The Influence of Sex, Parasitism, and Ontogeny on the Physiological Response of European Eels (*Anguilla anguilla*) to an Abiotic Stressor

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]Norwegian Institute for Nature Research (NINA), 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.

ABSTRACT

Migration of adult European eels (*Anguilla anguilla*) from freshwater feeding grounds to oceanic spawning grounds is an energetically demanding process and is accompanied by dramatic physiological and behavioral 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 eels. In this study, mixed-effects 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 after exposure to the standardized stressor and remained elevated after 4 h. Variation in plasma cortisol concentrations after exposure to the stressor were found to be time dependent, which was exacerbated by life stage and parasitism condition. Males and nonparasitized 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 that 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 2016). 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 and Wingfield 2013), thus promoting the survival and recovery of individuals (Sapolsky 1999). The stress response is characterized by the production and release of glucocorticoid steroid hormones (i.e., cortisol in fish) shortly after 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 can 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 life in seawater (SW) is a very important and challenging period usually characterized by high levels of mor-

tality (Bruijs and Durif 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 species of eels (Aoyama 2009) and is performed without feeding (Righton et al. 2012). Before migrating to SW, an eel's life is spent feeding in FW (for up to 25 y) to store enough fat (>20% of the body mass; Tesch 2003; yellow-eel stage) to fuel a 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 (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 in length) usually start their migration in August, while females (on average longer 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 FW to SW (Gollock et al. 2005; Iversen et al. 2013; Trischitta et al. 2013; Wilson 2013). Durif et al. (2005) described five stages of the silvering process in female eels according to their physiological changes as they prepare for their spawning migration: two growth phases (I and II), a premigration phase (III), and two migration phases (IV and V). In part because of their catadromous lifestyle, European eel populations have seen marked declines throughout their natural range in the past few decades; the species is currently classified as critically endangered (Jacoby and Gollock 2014) and is listed under Appendixes 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 (the European Union implemented the Eel Recovery Plan 2007 [Council Regulation no. 1100/2007/EC] and the International Council for the Exploration 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 and 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 analyzed in this study. The main goal of this study was to analyze how individual factors such as sex, parasite load (nonparasitized vs. parasitized with *A. crassus*), and ontogenetic phase (yellow, silver, and the 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-effects and linear models to quantify the physiological responses of eels. We measured blood parameters (i.e., plasma cortisol and body glucose) immediately (baseline) and 1 h (stress response) and 4 h (recovery period) after 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 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 the Gudenå River 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 $[L_t] \pm SD = 51.9 \pm 8.3$ cm; mean total weight $[W_t] \pm SD = 249.7 \pm 127.3$ g) were transported and held in three 8,000-L holding tanks (water temperature 12°–15°C) at the National Institute of Aquatic Resources, Technical University of Denmark, in Silkeborg, until the experiments were carried out (holding time of between 5 and 9 d). To minimize stress during holding and facilitate recovery from capture, transportation, and handling, shelter was provided for the eels. This shelter consisted of 3.0- and 4.5-cm diameter, 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 $L_t \pm SD = 54.9 \pm 6.1$ cm; mean $W_t \pm SD = 289.7 \pm 111.9$ g) and 15 males (mean $L_t \pm SD = 40.5 \pm 3.4$ cm; mean $W_t \pm SD = 105.13 \pm 27.2$ 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 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 1 and 4 h after the baseline sample. Eels were not anesthetized during this procedure, because anesthesia has been shown to influence gill Na^+/K^+-ATPase activity (Toni et al. 2014)—another parameter measured in this study (details provided in "Plasma and Gill Sample Analysis")—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-h time point would have served as a stressor that had the potential to influence the stress levels measured at the 4-h time point, but all fish were handled similarly, and the sampling 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 minimize disturbance of fish during blood sampling, this procedure was always conducted by the same operator. Fish were euthanized via decapitation with 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 no. 2013-15-2934-00808).

Individual Condition

At the end of each experiment eels were killed 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 (Bolger and Connolly 1989; Durif et al. 2005). These indices were used, together with the external morphological characteristics of a silver-phase eel (presence of black corpuscles in the lateral line; a dark dorsal region of the body and a lighter "silver" ventral region; 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 among silvering stages I–V (Pankhurst 1982; Durif et al. 2005). The swim bladder of each eel was also removed, and any *Anguillicola crassus* present in the swim bladder lumen were removed and enumerated.

Plasma and Gill Sample Analysis

Blood samples were obtained by puncture of the caudal vasculature with preheparinized (10,000 USP units/mL heparin sodium; Sandoz, Boucherville, Quebec), needles (25 G, 1/2 inch) and 1-mL syringes (BD Plastipak), 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, subsamples were removed for immediate determination of blood glucose concentrations with 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 aliquotted 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 accord-

ing 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 anticortisol (IgG, Nashville), and tracer ($[1,2,6,7]^3$H-cortisol, 2.59 TBq/mmol; Perkin-Elmer, Beaconsfield, UK) 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_2 EDTA, 50 mM imidazole; pH 7.3), frozen in N_2, and stored at $-80°C$ until analyzed. Gill homogenates were centrifuged at 1,000 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 et al. (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 analyzed for normality with the Shapiro-Wilcoxon test. To meet the normality requirements of parametric analysis, cortisol and glucose data were log(x) transformed as log cortisol (log C) and log glucose (log G), respectively.

Response variables log C and log G were fitted with linear mixed-effects (LME) models with individual fish as a random factor and time (baseline, 1 h, and 4 h), sex (male or female), life stage (yellow or silver), and parasite condition (nonparasitized or parasitized with *A. crassus*) as fixed effects. Silvering stages (I–V) could not be compared independently because of the small number of individual females in each stage; therefore, individuals were classified into three groups according to their similarities of development (after Durif et al. 2005). Group 1 included all the individuals belonging to the silvering stages I and II, group 2 had individuals in stage III, and group 3 had individuals in stages IV and V. To understand the effects of silvering stage on log C and log G, 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 were used to assess the effect of sex, life stage, parasite condition, and silvering stage on gill Na^+/K^+-ATPase activity. Data were analyzed with the nlme function in the R statistical environment (package ver. 3.1-117; Pinheiro et al. 2017). To compare model fits objectively and to determine which was the most appropriate, an information-theoretic approach was performed to compare models via 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 via maximum likelihood. Mean values are reported with standard error (mean $\pm$ SE), and results are considered significant at $\alpha < 0.05$.

Results

Parasitism

Overall, 20 eels were parasitized with *Anguillicola 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-stage group (group 1: $N = 7$; group 2: $N = 8$; group 3: $N = 4$). 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 ($\log G$) contained time and parasite condition as the main explanatory factors ($\log G \sim$ 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 $\pm$ SE) in unstressed eels at 0 h to 7.6 ± 0.4 mmol/L at 1 h after the stressor and 10.2 ± 0.5 mmol/L 4 h after the stressor (fig. 1). Temporal variation of glucose was similar between parasitized and nonparasitized eels, with parasitized eels exhibiting slightly higher overall glucose levels (7.5 ± 0.4 mmol/L) than nonparasitized 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 ($\log G \sim$ time + parasitism × silvering; AIC $= -145.53$; df $= 8$). Plasma glucose levels at 1 h (7.7 ± 0.5 mmol/L) and 4 h (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, a similar number of parasites in each silvering stage led to approx-imately the same values for plasma glucose in eels. Although the effect was minor, still, blood glucose levels increased with the number of *A. crassus* in each eel, particularly in eels parasitized with more than 4 individuals (table 1). Overall, mean glucose levels in nonparasitized 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 ($\log C \sim$ 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 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 nonparasitized 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 were 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 to 54.2 ± 3.2 ng/mL (table 2; fig. 2A). Overall, nonparasitized silver eels had higher plasma cortisol levels (58.6 ± 6.7 ng/mL) than nonparasitized 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 ($\log C \sim$ silvering + time × parasitism; AIC $= 36.06$; df $= 10$). Female eels belonging to the third silvering-stage group exhibited higher levels of plasma cortisol (58.02 ± 6.3 ng/mL) than eels of the second (27.9 ± 2.3 ng/mL) and first (35.5 ± 3.3 ng/mL) groups (table 2). Parasitized female eels exhibited lower levels of plasma cortisol (32.3 ± 2.8 ng/mL) than

Table 1: Glucose, cortisol, and Na^+/K^+-ATPase activity in female parasitized eels in different silvering conditions according to parasite range (mean $\pm$ SE)

Silvering-stage group, parasite range	N	Variable		
		Cortisol	Glucose	Na^+/K^+-ATPase activity
1:				
(0,4]	5	13.04 ± 2.69	4.08 ± 1.07	4.76 ± .63
(4,8]	2	26.60 ± 13.30	4.35 ± 1.65	3.53 ± 1.02
2:				
(0,4]	4	9.60 ± 3.81	4.15 ± .57	7.06 ± .50
(4,8]	3	11.90 ± 1.83	3.96 ± .94	4.89 ± 1.80
(8,12]	1	14.80	4.30	7.56
3:				
(0,4]	3	21.53 ± 8.38	4.60 ± 1.80	7.95 ± 1.42
(4,8]	1	7.80	9.60	10.05

Note. Silvering-stage group 1 consists of silvering stages I and II, group 2 of silvering stage III, and group 3 of silvering stages IV and V.

Table 2: Statistical outputs from linear mixed-effects models: random-effects model (glucose and cortisol) and fixed effects (Na$^+$/K$^+$-ATPase activity)

Variables, parameter	Value	SE	t value	P value
Glucose				
General:				
Time (1 h)	.269	.022	12.165	<.0001
Time (4 h)	.410	.022	18.356	<.0001
Parasitized	.060	.048	1.239	.220
Time (1 h) × parasitized	.018	.034	−.520	.604
Time (4 h) × parasitized	.041	.034	−1.186	.238
Females in different silvering stages:				
Time (1 h)	.260	.019	13.352	<.0001
Time (4 h)	.388	.020	19.747	<.0001
Parasitized	.044	.088	.511	.612
Silvering-stage group 2	.035	.097	−.361	.720
Silvering-stage group 3	−.075	.080	−.944	.351
Parasitized × silvering-stage group 2	−.050	.134	−.379	.707
Parasitized × silvering-stage group 3	.134	.127	1.057	.297
Cortisol:				
General:				
Time (1 h)	.315	.050	6.260	<.0001
Time (4 h)	.095	.050	1.881	.062
Parasitized	−.139	.097	−1.428	.159
Life stage (silver)	.117	.073	1.601	.115
Time (1 h) × parasitized	.255	.078	3.281	.001
Time (4 h) × parasitized	.139	.078	1.783	.077
Parasitized × life stage (silver)	−.246	.115	−2.140	.037
Females in different silvering stages:				
Silvering-stage group 2	−.069	.087	−.791	.433
Silvering-stage group 3	.142	.082	1.717	.093
Parasitized	−.285	.091	−3.120	.003
Time (1 h)	.299	.063	4.731	<.0001
Time (4 h)	.095	.063	1.509	.135
Time (1 h) × parasitized	.325	.093	3.498	.0008
Time (4 h) × parasitized	.188	.093	2.014	.047
Na$^+$/K$^+$-ATPase activity:				
General:				
Sex (males)	2.717	.755	3.599	.0006
Parasitized	1.051	.972	1.082	.284
Life stage (silver)	6.046	.818	7.395	<.0001
Parasitized × life stage (silver)	−3.506	1.308	−2.681	.0095
Females in different silvering stages:				
Silvering-stage group 2	1.973	.937	2.106	.0412
Silvering-stage group 3	5.163	.874	5.908	<.0001
Parasitized	.211	.758	.278	.782

Note. Silvering-stage group 2 consists of silvering stage III and group 3 of silvering stages IV and V. The P values of significant parameters are indicated.

nonparasitized 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 increased plasma cortisol levels. Nevertheless, in the last silvering stage even a small number *A. crassus* appeared to elicit a strong increase in plasma cortisol. Variation in plasma cortisol was also time dependent (table 2): plasma cortisol increased significantly, from 27.9 ± 5.0 to 56.5 ± 4.1 ng/mL, in the first hour after the stressor, decreasing to values close to the baseline levels 3 h later (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 than nonparasitized eels (table 2). This variation was clearly evident in the
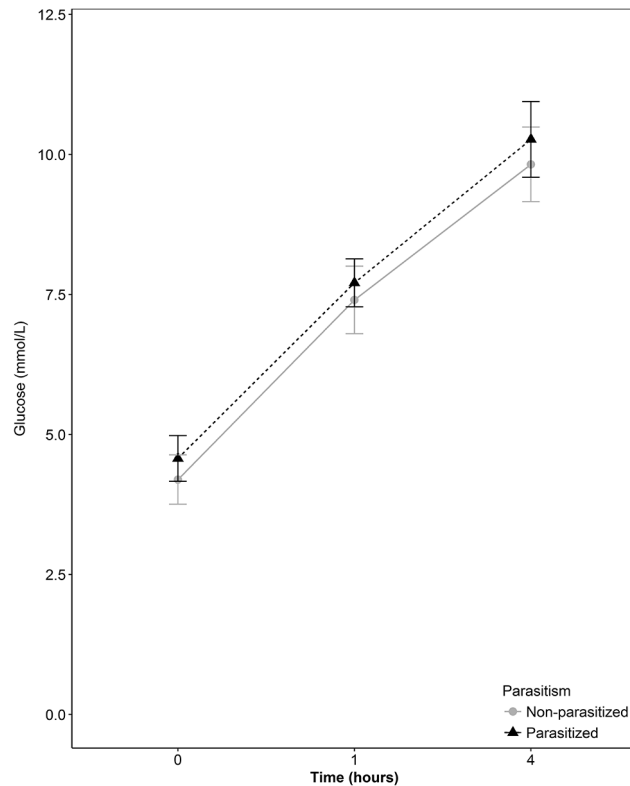
Figure 1. Variation of glucose in nonparasitized and parasitized eels through time: baseline (0 h), 1 h, and 4 h, mean ± SE.

first hour after disturbance (nonparasitized eels: 61.9 ± 7.1 ng/mL; parasitized eels: 53.9 ± 3.5 ng/mL; table 2). However, by the 4-h time point, plasma cortisol levels had recovered to near the levels seen in nonparasitized eels (4 h: 44.4 ± 8.0 ng/mL; baseline: 39.9 ± 9.7 ng/mL), but not in parasitized eels (4 h: 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 parasitism 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 higher values found in silver eels (7.97 ± 0.6 μmol ADP/mg protein/h) than in 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 nonparasitized individuals exhibited significantly higher Na^+/K^+-ATPase activity (10.26 ± 0.8 μmol ADP/mg protein/h) than parasitized ones (7.22 ± 0.5 μmol ADP/mg protein/h; fig. 3; table 2).

In female eels, gill Na^+/K^+-ATPase activity increased through the silvering stages (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); they were lower in the second group (5.79 ± 0.6 μmol ADP/mg protein/h) and lowest in the first group (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 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 mobilization of energy to provide short-term support for immediate coping activities to promote survival. Parasitized and nonparasitized eels showed similar levels of glucose, a result consistent with the Gollock et al. (2004) study on parasite-mediated stress responses to handling stressors in European eel. Moreover, we observed that the number of Anguillicola crassus in female parasitized eels led to slightly higher concentrations of blood glucose, but this was not significantly different in 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 h. 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-h time point. Despite that, cortisol recovery was still evident at the 4-h time point. When considering both males and females, the temporal variation in cortisol was similar in both parasitized and nonparasitized eels; parasitized eels exhibited a stronger response in terms of increment of cortisol than nonparasitized eels. This finding suggests that parasitism state plays an important role in the stress response of eels. The similarity in 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
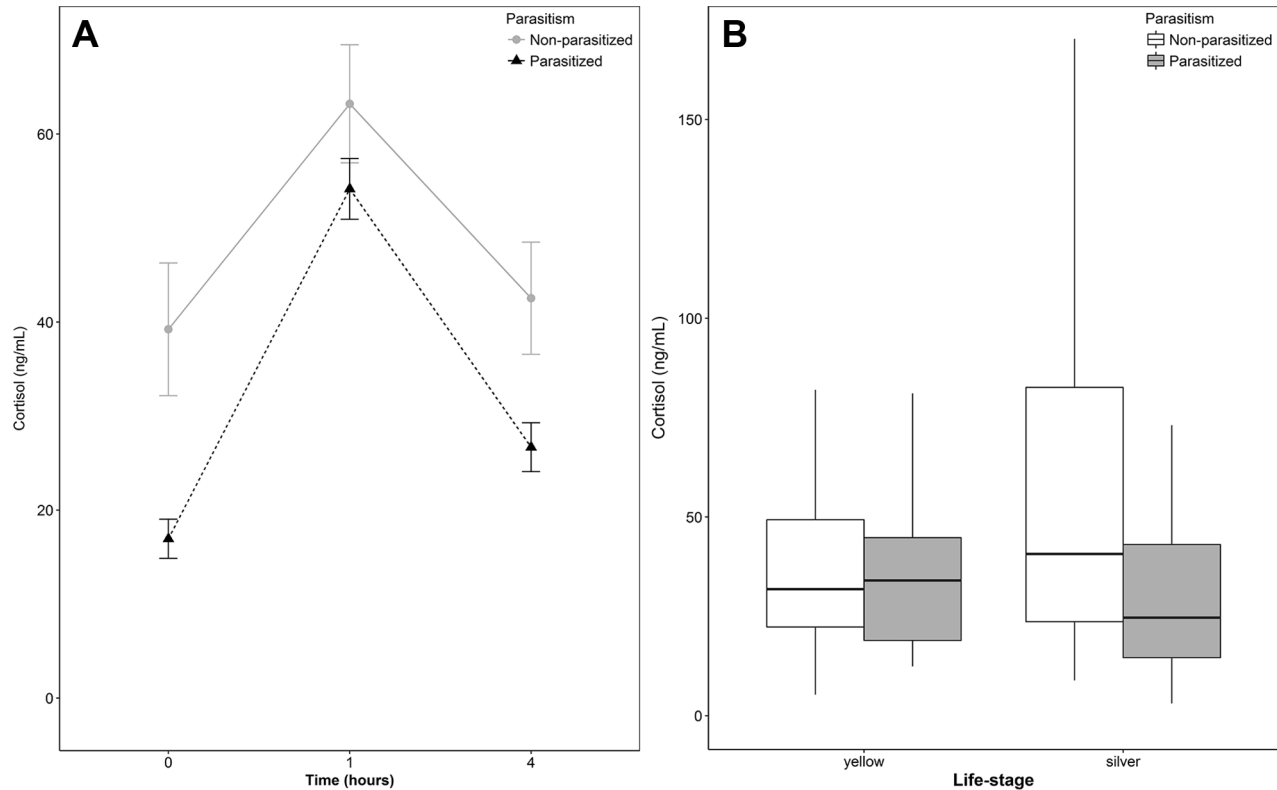
Figure 2. Variation of plasma cortisol in nonparasitized and parasitized eels: by time (baseline [0 h], 1 h, and 4 h; mean $\pm$ SE; A) and between life stages (yellow and silver; B). In B, the thick black line represents the median (50th percentile), and the bottom and top box edges the 25th (Q1) and 75th (Q3) percentiles, respectively. The bottom whisker represents the $\max(\min(x), Q1 - 1.5 \times IQR)$, where $IQR = Q3 - Q1$, whereas the top whisker represents the $\min(\max(x), Q3 + 1.5 \times IQR)$.

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 and 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* are 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 nonparasitized and parasitized specimens were strongly evident in female silver eels, even if overall no significant differences were found between the two life stages. This is evidence that there is a synergistic influence of multiple stressors on the stress response. Female eels categorized as being in the last stage of silvering (group 3) exhibited the highest levels of plasma cortisol, which may have some implications for reproductive function. Moreover, eels in this silvering stage were found to be more susceptible to the presence of parasites, as the highest levels of plasma cortisol were found even when the number of parasites was low (<4 individuals). Parasitism in the last silvering stage may negatively influence migration and reproduction of the eels.

High levels of cortisol for prolonged periods of time have been shown to play a role in energy mobilization as result of its lypolytic effect increasing free fatty acid levels, to reduce growth rate by increasing the pituitary gonadropin, to reduce immune function, and to disrupt fish reproduction function by depressing sex steroid levels (Huang et al. 1999). The implications of high

levels of cortisol for prolonged periods during exposure to chronic or frequent intermittent acute stressors on eel reproduction are therefore potentially important. The morphological and physiological transformation of yellow eels to the silver phase and the initiation of their spawning migration is triggered only when lipids constitute more than 20% of the body mass (van den Thillart and Dufour 2009; Palstra and van den Thillart 2010). Consequently, elevations in cortisol have the potential to influence both maturation and spawning migrations. Cortisol is also known to be related to SW adaptation of fish, helping them to acclimate to a hyperosmotic environment (SW) by increasing hypo-osmoregulatory capacity (Mommsen et al. 1999). Cortisol mediates SW acclimation by stimulating the gill chloride cell proliferation and Na^+/K^+-ATPase activity ensuring the transmembrane transfer of the cations Na^+ and K^+ and affecting the transepithelial movements of cations in gills (Madsen 1990*a*; McCormick 1995; Sancho et al. 1997). The stimulatory role of cortisol on gill Na^+/K^+-ATPase activity in the American eel (*Anguilla rostrata*) was previously shown by Butler and Carmichael (1972), in their study of the effects of environmental salinity 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 1990*a*), and sea trout *Salmo trutta* (Madsen 1990*b*; Fontaínhas-Fernandes et al. 2003).
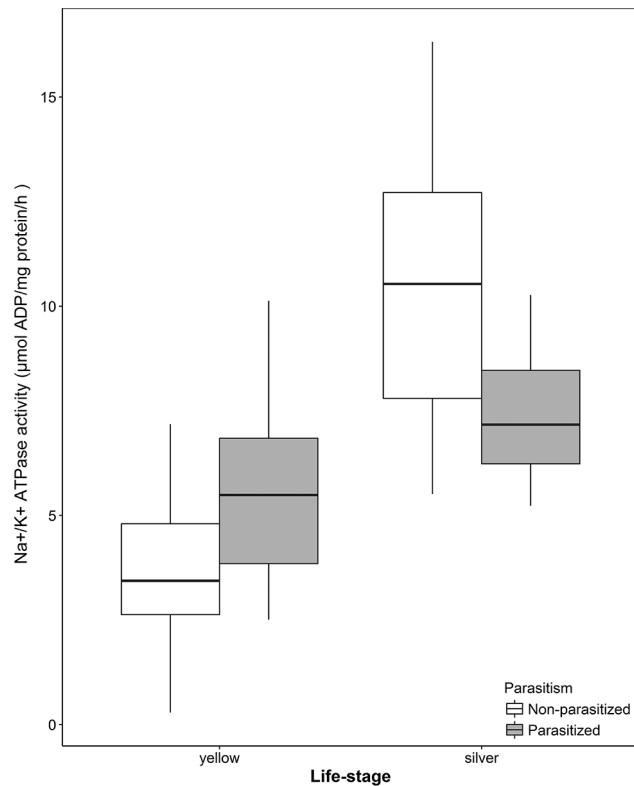
Figure 3. Gill Na$^+$/K$^+$-ATPase activity variation of nonparasitized and parasitized yellow and silver eels. The thick black line represents the median (50th percentile), and the bottom and top box edges the 25th (Q1) and 75th (Q3) percentiles, respectively. The bottom whisker represents the max(min(x), Q1 − 1.5 × IQR), where IQR = Q3 − Q1, whereas the top whisker represents the min(max(x), Q3 + 1.5 × IQR).

Gill Na$^+$/K$^+$-ATPase activity was significantly higher in silver eels than in yellow eels. This result was particularly evident in nonparasitized silver eels, since parasitized silver eels seem 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 this 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 particular in 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 endangered. Considering the well-known effects of cortisol on Na$^+$/K$^+$-ATPase activity (McCormick 1995), the highest levels of this parameter in nonparasitized silver eels may have been related to the high levels of cortisol found in these specimens. Furthermore, nonparasitized yellow eels exhibited the lowest levels of gill Na$^+$/K$^+$-ATPase activity. Once again, parasitism acting synergistically with other biotic factors affects 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; Palstra and van den Thillart 2010) and that the experiments were carried out at the end of October through the beginning of November, our findings may thus be related to 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 that the success of spawning of males becomes more susceptible and/or compromised by environmental conditions (e.g., parasite load) earlier than that of females. Gill Na$^+$/K$^+$-ATPase activity increased with silvering stage. Nevertheless, this variation was exacerbated in nonparasitized eels, which exhibited an elevation of gill Na$^+$/K$^+$-ATPase activity between silvering stages 2 and 3. Again, this points toward the idea that parasitized eels can adapt to deal with stress and that therefore their response to stress would be less severe.

Conclusions

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

Acknowledgements

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