Silva, Ana T.; Midwood, Jonathan D.; Aarestrup, Kim; Pottinger, Tom G.; Madsen, Steffen S.; Cooke, Steven J.. 2018. **The influence of sex, parasitism, and ontogeny on the physiological response of European eels (Anguilla anguilla) to an abiotic stressor.** *Physiological and Biochemical Zoology*, 91 (4). 976-986. https://doi.org/10.1086/698689

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*

Ana T. Silva[1,2 *], Jonathan D. Midwood [2,3], Kim Aarestrup[4], Tom G. Pottinger [5], Steffen S. Madsen [6], Steven J. Cooke[2]

[1]*NINA-The Norwegian Institute for Nature Research, Trondheim, Norway*
[2]*Fish Ecology and Conservation Physiology Laboratory, Department of Biology, Carleton University, Ottawa, Ontario, K1S 5B6, Canada,*
[3]*Great Lakes Laboratory for Fisheries and Aquatic Science, Fisheries and Oceans Canada, Burlington, Ontario, L7S 1A1, Canada,*
[4]*National Institute of Aquatic Resources, Freshwater Fisheries, Technical University of Denmark, Vejlsøvej 39,8600 Silkeborg, Denmark*
[5]*Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP, United Kingdom*
[6]*Department of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark*

*Corresponding author. Email: ana.silva@nina.com, anamftsilva@gmail.com

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.

Keywords: silver eel, yellow eel, stress response, *Anguillicola crassus*, cortisol, glucose, Na^+/K^+-ATPase activity

INTRODUCTION

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

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

sufficient energy to produce offspring. After attaining an adequate lipid reserve, eels start lipid mobilization (EELREP 2005; Trischitta et al. 2013) and sexual maturation, metamorphosing into "silver eels". During this stage, eels stop feeding, and begin the long migration back to the Sargasso Sea for spawning (Righton et al. 2012). Males (on average 40 cm) usually start their migration in August while females (on average bigger than 40 cm) leave later, during October and December (Tesch 2003).

Spawning migration of eels is a complex and energetically demanding process during which eels are very vulnerable to natural and anthropogenic challenges that can impair their migratory capacity as they transition from freshwater to saline water (Gollock et al. 2005, Iversen et al. 2013, Trischitta et al. 2013, Wilson 2013). Durif et al. (2005) described five different stages of the silvering process in female eels according to their physiological changes as they prepare for their spawning migration: a growth phase (I and II) a pre-migration phase (II) and two migration phases (IV and V). In part due to their catadromous lifestyle, European eel populations have seen marked declines throughout their natural range in the past few decades and are currently classified as Critically Endangered (Jacoby and Gollock 2014) and listed under Appendix I-III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2013). Several factors are thought to have contributed to these declines including barriers to migration, habitat loss, parasites (e.g. *Anguillicola crassus*), disease, climate change, bioaccumulation of toxins, predation, changes in ocean currents and overfishing (Dekker 2003; Knights 2003; Van Ginneken et al. 2005; Belpaire et al. 2009; Geeraerts and Belpaire 2010; Durif et al. 2011; Kettle et al. 2011; Wahlberg et al. 2014). The drastic decline of European eel populations has hastened the implementation of management measures aimed at restoring stocks by preventing mortality during migration (European Union implemented the Eel Recovery Plan 2007- Council Regulation No. 1100/2007/EC and the International Council for the Exploitation of the Sea -ICES 2014).

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

MATERIAL AND METHODS

Animals and experimental design

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

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

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

Individual condition

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

Plasma and Gill sample analysis

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

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

Statistical Analysis

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

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

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

RESULTS

Parasitism

Overall, 20 eels were parasitized with *A. crassus* . The number of parasites in the eels varied between 1 and 11 individuals per specimen, and it was different according to the : sex (females: N= 19, males N=1), life-stage (yellow eels N= 7, silver eels N=13) and silvering condition (life stage I: N= 7, life stage II, N= 8, Life stage III: N= 4). The different number of parasites in each silvering stage resulted in different levels of Glucose, Cortisol and Gill Na+/K+-ATPase activity (Table 1).

Glucose

The final model for blood glucose (logG) contained time and parasite condition as the main explanatory factors (logG~time*parasitism; AIC : -222.05, dF: 8). Blood glucose varied significantly with time (Table 2), and increased from 4.5 ± 0.3 mmol/L (mean ± S.E) in 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 4 hours following the stressor (Fig. 1). Temporal variation of glucose was similar between parasitized and non-parasitized eels, with parasitized eels exhibiting slightly higher overall glucose levels (7.5 ± 0.4 mmol/L) than non-parasitized eels (7.1 ± 0.3 mmol/L; Fig. 1), although this difference was not significant.

Variation of plasma glucose in female eels was best explained by a model that included both time and the interaction of parasite condition with silvering (logG~ time+parasitism*silvering; AIC: -145.53, dF: 8). Plasma glucose levels at 1h (7.7 ± 0.5 mmol/L) and 4h (10.2 ± 0.6 mmol/L) after the stressor were significantly different from the values in unstressed eels (4.5 ± 0.4 mmol/L) (Table 2). Parasitism and life-stage were also important covariates in explaining the variation of plasma glucose in female eels, improving

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

Plasma cortisol

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

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

and first group (35.5 ± 3.3 ng/mL) (Table 2). Parasitized female eels exhibited the lowest levels of plasma cortisol (32.3 ± 2.8 ng/mL) when compared to non-parasitized eels (49.1 ± 5.0 ng/mL). The variation of plasma cortisol in female parasitized eels was found to increase with the number of *A. crassus* (Table 1). When parasitized with more than 4 individuals eels had an increased on plasma cortisol levels. Nevertheless when in the last silvering stage, even a small number *A. crassus* appears to elicit a strong increase of plasma cortisol.Variation in plasma cortisol was also time dependent (Table 2); plasma cortisol significantly increased from 27.9 ± 5.0 ng/mL to 56.5 ± 4.1 ng/mL in the first hour following the stressor, decreasing to values close to the baseline levels after 3h (38.6 ± 4.5 ng/mL) (Table 2). This temporal variation was found to be related to the parasitism status of the individual (Table 2). After exposure to a stressor, parasitized eels exhibited a stronger increase in cortisol levels when compared to non-parasitized eels (Table 2). This variation was clearly evident in the first hour following disturbance (non- parasitized eels: 61.9 ± 7.1 ng/mL, parasitized eels: 53.9 ± 3.5 ng/mL) (Table 2). However, by the 4h time point, plasma cortisol levels had recovered to near the levels seen in non-parasitized eels (4h: 44.4 ± 8.0 ng/mL, baseline: 39.9 ± 9.7 ng/mL), but not in parasitized eels (4h: 26.2 ± 3.0 ng/mL, baseline: 14.7 ± 2.2 ng/mL) (Table 2).

Gill Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase activity varied between individuals of different sexes, life stages and parasitisim levels (Na^+/K^+-ATPase activity~ sex + life-stage*parasitism; AIC: 293.97, dF: 6). Males exhibited higher levels of Na^+/K^+-ATPase (8.71 ± 0.8 μmol ADP/mg protein/h) than females (6.02 ± 0.5 μmol ADP/mg protein/h) (Table 2). Na^+/K^+-ATPase levels were found to vary with the life-stage of eels, with the highest values found in silver eels (7.97 ± 0.6 μmol ADP/mg protein/h) when compared to yellow eels (4.74 ± 0.5 μmol ADP/mg protein/h) (Fig. 3, Table 2). Within life stages the variation of Na^+/K^+-ATPase activity was conditioned by the

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

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

DISCUSSION

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

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

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

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

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

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

The highest levels of Na^+/K^+ - ATPase activity were found in males and can be a consequence of different stages of sexual maturation achieved by the specimens. Considering that males initiate their migration earlier than females (Palstra et al. 2007; 2010), and that the experiments were carried out at the end of October through the beginning of November, our findings may then be related with the fact that most males could have been in a more advanced 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

435 **ACKNOWLEDGEMENTS**

436 This research was supported by NSERC HydroNet, the NSERC Discovery Grant Program and
437 the Canada Research Chairs Program. Ana T. Silva was also financed by the SafePass project
438 (no. 244022) funded by the Research Council of Norway (RCN) under the ENERGIX program.
439 We thank Alexander Wilson from University of Sydney for the useful suggestions, Ola Diserud
440 and Richard Hedger from NINA for their help in the statistical analysis. We also thank Bengt
441 Finstad from NINA and Mike Lawrence from Carleton University for providing constructive
442 comments on the manuscript. We would like to thank Martin Larsen and the technical staff of
443 the National Institute of Aquatic Resources, Freshwater Fisheries, DTU, for technical support.

444

445 **REFERENCES**

446 Aarestrup K., F. Økland, M.M. Hansen, D. Righton, P. Gargan, M. Castonguay, L. Bernatchez,
447 P. Howey, H. Sparholt, M.I. Pedersen, and R.S. McKinley. 2009. Oceanic spawning migration
448 of the European Eel (*Anguilla anguilla*). Science 325: 1660.

449 Angelier F. and J.C. Wingfield. 2013. Importance of the glucocorticoid stress response in a
450 changing world: theory, hypotheses and perspectives. General and Comparative
451 Endocrinology, 10th International Symposium on Avian Endocrinology 190: 118–128.

452 Aoyama J. 2009. Life history and evolution of migration in Catadromous Eels (Genus
453 *Anguilla*). Aqua Biosci. Monogr. 2:1–42.

454 Arthington A.H., N.K Dulvy, W. Gladstone, and I. J. Winfield. 2016. Fish conservation in
455 freshwater and marine realms: status, threats and management. Aquat. conserv. mar. freshw.
456 ecosys 26:838-857.

457 Axelrod J. and T. D. Reisine. 1984. Stress hormones: Their interaction and regulation. Science
458 224: 452-459.

459 Barton B.A. 2002. Stress in fishes: a diversity of responses with particular reference to changes
460 in circulating corticosteroids. Integr. Comp. Biol. 42: 517–525.

461 Belpaire C.G.J., G. Goemans, C. Geeraerts, P. Quataert, K. Parmentier, and P. Hagel, and J. De
462 Boer. 2009. Decreasing eel stocks: survival of the fattest? Ecol. Freshw. Fish 18: 197–214.

463 Bisbal GA. and J. L. Specker. 1991. Cortisol stimulates hypo-osmoregulatory ability in Atlantic
464 salmon, *Salmo salar* L. J. Fish Biol. 39: 421–432.

465 Boetius I. and J. Boetius. 1980. Experimental maturation of female silver eels, *Anguilla*
466 *anguilla*. Estimates of fecundity and energy reserves for migration and spawning. Dana 1: 1–
467 28.

468 Bolger T. and P.L. Connolly. 1989. The selection of suitable indices for the measurement and
469 analysis of fish condition. J. Fish Biol. 34:171-182.

470 Bruijs M. and C. Durif. 2009. Silver eel migration and behaviour. In Spawning Migration of
471 the European Eel, ed. G. Van den Thillart, S. Dufour and J.C. Rankin, 65–95. New York:
472 Springer.

473 Burnham K.P. and D.R. Anderson. 2002. Model Selection and Multimodel Inference Springer
474 Science, Business Media

475 Butler D.G. and F.J. Carmichael. 1972. (Na^+/ K^+)-ATPase activity in eel (*Anguilla rostrata*)
476 gills in relation to changes in environmental salinity: Role of adrenocortical steroids, Gen.
477 Comp. Endocrinol. 19: 421-427.

478 CITES. 2013. Convention on International Trade in Endangered Species of Wild Fauna and
479 Flora. Bangkok: CITES, 1973–2013.

480 Cook K.V., C.M. O'Connor, S.H. McConnachie, K.M. Gilmour, and S.J. Cooke. 2012.
481 Condition dependent intra-individual repeatability of stress-induced cortisol in a freshwater
482 fish. Comp. Biochem. Physiol., Part A 161:337-343

483 Cooke S.J., G.T. Crossin, D. Patterson, K. English, S.G. Hinch, J.L. Young, R. Alexander, M.C.
484 Healey, G. Van Der Kraak, and A.P. Farrell. 2005. Coupling non-invasive physiological and
485 energetic assessments with telemetry to understand inter-individual variation in behaviour and
486 survivorship of sockeye salmon: development and validation of a technique. J. Fish Biol.
487 67:1342-1358.

488 Dekker W. 2003. Did lack of spawners cause the collapse of the European eel, *Anguilla*
489 *anguilla*? Fish. Manag. Ecol. 10: 365–376.

490 Durif C., S. Dufour, and P. Elie. 2005.The silvering process of the eel: a new classification from
491 the yellow resident stage to the silver migrating stage. J Fish Biol 66:1–19

492 Durif C. M. F., J. Gjosaeter, and L.A. Vollestad. 2011. Influence of oceanic factors on *Anguilla*
493 *anguilla* (L.) over the twentieth century in coastal habitats of the Skagerrak, southern Norway.
494 Proc. R. Soc. Lond. B Biol. Sci. 278: 464–473.

495 EELREP. 2005. Estimation of the reproduction capacity of European eel. Final report.
496 Available via http://www. fishbiology.net/eelrepsum.html. Accessed 13 Oct 2009

497 Fontaínhas-Fernandes A., E. F. Gomes, M. A. Reis-Henriques, and J. Coimbra. 2003. Effect of
498 cortisol on some osmoregulatory parameters of the teleost, *Oreochromis niloticus* L., after
499 transference from freshwater to seawater. Arq Bras Med Vet Zootec 55:562–567.

500 Fuzzen M., N. J Bernier, and G. Van Der Kraak. 2011. Stress and reproduction. Hormones and
501 reproduction in vertebrates, 1: 103-117.

502 Geeraerts C. and C. Belpaire 2010. The effects of contaminants in European eel: a review.
503 Ecotoxicology 19: 239–266.

504 Gollock M.J., C.R. Kennedy, E.S. Quabius, and Brown J.A. 2004. The effect of parasitism of
505 European eels with the nematode, *Anguillicola crassus* on the impact of netting and aerial
506 exposure. Aquaculture 233: 45–54.

507 Gollock M.J., C.R. Kennedy, and J.A. Brown. 2005. European eels, *Anguilla anguilla* (L.),
508 infected with *Anguillicola crassus* exhibit a more pronounced stress response to severe hypoxia
509 than uninfected eels. J. Fish. Dis. 28: 429–436.

510 Gorissen, M. and G. Flik. 2016. 3- The endocrinology of the stress response in fish: an
511 adaptation-physiological view. Fish physiology. 35 :75-111.

512 Howey P., H. Sparholt, M.I. Pedersen, and R.S. McKinley. 2009. Oceanic spawning migration
513 of the European Eel (*Anguilla anguilla*). Science 325: 1660.

514 Huang Y.S., K. Rousseau, M. Sbaihi, N. Le Belle, M. Schmitz, and S. Dufour. 1999. Cortisol
515 Selectively Stimulates Pituitary Gonadotropin β-Subunit in a Primitive Teleost, *Anguilla*
516 *anguilla*. Endocrinol. 140: 1228–1235.

517 ICES. 2014. Report of the joint EIFAAC/ICES/GFCM working group on eel. 3–7 November
518 2014, Rome, Italy.

519 Iversen M.H., F. Økland, E.B. Thorstad, and B. Finstad. 2013. The efficacy of Aqui-S vet. (iso-
520 eugenol) and metomidate as anaesthetics in European eel (*Anguilla anguilla* L.), and their
521 effects on animal welfare and primary and secondary stress responses. Aquacult. Res 44: 1307-
522 1316.

523 Jacoby D. and M. Gollock. 2014. *Anguilla anguilla*. The IUCN red list of threatened species.
524 Version 2014.3. www.iucnredlist.org.

525 Kettle A.J., L. Asbjorn Vollestad, and J. Wibig. 2011. Where once the eel and the elephant were
526 together: decline of the European eel because of changing hydrology in southwest Europe and
527 northwest Africa? Fish Fish. 12: 380–411.

528 Knights B. 2003. A review of the possible impacts of long-term oceanic and climate changes
529 and fishing mortality on the recruitment of anguillid eels of the Northern Hemisphere. Sci. Total
530 Environ. 310: 237–244.

531 Korte S. M., J.M. Koolhaas, J.C. Wingfield, and B.S. McEwen. 2005. The Darwinian concept
532 of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and
533 disease. Neurosc Biobehav R. 29: 3-38.

534 Lawrence, M.J., S. Jain-Schlaepfer, A.J. Zolderdo, D.A. Algera, K.M. Gilmour, A.J. Gallagher,
535 and S.J. Cooke. 2018. Are 3-minutes good enough for obtaining baseline physiological
536 samples from teleost fish? Can. J. Zool. 00:000-000.

537 Lefebvre F. and A.J. Crivelli. 2012. Salinity effects on anguillicolosis in Atlantic eels: a natural
538 tool for disease control. Mar Ecol-Prog Ser. 471:193-202.

539 Lowry O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with
540 the Folin Phenol reagent. J. Biol. Chem. 193: 265-275

541 Madsen S.S. 1990a. Enhanced hypoosmoregulatory response to growth hormone after cortisol
542 treatment in immature rainbow trout, Salmo gairdneri. Fish Physiol. Biochem. 8: 271–279.

543 Madsen S.S. 1990b. The role of cortisol and growth hormone in seawater adaptation and
544 development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*). Gen.
545 Comp. Endocrinol. 79:1–11.

546 McCormick S.D. 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase
547 activity. Can. J. Fish. Aquat. Sci. 50: 656–658.

548 McCormick S.D. 1995. Hormonal control of gill Na1, K1-ATPase and chloride cell function.
549 In C. M. Wood and T.J. Shuttleworth (eds.), Fish physiology, Vol. XIV, Ionoregulation:
550 Cellular and molecular approaches, pp. 285–315. Academic Press, New York.

551 McEwen B.S. and J.C. Wingfield. 2003. The concept of allostasis in biology and bio-medicine.
552 Horm. and Behav. 43: 2–15.

553 Mommsen T.P., M.M. Vijayan, and T.W. Moon. 1999. Cortisol in teleosts: dynamics,
554 mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9: 211–268.

555 Neiffer D. L. and M.A. Stamper. 2009. Fish sedation, anesthesia, analgesia, and euthanasia:
556 considerations, methods, and types of drugs. ILAR journal, 50: 343-360.

557 Palstra A.P., D.F.M. Heppener, V.J.T. van Ginneken, C. Szekely, G.E.E.J. van den Thillart.
558 2007. Swimming performance of silver eels is severely impaired by the swim-bladder parasite
559 *Anguillicola crassus*. J. Exp. Mar. Biol. Ecol. 352: 244–256.

560 Palstra A. P. and G.E.E.J. van den Thillart. 2010. Swimming physiology of European silver
561 eels (*Anguilla anguilla* L.): energetic costs and effects on sexual maturation and reproduction.
562 Fish Physiol. Biochem. 36: 297–322.

563 Pankhurst N.W. 1982. Relation of visual changes to the onset of sexual maturation in the
564 European eel *Anguilla anguilla* (L.). J. Fish Biol. 21: 127–140.

565 Pankhurst N.W. 2011. The endocrinology of stress in fish: an environmental perspective.
566 General and Comparative Endocrinology 170: 265–275.

567 Pelster B. 2015. Swimbladder function and the spawning migration of the European eel
568 *Anguilla anguilla*. Frontiers in Physiology 5: 486.

569 Pinheiro J., D. Bates, S. DebRoy S., D. Sarkar and R Core Team (2017). nlme: Linear and
570 Nonlinear Mixed Effects Models. R package version 3.1-131, https://CRAN.R-
571 project.org/package=nlme.

572 Piper A.T., C. Manes, F. Siniscalchi, A. Marion, R.M. Wright, and P.S. Kemp. 2015. Response
573 of seaward migration European eel (*Anguilla anguilla*) to manipulated flow fields. Proc. R. Soc.
574 B. 282.

575 Pottinger T.G. and T.R. Carrick. 2001. Stress responsiveness affects dominant-subordinate
576 relationships in rainbow trout. Hor. Behav. 40: 419-427

577 Righton D., K. Aarestrup, D. Jellyman, P.Sébert, G. van den Thillart, and K. Tsukamoto. 2012.
578 The *Anguilla* spp. migration problem: 40 million years of evolution and two millennia of
579 speculation. J. Fish Biol. 81: 365–386.

580 Righton D., H. Westerberg, E. Feunteun, F. Økland, P. Gargan, E. Amilhat, Julian Metcalfe, J.
581 Lobon-Cervia, N. Sjöberg, J. Simon, A. Acou, M. Vedor, A. Walker, T. Trancart, U. Brämick,
582 and K. Aarestrup. 2016. Empirical observations of the spawning migration of European eels:
583 The long and dangerous road to the Sargasso Sea. Science Advances, 2, e1501694.

584 Sancho E., M.D. Ferrando, and E. Andreu. 1997. Inhibition of gill Na,K -ATPase activity in
585 the eel, *Anguilla anguilla*, by fenitrothion. Ecotoxicol. Environ Saf. 38: 132–136.

586 Sapolsky R.M. 1999. Glucocorticoids, stress, and their adverse neurological effects: relevance
587 to aging. Exp Gerontol 34: 721–732.

588 Schreck C.B. 1981. Stress and compensation in teleostean fishes: Response to social and
589 physical factors. In A. D. Pickering (ed.), Stress and fish, pp. 295–321. Academic Press, New
590 York.

591 Schreck C.B. 2000. Accumulation and long-term effects of stress in fish. In G. P. Moberg and
592 J. A. Mench (eds.), The biology of animal stress, pp. 147–158. CABI Publishing, Wallingford,
593 U.K

594 Schreck C.B. 2010. Stress and fish reproduction: The roles of allostasis and hormesis. Gen.
595 Comp. Endocrinol. 165, 549-556.

596 Stoot, L. J., N.A. Cairns, F. Cull, J. J. Taylor , J. D. Jeffrey, F. Morin, J.W. Mandelman, T.D.
597 Clark and S.J. Cooke .2014. Use of portable blood physiology point-of-care devices for basic
598 and applied research on vertebrates: a review. Conserv. Physiol. 2:1.

599 Sures B., K. Knopf, W. Kloas. 2001. Induction of stress by the swimbladder nematode
600 *Anguillicola crassus* in European eels, *Anguilla anguilla*, after repeated experimental infection.
601 Parasitology 123: 179–184.Tesch F.W. 2003 The eel, 5th edn. Oxford, UK: Blackwell Science.

602 Toni C., A.G. Becker, L.N. Simões, C.G. Pinheiro, L.L. Silva, B.M. Heinzmann, and B.O.
603 Caron. 2014. Fish anesthesia: effects of the essential oils of Hesperozygis ringens and Lippia
604 alba on the biochemistry and physiology of silver catfish (*Rhamdia quelen*). Fish Physiol.
605 Biochem. 40: 701–714.

606 Towle D.W. 1981. Role of Na^+, K^+-ATPase in ionic regulation by marine and estuarine animals.
607 Mar. Biol. Lett. 2:107–122.

608 Trischitta F., Y. Takei, and P. Sébert. 2013. Eel Physiology Editora CRCPress, pp. 378.

609 van den Thillart G. and S. Dufour. 2009. How to estimate the reproductive success of European
610 silver eels, in: van de Thillart, G. et al. (Ed.) Spawning migration of the European eel:
611 reproduction index, a useful tool for conservation management. Fish & Fisheries Series, 30: pp.
612 3-9.

613 Wendelaar Bonga S.E. 1997. The stress response in fish. Physiol. Rev. 77: 591–625

614 van Ginneken V., E. Antonissen, U.K. Müller, R. Booms, E. Eding, J. Verreth, and G. van den
615 Thillart. 2005. Eel migration to the Sargasso: remarkably high swimming efficiency and low
616 energy costs. J. Exp. Biol. 208: 1329–1335.

617 Wahlberg M., H. Westerberg, K. Aarestrup, E. Feunteun, P. Gargan, and D. Righton. 2014.
618 Evidence of marine mammal predation of the European eel (*Anguilla anguilla* L.) on its marine
619 migration. Deep-Sea Res I 86: 32−38.

620 Wilson J.M. 2013. Stress physiology. In: Trischitta, F, Takei, Y, Sebert, P (Eds). Eel
621 Physiology. CRC Press, Boca Raton, pp. 320-359. ISBN: 9781466598270

622 Wilson J.M., J.C. Antunes, P.D. Bouça, and J. Coimbra. 2004. Osmoregulatory plasticity of the
623 glass eel of *Anguilla anguilla*: freshwater entry and changes in branchial ion-transport protein
624 expression. Can. J. Fish. Aquat. Sci 61: 432–442.

625

626

627

628

FIGURE LEGENDS

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

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

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

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

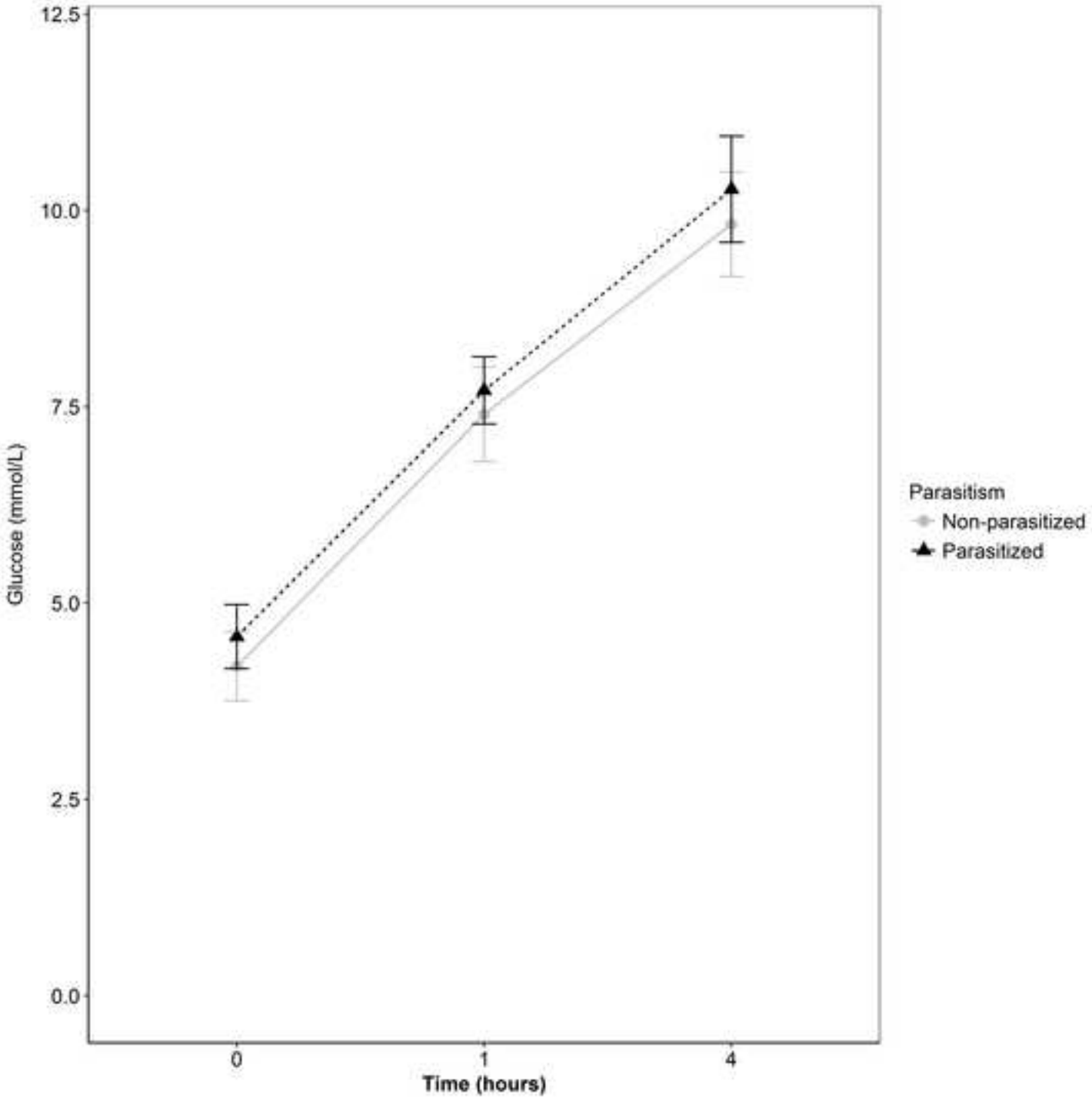

12.5
10.0
7.5
5.0
2.5
0.0
Glucose (mmol/L)
0
1
4
Time (hours)
Parasitism
Non-parasitized
Parasitized

Figure 2a

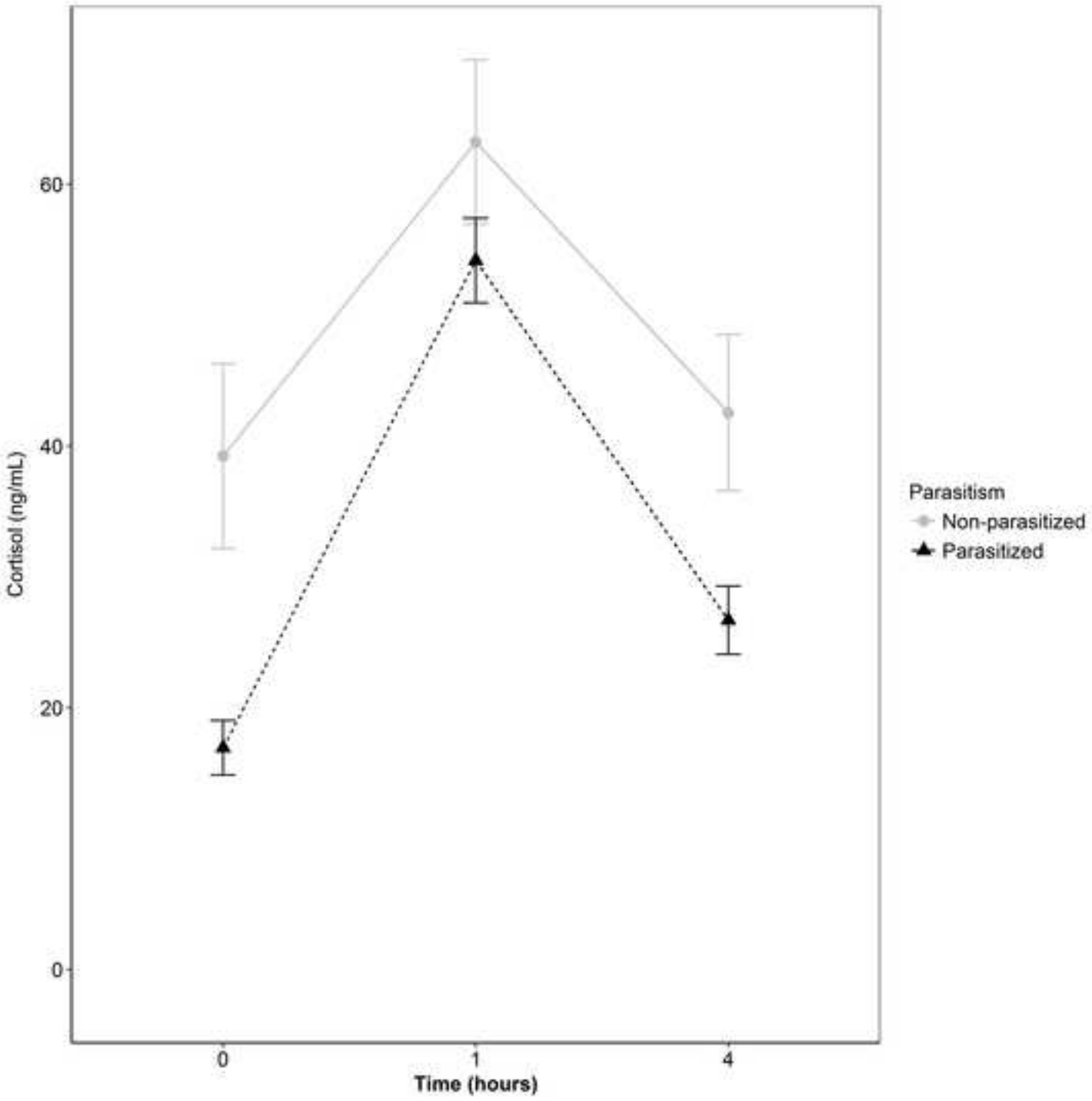

Cortisol (ng/mL)
60
40
20
0
0
1
4
Time (hours)
Parasitism
Non-parasitized
Parasitized

Figure 2b

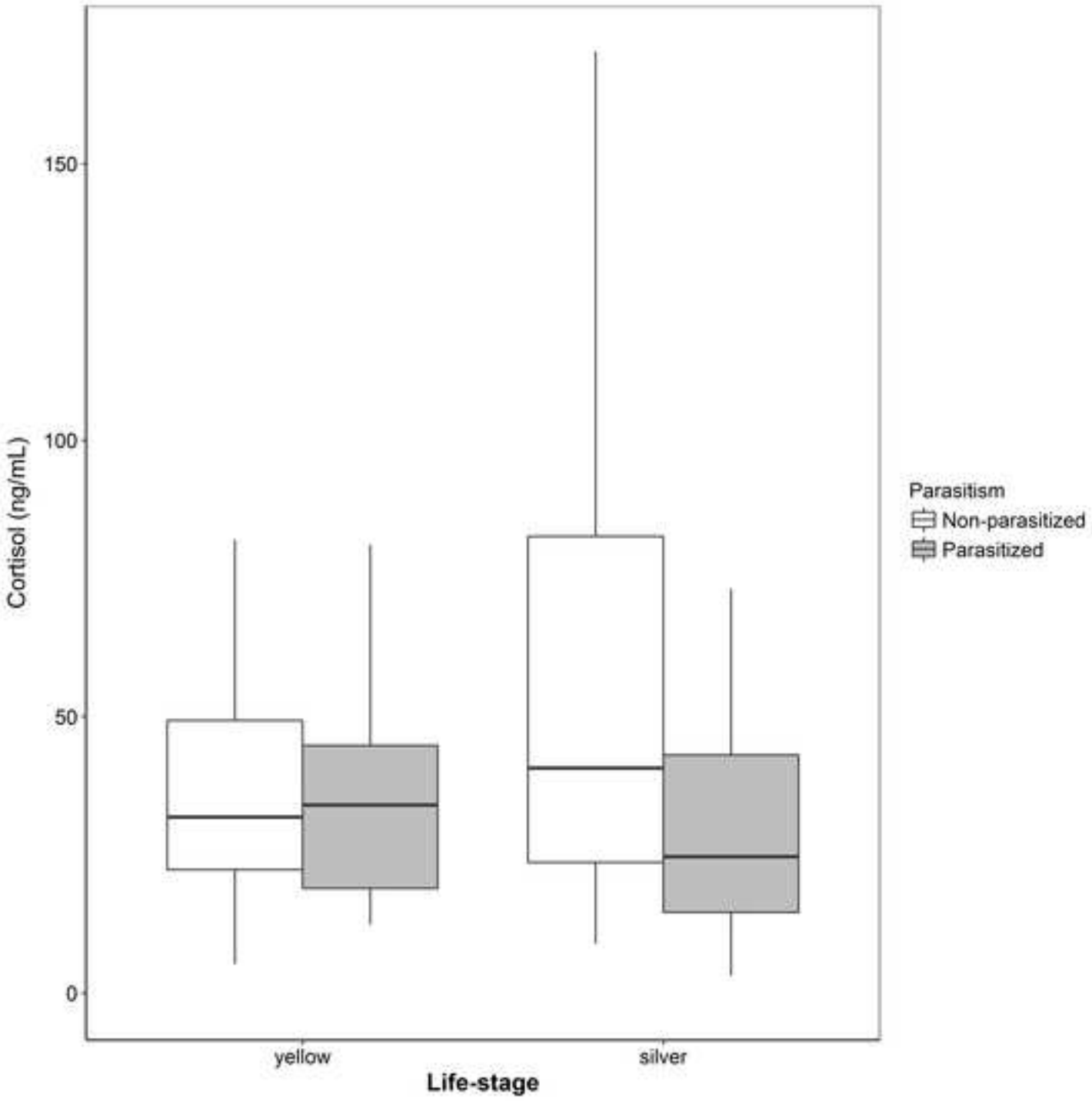
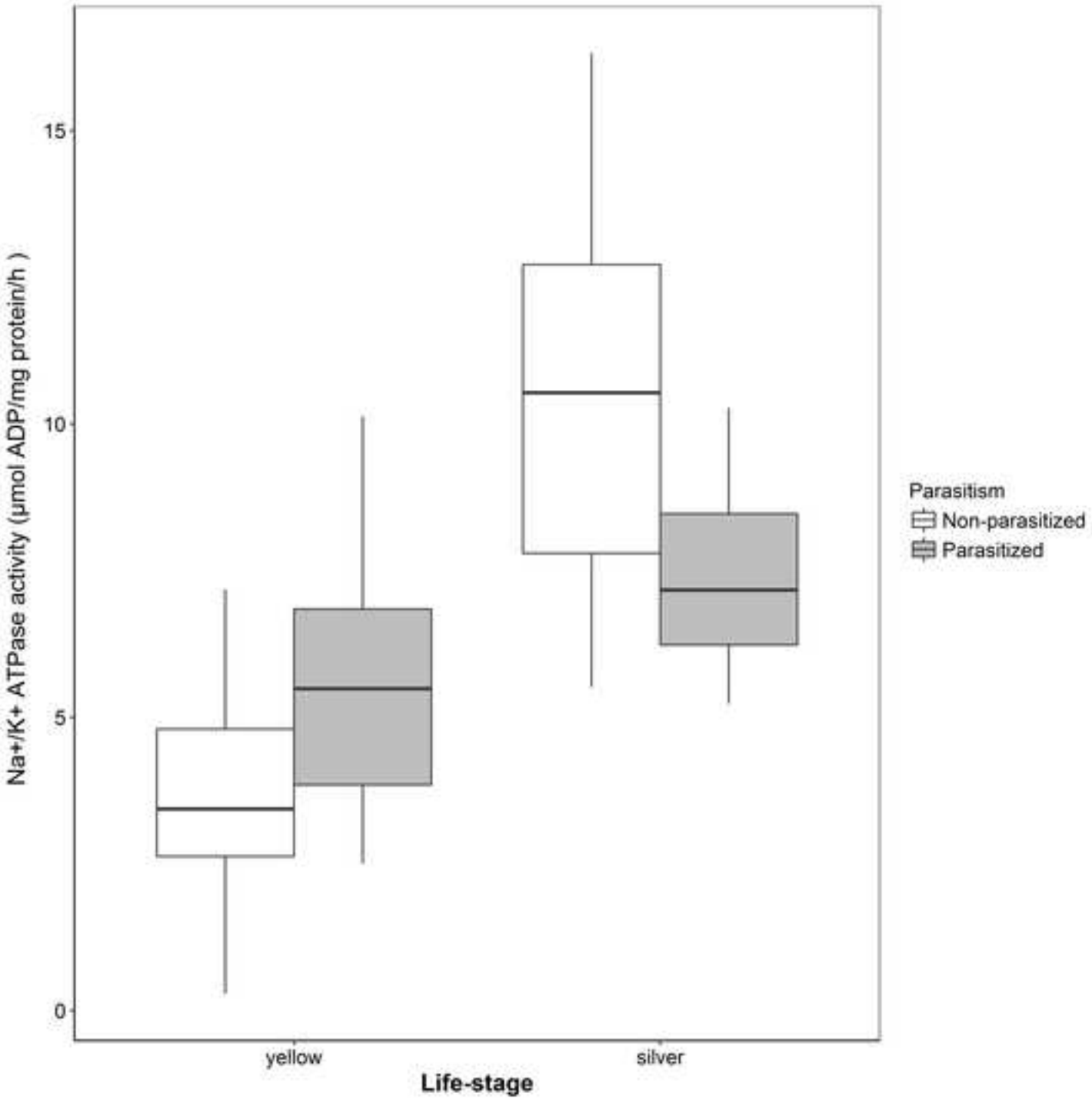

150
100
50
0
Cortisol (ng/mL)
yellow
silver
Life-stage
Parasitism
Non-parasitized
Parasitized

Figure 3


Na+/K+ ATPase activity (µmol ADP/mg protein/h)
15
10
5
0
yellow
silver
Life-stage
Parasitism
Non-parasitized
Parasitized