 individuals). Parasitism on the last silvering stage may negatively influence migration and
368 reproduction of the eels.

369 High levels of cortisol for prolonged periods of time have been shown to play a role in
370 energy mobilization as result of its lypolytic effect increasing free fatty acid levels, reduce
371 growth rate by increasing the pituitary gonadropin, reduce immune function, and disrupt fish
372 reproduction function by depressing sex steroid levels (Huang et al. 1999). The implications of
373 high levels of cortisol for prolonged periods during exposure to chronic or frequent intermittent
374 acute stressors on eel reproduction are therefore potentially important. The morphological and
375 physiological transformation of yellow eels to the silver phase and the initiation of their
376 spawning migration is only triggered when the levels of lipids is >20% of the body mass (Palstra
377 et al. 2010; van den Thillart et al. 2009). As such, elevations in cortisol have the potential to
378 influence both maturation and spawning migrations. Cortisol is also known to be related to SW
379 adaptation of fish helping them to acclimate to a hyperosmotic environment (SW) by increasing
380 hypoosmo-regulatory capacity (Mommsen et al. 1999). Cortisol mediates SW-acclimation by
381 stimulating the gill chloride cell proliferation and Na^+/ K^+-ATPase activity ensuring the
382 transmembrane transfer of the cations Na^+ and K^+ and affecting the transepithelial movements
383 of cations in gills (Madsen 1990a; Sancho et al 1997; McCormick 1995). The stimulatory role
384 of cortisol on gill Na^+/K^+-ATPase activity in the American eel (*Anguilla rostrata*) was
385 previously shown by Butler et al. (1972), on their study of the effects of environmental salinity
386 and adrenocortical steroids on Na^+/ K^+-ATPase activity. Also, studies on salmonids showed

387 that gill Na^+/ K^+-ATPase activity responds positively to injections of cortisol in Atlantic
388 salmon, *Salmo salar* (Bisbal and Specker 1991), rainbow trout, *Oncorhynchus mykiss* (Madsen
389 1990a), and sea trout *Salmo trutta* (Madsen 1990b; Fontaínhas-Fernandes et al. 2003).

390 Gill Na^+/ K^+-ATPase activity was significantly higher in silver eels than in yellow eels.
391 This result was particularly evident in non-parasitized silver eels since parasitized silver eels
392 appeared to have suppressed Na^+/K^+ - ATPase activity. Despite the low values of Na^+/K^+ -
393 ATPase activity in female parasitized eels, it was observed that these values increased with
394 silvering stage as well as with the number of *A. crassus*. It is known that Na^+/ K^+-ATPase
395 activity plays a crucial role in the osmoregulation of eels, thus the suppression of such protein
396 will limit the success of eels in salt water and therefore compromise their migration,
397 reproduction and concomitant survival. Control and mitigation of the levels of *A. crassus* in
398 eels, in particularly on the latest stages of maturation of this species are critical and must be
399 developed. Such conservation measures will contribute to the reduction of the decline of the
400 European eel, currently classified as critically. Considering the well-known effects of cortisol
401 on Na^+/ K^+-ATPase activity (McCormick, 1995), the highest levels of this parameter on non-
402 parasitized silver eels may have been related to the high levels of cortisol found in these
403 specimens. Furthermore, non-parasitized yellow eels exhibited the lowest levels of gill Na^+/
404 K^+-ATPase activity. Once again, parasitism acting synergistically with other biotic factors
405 affect ion regulation via an indirect effect on gill Na^+/ K^+ regulation.

406 The highest levels of Na^+/K^+ - ATPase activity were found in males and can be a
407 consequence of different stages of sexual maturation achieved by the specimens. Considering
408 that males initiate their migration earlier than females (Palstra et al. 2007; 2010), and that the
409 experiments were carried out at the end of October through the beginning of November, our
410 findings may then be related with the fact that most males could have been in a more advanced
411 silvering stage than the females. Although expected, this is an interesting result as it indicates

412 that the success of spawning of males become more susceptible and/or compromised by
413 environmental conditions (e.g. parasite load) earlier than females. Gill Na^+/K^+ - ATPase activity
414 increased with silvering stage. Nevertheless, this variation was exacerbated in non-parasitized
415 eels, which exhibited an elevation of Gill Na^+/K^+ - ATPase activity between silvering stage 2
416 and 3. Again, this points toward the idea that parasitized eels can be adapted to deal with stress,
417 and therefore their response to stress would be less severe.

418

419 **CONCLUSIONS**

420 This paper documented a strong glucose and cortisol response of European eels to a holding
421 stressor (netting confinement in air) that was mediated by the interaction of several biotic
422 factors. Such biotic interactions were also found to play an important role in the variation of
423 Na^+/ K^+-ATPase activity. Because we assessed the role of multiple biotic factors
424 simultaneously we had the ability to test their influence alone and in combination which is a
425 robust approach relative to examining them individually (e.g., just parasite burden) which has
426 been the typical approach in the literature thus far. Indeed, we revealed that the stress response
427 of eels was found to differ between life stage, sex and parasitism condition, as well as, with the
428 number of parasites. Parasitism, mainly when acting together with other biotic stressors, plays
429 an important role in the physiological response of the eels to stressors and presumably has the
430 potential to influence the maturation, reproductive and osmoregulatory processes in this
431 species. Future studies that examine the influence of biotic factors acting alone and interacting
432 under different abiotic conditions are needed to better understand the role of stress in the
433 different life stages, sex, health conditions and other physiological characteristics of wild fish.

434

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444

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628

629 **FIGURE LEGENDS**

630

631 Figure 1: Variation of glucose on non- parasitized and parasitized eels through time (baseline,
632 1 and 4 hours) (mean ± standard error).

633

634 Figure 2: Variation of plasma cortisol on non- parasitized and parasitized eels: (a) among time
635 (baseline, 1 and 4 hours) (mean ± standard error) and (b) between life-stages (yellow and silver).
636 The solid black line represents the median (50th percentile) and the bottom and top box edges
637 are the 25th (Q1) and 75th (Q3) percentile, respectively. The bottom wisker is the
638 max(min(x),Q1-1.5*IQR) with IQR=Q3-Q1, whereas the top wisker is the
639 min(max(x),Q3+1.5*IQR).

640

641 Figure 3: Gill Na^{+}/K^{+}-ATPase activity variation of non-parasitized and parasitized yellow and
642 silver eels. The solid black line represents the median (50th percentile) and the bottom and top
643 box edges are the 25th (Q1) and 75th (Q3) percentile, respectively. The bottom wisker is the
644 max(min(x),Q1-1.5*IQR) with IQR=Q3-Q1, whereas the top wisker is the
645 min(max(x),Q3+1.5*IQR).

646

TABLES

Table 1. Glucose, Cortisol and Na^+/K^+-ATPase activity on female parasitized eels in different silvering conditions according parasite range (mean ± standard error).

			Variable		
Silvering stage	Parasite range	N	Cortisol	Glucose	Na^+/K^+-ATPase activity
I	(0,4]	5	13.04± 2.69	4.08 ± 1.07	4.76 ± 0.63
	(4,8]	2	26.60 ± 13.30	4.35 ± 1.65	3.53 ± 1.02
II	(0,4]	4	9.60 ± 3.81	4.15 ± 0.57	7.06 ± 0.50
	(4,8]	3	11.90 ± 1.83	3.96 ± 0.94	4.89 ± 1.80
	(8,12]	1	14.80 ± -	4.30 ± -	7.56 ± -
III	(0,4]	3	21.53 ± 8.38	4.60 ± 1.80	7.95 ± 1.42
	(4,8]	1	7.80 ± -	9.60 ± -	10.05 ± -

TABLES

Table 2. Statistical outputs from linear mixed effects models: random effects model (Glucose and Cortisol) and fixed-effects (Na^+/K^+-ATPase activity). *P* values of significant parameters are indicated.

Variables	Parameter	Value	Std.Error	t-value	p-value
Glucose					
a) General					
	Time (1h)	0.269	0.022	12.165	<0.0001
	Time (4h)	0.410	0.022	18.356	<0.0001
	Parasitized	0.060	0.048	1.239	0.220
	Time (1h) x Parasitized	0.018	0.034	-0.520	0.604
	Time (4h) x Parasitized	0.041	0.034	-1.186	0.238
b) Females in different silvering stages					
	Time (1h)	0.260	0.019	13.352	<0.0001
	Time (4h)	0.388	0.020	19.747	<0.0001
	Parasitized	0.044	0.088	0.511	0.612
	Silvering stage II	0.035	0.097	-0.361	0.720
	Silvering stage III	-0.075	0.080	-0.944	0.351
	Parasitized x Silvering stage II	-0.050	0.134	-0.379	0.707
	Parasitized x Silvering stage III	0.134	0.127	1.057	0.297
Cortisol					
a) General					
	Time (1h)	0.315	0.050	6.260	<0.0001
	Time (4h)	0.095	0.050	1.881	0.062

Parasitized	-0.139	0.097	-1.428	0.159
Life-stage (silver)	0.117	0.073	1.601	0.115
Time (1h) x Parasitized	0.255	0.078	3.281	0.001
Time (4h) x Parasitized	0.139	0.078	1.783	0.077
Parasitized x Life-stage (silver)	-0.246	0.115	-2.140	0.037
b) Females in different silvering stages				
Silvering stage II	-0.069	0.087	-0.791	0.433
Silvering stage III	0.142	0.082	1.717	0.093
Parasitized	-0.285	0.091	-3.120	0.003
Time (1h)	0.299	0.063	4.731	<0.0001
Time (4h)	0.095	0.063	1.509	0.135
Time (1h) x Parasitized	0.325	0.093	3.498	0.0008
Time (4h) x Parasitized	0.188	0.093	2.014	0.047
Na^+/K^+-ATPase activity				
a) General				
Sex (males)	2.717	0.755	3.599	0.0006
Parasitized	1.051	0.972	1.082	0.284
Life-stage (silver)	6.046	0.818	7.395	<0.0001
Parasitized x Life-stage (silver)	-3.506	1.308	-2.681	0.0095
b) Females in different silvering stages				
Silvering stage II	1.973	0.937	2.106	0.0412
Silvering stage III	5.163	0.874	5.908	<0.0001
Parasitized	0.211	0.758	0.278	0.782

Figure 1

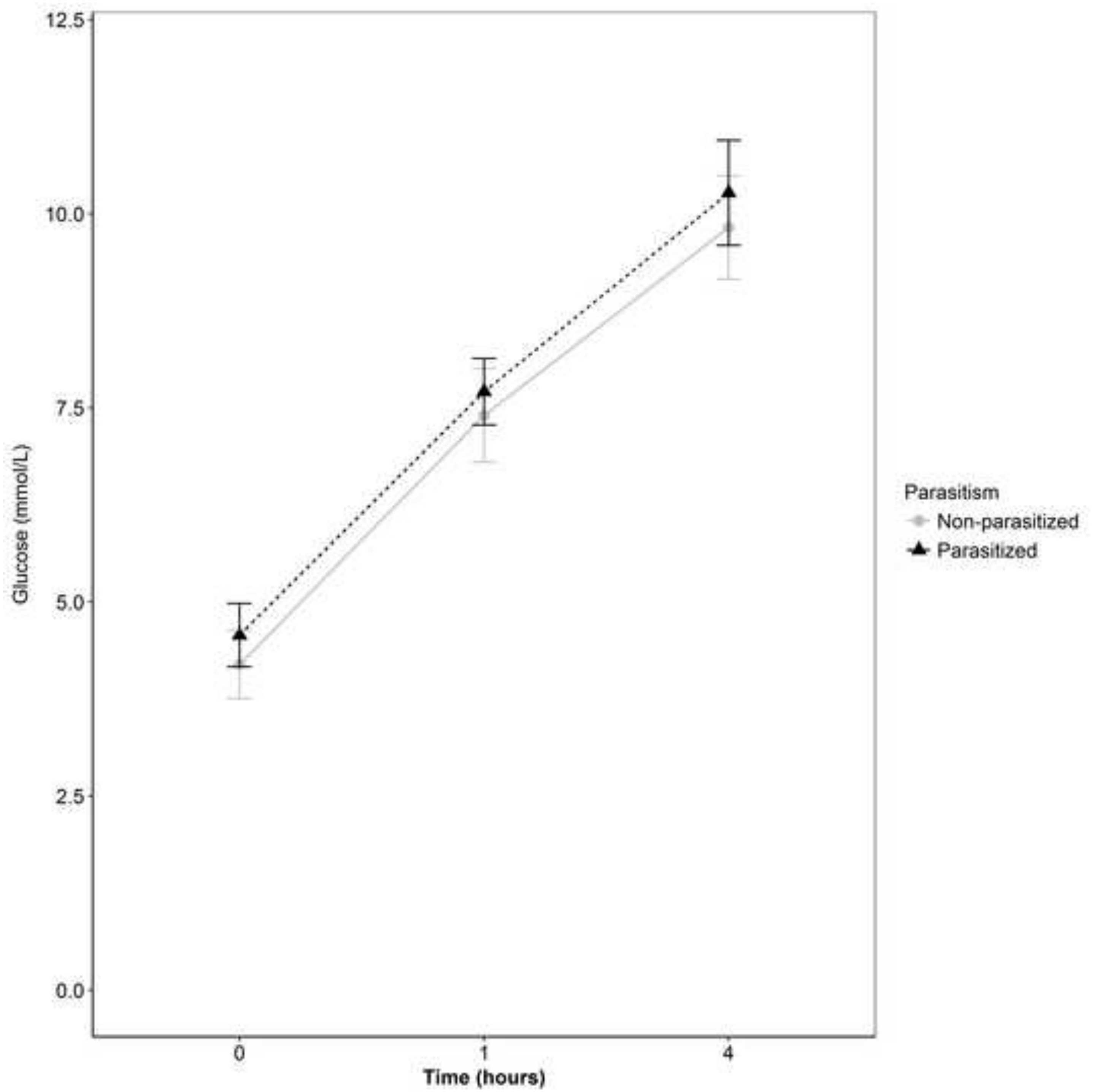

Figure 2a

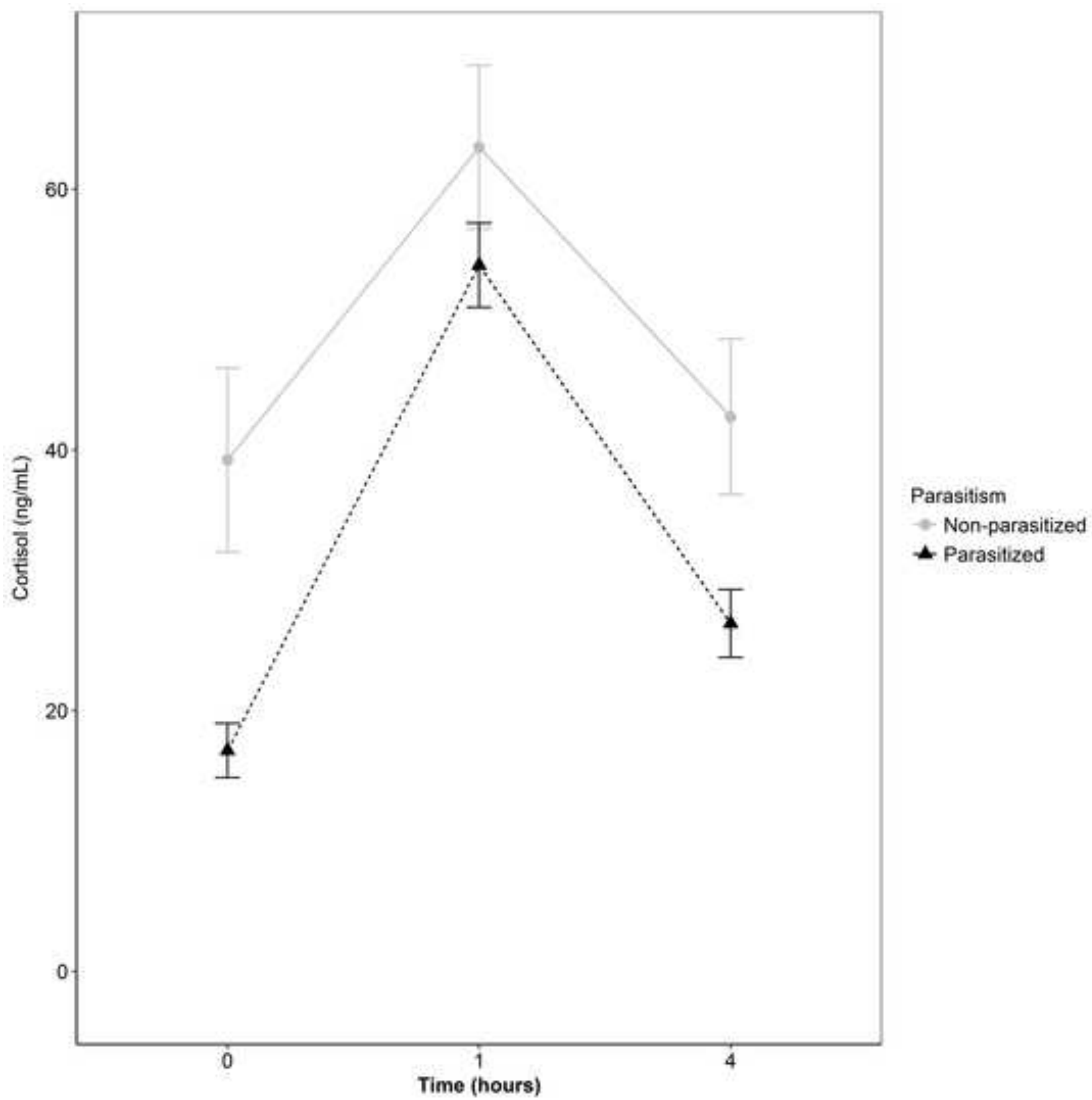

Figure 2b

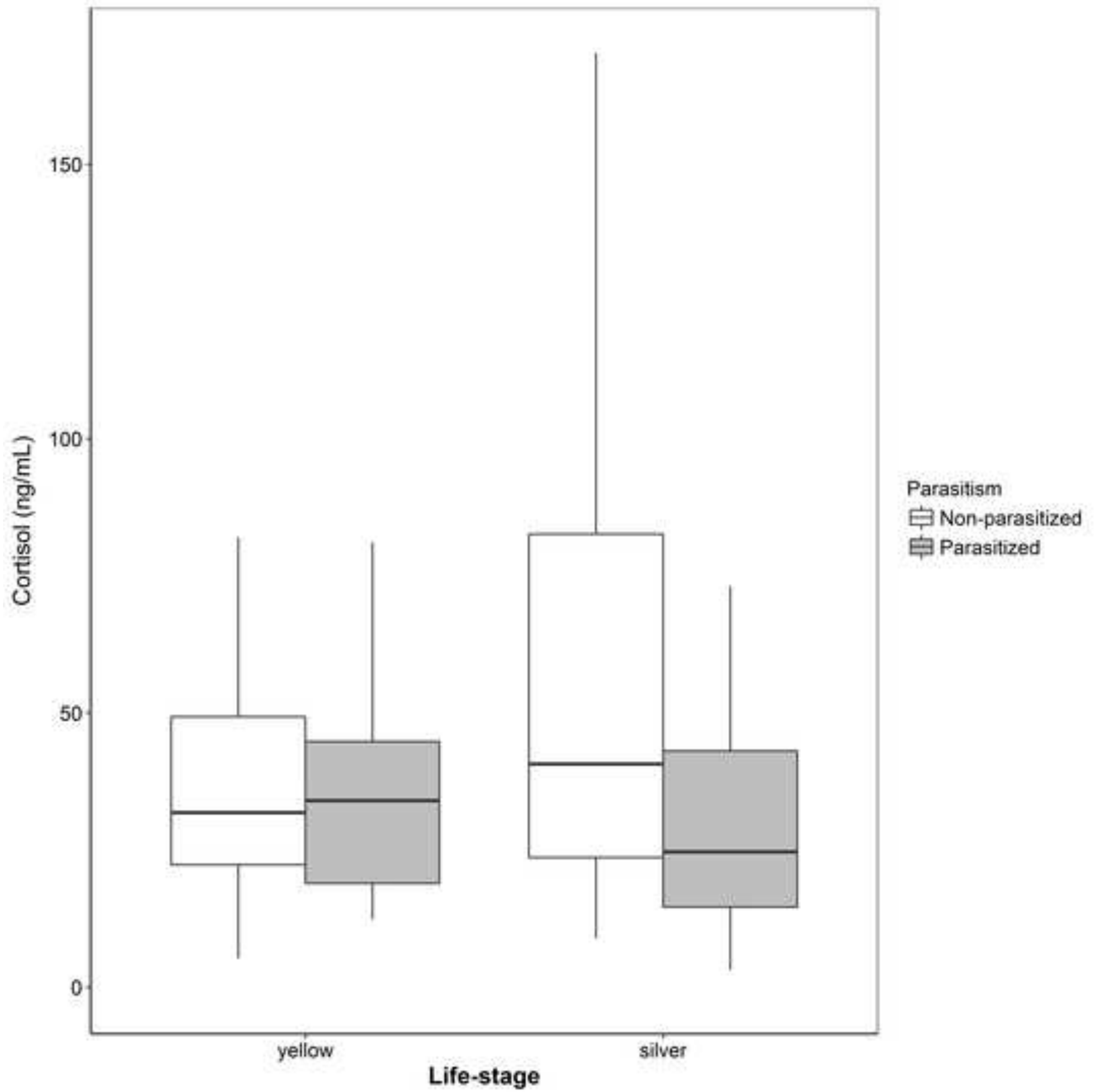

Figure 3