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Cortisol, cortisone and DHEAS in epidermis and scales of fish *Aphanius fasciatus*: HPLC-MS/MS measurement of stress indicators as proxies for natural and human-induced factors

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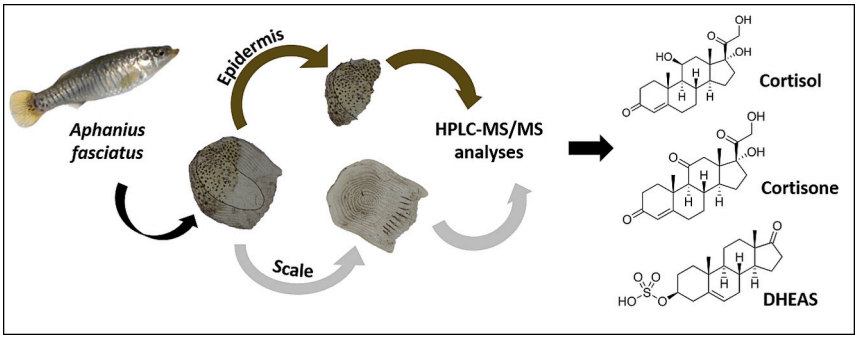
HIGHLIGHTS

- Environmental stressors can severely affect fish life and new procedures for stress measurement are required.
- An innovative, fast and sensitive method for the quantification of stress hormones in fish epidermis and scales is reported.
- Preanalytical procedure is fast, simple and allows to separate acute and chronic stress of *Aphanius fasciatus*.
- The quantification of stress hormones in fragile local fauna allows to study the environment quality.

GRAPHICAL ABSTRACT



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ABSTRACT

Fish health can be affected by a multitude of stressors. Acute and chronic stress assessment via specific hormones monitoring has become a trending research topic. Common investigated matrices are blood and plasma, but recently less invasive substrates have been identified. As chemical composition of skin mucus/epidermis has been demonstrated to link with acute stress, and of scales with chronic stress in fish, the aim of the study was firstly to improve the determination of three stress hormones, namely cortisol (COL), cortisone (CON), and dehydroepiandrosterone-3-sulfate (DHEAS), in skin mucus/epidermis and scales of *Aphanius fasciatus*. Secondly, an evaluation of the impact of different environments on hormones concentrations was carried out. A liquid chromatography coupled to tandem mass spectrometry method (HPLC-MS/MS) and a preanalytical procedure were validated to determine COL, CON and DHEAS. This methodology was applied to compare a pull of field-collected fish with a pull of fish housed in the laboratory for one year. Our results highlighted a significant

Abbreviations: 11β-HSD, 11-βhydroxysteroid dehydrogenase; AP, freshly caught fish; AVMA, American Veterinary Medical Association; COL, cortisol; CON, cortisone; DHEAS, dehydroepiandrosterone-3-sulfate; EIA, enzymatic immunoassay; HPI, hypothalamus-pituitary-interrenal axis; LOD, instrumental limit of detection; LOQ, instrumental limit of quantification; MDL, method detection limit; MQL, method quantification limit; OP, laboratory-housed fish; PSU, Practical Salinity Unit.

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presence of cortisol and cortisone in epidermis of the latter pull (averagely 0.10 and 0.14 ng mg^{-1}, respectively), while in the first pull both hormones were much less concentrated (averagely 0.006 and 0.008 ng mg^{-1}, respectively). Scales of both pulls showed presence of hormones, with a higher concentration for fish housed in the laboratory, although a relevant difference in concentration was found only for cortisone. DHEAS was always below the limit of detection.

1. Introduction

During their lifetime, animals experience and react to a wide array of situations and stimuli, which often induce stress. Animals can undergo two types of stress: acute stress, which is correlated to sudden events, and chronic stress, caused by prolonged and persistent situations such as presence of pollution and intensive farm and aquaculture (Barreto et al., 2022; Tort, 2011). The overall effects of stress can be highly deleterious: for instance, fishes can manifest development issues, immune suppression, osmo-regulatory problems, and infertility (Kennedy and Janz, 2023; Wu et al., 2015).

Generally, stressors can be divided into two main categories: biotic and abiotic. While the first includes biological sources, i.e., bacteria, viruses, parasites, and predators, the latter consists of non-living factors and anthropic-derived conditions, such as the presence of pollutants, fertilizers, and nutrients, the increase in Earth's average temperature, the extreme weather events, and other phenomena related to climate change (Khoshru et al., 2023). For what concerns aquatic environment, abiotic stressors include the variation of physical-chemical properties of water, i.e., pH, salinity, temperature, concentration of dissolved oxygen (Li et al., 2022), presence of pollutants, fertilizers, and phytopharmaceutical products (Fernandes et al., 2008; Korkmaz and Örün, 2022; Yang et al., 2023) and also specific human activities such as the noise pollution due to a music festivals (Cartolano et al., 2020).

Stress responses of fish can be roughly divided in primary, secondary and tertiary (Wu et al., 2015). The first category includes rapid production and release of stress hormones, i.e., catecholamine and cortisol (COL). These molecules trigger the secondary response, consisting in several physiological and biochemical modifications such as alteration of carbohydrate metabolism and other metabolic changes (Eissa and Wang, 2016; Nakano et al., 2014). These conditions lead to the tertiary response, which is expressed in a much more dramatic behavioural effect such as abnormal performance (Eissa and Wang, 2016).

COL is the most secreted glucocorticoid hormone released into the bloodstream via the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Sadoul and Geffroy, 2019). It is frequently investigated as stress indicator to evaluate animal wellness since i) the notable concentration allows a facile monitoring and ii) its secretion is strongly connected to stressful situations and can provide information on specific conditions of the animal (Mommsen et al., 1999; Sadoul and Geffroy, 2019). Indeed, cortisol levels are often employed to correlate the impact of anthropogenic stressors on animal wellness (Kennedy and Janz, 2023, 2022; Sadoul and Geffroy, 2019), since they can be altered also by endocrine disrupters and toxicants (Mommsen et al., 1999).

Common methodologies to evaluate COL concentrations require collecting blood and plasma (Li et al., 2022; Nakano et al., 2014; Wu et al., 2015). Nevertheless, this sampling procedure has some drawbacks. First of all, it is an invasive technique that causes extra stress to the animal. Secondly, the matrices involved do not accumulate hormones for a long amount of time, thus analyses provide information on acute stress only (Kennedy and Janz, 2023). Therefore, alternative matrices for cortisol evaluation have been successfully tested and include urine, faeces, surrounding water, skin mucus, and scales (Carbajal et al., 2019; Sadoul and Geffroy, 2019). It has been demonstrated that skin mucus cortisol correlates positively to cortisol in plasma, suggesting that mucus and epidermis are good matrices to study acute stress (Bertotto et al., 2010; De Mercado et al., 2018). On the contrary, scales are a matrix mainly made of calcium phosphate material similar to hydroxyapatite, with some inner layers of collagen (Kerr and Campana, 2014; Metz et al., 2012). Thus, they are suitable for the bioaccumulation of cortisol through diffusion from blood during the individual's life and allow to study chronic stress (Carbajal et al., 2019; Kennedy and Janz, 2023, 2022; Sadoul and Geffroy, 2019). Therefore, by separating the skin mucus and the epidermis that cover the scale from the scale itself, it should be possible to evaluate separately acute and chronic stress. Information provided by these two matrices are different, and our aim is to understand if these two types of stress can act as efficient indicators for the monitoring of the sampling environment wellness.

The study of a single biomarker can be insufficient for a good evaluation of animal stress. Indeed, although cortisol is the most secreted glucocorticoid, the study of its hormonally inactive form, cortisone (CON), can furnish additional information on the functioning of HPI-axis in fish (Mommsen et al., 1999). CON derives from the degradation of COL by the enzymatic activity of 11-βhydroxysteroid dehydrogenase (11β-HSD), which can be also increased by environmental stressors such a rise in temperature (Kennedy and Janz, 2023). In mammals, the ratio cortisone-to-cortisol (CON:COL) is often used to assess stress condition, as it is a good evaluation of COL inactivation and of enzymatic activity, which correlates to potential diseases (Andrews et al., 2002; Tiosano et al., 2003). Another molecule strongly linked to stress is dehydroepiandrosterone-3-sulfate (DHEAS), the sulphated and inactive precursor of dehydroepiandrosterone (DHEA), an anti-glucocorticoid hormone with anti-stressor function (Kennedy and Janz, 2022). DHEAS circulates as a DHEA reserve, indeed it can be de-sulphated via enzymatic activity when levels of stress hormones become dangerous for the organism (Kalimi et al., 1994). As it was demonstrated in mammals, levels of COL and DHEAS vary inversely proportionally with age – indeed COL tends to increase, while DHEAS decreases – thus it was suggested that ratio DHEAS:COL could be useful to evaluate state of organism aging (Kalimi et al., 1994). Nevertheless, studies on fish are still limited. Hence, our aim was not only to assess COL levels, which is already been largely investigated, but to have a more complete understanding of the stress response in fish by taking into account also CON, DHEAS, and their ratios with COL, allowing to understand more globally stress response in fish.

To produce useful data, the monitoring of stress levels should be applied to relevant fish. Annex II of the Habitat Directive (HD) provides a list of animals and plants species of Community interest that require specific conservation zones, citing >60 fish species. However, in the Italian transitional environments, few fish species spend the whole life cycle in this kind of habitats, including the Mediterranean killifish *Aphanius fasciatus* (Valenciennes, 1821). This species, together with two small goby species (the black spotted goby *Ninnigobius canestrinii* (Ninni, 1883) and the lagoon goby *Knipowitschia panizzae* (Verga, 1841), belong to the ecological guild of estuarine residents. *A. fasciatus* was listed by the Italian Legislation in the HD as an indicator of complex environmental structures in need of specific conservation areas (Council Directive 92/43/CEE). It was recently proposed as an ecological indicator of habitat quality in the context of European transitional waters since it can provide information about the ecosystem status based on their abundance and presence (Facca et al., 2020). As such, this fish is a suitable candidate for environmental wellness monitoring (Cavraro et al., 2014). *A. fasciatus* is a small fish (2–9 cm, Supporting Fig. S1) with a short life cycle that belongs to the Killifish group (Actinopterygii: Cyprinodontiformes). It is sedentary throughout the year, with low

dispersal capacities, and the population is supposed to move in a deeper part of the creek to winter. Although this fish can tolerate a wide range of salinity conditions, from 10 to 180 PSU (Practical Salinity Unit), it prefers brackish or hypersaline water bodies, subjected to tidal excursion and with strong fluctuations in chemical or physical parameters such as pH, turbidity, temperature, and salinity (Cavraro et al., 2014; Triantafyllidis et al., 2007). Despite its resilience, this fish is threatened by anthropogenic and natural stressors, i.e., habitat degradation, eutrophication, invasive species, and pollution, that might rapidly change the environmental condition and cause the local extinction of populations(Facca et al., 2020).

The species was studied to detect the environmental impact of chemical pollutants in Tunisia, investigating changes in liver mRNA (Kessabi et al., 2010). Another example of resident fish used as an ecological indicator can be found in the USA, where *Fundulus* sp., the ecological equivalent of *A. fasciatus* in the American salt marshes, was studied to understand the impact of an oil spill disaster in the northern Gulf of Mexico (McDonald et al., 2022). Still, to the best of our knowledge, no stress monitoring studies have been performed on *A. fasciatus*.

In the context of wider research program, using the Mediterranean killifish *A. fasciatus*, as an ecological indicator and a sentinel species for the Venice lagoon and the Mediterranean transitional waters, this paper aims to develop a sensitive and rapid method to measure and evaluate stress-related biomolecules extracted from fin scales in a fish with small size and short life cycle. The specific aims of the study herein proposed are: i) to develop and validate a fast, efficient and simple methodology to study acute and chronic stress on epidermis/skin mucus and scales of a fish of communitarian interest, such as *A. fasciatus,* based on the quantification of three stress-related biomolecules, namely COL, CON and DHEAS (Supporting Table S1) via liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS); ii) to study if the monitoring of stress hormones in *A. fasciatus* is a suitable manner to assess venetian lagoon wellness; iii) to widen knowledge on the study of animal stress using less invasive matrices compared to blood and plasma, which is still an underrated field.

2. Materials and methods

Ultrapure water (18.2 MΩ-cm, 1 ppb TOC) was produced using a Purelab Ultra System (Elga®, High-Wycombe, UK) and ultra-grade methanol (MeOH) was purchased from VWR® (Radnor, PA, USA). Cortisol (COL), Cortisone (CON) and Dehydroepiandrosterone-3-sulfate (DHEAS) were purchased from Sigma-Aldrich (Darmstadt, Germany). As internal standard, Cortisol-D4 (COL*, Sigma-Aldrich, Darmstadt, Germany) was employed. All materials were first decontaminated with ultra-grade MeOH. Samples were weighted with a microanalytical balance. Samples were stored in 1.5 mL plastic tubes. Scales extraction was performed with an ultrasound bath (Ceia CP104). Powdering of the scales was performed with a Rotary Tool (model PCG01B, TACKlife) with two different abrasive drills (Supporting Fig. S2). Filtration of supernatant was performed with a 3 mL plastic syringe (Braun, Melsugen, Germany) equipped with a Cellulose Acetate syringe filter (⌀ 0.22 μm, 13 mm, GVS Sanford, USA). Low volume glass vial and amber glass vial were used for the analyses.

Prior to any statistical testing, data were tested for normality and homoscedasticity using the Shapiro–Wilk test and the F test respectively. Statistical significance was taken as $p < 0.05$. All the hypothesis tests were run using g R-studio (version 1.1.463) base packages (version 3.5.1). Student *t*-test was employed to identify the significant mean differences between the data distributions.

2.1. Ethics declarations

Animals were handled in accordance with the principles stated by the EU Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes. All individuals studied in the laboratory were released at the sampling site as well as new-borns during laboratory operation.

Fishes euthanasia was conducted following the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals (Leary et al., 2020). Fishes were anaesthetised by immersion in a low concentrated ethanol solution (20 mL/L) and maintained in the solution until death occurred.

2.2. Fish sampling

Sampling site is located near the Venice Lagoon (Veneto, Italy), more specifically at Forte Marghera (45°28′31.7″N 12°15′42.0″E, Supporting Fig. S3). Canals of Forte Marghera are subjected to tidal excursion due to their proximity to the Lagoon and receive water from the Venice Lagoon and the Canal Salso, an anthropised canal characterised by salt and brackish water. In a contest of a different investigation, about 4500 fish were collected using a small rectangular fyke net (25 × 25 × 45 cm, 5 mm mesh) with a metallic frame. At both sides, a circular hole entrance with a diameter of 65 mm is placed at the end of a conical invitation turned towards the trap's centre (Supporting Fig. S4). "Algae wafer mini, Prodac" (composed of cereals, vegetables, algae, spirulina, and soybeans) was put into the trap in a small net bag to attract the fish. Fyke net was lowered with a sampling effort of three hours, the net was released during the central hours of the day (10–13 am). This capture method was chosen to reduce as much as possible the injuries on animals, as they entered the trap on their own. A sub-sample of the collected fish was transported to the zoology laboratory (at the Ca' Foscari University Scientific Campus, Via Torino 155, 30172, Mestre, Italy) for further analysis and kept in the aquariums for one year, where salinity tolerance and spawning capacity at different abiotic conditions were tested. The photoperiod kept in the aquarium lab followed the natural conditions. During this period, fish were frequently moved to different tanks and subjected to external stressors, such as changes in salinity and temperature, allowing us to check possible differences in chronic stress between field-collected individuals and fish kept in the laboratory. <0.5 % of the sampled population was sacrificed for the purpose of this study.

2.3. Scale removal and sample preparation

Scales were removed from the entire body by scraping the length of the body from head to tail with stainless steel tweezers, collected into a 1.5 mL snap cap Eppendorf and stored at −20 °C until analyses. All the fish were captured from the tanks during the day.

An average amount of 45–50 mg of wet scales (corresponding to slightly more than two fishes) was weighted into a 1.5 mL snap cap plastic tube and dried in oven at 60 °C for 1 h. Hormones degrade at a temperature higher that 200 °C, therefore warming up the samples is not problematic for molecule stability (The Merck Index, 2007). Next, 1 ng of internal standard COL* was spiked to the dry scales and 500 μL of ultrapure water were added. Removal of skin mucus/epidermis from scales was performed by vortexing the samples for 5 min at 1800 rpm with an Ika-Vibrax-VRX Type VX8 (Janke & Kunkel GmbH & Co KG, IKA Labortechnik Staufen, Germany). Absence of skin residuals on the scale was ensured via microscope analysis. Supernatant was then removed, filtered using a plastic syringe equipped with a 0.22 μm Acetate Cellulose filter and collected in a low-volume injection glass vial. The scales were added with 1 mL of ultrapure water and vortexed for few seconds. Supernatant was removed, filtered and collected into a 1.5 mL glass vial. This operation was repeated twice, for a total of three rinsing. These steps were needed to ensure that the scales were clean from the internal standard used to quantify hormones in epidermis, and disappearance of COL* was confirmed by HPLC-MS/MS analysis. Wet scales were transferred into a pre-weighted low-volume injection vial and dried in oven at 60 °C for 1 h; next, to ascertain the steadiness of weight after the dryness, scales were weighted three times. Scales were then pulverised with

a Rotary Tool. The powder (an average amount of 20 mg) was then spiked with 0.6 ng of internal standard COL*and 300 μL of ultrapure water were then added. Extraction was performed in a sonication bath for 30 min at 50 °C. The supernatant was filtered into a low-volume injection vial and analysed.

2.4. Instrumental analyses

The determination of COL, CON and DHEAS was performed by an Ultimate 3000 UHPLC system (Thermo Scientific™, USA) coupled with a TSQ Altis™ Plus Triple Quadrupole Mass Spectrometer (Thermo Scientific™, USA) using a heated-electrospray source (H-ESI).

Chromatographic separation was performed using a Synergi™ 2.5 μm Hydro-RP column 30 × 2 mm (Phenomenex, CA, USA) equipped with a 0.2 μm on-line filter. The mobile phase used during the elution was a mixture of ultrapure water (phase A) and methanol (phase B), with a flow rate of 0.25 mL min^{-1}. Chromatographic gradient was set as follow: 0–1 min isocratic step at 5 % phase B; 1–8 min gradient from 5 % to 85 % phase B; 8 min 100 % phase B; 8–10 min isocratic step at 100 % phase B; 10–12 equilibration stage at 5 % eluent B. The sample volume was set at 50 μL. A post-column reactive (0.025 mL min^{-1}, 0.7 % NH_3 in MeOH) was required to improve the ionisation of analytes into the H-ESI source. The mass spectrometer's source parameters were set as follow: negative ion potential −2800 V; sheath gas 53 Arb; auxiliar gas 4 Arb; sweep gas 0 Arb; ion transfer tube temperature 200 °C; vaporizer temperature 300 °C. All experimental parameters are summarized in Supporting Tables S2 and S3.

2.5. QA/QC

Quality control was performed by comparing the native compound peak area with that of the internal standard. We corrected the results using the instrumental response factor by analysing a solution with a mean concentration of 2 μg L^{-1} of COL, CON, DHEAS and COL*. The analytical procedure was validated by determining the linear ranges, instrumental limit of detections (LODs), instrumental limit of quantification (LOQs), procedural blanks, method detection and quantification limits (MDLs and MQLs), repeatability and trueness. The linearity was assessed from 0.2 ng L^{-1} to 10 μg L^{-1}, good linearity was obtained with R^2 values between 0.9981 and 0.9988. The slope, intercept, R^2, LOD and LOQ are reported in Supporting Table S4. Supporting Table S5 reports a summary of blank levels, MDL, MQL, trueness, recovery, and precision expressed as the relative percentual standard deviation (RSD%). *A. fasciatus* scales were used as the matrix for the procedure validation. Preliminary analyses of samples showed that DHEAS was never present neither in both skin mucus/epidermis and scales. Since DHEAS, COL and CON are close in backbone chemical structure and steric hindrance, and all three have polar moieties, DHEAS was chosen as the representative compound to validate the method. We decided to avoid the fortification of analytes for the method validation as it would have required quantities of COL and CON considerably higher than the concentration in the real sample. QA/QC was performed following the same procedure described in section Scale removal and sample preparation; some additional analyses were introduced to ensure the consistency of each step of the method, as reported in Section 3 (Results and Discussion).

For the skin mucus/epidermis quality control sample determination, a 1.5 mL snap cap plastic tube was put in oven at 60 °C for 1 h; no scales were added. 1 ng of internal standard (COL*) was spiked and 500 μL of ultrapure water were added. The solution was vortexed for 5 min at 1800 rpm, then the liquid was filtered and transferred into a low-volume HPLC injection vial, previously decontaminated with MeOH. Washing of the plastic tube was performed by adding 1 mL of ultrapure water, vortexing for few seconds and removing the liquid. This procedure was repeated two more times, for a total of three rinsing. Each aliquot was collected, filtered, and transferred into an HPLC injection vial, previously decontaminated with MeOH.

The blank determination of scales was performed transferring of remaining liquid of the 1.5 mL snap cap plastic tube into a low volume injection vial, which was next put in oven at 60 °C for 1 h. Using the Rotary Tool, glass was slightly scratched, then 0.6 ng of internal standard was spiked and 300 μL of ultrapure water were added. The vial was extracted via bath sonication for 30 min, at 50 °C. Liquid was then filtered and transferred into a low volume injection vial and analysed for MDL and MQL determination.

3. Results and discussion

3.1. Preanalytical method development and validation

3.1.1. Samples weighting and drying

After collecting the wet scales, microscope images were taken to ascertain the presence of mucus and epidermis film covering the scale itself (Fig. 1a). The film is supposed to receive the acute stress (Bertotto et al., 2010; De Mercado et al., 2018) and therefore must be separated from the scale, which in turn accumulates the chronic stress (Carbajal et al., 2019; Kennedy and Janz, 2023, 2022; Sadoul and Geffroy, 2019).

Literature (Carbajal et al., 2019, 2018; Kennedy and Janz, 2023; Roque d'orbcastel et al., 2021) collects several articles regarding fish scales analyses, however animals involved are usually bigger than *A. fasciatus* and a generous amount of 200 mg can be easily reached with a single fish. As *A. fasciatus* is a small fish, an average amount of 20 mg of wet scales can be collected from a single specimen. After different trials, it was proved that the lowest weight of wet scales that could guarantee concentrations of hormones above the quantification limit (Supporting Table S4) was 45–50 mg., which corresponds to ca. two fishes. As such, this amount of wet scales was put into a pre-decontaminated and pre-weighted 1.5 mL snap cap plastic tube and dried in oven. This drying step was performed in previous works by either 24 h of air dryness (Carbajal et al., 2018) or by padding (Kennedy and Janz, 2023). Both methods are extremely time-consuming, especially if a great number of samples has to be treated, and increase the chance to lose and contaminate the sample. Therefore, as hormones are thermally stables, it was decided to cut preanalytical times by performing the drying step in oven at a mild temperature of 60 °C, for 1 h. This combination was found to be the most suitable to avoid time-consuming steps and to guarantee the efficiency of the drying, without risking losing analytes due to thermal degradation.

3.1.2. Separation of epidermis and mucus from scales

Dry scales were weighted, 1 ng of internal standard COL* and 1 ng of DHEAS were spiked into the sample and 500 μL of ultrapure water were added. After 5 min of vortex at 1800 rpm, supernatant was filtered and transferred to a low-volume injection vial. Ultrapure water (1 mL) was added to the scales into the plastic tube and, after few seconds of vortex, it was removed. This operation was repeated twice, for a total of three rinsing. All the washing aliquots were collected and filtered in a 1.5 mL HPLC glass vial.

Preliminary washing trials consisted in agitating the scale for 2 min in ultrapure water and rinsing it with ultrapure water 3 times. Microscope images showed that some scales were perfectly clean, but others still presented epidermis. A big issue was the presence of scales stuck together, which somehow trapped water with epidermis and limited the cleaning efficiency (Fig. 1b). When vortexing time was extended to 5 min, all scales appeared clean (Fig. 1c). Carbajal et al. (2019) conducted a study to compare water and *i*-propanol washing efficiency. Their findings showed that the alcohol was able to clean scales from skin mucus without affecting cortisol concentration into scales, but on the contrary water was too aggressive (Carbajal et al., 2019). Our preliminary washing trials with *i*-propanol showed that, after three washings, scales were still covered by epidermis (Fig. 1d). The inefficiency of *i*-PrOH as washing solvent is in accordance with Kennedy and Janz (2023) findings (Kennedy and Janz, 2023). Other trials were performed

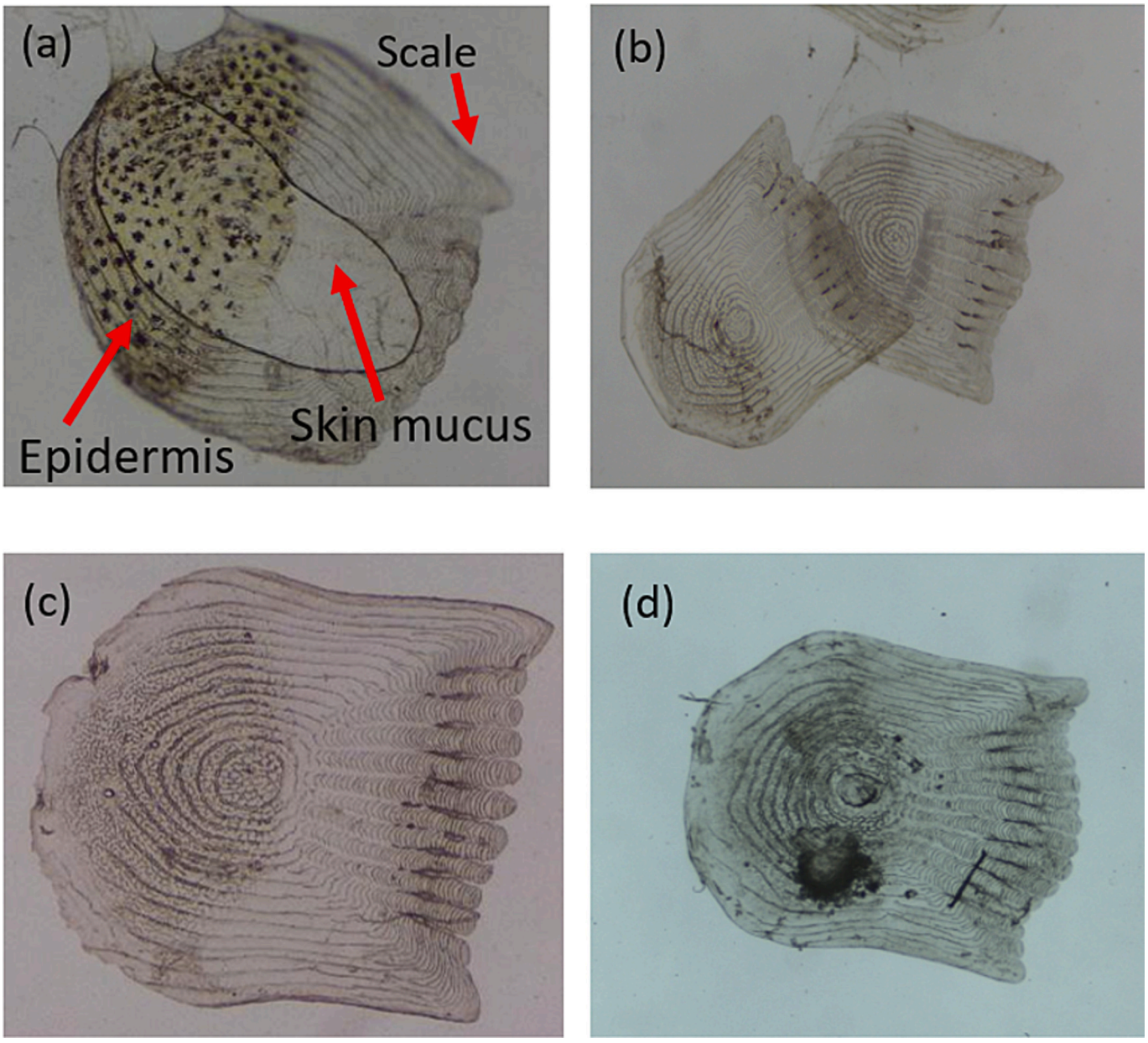

Fig. 1. Microscope images of: (a) *A. fasciatus* scale with epidermis and skin\ mucus, (b) Scales stuck together, (c) Scales washed three times with ultrapure water, (d) Scales washed three times with *i*-PrOH.

using less polar solvents, such as dichloromethane, heptane, and hexane, but results confirmed the need of a polar solvent to better clean the substrate. Therefore, it was decided to keep ultrapure water as cleaning solvent. To the best of our knowledge, this is the first work that ensures the absence of skin and mucus on the scale after the washing steps, which allows for major confidence in scale analyses and results.

3.1.3. Skin and mucus extraction validation

The washing and rinsing liquids were analysed. As can be seen from Fig. 2, almost all the spiked DHEAS is extracted in the first step. Indeed, an average of 92 % was recovered, confirming the efficiency of the procedure. For what concerns the three rinsing, as can be seen from Supporting Figs. S5 and S6 the amount of DHEAS and internal standard COL*, respectively, decreases. From the analysis of the third rinsing the signal of both compounds is comparable to the ones of the blanks.

3.1.4. Scales homogenization and extraction

After the cleaning process, the next step was to extract hormones from scales which are made principally of calcium phosphate material, similar to hydroxyapatite, with some inner layers of collagen as reported by Kerr and Campana (2014) (Kerr and Campana, 2014). Since smaller particles have greater surface area and allow a better interaction with the extraction medium, many previous reported works included a powdering step in their scale treating protocol employing either scissors (Aerts et al., 2015) or ball milling (Carbajal et al., 2018; Kennedy and Janz, 2023). In our case the amount of scales was not enough to successfully employ these tools or other instruments such as mortars or grinders. The solution was to use a Rotary Tool (TACKlife), which smashed and grinded the scales. Briefly, after the third rinsing, wet scales were transferred into a low-volume injection vial, dried at 60 °C for 1 h in oven and weighted. Subsequently, scales were pulverised with the Rotary Tool and weighted again. 1 ng of internal standard COL* and 1 ng of DHEAS were spiked into the sample and 300 μL of ultrapure

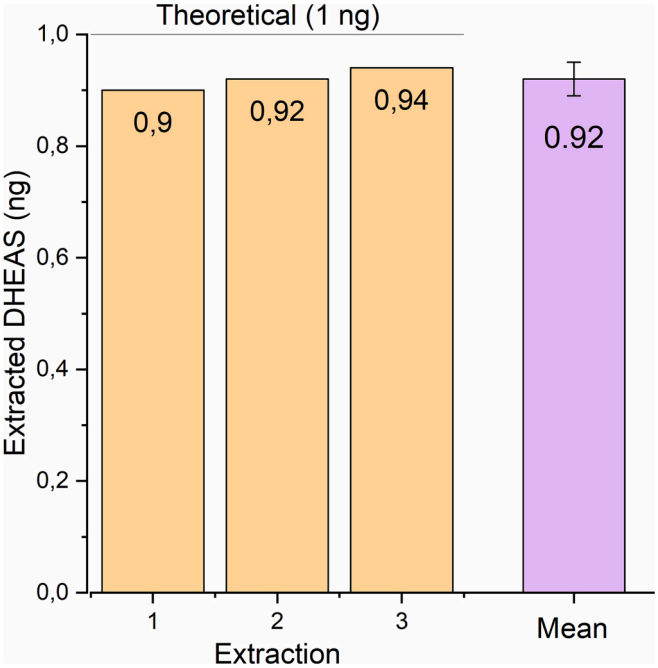

Fig. 2. Amount of spiked DHEAS extracted from skin mucus + epidermis.

water were added, then the powder was extracted with bath sonication for 30 min, at 50 °C. Compared to previous works, which performed extraction for 18 h using an incubator shaker (Carbajal et al., 2018) or a rotator (Kennedy and Janz, 2022), the use of ultrasonic bath for half an hour drastically shortens sample treatment procedure, while guaranteeing high performances. In addition, the limited volume of solvent employed for the extraction of both epidermis and scales avoids to concentrate the extract via evaporation of solvent, which can also require a nitrogen stream and can cause the loss of analytes (Aerts et al., 2015; Carbajal et al., 2018; Kennedy and Janz, 2023).

3.1.5. *Scales extraction validation*

After the ultrasound bath, supernatant was filtered into a low volume injection vial and analysed. Fig. 3 shows the absolute ng recovered after extraction of spiked DHEAS (0.6 ng). Although there was some variation, extraction of DHEAS from scales was overall efficient, with a mean recovery of the 95 % (0.57 ng) of the initially spiked DHEAS.

Generally, the preanalytical procedure herein reported allows to easily and efficiently separate acute from chronic stress matrices, giving the chance to study more globally the stress in *A. fasciatus*.

3.1.6. *Analytical method comparison*

To our knowledge, this is the first study quantifying cortisol, cortisone and DHEAS in skin mucus/epidermis (acute stress) and in fish scales (chronic stress) using a simple preanalytical procedure and HPLC-MS/MS analyses. A remarkable feature of this methodology is the extremely low values of LODs and LOQs achieved for COL (4 ng L^{-1}, 15 ng L^{-1}), CON (3 ng L^{-1}, 11 ng L^{-1}), and DHEAS (8 ng L^{-1}, 25 ng L^{-1}) (Supporting Table S6). Indeed, assessment of cortisol is often performed

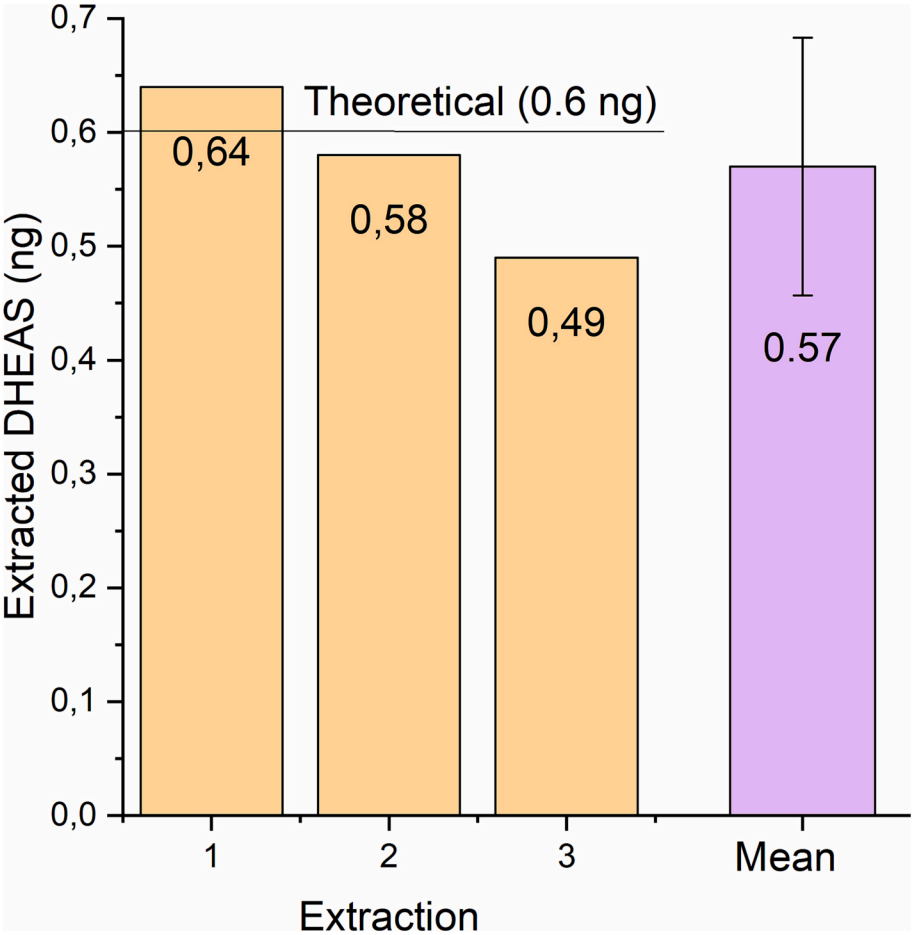

Fig. 3. Amount of spiked DHEAS extracted from scales.

with enzymatic immunoassays (EIA) such as the ELISA Kit (Carbajal et al., 2019, 2018; Kennedy and Janz, 2023; Sadoul and Geffroy, 2019), which is commercially available, thus more practical, but might be less sensitive. For instance, the Neogen® Corporation Cortisol ELISA Kit used by Carbajal et al. (2019) is reported to have a minimum assay range of 39 ng L^{-1}. Moreover, cross-reactivity must be taken into account as it might affect the signals (Blahová et al., 2007). Aguiar et al. (2023) tested several HPLC procedures for the quantification of cortisol in different matrices (i.e., blood plasma, larvae, faeces, liver) and the LOD/LOQ are reported (Aguiar et al., 2023). The lowest LOD (40 ng L^{-1}) was achieved with HPLC-MS/MS technique.

3.2. Stress measurement application

To study how different surrounding environments can affect fish stress, two pulls of fish were taken into account and compared. The first consisted of ten freshly caught (AP) *A. fasciatus*, the second was made of ten *A. fasciatus* housed in the laboratory for one year (PO). For both of the groups, scales of the ten specimens were removed, collected, mixed to homogenize the pull, and divided into four samples of an average weight of 50 mg. Overall, we analysed four samples representing the AP group and four samples representing the PO group. Results are shown in Fig. 4. DHEAS was always below the detection limit (8 ng L^{-1}, Supporting Table S4) both in epidermis and in scales; this is plausible as it is acknowledged that DHEAS circulates in much lower concentrations in fish compared to mammals (Kennedy and Janz, 2023). In turn, COL and CON were always above the detection and quantification limits (Supporting Table S4).

3.2.1. Acute stress quantification

For what concerns acute stress (Fig. 4a and c), a much higher concentration of COL and CON was found in epidermis/skin mucus for both pulls compared to scales. This result, already reported in literature (Bertotto et al., 2010; Kennedy and Janz, 2023), could be correlated to the acute stress that fish is subjected to when it is collected and euthanized (Dunlap et al., 2016; Ramsay et al., 2009). Nevertheless, both stress hormones were much more concentrated in the PO epidermis pull (0.10 ng mg^{-1} of COL and 0.14 ng mg^{-1} of CON) compared to the AP pull (0.006 ng mg^{-1} of COL and 0.009 ng mg^{-1} of CON). A possible explanation to these findings can be the different capture technique of fish in the tank compared to fish freshly collected, before anaesthesia and euthanasia. Indeed, the firsts are taken from the tank with a small rectangular net that can somehow simulate a predator. Thus, the attempt to escape might generate acute stress. On the contrary, the AP group was captured by a passive methos using fyke nets and subsequently euthanized, thus the acute stress is limited. In addition, it might be also possible that the laboratory surroundings itself has a negative effect, causing extra stress to the fish.

3.2.2. Chronic stress quantification

Regarding the chronic stress (Fig. 4b and d), no statistically significant difference was found in COL concentration between the two groups analysed ($p > 0.18$). The average concentration of COL in scales of AP and PO was found to be 0.2 and 0.3 pg mg^{-1} respectively. On the contrary, it was confirmed a statistically significant difference in CON concentration between the AP and PO groups ($p < 0.002$). The average concentration of CON was 0.09 and 0.2 pg mg-1, respectively. A possible explanation to these findings is that the PO group was subjected to captivity conditions in a contest of a different investigation.

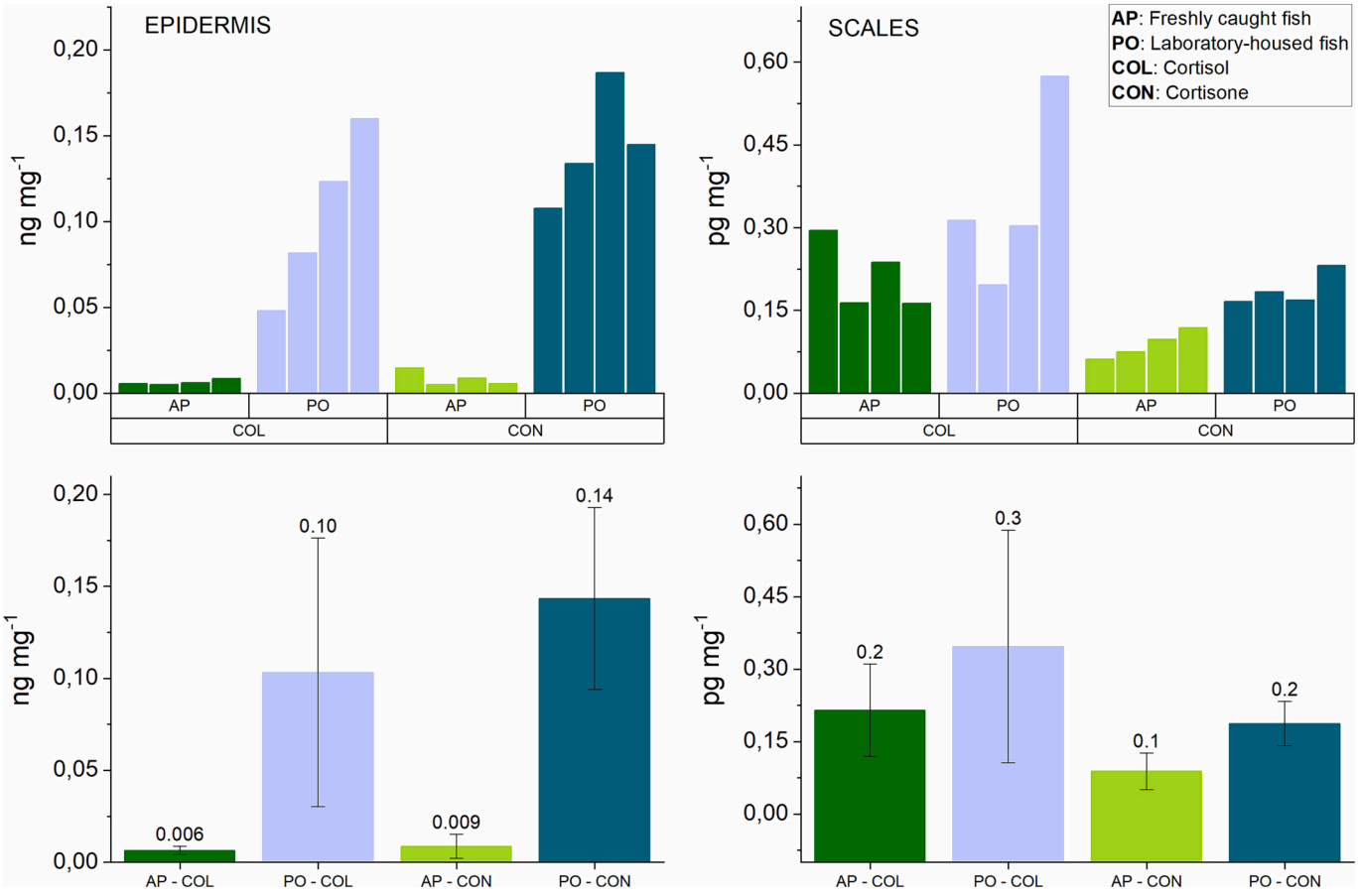

Fig. 4. Comparison of freshly caught fish (AP) and fish housed in laboratory for 1 year (PO) levels of cortisol (COL) and cortisone (CON) concentration in (a) epidermis/skin mucus and (b) scales. Average values are reported in (c) and (d), respectively.

Modification through the year of salinity conditions, food intake, and fish abundance in the housing tank might have affected stress response. Overall, it was expected that these conditions increased the scales' COL level in this group compared to AP. Unstable conditions might promote higher production of COL, which is then released in blood and plasma. Then, enzymatic activity converts COL into CON, as reported by Patiño et al. (1987), which is ultimately bioaccumulated in scales.

3.2.3. Cortisone:cortisol ratio

CON derives from the inactivation of COL caused by the enzyme 11-βhydroxysteroid dehydrogenase. As its activity can be modified by several environmental factors, the ratio CON:COL could give a more complete understanding of the regulation of the enzymatic activity caused by environmental stressors. For instance, levels of plasma CON:COL was reported to depend on water temperature for juveniles coho salmons, with a higher rate of conversion during June (16–18 °C) compared to April (12–13 °C) (Patiño et al., 1987). In our case (Fig. 5), it was found a ratio CON:COL of 1.3 and 1.6 for epidermis of AP and PO, respectively, and a ratio of 0.4 and 0.6 for the scales of AP and PO respectively. A higher ratio in epidermis could suggest that acute cortisol in blood and plasma is readily converted to cortisone to avoid tissue damage caused by a high concentration of the secreted glucocorticoid (Pottinger and Moran, 1993). On the contrary, the low ratio in scales might be explained by the constant presence of low levels of basal cortisol during fish life, which is less concentrated – and less dangerous – than the acute one, and therefore more slowly converted to cortisone.

Although the ratio CON:COL in epidermis and scales of the PO pull was higher compared to the AP group, the difference was not statistically significant ($p = 0.53$ for epidermis, $p = 0.18$ for scales). Anyways, the results we obtained might suggest that the two living environments (laboratory and open field) impact differently on fish wellness. Temperature of the tanks was kept similar to the Forte Marghera canals, therefore in our case it can be excluded as a major regulator of enzymatic activity. Other stressors, such as difference in salinity and confinement, might have stimulated an increase in conversion rate of cortisol to cortisone in the PO group. This trend is visible both for epidermis and for scales.

4. Conclusions

The study presents an innovative method for the investigation of acute and chronic stress in fish through the quantification of three stress hormones, namely cortisol, cortisone and dehydroepiandrosterone-3-sulfate. The separation of skin mucus/epidermis (acute stress) from scales (chronic stress) allows to evaluate how sudden events, recurrent phenomena and in general surrounding environment impact on fish stress response, governed by the HPI-axis.

Overall, this procedure comprises many remarkable innovations compared to previous works, starting from the small amount of scales required for the analyses (ca. 20–25 mg of dry scales) and the use of ultrapure water as cleaning and extraction solvent. Other improvements are the short time required for the dryness (1 h compared to 24 h), the small extraction volume employed, which avoid the concentration step, and the low LOD and LOQ achieved using the HPLC-MS/MS analyses.

This methodology was tested on a total of eight samples, four deriving from freshly caught fish and four coming from fish housed in the laboratory for one year. Results showed that the skin mucus/epidermis of laboratory housed fish had higher concentration of both cortisol (0.10 ng mg^{-1}) and cortisone (0.14 ng mg^{-1}) compared to freshly caught group (0.006 and 0.009 ng mg^{-1} respectively). Less significant was the difference in cortisol concentration between freshly caught and laboratory housed fish scales (0.2 and 0.3 pg mg^{-1} respectively), which could also be ascribed to the limited number of samples analysed. However, the housed fish demonstrated to have higher concentration of cortisol and cortisone compared to freshly caught fish, suggesting that the laboratory environment and the salinity experiments could negatively influence fish health. Nevertheless, for both the matrices and pulls, dehydroepiandrosterone-3-sulfate was always below the detection limit. Overall, although this study comprises a limited pull of samples, the method herein described can be a valid tool for the monitoring of environment wellness through the quantification of stress hormones in local fragile fauna. Since it is not in our interest to study stress indicators just in a single fish, as it is not representative of its environment, our future aim is to focus on the collection of a non-killing amount of scales from a pull of few fish to avoid their sacrifice and to

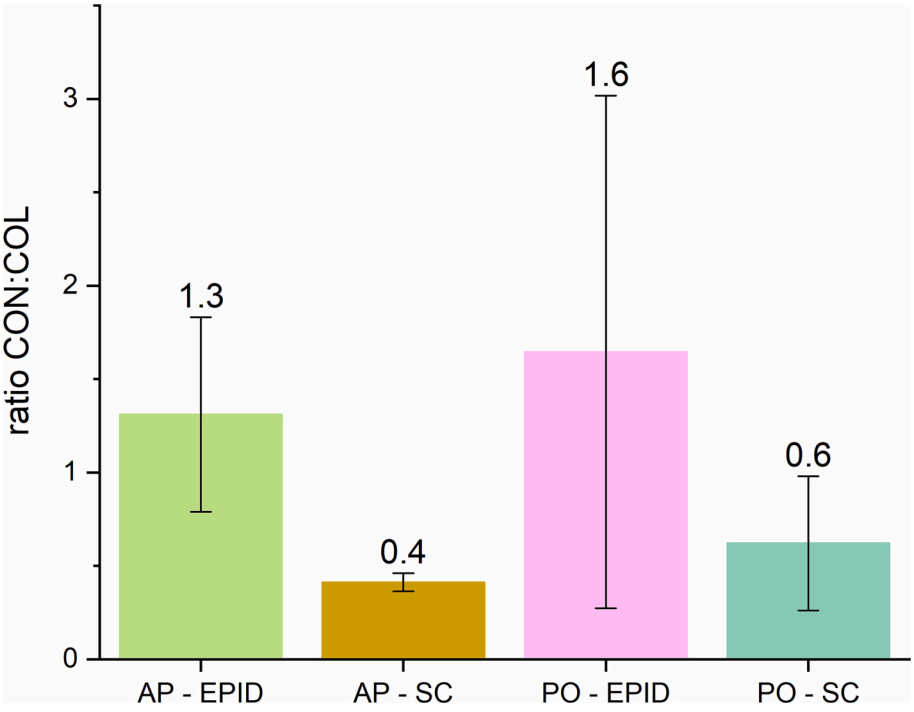

Fig. 5. Average ratio CON:COL in epidermis and scales of freshly caught fish (AP) and housed fish (PO).

have a statistically relevant sample. Moreover, an insight in the difference of stress indicators depending on sex, age, life stage, reproductive state, food intake, social status and other parameters should be considered as an input for future research.

CRediT authorship contribution statement

All authors conceived the project; A.L. performed the sampling and fish processing; M.G., F.M. and A.A. developed the analytical methodology and analysed the data; B.M. performed the statistical analyses. All authors contributed to manuscript editing and reviewing.

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 will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166900.

References

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G., Saeger, S.D., 2015. Scales tell a story on the stress history of fish. PloS One 10, e0123411. https://doi.org/10.1371/journal.pone.0123411.

Aguiar, D., Marques, C., Pereira, A.C., 2023. The importance of monitoring cortisol in the agri-food sector—a systematic review. Metabolites 13, 692. https://doi.org/10.3390/metabo13060692.

Andrews, R.C., Herlihy, O., Livingstone, D.E.W., Andrew, R., Walker, B.R., 2002. Abnormal cortisol metabolism and tissue sensitivity to cortisol in patients with glucose intolerance. J. Clin. Endocrinol. Metabol. 87, 5587–5593. https://doi.org/10.1210/jc.2002-020048.

Barreto, M.O., Rey Planellas, S., Yang, Y., Phillips, C., Descovich, K., 2022. Emerging indicators of fish welfare in aquaculture. Rev. Aquac. 14, 343–361. https://doi.org/10.1111/raq.12601.

Bertotto, D., Poltronieri, C., Negrato, E., Majolini, D., Radaelli, G., Simontacchi, C., 2010. Alternative matrices for cortisol measurement in fish. Aquacult. Res. 41, 1261–1267. https://doi.org/10.1111/j.1365-2109.2009.02417.x.

Blahová, J., Dobšíková, R., Svobodová, Z., Kaláb, P., 2007. Simultaneous determination of plasma cortisol by high pressure liquid chromatography and radioimmunoassay methods in fish. Acta Vet. Brno 76, 59–64. https://doi.org/10.2754/avb200776010059.

Carbajal, A., Monclús, L., Tallo-Parra, O., Sabes-Alsina, M., Vinyoles, D., Lopez-Bejar, M., 2018. Cortisol detection in fish scales by enzyme immunoassay: biochemical and methodological validation. J. Appl. Ichthyol. 34, 967–970. https://doi.org/10.1111/jai.13674.

Carbajal, A., Reyes-López, F.E., Tallo-Parra, O., Lopez-Bejar, M., Tort, L., 2019. Comparative assessment of cortisol in plasma, skin mucus and scales as a measure of the hypothalamic-pituitary-interrenal axis activity in fish. Aquaculture 506, 410–416. https://doi.org/10.1016/j.aquaculture.2019.04.005.

Cartolano, M.C., Berenshtein, I., Heuer, R.M., Pasparakis, C., Rider, M., Hammerschlag, N., Paris, C.B., Grosell, M., McDonald, M.D., 2020. Impacts of a local music festival on fish stress hormone levels and the adjacent underwater soundscape. Environ. Pollut. 265, 114925 https://doi.org/10.1016/j.envpol.2020.114925.

Cavraro, F., Varin, C., Malavasi, S., 2014. Lunar-induced reproductive patterns in transitional habitats: insights from a Mediterranean killifish inhabiting northern Adriatic saltmarshes. Estuar. Coast. Shelf Sci. 139, 60–66. https://doi.org/10.1016/j.ecss.2013.12.023.

COUNCIL DIRECTIVE 92 /43 / EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. Website: https://eur-lex.europa.eu/legal-content/IT/TXT/?uri=celex%3A31992L0043, Document: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31992L0043.

De Mercado, E., Larrán, A.M., Pinedo, J., Tomás-Almenar, C., 2018. Skin mucous: a new approach to assess stress in rainbow trout. Aquaculture 484, 90–97. https://doi.org/10.1016/j.aquaculture.2017.10.031.

Dunlap, K.D., Tran, A., Ragazzi, M.A., Krahe, R., Salazar, V.L., 2016. Predators inhibit brain cell proliferation in natural populations of electric fish, Brachyhypopomus occidentalis. Proc. R. Soc. B Biol. Sci. 283, 20152113. https://doi.org/10.1098/rspb.2015.2113.

Eissa, N., Wang, H.-P., 2016. Transcriptional stress responses to environmental and husbandry stressors in aquaculture species. Rev. Aquac. 8, 61–88. https://doi.org/10.1111/raq.12081.

Facca, C., Cavraro, F., Franzoi, P., Malavasi, S., 2020. Lagoon resident fish species of conservation interest according to the habitat directive (92/43/CEE): a review on their potential use as ecological Indicator species. Water 12, 2059. https://doi.org/10.3390/w12072059.

Fernandes, C., Fontaínhas-Fernandes, A., Rocha, E., Salgado, M.A., 2008. Monitoring pollution in Esmoriz–Paramos lagoon, Portugal: liver histological and biochemical effects in *Liza saliens*. Environ. Monit. Assess. 145, 315–322. https://doi.org/10.1007/s10661-007-0041-4.

Kalimi, M., Shafagoj, Y., Loria, R., Padgett, D., Regelson, W., 1994. Anti-glucocorticoid effects of dehydroepiandrosterone (DHEA). Mol. Cell. Biochem. 131, 99–104. https://doi.org/10.1007/BF00925945.

Kennedy, E.K.C., Janz, D.M., 2022. First look into the use of fish scales as a medium for multi-hormone stress analyses. Fishes 7, 145. https://doi.org/10.3390/fishes7040145.

Kennedy, E.K.C., Janz, D.M., 2023. Chronic stress causes cortisol, cortisone and DHEA elevations in scales but not serum in rainbow trout. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 276, 111352 https://doi.org/10.1016/j.cbpa.2022.111352.

Kerr, L.A., Campana, S.E., 2014. Chemical composition of fish hard parts as a natural marker of fish stocks. In: Stock Identification Methods. Elsevier, pp. 205–234. https://doi.org/10.1016/B978-0-12-397003-9.00011-4.

Kessabi, K., Navarro, A., Casado, M., Saïd, K., Messaoudi, I., Piña, B., 2010. Evaluation of environmental impact on natural populations of the Mediterranean killifish Aphanius fasciatus by quantitative RNA biomarkers. Mar. Environ. Res. 70, 327–333. https://doi.org/10.1016/j.marenvres.2010.06.005.

Khoshru, B., Mitra, D., Joshi, K., Adhikari, P., Rion, M.S.I., Fadiji, A.E., Alizadeh, M., Priyadarshini, A., Senapati, A., Sarikhani, M.R., Panneerselvam, P., Mohapatra, P.K. D., Sushkova, S., Minkina, T., Keswani, C., 2023. Decrypting the multi-functional biological activators and inducers of defense responses against biotic stresses in plants. Heliyon 9, e13825. https://doi.org/10.1016/j.heliyon.2023.e13825.

Korkmaz, N., Örün, İ., 2022. Effects of pesticide NeemAzal-T/S on thyroid, stress hormone and some cytokines levels in freshwater common carp, Cyprinus carpio L. Toxin Rev. 41, 496–505. https://doi.org/10.1080/15569543.2021.1895841.

Leary, S., Pharmaceuticals, F., Underwood, W., Anthony, R., Cartner, S., Johnson, C.L., Patterson-Kane, E., 2020. AVMA Guidelines for the Euthanasia of Animals, 2020 edition.

Li, D., Wang, G., Du, L., Zheng, Y., Wang, Z., 2022. Recent advances in intelligent recognition methods for fish stress behavior. Aquac. Eng. 96, 102222 https://doi.org/10.1016/j.aquaeng.2021.102222.

McDonald, A.M., Martin, C.W., Rieucau, G., Roberts, B.J., 2022. Prior exposure to weathered oil influences foraging of an ecologically important saltmarsh resident fish. PeerJ 10, e12593. https://doi.org/10.7717/peerj.12593.

Metz, J.R., de Vrieze, E., Lock, E.-J., Schulten, I.E., Flik, G., 2012. Elasmoid scales of fishes as model in biomedical bone research. J. Appl. Ichthyol. 28, 382–387. https://doi.org/10.1111/j.1439-0426.2012.01990.x.

Mommsen, T., Vijayan, M., Moon, T., 1999. Cortisol in teleost dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268. https://doi.org/10.1023/A:1008924418720.

Nakano, T., Kameda, M., Shoji, Y., Hayashi, S., Yamaguchi, T., Sato, M., 2014. Effect of severe environmental thermal stress on redox state in salmon. Redox Biol. 2, 772–776. https://doi.org/10.1016/j.redox.2014.05.007.

Patiño, R., Redding, J.M., Schreck, C.B., 1987. Interrenal secretion of corticosteroids and plasma cortisol and cortisone concentrations after acute stress and during seawater acclimation in juvenile coho salmon (Oncorhynchus kisutch). Gen. Comp. Endocrinol. 68, 431–439. https://doi.org/10.1016/0016-6480(87)90082-7.

Pottinger, T.G., Moran, T.A., 1993. Differences in plasma cortisol and cortisone dynamics during stress in two strains of rainbow trout (Oncorhynchus mykiss). J. Fish Biol. 43, 121–130. https://doi.org/10.1111/j.1095-8649.1993.tb00415.x.

Ramsay, J.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L., Schreck, C.B., 2009. Whole-body cortisol response of zebrafish to acute net handling stress. Aquaculture 297, 157–162. https://doi.org/10.1016/j.aquaculture.2009.08.035.

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. Fishes 104, 725–732. https://doi.org/10.1007/s10641-021-01107-6.

Sadoul, B., Geffroy, B., 2019. Measuring cortisol, the major stress hormone in fishes. J. Fish Biol. 94, 540–555. https://doi.org/10.1111/jfb.13904.

The Merck Index, 2007. An Encyclopedia of Chemicals, Drugs, and Biologicals, 14th ed. Edited by Maryadele J. O'Neil (Editor), Patricia E. Heckelman (Senior Associate Editor), Cherie B. Koch (Associate Editor), and Kristin J. Roman (Assistant Editor).

Merck and Co., Inc.: Whitehouse Station, NJ. 2006. 2564 pp. $125.00. ISBN 0-911910-00-X. J. Am. Chem. Soc. 129, 2197. https://doi.org/10.1021/ja069838y.
Tiosano, D., Eisentein, I., Militianu, D., Chrousos, G.P., Hochberg, Z., 2003. 11β-Hydroxysteroid dehydrogenase activity in hypothalamic obesity. J. Clin. Endocrinol. Metabol. 88, 379–384. https://doi.org/10.1210/jc.2002-020511.
Tort, L., 2011. Stress and immune modulation in fish. Dev. Comp. Immunol. 35, 1366–1375. https://doi.org/10.1016/j.dci.2011.07.002.
Triantafyllidis, A., Leonardos, I., Bista, I., Kyriazis, I.D., Stoumboudi, M.Th., Kappas, I., Amat, F., Abatzopoulos, T.J., 2007. Phylogeography and genetic structure of the Mediterranean killifish Aphanius fasciatus (Cyprinodontidae). Mar. Biol. 152, 1159–1167. https://doi.org/10.1007/s00227-007-0760-7.
Wu, H., Aoki, A., Arimoto, T., Nakano, T., Ohnuki, H., Murata, M., Ren, H., Endo, H., 2015. Fish stress become visible: a new attempt to use biosensor for real-time monitoring fish stress. Biosens. Bioelectron. 2014 (67), 503–510. https://doi.org/10.1016/j.bios.2014.09.015. Special Issue: BIOSENSORS.
Yang, J., Guo, C., Luo, Y., Fan, J., Wang, W., Yin, X., Xu, J., 2023. Effect of thiamethoxam on the behavioral profile alteration and toxicity of adult zebrafish at environmentally relevant concentrations. Sci. Total Environ. 858, 159883 https://doi.org/10.1016/j.scitotenv.2022.159883.