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Effects of temperature and feeding regime on cortisol concentrations in scales of Atlantic salmon post-smolts

Christina O'Toole [a,b,1,*], Philip White [a], Katie Thomas [a], Niall O'Maoiléidigh [c], Per Gunnar Fjelldal [d], Tom Johnny Hansen [d], Conor T. Graham [a], Deirdre Brophy [a]

[a] Marine and Freshwater Research Centre, Atlantic Technological University, Galway, Ireland
[b] Ecosystem Approach to Fisheries Management, Fisheries Ecosystem Advisory Services, Marine Institute, Rinville, Oranmore, Co. Galway, Ireland
[c] Fisheries and Ecosystems Advisory Services, Marine Institute, Furnace, Newport, Co. Mayo, Ireland
[d] Institute of Marine Research (IMR), Matre Aquaculture Research Station, Matredal, Norway

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ABSTRACT

Anadromous fish are vulnerable to anthropogenic and environmental stressors including pollution, rising temperatures, and changes in food availability. Knowledge of how fish respond to specific stressors aid our understanding of population declines and inform predictions of how populations will react to future environmental change. There is increasing interest in using cortisol measurements from fish scales to measure chronic stress in fish, however the effects of increased metabolic rate on scale cortisol dynamics must also be considered. This study examines the effect of temperature and feeding conditions on scale cortisol in Atlantic salmon (*Salmo salar*). Post-smolts were subjected to three different temperatures (6, 10.5 and 15 °C) and four feeding/starvation treatments over a 12-week period. Females held at 6 and 15 °C had significantly higher scale cortisol levels than those held at 10.5 °C, while rearing temperature had no effect on scale cortisol in males. The increase in scale cortisol at 6 °C indicated that temperature related differences were not driven solely by metabolic rate. A two-week starvation period produced an increase in scale cortisol in males and females held at 10.5 °C but not at 6 °C or 15 °C. The study demonstrates that scale cortisol fluctuations can be detected in a low amount (~10 mg) of Atlantic salmon scales for monitoring of the physiological stress response. Scale cortisol shows potential for monitoring physiological responses during the marine phase in Atlantic salmon. However, the influence of environmental stressors on scale cortisol needs to be better understood, with consideration for sex-specific and interactive effects.

1. Introduction

Wild fish are regularly exposed to environmental factors that may cause stress, including varying food availability, risk of predation, infection, temperature changes, and pollutants (Schulte, 2014). Many of these stressors are influenced by climate change, which is expected to have a negative effect on numerous fish species (Free et al., 2019), with impacts varying by species and life stage (Barbeaux and Hollowed, 2018). Chronic exposure to stressors can be detrimental to the long-term coping capacity of fish, as prolonged physiological stress has consequences for growth, survival, and reproduction (Alfonso et al., 2021). Physiological responses to climate change and other anthropogenic pressures have consequences for the management of commercial fisheries and the design of conservation measures to protect culturally, ecologically and economically important species such as the Atlantic salmon (*Salmo salar*, L.).

Cortisol, a steroid hormone released in response to stress, is an important biomarker that can be used to examine the effect of anthropogenic changes in the environment on fish (Sadoul and Geffroy, 2019) and can serve as a sensitive indicator to the severity of stressors (Baker and Vynne, 2014). Cortisol measurement is an essential tool for investigating coping mechanisms in fish species and is often used in an aquaculture setting (Barton and Iwama, 1991; Barton, 2002; Bertotto et al., 2010). Cortisol is usually measured in blood plasma (Cook, 2012),

* Corresponding author.
E-mail address: Christina.ODonnell@marine.ie (C. O'Toole).
[1] Present address: Ecosystem Approach to Fisheries Management, Fisheries Ecosystem Advisory Services, Marine Institute, Rinville, Oranmore, Co. Galway, H91 R673, Ireland

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however this approach is impractical for wild fish which can be widely dispersed and highly sensitive to disturbances (Pankhurst, 2011). As well as being invasive, blood sampling can interfere with real-time cortisol levels and provides a measure of acute rather than chronic stress (Bertotto et al., 2010; Cook, 2012; Aerts et al., 2015; Sadoul and Geffroy, 2019). This restricts the use of cortisol as a marker of stress, particularly in wild populations due to the capture and sampling requirements of obtaining a timely blood sample (Pankhurst, 2011).

Fish scales incorporate glucocorticoids, including cortisol, as they grow and provide an ideal matrix for monitoring chronic circulating cortisol levels due to their slow, persistent growth, and their ease of sampling (Aerts et al., 2015). Cortisol in fish scales becomes elevated in response to chronic stress (Aerts et al., 2015; Laberge et al., 2020), is not incorporated quickly enough to be affected by acute stress during sampling, and remains elevated longer than plasma cortisol (Carbajal et al., 2019; Laberge et al., 2020). Scale cortisol levels have been shown to increase in response to chronic stress in milkfish (*Chanos chanos,* Forsskål (Hanke et al., 2019), rainbow trout (*Oncorhynchus mykiss,* Walbaum (Carbajal et al., 2019), goldfish (*Carassius auratus,* L. (Laberge et al., 2020)) and European sea bass (*Dicentrarchus labrax*, L. (Goikoetxea et al., 2021)). Scale cortisol has been proposed as an indicator of thermal stress and an appropriate biomarker for examining stress responses of fish to environmental change (Goikoetxea et al., 2021).

As a hormone involved in the regulation of normal physiological processes including growth, cortisol production will increase at high temperatures due to the increased rate of metabolic reactions (Bonga, 1997; Mommsen et al., 1999; Schulte et al., 2011). Disentangling this effect from increases in cortisol generated by a chronic stress response to elevated temperature is difficult (Alfonso et al., 2021). Basal levels of cortisol in unstressed fish have been shown to increase with temperature both between and within species (Alfonso et al., 2023), suggesting that metabolic rate is an important driver and increases in scale cortisol at elevated temperatures can coincide with an increase in growth rate (Hanke et al., 2019). Goikoetxea et al. (2021) demonstrated that during short-term (4-day) exposure to elevated temperatures, increases in plasma cortisol in European sea bass larvae were higher than levels predicted due to the increase in metabolic rate. Over longer time periods at high temperatures it is not known if cortisol concentrations in scales, plasma or other matrices reflect metabolic rate or indicate mild physiological stress.

Anadromous fish such as Atlantic salmon are exposed to many environmental stressors during their dispersive life history and are particularly vulnerable to the effects of climate change (Graham and Harrod, 2009; Lassalle and Rochard, 2009). In recent decades, wild Atlantic salmon stocks in Europe have been declining. This decline has been attributed to many interacting factors including climatic conditions (Almodóvar et al., 2018; Nicola et al., 2018), food availability (Renkawitz et al., 2015), interactions with farmed salmon (McGinnity et al., 2009; Vollset et al., 2016; Forseth et al., 2017) and illegal fishing (Dadswell et al., 2021). Future climate change could pose a further threat to the species; predictive modelling indicates a contraction of Atlantic salmon distribution by the end of the century (Lassalle and Rochard, 2009). The post-smolt period, when salmon first migrate into the marine environment, is considered a critical stage during which growth and survival has declined dramatically (Friedland et al., 2009; Soto et al., 2018). If exposure to elevated temperatures, reduced food availability or other stressors during the post-smolt period is reflected in scale cortisol levels, this biomarker could be used to examine physiological stress responses to environmental change during the marine migration. Scales are routinely sampled for many salmon populations across Europe and multi-decadal collections exist (Tray et al., 2020). These archives provide material to investigate physiological stress responses to environmental change in salmon across broad temporal and spatial scales.

This study used an artificial rearing experiment to investigate if temperature and feeding conditions during the post-smolt stage of Atlantic salmon affect scale cortisol levels. If temperature-related variation in scale cortisol primarily reflects increasing metabolic rate at high temperatures, then we would expect to see an increase when fish are held above optimal temperatures, but not when they are held below optimal temperatures or when feeding is restricted. If fluctuations in metabolic activity associated with normal growth and development were the main driver of changes in scale cortisol we would expect scale cortisol levels to also covary with measures of fish growth, size and condition. If on the other hand scale cortisol levels can provide an indicator of chronic stress they should fluctuate in response to a range of stressors, including reduced temperature and restricted feeding in addition to elevated temperature.

The specific aims of this study were:

(1) To test if cortisol could be reliably detected in the scales of Atlantic salmon post-smolts
(2) To investigate if cortisol levels in the scales of Atlantic salmon post-smolts are related to temperature and feeding conditions
(3) To examine the role of metabolism in determining scale cortisol levels by investigating the relationship between scale cortisol and fish size, growth and condition

2. Materials and methods

2.1. Rearing experiment

The fish used for this experiment were one-year-old (1+) Atlantic salmon post-smolts from a Norwegian hatchery strain (AquaGen, Trondheim, Norway), reared as part of a separate study by Thomas et al. (2019). The fish were reared in 6 °C ambient freshwater at the Institute of Marine Research in Matredal, Norway, and were transferred to seawater at the beginning of the experiment, having completed smoltification. For the duration of the 12-week experiment, the fish were kept in 1 × 1 m tanks with a salinity of 35‰ and a dissolved oxygen level of over 90%. The photoperiod used (24 h of light and 0 h of darkness (LD 24:0)) represented the conditions experienced in the Norwegian Sea in the month of May. Post-smolts were kept at three temperatures: 6, 10.5 and 15 °C. Temperatures were gradually increased from the initial 6 °C over 48 and 96 h to reach 10.5 and 15 °C respectively, to avoid the shock of a fast temperature increase. Four feeding treatments were used in the experiment: the control group were fed to excess for the duration (Constant), one group was starved for 7 days in week 8 (ST7), another group was starved for 14 consecutive days in weeks 7 and 8 (ST14), and the final group was starved for 28 days total in 7-day increments (weeks 4, 6, 8 and 10; ST28). A fractional factorial design was used; all feeding treatments were crossed with the 10.5 °C temperature treatment, and two feeding treatments (Constant and ST14) were crossed with the 6 and 15 °C temperature treatments (Fig. 1). A total of 16 tanks with 60 fish

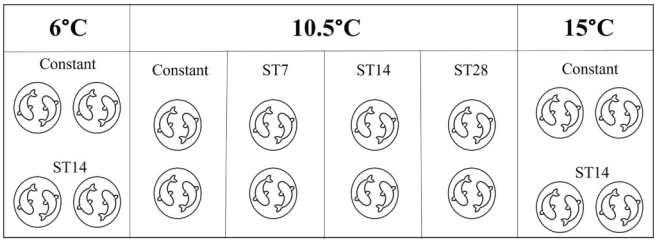

Fig. 1. Schematic of the rearing experiment for *Salmo salar* post-smolts. Each fish-pond icon represents a tank, with two replicate tanks for each temperature and feeding treatment combination (16 tanks total; some 10.5 °C tanks are duplicated between the two experiments). Three temperature treatments were used (6, 10.5, and 15 °C), and four feeding treatments: Constant (control group that was fed constantly), ST7 (starved for 7 days during week 8), ST14 (starved for 14 consecutive days in weeks 7 and 8) and ST28 (starved for 28 days total; 7 days in each of weeks 4, 6, 8 and 10).

per tank were included in the experiment with two replicate tanks held at each temperature × feeding combination (Fig. 1). Further detail of the experimental set-up can be found in Thomas et al. (2019). All experimental work was conducted ethically in accordance with the Norwegian Regulation on Animal Experimentation 1996.

2.2. Post-smolt sampling

Three fish were randomly selected from each tank and killed at the beginning of each experimental week using a lethal dose of 2-Phenoxyethanol solution (0.6 ml l^{-1}). Fork length (as an estimate of skeletal growth) and weight measurements were recorded. Scale samples were removed from each fish from the standard scale sampling location on the left side posterior of the dorsal fin and above the lateral line (Shearer, 1992), then stored in paper envelopes.

Scales were stored at room temperature in a sealed airtight container for six years prior to analysis. A separate study by the authors confirmed that cortisol in scales remains stable during storage relative to concentrations in freshly sampled scales (O'Toole, 2022). To investigate the effect of temperature and feeding conditions on cortisol in scales, a subset of scale samples was prepared for cortisol analysis ($n = 156$). The investigation was conducted in two parts. In part 1, the effects of temperature and feeding regime were examined using samples from all three temperature treatments (6, 10.5 and 15 °C) held under constant and interrupted (ST14) feeding conditions using scales collected in weeks 2, 9 and 12 of the experiment. All of these fish had been fed constantly until week 7 when the first starvation period was initiated for the ST14 treatment. Therefore, scale samples collected in week 2 were all assigned to the Constant feeding treatment. In part 2, the effect of feeding conditions on scale cortisol were examined in more detail using scales sampled in weeks 9, 11 and 12 from the four feeding treatments (Constant, ST7, ST14 and ST28) held at a temperature of 10.5 °C. The number of samples analysed from each temperature and feeding treatment is shown in Table 1. There was some overlap in the samples used in parts 1 and 2; the fish from the Constant and ST14 treatments at 10.5 °C are included in both parts.

2.3. Scale preparation

Prior to analysis, all scales from each sample were cleaned by soaking for a minimum of 3 min in ultrapure water (18.2 mΩ) and then manipulated using clean forceps to gently remove mucus before cleaned scales were allowed to air dry. Each clean, dry scale was cut into small pieces in a glass petri dish using a scalpel. Cut scales were placed into pre-weighed centrifuge tubes and the weight (target weight 10 mg) of each sample was recorded.

2.4. Scale cortisol analysis

A reference material was fabricated by cleaning and cutting the scales (8.63 g) from a farmed adult salmon in accordance with the above method and then spiking with a solution containing 76 ng.g^{-1} of cortisol (Cerilliant). The spiked scales were kept in the freezer at −18 °C prior to analysis as a reference material.

The extraction procedure was adapted from Aerts et al. (2015), with each extraction batch including 14 post-smolt scale samples, a procedural blank that did not contain any scales, and 10 mg of the spiked scale standard in accordance with quality control procedures. 100 mg of an internal standard containing 157.3–160.1 ng.g^{-1} cortisol-d_4 (Cerilliant) was added to all samples and to the calibration standards to account for variability in the extraction and analysis processes. The internal standard was added dropwise by weight, followed by 8 ml of ROMIL Methanol 215 SpS (>99.9% assay). Samples were vortexed (VWR S42 and S040 Lab Dancer) for 30 s and then placed on a shaker (Stuart Scientific Flask Shaker SF1) at 800osc/min for one hour at ambient temperature. Samples were then placed in a Thermo Scientific Megafuge 8 benchtop centrifuge for 10 min at 3260 *g*. The supernatant was carefully pipetted off into glass universal tubes and evaporated to almost dryness under nitrogen at 60 °C in a TurboVap LV Concentration Workstation, until approximately 0.5 ml remained in each sample. Samples were reconstituted in 5 ml of H_2O/MeOH (80:20, *v*/v). GracePure SPE C_{18}-Max (500 mg, 6 ml) solid phase extraction (SPE) columns were conditioned on vacuum with 3 ml MeOH followed by 3 ml of ultrapure water. Samples were loaded using glass Pasteur pipettes, followed by 4.5 ml of H_2O/MeOH (65:35, v/v). The samples were eluted

Table 1
Mean and range of scale cortisol content (ng/g), average fork length (cm), and sex of *Salmo salar* post-smolts at each temperature and feeding treatment combination at weeks 2, 9, 11 and 12 of the 12-week experiment. The four feeding treatments were as follows: Constant (control group that was fed constantly), ST7 (starved for 7 days during week 8), ST14 (starved for 14 consecutive days in weeks 7 and 8) and ST28 (starved for 28 days total; 7 days in each of weeks 4, 6, 8 and 10). Samples below the LOD (limit of detection) of the GCMS ($n = 5$) were estimated using regression. * denotes a group that includes an estimated value.

Week	Feeding	Temp. (°C)	Tanks (N)	Fish (N)	Cortisol (ng/g)		Length (cm)		Sex	
					Mean	Range	Mean	Range	M	F
2	Constant	6	4	12	48.86	10.91–90.61	20.2	19.5–21.3	6	6
		10.5	4	12*	18.63	5.09–45.75	19.8	19.0–20.6	4	8
		15	4	12	51.60	20.09–86.40	20.3	17.9–22.1	7	5
9	Constant	6	2	6	69.36	9.05–126.47	21.3	20.3–22.5	4	2
		10.5	2	6*	32.92	5.20–73.21	23.2	21.9–25.0	4	2
		15	2	6	69.35	47.69–118.92	24.4	22.7–26.8	2	4
	ST7	10.5	2	6	49.72	6.81–78.99	23.8	22.9–24.5	3	3
		6	2	6*	87.91	5.95–169.00	20.6	19.2–22.0	3	3
	ST14	10.5	2	6	54.63	5.28–124.31	22.8	21.5–24.1	3	3
		15	2	6	27.65	7.77–71.72	23.3	21.3–25.0	3	3
	ST28	10.5	2	6	49.29	27.97–91.51	23.1	20.5–25.7	2	4
11	Constant	10.5	2	6	69.16	21.95–168.42	24.5	22.6–27.0	4	2
	ST7	10.5	2	6*	35.72	6.58–63.95	23.9	23.0–25.3	3	3
	ST14	10.5	2	6	67.64	29.30–215.93	23.5	21.5–26.1	5	1
	ST28	10.5	2	6	54.31	31.32–74.68	22.6	20.8–25.0	3	3
12	Constant	6	2	6	37.78	6.00–50.11	21.4	19.9–22.4	4	2
		10.5	2	6	35.17	6.43–91.11	24.6	23.9–25.1	2	4
		15	2	6	48.23	16.26–85.50	26.2	25.2–27.5	3	3
	ST7	10.5	2	6	49.63	22.42–92.74	24.1	21.5–27.1	3	3
		6	2	6	59.27	5.98–140.86	21.0	19.2–22.4	2	4
	ST14	10.5	2	6	90.28	21.00–162.94	24.4	23.0–25.6	2	4
		15	2	6	57.37	20.99–191.16	24.9	22.6–27.1	2	4
	ST28	10.5	2	6*	48.76	7.14–65.36	23.8	22.6–26.0	3	3

with 3.5 ml of H_2O/MeOH (20:80, v/v) and evaporated to almost dryness (approximately 0.5 ml) at 60 °C under nitrogen before being transferred to gas chromatography (GC) vials for analysis. Any samples with a volume less than the quarter delineation on the GC vial were topped up with some H_2O/MeOH (20:80, v/v). Equipment was cleaned and acid-washed between samples to prevent cross-contamination.

Samples and standards were analysed on an Agilent 6890 N Network GC (gas chromatography) system with an Agilent Tech 5975 Mass Selective Detector with helium as the carrier gas and a Trajan (SGE) capillary GC column HT8 (25 m × 0.2 mm, 0.25 μm film thickness). The 189.1 *m/z* ion was used to identify cortisol, and the 306.1 *m/z* ion was used to identify cortisol-d_4. The GCMS was calibrated by analysing a series of standard solutions containing cortisol (2–2000 ng.g^{-1}) and 100 mg of the internal standard. The ratio of the cortisol and cortisol-d_4 peak areas recorded by the instrument was plotted against the ratio of cortisol concentration to internal standard in the calibration standards. During analysis of the samples, the calibration plot was used to convert recorded cortisol:cortisol-d_4 peak area ratios to cortisol concentration (ng.g^{-1}) based on linear regression. The inclusion of a constant known weight of cortisol-d4 as an internal standard in the calibration standards and samples controlled for variability in the extraction and analysis processes. Reference scales and system suitability standards were used as quality control to check instrument consistency across runs, and procedural blank concentrations were used to calculate the limit of detection (LOD) of the GCMS (gas chromatography mass spectrometer). The samples ($n = 5$) with cortisol values below the LOD of 5.1 ng.g^{-1} were estimated using robust linear regression following the method of Helsel (1990) and Higgins et al. (2013).

2.5. Statistical analysis

Initially, linear mixed effects models were used to investigate the effects of temperature and feeding on scale cortisol. Treatments (*Temperature, Feeding*) and *Week* were included as fixed effects and *Tank* was included as a random effect to account for non-independence of measurements from the same tank. In all analyses, the estimated variance for the tank effect was zero. It was concluded that scale cortisol levels did not vary between tanks held under the same treatment. Therefore, the analyses were rerun using General Linear Models (excluding the tank effect). In all cases, the conclusions from both modelling approaches were the same. During post-hoc testing, a Holm adjustment (Holm, 1979) was used to minimise the chance of a Type I error. The adjustment accounted for the total number of pair-wise comparisons made across parts 1 and 2 of the analysis.

2.5.1. Experiment 1: Effect of temperature and feeding conditions on scale cortisol

To investigate the combined effects of temperature and a two week starvation period on scale cortisol, data from the Constant (weeks 2, 9 and 12) and ST14 (weeks 9 and 12) treatments held at each of the three temperatures (6 °C, 10.5 °C and 15 °C) were analysed. *Temperature*, *Feeding, Week* and *Sex* were included as fixed effects. Cortisol data were transformed to achieve a normal distribution by raising to the power of 0.26, as determined by the Box Cox procedure. The response variable was *Cortisol* $(ng.g^{-})^{0.26}$, and the full model included all terms and all possible interactions between the fixed effects. The Feeding*Week interaction was excluded as fish had not been exposed to the ST14 treatment in week 2.

Cortisol $(ng.g^{-1})^{0.26}$ ~ *Temperature *Week*Sex + Temperature*Feeding*Sex.*

The full model was compared to a series of less complex models and the best fitting model was selected based on Akaike Information Criteria (AIC) values and loglikelihood tests. Model diagnostics were used to confirm independence and normality of the residuals and to screen for highly influential observations.

2.5.2. Experiment 2: Effect of four feeding regimes on scale cortisol at a constant temperature

To examine the effects of feeding on scale cortisol in more detail, data from the Constant, ST7, ST14 and ST28 held at 10.5 °C in weeks 9, 11 and 12 were analysed. *Feeding, Week* and *Sex* were included as fixed effects. The response variable was *Cortisol* $(ng.g^{-1})^{0.26}$. The full model included the three fixed effects and their three-way interaction:

$$Cortisol\ \left(ng.g^{-1}\right)^{0.26} \sim Feeding^{*}Week^{*}Sex$$

The best-fitting model was selected using the same procedure as described above.

2.5.3. Relationship between scale cortisol levels and fish size, growth and condition

Changes in length and fish condition during the experiment were plotted using the full dataset of 960 fish from Thomas et al. (2019) for comparison with changes in scale cortisol. To examine the extent to which changes in scale cortisol reflected fluctuations in metabolic activity, correlations with available measures of size, growth and condition were examined. Fish total fork length (cm) and weight (g) provided measures of total size. Condition was calculated using residual values from the regression of ln(weight) against ln(length) (Fechhelm et al., 1995; Blackwell et al., 2000). A fish with a large positive mean residual value was considered to have a higher-than-average weight for its length. Scale growth measurements were available for 149 of the 156 fish included in the cortisol analysis. Scale growth rate during the experimental marine rearing period was used as a proxy for fish growth rate and was calculated. A measure of instantaneous scale growth rate (g_s) was calculated using the formula:

$$g_s = \frac{lnR_t - lnR_0}{t}$$

where R_t is the total scale radius at the time of sampling, R_0 is the scale radius at the beginning of the experiment (as indicated by a calcein mark on the scale) and t is the number of days since the beginning of the experiment. The scale growth analysis is fully described in Thomas et al. (2019). Pearson's product-moment correlations were used to test for correlations between the transformed cortisol values and each of the size, growth and condition measures.

All statistical analyses were carried out using R Version 4.1.2 in R Studio Version 2022.02.0 + 443. The NADA package (Lee, 2020) was used to estimate values for samples below LOD based on robust linear regression (Helsel, 1990). Linear mixed effects models were run using the lme4 package (Bates et al., 2015), and the MASS package (Ripley et al., 2022) was used for Box Cox transformation. The MuMIn package (Bartoń, 2020) was used to test all model combinations using the "dredge" function, and to determine marginal and conditional r^2 values. Model diagnostics was performed using the car package (Fox and Weisberg, 2019). The emmeans package (Lenth, 2021) was used for post-hoc testing. Data visualisation was carried out using the ggplot2 package (Wickham, 2016).

3. Results

3.1. Cortisol analysis

Cortisol was successfully extracted from the scales of 156 Atlantic salmon post-smolts and quantified using GCMS. Cortisol content of the samples ranged from 5.28 ng.g^{-1} to 215.93 ng.g^{-1} (with the exception of five samples that were below the LOD and were replaced with estimated values) (Table 1).

3.2. Experiment 1: Effect of temperature and feeding conditions on scale cortisol

The best fitting GLM model included *Temperature*, *Sex, Feeding* and two two-way interactions: *Temperature*Sex* and *Temperature*Feeding* (Table 2, Fig. 2). The model explained 19.1% of the variance in cortisol concentrations, according to the adjusted R^2 value. Post-hoc tests of the significant interactions showed that constantly fed females held at 10.5 °C had significantly higher scale cortisol values than constantly fed females held at 6 °C or 15 °C ($p = 0.001$, Table 3). There were no significant differences in scale cortisol values between the temperature treatments for females from the ST14 feeding treatment or for males held at either the Constant or ST14 feeding treatment. Comparisons of the Constant and ST14 feeding treatments showed that the 2 week starvation period produced an increase in scale cortisol at 10.5 °C ($p = 0.005$) but not at 6 °C or 15 °C (Table 2, Fig. 3).

3.3. Experiment 2: Effect of four feeding regimes on scale cortisol at a constant temperature

When data from the four feeding treatments at 10.5 °C were analysed, the null model provided the best fit to the data, indicating that when all feeding treatments were considered together, the feeding effect does not contribute to the variation in scale cortisol concentrations. Variation in scale cortisol across the four feeding treatments is plotted in Fig. 4.

3.4. Relationship between scale cortisol levels and fish size, growth and condition

According to Pearson's product moment correlations there were no significant correlations between scale cortisol and fish length ($r = 0.10$, $p = 0.20$), weight ($r = 0.10$, $p = 0.20$), condition ($r = -0.04$, $p = 0.61$) or instantaneous scale growth rate ($r = -0.01$, $p = 0.89$), indicating changes in metabolic activity associated with normal growth and development were not the main driver of variation in scale cortisol. Changes in fish length and condition in each treatment during the course of the experiment are shown in Fig. 5. Responses to starvation varied between treatments and with temperature. The ST14 and ST28 starvation treatments produced a reduction in condition and length in all treatments relative to the control. At 6 °C starved fish (ST14) had recovered their length and condition deficits relative to the control by the end of the experiment. At 10.5 °C length and condition of fish in the control treatment were equal to that of the fish in the ST14 treatment by week 12 while condition in the ST28 treatment was still reduced and fish in ST7 treatment had the same length and higher condition than the control fish. At 15 °C starved fish had recovered in terms of condition relative to the control but not length by week 12.

4. Discussion

This research determined that cortisol can be detected and quantified in small quantities (down to 10 mg) of scales from Atlantic salmon post-smolts. Scale cortisol concentrations were related to both temperature and feeding conditions, although the effect of temperature was only evident in females and not in males. Importantly, elevated scale cortisol levels were observed in females reared at low temperatures as well as in those reared at high temperatures, indicating that temperature related increases were not driven solely by increases in metabolic rate. The assertion that fluctuations in scale cortisol are not related to variations in metabolic activity is further supported by the fact that scale cortisol did not correlate with fish size, condition or scale growth. The methods used here to analyse small volumes of scale material could also be used to quantify cortisol in isolated portions of adult scales, potentially providing a non-lethal method of monitoring physiological responses to stressors at different stages in the migration. However, sex-specific and interactive effects must be considered.

Table 2
Anova table for the best fitting model of scale cortisol as a function of temperature and feeding (*Temperature*Sex*, *Temperature*Feeding*) showing the degrees of freedom (*df*), Sum of Squares (SS), Mean Square (MS) F ratio and associated *p* value for each term in the model. Significant terms are indicated with an asterisk.

Model Term	df	SS	MS	F ratio	p value
Temperature	2	2.29	1.14	3.85	0.02*
Food	1	0.83	0.83	2.79	0.09
Sex	1	0.17	0.17	0.57	0.45
Temperature*Food	2	4.32	2.16	7.27	0.001*
Temperature*Sex	2	2.26	1.13	3.81	0.03*
Residuals	99	29.43	0.29		

The fish in this study completed the process of smoltification in freshwater and were transferred to saltwater at the beginning of the 12-week experiment. Corticosteroids have been shown to increase during the smoltification process in coho salmon (Specker and Schreck, 1982; Barton et al., 1985; Björnsson et al., 2011) and may play an important role in maintaining sodium transport and increasing Na-K-ATPase (sodium-potassium-stimulated adenosine-triphosphatase) (Pickford et al., 1970) which is involved in salinity tolerance (Epstein et al., 1967). Plasma cortisol increases occurred simultaneously between February and May in Atlantic salmon pre-smolts exposed to three different photoperiods (Stefansson et al., 1989), which correlated with previously recorded cortisol increases in spring associated with smoltification (Thorpe et al., 1987). Plasma cortisol levels can remain elevated up to one month after transfer to saltwater (Nilsen et al., 2008). We did not observe a uniform increase or decrease in cortisol over the duration of the experiment in any of the treatments, suggesting that cortisol increases associated with smoltification had stabilized by the start of the experiment. While we cannot rule out that baseline cortisol levels in the experimental fish were higher than they were in freshwater prior to the onset of smoltification, this cannot explain the observed differences between treatments which were consistent across duration of the experiment (i.e. the effect of *Week* did not make a statistically significant contribution to the observed variation in scale cortisol as a main effect, or in interaction with the other factors).

Several previous studies have demonstrated sexual dimorphism in physiological stress responses, which varies in magnitude and direction depending on the species, life-stage and context (Campbell et al., 2021). In some cases, increases in cortisol and other stress markers are more pronounced in females compared to males (Mehdi et al., 2018; Pottinger and Matthiessen, 2016) while in other cases, the reverse is true (Afonso et al., 2003). In salmonids, investigations of sex-specific differences in the stress response have focused primarily on mature adults during the spawning migration (Cook et al., 2011; Donaldson et al., 2014; Hruska et al., 2010; Kubokawa et al., 2001). To the best of our knowledge, temperature-related differences in cortisol production between male and female salmon smolts have not previously been reported. If elevated cortisol levels in scales reflect chronic stress, our results would suggest that female Atlantic salmon smolts are more vulnerable to temperatures above and below the optimal than their male counterparts (although both sexes showed a similar response to a two-week starvation period). The results highlight the importance of considering sexual dimorphism when using scale cortisol as an indicator of chronic stress. Further investigation of sex-specific responses to elevated temperatures in Atlantic salmon post smolts is warranted as increased sensitivity of females to thermal stress would have implications for population level responses to climate change.

The temperature treatments in this study were within the range experienced by Atlantic salmon in the wild and were far from the limits critical for survival (Elliott and Elliott, 2010). However, at temperatures outside of a species' physiological optimum, traits such as growth, fecundity and locomotion can be impaired (Asheim et al., 2020) and

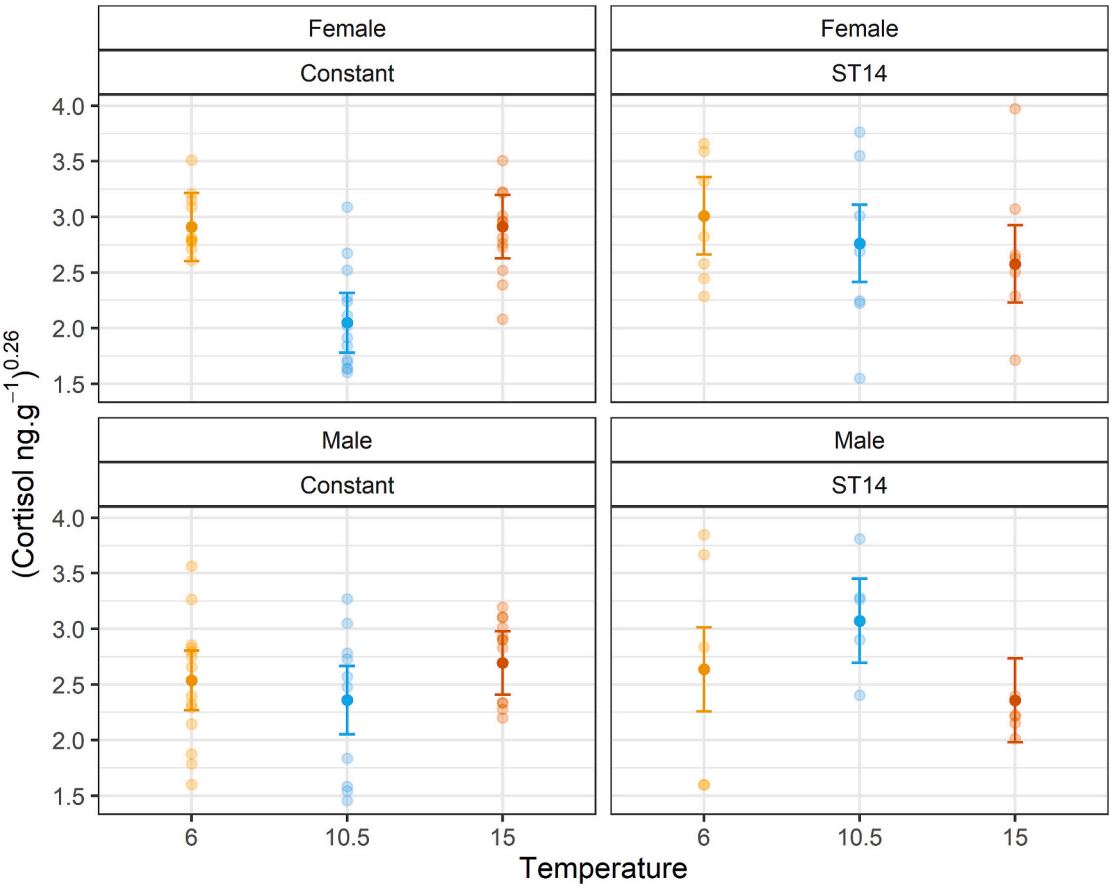

Fig. 2. Plot showing predicted Cortisol (ng.g^{-1})$^{0.26}$ concentrations in *Salmo salar* post-smolt scales (solid points) with 95% confidence intervals (error bars) for Males and Females from each temperature and feeding treatment. Predictions are from the best fitting model which included *Temperature*, *Sex*, *Feeding* and two two-way interactions: *Temperature*Sex* and *Temperature*Feeding*. The data are shown as semitransparent points. Fish were kept at 6 °C, 10.5 °C and 15 °C for a period of 12 weeks and were fed to excess throughout (Constant) or starved for 14 days at weeks 7 and 8 (ST14). Cortisol was transformed to achieve normal distribution.

Table 3
Results from post-hoc comparisons of the significant interaction terms from the best fitting model of scale cortisol as a function of temperature and feeding (*Temperature*Sex*, *Temperature*Feeding*). Adjusted (Adj.) p value refers to the p value following Holm adjustment for multiple comparisons. Contrasts that are significant after correction are indicated with an asterisk. Cortisol values were transformed to achieve a normal distribution (Cortisol (ng.g^{-1})$^{0.26}$).

Contrast	Estimate	S.E.	t ratio	p value	Adj. p value
Constant feeding, Female					
10.5 °C – 6 °C	−0.86	0.21	−4.19	0.0001	0.001*
10.5 °C – 15 °C	−0.87	0.20	−4.38	<0.0001	0.001*
6 °C – 15 °C	−0.0038	0.21	−0.18	0.99	1.00
Constant feeding, Male					
10.5 °C – 6 °C	−0.18	0.21	−0.86	0.39	1.00
10.5 °C – 15 °C	−0.33	0.21	−1.58	0.12	0.98
6 °C – 15 °C	−0.16	0.20	−0.80	0.42	1.00
ST14, Female					
10.5 °C -6 °C	−0.25	0.25	−1.00	0.32	1.00
10.5 °C – 15 °C	0.18	0.25	0.74	0.46	1.00
6 °C – 15 °C	0.43	0.25	1.75	0.08	0.92
ST14, Male					
10.5 °C -6 °C	0.44	0.27	1.62	0.11	0.98
10.5 °C – 15 °C	0.71	0.27	2.66	0.009	0.11
6 °C – 15 °C	0.28	0.27	1.03	0.30	1.00
Temperature 10.5 °C					
Constant-ST14	−0.71	0.19	−0.37	0.0004	0.005*
Temperature 6 °C					
Constant-ST14	−0.10	0.20	−0.51	0.61	1.00
Temperature 15 °C					
Constant-ST14	0.34	0.19	1.74	0.09	0.92

prolonged exposure to these temperatures can produce a chronic stress response (Tromp et al., 2018). In Atlantic salmon, temperature tolerance varies between life stages (Elliott and Elliott, 2010). Post-smolts exhibit a preference for an 8–12 °C temperature range (Friedland et al., 2000; Holm et al., 2000), and post-smolt growth and survival declines with increasing temperature (Hansen and Quinn, 1998; Friedland et al., 2000). The upper threshold for growth and development of Atlantic salmon post-smolts in seawater is around 18 °C; however, food conversion efficiency and food intake have been shown to decline from 12.8 °C for small fish (70-150 g) and 14 °C for larger fish (170-300 g) (Handeland et al., 2000; Handeland et al., 2008). The fish analysed for scale cortisol in this study ranged from 32 to 250 g. In Atlantic salmon post-smolts acclimated to four different temperatures (4, 8, 12 and 17 °C) baseline cortisol levels (measured in water) increased with temperature (Madaro et al., 2018) as did the speed and intensity of reactions to additional stressors (Madaro et al., 2018). Increases in cortisol in response to low temperatures have also been observed in Atlantic salmon post-smolts; Tang et al. (2022) recorded an increase in plasma cortisol levels in Atlantic salmon post-smolts exposed to sudden temperature reductions (from 13 °C to 10, 7, and 4 °C). After a 58 day acclimation period plasma cortisol at the lower temperatures were the same as at the control temperature of 13 °C, although other stress markers associated with neural functioning showed evidence of prolonged stress at 7 and 4 °C. Vadboncoeur et al. (2023) detected a physiological stress response at 4–5 °C in Atlantic salmon post-smolts exposed to gradually reducing water temperatures, although increases in plasma cortisol were not observed until temperatures fell to 1 °C. Both studies were conducted using post-smolts that were larger and several

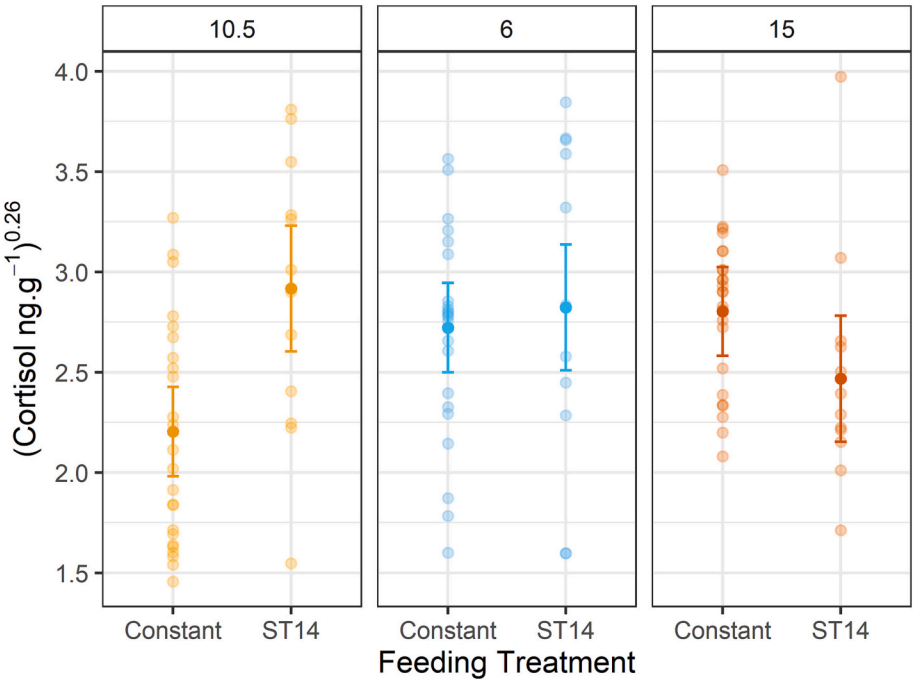

Fig. 3. Plot of the marginal effects of the *Temperature*Feeding* interaction from the best fitting general linear model of Cortisol (ng.g^{-1})$^{0.26}$ concentrations in *Salmo salar* post-smolt scales. Model predicted values are indicated by solid points. The vertical bars show the 95% confidence intervals of the estimates. The data are shown as semitransparent points. Fish were kept at 6 °C, 10.5 °C and 15 °C for a period of 12 weeks and were fed to excess throughout (Constant) or starved for 14 days at weeks 7 and 8 (ST14). Cortisol was transformed to achieve normal distribution.

months older at the start of the experiment than the fish used in this study. Smolts from Norwegian rivers (the genetic origin of the experimental fish) usually enter seawater that is 8–10 °C (Friedland et al., 1998; Hvidsten et al., 1998). A temperature of 6 °C is below the size-dependent optima for food conversion efficiency and growth for Atlantic salmon post-smolts and would therefore cause food intake and digestion rates to decrease (Handeland et al., 2008). A more detailed investigation of a suite of physiological and behavioural markers would be needed to fully understand the effect of rearing temperature on chronic stress in Atlantic salmon. This study provides evidence that exposure to temperatures above (15 °C) and below (6 °C) the optimum can produce a physiological response that is recorded as an increase in cortisol concentrations in the scale and may be indicative of chronic stress.

The evidence for a stress response to starvation in fish is equivocal, with various studies reporting increases (Barcellos et al., 2010; Blom et al., 2000; Varnavsky et al., 1995) decreases (Barton et al., 1988; Small, 2005) and no change (Holloway et al., 1994; Vijayan et al., 1993) in cortisol during fasting. It is thought that fish held at low temperatures are resistant to starvation induced stress due to reduced metabolism and slowed gut evacuation rates (Waagbo et al., 2017). As metabolic demand increases with temperature (Eliason and Farrell, 2016), the ability of fish to withstand food deprivation is likely lower at higher temperatures (Hvas, 2022). In this study, a two-week starvation period led to elevated levels of cortisol in the scale in males and females, but only at 10.5 °C. The cause of the interaction is not clear. It may be in some way related to the effects of starvation on length and condition, which also varied between temperature treatments. After starvation, fish held at 10.5 °C showed a more rapid recovery in length compared to the other temperature treatments but a slower recovery in condition, suggesting a greater allocation of resources to structural growth than to energy replenishment. Cortisol is known to play a role in these processes, although its precise metabolic role is uncertain (Mommsen et al., 1999; Pottinger et al., 2003; Waagbo et al., 2017).

In this experiment, scale cortisol values showed a high degree of variability within each treatment and a large proportion of the variance was not explained by the experimental treatments, indicating that individual variability played an important role. This could be related to social dynamics within the tanks as dominant and subordinate fish react differently to stressors and have different baseline cortisol levels (Doyon et al., 2003; Jeffrey et al., 2014). Fish with a high standard metabolic rate (SMR), often dominant fish, tend to utilise food reserves more quickly than fish with a low SMR, making food deprivation a greater threat (O'Connor et al., 2000). While similar effects may be observed in wild fish exposed to periods of food shortage or temperature stress, the effects of competition and individual dominance traits would likely be different for wild fish compared to a laboratory reared population confined to tanks. Some of the individual variability in scale cortisol may have arisen during handling and processing if traces of mucus remained on the scales. Previous studies have employed different methods for removing skin and mucus from scales prior to analysis. The approach used in this study and by Aerts et al. (2015) involved rinsing scales in ultrapure water before air drying at room temperature. Other studies report more vigorous cleaning including vortex-washing followed by drying at 50 °C (Laberge et al., 2020) or washing with isopropanol (Carbajal et al., 2019). Further comparative studies are needed to establish the best method for removing contaminants without altering the levels of cortisol contained in the scale, particularly when handling small volumes of scale material or dealing with archived scales which are not usually washed prior to storage.

A significant advantage of using scales to monitor cortisol levels is that they can be sampled non-lethally. Other authors have expressed a concern that scale cortisol analysis is not entirely non-invasive due to the amount of scale needed, particularly if sampled from a small fish (Sadoul and Geffroy, 2019). In previous studies, 100 mg (Aerts et al., 2015) and 40-50 mg (Carbajal et al., 2019; Laberge et al., 2020) of scale material were analysed. In our research, the method was optimised for use with 10 mg of scale material, requiring fewer scales per sample which would therefore result in less intrusion on fish during non-lethal sampling. The lower sample weight makes the method more suitable for

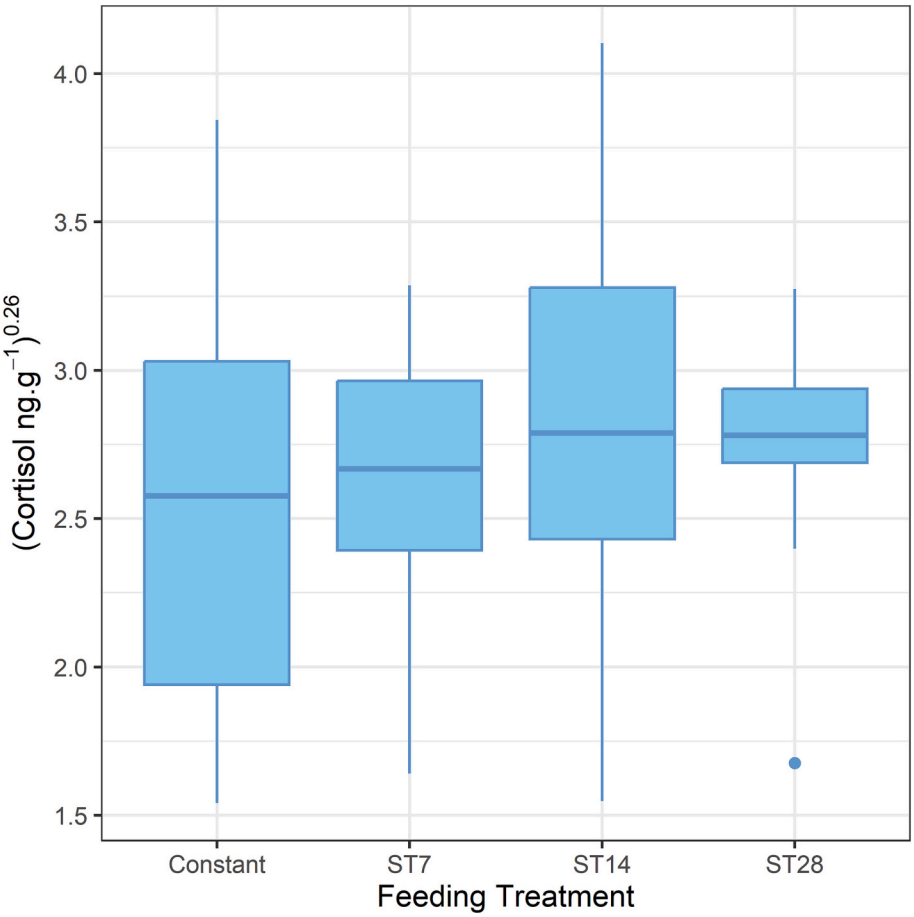

Fig. 4. Median and variation of Cortisol (ng.g^{-1})$^{0.26}$ in scales of *Salmo salar* post-smolts during weeks 9–12 of the study, after starvation treatments were initiated. Fish were kept at 10.5 °C and four feeding treatments: Constant (the control group that was fed constantly), ST7 (starved for 7 days), ST14 (starved for 14 days) and ST28 (starved for 28 days in four 7-day periods). Cortisol was transformed to achieve normal distribution.

use on wild fish and for scale archives, which contain limited amounts of material, and enables the analysis of isolated portions of adult scales. It also makes the approach more suitable to species with smaller scales and younger life stages. The use of higher specification GCMS instrumentation could enable the further reduction of required scale material which could support the analysis of scale cortisol across a wider variety of samples and populations.

For a biomarker to be useful as an indicator of environmentally induced stress, its natural range and sources of variability should be known, and it should be possible to sample without compromising baseline levels (Pankhurst, 2011). The second criterion is met, as scale cortisol levels are not altered by acute stress during sampling (Carbajal et al., 2019). However, much uncertainty remains with regards to baseline levels of scale cortisol in salmonids and other species, what causes them to vary and how they change in response to environmentally relevant stressors. As wild salmon are exposed to a wide range of interacting stressors including sea lice (*Lepeophtheirus salmonis*, Krøyer) infestation (Poole et al., 2000), maturation (Baker and Vynne, 2014), and climatic patterns (Friedland et al., 2009), direct stress responses to temperature will be difficult to establish. Nonetheless, this study has demonstrated that under laboratory conditions, scale cortisol levels can vary in response to temperature and feeding conditions during the post-smolt phase of Atlantic salmon, while highlighting sex-specific differences and interactive effects. Further investigation is warranted to improve understanding of the processes and factors influencing the uptake of cortisol into the scales of Atlantic salmon and to establish appropriate baselines against which potential stress responses can be evaluated.

With further refinement of the approach, the potential exists to use scale cortisol to monitor physiological responses during the marine phase of salmonids or to relate stress during the freshwater phase to subsequent survival. In Atlantic salmon and sea trout kelts, baseline blood cortisol levels are a good predictor of migration success (Birnie-Gauvin et al., 2019); if cortisol in scales shows a similar relationship, it may provide a useful index of pre-migration condition that can be collected less invasively than blood samples. In the future it may be possible to detect other markers of thermal responses such as heat shock proteins from scales, as is the case for fin tissue (Feldhaus et al., 2010). Future use of scales as biological recorders could enable retrospective analysis of chronic stress in Atlantic salmon over time using scale archives, to provide insight into how salmon have responded to environmental change in the past and informing predictions of responses to future change.

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Author statement

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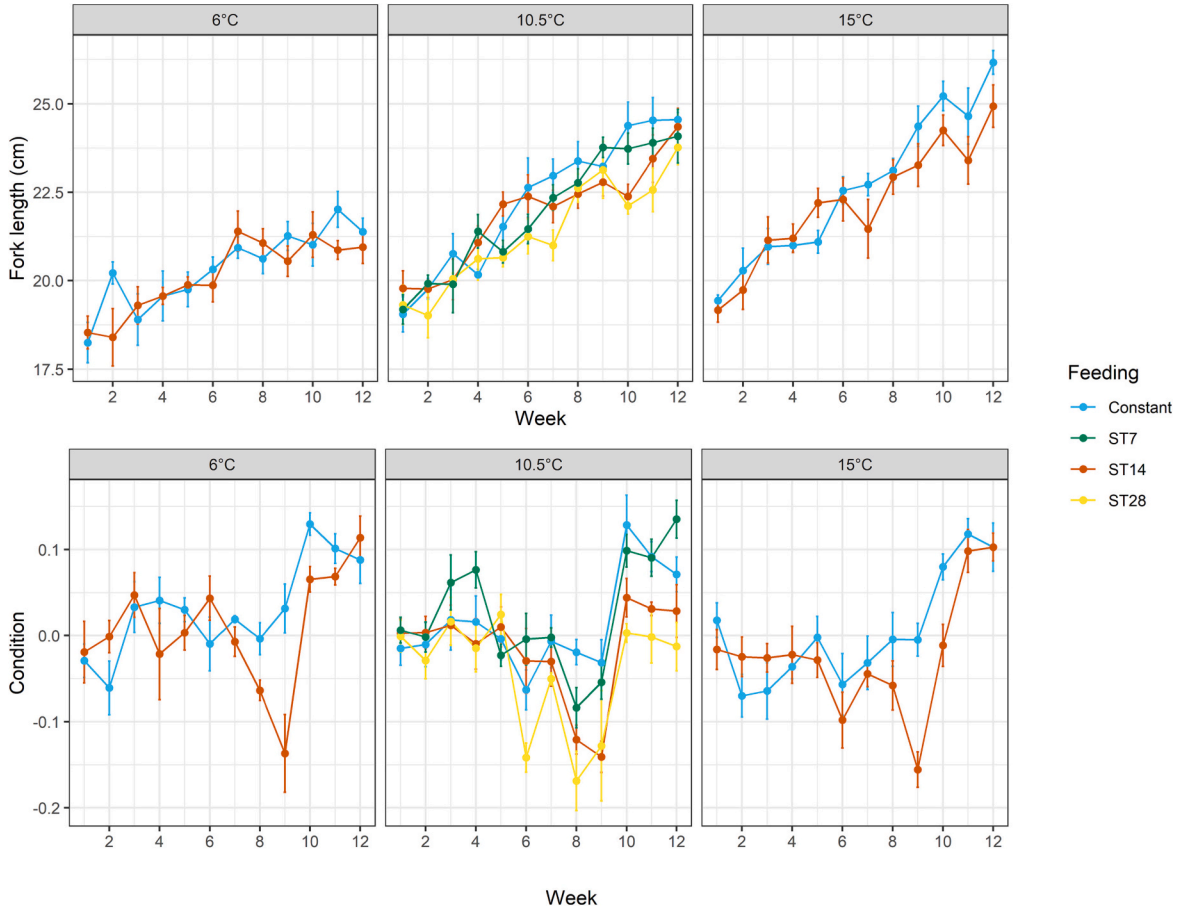

Fig. 5. Mean (±standard error) fork length and condition of *Salmo salar* post-smolts during the 12-week study. Fish were kept at three temperature treatments (6, 10.5 and 15 °C) and four feeding treatments: Constant (the control group that was fed constantly), ST7 (starved for 7 days), ST14 (starved for 14 days) and ST28 (starved for 28 days in four 7-day periods). Fork length data was collected by Thomas et al. (2019).

approved by all authors and has not been previously published and is not being considered for publication in another journal.

CRediT authorship contribution statement

Christina O'Toole: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Philip White:** Conceptualization, Methodology, Validation, Writing – original draft, Supervision. **Katie Thomas:** Conceptualization, Writing – review & editing. **Niall O'Maoiléidigh:** Conceptualization, Writing – review & editing. **Per Gunnar Fjelldal:** Conceptualization, Writing – review & editing. **Tom Johnny Hansen:** Conceptualization, Writing – review & editing. **Conor T. Graham:** Conceptualization, Writing – original draft, Supervision. **Deirdre Brophy:** Conceptualization, Formal analysis, Writing – original draft, Supervision.

Declaration of Competing Interest

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

Data availability

Data are available from the author on request.

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