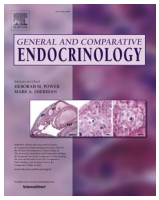
General and Comparative Endocrinology 362 (2025) 114671

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Research paper

Influence of season, capture method, sample age and extraction protocols on the scale cortisol concentrations of three species of freshwater fish

Ahmad Ghazal [a], Richard Paul [a], A. Serhan Tarkan [a,b,c], J. Robert Britton [a,*]

[a] Department of Life and Environmental Sciences, Bournemouth University, Poole BH12 5BB United Kingdom
[b] University of Lodz, Faculty of Biology and Environmental Protection, Department of Ecology and Vertebrate Zoology, Lodz, Poland
[c] Department of Basic Sciences, Faculty of Fisheries, Muğla Sıtkı Koçman University, Muğla, Turkey

ARTICLE INFO

Keywords:
Fish scales
Stressor
Environmental change
Scale archive
LCMS

ABSTRACT

Scale cortisol concentration (SCC) is increasingly applied as a biomarker of chronic stress in fish, but knowledge gaps remain on how SCC is affected by the sampling season and method of fish capture, the time since sample collection, and the cortisol extraction protocol. Here, working with three freshwater fishes (common carp *Cyprinus carpio*, European chub *Squalius cephalus* and Northern pike *Esox lucius*), a robust extraction protocol was developed and then applied to identifying how scale cortisol levels can vary in fish populations according to aspects of the fish capture events. Across five scale cortisol extraction protocols, three provided relatively low yields, so their application would result in erroneously low SCC. Application of the extraction protocol providing the highest yields to scale samples indicated that fish sampled in winter have significantly lower SCC than those collected in spring and summer, while fish captured by angling have significantly lower SCC than fish collected from the same population by electric fishing. There were no significant differences in SCC measured from populations across 40 years, suggesting that archived scales potentially provide a valuable resource for measuring temporal changes in SCC. Future studies based on using scale cortisol for analyses of fish chronic stress should consider these issues in their study designs and evaluations to ensure measured differences in cortisol across time and space are due to differences in how the fish are responding to their environment rather than being an artefact of study design.

1. Introduction

Circulating cortisol levels in fish can provide information on acute stress responses in fish (Samaras et al., 2021; Madaro et al., 2023). Their application as chronic stress indicators are less reliable (Aerts et al., 2015; Samaras and Pavlidis, 2022), meaning alternative methods are needed for evaluating chronic stress (Bertotto et al., 2010; Sadoul and Geffroy, 2019). Fish scale cortisol concentration is increasingly being applied as a chronic stress biomarker (Harris and Carr, 2016; Roque d'Orbcastel et al., 2021; Kennedy and Janz, 2023). This is through the accumulation and clearance rates of cortisol in scales being much slower than in, for example, plasma (Laberge et al., 2019; Britton et al., 2023). Evidence suggests scale cortisol levels are influenced by energetically intense periods of intermediate duration, suggesting they can provide a retrospective measure of stress experience of up to 30 days (Carbajal et al., 2018, Carbajal et al., 2019a, Carbajal et al., 2019b).

Scale cortisol analyses have been applied to assessments of chronic stress in a wide range of fish species, including rainbow trout *Oncorhynchus mykiss* (Carbajal et al., 2019a), goldfish *Carassius auratus* (Carbajal et al., 2018; Laberge et al., 2019), Catalan chub *Squalius laietanus* (Carbajal et al., 2019b), sea bass *Dicentrarchus labrax* (Lebigre et al., 2022), dab *Limanda limanda* (Vercauteren et al., 2022), common carp *Cyprinus carpio* and European barbel *Barbus barbus* (Britton et al., 2023). While commonly applied to identifying patterns in chronic stress in these species, it is also important to note that changes in cortisol levels (including in scales) will also occur in relation to environmental cycles, circadian rhythms, sex, maturity, and reproductive stages, as well as for supporting adaptive behaviours or eustress, where the response elicited is positive (Lemos et al., 2023). Consequently, it is important to develop understandings of what might represent typical cortisol values for a population versus those of that are elevated and so represent stress related values (Cyr and Romero, 2009; Balasch and Tort, 2019; Lemos et al., 2023).

Understanding typical scale cortisol levels requires knowledge on

* Corresponding author.
E-mail address: rbritton@bournemouth.ac.uk (J.R. Britton).

https://doi.org/10.1016/j.ygcen.2025.114671
Received 20 November 2024; Received in revised form 26 January 2025; Accepted 30 January 2025
Available online 31 January 2025

how these levels vary between seasons and different sampling methods, vary across different storage times, and according to different preparation methods in the laboratory (O'Toole et al., 2024). Given that fish tend to express higher levels of activity and metabolism in warmer versus colder months then their daily adaptive responses to their heterogeneous environments will differ between summer and winter, potentially increasing their cortisol levels in summer irrespective of stressor presence/absence (Boonstra, 2013; Mehdi et al., 2021). As fish behavioural traits consistently map on to their physiological traits (Damsgård et al., 2019) then the sampling method used to capture the fish could potentially affect the scale cortisol data collected. For example, angling can be selective for specific phenotypes (e.g. for bolder, more active individuals; Klefoth et al., 2017), whereas electric fishing tends to be less biased by phenotype so potentially provides samples that are more phenotypically variable, including in scale cortisol levels (Gutmann Roberts et al., 2019). Archives of fish scales collected in previous decades have already been used to extract DNA to understand population genetics (e.g. Iwamoto et al., 2012) and apply stable isotope analysis (e.g. Vašek et al., 2021). Accordingly, there is scope for scales held in archives to be applied to understanding temporal patterns in scale cortisol concentrations. Indeed, O'Toole et al., (2024) recently indicated that for Atlantic salmon *Salmo salar*, there was no significant relationship between scale cortisol concentration and storage time for scales stored between 3 and 32 years. In addition, there also remains an absence of a standardised methodology for initial extraction of cortisol from scales, potentially leading to concentrations in some studies being under-estimated.

The aim here was to develop a robust protocol of scale cortisol extraction and apply this protocol to developing new understandings of how the season, method and decade of scale sample collection influenced the derived scale cortisol concentrations in three temperate freshwater fishes. Methods that have been used to quantify scale cortisol concentrations include high-performance liquid chromatography-fluorescence (HPLC-FL) (Kulczykowska et al., 2018), liquid chromatography-mass spectrometry (LC-MS) (Sadoul and Geffroy, 2019), and gas chromatography-mass spectrometry (GC–MS) (O'Toole et al., 2023). LC-MS/MS was used here to test the extraction protocols as it is considered to be the most precise and accurate (Geis-Asteggiante et al., 2011; Nagae et al., 2021).

2. Materials and methods

2.1. Scale sample collection

Scales used in the study were collected from common carp ("carp"), European chub *Squalius cephalus* ("chub") and Northern pike *Esox lucius* ("pike"). For comparing the protocols of scale cortisol extraction, carp scales were used from Lake Naivasha, Kenya, which were collected using multi-mesh gillnets as described in Oyugi et al., (2011). The carp were measured (fork length, nearest mm) and up to 5 scales removed and stored dry in a paper envelope at room temperature.

For testing the effect of season on scale cortisol levels, carp and pike were used. Carp were sampled using seine nets across the spring, summer and winter 2022 and 2023 from three recreational angling ponds (catch-and-release) in Southern England (exact location cannot be provided for business confidentiality reasons as the fisheries are going concerns). The ponds were up to 4 ha in area and up to 2 m deep, with carp being highly abundant in them all following stocking exercises up to 2 years prior to the start of sample collection. Pike scales were collected from the lower River Severn basin, western England (comprising the main River Severn, Warwickshire Avon tributary and connected boat marinas) where samples were collected in summer and winter 2017 to 2019, with fish captured using both electric fishing and catch and release angling. Electric fishing was completed from a boat using DC at a power typically of 200 to 250 V but varying depending on the depth of the water being sampled in accordance with the response of the fish to that power. On their removal from the water, the fish were transferred to water filled tanks where their recovery to normal behaviour was rapid, with no obvious injuries observed. As scales were collected within 3 h of capture, and responses of cortisol levels in scales resulting from a stressor event take considerably longer to occur (Laberge et al. 2019), then the effect of capture on scale cortisol levels was considered negligible. For both species, up to 5 scales were collected from the fish and stored dry in paper envelopes, with fish fork length (to nearest mm) and capture date recorded.

Comparison of scale cortisol concentrations between angled and electric fished individuals used chub from the River Teme, Western England, sampled in September 2020. Electric fishing was completed using generator powered equipment, with the operators fishing in an upstream direction by wading, with the equipment being towed in a small boat behind them, with all other aspects as described above. Captured chub were transferred to water-filled tanks prior to being processed, where fish fork length was recorded, and 3 scales were collected and stored in paper envelopes. The angled chub were captured by rod and line from the same reach of river in the period immediately prior to electric fishing, with the same data and number of scales collected from each fish.

For comparisons of scale cortisol concentrations between historical and contemporary scales, it was not possible to generate data from the historical samples at their time of collection. O'Toole et al., (2024) overcame this issue by comparing real-time scale cortisol concentrations from Atlantic salmon that had been stored in paper envelopes at room temperature for between 3 and 32 years. Correspondingly, we applied a similar method for chub were used from the Rivers Colne and Wensum, Eastern England, and River Teme, western England, which were all collected by electric fishing. Scales were available from samples collected between 1983 and 1999 ("historical") and 2018 to 2022 ("contemporary"), with fish length, year of sampling and fish length available for each individual chub used in analyses.

2.2. Ethical declarations

Scales collected in England were from fish sampled following ethical approvals and under UK Home Office licences 700/8063 and P47216841. Scales collected from Lake Naivasha were under the Kenya Government Research Permit NCST 5/002/R/020-D.

2.3. Protocols of scale cortisol extraction

Based on pilot studies using common carp scales from Lake Naivasha, the scale mass used in the analysis of cortisol concentration that produced a quantifiable response through LC-MS/MS analysis was between 30 and 150 mg of scale material. Prior to cortisol extraction, the scales were washed using deionized water three times. Five extraction protocols were used to test their effect on subsequent scale cortisol concentrations derived from the LC-MS/MS analyses. Protocol 1 involved taking 100 mg of scale sub-samples from an individual fish, cutting these into smaller pieces and transferring these into a Fisherbrand reinforced 2 ml tube, with the tube containing 2.8 mm metal beads. Cortisol extraction was performed by adding 0.25 ml internal standard (cortisol d4) and HPLC grade methanol to make up the volume to 1 ml, followed by wet grinding the fish scales using a Fisherbrand™ Bead Mill 24 Homogenizer for 1 h. The samples were then centrifuged for 3 mins, and then 200ul of supernatant was collected and transferred into HPLC vials. Protocols 2 to 5 were then taken from published methods on cortisol extraction in fish scales and compared with cortisol concentrations from Protocol 1. To enable direct comparisons across all five protocols, scale sub-samples were always used from the same fish. Protocol 2 involved grinding the scales using a Fisherbrand Reinforced 2 ml tube (metallic 2.8 mm) and a bead ruptor (Fisherbrand™ Bead Mill 24 Homogenizer). HPLC-gradient grade methanol was used as extraction solvent and purification was done using SPE C18 500 mg/6 ml solid-phase extraction

(SPE) columns. After resuspension, ultra-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to quantify scale cortisol (Hanke et al., 2019). Protocol 3 was based on Aerts et al., (2015), where the scale sub-samples were cut into fine pieces using scissors before 1 ml of methanol was added as extraction solvent and cortisol-d4 solution was added as internal standard. The sample was vortex-mixed for 30 s, placed on an overhead shaker at 60 rpm for 1 h at room temperature, and centrifuged for 10 min at 3500 g.

In Protocol 4, the homogeneity of minced scale particles required the scales to be dried and ground for 2.5 min at 4 m/s using a Fisherbrand™ Bead Mill 24 Homogenizer. The powdered scale was then incubated at 30 °C for 18 h with 1.5 mL of methanol. Samples were then centrifuged (7000 × g, 10 min) after extraction as described by Carbajal et al., (2019). Finally, Protocol 5 involved the scale sub-samples cut into smaller pieces and further homogenized using a Fisherbrand™ Bead Mill 24 Homogenizer after which cortisol was extracted with methanol. After vortex-mixing and centrifugation (10 min, 3500 × g), the supernatant was collected and evaporated. The dried pellet was re-suspended and purified through SPE after which LC-MS/MS analysis was performed, this protocol was based on Vercauteren et al., (2022).

2.4. Scale cortisol analyses, including calibration and accuracy

The extracted scale cortisol samples were then analysed on an Agilent 1290 Infinity II UHPLC system (Agilent Technology, Palo Alto, CA, USA) connected to an Agilent 6546 Quadrupole Time-of-Flight Mass Spectrometry instrument equipped with an electrospray ionization (ESI) source for the targeted MS/MS analysis. The column used for compound separation was a 2.1 × 50 mm 1.8 μm C18 analytical column (Zorbax Eclipse Plus C18, Agilent) which was protected by a 2.1 mm × 5 mm 2.7 μm C18 guard cartridge (Agilent). For the chromatography, the injection volume was 10 μL, and the mobile phases were 0.1 % of formic acid in deionized water (phase A) and methanol (phase B) at a constant flow rate of 0.5 mL/min. The gradient used was as follows: 5 % B at 0 min; gradient elution changes from 5 % to 55 % B in 0.3 min; from 55 % to 80 % B in 3.7 min; from 80 % to 100 % B for 1 min. After analysis, the column was equilibrated to the initial conditions within 1 min. The dual ESI source operated in negative ionization mode under the following conditions: nebulizer gas at 35 psi, drying gas flow rate and temperature at 12 L/min and 250 °C, respectively. The sheath gas was set at 350 °C with a flow rate of 12 L/min. The capillary voltage was set at 2500 V, while the fragmentor, skimmer, and octapole voltages were fixed at 150, 65, and 750 V, respectively. The data were acquired in centroid mode and full scan was carried out at 2 spectra/s within the *m/z* range of 100–1700. Subsequently targeted MS/MS with a set precursor ion (cortisol: 407.2 and cortisol-d4: 411.2 *m/z*; Table 1) in negative mode using a collision energy of 20 eV at 2 spectra /s within the *m/z* range 100–500 was carried out. Due to the fact that both compounds can form a very abundant and stable adduct with formic acid in negative mode ([M + HCOO] −), the MS parameters were optimized using the cortisol and cortisol-d4 adduct with formic acid (Molecular weight (MW) = 407 Da and MW = 411.2, respectively) as precursor ion.

The needle wash was set to 10 s flush port with 100 % methanol. At the beginning of each day, the Quadrupole Time-of-flight mass spectrometry was calibrated with the Agilent ESI-L Low Concentration Tuning Mix to maintain the accuracy of high-resolution mass. The MassHunter Quant Workstation software was used to process the data obtained by UHPLC–QTOF in targeted MS/MS mode.

For linearity evaluation, seven matrix-matched calibration standards with increasing concentrations of cortisol in the range of 0.1 ng/mL to 10 ng/mL were analysed. Each sample was prepared with a set amount of internal standard. Next, the regression plots were built for each analyte using the response factors of the ratio of the analyte peak area over cortisol-D4 peak area. Linearity was acceptable when $R^2 \geq 0.995$. The limit of detection (LOD; lowest level at which a compound could be identified with a signal-to-noise (S/N) ratio greater than 3) and the limit of quantification (LOQ; the lowest level at which a compound could be identified and quantified with a signal-to-noise ratio greater than 10) were calculated for each compound. The intra- and inter-day accuracy and precision measurements were then assessed across a 5 day method validation, using measurements of three quality control (QC) cortisol standards dissolved in matrix on a single assay, repeated (with triplicates) daily for 5 days, calculated for each QC (low, medium and high).

This LC-MS-MS protocol detected and quantified scale cortisol levels using multiple-reaction monitoring (MRM) mode. Negative electrospray ionization mass spectrometry has been used for analysing glucocorticoids (mainly cortisol), resulting in an established fragmentation pattern with product ion *m/z* 311.19 and qualifier ion at *m/z* 297.14 (Spectrum MoNA037977 in MassBank of North America, SPLASH: splash10-001i-0249000000-748cf1515e3983d16150). The same pattern was observed in our analyses. Calibration curves for both MRM transitions were linear ($R^2 > 0.99$; Table 1B). To determine LOD and LOQ, a second calibration curve (calibrators ranging from 0.1 to 1 pg/mg) indicated that LOD and LOQ were 0.4 pg/mg and 1 pg/mg for cortisol (assuming a 100 mg scale sample) with a signal to noise ratio of 3:1 (LOD) and 10:1 (LOQ) respectively. Overall, cortisol showed linearity from the LOD (0.4 pg/mg) to 10 pg/mg. All coefficient of variations (CV) for intra-day and inter-day were less than 10 % for cortisol when spiked with low, medium and high standard concentrations (Table 1).

Table 1
Scale cortisol assay parameters: (A) Ionization and fragmentation conditions (CE: collision energy; RT: retention time; and (B) validation parameters (*P < 0.001).

(A)

Compound name	Precursor ion (Da)	Product ion (Da)	Qualifier ion (Da)	CE (V)	RT (min)
Cortisol	407.2	331.19	297.14	20	1.6
Cortisol − d4	411.2	335.21	–	20	1.6

(B)

Linearity (cortisol range, R^2)	0.1–10 ng/ml, 0.99*
Inter-day CV (n = 5): Low cortisol (1ng/ml)	4.4 %
Inter-day CV (n = 5) Medium cortisol (4.5 ng/ml)	2.1 %
Inter-day CV (n = 5) High cortisol (9 ng/ml)	4.0 %
Intra-day CV (n = 5): Low cortisol (1ng/ml)	4.5 %
Intra-day CV (n = 5) Medium cortisol (4.5 ng/ml)	1.6 %
Intra-day CV (n = 5) High cortisol (9 ng/ml)	5.4 %
LOD (ng/ml, pg/mg)	0.04, 0.4

2.5. Analyses of scale cortisol: Variation by season, sampling method and time

Assessing the influence of season, sampling method and time since scale collection on scale cortisol levels used Gamma Generalized Linear Modelling (GLM) in the R environment (v.4.3.2; R Core Team, 2024), with use of the `glmmTMB` package (Brooks et al., 2017). Prior to model fitting, data exploration following the Ieno and Zuur (2015) protocol, which included checking for missing values, identifying outliers in both response and explanatory variables, assessing homogeneity and zero inflation in the response variable, evaluating collinearity between explanatory variables, ensuring balance in categorical variables, and examining the nature of relationships between the response and explanatory variables. The data for cortisol levels were positively skewed but free of outliers. In the first model, the effect of season, fish length, year and site were included as fixed effects. In the second model, sampling period (i.e. historical or contemporary), fish length, sampling method (electric fishing or angling), and river were fixed factors. As scales were collected from some rivers across numerous sites, then site was included as a random factor. The *dredge* function from the `MuMIn` R package (Bartoń, 2022) was used to generate a subset of candidate models by incorporating various combinations of fixed effects and random factors, thereby achieving an optimal balance between model

complexity and fit. Models with a ΔAIC < 2 (i.e., the difference in AIC between the best candidate model and the model under consideration) were retained. All candidate models were subsequently validated using the DHARMa package in R (Hartig, 2023).

3. Results

3.1. Comparison of scale cortisol extraction methods

Mean scale cortisol yield and concentrations were highest in protocol 1 (0.79 ng/ml; 7.93 pg/mg), with similar values obtained from protocol 4 (0.76 ng/ml; 7.56 pg/mg (Table 1B; Fig. 1). However, Protocol 2 and 5 provided lower yields and concentrations (2: 0.45 ng/ml; 4.53 pg/mg; 5: 0.60 ng/ml; 6.67 pg/mg, with the lowest values from Protocol 3 (0.19 ng/ml; 1.9 pg/mg) (Fig. 1). Protocol 1 was used in all subsequent analyses.

3.2. Effects of season, sampling method and sample age

The carp scale cortisol concentrations from the three recreational fisheries ranged between 0.72 and 11.55 pg/mg with a mean of 3.58 ± 0.36 pg/mg. There was considerable variability within and between the sites (Table 2A), with the distribution of values suggesting an overall effect of season (Fig. 2). The GLM revealed that in winter, carp scale

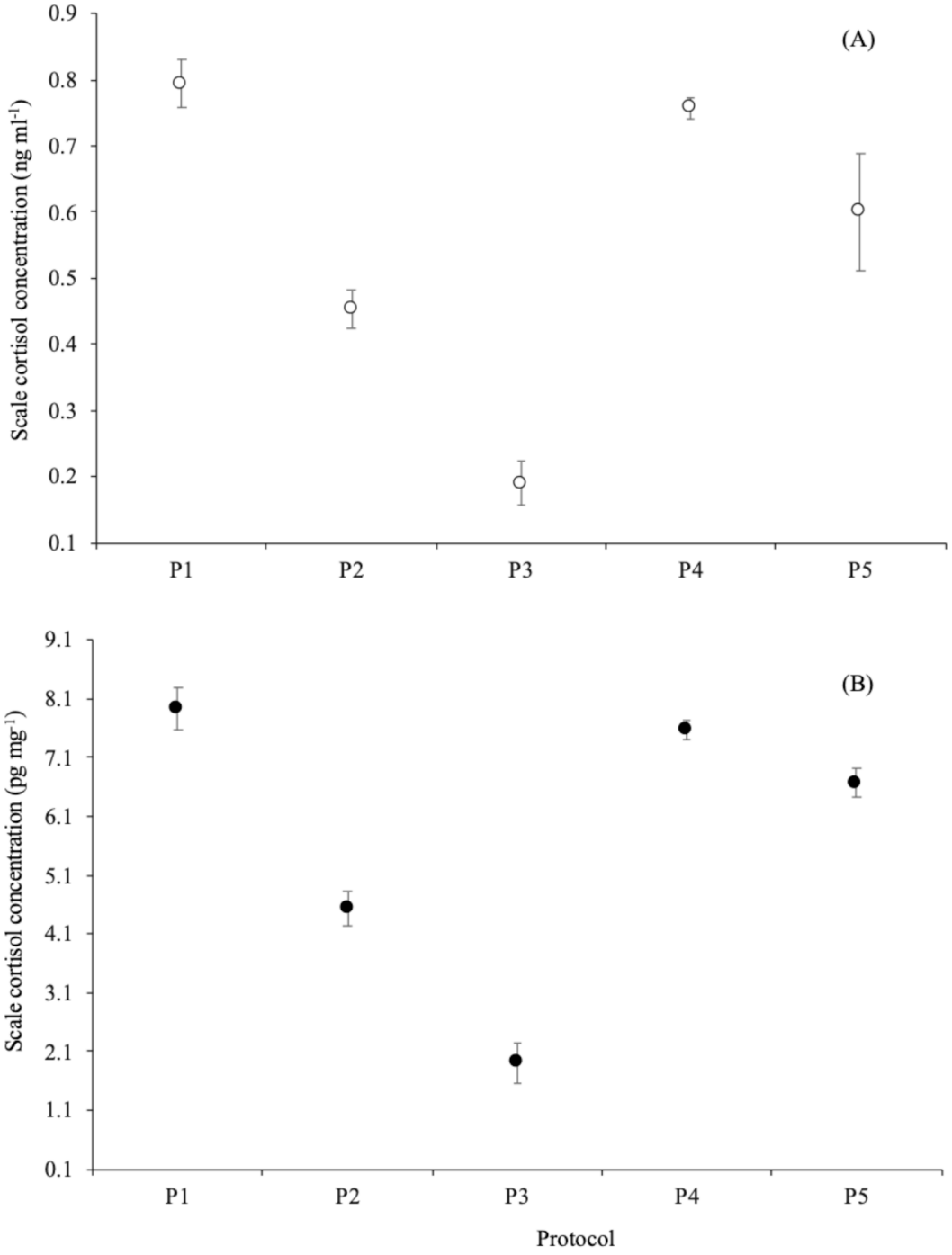


Fig. 1. Mean (± 95 % CI) cortisol concentrations as (A) volume and (B) mass of common carp scales, where cortisol was extracted from scales using Protocols 1 to 5 (*cf.* Methods).

Table 2
(A) Carp samples sizes (as the number of fish sampled, "n") and mean scale cortisol data ("SC"; ± 95 % CI, all values in pg/mg) sampled from three recreational fisheries in southern England (referred to as "Site 1, "Site 2" and "Site 3"). (B) Output of the best fitting GLM testing seasonal differences in carp scale cortisol concentrations (scale cortisol ~ season + year + length + site). Bold denotes significant values.

(A)

	All		Spring		Summer		Winter	
Site	n	Mean SC	n	Mean SC	n	Mean SC	n	Mean SC
1	61	4.10 ± 0.48	21	4.56 ± 1.04	20	4.62 ± 0.70	20	3.10 ± 0.50
2	48	2.69 ± 0.50	1	1.34	38	2.81 ± 0.61	9	2.36 ± 0.80
3	29	3.93 ± 0.97	9	4.22 ± 1.75	19	3.82 ± 1.25	1	3.44

(B)

Coefficient	Estimate (± SE)	Z	P
(Intercept)	−48.29 ± 222.90	−0.22	0.83
Site 2	−0.62 ± 0.15	−4.28	**< 0.001**
Winter	−0.30 ± 0.15	−2.06	**0.04**
Fish length	0.001 ± 0.0001	2.34	**0.02**
Site 3	−0.15 ± 0.13	−1.13	0.26
Summer	−0.05 ± 0.13	−0.43	0.67
Year	0.02 ± 0.11	0.22	0.83

cortisol levels were significantly decreased (P = 0.04), with a strong negative effect on scale cortisol levels from Site 2 (P < 0.001) and a positive effect by carp length (P = 0.02) (Table 2B). A similar pattern was apparent in pike, with significantly lower scale cortisol values derived in samples collected in winter versus summer (Table 3).

Chub captured by angling generally had lower scale cortisol concentrations than those sampled by electric fishing (Fig. 3). The best fitting GLM was scale cortisol ~ sampling method + chub fork length, with sampling method being the significant factor influencing cortisol concentrations (estimate: 0.57 ± 0.55, P = 0.04; Fig. 3). Scale cortisol levels were also more varied and generally higher in pike sampled by electric fishing than angling, with the effect of sampling method being significant (Table 3). There was a similar distribution in scale cortisol concentrations of chub between the historical and contemporary time periods (Fig. 4), with the GLM indicating no significant effect of time period on cortisol concentrations (P = 0.67; Table 4). In the model, chub length (P = 0.05) and the River Wensum (P = 0.03) both had significant and negative effects on scale cortisol levels (Table 4).

4. Discussion

Scale cortisol has been analysed in numerous species and applied to identify patterns in chronic stress (e.g. Carbajal et al., 2018; Laberge et al., 2019; Britton et al., 2023). Here, we identified that the effect of season of sampling, the sampling method used to capture the fish, and the cortisol extraction method used during scale preparation, all affect derived scale cortisol concentrations. Accordingly, unless accounted for in future studies, these issues will potentially present confounding factors. There was, however, no significant difference in chub scale cortisol concentrations across an extended time period, suggesting that the method could potentially be applied to historical scale samples, as also recently suggested by O'Toole et al., (2024) for Atlantic salmon. However, this comes with the caveat that the original cortisol concentrations of these scales are unknown and were only compared using values derived in real time and thus this result should be treated with some

Table 3
(A) Pike sample sizes and mean scale cortisol data ("SC"; ± 95 % CI, all values in pg/mg) for the rivers sampled. (B) Output of the best fitting GLM testing differences in pike scale cortisol concentrations according to season, fish length and sampling method (scale cortisol ~ season + length + sampling method + river as random variable). Bold denotes significant values.

(A)

	All		Summer		Winter	
River	N	Mean SC	n	Mean SC	n	Mean SC
Severn	16	4.72 ± 1.65	16	4.72 ± 1.65	–	–
Stour	64	1.69 ± 0.33	16	2.38 ± 0.87	48	1.46 ± 0.31
W Avon	14	3.20 ± 1.55	14	3.20 ± 1.55	–	–

(B)

Coefficient	Estimate (± SE)	Z	P
(Intercept)	1.94 ± 0.40	4.88	**<0.001**
Season (winter)	−0.72 ± 0.16	−4.41	**< 0.001**
Fish length	−0.001 ± 0.0005	−2.45	**0.02**
Sampling method (electric fishing)	0.66 ± 0.28	2.40	**0.02**

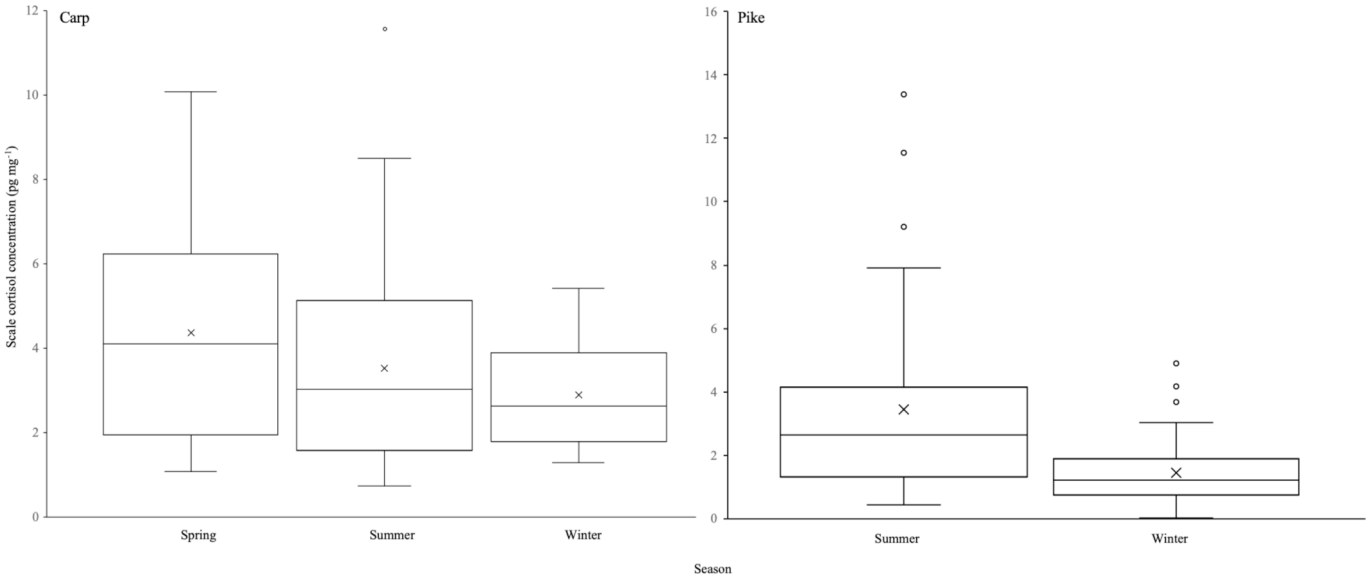


Fig. 2. Box plots comparing the distribution of scale cortisol concentrations of common carp *Cyprinus carpio* ("Carp") sampled in spring, summer and winter across three pond fisheries and Northern pike *Esox lucius* ("Pike") sampled in summer and winter across three rivers, where horizontal lines represent 10, 25, 50, 75 and 90 percentiles, x is the mean and clear circles are outliers. Note differences in the scale on the Y axes.

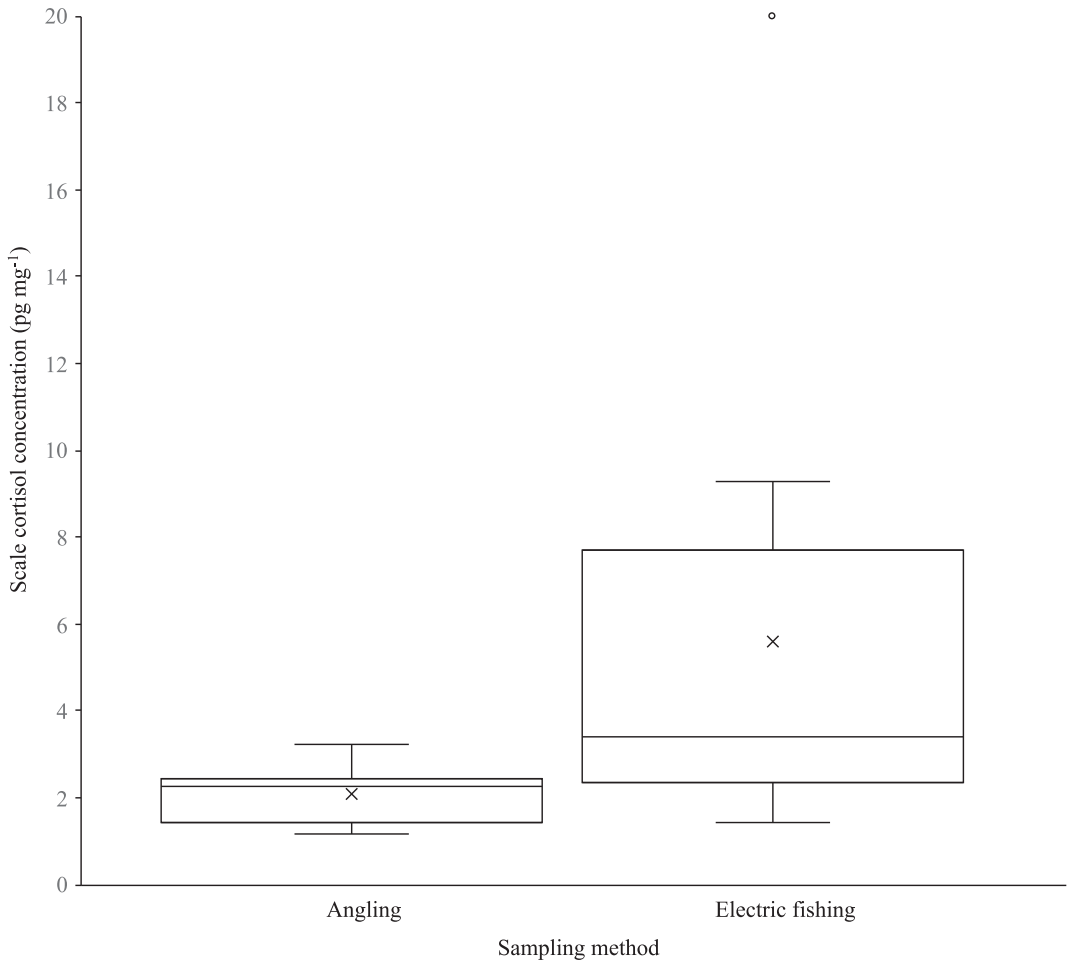


Fig. 3. Box plot comparing the distribution of scale cortisol concentrations of chub *Squalius cephalus* sampled from the River Teme, Western England, by angling and electric fishing, and where horizontal lines represent 10, 25, 50, 75 and 90 percentiles, x is the mean and clear circles are outliers.

caution. Also, the three fish species used demonstrated considerable variability in scale cortisol concentrations between individuals. As these fish were captured from the wild then knowledge on their movements and behaviours in the days prior to their capture was unknown and thus it is beyond this study to suggest whether fish with relatively high scale cortisol concentrations were chronically stressed or had just been more active in this pre-capture period (Boonstra, 2013). The direction of the effect of fish length also varied across the three species and so it is difficult to conclude how it might affect individual differences in stress responses and, thus, the scale cortisol concentrations. Nevertheless, the derived scale cortisol values arguably represent typical values derived for these species in temperate freshwaters.

Scale cortisol concentrations of carp were significantly lower in winter when compared with spring and summer across both carp and pike, suggesting that chronic stress levels are relatively low in winter versus warmer seasons. The low temperatures of winter generally decrease fish metabolic rates, swimming capacity and foraging rates (Marsden et al., 2021; Sutton et al., 2021). These reduced activity levels are associated with suppressed energy intake and expenditure when compared to levels in summer (Marsden et al., 2021; Sutton et al., 2021). Carp is also a species of relatively high temperature preferences and tolerances (Chatterjee et al., 2004), with the fastest somatic growth rates achieved at 23 to 30 °C (Ahmad et al., 2011). While pike tend to prefer cooler temperatures, their optimal growth conditions are at temperatures up to 21 °C (Margenau et al., 1998), with this temperature rarely exceeded in British rivers at present (Amat-Trigo et al., 2024). Consequently, in cold winter temperatures in Southern England (< 8°C), most carp and pike are likely to be in a low activity state and, in very cold periods, might enter periods of winter quiescence (especially carp; Block et al., 2020). In both species, these low activity levels appear to result in reduced scale cortisol levels compared to warmer seasons when it is expected that the fish have higher activity levels and metabolic rates. Additionally, with carp in England not being able to reproduce successfully each year due to temperature constraints (Britton et al., 2010), this potentially adds an additional physiological stressor relating to reproduction in late spring/ early summer. Nevertheless, carp can reproduce in England occasions, with some recruitment evident (Skeate et al., 2022), and thus the elevated summer cortisol levels might reflect spawning activity more than the increased fish activity, although decoupling this would be challenging. Irrespective, these results demonstrate that studies comparing scale cortisol measurements across populations must consider the season of sample collection in their analyses. Notwithstanding the importance of this result, it should also be noted that in the carp samples, only one fish was available for analysis at Site 2 in spring and Site 3 in winter, limiting the robustness of these results. However, this is offset by the higher sample sizes in each season of the carp samples from Site 1, which were a minimum of 20 in each analysed season and that revealed the distinct seasonal pattern in the scale cortisol concentrations.

The fish sampling method also had a strong influence on scale cortisol levels, with chub and pike sampled from a population by electric fishing exhibiting wider ranges of scale cortisol than those sampled from the same populations by angling. It is recognised that in general, angling is a non-random method of fish capture, where selection is usually for

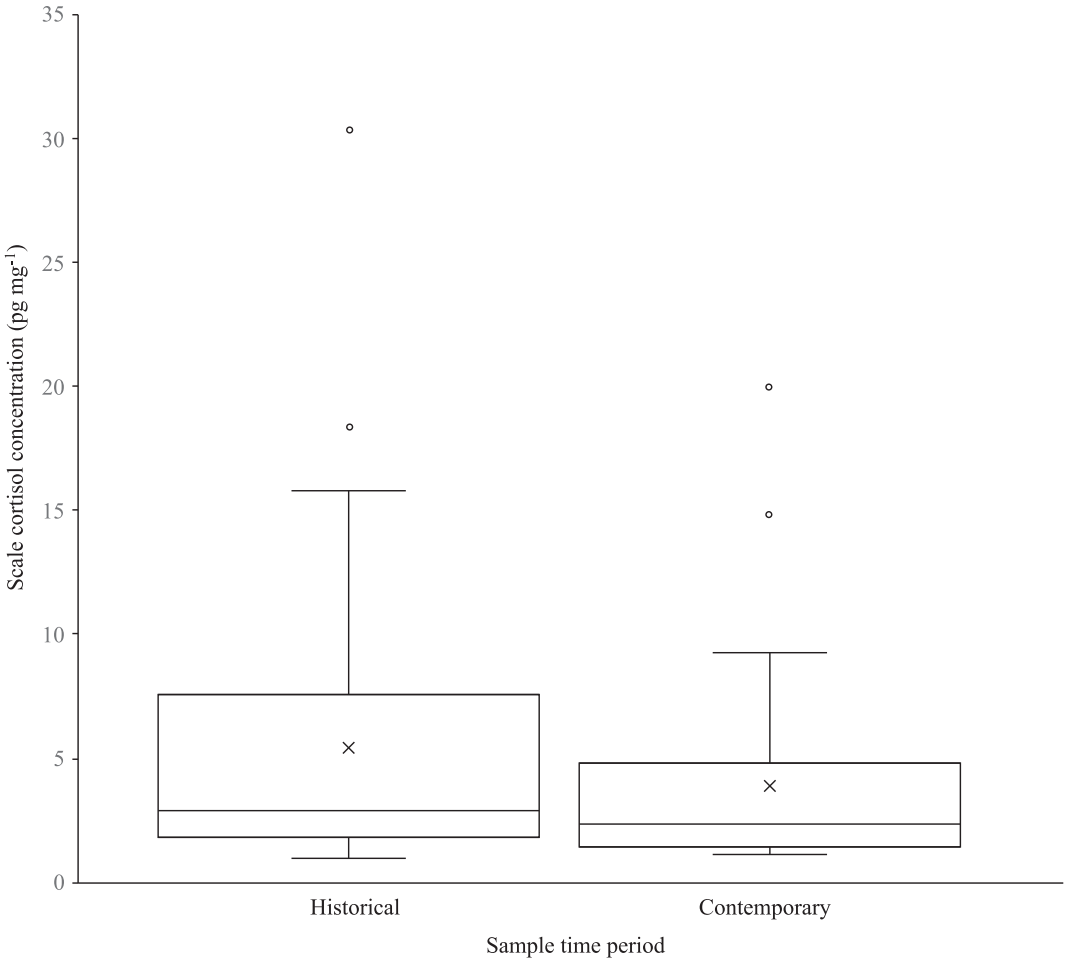


Fig. 4. Box plot comparing the distribution of scale cortisol concentrations of chub *Squalius cephalus* sampled from across three rivers in England (cf. Materials and Methods) between 1983 and 1999 ("Historical") and 2018 and 2022 ("Contemporary"). Horizontal lines represent 10, 25, 50, 75 and 90 percentiles, x is the mean and clear circles are outliers.

Table 4
Output of the best fitting GLM testing differences in chub scale cortisol concentrations across historical and contemporary samples (scale cortisol ~ time period (historical/ contemporary) + chub length + sampling method (angling/ electric fishing) + (1 | Year) + River). Bold denotes significant values.

Coefficient	Estimate	95 % CI of estimate	P
(Intercept)	1.59	−0.05 to 3.22	0.06
River Wensum	−0.53	−1.01 to −0.05	**0.03**
Chub length	−0.00	−0.01 to −0.00	**0.05**
Electric fishing	0.43	−0.22 to 1.09	0.20
Historical	0.22	−0.80 to 1.23	0.67
River Teme	0.03	−0.81 to 0.87	0.95
Random effects			
σ^2	0.43		
$\tau_{00\ Year}$	0.00		
N_{Year}	11		

population sub-groups with specific trait combinations that are most vulnerable to capture (Britton et al., 2023). Although there can be variability between species in the trait combinations most vulnerable to angling, there is a general pattern that trait combinations involving high activity and boldness result in relatively high vulnerability, with these traits also usually aligning to high stress resilience (Castanheira et al., 2017; Vindas et al., 2017; Villegas-Ríos et al., 2018). This was apparent in rainbow trout, where individuals that had low physiological responses to an experimental stressor were more vulnerable to angling capture than those with higher stress responses (Koeck et al., 2019; Monk et al., 2021). Phenotypic differences between angled and electric fished individuals were also apparent in European barbel, where individuals with smaller home ranges were primarily sampled by angling, whereas those with larger home ranges were captured by electric fishing, with electric fishing considered as less biased in fish capture than angling (Vehanen et al., 2013; Radinger et al., 2019). Consequently, comparisons of scale cortisol levels within and between populations must consider the capture method to avoid erroneous evaluations and, where representative profiles of scale cortisol levels are desired for a population, then sampling methods used must be able to capture all phenotypes present.

The cortisol extraction method is also important, given that across five methods, one provided very low yields and concentrations, with two others also providing relatively low values. The results here indicate that in the extraction protocols, the grinding step played an important role in the extraction of cortisol from scales, given the very low yield from protocol 3 that lacked grinding. It is recommended that extraction protocols use a wet grinding / extraction approach (enabling the grinding and extraction step to be completed at the same time) for a duration of 1 h, as this produced the highest cortisol yield.

In summary, scale cortisol concentrations have increasingly been used as a method to investigate chronic stress levels in fish, although issues relating to extraction method and sample collection have inhibited the wider use of the method. A series of these issues have been overcome here, where the most effective extraction methods were identified, and the importance of season and method of fish capture was revealed. Given that measures of chronic stress levels in fish can provide

a valuable metric to determine the chronic response of fishes to stressors in their environment, we argue that the use of the information provided here should generate more reliable data that assist comparisons over time and space.

CRediT authorship contribution statement

Ahmad Ghazal: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Richard Paul:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **A. Serhan Tarkan:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **J. Robert Britton:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding

The work was supported by an internal grant awarded by Bournemouth university under the Research Capacity Transformation Scheme.

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.

Acknowledgements

We thank the owners of the fisheries who provided permissions for the collection of the scales.

Data availability

Data will be made available on request.

References

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G., De Saeger, S., 2015. Scales tell a story on the stress history of fish. PLoS One 10 (4).
Ahmad, S.M., Shah, F.A., Bhat, F.A., Bhat, J.I.A., Balkhi, M.H., 2011. Thermal adaptability and disease association in common carp (Cyprinus carpio communis) acclimated to different (four) temperatures. J. Therm. Biol. 36 (8), 492–497.
Amat-Trigo, F., Tarkan, A.S., Andreou, D., Aksu, S., Bolland, J.D., Gillingham, P.K., Roberts, C.G., Yeldham, M.I., Britton, J.R., 2024. Variability in the summer movements, habitat use and thermal biology of two fish species in a temperate river. Aquat. Sci. 86 (3), 1–15.
Balasch, J.C., Tort, L., 2019. Netting the stress responses in fish. Front. Endocrinol. 10, 62.
Bartoń, K. (2022). MuMIn: Multi-Model Inference. R package version 1.47.1. Available at https://CRAN.R-project.org/package=MuMIn.
Bertotto, D., Poltronieri, C., Negrato, E., Majolini, D., Radaelli, G., Simontacchi, C., 2010. Alternative matrices for cortisol measurement in fish. Aquacult. Res. 41 (8), 1261–1267.
Block, B.D., Stockwell, J.D., Marsden, J.E., 2020. Contributions of winter foraging to the annual growth of thermally dissimilar fish species. Hydrobiologia 847 (20), 4325–4341.
Boonstra, R., 2013. Reality as the leading cause of stress: rethinking the impact of chronic stress in nature. Func. Ecol. 27 (1), 11–23.
Britton, J.R., Cucherousset, J., Davies, G.D., Godard, M.J., Copp, G.H., 2010. Non-native fishes and climate change: predicting species responses to warming temperatures in a temperate region. Freshwater Biol. 55 (5), 1130–1141.
Britton, J.R., Andreou, D., Lopez-Bejar, M., Carbajal, A., 2023. Relationships of scale cortisol content suggest stress resilience in freshwater fish vulnerable to catch-and-release angling in recreational fisheries. Fish. Res. 266.
Carbajal, A., Monclús, L., Tallo-Parra, O., Sabes-Alsina, M., Vinyoles Cartanyà, D., López Béjar, M., 2018. Cortisol detection in fish scales by enzyme immunoassay: biochemical and methodological validation. J. Appl. Ichthyol. 34, 967–970.
Carbajal, A., Reyes-López, F.E., Tallo-Parra, O., Lopez-Bejar, M., Tort, L., 2019a. Comparative assessment of cortisol in plasma, skin mucus and scales as a measure of the hypothalamic-pituitary-interrenal axis activity in fish. Aquacult. 506, 410–416.
Carbajal, A., Tallo-Parra, O., Monclús, L., Vinyoles, D., Solé, M., Lacorte, S., López-Béjar, M., 2019b. Variation in scale cortisol concentrations of a wild freshwater fish: Habitat quality or seasonal influences? Gen. Comp. Endocrinol. 275, 44–50.
Castanheira, M.F., Conceição, L.E., Millot, S., Rey, S., Bégout, M.L., DamsgAard, B., Kristiansen, T., Höglund, E., Øverli, Ø., Martins, C.I., 2017. Coping styles in farmed fish: consequences for aquaculture. Review. Aquacult. 9 (1), 23–41.
Chatterjee, N., Pal, A.K., Manush, S.M., Das, T., Mukherjee, S.C., 2004. Thermal tolerance and oxygen consumption of Labeo rohita and Cyprinus carpio early fingerlings acclimated to three different temperatures. J. Therm. Biol. 29 (6), 265–270.
Cyr, N.E., Romero, L.M., 2009. Identifying hormonal habituation in field studies of stress. Gen. Comp. Endocrinol 161 (3), 295–303.
Damsgård, B., Evensen, T.H., Øverli, Ø., Gorissen, M., Ebbesson, L.O., Rey, S., Höglund, E., 2019. Proactive avoidance behaviour and pace-of-life syndrome in Atlantic salmon. Roy. Soc. Open Sci. 6 (3).
Geis-Asteggiante, L., Lehotay, S.J., Fortis, L.L., Paoli, G., Wijey, C., Heinzen, H., 2011. Development and validation of a rapid method for microcystins in fish and comparing LC-MS/MS results with ELISA. Analyt. Bioanalyt. Chem. 401, 2617–2630.
Gutmann Roberts, C., Hindes, A.M., Britton, J.R., 2019. Factors influencing individual movements and behaviours of invasive European barbel Barbus barbus in a regulated river. Hydrobiologia 830, 213–228.
Hanke, I., Ampe, B., Kunzmann, A., Gärdes, A., Aerts, J., 2019. Thermal stress response of juvenile milkfish (Chanos chanos) quantified by ontogenetic and regenerated scale cortisol. Aquaculture 500, 24–30.
Harris, B.N., Carr, J.A., 2016. The role of the hypothalamus-pituitary-adrenal/interrenal axis in mediating predator-avoidance trade-offs. Gen. Comp. Endocrinol. 230, 110–142.
Hartig, F., 2023. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.4.6. Available at: https://CRAN.R-project.org/package=DHARMa.
Ieno EN, Zuur AF., 2015. A Beginner's guide to data exploration and visualisation with R.
Kennedy, E.K., Janz, D.M., 2023. Can scale cortisol concentration be quantified non-lethally in wild fish species? Conserv. Physiol. 11 (1), p.coac081.
Klefoth, T., Skov, C., Kuparinen, A., Arlinghaus, R., 2017. Toward a mechanistic understanding of vulnerability to hook-and-line fishing: Boldness as the basic target of angling-induced selection. Evol. Appl. 10 (10), 994–1006.
Koeck, B., Závorka, L., Aldvén, D., Näslund, J., Arlinghaus, R., Thörnqvist, P.O., Winberg, S., Björnsson, B.T., Johnsson, J.I., 2019. Angling selects against active and stress-resilient phenotypes in rainbow trout. Can. J. Fish. Aquat. Sci. 76 (2), 320–333.
Kulczykowska, E., Kalamarz-Kubiak, H., Gozdowska, M., Sokołowska, E., 2018. Cortisol and melatonin in the cutaneous stress response system of fish. Comp. Biochem. Physiol. a. 218, 1–7.
Laberge, F., Yin-Liao, Bernier, N.J., 2019. Temporal profiles of cortisol accumulation and clearance support scale cortisol content as an indicator of chronic stress in fish. Conserv. Physiol. 7 (1), p.coz052.
Lebigre, C., Woillez, M., Barone, H., Mourot, J., Drogou, M., Le Goff, R., Servili, A., Hennebert, J., Vanhomwegen, M., Aerts, J., 2022. Temporal variations in scale cortisol indicate consistent local-and broad-scale constraints in a wild marine teleost fish. Mar. Env. Res. 182.
Lemos, L.S., Angarica, L.M., Hauser-Davis, R.A., Quinete, N., 2023. Cortisol as a stress indicator in fish: sampling methods, analytical techniques, and organic pollutant exposure assessments. Int. h. Environ. Res. Pub. Health 20 (13), 6237.
Madaro, A., Nilsson, J., Whatmore, P., Roh, H., Grove, S., Stien, L.H., Olsen, R.E., 2023. Acute stress response on Atlantic salmon: a time-course study of the effects on plasma metabolites, mucus cortisol levels, and head kidney transcriptome profile. Fish Physiol. Biochem. 49 (1), 97–116.
Marsden, J.E., Blanchfield, P.J., Brooks, J.L., Fernandes, T., Fisk, A.T., Futia, M.H., Hlina, B.L., Ivanova, S.V., Johnson, T.B., Klinard, N.V., Krueger, C.C., 2021. Using untapped telemetry data to explore the winter biology of freshwater fish. Rev. Fish Biol. Fish. 31, 115–134.
Margenau, T.L., Rasmussen, P.W., Kampa, J.M., 1998. Factors affecting growth of northern pike in small northern Wisconsin lakes. N. Am. J. Fish. Man. 18 (3), 625–639.
Mehdi, H., Lau, S.C., Synyshyn, C., Salena, M.G., Morphet, M.E., Hamilton, J., Muzzatti, M.N., McCallum, E.S., Midwood, J.D., Balshine, S., 2021. A comparison of passive and active gear in fish community assessments in summer versus winter. Fish. Res. 242.
Monk, C.T., Bekkevold, D., Klefoth, T., Pagel, T., Palmer, M., Arlinghaus, R., 2021. The battle between harvest and natural selection creates small and shy fish. Proc. Nat. Acad. Sci. 118 (9).
O'Toole, C., White, P., Thomas, K., O'Maoiléidigh, N., Fjelldal, P.G., Hansen, T.J., Graham, C.T., Brophy, D., 2023. Effects of temperature and feeding regime on cortisol concentrations in scales of Atlantic salmon post-smolts. J. Exp. Mar. Biol. Ecol. 569.
O'Toole, C., White, P., Graham, C.T., Conroy, C., Brophy, D., 2024. Cortisol in fish scales remains stable during extended periods of storage. Conserv. Physiol. 12 (1), p. coae065.
Oyugi, D.O., Cucherousset, J., Ntiba, M.J., Kisia, S.M., Harper, D.M., Britton, J.R., 2011. Life history traits of an equatorial common carp Cyprinus carpio population in relation to thermal influences on invasive populations. Fish. Res. 110 (1), 92–97.
R Core Team, 2024 R: A Language and Environment for Statistical Computing.
Radinger, J., Britton, J.R., Carlson, S.M., Magurran, A.E., Alcaraz-Hernández, J.D., Almodóvar, A., Benejam, L., Fernández-Delgado, C., Nicola, G.G., Oliva-Paterna, F. J., Torralva, M., 2019. Effective monitoring of freshwater fish. Fish Fish. 20 (4), 729–747.

Roque d'Orbcastel, E., Bettarel, Y., Dellinger, M., Sadoul, B., Bouvier, T., Amandé, J.M., Dagorn, L., Geffroy, B., 2021. Measuring cortisol in fish scales to study stress in wild tropical tuna. Environ. Biol. Fish. 104 (6), 725–732.
Sadoul, B., Geffroy, B., 2019. Measuring cortisol, the major stress hormone in fishes. J. Fish. Biol. 94 (4), 540–555.
Samaras, A., Pavlidis, M., 2022. Fish scales produce cortisol upon stimulation with ACTH. Animals 12 (24), 3510.
Samaras, A., Dimitroglou, A., Kollias, S., Skouradakis, G., Papadakis, I.E., Pavlidis, M., 2021. Cortisol concentration in scales is a valid indicator for the assessment of chronic stress in European sea bass, Dicentrarchus Labrax l. Aquaculture 545.
Skeate, E.R., Perrow, M.R., Tomlinson, M.L., Madgwick, G., Harwood, A.J., Ottewell, D., Berridge, R., Winfield, I.J., 2022. Fish stocking for recreational angling is culpable for the poor condition of many English lakes designated for conservation purposes. Inland Waters 12 (1), 19–32.
Sutton, A.O., Studd, E.K., Fernandes, T., Bates, A.E., Bramburger, A.J., Cooke, S.J., Hayden, B., Henry, H.A., Humphries, M.M., Martin, R., McMeans, B., 2021. Frozen out: Unanswered questions about winter biology. Env. Rev. 29 (4), 431–442.
Vašek, M., Souza, A.T., Říha, M., Kubečka, J., Znachor, P., Hejzlar, J., 2021. Stable isotope evidence from archived fish scales indicates carbon cycle changes over the four-decade history of the Římov Reservoir (Czechia). Sci. Tot. Environ. 755.
Vehanen, T., Sutela, T., Jounela, P., Huusko, A., Mäki-Petäys, A., 2013. Assessing electric fishing sampling effort to estimate stream fish assemblage attributes. Fish. Man. Ecol. 20 (1), 10–20.
Vercauteren, M., Ampe, B., Devriese, L., Moons, C.P.H., Decostere, A., Aerts, J., Chiers, K., 2022. Explorative study on scale cortisol accumulation in wild caught common dab (Limanda limanda). BMC Vet. Res. 18 (1), 324.
Villegas-Ríos, D., Réale, D., Freitas, C., Moland, E., Olsen, E.M., 2018. Personalities influence spatial responses to environmental fluctuations in wild fish. J. Anim. Ecol. 87 (5), 1309–1319.
Vindas, M.A., Gorissen, M., Höglund, E., Flik, G., Tronci, V., Damsgård, B., Thörnqvist, P. O., Nilsen, T.O., Winberg, S., Øverli, Ø., Ebbesson, L.O., 2017. How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. J. Exp. Biol. 220 (8), 1524–1532.