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Using novel methodologies to examine the impact of artificial light at night on the cortisol stress response in dispersing Atlantic salmon (*Salmo salar* L.) fry

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Artificial light at night (ALAN) is gaining recognition as having an important anthropogenic impact on the environment, yet the behavioural and physiological impacts of this stressor are largely unknown. This dearth of information is particularly true for freshwater ecosystems, which are already heavily impacted by anthropogenic pressures. Atlantic salmon (*Salmo salar* L.) is a species of conservation and economic importance whose ecology and behaviour is well studied, making it an ideal model species. Recent investigations have demonstrated that salmon show disrupted behaviour in response to artificial light; however, it is not yet clear which physiological processes are behind the observed behavioural modifications. Here, two novel non-invasive sampling methods were used to examine the cortisol stress response of dispersing salmon fry under different artificial lighting intensities. Fish egg and embryos were reared under differing ALAN intensities and individual measures of stress were subsequently taken from dispersing fry using static sampling, whereas population-level measures were achieved using deployed passive samplers. Dispersing fry exposed to experimental confinement showed elevated cortisol levels, indicating the capacity to mount a stress response at this early stage in ontogenesis. However, only one of the two methods for sampling cortisol used in this study indicated that ALAN may act as a stressor to dispersing salmon fry. As such, a cortisol-mediated response to light was not strongly supported. Furthermore, the efficacy of the two non-invasive methodologies used in this study is, subject to further validation, indicative of them proving useful in future ecological studies.

Key words: Artificial light at night, Atlantic salmon, cortisol

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Introduction

Since the Industrial Revolution, and particularly over the last 60 years, the number of outdoor lights has increased rapidly across the UK. Globally, the number of artificial lights is increasing by 6% each year (Hölker *et al.*, 2010) and 3% annually in the UK (Royal Commission Report, 2009). Despite growing concerns (Royal Commission Report, 2009),

there are few systematic data demonstrating the ecological effects of artificial light at night (ALAN; Rich and Longcore, 2006). Artificial light at night is primarily, but not entirely, as a result of streetlights in public areas, along roads and highways (Gaston and Bennie, 2014). Artificial light at night is increasingly thought to alter the behaviour and/or physiology of a broad range of species and taxa (Rich and Longcore, 2006). Under ALAN, disruptions have been documented in the daily rhythms of nocturnal primates (LeTallec *et al*., 2013), bird singing behaviour (Miller, 2006) and the community composition of terrestrial invertebrates (Davies *et al*., 2012). There is, however, a notable paucity of information on the impacts of ALAN on aquatic systems (Perkin *et al*., 2011; Kronfeld-Schor *et al*., 2013). There has been a recent upsurge in interest in determining whether light may be having a detrimental impact on the health and functioning of organisms (Gaston *et al*., 2014), but successful conservation and mitigation requires that the impact of ALAN is better understood across a range of taxa and ecosystems.

While the physiological effects of artificial lights used in aquaculture systems are well known and often intended (Boeuf and LeBail, 1999), there is evidence that ALAN can cause physiological stress (increased plasma cortisol and glucose) in farmed Atlantic salmon (*Salmo salar* L.; Migaud *et al*., 2007). Artificial light at night may, therefore, impact upon the physiology of wild fish species (McConnell *et al*., 2010). Artificial lighting can be over a million times brighter than natural nocturnal illumination and, as such, changes to the lighting regimen to which animals are adapted will be likely to result in large-scale behavioural changes (Perry *et al*., 2008). Artificial light at night has been shown to have a negative effect on the behaviour of a number of important stages in the life cycle of Atlantic salmon, such as the delayed dispersal of fry (Riley *et al*., 2013, 2015) and the migration of smolts (Riley *et al*., 2012a), because these occur primarily at night (Riley and Moore, 2000; Riley *et al*., 2012b). However, it is not known how these behaviours are mediated physiologically, despite suggestions from previous studies that cortisol stress responses are mounted by teleost fish exposed to daytime aquarium light of different type, colour and intensity (Migaud *et al*., 2007). On the contrary, a recent study found no effect of ALAN on the cortisol response of European perch (*Perca fluviatilis*; Brüning *et al*., 2015).

Anthropogenic impacts on the environment have increased in number and diversified greatly to include a number of chemical, biological and physical factors (Fair and Becker, 2000) and should often be considered as stressors to the species they impact upon. Freshwater ecosystems are the most heavily debased ecosystem globally and are subject to many stressors, both anthropogenic (pollution, habitat alteration and invasive species) and as a result of climate change. For this reason, it is of the utmost importance to gain a full understanding of the influence of any anthropogenic impacts on juvenile Atlantic salmon. This study sought to apply novel methodology to test whether the behavioural delay seen in dispersing Atlantic salmon fry under ALAN (Riley *et al*., 2013, 2015) is mediated by a cortisol stress response. Previous research suggests strong species specificity regarding the ontogeny, magnitude and duration of the cortisol response (Feist and Schreck, 2002; Fanouraki *et al*., 2011). Specifically, the developmental stage at which fish are able to mount a response to stressors is dependent upon both species and environment (De Jesus and Hirano, 1992; Barry *et al*., 1995; Stephens *et al*., 1997; Stouthart *et al*., 1998; Feist and Schreck, 2002; Jentoft *et al*., 2002; Auperin and Geslin, 2008). Two methodologies were applied to this ecological concern: firstly, deployable passive samplers were used to determine the population-level response; and secondly, individual measures of stress were assessed using static water samples. If these studies provide viable methods for non-invasively determining the stress response of Atlantic salmon, and other vulnerable freshwater fish species, to a given external stimulus, their use in conservation research will be an extremely interesting subject for further validation.

Materials and methods

Experimental design

Experimental work was conducted at the Centre for Environment Fisheries and Aquatic Science (Cefas) laboratory aquarium, Lowestoft, UK (52°27′33″N, 1°44′22″E). For full details of the aquarium set-up see Riley *et al*. (2015). Daytime lighting was representative of natural daylight (1177–728 lux, 14 h light–10 h dark), provided by daylight-mimicking, low-pressure mercury discharge fluorescent lamps. Nocturnal illumination was provided by a metal halide streetlight (Philips Master Cosmo White) mounted in a luminaire (Philips 'iridium series' opti-C unit). Neutral density filters were attached to the streetlight to reduce the intensity to levels measured in the field without altering the spectrum. The incubators (test tanks) were 1.7 m below the streetlight, positioned to create different nocturnal light intensities at the surface of the water, as follows: 8, 4, 2, 1 and 0.1 lux, with two replicates at each light level treatment. The 0.1 lux level is representative of moonlight (Riley *et al*., 2013) and was considered a control treatment.

On 22 February 2012, 500 Atlantic salmon fertilized eggs (development ~260 deg days) sourced from wild-caught broodstock (Kielder Hatchery, Northumberland, UK) were implanted into each of ten 75 l black plastic deep substrate incubators. Eggs developed in the gravel (as in the wild) under the different nocturnal light treatments until they hatched, emerged from the gravel and swam up into the water column to disperse. Fish dispersing through the outflow of the incubators were retained in mesh collecting boxes over a 9 day period of 24 h monitoring (10–19 April 2012; see Riley *et al*., 2015).

The stress status of these fry from different light treatments was assessed by non-invasive measurement of cortisol released into the surrounding water via the gills, a technique previously applied to salmon, although not at this early life-history

stage (Ellis *et al.*, 2007, 2012; Kittilsen *et al.*, 2009). The surrounding water, into which fish release free steroids (including cortisol) via the gills, is the most common matrix used for non-invasive monitoring of fish hormones (see Ellis *et al.*, 2013). The following two independent sampling methods were used to collect free cortisol: (i) water from the flow-through incubators; and (ii) static water from separate containers.

Population cortisol sampling from the incubators

Owing to the low expected concentration of cortisol in the water of the incubator, direct point sampling was not attempted; instead, a novel method was used, in which cortisol is absorbed by a passive sampler to provide an integrated hormonal history of a fish population over time (Scott and Ellis, 2007). Although it is assumed that passive samplers absorb steroids at a rate dependent upon their concentration in the water, their use for cortisol has not yet been validated. However, as all samplers were treated identically in all experimental incubators, for the purpose of this study, any differences in the recovery rates of the individual Polar Organic Chemical Integrated Samplers (POCIS) will not have influenced the overall results. It must also be recognized that tank-specific factors, such as water flow (mean 252.54 l h^{-1}, minimum 200 l h^{-1} and maximum 300 l h^{-1}) and biofilm growth on samplers, could affect uptake rate.

The POCIS were prepared by a standard method (see Alvarez *et al.*, 2004), and one was deployed in each incubator, in similar positions on the surface of the gravel. Each incubator was implanted with 500 fertilized eggs at the start of the experiment. The POCIS were deployed when it was calculated that the embryos had absorbed the majority of their yolk sac and were close to dispersal (based on predicted development using degree-days) to limit uptake of any residual maternal cortisol and reflect any cortisol response to treatment. The POCIS were placed in each of the incubators on 25 March 2012, removed on 21 April 2012 and stored at –20°C. Cortisol was extracted from the sorbent by methanol elution, followed by solid-phase extraction (see Alvarez *et al.*, 2004) with ethyl acetate as the final eluate. Extracts were stored at –20°C until evaporation (under nitrogen) and reconstitution in 1 ml of radioimmunoassay (RIA) buffer for assay.

Individual cortisol sampling using separate static containers

In this method, individual fish were removed from the mesh collecting boxes and placed in a small container of clean water for a standard time. Sampling of water from static containers has been used to assess the release of a variety of steroids in a diverse range of fish species (Ellis *et al.*, 2013). This technique, however, has not previously been applied to fish <0.5 g in weight, at an early ontogenetic stage. As such, the static sampling technique suffers from the potential disadvantage that the procedure itself (handling and confinement) may affect the amount of cortisol released into the water; this impact can be limited by restricting the duration of the collection period.

Immediately after entry into the mesh collecting box, individual fry were netted and placed in a beaker containing 10 ml of clean inflow water. After 30 min, the fry were removed and weighed. Water samples were placed temporarily on ice (maximum 2 h) before storage at –20°C. Thawed water samples were passed through solid-phase extraction cartridges [Sep-pak® Plus, C18 (360 mg), Waters Ltd., UK], the cortisol was retrieved by ethyl-acetate elution, and this eluate was evaporated under nitrogen before reconstitution in 0.5 ml of RIA buffer for assay (Ellis *et al.*, 2004). Additional quality-control samples (blank samples of clean inflow water and cortisol-spiked inflow water samples) were prepared and processed contemporaneously. The spiked samples were prepared by addition of cortisol (Sigma Aldrich, UK) dissolved in ethanol; the genuine (rather than nominal) spike was determined by RIA of equivalent 50 µl aliquots of the spiking solution (after evaporation under nitrogen and reconstitution in RIA buffer).

As this method has not previously been applied to small fish at an early ontogenetic stage, three individual fry were placed in beakers of clean inflow water for 1 h (ascribed as positive control, PC). This was to determine whether fry at the dispersal stage mount a cortisol response to a known stressor of older fish (i.e. handling and confinement). Although a number of species appear able to synthesize cortisol at the time of hatching, the development of a cortisol response to stressors appears later in development (Jentoft *et al.*, 2002). It has previously been demonstrated that Atlantic salmon are not capable of mounting a stress response until 72 days post-hatching at 6°C (432 deg days; Neachev *et al.*, 2006).

Radioimmunoassay

Amounts of cortisol in extracts were measured with an in-house radioimmunoassay (Ellis *et al.*, 2004). A 100 µl aliquot of extract (or dilution of extract) was added to duplicate glass tubes, and nine standards [2, 4, 8, 16, 32, 64, 125, 250 and 500 pg (100 µl)$^{-1}$] were made up by serial dilution. To all tubes was then added 100 µl of assay buffer containing ~5700 d.p.m. of tritiated cortisol and sufficient antibody to bind 48% of the radiolabel in the absence of radioinert steroid. Tubes were left to equilibrate overnight (≥16 h, 4°C), and unbound steroid was removed by addition of dextran-coated charcoal (30 min on ice). After centrifugation (12 min at 1000*g*), the liquid was decanted and the remaining tritiated cortisol counted (Beckman LS6500 scintillation counter). Amounts of cortisol in extracts were determined from the standard curve. The intra-assay coefficient of variation was 6%, and the inter-assay coefficient of variation has previously been assessed at 11% (Ellis *et al.*, 2004).

Data analysis

The cortisol RIA provides estimates of the amount of cortisol (in picograms) in an extract. For statistical comparisons,

individual water cortisol sample values were converted to a release rate (in picograms per gram per hour) to standardize for fish size (Ellis *et al.*, 2013); population water cortisol values (from POCIS samples) were not converted because it is not known how sampler uptake relates to the amount in the water. Differences in fish biomass and exposure time between tanks and treatments may therefore affect the population water cortisol values. Statistical analyses were conducted in R (version 2.13.2; R Development Core Team, 2012). Factors (light, fish mass, day and incubator) influencing the cortisol release rate of the dispersing fry were evaluated using generalized linear models, refined using Akaike information criterion comparisons between candidate model structures, combined with deletions of non-significant terms to identify a minimal adequate model containing only significant factors.

Results

A total of 297 fish were sampled individually during the dispersal period of 9 days. A generalized linear model revealed no significant differences in the body mass of sampled fish over time ($F_{1,31} = 1.722$, $P = 0.199$) or between individual experimental incubators ($F_{9,31} = 0.942$, $P = 0.504$) or light intensities ($F_{1,36} =$, $P = 0.538$). A representative subset of 48 treatment samples from across the sampling period and experimental incubators was initially assayed, plus three PC and six quality-control samples. The remaining samples were not processed owing to the lack of significant treatment effects.

Population cortisol sampling from the incubators

Cortisol was readily measurable in all 10 POCIS samples, falling in the middle of the RIA standard curve (median 26 pg). Light intensity had a marginally significant influence on the cortisol content of the POCIS samples, with cortisol content in the POCIS sample shown to increase as light intensity increased (Fig. 1).

Individual cortisol sampling using separate static containers

Cortisol was readily measurable in the three PC samples (confined for 1 h), with samples falling in the middle of the RIA standard curve (median 34 pg). The amounts of cortisol in the 48 treatment samples (confined for 30 min) were lower, largely falling within the upper third of the RIA standard curve (median 15 pg). The three quality-control blank samples returned zero or negligible cortisol values. The three spiked quality-control samples indicated highly variable recoveries of 125–350% of the cortisol spike (21 pg) added.

Cortisol release rates (i.e. adjusted for duration) were significantly greater in the PC, actively stressed, fry than treatment fry ($F_{1,43} = 12.37$, $P = 0.001$), indicating that the PC fry mounted a cortisol response to handling and/or confinement (Fig. 2). Within the light treatment groups, there was no significant difference in cortisol release rate between the control (0.1 lux) and elevated light groups (1, 2, 4 and 8 lux, $F_{4,36} = 2.006$, $P = 0.114$). There was a significant effect of sampling day on cortisol release rates ($F_{1,40} = 11.351$, $P = 0.002$), with cortisol release rates decreasing in fish sampled later in the study period (Fig. 3).

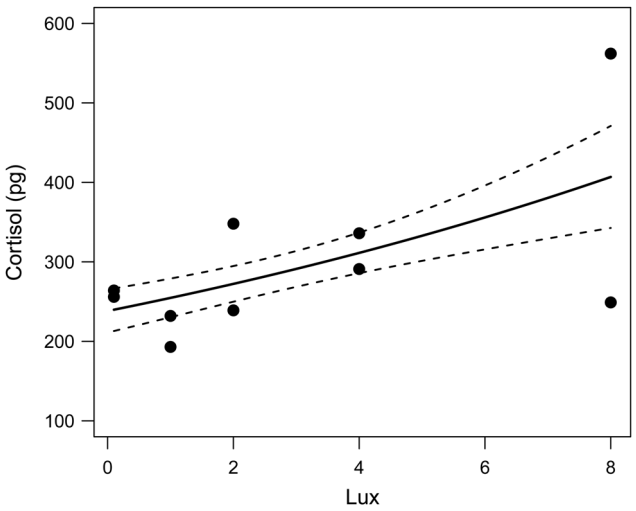

Figure 1: Amounts of cortisol (in picograms) retrieved from the Polar Organic Chemical Integrated Samplers at each of the experimental light intensities (in lux), with a line of best fit (±1 SEM) generated from a generalized linear model of cortisol (dependent variable) in relationship to light intensity (independent variable) using a γ error family and a log-link function ($F_{1,8} = 5.979$, $P = 0.0415$).

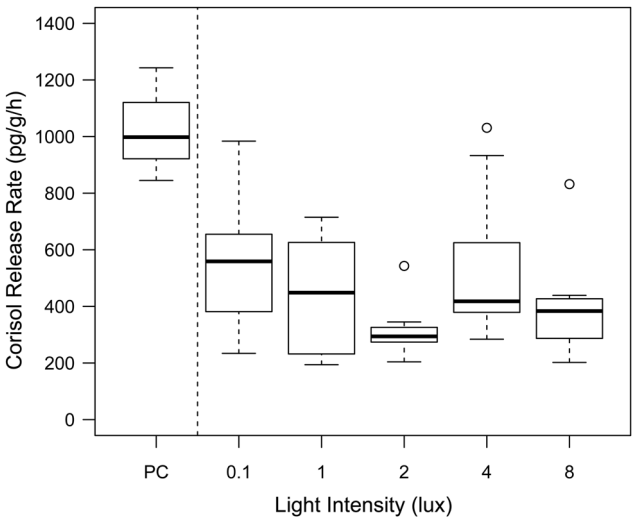

Figure 2: Cortisol release rate of individual fry in relationship to nocturnal light level (0.1–8 lux) in each of the experimental treatments ($n = 48$; number of samples from each treatment: 0.1 lux $n = 9$, 1 lux $n = 8$, 2 lux $n = 8$, 4 lux $n = 9$ and 8 lux $n = 10$) and in the positive control (PC, $n = 3$). Significantly different cortisol release rates in the actively stressed fry ($F_{1,43} = 12.37$, $P = 0.001$) are indicated (*).

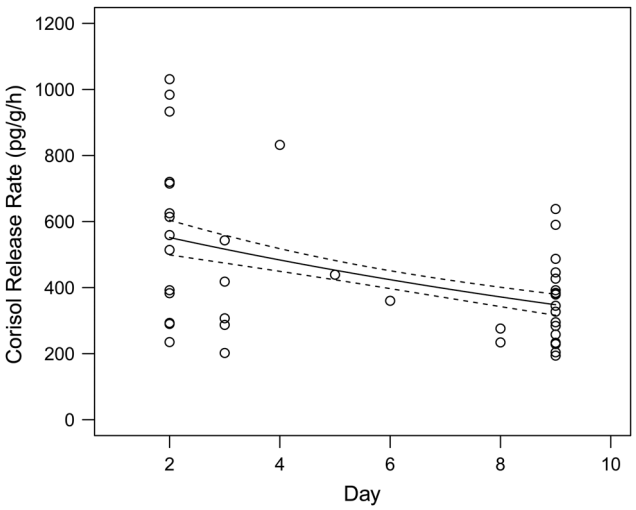

Figure 3: Cortisol release rate declined significantly across the nine sampling days. The line of best fit (±1 SEM) was generated from the generalized linear model with a γ error family and a log-link function ($F_{1,36} = 9.793$, $P = 0.003$).

Discussion

The work described here demonstrates two important points. Firstly, the results of this study showed no significant effect of ALAN, at varying intensities, on the cortisol stress response of individually sampled fry. As such, the results do not clearly support the hypothesis that the previously observed delay in dispersal behaviour caused by ALAN (Riley *et al.*, 2013, 2015) is associated with a cortisol stress response. The results show that ALAN did not significantly affect the levels of cortisol sampled in individual fish, although there was a marginally significant effect in the population data as sampled using the POCIS method. The lack of a significant result seen here suggests that although the dispersal behaviour of Atlantic salmon fry is disrupted by ALAN, this behaviour does not appear to be mediated by a cortisol stress response. However, an alternative explanation for the lack of response seen to dispersing under ALAN is that the very act of dispersing may itself produce a stress response in the fry, which could have masked any treatment effect. There was, however, a significant effect of sampling day on the cortisol release rate of the dispersing fry, with fish dispersing later in the sampling period having a lower cortisol release rate than those dispersing earlier. The finding that fry dispersing under ALAN had a delayed dispersal compared with the control fry (Riley *et al.*, 2015), but with no increase in cortisol seen, could possibly suggest acclimation to ALAN; as such, the cortisol response of an individual fry may be negatively correlated with the length of time they are exposed to the light. The relatively small sample size limits the power of these tests, and further samples would clarify the result.

Secondly, we have demonstrated that non-invasive sampling of cortisol status is possible at this early stage in salmon development. Previous studies examining the ontogeny or presence of a cortisol response have measured total body cortisol or plasma cortisol levels. Passive sampling and separate static container sampling proved to be viable non-invasive techniques for investigating the cortisol status of fry at the dispersal stage. Nevertheless, additional refinement and validation is required (see Ellis *et al.*, 2013) to ensure that methodological issues are not masking or confounding experimental conclusions.

For the passive sampling, it remains to be seen that cortisol uptake is related to the concentration in the water, and corrections for changes in biomass during the exposure period must be incorporated. For the separate static container method, an appropriate duration of confinement must be determined. The duration needs to be short enough to minimize any impact of handling and confinement on cortisol release, but long enough to ensure that sufficient cortisol is collected for assay. The observed difference in release rate between the 30 and 60 min periods of confinement indicates that this initial selection (based upon previous studies) was appropriate. Nevertheless, the unexplained high and variable recovery of the spiked samples within this study needs to be addressed because it may be associated with the low water volume and amounts of cortisol.

A final point to note is that through the positive controls used, this is the first study to reveal a cortisol stress response to an external stimulus in Atlantic salmon at this early stage of development. Juvenile Atlantic salmon were previously thought to be unable to mount a stress response except in response to an induced stimulus, such as injection of adrenaline (Neachev *et al.*, 2006). Here we demonstrate that, in line with other salmonid species (*Oncorhynchus mykiss*, Barry *et al.*, 1995, Karakatsouli *et al.*, 2008; *Oncorhynchus tshawytscha*, Feist and Schreck, 2002), Atlantic salmon can mount a cortisol response to an external stimulus at the dispersal stage.

In summary, further work is required to elucidate whether the physiology of dispersing Atlantic salmon fry is influenced by ALAN; however, the techniques described here provide valuable non-destructive methodologies to assess the hormonal status of fish at this early ontological stage. The methodologies have proved capable of detecting cortisol in dispersing salmon and will no doubt prove useful in future ecological studies subject to subsequent validation, as outlined previously. Artificial light at night is only one of many anthropogenic pressures that impact upon freshwater ecosystems, and a full understanding of the physiological effect that these pressures have on freshwater species is required for conservation and management purposes. Furthermore, the methodologies provided in the present study allow for the response to anthropogenic stressors to be characterized over time and could, for example, determine whether acclimatization occurs when fish are exposed to these stressors for a sustained period. In the case of ALAN, an understanding of whether acclimatization or a reduced response with age occurs in salmon would be of use when attempting to mitigate its impact.

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