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A preliminary approach on the stress assessment through harmless procedures in farmed seabream (Sparus aurata)

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Abstract

*Fish welfare is a key factor in ensuring successful cultures. Farmed fish that are stressed have been shown to be susceptible to pathologies and present lower growth rates. The present work seeks to check the efficacy of faecal cortisol as a non-invasive method of assessing acute stress in a commercial cultured fish, the gilthead seabream (*Sparus aurata*). Typical stress markers (plasma cortisol, glucose and lactate) and faecal cortisol were measured in basal and post-stress (air exposure) state. Plasma and faecal cortisol, and plasma lactate after acute stress varied significantly compared to basal levels. Moreover, faecal cortisol showed a significant correlation with plasma cortisol and lactate. In conclusion, this work describes an easy, non-invasive and practical technique to assess acute stress in farmed fish. Further studies are needed to focus on other practical procedures for chronic stress measurements in sea-farms in order to improve the welfare of these animals.*

Keywords: *animal welfare, aquaculture, faecal cortisol, gilthead seabream, sea-farm, stress*

Introduction

Fish welfare is a key factor in ensuring successful cultures. Stressed farmed fish have been shown to be prone to pathologies and present lower growth rates (Salas-Leiton *et al* 2010; Herrera *et al* 2012). Moreover, stressful culture procedures affect the flesh quality; for instance, fish stressed before slaughter show lower muscle pH and faster deterioration in meat quality (Acerete *et al* 2004; Matos *et al* 2010).

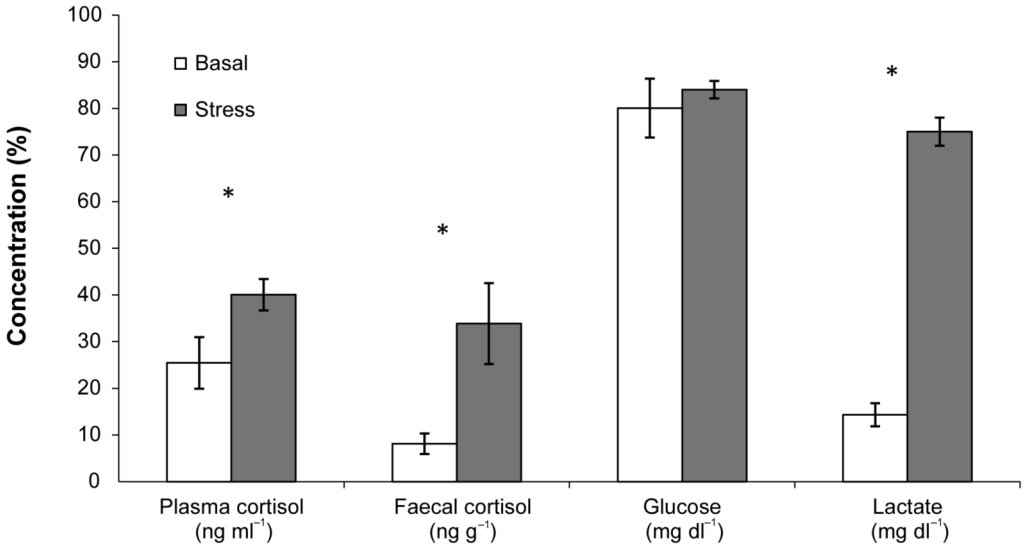
European and Spanish legislation on the welfare of farmed animals is developing to include fish and, accordingly, sea-farm production protocols are compelled to adopt legal requirements focusing on the maintenance of welfare. Fish welfare tends to be assessed through stress indicators, such as plasma cortisol, glucose and lactate (Pottinger 2008). However, blood extraction can be difficult and harmful for fish, since it requires experienced personnel and can provoke internal and external injuries, and haemorrhages.

In fish, as in mammals, the main excretory pathways for corticosteroids (including cortisol) are water-soluble conjugates (glucuronidates and sulphates) in the faeces (via bile) and urine (Ellis *et al* 2013). Therefore, a number of non-invasive methods for steroid measurement in fish held in tanks have been based on different matrixes, such as faeces, urine, water and mucus; however, several of these are yet to determine cortisol concentration and its relationship with plasma cortisol (Oliveira *et al* 1996; Turner *et al* 2003; Ellis *et al* 2004; Schultz *et al* 2005; Scott & Ellis 2007; Simontacchi *et al* 2008; Kittilsen *et al* 2009). The assay in culture water appears the least invasive and has been widely accepted as a valid method since the reporting of standard protocols began (Ellis *et al* 2013), despite being unsuitable in individual measurements for fish kept in groups. Ellis *et al* (2013) reported that standard protocols for mucus sampling are yet to be developed. For urine samples, Oliveira *et al* (1996) elucidated sex steroid concentrations in urine extracted through stripping but did not measure cortisol.

Faecal corticosteroids, including cortisol, have been measured by a number of researchers and many of these studies have been carried out in the field; a technique which allows for assessment of physiological status in wild populations (Laver *et al* 2012; Shutt *et al* 2012; Narayan *et al* 2013). A few studies have sought to assess fish stress through faecal analysis (Karsten & Turner 2003; Turner *et al* 2003; Lupica & Turner 2009) and this seems the least stressful procedure given that animals are not handled. However, fish need to be large enough to procure suitable samples (solid faeces) and, also, faeces must be collected quickly, immediately following excretion. In sea-farms, fish tend not to be particularly large and high stocking densities ensure faeces do not remain compact as a result of water and fish movements. Thus, it would not be physically feasible to obtain individual samples and, accordingly, individual intestinal faecal extraction via abdominal pressure remains an option.

Species' differences as regards glucocorticoid metabolism and excretion make it necessary to ensure such non-invasive methods are compatible, physiologically, with each species (Konjević *et al* 2011). To our knowledge, cortisol measurement in the faeces of cultured fish is yet to be described. The

Figure 1



Post-stress (air exposure) and mean (± SEM) basal variables in seabream (*Sparus aurata*): plasma glucose (t = –0.60; df = 14; P = 0.556), lactate (t = –15.59; df = 12; P = 0.00) and cortisol (t = –2.72; df = 13; P = 0.04) and faecal cortisol (t = –2.88; df = 11; P = 0.02). Asterisks indicate significant differences between states for every variable.

gilthead seabream (*Sparus aurata*) is one of the most important species cultured in the Mediterranean area; hence, a simple sampling method to detect stress would be useful both in terms of production and welfare legislation. As such, the present study evaluates the efficacy of a non-invasive method to assess acute stress in cultured fish.

Materials and methods

Ethical statement

This experiment complied with the Guidelines of the European Union Council (2010/63/EU) and the Spanish Government (RD1201/2005; RD53/2013 and law 32/2007) for the use of laboratory animals. According to RD1201/2005, the experimental procedures were reviewed by the IFAPA's Ethics Committee, which approved it. M Herrera is certified (type C) for working and designing experiments with animals, and the aquaculture technicians involved in this work have the A-type Certificate, necessary for laboratory animal maintenance and care.

Study animals

Fourteen gilthead seabream with a mean (± SEM) weight of 368.7 (± 13.2) g and a total length of 27.6 (± 0.38) cm were purchased from MARESA sea-farm, Ayamonte, Spain. They were individually tagged with PIT tags (Trovan®-DorsetID, Weverij 26, 7122 MS Aalten, The Netherlands) and stocked in a rectangular PRFV tank (1,500 L) at 3.5 kg m^{-3}. The culture conditions were: dissolved oxygen above saturation, mean (± SEM) temperature 19.6 (± 0.9)ºC, mean (± SEM) salinity 34.5 (± 0.31) ppt, and natural photoperiod. They were fed *ad libitum* commercial fish feed (SKRETTING, D-6®, Skretting, Burgos, Spain) with belt-feeders for 24 h (2% biomass daily) prior to the time of sampling.

After a two-week period of acclimation, blood and faecal samples were taken. These readings provided a basal rate for the study. After sampling, fish were released into the same tank. The second sampling tested the stressed state and was performed two weeks later. For this, fish were subjected to acute stress through air exposure for 3 min; a condition shown to be stressful to seabream (Arends *et al* 1999). They were then sampled 30 min later at peak cortisol (Arends *et al* 1999). Prior to being sampled, fish were anaesthetised (0.2 ml L^{-1} of 2-phenoxyethanol). Briefly, the sampling of stressed fish went as follows: capture, air stress (3 min), sampling tank (30 min), anaesthetic tank (~1 min), blood and faecal extraction (< 1 min), recovery tank (~7 min), and housing tank. The sequence for non-stressed fish was identical apart from the omission of air stress.

Intestinal faeces were extracted through gentle abdominal pressure in each individual, in a technique similar to the protocol of Oliveira *et al* (1996) for urine samples. Samples were collected with a micro spatula, quickly put into a 1.5-ml plastic tube, snap frozen in liquid nitrogen, and stored at –80ºC. Samples contaminated with urine were discarded. Blood was collected via puncture from the caudal peduncle into 1-ml heparinised syringes (25,000 units of ammonium heparin per 3 ml saline solution 0.6% NaCl, Sigma H6279, Sigma-Aldrich, St Louis, MO, USA). Plasma was separated from cells by centrifugation of the whole blood (3 min, 10,000 × g, 4°C), snap frozen in liquid N_2 and stored at –80ºC until analysis of metabolites (glucose and lactate concentrations) and cortisol.

Plasma glucose and lactate were measured using commercial kits (Spinreact Lactate 1001330, QCA Glucose ref 998225, QCA, Barcelona, Spain), adapted to 96-well microplates,

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according to Herrera *et al* (2012). Plasma-free cortisol (not bound to transcortin or conjugated) levels were quantified by an ELISA kit (EA65, Oxford Biomedical Research, MI, USA) modified and adapted to fish (Herrera *et al* 2015). Cortisol was extracted from 20 µl plasma in 200 µl diethyl ether. The lower limit of detection (81% binding) was 0.1 ng ml^{-1}. The inter- and intra-assay coefficients of variation were 10.9 and 5.8%, respectively. The mean percentage of recovery was 90%. The linearity was checked by diluting a known plasma sample (10 ng ml^{-1}) in cortisol-stripped plasma: previously incubated for 24 h with active charcoal (C5385, Sigma Chemical Co, St Louis, MO, USA), centrifuged and filtered. The correlation coefficient between theoretical (calculated from dilutions) and measured cortisol was 0.970.

Faecal samples were homogenised with PBS (2 µl mg^{-1}) by sonication, dissolved in diethyl ether (10 µl mg^{-1}), centrifuged (10 min, 1,200 × g, 4ºC), the supernatant extracted and evaporated with N_2 stream, and finally dissolved in a buffer provided by the kit manufacturer, according to Simontacchi *et al* (2009). Faecal cortisol concentration in extracts was measured as above. The validation parameters were 11.45%, 6.9%, 88.6%, and 0.962 for intra- and inter-assay coefficient of variation, recovery and correlation coefficient, respectively (see above).

All data groups were analysed using the Kolmogorov-Smirnov test in order to check for normality. Individual differences between groups (basal and stressed) were detected with a Student's *t*-test. Pearson's test was used to check correlations among the variables. The significance level was $P = 0.05$.

Results and discussion

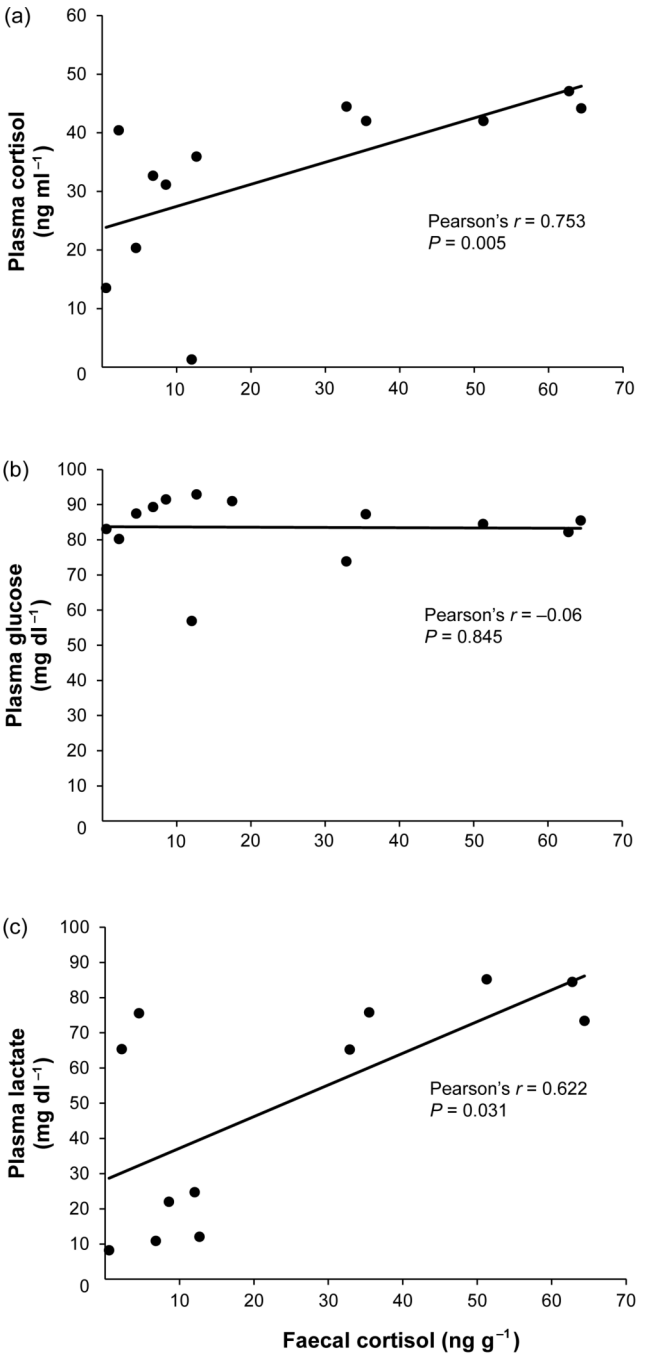
Acute stress was shown to cause plasma and faecal free cortisol as well as plasma lactate to vary significantly compared to basal levels (Figure 1). In fact, faecal cortisol was significantly correlated with plasma cortisol and lactate (Figure 2).

Nevertheless, basal and stressed plasma glucose levels did not differ significantly.

Plasma cortisol is generally considered to be a good indicator of acute stress despite failing in conditions of chronic stress as a result of habituation of the HPI axis (Haukenes & Barton 2004; Barton *et al* 2005). Here, fish were subjected to acute stress so we could demonstrate that our fish responded to stress in such a way. Nevertheless, significant stress responses in plasma glucose were not found. Indeed, that response (secondary) tends to occur later than the cortisol effect (primary) (Iwama *et al* 2005). No significant plasma glucose changes were reported in the spotted wolfish (*Anarhichas minor*) within 4 h post-stress (Lays *et al* 2009), although Ishibashi *et al* (2002) identified a plasma glucose peak 6 h after stress in the Nile tilapia (*Oreochromis niloticus*). Therefore, it appears species-specific differences exist as regards the mechanisms related to energy mobilisation under stress.

In our work, plasma free cortisol concentrations increased significantly after subjecting fish to air exposure (3 min). In addition, faecal cortisol levels increased, however, this increase was higher than that seen in the plasma assay; 4-fold as opposed to approximately 1.5-fold. Arends *et al* (1999) reported a 50-fold plasma cortisol increase after 0.5 h. However, experimental conditions differed in this study as fish underwent starving prior to sampling. Therefore, the greater increase could be related to an increased energy demand brought about by starvation and subsequent plasma cortisol elevation as metabolic pathways are enhanced to use energy reserves.

Figure 2



Correlation lines (including statistical parameters from Pearson's *r* test) between faecal cortisol and plasma variables (a) cortisol, (b) glucose and (c) lactate after air exposure stress. Only plasma glucose was not correlated significantly to faecal cortisol.

Elevated faecal cortisol levels after acute stress in fish have not previously been reported, although faecal corticoids have been shown to increase up to 25-fold in other vertebrates (Palme *et al* 2000; Wasser *et al* 2000). In mammals, glucocorticoids have been found to be metabolised and excreted with intact hormones as well as their metabolites present in faeces (Touma *et al* 2003). Although it has been reported that the fraction of free cortisol in faeces is low in comparison with conjugated forms (Chelini *et al* 2006), it is the most frequently used compound in faecal analysis since, unlike the conjugated sulphates and glucuronides, free glucocorticoids are the physiologically active forms and readily extractable from faeces in organic solvents with cheaper and faster analytical methods (Lupica & Turner 2009; Ellis *et al* 2013).

Although not our primary objective, we noted that elevated cortisol levels in faeces could be identified 30-min post-stress in the gilthead seabream. The post-stress time for faecal cortisol to peak is species-dependent and appears short compared to ruminants (10–12 h), and chickens (2 h) (Rettenbacher & Palme 2009; Konjević *et al* 2011; Palme 2012). These data originate mainly from free cortisol peaks, and no information exists as to the exact time taken for faecal free cortisol to increase significantly. In this way, perhaps we could have obtained higher differences in our later samplings.

In spite of there being no information on cortisol metabolising rate in fish; a closely related experiment reported that injected sexual steroids (not cortisol) were excreted after 5–7 h (Scott & Sorensen 1994). Their data emerged from analysis of water, therefore excretion had to be earlier. Our data came from samples collected over 3-h periods, so it was impossible to determine the minimum time at which faecal free cortisol reaches significance. Similarly, this time could also be reduced if Scott and Sorensen's (1994) samples were, in fact, intestinal faeces (prior to excretion), which was where our samples originated in the current study.

In fish, very few works have described the assessment of cortisol or costicosteroids in faeces collected, both from the wild or experimental aquaria. Karsten and Turner (2003), and Lupica and Turner (2009) developed a procedure for measuring faecal cortisol in the epaulette shark (*Hemiscyllium ocellatum*) and parrotfish (*Sparisoma viride* and *Scarus vetula*); though fish were not subjected to previous stress. However, Turner *et al* (2003) measured faecal corticosteroids both in wild and captive (aquaria) parrotfish (*S. viride*, *S. vetula* and *Scarus guacamaia*), noting that cortisol in faeces can be used as an indicator of the stress-response which is unlikely to be masked by intrinsic variability in the sample source, environment or methodology. In fact, these authors stated that cortisol present in faecal samples and maintained at 28ºC in seawater remains100% intact for 8 h and 93% intact after 16 h. Nevertheless, cultured fish are kept at moderate or high stocking densities, hence faeces do not stay consistent due to the water movement that arises from fish swimming and, also, identification of individual faeces is impossible. Fish size also limits faecal collection in culture water as small or medium fish excrete small particles which dissolve into water quickly.

All previous studies were based on sample collection after deposition, and our work was based on extraction through abdominal pressure, with no interference from environmental water or other factors. Moreover, this procedure can be easily performed by non-experienced personnel and is faster than blood extraction, which requires the preparation of syringes, heparin, centrifugation, and plasma extraction.

In conclusion, the work described here is an easy, minimally invasive and practical technique to assess acute stress in farmed fish. Further studies should focus on other practical procedures for chronic stress measurement in sea-farms, and the time course of cortisol metabolism depending on different culture conditions.

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