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Annaïs Carbajal Brossa

Universitat Autònoma de Barcelona

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Annaïs Carbajal Brossa

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Dr. Manel López-Béjar

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Manel López Béjar, professor titular d'universitat del Departament de Sanitat i Anatomia Animals de la Universitat Autònoma de Barcelona,

Certifica:

Que la memòria titulada "**Cortisol in skin mucus and scales as a measure of fish stress and habitat quality**", presentada per Annaïs Carbajal Brossa amb la finalitat d'optar al grau de Doctor amb Menció Internacional en Medicina i Sanitat Animals, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

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Dr. Manel López-Béjar

Annaïs Carbajal Brossa

(Doctorand)

SUMMARY

The analysis of circulating cortisol, the end product of the hypothalamic-pituitary-interrenal (HPI) axis activation, has been by far the most common method used as a means to assess fish stress responses. To avoid the drawbacks inherent to blood sampling, cortisol can be less-invasively analysed in fish skin mucus. The measurement of cortisol concentrations in skin mucus has been shown to be a good stress indicator in farm fish exposed to different acute stressors. Given that this type of sample reflects the short-term activity of the HPI axis, and the growing interest in assessing chronic increases of cortisol, fish scales have been recently recognized as a biomaterial able to reflect the long-term HPI axis activity. There are, however, primary gaps in the knowledge on the degree to which the cortisol content in these samples represents the activity of the HPI axis. In addition, cortisol measurement in both skin mucus and scale samples could offer many practical and conceptual advantages when being applied in wildlife. Nevertheless, before being employed in free-ranging animals for conservation, management or other purposes, each of these new matrices needs to be fully biologically validated. Accordingly, the present thesis was conducted with the general objective to validate the measurement of skin mucus cortisol concentrations (MCC) and scale cortisol concentrations (SCC) and evaluate the applicability of these methods as potential tools to assess habitat quality. We first validated a protocol for hormone extraction in these matrixes and the quantification of cortisol by enzyme immunoassay. After methodological validation, we aimed to examine whether MCC and SCC reflect biological events of interest. Levels of MCC demonstrated to reliably reflect acute stress responses, however, we suggest caution when applying this method for chronic stress assessments. Findings also revealed that MCC could be used as an alternative, non-invasive approach to assess the HPI axis activity in a wild freshwater fish (Catalan chub, *Squalius laietanus*) within its natural environment. Finally, measurements of SCC proved to reliably offer a retrospective, long-term, integrated measure of the HPI axis activity in fish subjected to long-term continuous stress. Having demonstrated the biological significance of both matrices, it is possible to study whether these measures are equally valid as potential bioindicators of habitat quality. Levels of MCC differed between habitats of different pollution gradient revealing that this metric could be potentially used as a bioindicator of habitat quality in fish residing contaminated streams. Although SCC remained unchanged between habitats, we observed an increase in SCC concurring with a temporarily stressful period, suggesting that transient energetically demanding periods could influence the deposition of cortisol in fish scales. Overall, this thesis offers guidance on the future measurement of cortisol levels in both skin mucus and scales, and its potential use in evaluating environmental impacts in free-living organisms. Moreover, it opens up new relevant research questions that should be addressed to further illustrate the high potential of skin mucus and scale cortisol as a proxy measure for stress.

RESUM

L'anàlisi del cortisol circulant, el producte final de l'activació de l'eix hipotalàmic-pituïtari-interrenal (HPI), ha sigut la metodologia més usada en els estudis d'estrès en peixos. Per evitar els inconvenients inherents al mostreig de sang, el cortisol es pot analitzar de forma menys invasiva en el mucus cutani dels peixos. Les concentracions de cortisol mesurades en el mucus han demostrat ser un bon indicador d'estrès en peixos de piscifactoria exposats a diferents tipus d'estrès agut. Atès que aquest tipus de mostra reflexa l'activitat de l'eix HPI a curt termini, i el creixent interès de la comunitat científica en estudiar increments crònics de cortisol, recentment les escates dels peixos han resultat ser un biomaterial amb capacitat de reflectir l'activitat de l'eix HPI a llarg termini. Al tractar-se de matrius relativament noves, encara falten molts aspectes per conèixer, principalment aquells relacionats amb la capacitat d'aquestes matrius per reflectir l'activitat de l'eix. A més, la mesura de cortisol en mucus i en escata de peixos en estat salvatge podria oferir avantatges tant pràctics com conceptuals. Tot i així, abans d'usar aquestes mesures en fauna salvatge amb finalitats de conservació i/o gestió, entre d'altres, cal fer una validació biològica exhaustiva de cada una d'elles. Així doncs, aquesta tesis es va realitzar amb l'objectiu general de validar la mesura de cortisol en mucus cutani (MCC per les seves sigles en anglès) i en escates (SCC) i avaluar l'ús d'aquests mètodes com a eines potencials per estudiar la qualitat de l'hàbitat. Primer, es va validar satisfactòriament el protocol d'extracció d'hormones en aquestes mostres i la quantificació de cortisol mitjançant enzim immunoassaig. Un cop validada la part metodològica, es va estudiar la capacitat de les MCC i les SCC per representar esdeveniments biològics d'interès. Les MCC van demostrar reflectir amb més exactitud les respostes agudes d'estrès que les cròniques. Els resultats també van revelar que les MCC es poden usar com una metodologia alternativa i no invasiva per avaluar l'activitat de l'eix HPI del peix salvatge de riu de l'espècie *Squalius laietanus* (bagra catalana) en el seu hàbitat natural. Finalment, les SCC van demostrar oferir informació integrada, retrospectiva i a llarg termini de l'activitat de l'eix HPI en peixos sotmesos a situacions d'estrès crònic i continuat. Un cop demostrada la importància biològica d'ambdós matrius, es possible estudiar si aquestes dues mesures són igualment vàlides per a ser potencialment usades com a bioindicadores de la qualitat de l'hàbitat. Es va observar que els peixos que residien en habitats amb un grau de pol·lució diferent presentaven diferències significatives en les MCC, suggerint que aquesta eina podria ser usada com a bioindicador de la qualitat de l'hàbitat en peixos que habiten aigües contaminades. Tot i que no es van observar diferències en SCC entre hàbitats, les SCC van augmentar de forma significativa coincidint amb un període potencialment estressant per l'espècie. Aquests resultats podrien indicar que les SCC estan més influenciades per períodes transitoris energèticament exigents que per factors estressants crònics. En general, aquesta tesi ofereix una base per futurs estudis que usin el mucus cutani i les escates per mesurar les concentracions de cortisol en peixos, així com també demostra el seu potencial per a ser usada com a eina d'avaluació d'impacte ambiental en fauna salvatge. A més, obre noves línies de recerca que s'haurien d'abordar per tal de clarificar encara més l'elevat potencial d'aquestes matrius com a mesures d'estrès en peixos.

RESUMEN

El análisis del cortisol circulante, el producto final de la activación del eje hipotalámico-pituitario-interrenal (HPI), ha sido la metodología más usada en los estudios de estrés en peces. Para evitar los inconvenientes inherentes al muestreo de sangre, el cortisol se puede analizar de forma menos invasiva en la secreción mucosa cutánea de los peces. Las concentraciones de cortisol en mucus han demostrado ser un buen indicador de estrés en peces de piscifactoría expuestos a diferentes tipos de estrés agudo. Dado que este tipo de muestra refleja la actividad del eje HPI a corto plazo, y el creciente interés de la comunidad científica en estudiar los incrementos crónicos de cortisol, recientemente, las escamas de los peces han resultado ser un biomaterial con la capacidad de reflejar la actividad del eje HPI a largo plazo. Al tratarse de medidas relativamente nuevas, aún faltan muchos aspectos por estudiar, principalmente los relacionados con la capacidad de estas muestras para reflejar la actividad del eje. Además, la medición de cortisol en mucus y en escamas de peces en estado salvaje podría ofrecer ventajas tanto prácticas como conceptuales. Aún así, antes de ser usadas con finalidades de conservación o gestión, entre otras, es necesario hacer una validación biológica exhaustiva de cada una de estas matrices. Por lo tanto, esta tesis se realizó con el objetivo general de validar la medición de cortisol en mucus cutáneo (MCC por sus siglas en inglés) y en escama (SCC), y evaluar el uso de estos métodos como potenciales herramientas para estudiar la calidad del hábitat. En un primer lugar, se validó satisfactoriamente el protocolo de extracción de hormonas en estas matrices y la cuantificación de cortisol mediante inmunoensayo enzimático. Con la metodología validada, estudiamos la capacidad de MCC y SCC para representar eventos biológicos de interés. La MCC demostró reflejar con más exactitud la respuesta aguda de estrés que la crónica. Además, los resultados también revelaron que las MCC podrían ser usadas como una metodología alternativa y no invasiva para evaluar la actividad del eje HPI en peces salvajes de río de la especie *Squalius laietanus* (bagra catalana) en su hábitat natural. Finalmente, las SCC demostraron ofrecer información integrada, retrospectiva y a largo plazo de la actividad del eje HPI en peces sujetos a situaciones de estrés crónico y continuado. Habiendo demostrado la importancia biológica de ambas matrices, pudimos estudiar si estas dos medidas son igualmente válidas para ser potencialmente utilizadas como bioindicadoras de la calidad del hábitat. Se observó que los peces residentes de hábitats con diferente grado de polución presentaban diferencias significativas en las MCC, sugiriendo que esta herramienta podría ser usada como bioindicador de la calidad del hábitat en peces que habitan aguas contaminadas. Aunque no se detectaron diferencias en las SCC entre hábitats, las SCC aumentaron de forma significativa coincidiendo con un periodo potencialmente estresante para la especie. Estos resultados podrían indicar que las SCC están más influenciadas por periodos transitorios energéticamente exigentes que por factores estresantes crónicos. En general, esta tesis ofrece una base para futuros estudios que utilicen el mucus cutáneo y las escamas para medir las concentraciones de cortisol en peces, a la vez que demuestra el potencial de estas matrices para poder ser usadas como herramientas de evaluación de impacto ambiental en fauna salvaje. Además, abre nuevas líneas de investigación cuyo abordaje puede clarificar, aún más, el elevado potencial de estas matrices como medidas de estrés en peces.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
CEC	contaminants of emerging concern
COD	chemical oxygen demand
CV	coefficients of variation
EIA	enzyme immunoassays
ENA	erythrocytic nuclear abnormalities
GC	glucocorticoid
GSI	gonadosomatic index
HPA	hypothalamic-pituitary-adrenal
HPI	hypothalamic-pituitary-interrenal
HPLC	liquid chromatography
IE	immature erythrocytes
K	Fulton's condition factor
LOD	limit of detection
MCC	skin mucus cortisol concentrations
MN	micronuclei
MPFOA	perfluoro-n-(1, 2, 3, 4-13C4) octanoic acid
MPFOS	sodium perfluoro-1-(1, 2, 3, 4-13C4) octane sulfonic acid
N:L ratio	relative proportion of neutrophils to lymphocytes
NTU	nephelometer turbidity units
PCC	plasma cortisol concentrations
PFAS	per- and polyfluoroalkyl substances
PFBS	perfluorobutane sulfonic acid
PFCA	perfluoroalkylcarboxylic acid
PFDA	perfluorodecanoic acid
PFDoA	perfluorododecanoic acid
PFDS	perfluorodecane sulfonic acid
PFHpA	perfluoroheptanoic acid
PFHS	perfluorohexane sulfonic acid
PFHxA	perfluorohexanoic acid
PFHxDA	perfluorohexadecanoic acid
PFNA	perfluorononanoic acid
PFOA	perfluorooctanoic acid
PFDOA	perfluorooctadecanoic acid
PFOS	perfluorooctane sulfonic acid
PFPA	perfluoropentanoic acid
PFSA	perfluoroalkylsulfonates
PFTeDA	perfluorotetradecanoic acid

PFTriDA	perfluorotridecanoic acid
PFUnA	perfluoroundecanoic acid
RBC	red blood cells
RIA	radioimmunoassay
SCC	scale cortisol concentrations
SD	standard deviation
SE	senescent erythrocytes
SS	suspended solids
TOC	total organic carbon
UPLC-MS	ultra-performance liquid chromatography-tandem mass spectrometry
WBC	white blood cell
WCC	wash cortisol concentrations

LIST OF SPECIES MENTIONED

Common name	Scientific name
Brown bullhead	*Ameiurus nebulosus*
Catalan chub	*Squalius laietanus*
European sea bass	*Dicentrarchus labrax*
Gilthead seabream	*Sparus aurata L.*
Goldfish	*Carassius auratus*
Largemouth bass	*Micropterus salmoides*
Logperch	*Percina caprodes*
Pumpkinseed	*Lepomis gibbosus*
Rainbow darter	*Etheostoma caeruleum*
Rainbow trout	*Oncorhynchus mykiss*
Silver carp	*Hypophthalmichthys molitrix*
Three-spined sticklebacks	*Gasterosteus aculeatus L.*
White sucker	*Catostomus commersonii*
Zebrafish	*Danio rerio*

INTRODUCTION

There is growing evidence that human activities have a variety of negative impacts on wildlife (Tarlow and Blumstein, 2007). Furthermore, as a result of climate change, free-ranging populations are increasingly challenged by extreme weather and climate events (Wingfield et al., 2011). The stress response plays a key role in allowing an organism to cope with those challenges that can potentially threaten its homeostasis (Sapolsky et al., 2000). Therefore, the study of stress responses to environmental disturbances are of growing interest since they can be used to identify natural and anthropogenic impacts on wildlife (Cockrem, 2005; Wikelski and Cooke, 2006). Measurement of glucocorticoids is probably the most accepted metric as a proxy for stress (Cook, 2012; Johnstone et al., 2012). Cortisol, as the main glucocorticoid in teleost fish, has been commonly measured in blood (Baker et al., 2013). Nevertheless, cortisol levels in fish can be less invasively quantified in skin mucus, the surrounding water and faeces, or in more intrusive samples such as in whole-body homogenates, gut content, bile and gill filaments (Ellis et al., 2013; Gesto et al., 2015; Pottinger and Mosuwe, 1994). Among them, skin mucus appears as an excellent tool to evaluate cortisol fluctuations with minimal restraint and disruption to the animal. In addition, samples can be easily and individually collected, but as blood cortisol, this tool provides a very narrow window into the animal's cortisol status. To evaluate the long-term retrospective cortisol secretion, fish scales have been recently recognized as an exciting new matrix able to offer this knowledge. Importantly, before being applied for research purposes, each of these new techniques needs to be fully validated (Buchanan and Goldsmith, 2004). This process should start by selecting the appropriate preparation technique (Cook, 2012), and subsequently ensuring that other compounds present in the matrix do not interfere with the analytical assay (Möstl et al., 2005). The applicability of the novel matrix as indicator of stress relies upon the degree to which the cortisol content represents the HPI axis activity (Cook, 2012). Accordingly, the validation process usually concludes by demonstrating that cortisol fluctuations in the target matrix have a clear biological significance (Mormède et al., 2007). The quantification of cortisol in skin mucus and scales, as well as its ability to reflect physiological events of interest has been partially validated. While skin mucus cortisol has been positively correlated to plasma levels (Guardiola et al., 2016), the correspondence between scales

and plasma cortisol is still unknown. Indeed, whether these relationships differ across resting, acute or chronic cortisol secretion has never been addressed before. The importance to further validate these novel matrices as a means to assess the stress response in fish is, therefore, evident. Most notably, none of the published reports measuring cortisol in skin mucus or scales have been performed in free-ranging animals. There are several examples revealing the usefulness of cortisol measurements in wild freshwater fish. Cortisol detections in blood, whole-body homogenates and the surrounding water have been successfully applied to reveal whether, when and how animals are impacted by environmental disturbances (King et al., 2016; Norris et al., 1999; Pottinger et al., 2016). Despite the very clear practical and conceptual advantages inherent to skin mucus and scale cortisol analysis, the question raises as to whether these two measures could be equally valid as potential biological indicators of habitat quality.

LITERATURE REVIEW

GLUCOCORTICOID PHYSIOLOGY AND STRESS RESPONSES

Glucocorticoids (GC) are steroid hormones secreted as an end product of the hypothalamic-pituitary-adrenal (HPA) axis in mammals, birds and reptiles or the hypothalamic-pituitary-interrenal (HPI) axis in fish (Barton, 2002; Moberg and Mench, 2000). Although GC are typically considered "stress hormones", their actions will largely depend on a dynamic and complex profile of ultradian, circadian and stress reactive circulating levels of the hormone (Spencer and Deak, 2016). At low or resting levels, GC have the continuous role of maintaining several life tasks such as growth, development, reproduction and disease resistance (Nader et al., 2010; Sapolsky, 2002). Stress results when GC values increase above baseline levels in response to unpredictable or challenging events, also referred to as stressors, with the primary function to overcome the threat (Mommsen et al., 1999; Möstl and Palme, 2002; Schreck et al., 2016). The hippocampus and the amygdala decide whether the stimulus is, in fact, a stressor or not. Afterwards, if perceived as a stressor, the hypothalamus releases corticotropin-releasing factor, which in turn stimulates the pituitary to release adrenocorticotropic hormone (ACTH). Circulating ACTH travels to the adrenal tissue and increases the synthesis of GC, which will act on various target organs and tissues (Moberg and Mench, 2000; Wendelaar Bonga, 1997). The primary GC varies among and within taxa: most mammals and fish generally synthesize cortisol, whereas birds, reptiles, amphibians, and many rodents release corticosterone (Cockrem, 2013; Romero, 2004). A crucial function of these hormones is to curtail the HPA/HPI axis activity (hereafter only "HPI" will be mentioned) through negative feedback at the hypothalamic and pituitary levels and restore homeostasis (Samuel Bradford et al., 1992; Wendelaar Bonga, 1997). Usually, after brief and not overly injurious stressors healthy individuals tend to fully recover, but in the face of a severe acute or long-lasting stressors, restoration of homeostatic equilibrium can be challenging (Branson, 2008; Schreck et al., 2016). Therefore, the stressors' severity and duration will characterize the nature (magnitude and

duration) of the stress response (Barton, 2002). Brief stressors usually trigger acute stress responses characterized by short-term GC increases, which are mostly positive and adaptive. When the stressor is prolonged or repeated, the initial adaptive response is extended over time resulting in a chronically extended stress response. In such situations, the entire functioning of the HPI axis can change, the stress response may lose its adaptive value and become dysfunctional, which can ultimately have detrimental and maladaptive effects for health (Schreck et al., 2016; Wendelaar Bonga, 1997). Given the direct link between chronic stress and animal health, there is a special interest in recognizing long-term stressed individuals, either in the wild for conservation issues (Wikelski and Cooke, 2006) or in domestic species to control for welfare status (Mormède et al., 2007; Ralph and Tilbrook, 2016). This is probably the reason why many researchers wonder how does a chronically stressed animal look like? There have been therefore many efforts to understand which levels of cortisol and corticosterone indicate that the animals' health is or will be detrimentally affected. Recent reviews in wild animals stated that a consistent, predictable GC response to chronic stress does not exist (Dickens and Romero, 2013; Vera et al., 2017). These authors pointed out that baseline and stress-induced circulating GC levels can increase, decrease or remain unchanged in response to chronic stress. Recognizing maladaptive stress responses appears to be particularly challenging yet an important concern for different research fields.

GLUCOCORTICOID MEASUREMENT IN FISH

As in the vast majority of other taxa, the traditional media used to measure cortisol levels in fish has been blood (Baker et al., 2013). Nevertheless, to avoid the invasive procedures inherent to blood sampling, particular attention has been paid on developing alternative matrices whose collection involves the minimum disruption of the animal's biology (Cook, 2012; Goymann, 2012; Sheriff et al., 2011). Although the impetus on developing non-invasive methods for cortisol measurement should be stronger in fish, such efforts in this vertebrate group have been comparatively lower. Levels of cortisol can be less invasively quantified in skin mucus, the surrounding water and faeces (Guardiola et al., 2016; Oliveira et al., 1999; Scott and Ellis, 2007). The analysis of cortisol in whole-body homogenates, gut content, bile and gills has also been described despite the inherent intrusiveness of these methods (Bertotto et al., 2010; Gesto et al., 2015; Lupica and Turner, 2009; Pottinger et al., 1992; Ramsay et al., 2006). In this section we will review the most commonly employed methods for fish cortisol measurement, and we will introduce a novel integrative measure of the fish HPI axis activity.

BLOOD

As above mentioned, cortisol levels in fish have been, and still are routinely determined from blood (Baker et al., 2013). Hormones are released into the bloodstream right after being synthesized, thus blood is the ideal media to assess real cortisol changes (Kersey and Dehnhard, 2014). Levels of cortisol in blood provide an immediate snapshot of the HPI axis activity at the instant the sample is taken (Scott and Ellis, 2007; Sheriff et al., 2011). Once in the laboratory, blood samples are spun to collect serum or plasma wherein GCs are extremely stable over time (Stroud et al., 2007). Additionally, plasma is a conventional tissue for measuring a wide range of other physiological parameters, thus several variables can be simultaneously assessed in a single blood sample (e.g. Clauss et al., 2008; Witeska, 2005). The constraint of this method is the difficulty of obtaining blood samples without triggering some activation of the HPI axis (Pottinger, 2008; Spencer and Deak, 2016), not to mention the intrusiveness of the sampling technique. Also, given that this practice can damage the vascular beds, particularly in small species, repeated blood sampling is generally not recommended.

WATER

Considerable amounts of hormones are excreted into the water through the urine, faeces, seminal fluids, skin, and gills (Vermeirssen and Scott, 1996). Accordingly, measurement of cortisol levels in the surrounding water would certainly solve the problem of fish manipulation.
Water cortisol measures are hypothesized to represent integration over the time that the hormones were accumulating in the water (Ellis et al., 2013), accordingly, the time period they reflect will largely depend on the methodology applied and the container type. Plasma cortisol levels have been demonstrated to significantly correlate to water concentrations (Ellis et al., 2004; Félix et al., 2013). Furthermore, cortisol levels in water increase in a dose-dependent manner after applying single and multiple acute (Ellis et al., 2004; Scott et al., 2001) or chronic stressors (Fanouraki et al., 2008), as well as after an ACTH challenge (Félix et al., 2013; Kim et al., 2018). The method has been successfully applied in laboratory conditions to assess, for example, the influence of high density on growth (Ruane and Komen, 2003), or to show the importance of the aquaculture systems' design in the accumulation of water steroids (Mota et al., 2016). Few researchers have used it in the wild (e.g. Pottinger, 2017; Pottinger et al., 2016; Pottinger and Matthiessen, 2016), probably because of the drawbacks related to its collection in field conditions, and eventually, the interpretation of results related to the collection method applied (Ellis et al., 2013; Guardiola et al., 2016).

WHOLE-BODY HOMOGENATES

In an attempt to obtain endocrine data from small animals or species with very low blood volumes, Pottinger and Mosuwe (1994) validated the use of whole-body cortisol concentrations

as an indicator of stress. Fluctuations in whole-body cortisol levels correlate well to blood levels during acute and chronic crowding stress (King and Berlinsky, 2006; Ramsay et al., 2006). Curiously, this method has been considerably applied in free-ranging species as a means to identify environmental stressors or assess habitat quality (Belanger et al., 2016; Pottinger et al., 2013, 2002), to determine the effects of hypoxia (O'Connor et al., 2011) or to assess the salinity tolerance for an invasive species (Scott et al., 2007). Nevertheless, several studies have shown that steroids within the sample tissue other than cortisol significantly cross-react with the antisera, causing inconsistency when using this method (King and Berlinsky, 2006; Pottinger and Mosuwe, 1994). In addition, for efficient whole-body homogenization this method can only be applied in small individuals (Guest et al., 2016), and for steroid extraction, a relatively large amount of laboratory resources is needed. A significant drawback of the method is that it involves the sacrifice of the specimens, therefore applying this method in species of significant financial or conservation value may be questionable.

SKIN MUCUS

Steroids like GC may diffuse from the blood into the skin mucus thanks to its lipophilic nature (Bertotto et al., 2010), therefore, cortisol levels have been successfully measured in this matrix. Cortisol values in skin mucus provide a snapshot at a single time point, with a time lag between secretion and excretion of the hormone of about 10 min in rainbow trout (*Oncorhynchus mykiss*) (De Mercado et al., 2018). A positive relationship has been demonstrated between cortisol in blood and skin mucus (Bertotto et al., 2010; Simontacchi et al., 2008), although to date if such correlation differs between baseline and stress-induced cortisol levels remain to be explored. Furthermore, skin mucus cortisol concentrations have been shown to be good acute stress indicators in fish exposed to crowding (Simontacchi et al., 2008), to anaesthetic agents (Guardiola et al., 2016), to transport stress (Bertotto et al., 2010) and to hypoxia (De Mercado et al., 2018). Nevertheless, there is still a lack of knowledge regarding the temporal pattern of cortisol secretion in skin mucus far from short-term evaluations or after applying single acute stressors. Despite the technique is simple and fast to perform, relatively non-invasive and comparably inexpensive, very few researchers have selected this method for stress assessments, and to the author's knowledge, none of them has applied the technique in free-ranging species.

SCALES

While cortisol levels measured in the above-mentioned samples represent from a single time-point estimate of the HPI axis activity to integration over a short time period, scale cortisol levels are assumed to provide a retrospective view of integrated hormone secretion over longer periods (Aerts et al., 2015). Scales are formed late in ontogeny (Sire and Akimenko, 2004), and their growth generally continues throughout life (Elliott, 2000). Therefore, cortisol may be

incorporated constantly into the fish scale matrix through diffusion from blood, similarly to the process previously described for corticosterone deposition on feathers (Bortolotti et al., 2008) or cortisol diffusion into the hair shaft (Russell et al., 2012). So far, there is only one study showing that fish subject to chronic intermittent stress presents higher scale cortisol levels than undisturbed fish (Aerts et al., 2015). Additionally, physiological validation of the technique was successfully carried out by the same authors. Nevertheless, given the preliminary nature of those results, there are still elemental concerns related to the methodology and the biological significance of cortisol deposition in fish scales that need further validation.

METHODS VALIDATION

Every technique should be fully validated before being applied to monitor the endocrine system, especially when alternative samples are being used as a proxy for stress (Buchanan and Goldsmith, 2004). This is particularly important since the performance characteristics of any technique will influence the correct interpretation of GC levels in relation to stress (Cook, 2012). Therefore, the initial step before quantification can be undertaken involves the selection of appropriate preparation techniques (Cook, 2012; Palme et al., 2013). Afterwards, when determining concentrations of the hormone by the assay method selected, we should guarantee that other compounds present in the matrix do not interfere with the analysis (Möstl et al., 2005). Once the methodological components have been thoroughly validated, it is crucial to verify that changes in GC levels in the target matrix relate to changes in the adrenocortical activity (Mormède et al., 2007; Palme, 2005). More detailed information regarding these three validation steps is provided in the sections below.

PROTOCOL VALIDATION

Factors related to the technical part of the overall analysis have an important function in the successful assessment of hormone levels. Fundamental matters that every researcher should take into account include: the material used for sample collection (Ellis et al., 2004; Scott and Ellis, 2007); the storage conditions to keep hormone levels unaffected (Burgess et al., 2016; Todini et al., 2010); and the efficiency of the technique to extract the hormones (Davenport et al., 2006; Palme et al., 2013). The essential steps that can influence the end concentrations, however, will clearly rely upon the type of matrix studied. An illustrative example is given by the analysis of cortisol in mammal hair. Because cortisol from blood, sweat and/or saliva can be externally deposited on hair, samples should be washed prior to assay in order to avoid artifactual elevations of cortisol levels (Davenport et al., 2006; Kroshko et al., 2017). The protocol for

decontaminating hair samples has been extensively evaluated and adapted to different species accordingly (Bechshøft et al., 2011; Davenport et al., 2006; Hamel et al., 2011; Raul et al., 2004). In line with these examples, the sample preparation technique acquires crucial importance for the present thesis given that the detection of cortisol in scales is a recent finding. There is little information available concerning validation of the technical methodologies applied, highlighting the need to further validate essential methodological factors before quantifying levels of cortisol in fish scales.

BIOCHEMICAL VALIDATION

Once the preparation techniques have been established, the assay method applied for hormone detection should be fully validated (Buchanan and Goldsmith, 2004; Möstl et al., 2005). In other words, we should verify that the assay method selected provides an accurate measurement of the hormone in the target matrix.

In fish' endocrine-related studies, several analytical techniques have been applied and properly validated for the quantification of cortisol in plasma, such as radioimmunoassays (RIA; Gamperl et al., 1994), enzyme immunoassays (EIA; Mills et al., 2010) and chromatographic methods (Blahová et al., 2007). Although RIA is the preferred analytical technique for whole-body and water cortisol detection (Ellis et al., 2004; Pottinger et al., 2002; Pottinger and Mosuwe, 1994; Ruane and Komen, 2003) also the use of EIA has been properly validated (Fanouraki et al., 2008; Guest et al., 2016; Sutherland et al., 2008). Surprisingly, only RIA methods for cortisol quantification in skin mucus have been accurately validated (Bertotto et al., 2010), whereas EIA, a popular assay technique (Sheriff et al., 2011), has been applied without apparent validation (De Mercado et al., 2018; Guardiola et al., 2016). Regarding scale cortisol quantification, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) has only been used despite having easier, faster and cheaper assays for steroid determination within reach (Kersey and Dehnhard, 2014).

In many vertebrate species other than teleost fish, the use of EIA is gaining popularity, probably because it does not require radioactive reagents, being, therefore, safer and more economical than RIA (Blahová et al., 2007; Sheriff et al., 2011). Commercial EIA kits developed mainly for human and laboratory animal use are also readily available (Kersey and Dehnhard, 2014; Touma and Palme, 2005). Interestingly, many commercial EIA kits have been successfully applied for measuring cortisol levels in other species than the one being certified by the manufacturer (e.g. Carbajal et al., 2014; Macbeth et al., 2010; Sutherland et al., 2008; Tallo-Parra et al., 2014). Although they come already species-specifically validated, each assay needs an exhaustive biochemical validation for the species and sample of interest (Buchanan and Goldsmith, 2004). Validation of EIA follows the criteria for an immunological validation by determination of assay specificity, accuracy, precision and sensitivity (Food and Drug Administration, 2015; Midgley et

al., 1969; Reimers and Lamb, 1991). Specificity refers to the ability of the assay to measure what it is intended to measure because other substances present in the sample do not cross-react significantly with the antibody (Food and Drug Administration, 2018; Midgley et al., 1969). The EIA kit manufacturer provides information regarding cross-reaction with other steroids, nevertheless, specificity should be tested for each matrix and target species (Buchanan and Goldsmith, 2004; Mormède et al., 2007). Accuracy provides a measure of the exactness of the analytical assay by measuring the closeness between the obtained value and the value accepted as true or reference (Davidian et al., 2015; Food and Drug Administration, 2018). Precision, also regarded as repeatability, is the measure of variation of repeated determinations of the same sample, either within an assay (intra-assay precision), between different assays (inter-assay precision) or between laboratories (Food and Drug Administration, 2018; Midgley et al., 1969). Finally, the sensitivity of the assay refers to the capacity to distinguish and measure the smallest amount of target hormone (Midgley et al., 1969; Möstl et al., 2005).

BIOLOGICAL VALIDATION

After analytical validation of the assay, it is imperative to verify whether GC levels in the chosen matrix reflect reliable biological changes in the animal (Kersey and Dehnhard, 2014; Palme, 2005). Although there are several approaches applied to verify this relationship, the most commonly employed in fish is to prove a cause-and-effect relationship. Establishing a cause-and-effect relationship means to expose an animal to a known biological stressor (e.g. capture and handling, food deprivation, crowding stress, predation risk) and measure the amount of cortisol present in the sample. This test is particularly useful for subsequent field studies to ensure that the method will be sensitive to genuine stressors typically encountered in the wild (Sheriff et al., 2011). Every alternative matrix in fish has been properly validated by establishing a relationship of cortisol fluctuations to, at least, one stressor type. For instance, cortisol levels in gut content increased after transport in rainbow trout but not in European sea bass (*Dicentrarchus labrax*) (Bertotto et al., 2010). Another illustrative case is given by the study of Guardiola and colleagues (2016) in gilthead seabream (*Sparus aurata L.*), who demonstrated that cortisol concentrations in skin mucus were affected by crowding stress but not by hypoxia. These examples emphasize the need to evaluate the influence of different stressor types on the same metrics by using different species. In order to assure that the event will be perceived as a stressor, some authors have applied a combination of different stressors, such as a physical disturbance in tandem with a confinement stress (Pottinger and Mosuwe, 1994). The deposition of cortisol in fish scales has also been biologically tested by establishing a cause-and-effect link (Aerts et al., 2015). In these sorts of studies the procedures applied to induce chronic stress are crucial since they can influence the resultant physiological effects (Dickens and Romero, 2013). For instance, in that experiment, fish were daily subjected to acute stressful

events throughout the study (Aerts et al., 2015). Nevertheless, to further evaluate the effectiveness of the method as a bioindicator of long-term HPI axis activity, the effect of a continuous and prolonged stressor on fish scale cortisol deposition should be studied, rather than applying single acute stressors.

Stronger methods to evaluate the biological relevance of alternative matrices for GC measurement involve pharmacological stimulations or inhibitions of hormone release (Mormède et al., 2007). Although this method has proven to be very useful in mammals, few researchers have externally manipulated cortisol levels in fish with this specific purpose. Recent studies certified that levels of cortisol detected in fish holding-water were positively correlated to the increase in circulating levels after stimulation with an ACTH challenge (Félix et al., 2013; Kim et al., 2018). Another successful example is given by the study performed by Aerts and colleagues (2016) who validated the measurement of cortisol in scales by feeding fish with either cortisol (stimulation) or dexamethasone (inhibition).

To further increase the applicability of non-conventional matrices as a sensitive-individual measure of fish stress, validations should prove that hormone concentrations in these media are proportional to their abundance in the bloodstream (Cook, 2012; Sheriff et al., 2011). This correlation in cortisol levels between matrices is usually performed in tandem with the previously described biological validations. These approaches are actually very common in fish endocrine-related studies, probably because results can be easily interpreted and they provide meaningful information. As the study of Bertotto and colleagues (2010) proves, a between-matrix correlation successfully detected in a certain species does not mean that all species will show the same correspondence, highlighting the need to evaluate these relationships for each species and matrix.

GLUCOCORTICOIDS AS BIOINDICATORS OF HABITAT QUALITY

Glucocorticoid responses to environmental disturbances are of growing interest since they can be used to quantify the impacts of stressors on individuals (Cockrem, 2005; Wikelski and Cooke, 2006) and ultimately, be used as bioindicators for conservation purposes (Jeffrey et al., 2015). To date, a considerable number of studies have applied the measurement of cortisol to evaluate whether and how habitat quality impact on fish populations (Baker et al., 2013; Pankhurst, 2011), probably because of the significant anthropogenic pressure on the aquatic environment (Jeffrey et al., 2015; Rhind, 2009). Habitat quality, here understood as the *"goodness" of the physical environment of individual fish that is required to carry out its entire life cycle*

(Bálint et al., 2014; Jeffrey et al., 2015; Johnson, 2007), is typically altered by temperature and pH modifications, changes in dissolved oxygen, as well as exposure to contaminants (Barton, 2002) which, in different occasions, are known to generate environmental stress.

One of the primary issues that should be considered before studying the influence of environmental stress on fish is whether individuals actually perceive the target event as stressful (Busch and Hayward, 2009; Pankhurst, 2011; Schulte, 2014). An extreme condition potentially stressful from a certain species or population might not be for another. For example, largemouth bass (*Micropterus salmoides*), brown bullhead (*Ameiurus nebulosus*) and logperch (*Percina caprodes*) from a degraded habitat presented altered cortisol responses compared to the reference site, while white sucker (*Catostomus commersonii*) and pumpkinseed (*Lepomis gibbosus*) inhabiting the same stream showed unaltered cortisol levels (King et al., 2016). The effects cannot only differ among species or populations, they can also be sex-specific, as demonstrated on rainbow darter (*Etheostoma caeruleum*) and three-spined sticklebacks (*Gasterosteus aculeatus L.*) from effluent-contaminated sites (Mehdi et al., 2017; Pottinger and Matthiessen, 2016) or in zebrafish (*Danio rerio*) exposed to pesticides (Zhang et al., 2015). Abrupt and fleeting habitat changes, events such as drought (Baker et al., 2013), variation in food availability (Chase et al., 2016; Pottinger et al., 2002) or extreme temperatures (Davis and Peterson, 2006; Rotllant et al., 2000; Schulte, 2014) are known to considerably stimulate the HPI axis activity. But also gradual and long-term changes can influence the fish stress response. The standard example of a prolonged environmental stressor is habitat degradation and usually linked to high exposure to contaminants (e.g. Marentette et al., 2012; Oliveira et al., 2011; Pottinger et al., 2013).

A second requirement for the successful use of cortisol concentrations in fish as a bioindicators is the establishment of reference values for each species (Pankhurst, 2011; Sanchez et al., 2010). In other words, the interpretation of GC responses requires of controls. In the field, the usual practice is to compare exposed to unexposed (reference) populations to a certain environmental challenge (Matthiessen et al., 2018; Rhind, 2009). To assess the HPI axis, cortisol levels are commonly measured in baseline samples and/or after inducing a stress response (Homyack, 2010; Romero, 2004). In this context, laboratory studies are widely used to first obtain a validation, which will afterwards, be extrapolated to wild conditions. Examples of these experimental approaches are numerous, and include from drought simulation (Flodmark et al., 2002; Sloman et al., 2001) or food deprivation (Chase et al., 2016) to pollutants exposure (Gandar et al., 2017; Ghasemzadeh et al., 2015; Teles et al., 2017; Zhang et al., 2015). Besides, cortisol manipulations in free-living fishes have shown to be effective in reflecting how individuals cope with extreme environmental conditions (Dekoning et al., 2004; Nagrodski et al., 2013).

Adequate temporal sampling is a crucial concern when evaluating variation in physiological indices (Lennox et al., 2018). First, if cortisol secretion is subjected to circadian rhythm

(Dickmeis, 2009), it is imperative to strictly control for sampling time especially when using single-point matrices. Further, the time-point at which the sample is collected after stressor exposure cannot be chosen arbitrarily (Schreck et al., 2016). For example, two individuals can show the same magnitude in the stress response at the sampling time-point, but one of them could present afterwards, a shorter stress response compared to the other (Norris et al., 1999). Also, in response to an acute stressor the short-term capacity to respond may not be compromised but could have longer-term implications (Jorgensen et al., 2017, 2002), thus this will require for future samplings. It is well known that an environmental change perceived as a stressor on a certain context may not be stressful in another (Homyack, 2010; Killen et al., 2016; Madliger and Love, 2014; Schulte, 2014). Therefore, care must be taken when studying fish at different life-history stages given the substantial evidence that vertebrates are able to temporarily modify their sensitivity to stress (Romero, 2002; Wingfield and Sapolsky, 2003). In fish, a remarkable illustration of this temporal change is given by the across season variation in cortisol levels, which in several occasions, these GC fluctuations have been successfully linked to the breeding period (Belanger et al., 2016; Carruth et al., 2000; Pankhurst and Sharpies, 1992).

Understanding cortisol fluctuations in wild animals is undoubtedly a complex process (Dantzer et al., 2014; Johnstone et al., 2012). For this reason, interpretation of cortisol levels is greatly enhanced if they are accurate in predicting population viability (Tarlow and Blumstein, 2007). Linking results to mortality, disease, reproduction and survival (Busch and Hayward, 2009; Cooke and O'Connor, 2010), and including additional indices such as haematological parameters (Hedayati and Hassan Nataj Niazie, 2015) or other endpoints of the stress response (Blevins et al., 2013; Pérez-Casanova et al., 2010) will provide a more complete insight about the ecology of stress (Boonstra, 2013; Schulte, 2014).

The nature of the environmental stressor, whether the aim is to evaluate acute or chronic stress, the target species, as well as the desired degree of invasiveness, will determine the adequate media wherein cortisol levels will be analysed (Sheriff et al., 2011). Blood and more recently whole-body homogenates have been the substrate of choice for cortisol measurement in wild fish. These methods have demonstrated to reliably quantify the impact of certain environmental stressors (Blevins et al., 2012; Hontela et al., 1992; Pottinger et al., 2013) and predict fitness (Cook et al., 2014, 2011; Midwood et al., 2016). However, they involve a substantial degree of invasiveness that may not always be possible or desirable (Cooke and O'Connor, 2010). Cortisol measures in skin mucus seem to be a good alternative for measuring fish stress with minimal restraint and disruption to the animal (Bertotto et al., 2010; Guardiola et al., 2016; Simontacchi et al., 2008). This technique is far less invasive than the aforementioned and the sample is easier and faster to collect. Nevertheless, its applicability in wild environments still needs to be addressed. Likewise, the assessment of cortisol in fish scales would probably provide many

advantages in wildlife studies. The method could offer the measurement of hormones from a wide range of species, in locations, and time periods that are inaccessible for blood collection. As previously observed in feather and hair GC analyses (Ashley et al., 2011; Fairhurst et al., 2013; Tallo-Parra et al., 2017), probably the contribution of single acute stressors to scale cortisol is likely small. The technique, therefore, may not be able to detect acute stressful events, but it is a promising useful tool for assessing responses to stressors or to energy demanding situations that take place over longer time periods (e.g. extreme climate fluctuations, pollution, life-history stages, and seasons).

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OBJECTIVES

The general objective of this thesis is to validate the measurement of cortisol in fish skin mucus and scales and evaluate the applicability of these methods as potential tools to assess habitat quality.

The specific objectives are as follows:

1. To validate a protocol for the extraction of cortisol from fish scales and skin mucus and the quantification of cortisol concentrations in these matrices by enzyme immunoassay.

2. To evaluate whether the quantification of cortisol in skin mucus represents a short-term measure of fish hypothalamic-pituitary-interrenal axis activity.

3. To evaluate whether the quantification of cortisol in scales represents a long-term integrated measure of fish hypothalamic-pituitary-interrenal axis activity.

4. To evaluate the potential use of skin mucus and scale cortisol as bioindicators of habitat quality in a wild freshwater fish.

CHAPTER I

Cortisol detection in fish scales by enzyme immunoassay: Biochemical and methodological validation

A. Carbajal[1], L. Monclús[1], O. Tallo-Parra[1], M. Sabes-Alsina[1], D. Vinyoles[2], and M. Lopez-Bejar[1]

[1] *Department of Animal Health and Anatomy, Veterinary Faculty, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

[2] *Department of Evolutive Biology, Ecology and Environmental Sciences, Universitat de Barcelona, Avinguda Diagonal 643, 08028, Barcelona, Spain*

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ABSTRACT

The study of fish stress is usually assessed by measuring blood cortisol. Nevertheless, blood provides only a snapshot of the hormonal profile at one point in time. An alternative source of cortisol may be found in scales, providing a new approach for assessing long-term hormonal levels. The present study aimed to develop and validate a methodology for detecting cortisol in scales of goldfish (Carassius auratus). The study highlights the importance of an initial isopropanol washing procedure to completely eliminate external contaminations of cortisol. Additionally, the biochemical validation of the enzyme immunoassay verified the possibility to detect cortisol with repeatability and reliability in goldfish scales. In conclusion, this study provides validated information about a new methodology to measure cortisol in scales. The incorporation of this biomarker could provide retrospective hormonal measurements from species and time periods that are usually difficult or impossible to obtain, thus offering key data of an animal's physiology.

INTRODUCTION

In fish the response to stress is usually assessed by quantification of short-term changes in blood cortisol levels (Baker et al., 2013), the main glucocorticoid (GC) in most teleosts (Mommsen et al., 1999). Blood provides only a snapshot of the hormonal profile at one point in time. An alternative source for cortisol measurement may be fish scales. The analysis of cortisol in scales could constitute a method for retrospective assessment of fish GC secretion over extended periods of time (Aerts et al., 2015). However, little information concerning the laboratory processing and validation of cortisol measurement in scales is available. Accordingly, the present study was focused on developing and biochemically validating the method for detecting cortisol deposited inside the scales of goldfish *Carassius auratus* (L. 1758).

External sources of cortisol, presumably coming from the fish skin mucus (Bertotto et al., 2010), should be removed in order to study a more tightly bound fraction incorporated in the interior of the scale matrix. While isopropanol has been the wash solvent of choice to remove external contaminations of GC in mammal hair (Davenport et al., 2006; Tallo-Parra et al., 2015), in fish, only ultrapure water has been employed (Aerts et al., 2015). Hence, the first experiment explored the appropriateness of water and isopropanol as solvents to decontaminate the scale without influencing the hormone contents inside the scale.

We additionally aimed to test the suitability of an enzyme immunoassay (EIA) in the quantification of cortisol concentrations in scales, since the assay procedure should always be properly validated for each new species and matrix (Buchanan and Goldsmith, 2004).

MATERIALS & METHODS

ANIMALS AND SAMPLING

Twenty goldfish were euthanized with an overdose of MS-222 (100 mg/L). Immediately after sacrifice, whole body scales were removed and thoroughly mixed to create two separate pools, one for each experiment.

WASHING PROCEDURE VALIDATION

The pool of scales was uniformly split into two groups given by the type of wash solvent evaluated; water and isopropanol. Scales from each group were divided into three treatment conditions; for one, two or three consecutive washes, with three replicates per treatment. Each replicate comprised 300 mg of pooled scales introduced into polypropylene tubes. Three millilitres of solvent were added to each tube and vortexed at 1800 r.p.m. for 2.5 min. Afterwards, the supernatant was separated for further analysis. The process was then repeated once or twice in accordance to the treatment assigned. After being washed, the scales were air-dried at room temperature for about 24 hours. Once the scales were dry they were minced with a ball mill (Retsch, MM2 type, Germany). Then, 75 mg of each powdered sample were incubated with 1.5 ml of methanol for 18 h at 30^{o}C in an incubator shaker with continuous mixing (G24 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc., Edison, USA). Following extraction, samples were centrifuged at 9500 x g for 10 minutes and 1 ml of the supernatant was evaporated and reconstituted with 0.2 ml of EIA buffer provided by the assay kit.

BIOCHEMICAL VALIDATION OF THE EIA

Cortisol concentrations and the validation tests were determined by using competitive EIA kits (Neogen® Corporation Europe, Ayr, UK). The assay was validated following the criteria for an immunological validation by determining assay specificity, accuracy, precision and sensitivity (Reimers and Lamb, 1991). Extracts from 10 samples were pooled for assay validation. Precision was assessed by calculating intra-assay coefficients of variation (CV) from all duplicated samples. The specificity was evaluated with the linearity of dilution, determined by using dilutions of pools with EIA buffer. Specificity was also evaluated with the parallelism test using the pooled extracts spiked with the standards of the kit and assayed to test for a parallel response to the same kit-based reference standards. Accuracy was assessed through the spike-and-recovery test, calculated by adding known volumes of pool to different volumes and concentrations of pure standard cortisol solution. Finally, the sensitivity of the test was given by the smallest amount of hormone concentration analysed.

STATISTICAL ANALYSIS

Data obtained were analysed using R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) with a p-value below 0.05 as a criterion for significance. The assumption of normality was checked using a Shapiro–Wilk test and concentrations were log transformed where necessary to achieve normality. A two-way analysis of variance (ANOVA) was used to test for differences between distilled water and isopropanol treatments using wash solvent and number of washes applied as main factors. When significant, ANOVA was followed by post hoc analysis (Tukey's test) in order to determine the source of significance. For the biochemical validation, statistical correlations in the dilution test (expected vs. obtained values) and parallelism test (standard vs. pool concentrations) were determined using the Pearson's Product correlation test.

RESULTS

WASHING PROCEDURE VALIDATION

No significant differences in the scale cortisol concentrations (SCC) were found between samples subjected to 1, 2 or 3 washings with isopropanol ($p > 0.05$; Fig. 1). Samples washed with distilled water showed a significant decrease in the SCC from the first to the second wash ($p < 0.05$), but not from the second to the third one ($p > 0.05$; Fig. 1). Tukey's test indicated significant differences in SCC between water and isopropanol when samples were washed twice and thrice ($p < 0.05$). Significant differences were found in wash cortisol concentrations (WCC) between the first, the second and the third wash supernatant of scales treated with either isopropanol ($p < 0.05$; Fig. 2) or distilled water ($p < 0.05$). No solvent-induced differences were detected in WCC ($p > 0.05$; Fig. 2).

BIOCHEMICAL VALIDATION OF THE EIA

The intra-assay CV was 6.30 %. In the linearity of dilution, the obtained cortisol concentrations correlated with the expected cortisol values ($r = 0.99$, $p < 0.05$; Fig. 3A). In the spike-and-recovery test, hormone standards spiked with the pool of scale extracts presented a mean recovery percentage of 89.6 ± 7.71 % (mean ± SD). Cortisol concentrations from the standard curve and the pool curve obtained in the parallelism test showed a high correlation ($r = 0.99$, $p < 0.05$; Fig. 3B). The sensitivity of the assay obtained was 0.22 pg of cortisol/mg of scale.

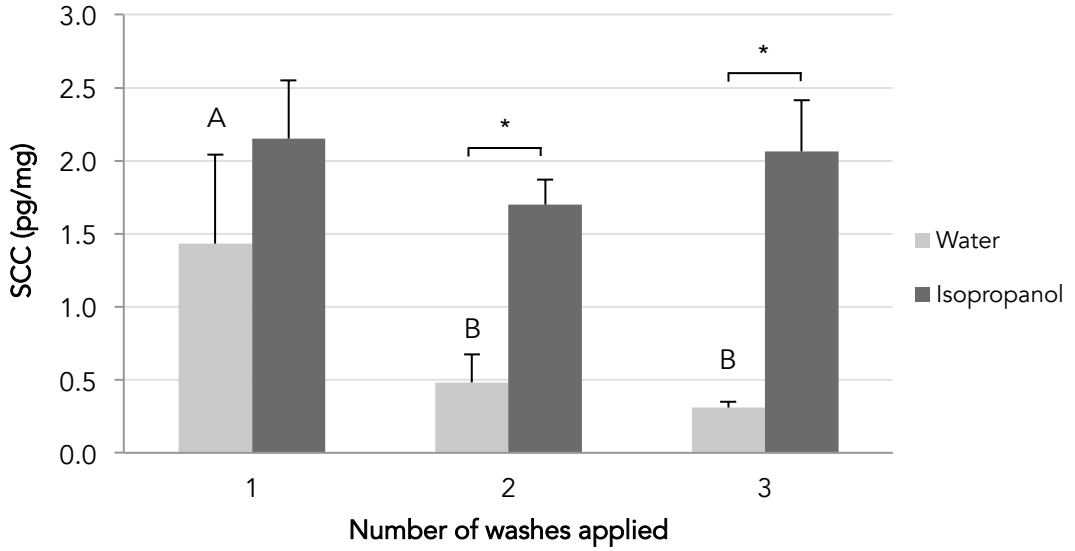

Figure 1. Mean (± SD) scale cortisol concentrations (SCC) in samples subject to 1, 2 and 3 washings with distilled water (light grey) and isopropanol (dark grey). Different uppercase letters indicate statistical differences in SCC among number of washes with distilled water ($p < 0.05$). No significant differences were detected in SCC when the scales were washed with isopropanol ($p > 0.05$). Solvent-induced differences are indicated by asterisks ($p < 0.05$).

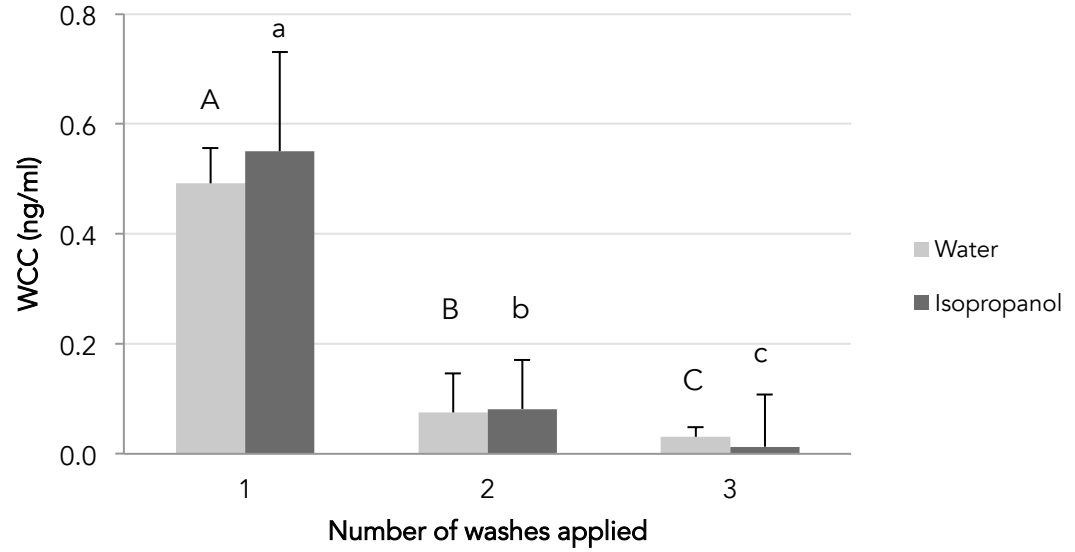

Figure 2. Mean (± SD) wash cortisol concentrations (WCC) in in the first, second and third supernatant of samples washed with distilled water (light grey) and isopropanol (dark grey). Different letters indicate statistical differences among water supernatants (uppercase) and among isopropanol supernatants (lowercase) ($p < 0.05$). No solvent-induced differences were found in WCC ($p > 0.05$).

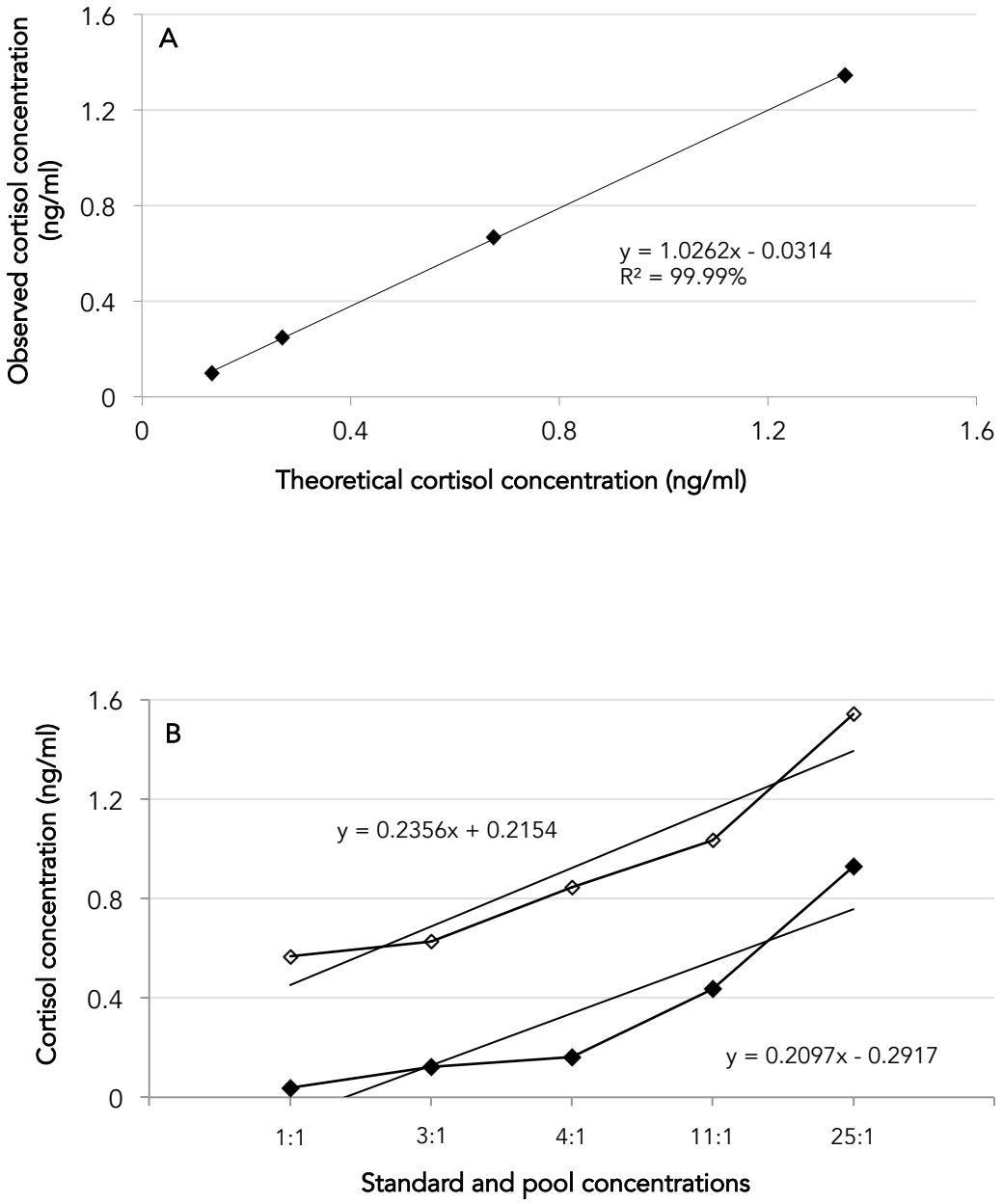

Figure 3. Results obtained in the biochemical validation of the enzyme immunoassay: (A) correlation between observed and theoretical cortisol concentrations obtained in the dilution test (Pearson's correlation; r = 0.99, $p < 0.05$) and (B) parallelism relation between lines from the standard (black diamonds) and sample pool (white diamonds) curves obtained in the parallelism test (Pearson's correlation; r = 0.99, $p < 0.05$).

DISCUSSION

Scale cortisol concentrations in samples washed with isopropanol, unlike scales washed with water, remained constant regardless of the number of washings. These results could indicate two phenomena: washing scales with isopropanol was not effective enough to remove the external contamination of cortisol, or water could be penetrating into the scale while removing the hormone deposited inside the matrix. WCC analysis showed that successive washes with either isopropanol or water were removing cortisol from the first to the third wash. Cortisol concentrations detected in the wash supernatant were three times higher than in scales for both isopropanol and distilled water washes, probably due to the presence of cortisol in the skin mucus. The presence of high cortisol concentrations in skin mucus has been described in different fish species (Bertotto et al., 2010). Therefore, cortisol levels detected in scales washed with isopropanol suggest that this solvent was effective in removing the cortisol coming from external sources. Additionally, successive washes with water could be removing endogenous cortisol from the scales, as observed in hair shaft washed with water (Hamel et al., 2011). Accordingly, to ensure a complete external decontamination, three 2.5-min isopropanol washings with 3 ml each were established as the washing protocol for subsequent assessments.
Performance of the EIA for cortisol analysis in fish scales was fully validated obtaining repeatable and reliable results. The values obtained in the dilution test were close to those expected, confirming that samples interacted with the assay antibody in a dose-dependent manner. Additionally, immunoreactivity of pooled extracts was parallel to the kit standards, suggesting that standard and samples have similar antibody-binding characteristics. Finally, in the spike-and-recovery test, various quantities of cortisol previously added to pooled extracts were quantitatively recovered, thus other components of the samples probably did not interfere with the estimation of the hormone.

In conclusion, the present work presents a validated method to detect cortisol in scales of goldfish. We highlight the importance of an isopropanol washing procedure to completely eliminate external contaminations of cortisol while preserving the steroid content inside the matrix. Additionally, we demonstrated the suitability of the EIA in the quantification of cortisol concentrations in scales processed through the aforementioned method. The incorporation of this biomarker could provide retrospective hormonal measurements from species and time periods that are usually difficult or impossible to obtain, thus offering key data of an animal's physiology.

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CHAPTER II

Comparative assessment of cortisol in blood, skin mucus and scales as a measure of the hypothalamic-pituitary-interrenal axis activity in fish

A. Carbajal[1], F. Reyes-López[2], O. Tallo-Parra[1], M. Lopez-Bejar[1], L. Tort[2]

[1] *Department of Animal Health and Anatomy, Veterinary Faculty, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

[2] *Department of Cellular Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

ABSTRACT

Cortisol, the end product of the hypothalamus-pituitary-interrenal (HPI) axis, has been traditionally measured in blood as indicator of stress in fish, however, the degree of invasiveness inherent to blood collection is not always possible or desirable. Instead, cortisol measurement in skin mucus is far less invasive, but as blood, this method provides a brief window of information of the HPI axis activity. The newly described method of cortisol measurement from scales may serve as a long-term, integrated measure of the HPI axis activity in fish. While skin mucus and scale cortisol measurement present practical and conceptual advantages, there are still several unclear issues related to their biological relevance that need deeper study. Accordingly, we aimed to evaluate whether skin mucus and scale cortisol levels can be reliably used as short- and/or long-term stress indicators by subjecting fish to prolonged, continuous stressful conditions. The present study demonstrates that the measurement of cortisol in skin mucus reflect better the circulating cortisol concentrations when fish are responding to stress with an intense activation of the HPI axis. Results also revealed that cortisol content in scales strongly correlates to circulating cortisol levels in chronically stressed fish. Besides, we provide further support that scale cortisol assessment offer a retrospective measure of the past stress experience in fish. While this study provides a good basis for future research applying the methods presented, our results open the question of whether these matrices have additional sources of cortisol other than blood, and the route of incorporation or diffusion. Further knowledge about the general robustness and stability of scale cortisol in fish subjected to prolonged stress would largely help strengthen the interpretation of hormone fluctuations in this matrix.

INTRODUCTION

When perceiving a potential threat to homeostasis, cortisol, the main glucocorticoid (GC) in most teleost fish, is released into the bloodstream through the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Mommsen et al., 1999; Pankhurst, 2011). The resulting secretion of cortisol triggers a progression of behavioural and physiological changes to promote survival (Moberg and Mench, 2000; Mommsen et al., 1999). However, when this activation known as the stress response, is prolonged or severe, it can have detrimental consequences on health, immune defences, growth and/or reproduction (Sapolsky et al., 2000; Schreck et al., 2016). The study of the stress response, therefore, is of growing interest both because of the assessment of the performance response of the fish but also because such an assessment can be used as a welfare indicator (Schreck et al., 2016). As in the vast majority of other vertebrates, blood cortisol measurement in fish has been the traditional method used to assess the activity of the HPI axis (Baker et al., 2013). Nevertheless, capturing, handling and collecting blood samples can elicit a stress response by itself that may confuse the interpretation of results (Marino et al., 2001). Recent advances in the use of minimally and non-invasive matrices to assess GC levels are shifting the use of blood (Ellis et al., 2013). In fish, such alternative samples include the skin mucus, the surrounding water, faeces and urine (Guardiola et al., 2016; Lupica and Turner, 2009; Scott and Ellis, 2007). Measurement of skin mucus cortisol levels have been shown to faithfully reflect acute stress responses in fish exposed to transport stress or to hypoxia among others (Bertotto et al., 2010; De Mercado et al., 2018). These previous studies have demonstrated that skin mucus cortisol is positively correlated to plasma levels (Guardiola et al., 2016). However, none of them have differentiated between control and stressed induced cortisol measurements, neither the temporal pattern of cortisol secretion in skin mucus far from short-term evaluations or after applying single acute stressors (Bertotto et al., 2010; De Mercado et al., 2018; Simontacchi et al., 2008). Thus the importance to further validate this novel matrix for fish stress response assessments is evident. While the measurement

of cortisol in these unconventional matrices can improve the understanding of fish hormonal response (Ellis et al., 2013; Guardiola et al., 2016), they present a clear limitation: they reflect circulating cortisol levels from a relatively short time frame. This important drawback has been recently compensated by developing a method able to inform about long-term HPI axis activity, the measurement of cortisol in fish scales (Aerts et al., 2015). Scales are formed late in ontogeny (Sire and Akimenko, 2004), and their growth generally continues throughout life (Elliott, 2000). Therefore, cortisol incorporates constantly into the fish scale matrix through diffusion from blood, similarly to the process previously described for corticosterone deposition on feathers (Bortolotti et al., 2008) or cortisol diffusion into the hair shaft (Russell et al., 2012). To guarantee that fish scales can be potentially used for chronic stress assessment, the measurement of cortisol in this matrix has to be biologically relevant. Biological validation of the method can be performed by several ways; by comparing independent measures of cortisol levels in different matrices, by applying a pharmacological challenge, or evaluating a cause-and-effect relationship (Cook, 2012; Kersey and Dehnhard, 2014). So far, the only published study revealing the properties of fish scale cortisol contrasted the hormone levels in this matrix from a group of fish subjected to daily acute stressors (Aerts et al., 2015), rather than applying a continuous prolonged stress throughout the study. In addition, information regarding the correlation between cortisol levels in scales to those in matched blood samples remains to be explored. Given that the measurement of cortisol in scales is a recent finding, there is the need to further study the potential of this matrix in order to obtain results that can be fully interpreted.

Accordingly, we subjected fish to long-term, uninterrupted stressful conditions in order to test whether skin mucus and scale cortisol levels can be reliably used as short- and/or long-term stress indicators. Specifically, we aimed (1) to test the hypothesis that cortisol levels in skin mucus reflect better short- than long-term stress responses, (2) to test the hypothesis that, at an individual level, cortisol levels in scales relate to those in matched blood samples when the HPI axis has been long-term activated, and (3) to test the hypothesis that the amount of cortisol deposited in scales provides a long-term, integrated measure of the HPI axis activity in fish held under chronic and continuous stress.

MATERIALS & METHODS

ANIMALS

Experimental protocols were conducted with approval from the Ethical Committee of the Universitat Autonoma de Barcelona. Juvenile rainbow trout (*Oncorhynchus mykiss*) (108.68 ±

27.54 g body weight) were obtained from a commercial supplier (Trout Factory, Peramola, Spain) and kept in stock tanks in the facilities at the Universitat Autonoma de Barcelona (AQUAB, Bellaterra, Spain) at a density of 3.2 kg/m^3. Fish were allowed to acclimatise for 30 days before starting the experiment.

EXPERIMENTAL SET-UP

A total of 64 rainbow trout were randomly divided into 8 groups. Five groups were confined at a high-density equivalent of 30 kg/m^3 (stressed groups), each group in a different keepnet (45 cm x 25 cm x 25 cm) thus maintaining the confinement but also not modifying the total water volume in the tank. Three groups were kept undisturbed in different holding tanks of 90 cm diameter and 110 cm depth containing 380 L of freshwater at a low-density of 2.6 kg/m^3 (control groups).

One control group was sampled for blood, skin mucus and scales at the start of the experiment in order to obtain pre-stress levels (0 h). To study the short-term stress response, three stress groups were sampled for blood and skin mucus at 1, 6 and 24 h after starting the confinement stress. For the long-term stress response assessment, fish were kept under low or high-density confinement for 14 and 30 days. At each period, blood, skin mucus and scales were sampled from a control and a stress group.

SAMPLE COLLECTION & LABORATORY PROCEDURES

Prior to sampling, fish were euthanized with an overdose of MS-222 (100 mg/L). Skin mucus samples were quickly collected according to the method described by Guardiola et al. (2016) with some modifications. Briefly, immediately after fish were anesthetized, the skin mucus was collected by gently scrapping the lateral surface of each individual with a cell scrapper. The collected sample was introduced in a 1.5 mL tube and vortexed for 5 min. Afterwards, samples were centrifuged (2000 x g, 10 min) at 4 ºC and the supernatant was stored at -20 ºC until analysis.

Blood samples were collected by puncture of the caudal vein with an insulin syringe. After clotting for 4 h in cold, samples were centrifuged (1500 x g, 5 min) at 4 ºC and the plasma collected was stored at -20 ºC until analysis.

Scales from the dorso-lateral flank were removed with a small scalpel. Once in the laboratory, 200 mg of scales were weighted and introduced in a polypropylene tube. Three successive washes of isopropanol were applied to each sample in order to remove external cortisol contamination (Carbajal et al., 2018). Scales were then allowed to dry at room temperature for about 24 h. Dried scales were pulverized with a ball mill (Retsch, MM200 type, Germany) for 2.5 min at 25 Hz to reach homogeneity on the minced particles. Between 40 and 50 mg of powdered scale was incubated in 1.5 mL of methanol for 18 h at 30 ºC. Following extraction,

samples were centrifuged (7000 x g, 10 min) and the supernatant was evaporated. Once dried, extracts were reconstituted with 0.2 mL of enzyme-immunoassay (EIA) extraction buffer and immediately stored at −20 ˚C until analysis.

CORTISOL ANALYSES & BIOCHEMICAL VALIDATION

Plasma cortisol concentrations (PCC), skin mucus cortisol concentrations (MCC) and scale cortisol concentrations (SCC) were analysed using a commercial EIA kit (Cortisol ELISA KIT; Neogen® Corporation, Ayr, UK). According to the manufacturer, cross-reactivity of the antibody with other steroids is as follows: prednisolone 47.4%, cortisone 15.7%, 11-deoxycortisol 15.0%, prednisone 7.83%, corticosterone 4.81%, 6β-hydroxycortisol 1.37%, 17-hydroxyprogesterone 1.36%, deoxycorticosterone 0.94%. Steroids with cross-reactivity < 0.06% are not presented.

The assay was biochemically validated for each matrix following the criteria for an immunological validation (Midgley et al., 1969; Reimers and Lamb, 1991). A pool of plasma, skin mucus and scale extracts was created for validation. Intra-assay and inter-assay coefficients of variation from all duplicated samples analysed were calculated for precision assessment. The specificity was evaluated with the linearity of dilution, determined by serially diluting the pool with EIA buffer. Accuracy was assessed through the spike-and-recovery test, calculated by adding known volumes of pool to different volumes and concentrations of pure standard cortisol solution. The sensitivity of the test was given by the smallest amount of hormone concentration analysed.

STATISTICAL ANALYSES

Statistical analyses were conducted using R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) with a p-value below 0.05 as a criterion for significance. Normality of data was checked using a Shapiro–Wilk test and accordingly, concentrations were log-transformed where necessary to improve homogeneity of variance. Data were analysed by two-way ANOVA with cortisol concentrations in serum, skin mucus, and scales as dependent variables, and group, total length and fish weight as factors. Where significant differences were detected, Tukey post-hoc tests were subsequently applied to identify the source of variance. Additionally, Pearson's Product Moment correlation tests were used to test for correlations in cortisol concentrations among all matrices evaluated.

When analysing the data obtained in the biochemical validation, also Pearson's correlation was used to evaluate the correlation between obtained and expected values from serial dilutions.

RESULTS

PLASMA, SKIN MUCUS AND SCALE CORTISOL LEVELS

A significant increase in PCC (Fig. 1A) and MCC (Fig. 1B) was detected one hour after starting the confinement stress. In blood, cortisol levels remained significantly higher than pre-stress levels up to 24 h after. Whereas in mucus, cortisol levels decreased significantly at 24 h compared to the initial cortisol peak, although they remained above the pre-stress levels. At day 14 and 30, in the groups exposed to the chronic continuous stress, PCC and MCC decreased significantly compared to the peak cortisol elevation detected at 1, 6 and 24 h.

No significant differences in PCC were detected between stressed and control groups neither at day 14 nor at day 30. In MCC, no differences were detected between controls and stressed fish at day 14, while at day 30 stressed fish presented higher MCC than controls. In addition, both at day 14 and 30 the stressed group presented significantly higher MCC than pre-stress levels. Significant differences in SCC were detected between stressed and control fish at day 14 and at day 30 (Fig. 1C). A decrease in SCC was detected from day 14 to day 30 of the study.

CORRELATION AMONG MATRICES

Correlations between matrices were performed categorizing the 8 groups of fish either by control groups (0 h, 14 d and 30 d), short-term stress groups (1, 6 and 24 h) and long-term stress groups (14 and 30 d), as shown in Table 1.

Table 1. Correlation (r) between blood, skin mucus and scale cortisol concentrations detected in control groups (0 h, 14 d and 30 d) and in short-term (1, 6 and 24 h) and long-term (14 and 30 d) stress groups

Matrix		Mucus	Scales
Blood			
	Control	r = 0.22	r = 0.19
	Short-term	r = 0.70*	-
	Long-term	r = 0.26	r = 0.84*
Mucus			
	Control	-	r = 0.73*
	Short-term	-	-
	Long-term	-	r = 0.01

Asterisks (*) denote significant correlations ($p < 0.01$)

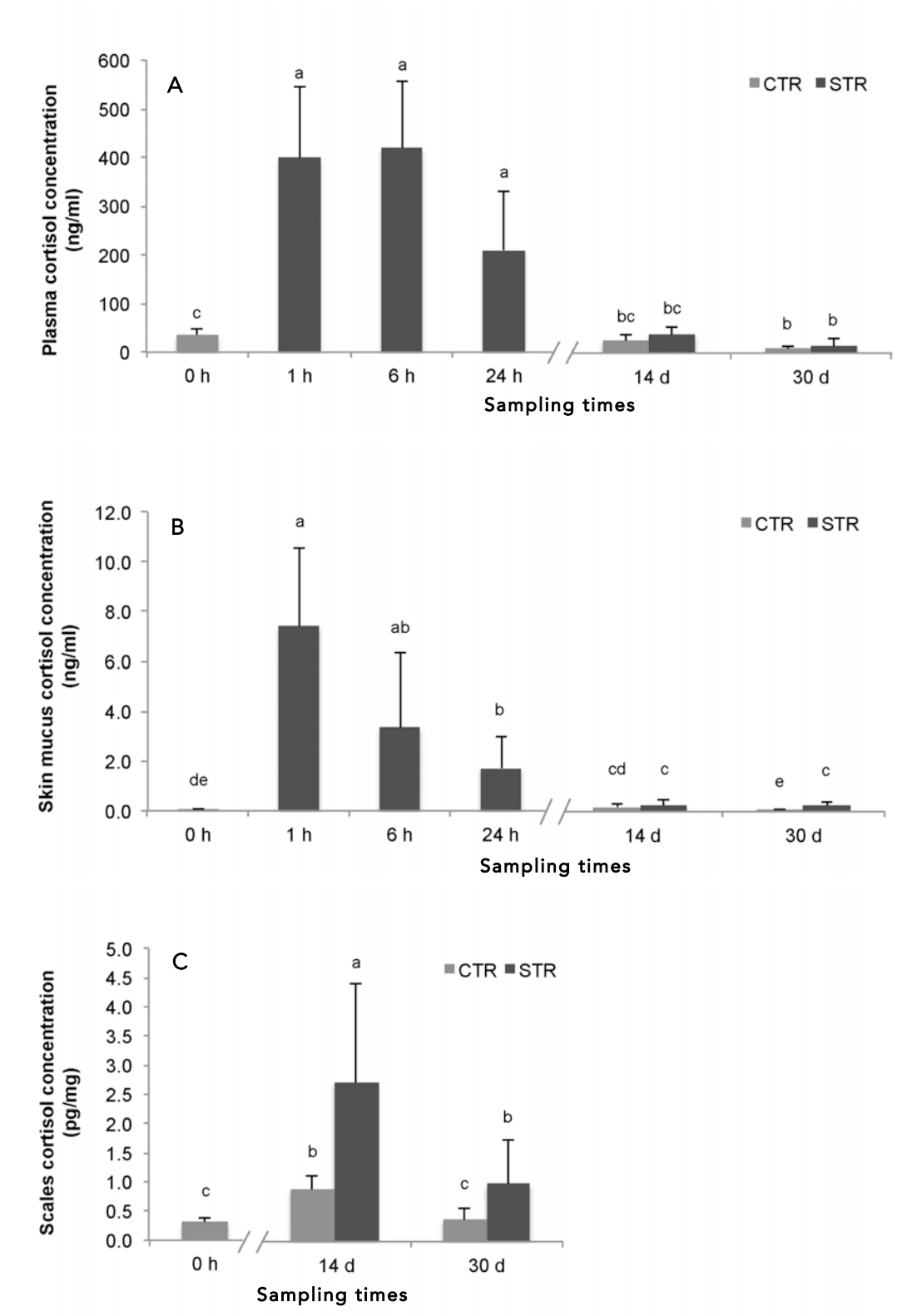

Figure 1. Cortisol levels in (A) plasma (ng/ml), (B) skin mucus (ng/ml) and (C) scales (pg/mg) in rainbow trout (*Oncorhynchus mykiss*). Cortisol concentrations were measured at different times (0 h or pre-stress levels, 1 h, 6 h, 24 h, 14 d and 30 d) in control (light grey) and stressed (dark grey) groups of fish throughout the 30-day experiment. Each bar represents the mean values (± SD). Different letters indicate significant differences ($p < 0.05$).

BIOCHEMICAL VALIDATION OF THE EIA

Results obtained in the EIA biochemical validation of each matrix studied are shown in Table 2. Serial dilutions of plasma, skin mucus and scale extracts displayed significant correlation coefficients between observed and expected values (for all three matrices; r = 0.99, $p < 0.01$). Results demonstrate that the EIA kit used has precision, specificity, accuracy and sensitivity detecting cortisol concentrations in the all the matrices evaluated.

Table 2. Biochemical validation of the EIA; results show the precision (intra and inter-assay coefficients of variation), specificity (dilution test), accuracy (spike-and-recovery test) and sensitivity of the assay.

Matrix	CV (%)		Dilution		Spike-recovery	Sensitivity (ng/ml)
	Intra-assay	Inter-assay	R^2 (%)	Mean error (%)	Mean recovery (± SD)	
Plasma	5.46	11.53	99.2	102.7 ± 2.7	115.0 ± 20.0	0.059
Mucus	2.79	8.85	98.2	114.2 ± 14.2	104.9 ± 19.7	0.042
Scales	5.01	19.24	98.5	104.8 ± 4.8	109.8 ± 7.4	0.039

CV, coefficient of variation; SD, standard deviation

DISCUSSION

Determination of plasma cortisol levels has been the traditional method used to assess the stress response in fish (Baker et al., 2013). However, due to its evident sampling limitations, especially in aquatic animals, other less invasive matrices are being recently evaluated (Ellis et al., 2013; Pottinger et al., 2016). Among them, skin mucus appears as a very useful tool to assess non-invasively the fish HPI axis activity. Given that MCC provides information about a narrow snapshot of an individual's endocrine status (De Mercado et al., 2018; Guardiola et al., 2016), the development of novel techniques to quantify long-term HPI axis activity in fish are indeed crucial. Recently, scales have been presented as a means to assess fish chronic stress (Aerts et al., 2015). In this direction the present study adds evidence and provides novel information about the usefulness of skin mucus and scales to assess the individual HPI axis activity in rainbow trout.

After placing fish into the confinement nets, a significant rise in PCC was detected, and circulating levels remained high up to 24 h after the start of the experiment. In response to an acute stressor, circulating cortisol levels in rainbow trout peak within 10 min to 1 h and return to basal within 8 h (De Mercado et al., 2018; Gesto et al., 2013; López-Patiño et al., 2014). While the later studies reported an increase of 2 to 4 times in blood cortisol values in response to acute stressors, in our study peak cortisol levels were about 11 times higher than pre-stress levels, probably given by the severity and the duration of the continuous stressor applied (Barton, 2002). Therefore, fish responded to the stressor with an initial peak of cortisol close to the maximum physiological acute response, but importantly, the time course activation of the HPI axis was similar to a chronic stress response. These results clearly suggest that rainbow trout triggered a prolonged stress response that was longer than the one commonly observed after applying an acute stressor in the same species.
After 14 days under stressful conditions, circulating cortisol levels from the stress group decreased to the point that PCC did not differ from the control group, neither at day 14 nor 30 of the study. This finding confirms that a single-point blood sample may not be a good assessment of the long-term stress experience in fish.

As expected, MCC exhibited a similar initial response pattern to PCC, confirming that cortisol fluctuations in skin mucus reflect those in blood. Most notably, these results further validate that increases in skin mucus cortisol levels relates to the individual short-term experience of stress in fish. Note that at 24 h, MCC decreased compared to the initial peak observed at 1 and 6h. Although this response is typically linked to the feedback effect exerted by cortisol itself (Barton, 2002; Moberg and Mench, 2000), blood levels remained elevated, thus the later hypothesis was ruled out. In addition, after long-term exposure to stressful conditions, the patterns of significances in MCC were inconsistent in relation to those detected in plasma. Chronically stressed fish presented higher MCC than pre-stress levels, and even at day 30, the stress group presented higher MCC than the 30-day control fish. We should emphasize that differences in MCC between control and stressed groups were much more evident in the short-term, concurring with the initial peak in circulating cortisol levels. Nevertheless, the inconsistency in the patterns of response between PCC and MCC deserves special attention. As several authors have demonstrated, different stressors can affect the structure and cellular composition of the fish epidermis (Tacchi et al., 2015; Vatsos et al., 2010), which in turn, can lead to physical and biological changes of the skin mucus (Elliott, 2000; Pérez-Sánchez et al., 2017; Shephard, 1994). Yet some of these changes have been observed as early as 5 h after subjecting fish to continuous stress (Tacchi et al., 2015). Our data, along with the studies mentioned, suggest that the severe generated stressful conditions could have modified the skin mucus production. It is therefore not surprising that correlation between PCC and MCC in long-term stressed fish was not detected, adding further evidence to the alleged mucus

alteration. Likewise, no correlation was detected between PCC and MCC in the control group. Conversely, a strong linear relationship between PCC and MCC became apparent when fish was responding to the stressor with an intense activation of the HPI axis. Therefore, these results show for the first time that circulating cortisol is better reflected quantitatively in skin mucus right after the HPI axis is activated, rather than in control conditions or after being subject to long-term stressful conditions. This relationship could be expected since correlation between cortisol levels in blood and saliva or milk, whose time lag in cortisol levels may be comparable to that between blood and skin mucus (De Mercado et al., 2018), is much grater following stimulation of the neuroendocrine stress axis compared to resting periods (Cook, 2012; Mormède et al., 2007). Accordingly, cortisol may diffuse to the skin mucus in proportion to the amount of circulating hormone when high amounts of cortisol are released in the bloodstream. Taken together these findings validate the measurement of cortisol in skin mucus as an innovative, non-invasive approach to assess acute stress in fish. In addition, these data also suggest caution in using baseline MCC measured in long-term stressed fish for chronic stress assessments, particularly in experimental models subjected to continuous and intense stressful conditions.

As demonstrated with the measurement of cortisol in skin mucus, showing that SCC correlate to PCC, represents a key step when testing the suitability of a method for assessment of the HPI axis activity (Cook, 2012). Nevertheless, this relationship has not yet been demonstrated. The present study verifies for the first time that cortisol content in scales is related to the hormone levels in matched plasma samples. Interestingly, we observed that SCC correlate better to the circulating cortisol concentrations of fish under chronic stress rather than in unstressed fish. Despite PCC from the stressed group decreased after 14 days compared to the initial acute stress response, levels in this matrix were sustained long enough to correlate with SCC on an individual basis. These findings reveal that SCC correlates to natural, endogenous cortisol fluctuations in individuals that have been subjected to stress over a certain period of time, therefore providing more evidence that SCC may integrate the duration of the stress responses.

In this study we also attempted to evaluate the correlation between cortisol levels in scales and skin mucus. Since the time lag in cortisol levels between blood and mucus is less than 10 minutes in rainbow trout (De Mercado et al., 2018) we expected to find a similar correspondence between matrices to that seen with PCC, but surprisingly, correlations presented switched results. While cortisol levels in skin mucus and scales were highly correlated in control groups, after experiencing chronic stress the correspondence between matrices disappeared. This later result reinforces the hypothesis of the potential alteration of the skin mucus production in long-term stressed fish. The high correlation observed in control fish is nonetheless, remarkable. As suggested by previous studies on mammal hair (Burnard et al.,

2017; Henderson, 1993; Skobowiat et al., 2011), different external or internal sources of cortisol content in scales, other than blood, should not be ruled out. Although this study was not specifically designed to investigate whether and how cortisol diffuses from blood to skin mucus or scales, it highlights the need to expand our knowledge on these issues in order to better interpret cortisol fluctuations in these matrices.

Furthermore, in order to test whether fish scales integrate the past HPI axis activity, SCC from the long-term stressed group were compared to those from control groups. We successfully detected a significant increase in SCC after 14 and 30 days under stressful conditions. These findings suggest that SCC are able to provide the recording of a stress experience in fish subjected to chronic continuous stress. Contrary to Aerts and colleagues (2016) who reported an increase in SCC from day 21 to day 42, we detected a decrease in SCC from day 14 to day 30 of the study. While those authors applied a daily acute stressor throughout the study, we generated an uninterrupted prolonged stress. Thus, as previous authors have described, the different type of stressor applied could potentially lead to the inconsistencies observed between studies (Dickens and Romero, 2013). These results, therefore, may suggest that cortisol in scales could not be metabolically inert. Many authors have addressed the question on whether calcified material in scales is stable, demonstrating that scales can be subject to biological, mechanical, and chemical erosion (Tzadik et al., 2017). Specifically, previous works demonstrated that under a prolonged physiological stress or extreme environmental conditions, fish can reabsorb minerals from the scales, modifying the previous content of the deposited materials (Metz et al., 2014; Mugiya and Watabe, 1977). The hypothesis that scales can give insight into long-term hormonal levels should be interpreted with caution, particularly in fish that has been under severe stressful conditions for prolonged periods. As observed here, we could deduce that SCC can inform about a chronic stress response up to 14 days, but the consequences on scale cortisol deposition in fish subjected to longer periods of stress needs further clarification.

The advantages of using skin mucus and scales over other samples are numerous. The main interest of analysing cortisol in skin mucus is probably given by its non-invasive nature compared to blood sampling. In addition, mucus samples are easily collected and the technique is fast to perform. Thus, obtaining baseline cortisol levels appears technically easier by using skin mucus than blood, although capture is also required in most cases. Likewise, scales samples can be collected non-lethally, and after, new scales will regenerate (Sire and Akimenko, 2004), being especially advantageous for the study of endangered or protected species. Additionally, as fish surface is totally covered by scales, different replicates can be achieved from the same specimen. Also, fish scales are easily collected with small tweezers, they can be stored frozen, and, eventually, the extraction and analysis of hormones can be simply performed, as it requires little specialized equipment. Here, we present a novel and easy-to-perform technique to clean, extract

and detect cortisol from fish scales (Carbajal et al., 2018). Note that scales were mechanically and automatically grinded with a ball mill in order to assure homogeneity of the powdered particles among samples. Previous studies on mammal hair demonstrated greater extraction efficiency when samples were mechanically grinded compared to minced with scissors (Davenport et al., 2006). Thus, the process followed here would also be advantageous when studying species with low circulating cortisol levels.

CONCLUSION

Overall, the current work provides a good basis for future research applying the analysis of cortisol either in skin mucus or scales for stress assessments in fish. This study demonstrates that cortisol levels in skin mucus reflect better the circulating cortisol concentrations when fish are responding to stress with an intense activation of the HPI axis, rather than in control or chronically stressed fish. Findings also verify the usefulness of skin mucus cortisol concentrations as a proxy measure for acute stress. We have shown that cortisol content in scales strongly correlates to endogenous circulating cortisol levels in long-term stressed fish, further suggesting that SCC may integrate the duration of the stress responses In addition, we provide evidence that the measurement of cortisol in scales can offer a long-term, integrated measure of the HPI axis activity in fish experiencing prolonged and continuous stress. In this context, we raise the question of whether the deposited cortisol is metabolically inert in scales. Further knowledge about the general robustness and stability of scale cortisol in fish subjected to prolonged stress or demanding events would largely help to strengthen the interpretation of hormone fluctuations in this matrix. We also encourage future studies assessing whether these matrices have additional sources of cortisol other than blood and the route of incorporation or diffusion.

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CHAPTER III

Measuring cortisol concentrations in fish skin mucus as a bioindicator of habitat quality

A. Carbajal[1], O. Tallo-Parra[2], P. Soler[3], D. Vinyoles[3], M. Lopez-Bejar[1]

[1] *Department of Animal Health and Anatomy, Veterinary Faculty, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

[2] *Department of Animal and Food Science, Veterinary Faculty, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

[3] *Department of Evolutive Biology, Ecology and Environmental Sciences, Universitat de Barcelona, Avinguda Diagonal 643, 08028, Barcelona, Spain*

ABSTRACT

Non-invasive collection techniques for hormone measurement have become increasingly used because they avoid many of the drawbacks inherent to blood sampling. In fish, skin mucus can be quickly collected with minimal restraint to minimize disturbance. Cortisol levels in this matrix have been shown to be good stress indicators in farm fish exposed to different acute stressors. Nevertheless, its applicability in free-ranging animals subject to long-term environmental stressors remains to be explored. Accordingly, the present study was designed to examine whether skin mucus cortisol levels could be reliably used as estimates of habitat quality in a wild freshwater fish (Catalan chub, *Squalius laietanus*). To better understand the cortisol fluctuations in this alternative non-invasive matrix, other physiological endpoints typically altered with exposure to pollutants were assessed in parallel. Fish inhabiting environments of different pollution gradient were sampled for blood and skin mucus. Several well-established haematological parameters and cortisol concentrations were measured in blood and compared to variations in skin mucus cortisol values across habitats. Levels of cortisol in blood and matched skin mucus samples were positively correlated suggesting that cortisol diffuses into the skin mucus in proportion to the amount of circulating hormone. Fluctuations of cortisol in skin mucus of Catalan chub varied across the streams of differing habitat quality following a similar pattern of response to that detected by the assessment of cortisol levels in blood and the haematological parameters. Furthermore, there was a close relationship between cortisol concentrations in skin mucus and several of the erythrocytic alterations and the relative proportion of neutrophils to lymphocytes. Taken together, results of this study provide first evidence that skin mucus cortisol levels could be potentially used as bioindicators of habitat quality in freshwater fish inhabiting polluted environments.

INTRODUCTION

Throughout their lifetime, wild fish faces many challenges of the aquatic environment that can impose considerable stress (Pankhurst, 2011). These challenges can be either natural or with an anthropogenic origin, and, depending on the magnitude and duration, they can cause acute or chronic stress responses (Schreck et al., 2016; Sheriff et al., 2011). Acute stress responses, such as those triggered by a predator attack or certain unpredictable weather conditions, can facilitate survival (Wingfield et al., 2011), whereas long-term stressors, like exposure to environmental pollution, are associated with a wide range of maladaptive effects (Scott and Sloman, 2004) that may, ultimately, led to loss of biodiversity (Dantzer et al., 2014; Hooper et al., 2012). Accordingly, understanding the causes and effects of environmental disturbances on fish physiology may help developing conservation strategies to enhance restoration and protect freshwater ecosystems (Jeffrey et al., 2015; Scott and Sloman, 2004).

Initially designed specifically for wastewater treatment, constructed wetlands are nowadays an important component of urban ecosystems since they play a crucial role in environmental pollution control (Babatunde et al., 2008; Brix et al., 1994). Constructed wetlands are macrophyte-based systems that remove pollutants through a combination of physical, chemical and biological processes (Brix et al., 1994). Wetlands' performances though, need to be periodically monitored (Guittonny-Philippe et al., 2014). The described methodologies for wetlands monitoring include physical and chemical techniques that provide information about the amount of pollutants present in the water. Nevertheless, these tools do not give insight into how living organisms cope with water contaminants (Guittonny-Philippe et al., 2014).
A cheap and practical option that can give substantial amount of information about the overall health status of individuals is the peripheral blood test (Johnstone et al., 2012; Maceda-Veiga et al., 2015). The analysis of red blood cells (RBCs) allows the detection of DNA damage and synthesis alterations by the assessment of erythrocytic nuclear abnormalities (ENA), circulating

micronuclei (MN), and senescent (SE) and immature (IE) erythrocytes (Colin et al., 2017; Hussain et al., 2018). The detection of RBCs abnormalities has actually been widely used as an indicator of exposure to genotoxic and mutagenic contaminants (Braham et al., 2017; Castaño et al., 2000; Ivanova et al., 2016). In parallel, relative white blood cell (WBC) count can be obtained, which offers a very common measure of stress and innate immune response (Davis et al., 2008). In particular, the relative proportion of neutrophils to lymphocytes has been successfully applied as a measure of prolonged pollutant exposure (Hedayati and Jahanbakhshi, 2012; Johnstone et al., 2012; Witeska, 2005). Other uses of blood samples in ecotoxicology include the quantification of glucocorticoid (GC) hormones to assess the cortisol stress response (Busch and Hayward, 2009; Homyack, 2010). Cortisol is the main GC in teleost fish secreted after the activation of the hypothalamic-pituitary-interrenal (HPI) axis in response to acute and chronic stress (Mommsen et al., 1999; Schreck et al., 2016). Analyses of cortisol levels in blood, and more recently, in whole-body and the surrounding water have been effectively used to monitor environmental stress responses (King et al., 2016; Norris et al., 1999; Pottinger et al., 2016). Blood, whole-body and the surrounding water sampling however, present clear limitations when being applied on wild population studies. First, blood collection is an invasive technique that the process by itself may provoke further stress. Whole-body cortisol analysis involves sacrifice of the specimens (King et al., 2016). And finally, collection of the holding water comprises wild fish retained in a bucket, otherwise the technique is difficult to be applied in the wild (Pottinger et al., 2016). Cortisol analysis in fish skin mucus has recently gained considerable attraction, especially because the sampling method is much less invasive compared to the aforementioned techniques (De Mercado et al., 2018; Guardiola et al., 2016; Simontacchi et al., 2008). Skin mucus cortisol levels have been shown to reflect circulating concentrations in several species of farm fish (Bertotto et al., 2010; Simontacchi et al., 2008), but there is yet no evidence of such a relationship in free-ranging species. As far as we are aware, this method has to date only been applied in strictly controlled environments. Additionally, increases in skin mucus cortisol levels have only been linked to the short-term stress triggered by transport (Bertotto et al., 2010) or by acute hypoxia (De Mercado et al., 2018; Guardiola et al., 2016).

Therefore, the present study was conducted with the general objective to examine whether skin mucus cortisol concentrations (MCC) from a wild freshwater fish residing in a wetland system could be reliably used to evaluate its habitat quality. It is well known that understanding changes in cortisol levels is not a simple process, especially when measuring cortisol in wild animals by using alternative samples others than blood (Dantzer et al., 2014; Johnstone et al., 2012). Given that the measurement of cortisol in skin mucus is a novel method, other physiological endpoints of the effects of pollution in fish were assessed to better interpret cortisol fluctuations wherein this matrix. Several haematological parameters (RBCs anomalies and altered WBCs counts)

were measured in parallel since they have already been successfully used as indicators of health condition in the species of interest (Colin et al., 2017), as well as in many others (reviewed above). Therefore, the first specific aim was to validate that those endpoints measured in Catalan chub (*Squalius laietanus*) accurately reflect their habitat quality. The second objective was to evaluate paralleling fluctuations in skin mucus cortisol to blood samples in relation to habitat quality. Furthermore, matched blood and skin mucus samples were correlated with the aim to test whether cortisol in skin mucus reflects blood hormone levels, and thus it can be potentially used as a reliable measure of the HPI axis activity in wild fish. The third specific objective was to contrast the patterns of response of all the endpoints assessed to skin mucus cortisol levels across the habitats of different pollution gradient.

MATERIALS & METHODS

STUDY AREA AND FIELD SAMPLING

Sampling areas were determined following the protocols from the European Committee for Standardization (CEN prEN 14011:2002). Fish were sampled at three different areas located in the Besòs River; two sites within the wetland system, each of which represents a different stage of biodegradation of water pollutants (P1 and P2), and a reference non-impacted upstream site located outside the wetlands that provides an optimal habitat for Catalan chub. In order to assess two contaminated habitats with different pollution gradient, P1 was placed at the beginning of the constructed wetland, and P2 was located at the end of the overall wetland system.

At the start of the study, several physico-chemical parameters and contaminants of emerging concern (CEC) were measured as a basis of the water quality from each sampling site (Table 1). Results of the water analyses provided evidence that the sampling sites classified as polluted presented features typically identified in disturbed environments (Colin et al., 2017; Maceda-Veiga et al., 2013; Stasinakis et al., 2012).

Samplings were performed from May 17th to June 15th 2017, once the constructed wetland system had been operative for 3 months (23st May, P1; 17th May, P2; 15th June, reference site). Unequal sample size was collected within the polluted sites probably due to sub-optimal habitat conditions in P1 (n = 6) compared to P2 (n = 17) or the reference site (n = 22). All procedures followed the national and institutional regulations of the Spanish Council for Scientific Research (CSIC) and the European Directive 2010/63/EU.

Table 1. Occurrence of contaminants of emerging concern (CEC) and physico-chemical data from river water samples collected within the wetland system (P1 and P2) and the reference non-impacted site located outside the system (Reference) during the study period

	Sites		
	P1	P2	Reference
CEC (µg/L)			
Volatil organic compounds			
Tetrachloroethene	<LOD	0.6	<LOD
Pesticides			
Simazine	0.13	0.13	<LOD
Diuron	<LOD	<LOD	<LOD
Isoproturon	0.04	0.04	<LOD
Pharmaceutical products			
Diclofenac	1.61	0.29	<LOD
Alkylphenols			
4-tert-octylphenol	0.025	<LOD	<LOD
Nonylphenol	0.14	<LOD	<LOD
PHYSICO-CHEMICAL DATA (mg/L)			
NH4+	12.6	10.6	0.07
NO3-	3.31	2.83	0.18
PO4-	0.8	1	0.4
TOC	9.25	6.39	2.16
COD	29.9	30.5	5.88
SS	7	9.5	0.5
Turbidity (NTU)	4.47	3.01	0.66

Concentrations of compounds under the instrumental detection limit (LOD, Limit of detection) are not included. TOC, Total organic carbon; COD, Chemical oxygen demand; SS, Suspended solids; NTU, Nephelometer turbidity units.

SAMPLE COLLECTION

Fish were sampled after applying the combined stressor of capture and a brief period of confinement given that stressed-induced cortisol levels offer a considerable understanding of the overall stress response (Romero, 2004). In this context, growing evidence suggest that circulating cortisol increases can be detected from as short as 1 – 2.5 min following exposure to stressors (Mommsen et al., 1999; Pankhurst, 2011). Accordingly, fish were caught using a

portable electrofishing unit (300 V), and confined in buckets of 20 L for 15 minutes approximately. Afterwards, specimens were anaesthetized with MS-222 and immediately after, blood and skin mucus were sampled. Blood was collected by caudal vein puncture with an insulin syringe. A drop of blood was smeared for haematological analyses, and the remaining fluid was left to clot at 4 ºC for 4 h. Samples were then centrifuged (1500 x g, 5 min) and plasma was collected and stored at -20 ºC. Skin mucus was collected following the method described by Schultz and colleagues (2005) with some modifications. Briefly, a polyurethane sponge was used to absorb the skin mucus by applying light pressure to the left and right flank as this method has been shown to be less stressful than using a spatula (Schultz et al., 2005). The sponge was then introduced into a cylinder of a syringe and compressed with the barrel to collect, into a centrifuge tube, the skin mucus. Afterwards, samples were centrifuged (2000 x g, 10 min) and the supernatant was stored at -20 ºC until analysis. Morphological variables, including length and weight were measured. Fulton's body condition factor was calculated according to the formula $K = 10^6 \cdot \text{body weight (g)} \cdot \text{total length (mm)}^{-3}$ (Goodbred et al., 2015).

HAEMATOLOGICAL ANALYSIS

Immediately after being collected, a drop of blood was placed on glass microscope slides, drawn across the surface and, once air-dried, slides were fixed in absolute methanol for 10 minutes. This procedure was run in duplicate for each specimen. Upon arrival in the laboratory, one of each duplicated slides was stained with Diff-Quick® to assess the frequency of abnormal RBCs and for the WBCs count. Out of 1000 RBCs of each individual slide were scored to calculate the frequency of ENA, SE and IE. The ENAs analysed were defined as lobed, kidney-shaped, fragmented and vacuolated nuclei following the directions of Pacheco and Santos (1996). The relative count of all types of WBCs (neutrophils, lymphocytes, monocytes, eosinophils and basophils) was performed out of 100 WBCs and following the directions of Tavares-Dias (2006). The neutrophil and lymphocyte count was used to calculate the relative proportion of neutrophils to lymphocytes (hereafter N:L ratio). The second duplicated slide of each individual was used to assess the number of micronucleus after performing an acridine orange staining. Out of 3000 RBCs of each slide were scored to calculate the frequency of MN.

CORTISOL EXTRACTION AND BIOCHEMICAL VALIDATION

To analyse cortisol levels from blood and skin mucus a commercial enzyme immunoassay (EIA) kit (Cortisol ELISA KIT; Neogen® Corporation, Ayr, UK) was used. The antibody' cross-reactivity with other steroids is as follows: prednisolone 47.4 %, cortisone 15.7 %, 11-deoxycortisol 15.0 %, prednisone 7.83 %, corticosterone 4.81 %, 6β-hydroxycortisol 1.37 %, 17-

hydroxyprogesterone 1.36 %, deoxycorticosterone 0.94 %. Steroids with a cross-reactivity < 0.06 % are not indicated.

Biochemical validation of the EIA was carried out following the methods described by Carbajal and colleagues (2018). Samples of plasma and skin mucus extracts from several individuals were first pooled and used in each validation test. Precision was evaluated with the intra-assay coefficient of variation (CV), calculated from all duplicated samples analysed. The dilution test was applied to assess the specificity of the EIA kit by comparing observed and theoretical values from pools diluted with EIA buffer. To test the assay's accuracy the spike-and-recovery test was used, where known volumes of pools were mixed with different volumes and concentrations of pure standard cortisol solution. Finally, we evaluated the sensitivity of the test, given by the smallest amount of cortisol concentration detected.

STATISTICAL ANALYSIS

The computer program R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) was used to analyse the data. All values are presented as mean ± SD, and $p < 0.05$ was considered statistically significant. Normality of the data waassessed using Shapiro-Wilk tests, and parametric and non-parametric tests were applied accordingly. Differences in cortisol levels and haematological data between sites were assessed using one-way ANOVA with Tukey's pairwise post hoc tests. Non-normally distributed data were assessed by using Kruskal-Wallis test, followed by a multiple comparison test. Body condition was introduced as a covariate in the models to test if the energy accumulated in the body could potentially influence changes in blood measures between sites. The covariate was subsequently deleted due to the lack of significance. Pearson and Spearman correlation tests were applied to test for correlations between skin mucus cortisol levels to levels of the hormone in blood and the haematological variables.

RESULTS

BIOCHEMICAL VALIDATION OF THE EIA

Plasma and skin mucus intra-assay CV was 8.8 % and 7.7 % respectively. The dilution test obtained for plasma showed an R^2 = 98.4 % and a mean percentage error of 104.1 ± 4.1%. In the skin mucus dilution test, an R^2 = 99.7 % and a mean percentage error of 108.7 ± 8.7% was obtained. Also, in the dilution test, obtained and theoretical concentrations of plasma and skin mucus extracts showed significant correlation coefficients (r = 0.99, $p < 0.01$). In the spike-and-

recovery test, the average of the recovery percentage was 107.6 ± 10.0 % for plasma and 109.6 ± 9.1 % for skin mucus. The sensitivity of the assay for plasma and skin mucus assessment was 0.07 ng cortisol/ml and 0.03 ng cortisol/ml respectively. The biochemical validation of the EIA showed reliable results that demonstrated the assay's precision, specificity, accuracy and sensitivity in measuring plasma and skin mucus cortisol levels of Catalan chub.

HAEMATOLOGICAL PARAMETERS AND CORTISOL LEVELS

Significant differences in plasma cortisol concentrations (PCC), mucus cortisol concentrations (MCC), RBCs alterations and WBCs counts were detected between polluted and reference sites (Table 2). Correlations between MCC, PCC and the haematological parameters are shown in Table 3. Significant relationships were identified between MCC and PCC, IE, ENA and N:L ratio.

Table 2. Mean values and standard deviation of cortisol levels (ng/ml), red (%) and white (‰) blood cell parameters determined in Catalan chub from polluted (P1 and P2) and reference sites in Ripoll River. Different letters indicate significant differences among sites ($p < 0.05$)

	Sites		
	P1	P2	Reference
Cortisol			
PCC	711.01 ± 228.41^{a}	566.75 ± 193.74^{a}	363.89 ± 123.91^{b}
MCC	46.07 ± 19.54^{a}	42.95 ± 23.88^{a}	25.36 ± 9.11^{b}
Red blood cells			
IE	74.50 ± 5.45^{ab}	85.56 ± 9.29^{a}	68.19 ± 10.23^{b}
SE	11.00 ± 1.83^{ab}	15.07 ± 6.09^{a}	10.25 ± 2.65^{b}
ENA	50.83 ± 22.66^{a}	58.94 ± 25.24^{a}	28.59 ± 12.90^{b}
MN	0.17 ± 0.18^{a}	1.37 ± 1.58^{b}	0.62 ± 0.66^{ab}
White blood cells			
N:L ratio	10.54 ± 4.49^{a}	8.00 ± 3.46^{a}	4.79 ± 2.91^{b}
Monocytes	4.50 ± 3.67^{a}	7.47 ± 3.83^{a}	6.05 ± 2.82^{a}
Eosinophils	0.67 ± 0.82^{a}	1.35 ± 1.17^{a}	1.18 ± 1.30^{a}
Basophils	0.33 ± 0.52^{ab}	1.24 ± 1.20^{a}	0.36 ± 0.58^{b}

PCC, plasma cortisol concentrations; MCC, mucus cortisol concentrations; IE, immature erythrocytes; SE, senescent erythrocytes, ENA, erythrocytic nuclear abnormalities; MN, micronucleus; N:L ratio, relative proportion of neutrophils to lymphocytes

Table 3. Correlation (r) and *p*-value between haematological variables and skin mucus cortisol concentrations (MCC)

Variable	MCC	*p-value*
Cortisol		
PCC	0.55	**< 0.01**
Red blood cells		
IE	0.40	**0.03**
SE	0.23	0.24
ENA	0.41	**0.02**
MN	- 0.001	0.99
White blood cells		
N:L ratio	0.34	**0.05**
Monocytes	- 0.01	0.97
Eosinophils	- 0.21	0.20
Basophils	0.24	0.17

PCC, plasma cortisol concentration; IE, immature erythrocytes; SE, senescent erythrocytes; ENA, erythrocytic nuclear abnormalities; MN, micronucleus; N:L ratio, relative proportion of neutrophils to lymphocytes

DISCUSSION

In this study, we first successfully validated that several physiological endpoints typically used as indicators of exposure to pollutants (abnormal RBCs and altered WBCs counts), were accurately related to the habitat quality in Catalan chub. Cortisol levels in blood were also compared between habitats and they were correlated to skin mucus cortisol concentrations. Finally, we contrasted the patterns of response of all the endpoints assessed to skin mucus cortisol levels across the habitats of different pollution gradient to evaluate the potential of this non-invasive tool to assess habitat quality.

ABNORMAL RBC FREQUENCIES

There was no consistent pattern in abnormal RBC frequencies when they were compared between the polluted and the reference habitats. As confirmed by the physico-chemical and CEC analysis, the site P1, located at the beginning of the wetlands system, presented slightly worse habitat conditions compared to the site P2, placed at the end of the same system.

Accordingly, we expected to identify further RBCs alterations in P1 than in P2. Nevertheless, relative to the reference site, P1 only exhibited significantly higher frequencies of ENA, while fish from P2 presented greater IE, SE and ENA frequencies. As mentioned earlier, the sub-optimal conditions of P1 was likely the cause of the small sample size collected in the site, which in turn, could have limited the statistical power in detecting potential differences.

Conversely, both polluted P1 and P2 sites showed clearly higher ENA levels than the reference habitat. Particularly on spring, greater frequencies of nuclear abnormalities in the same cyprinid species have already been identified (Colin et al., 2017). The ENA test has been demonstrated to be a highly sensitive parameter for pollution assessment (Hussain et al., 2018; Maceda-Veiga et al., 2010), probably explaining the clear variations detected in these nuclear abnormalities between habitats.

Besides the inconsistencies found between both polluted sites, higher frequencies of RBCs disorders detected in the polluted habitat with larger sample size further support the idea that IE, SE and ENA tests are reliable biomarkers of habitat quality in Catalan chub (Colin et al., 2016; Pacheco and Santos, 2002).

Although these commonly noted abnormalities are highly sensitive to pollution, they are not as widely accepted as the use of MN tests (Mussali-Galante et al., 2013; Pacheco and Santos, 2002). The influence of river status on the frequency of MN has been previously studied in Catalan chub, and, in accordance with our results, no changes were detected between degraded and reference streams (Colin et al., 2017). Nevertheless, in our study, the two polluted areas evaluated differed in MN levels between them, with the highest values detected at the end of the wetland system. Although this result could also be given by the different sample size between study sites, it should be noted that a different contaminant profile can also result in mismatch between habitats (Braham et al., 2017). For example, exposure to atrazine and ametrine herbicides resulted in increased MN (Botelho et al., 2015), in contrast, a different herbicide, pendimethalin, showed increased ENA but not MN (Ahmad and Ahmad, 2016). Tetrachloroethene, the only CEC analysed that presented higher concentrations in P2 than P1, is a dry-cleaning compound widely used in the textile industry known to have toxic effects in fish (Spencer et al., 2002; Wang et al., 2007). Although evidence in humans supports the link between the MN formation and this compound (Augusto et al., 1997), to the authors' knowledge there are no published studies demonstrating this association in fish. Despite that, other compounds not specifically analysed in this study could also be the consequence of the differing results between polluted habitats.

Taken together, these findings suggest that the detection of RBCs disorders can be potentially used to identify low-quality habitats in Catalan chub, while being an approach that could contribute to a better understanding of the species' health status than the MN test.

VARIATIONS IN WBC COUNTS

Characteristic changes in blood leukocyte counts have been generally linked to the continuous activation of the HPI axis (Gil Barcellos et al., 2004; Grzelak et al., 2017; Schreck et al., 2016; Wendelaar Bonga, 1997). Interestingly, prolonged exposure to environmental contaminants can cause neutrophilia and/or lymphopenia in fish (Davis et al., 2008; Hedayati and Jahanbakhshi, 2012; Witeska, 2005) likewise in other taxa (Johnstone et al., 2012; Letcher et al., 2010). In line with these published reports, different WBC counts were detected between the study sites. Most notably, the N:L ratio was significantly higher in both polluted sites compared to the reference stream, probably as a consequence of the sub-optimal environmental conditions in those habitats. The number of basophils, a cell type still not assessed in this species, was also higher in P2 compared to the reference site. Although the function of this cell type is poorly understood, probably because its occurrence in teleost fish seems to be very rare (Tavares-Dias, 2006a, 2006b), basophils have been related to acute inflammation processes (Davis et al., 2008). Besides that, neither the monocyte nor the eosinophil count appeared to differ between sites, similar to earlier findings on silver carp (*Hypophthalmichthys molitrix*) in response to pesticides (Hedayati and Hassan Nataj Niazie, 2015). The assessment of these cell types is not common in contemporary research, perhaps due to the controversy concerning the effect of stress on eosinophil and monocyte numbers (Davis et al., 2008; Schreck et al., 2016). When only WBC data is available, evaluation of these two leukocyte types can help distinguish stress from infectious responses (Davis et al., 2008), thus further research on monocyte and eosinophil changes is strongly encouraged.

CHANGES IN CORTISOL LEVELS

Cortisol levels detected in plasma and skin mucus within the same individuals displayed a close linear relationship, suggesting that cortisol diffuses to the skin mucus in proportion to the amount of circulating hormone. Validation of alternative matrices for HPI axis activity assessment should prove that hormone concentrations in these media are proportional to their abundance in the bloodstream (Cook, 2012; Sheriff et al., 2011), as the present study demonstrates for the measurement of cortisol in skin mucus. These results, therefore, increase the applicability of the method as a sensitive-individual measure of the HPI axis activity in wild freshwater fish within their natural environment.

In addition, both plasma and skin mucus cortisol levels differed significantly between habitats of different quality, with the highest hormone values observed in the polluted sites assessed. This association between cortisol concentrations and habitat quality suggests that variation in the HPI axis activity is likely to be related to the presence of environmental disturbances. Greater stress responses attributed to the effects of pollutants have been reported in several fish species (King et al., 2016; Knag and Taugbøl, 2013; Pottinger et al., 2016), as well as in other taxa

(Baos et al., 2006; Strong et al., 2015; Wikelski et al., 2002). Nevertheless, it is important to note that chronic exposure to certain aquatic contaminants can also have suppressive effects on the stress axis (Blevins et al., 2013; Gesto et al., 2008; Oliveira et al., 2011; Pottinger et al., 2013). In this context, investigating the toxic mechanisms underlying variation in the HPI axis alteration will be particularly informative.

INTEGRATED ASSESSMENT

Interpreting cortisol fluctuations in free-living vertebrates is certainly a complex practice, particularly when applying alternative matrices for hormone assessment (Dantzer et al., 2014; Johnstone et al., 2012). This is why linking cortisol levels to other endpoints of the stress responses can significantly enhance current understanding on the ecology of stress (Boonstra, 2013).

In the present study, fluctuations in skin mucus cortisol levels between habitats paralleled those detected in blood, the traditional matrix used for hormone assessments in fish (Baker et al., 2013). Relative to habitat quality, changes of the hormone in skin mucus also coincided with variations in the haematological parameters, except for MN levels. Furthermore, the amount of cortisol in skin mucus was directly proportional to frequencies of abnormal erythrocytes (IE and ENA) and to the well-established stress index N:L ratio. Red blood cells are highly sensitive to landscape disturbances (Houston, 1997) and more specifically, to environmental pollution (Farag and Alagawany, 2018; Nikinmaa, 1992). Accordingly, the measurement of abnormal erythrocytes has been successfully used to assess the health status of Catalan chub (Colin et al., 2017; Maceda-Veiga et al., 2013) and many other fish species (Braham et al., 2017; Hedayati and Jahanbakhshi, 2012; Hussain et al., 2018). In the same context, WBC counts, particularly the N:L ratio, increases in individuals exposed to heavy metals and other contaminants proportional, indeed, to the circulating cortisol levels (Davis et al., 2008). Given the very clear effect of pollution on leukocyte and erythrocyte profiles, the strong linkages detected in this study provide new evidence that the measurement of cortisol in skin mucus could be used as a potential bioindicator of habitat quality in freshwater fish residing polluted environments.

The demonstrated sensitivity of the methods evaluated to different pollution gradients could be exploited for biomonitoring the wetlands systems' performances. We should keep in mind that inter-disciplinary approaches using multiple indicators will provide a better insight about the overall habitat quality (Cooke et al., 2013; Dantzer et al., 2016; Romero et al., 2015). Despite this, combining several techniques may not always be possible, thus we should select the method with best field applicability. The collection of skin mucus over blood offers at least two important practical advantages: the technique is far less invasive and easier to perform. In addition, cortisol assessment by EIA is a relatively simple analysis based on an objective

appraisal, whereas estimation of RBCs and WBCs are usually long-lasting and laborious methodologies susceptible of the subjectivism of cell identification (Castaño et al., 2000; Maceda-Veiga et al., 2015).

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CHAPTER IV

Variation in scale cortisol concentrations of a wild freshwater fish: habitat quality or seasonal influences?

A. Carbajal[1], O. Tallo-Parra[1], L. Monclús[1], D. Vinyoles[2], M. Solé[3], S. Lacorte[4], M. Lopez-Bejar[1]

[1] *Department of Animal Health and Anatomy, Veterinary Faculty, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

[2] *Department of Evolutive Biology, Ecology and Environmental Sciences, Universitat de Barcelona, Avinguda Diagonal 643, 08028, Barcelona, Spain*

[3] *Institut de Ciències del Mar (ICM-CSIC), Pg. Marítim de la Barceloneta 37–49, 08003, Barcelona, Spain*

[4] *Department of Environmental Chemistry, IDAEA-CSIC, c/Jordi Girona 18, 08034, Barcelona, Spain*

ABSTRACT

A significant body of literature suggests that aquatic pollutants can interfere with the physiological function of the fish hypothalamic-pituitary-interrenal (HPI) axis, and eventually impair the ability to cope with subsequent stressors. For this reason, development of accurate techniques to assess fish stress responses have become of growing interest. Fish scales have been recently recognized as a biomaterial that accumulates cortisol, hence it can be potentially used to assess chronic stress in laboratory conditions. We, therefore, aimed to evaluate the applicability of this novel method for cortisol assessment in fish within their natural environment. Catalan chub (*Squalius laietanus*) were sampled from two sites; a highly polluted and a less polluted (reference) site, in order to examine if habitat quality could potentially influence the cortisol deposition in scales. We also evaluated the seasonal variation in scale cortisol levels by sampling fish at three different time points during spring-summer 2014. In each sampling, blood was collected to complement the information provided by the scales. Our results demonstrate that blood and scale cortisol levels are significantly correlated, therefore increasing the applicability of the method as a sensitive-individual measure of fish HPI axis activity. Scale cortisol concentrations were unaffected by habitat quality although fish from the polluted environment presented lower circulating cortisol levels. We detected a seasonal increase in scale cortisol values concurring with a potentially stressful period for the species, supporting the idea that the analysis of cortisol in scales reveals changes in the HPI axis activity. Taken together, the present study suggests that cortisol levels in scales are more likely to be influenced by mid-term, intense energetically demanding periods rather than by long-term stressors. Measurement of cortisol in fish scales can open the possibility to study novel spatio-temporal contexts of interest, yet further research is required to better understand its biological relevance.

INTRODUCTION

The analysis of circulating cortisol, the main glucocorticoid (GC) in teleost released after the activation of the hypothalamic-pituitary-interrenal (HPI) axis, has been by far the most common method used in stress response assessments (Mommsen et al., 1999; Schreck et al., 2016). Although acute stress responses, such as pursuit by predation or severe storms, are imperative for fish homeostasis and survival, chronic stressors can negatively affect fish growth, reproduction and the immune system (Moberg and Mench, 2000; Pankhurst, 2011). The difficulty of obtaining baseline blood samples in wildlife, and the growing interest of conservation physiology in assessing chronic increases of cortisol (Dantzer et al., 2014), makes imperative the development of novel techniques to quantify fish HPI axis activity. In this direction, fish scales have been recently recognized as a biomaterial that accumulates cortisol (Aerts et al., 2015; Carbajal et al., 2018). As scales grow during the entire life of the fish (Elliott, 2000), cortisol measurements in this mineralized tissue could potentially integrate a longer period than any other tissue available. Despite the use of fish scales is promising as it provides integrated measures of cortisol, this method is not yet fully validated. To date, only one study has demonstrated the usefulness of scales as an indicator of long-term HPI axis activity in fish subjected to laboratory conditions (Aerts et al., 2015). Although these authors verified the biological relevance of scale cortisol levels, whether hormone concentrations in this media are proportional to their abundance in the bloodstream still remains to be explored. Establishing the relationship between scales and blood cortisol levels is crucial to increase the applicability of this novel method as a sensitive-individual measure of fish HPI axis activity (Cook, 2012; Sheriff et al., 2011). In addition, this integrative technique has only been tested in farmed fish held under captivity. Nevertheless, given the structural characteristics of this matrix, the assessment of cortisol in fish scales is likely to present a promising applicability in natural environments.

Decline of wild fish populations, particularly those from freshwater systems, has been partly exacerbated by pollution (Ismail et al., 2017). Long-term exposure to pollutants, such as metals,

pesticides, and other organics, can cause the chronic activation of the HPI axis, which as mentioned, can have detrimental consequences on fish performance (Mommsen et al., 1999; Scott and Sloman, 2004). Many researchers have explored the effects of environmental contaminants on the fish stress response, either measuring cortisol in blood (Hontela et al., 1992; Jorgensen et al., 2017; Miller et al., 2009) the surrounding water (Pottinger et al., 2016) or using whole-body homogenates (Belanger et al., 2016; King et al., 2016; Pottinger et al., 2013). Besides this, measurement of cortisol in scales could be a better option when an integrated measure of the HPI axis activity over longer periods is needed to enhance the "snapshot" of cortisol measurement.

When designing an experiment, several factors must to be considered in order to yield valuable, biologically relevant results (Johnstone et al., 2012; Killen et al., 2016; Schreck et al., 2016), and this is especially important when new matrices for endocrine assessment are being developed (Cook, 2012; Sheriff et al., 2011). In this context, a considerable amount of research has reported seasonal variation on cortisol levels (Belanger et al., 2016; Madliger and Love, 2014; Palme, 2005). Given that the assessment of cortisol in fish scales is a recent contribution (Aerts et al., 2015; Carbajal et al., 2018), it is crucial to understand the potential seasonal variation in scale cortisol concentrations (SCC) before using this method as an indicator of HPI axis activity in wild specimens.

We therefore aimed to evaluate whether the quantification of cortisol in scales reflects the HPI axis activity by individually comparing blood and scale cortisol levels in specimens of Catalan chub (*Squalius laietanus*). Furthermore, the influence of long-term environmental pollution in the cortisol content in fish scales was explored. We hypothesized that fish from a polluted habitat would present lower SCC compared to fish from a less polluted habitat because of the long-term inhibitory effects of certain pollutants on the HPI axis activity (Gesto et al., 2008; Hontela et al., 1992; Leblond et al., 2001; Norris et al., 1999). Additionally, we evaluated whether seasonality could influence SCC. For that, fish were sampled at the beginning-spring, middle-spring and beginning-summer concurring with a potentially stressful period for this cyprinid (Colin et al., 2017).

MATERIALS & METHODS

STUDY AREA

Fish sampling was carried out in the Ripoll River (Besòs basin) located in the north-east of Spain. The polluted habitat (2°06′01.40″E 41°34′17.88″N) is located immediately after an industrial plant, strongly affected by the industry and urbanization. The less polluted site,

henceforth referred to as the reference site (2°03'24.07E" 41°38'45.05N"), was located on the same river but 2.7 km upstream from the highly degraded habitat. Analysis of physico-chemical water parameters and chemical analysis of per- and polyfluoroalkyl substances (PFAS) in the muscle of the fish collected at the two sites were performed in order to certify that the selected habitats were correctly classified according to their pollution gradient: high pollution (henceforth referred to as the "polluted habitat") and low pollution (henceforth referred to as the "reference habitat") habitats.

SAMPLING TIMES

In order to study whether habitat quality influences SCC without accounting for seasonal variation, fish were sampled at the beginning of spring 2014 from the reference (25/03/2014; n = 7) and from the polluted habitat (21/03/2014; n = 17). To examine the seasonal influence on SCC, three consecutive sampling efforts were carried out in the reference habitat in spring-summer 2014. Samplings were performed in the early spring (25/03/2014; n = 7), middle spring (08/04/2014; n = 8) and early summer (16/07/2014; n = 17).

In each sampling, blood was also collected in order to complement the information provided by the scales with the "snapshot" of blood cortisol measurement. We evaluated circulating cortisol levels after a period of confinement since stressed-induced cortisol concentrations are known to provide a better understanding of the overall stress response (Romero, 2004). Stress-induced cortisol increases have been detected from as short as 2.5 min to as long as 120-min in different fish species (Mommsen et al., 1999; Pankhurst, 2011). Accordingly, fish were caught with a portable electrofishing unit (300 V) and kept in tanks with the local river water for about 1 h in order to trigger a stress response by capture and confinement. Fish were then euthanized with an overdose of MS-222, and immediately after, blood and whole body scales were collected. A portion of muscle was sampled for chemical analysis of PFAS. Sex, body weight, gonad weight and total length were recorded during post-mortem examinations. Fulton's condition factor, considered to reflect an individual's energetic state (Barton et al., 1998) was calculated according to the formula, $K = 10^6 \cdot \text{body weight (g)} \cdot \text{total length (mm)}^{-3}$ (Goodbred et al., 2015). The reproductive stage of each individual was given by the gonadosomatic index, a broadly used indicator of reproductive periods (Brewer et al., 2008), which was calculated with the formula, $\text{GSI} = 100 \cdot \text{gonad weight (g)} \cdot \text{body weight (g)}^{-1}$ (Goodbred et al., 2015).

PHYSICO-CHEMICAL WATER PARAMETERS

In the physico-chemical analysis, altered water parameters were observed in the polluted habitat compared to the reference site (Table 1). These values provide evidence that the habitat classified as polluted exhibits features commonly observed in disturbed ecosystems (Colin et al., 2017; Maceda-Veiga et al., 2013; Stasinakis et al., 2012).

Table 1. Physico-chemical water parameters from polluted and reference habitats analyzed on spring and summer 2014

	Spring		Summer	
	Reference	Polluted	Reference	Polluted
	25/3/14	21/3/14	16/7/14	15/7/15
Flow (L/s)	239.5	241.2	28.4	124.0
Temperature (°C)	14.2	16.9	19.0	23.0
Oxygen (mg/L)	7.2	6.2	2.7	8.0
Conductivity (µS/cm)	728	3680	709	4777
pH	8.1	8.3	7.1	8.3
NH_3 (mg/L)	0.04	0.40	0.04	5.30
NO_2 (mg/L)	0.01	0.90	0.01	5.51
NO_3 (mg/L)	0.13	19.6	0.06	10.6
PO_4 (mg/L)	0.1	1.0	0.1	0.8
SO_4 (mg/L)	15.8	414.1	17.9	464.0
Cl (mg/L)	40.0	987	31.9	1088

PER- AND POLYFLUOROALKYL SUBSTANCES

Seven PFAS were detected in muscle tissue by UPLC; perfluorononanoic acid (PFNA), perfluorooctane sulfonic acid (PFOS), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorodecane sulfonic acid (PFDS), perfluorododecanoic acid (PFDoA) and perfluorotridecanoic acid (PFTriDA). Information regarding the chemicals and reagents, sample extraction and analysis technique applied is provided in the Supplementary material. The analysis of PFAS confirmed that, either on spring and summer, fish from the polluted habitat presented higher bioaccumulation of PFAS than the reference site (Table 2), further verifying that both habitats had been properly classified according to their habitat quality characteristics.

CORTISOL EXTRACTION

Blood - Blood was collected by puncture of the caudal vein with an insulin syringe. After clotting for 4 h, samples were centrifuged at 1500 x g for 5 min at 4 ºC and the plasma collected was stored at -20 ºC until analysis.

Scales - Whole body scales were removed with a small scalpel. Extraction of cortisol from scales was performed following the procedure described by Carbajal et al., (2018). Briefly, scales were washed three times with isopropanol and, once dry, they were minced with a ball mill (Retsch,

MM2 type, Germany). The powdered sample was incubated in methanol for 18h. Following extraction, samples were centrifuged and the supernatant was evaporated. Dried extracts were reconstituted with enzyme immunoassay (EIA) extraction buffer and immediately stored at −20 °C until analysis. Not enough scale sample mass could be collected from some specimens (reference n = 8; polluted n = 6) due to their small body size, consequently, from these individuals only cortisol from plasma was analysed.

Table 2. Per- and polyfluoroalkyl substances detected in muscle tissue by UPLC from individuals inhabiting polluted and reference habitats analysed on spring and summer 2014

	Spring		Summer	
	Reference	Polluted	Reference	Polluted
	25/3/14	21/3/14	16/7/14	15/7/15
PFNA	0.06	0.52	0.13	0.27
PFOS	2.32	13.9	2.27	8.07
PFDA	0	13.2	0	9.12
PFUnA	0.77	18.2	0.55	13.9
PFDS	0.01	0.06	0	0.06
PFDoA	0.96	24.5	0.6	43.4
PFTriDA	0.51	47.1	0.32	49.9
Total	**4.63**	**117.48**	**3.87**	**124.72**

PFNA, perfluorononanoic acid; PFOS, perfluorooctane sulfonic acid; PFDA, perfluorodecanoic acid; PFUnA, perfluoroundecanoic acid; PFDS, perfluorodecane sulfonic acid; PFDoA, perfluorododecanoic acid; PFTriDA, perfluorotridecanoic acid

CORTISOL ANALYSIS AND VALIDATION TESTS

Cortisol concentrations from plasma and scales were measured by enzyme immunoassay (Cortisol EIA KIT; Neogen® Corporation, Ayr, UK) . Biochemical validation was conducted using methods previously described for cortisol analysis in scales of goldfish (*Carassius auratus*) by EIA (Carbajal et al., 2018). Intra-assay coefficient of variation (CV) from all duplicated samples analysed was calculated for precision assessment. The specificity was evaluated with the linearity of dilution. Accuracy was assessed through the spike-and-recovery test. And the sensitivity of the test was given by the smallest amount of hormone concentration analysed.

STATISTICAL ANALYSIS

The computer program R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) was used to analyse the data. A $p < 0.05$ was considered statistically significant. Shapiro-Wilk tests were used to test for normality of data, and log-transformed when appropriate.

Pearson's correlation coefficients (r) were used to test the relationship between SCC and plasma cortisol concentrations (PCC) in the two sites separately. We explored whether season and habitat quality could potentially influence PCC and SCC using linear regression models with sex, *K* and GSI as covariates. Due to lack of influence, covariates were omitted from the models. We used Tukey post-hoc tests to distinguish the seasonal variations in SCC. We assessed seasonal and habitat differences in *K*, GSI and sex by applying ANOVAs and Student's t-test for quantitative variables, and chi-squared for sex. Additionally, sex differences in PCC and SCC were analysed with a Student's t-test.

In the biochemical validation, Pearson's correlation was used to evaluate the correlation between obtained and expected values from serial dilutions. The same statistical test was applied to calculate the relationship of the parallelism between cortisol standards and the serially diluted pool extract.

RESULTS

GENDER AND MORPHOLOGICAL VARIABLES

The morphological variables and gender distribution of Catalan chub in both assessments (habitat quality and seasonal variability) are shown in Table 3. Significant differences in *K* between individuals from the reference and polluted habitats were detected ($p < 0.01$). A seasonal change in *K* was also observed with a significantly higher values detected on the early summer ($p < 0.01$).

BIOCHEMICAL VALIDATION OF THE EIA

The sensitivity of the assay was 0.07 ng cortisol/ml for plasma and 0.08 ng cortisol/ml for scales extracts. Intra-assay CV for plasma and scales samples was 8.80 % and 6.60 % respectively. In the dilution test, obtained and expected cortisol concentrations were significantly correlated both in plasma and scales ($r = 0.99$, $p < 0.01$). The average of the recovery percentage from spike-and-recovery test was 107.6 ± 10.0 % (mean ± SD) for plasma and 101.1 ± 4.8 % (mean ± SD) for scales validation.

Table 3. Gender distribution (n (%)) and values of K and GSI (mean ± SD) of individuals sampled from reference and degraded habitats (habitat quality) and individuals sampled during the early spring, middle spring and early summer (seasonal variability). Different letters indicate statistical difference between sites (habitat quality) and among sampling efforts (seasonal influence) ($p < 0.01$).

Variable	Habitat quality		Seasonal influence		
	Reference	Polluted	Early spring	Middle spring	Early summer
Sex (males)	5 (62.5 %)[a]	10 (52.6 %)[a]	5 (62.5%)[a]	5 (62.5%)[a]	13 (65.0%)[a]
K	1.02 ± 0.05[a]	1.12 ± 0.08[b]	1.02 ± 0.05[a]	1.01 ± 0.06[a]	1.26 ± 0.11[b]
GSI	3.76 ± 2.17[a]	3.27 ± 1.98[a]	3.76 ± 2.17[a]	4.47 ± 2.30[a]	5.36 ± 2.76[a]

K, Fulton's condition factor; GSI, gonadosomatic index

CORTISOL LEVELS

Fish from the reference site displayed significant correlation between SCC and PCC ($r = 0.41$, $p = 0.04$), however, such correlation was not significant in fish inhabiting the polluted habitat ($r = 0.44$, $p = 0.20$). Comparison between habitats revealed that the degraded site presented significantly lower PCC levels ($p = 0.02$; Fig. 1A), but no differences on SCC between sites were detected ($p = 0.56$; Fig. 1B). Seasonal differences were detected on SCC ($p = 0.01$; Fig. 2B), although PCC remained constant ($p = 0.90$; Fig. 2A). Post-hoc tests revealed that in early summer, SCC were significantly higher compared to levels detected in early ($p = 0.04$) and middle spring ($p = 0.03$). Gender differences in PCC ($p = 0.09$) and SCC were not detected ($p = 0.76$).

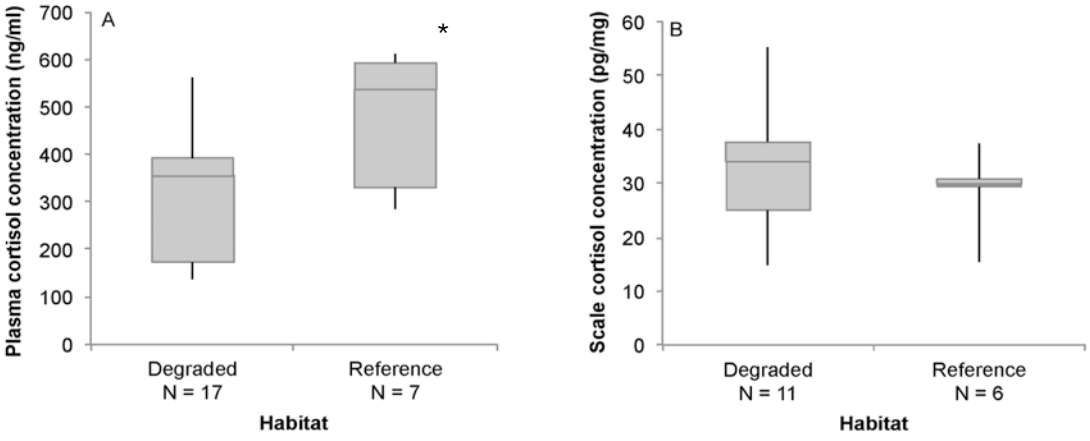

Fig. 1. Boxplots of (A) plasma cortisol concentrations (ng cortisol/ml plasma) and (B) scale cortisol concentrations (pg cortisol/mg scale) in Catalan chub from degraded and reference habitats. The asterisk indicates differences in plasma cortisol concentrations between habitats ($p = 0.02$).

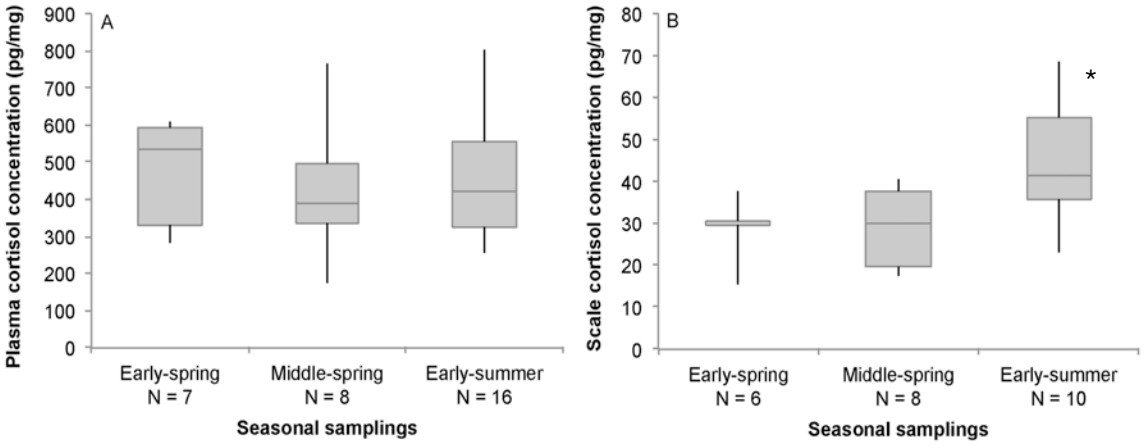

Fig. 2. Boxplots of seasonal comparisons on (A) plasma cortisol concentrations (ng cortisol/ml plasma) and (B) scale cortisol concentrations (pg cortisol/mg scale) in Catalan chub from the reference habitat. The asterisk indicates that at the early summer scales cortisol levels were significantly higher compared to early (p = 0.04) and mid (p = 0.03) spring levels.

DISCUSSION

Before its application in research, every laboratory should validate that the analytical method selected provides an accurate measurement of the hormone in the target matrix (Buchanan and Goldsmith, 2004). The commercial EIA kit used in the present study was therefore biochemically validated for measuring cortisol in plasma and scales of the cyprinid fish *S. laietanus*. Results demonstrated that the EIA is precise, specific, accurate and sensitive measuring cortisol levels both in plasma and scales of the Catalan chub, likewise demonstrated in other fish species (Carbajal et al., 2018). In fish, blood cortisol has been typically analysed by immunoassays, such as radioimmunoassay (RIA) and EIA, whereas in scales, chromatographic methods (Aerts et al., 2015) and also EIA (Carbajal et al., 2018) have been applied so far. Several advantages have led to the preference for EIA, such as its safety, ease of use, and cost-effective equipment, allowing to many researchers to conduct endocrine analysis (Mills et al., 2010; Sink et al., 2008).

After verifying that the analytical technique is accurate, the next step forward should validate whether scale cortisol reflect the HPI axis activity (Kersey and Dehnhard, 2014). An essential way to validate this relationship is to evaluate if hormone levels deposited in the matrix correlate with those detected in plasma from the same individuals (Cook, 2012; Sheriff et al., 2011). Documentation on this relationship, however, has never been reported before in free-ranging animals. We should point out that, although we could not specifically validate the time course

elevation in blood cortisol concentrations, as supported by the literature, we were probably measuring stress-induced PCC given that we collected blood 1 h following exposure to a stressor (Mommsen et al., 1999; Pankhurst, 2011). From our study, a significant correlation was found between cortisol levels in scales and blood in fish from the reference habitat. This connection between both matrices' cortisol levels provides evidence that scales could be integrating cortisol relative to bloodstream concentrations, at least in the non-stressful habitat since such relationship was not mirrored in fish from the polluted site.

The lack of correlation in the polluted habitat is indeed not surprising. When PCC were contrasted between fish from habitats of different contaminant load, fish from the polluted site exhibited lower PCC. This different response in either site could be due to the effect of certain aquatic contaminants, since there is strong evidence that can inhibit post-stress cortisol levels (Hontela et al., 1992; Jorgensen et al., 2017; Leblond et al., 2001; Quabius et al., 1997) or delay the stress response (Marentette et al., 2012; Norris et al., 1999). Importantly, altered PCC may suggest a reduced capacity of the fish to tolerate subsequent or additional stressors from their natural settings (Angelier and Wingfield, 2012; Odermatt et al., 2006). Consequently, any potential relationship between PCC and SCC could have been masked as a result of the pollutants' interference. Despite this, the possibility of a sample size with not enough statistical power to identify the relationship between matrices cannot be completely ruled out. Increasing sample size will probably aid in determining if such between-matrix relationship differs among populations due to the habitat characteristics.

Since scales are hypothesized to accumulate cortisol relative to concentrations in bloodstream (Aerts et al., 2015), this latter result observed on PCC should also be reflected in SCC. However, SCC remained unchanged between habitats. Previous studies have described that habitat degradation can affect the cortisol response to a stressor, while keeping baseline levels unaltered (Belanger et al., 2016; Blevins et al., 2013, 2012; King et al., 2016). Considering that basal cortisol concentrations in Catalan chub potentially remained unaffected by the habitat quality, our results are consistent with the idea that cumulative matrices, such as feather, hair and shed skin, are more influenced by basal levels of the hormone than by acute and non-recurrent stress responses (Ashley et al., 2011; Berkvens et al., 2013; Fairhurst et al., 2013a; Tallo-Parra et al., 2017). Understanding the influence of acute and short elevations of cortisol in SCC seems vital in order to deepen its value as a measure of long-term HPI axis activity. Although further experimental work is needed to clarify this effect, our findings may suggest that the contribution of single acute stressors to scale cortisol is probably small.

Despite the fact that SCC stayed the same between habitats, there was a seasonal change in SCC. A growing body of literature has demonstrated seasonality on GC levels in different taxa (Baker et al., 2013; Cockrem, 2013; Wingfield and Romero, 2015). In line with this assumption, the present study provides novel evidence that also SCC could vary seasonally. The

increment of SCC from middle spring to summer suggests that during the time between these two sampling efforts something promoted the activation of the HPI axis. As a consequence, circulating levels of the hormone could have been increased, incorporating higher amounts of cortisol into the scales. In agreement with previous reports on Catalan chub (Aparicio, 2016; Sostoa et al., 1990), the increment observed in GSI from spring to summer suggests that the period studied covered the species' breeding season. Breeding is a life-history stage energetically expensive (Bonier et al., 2011; Romero, 2002) largely known to influence the HPI axis activity (Dantzer et al., 2014; Wingfield and Sapolsky, 2003). In this context, the breading season in Catalan chub has already been described to be a stressful period distinguished by a high percentage of blood alterations (Colin et al., 2017). Therefore, the increase in SCC concurring with the breeding period of the species could be partly influenced by the common energetic needs of individuals during reproduction (Milla et al., 2009; Schreck, 2010).

Besides the biological demands driven by the breeding season, the period studied coincides with a series of short-term changes in the habitat conditions that worth mentioning. As demonstrated by the physico-chemical analysis, the water flow was drastically reduced from spring to summer in the reference site. Drought periods and consequently low water flow conditions are typically observed in this geographic area, and are related to reduced habitat quantity and quality (Jessop et al., 2003; Maceda-Veiga et al., 2009). Interestingly, events such as drought are known to trigger stress responses in many vertebrate species (Baker et al., 2013; Jessop et al., 2003; Tokarz and Summers, 2011; Wikelski et al., 2001). Variation in water temperature is another environmental variable that should be considered when studying wild fish, since several authors have demonstrated its influence on cortisol stress responses (Blevins et al., 2012; Cook et al., 2011; Meka and McCormick, 2005; Quinn et al., 2010). In order to cope with subtle changes in the environment, such as the above mentioned, healthy individuals are predicted to increase GC secretion (Wikelski and Cooke, 2006), leading to higher circulating cortisol levels (Bonier et al., 2009). Therefore, the seasonal differences observed in SCC could also be driven or exacerbated by short-term changes, probably of a certain intensity, in the environmental conditions (Wingfield et al., 2011). Some authors have concluded that in order to detect hormonal changes in cumulative matrices a more intense and/or prolonged activation of the HPI axis in needed (Fairhurst et al., 2013a, 2013b; Lattin et al., 2011). In agreement with these previous observations, our results largely suggest that fluctuations in SCC may become apparent once the HPI axis has been challenged or stimulated for a period of at least 3 months (period span between the 2nd and 3rd sampling), regardless of whether it is driven by intrinsic or extrinsic causes.

The relationship between cortisol levels and intrinsic factors related to the animals' biology such as body condition or the reproductive status has been emphasized by many authors

(Baker et al., 2013; Cook et al., 2012; Sheriff et al., 2011; Vera et al., 2017). Despite not detecting an influence of neither *K* nor GSI on SCC, fish from the polluted habitat presented higher body condition than those from the reference site. Although not common, higher body condition in fish inhabiting polluted environments has been described (Goodbred et al., 2015). Colin and colleagues (2017) reported similar findings in Catalan chub by using the Scaled Mass Index instead of the Fulton's condition index. As these authors suggested, eutrophication of fresh water can result in better food quality. Note that some pollutants, especially those with endocrine disrupting effects, have obesogenic activity in humans and other vertebrates (Holtcamp, 2012; Ismail et al., 2017) including fish (Lyche et al., 2010). Thus a differing contaminant profile between habitats could also drive to the contrasting body condition observed. Furthermore, fish at the early summer increased their body condition compared to the previous assessments. Several factors other than stress, such as seasonal and developmental modifications, can also induce changes in condition indices (Barton et al., 1998; Mahé et al., 2018), possibly explaining why body condition varied as the season progressed.

In fish, sex has been less frequently considered in comparison with studies in other vertebrates, yet it is known that cortisol levels can vary due to gender differences (Baker et al., 2013). In this study we did not detect differences in PCC nor SCC between males and females. While this is the first time that sex differences in SCC are evaluated, our results provide valuable data for studies in wildlife where sex is a factor usually difficult to control, especially in species without sexual dimorphism, and when the number of individuals collected needs to be kept low for ethical reasons.

In conclusion, while comparison of fish inhabiting habitats of different contaminant load suggests that SCC may not be a promising bioindicator of environmental quality, the SCC increase concurring with a stressful period for the fish species studied strongly supports the idea that the analysis of cortisol in scales could reveal changes in the HPI axis activity. This study, therefore, indicates that cortisol levels in scales are more likely to be influenced by mid-term, intense energetically demanding periods rather than by long-term stressors. The degree to which cortisol deposition in scales is affected by external (drought, temperature) and/or internal (reproduction) factors needs to be further explored. Studies including samples collected over extended periods of time (e.g. a year), along with the assessment of other physiological endpoints of stress responses would be of interest to determine whether, when and which factors influence the cortisol deposition in fish scales.

SUPPLEMENTARY MATERIAL

ANALYSIS OF PER- AND POLYFLUOROALKYL SUBSTANCES (PFAS)

Chemical and reagents

Mixture of native perfluoroalkylcarboxylic acids (PFCAs) (perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA) and perfluorooctadecanoic acid (PFODA)) and native perfluoroalkylsulfonates (PFSAs) (perfluorobutane sulfonic acid (PFBS), perfluorohexane sulfonic acid (PFHS), perfluorooctane sulfonic acid (PFOS) and perfluorodecane sulfonic acid (PFDS)) were purchased from Wellington Laboratories (Guelph, ON, Canada). Stock solutions of the target compounds were prepared in acetonitrile (5 ng/µl) and stored at – 18 ºC. Perfluoro-n-(1, 2, 3, 4-13C4) octanoic acid (MPFOA) and sodium perfluoro-1-(1, 2, 3, 4-13C4) octane sulfonic acid (MPFOS), also purchased from Wellington Laboratories, were used as surrogate standards. High performance liquid chromatography (HPLC) grade water and acetonitrile were bought from Merck (Darmstadt, Germany) and glacial acetic acid from Panreac (Barcelona, Spain).

Sample extraction

Extraction followed the methods of Vicente et al. (2012) with some modifications. Muscle sample (1 g) was incubated in polypropylene tubes with addition of the internal standard mixture (100 ng) for 18 hours at 4ºC. Afterwards, 9 ml of acetonitrile were added and the samples were thoroughly mixed using a vortex. Samples extraction was performed in an ultrasonic bath for 10 min at room temperature. Vortexing and ultrasonic extraction was repeated 3 times with the same solvent. Samples were then centrifuged (2500 rpm, 5 min) and the supernatant was transferred into a new vial and evaporated to dryness. Once dried, 1 ml of acetonitrile was added and incubated for 10 min in the ultrasonic bath. Purification of samples was performed by adding 25 mg of activated carbon and 50 µL of glacial acetic acid and vigorously mixed for 1 minute. Samples were then centrifuged (10000 rpm, 10 min) and the supernatant was transferred to a clean micro vial, evaporated and reconstituted with 250 µL of acetonitrile and 250 µL of water with 10 mM ammonium acetate buffer.

Sample analysis

Acquity Ultra Performance Liquid Chromatography (UPLC) system connected to a Triple Quadruple Mass Spectrometry Detector (Waters, USA) was used to measure PFASs. To remove any source of contamination from the mobile phases, an XBridge C18 column (3.5 µm particle size, 50 mm x 4.6 mm, Waters, USA) was used as mobile phase residue trap. The analysis was performed on an Acquity UPLC BEH C18 column (1.7 µm particle size, 100 mm x 2.1 mm, Waters, USA). Five µL of extract were injected. The mobile phase consisted of (A) HPLC water with 10 mM ammonium acetate/methanol (80:20) and (B) acetonitrile with 10 mM ammonium acetate. Gradient elution started from 50% A and 50% B, held for 3 min and increased to 100% B in 7 min and held for 2 min, at a flow rate of 0.3 ml/min. Afterwards, initial conditions were regained in 1 min and the system was stabilized for 2 min at initial conditions. The different PFASs were measured under negative electrospray ionization using two transitions from parent to product ion to identify each compound except for PFBA and PFPA where only one transition was used.

Extraction efficiency was assessed by calculating the recovery of surrogate standards in MPFOS (mean ± CV = 111 ± 18 %) and MPFOA (mean ± CV = 114 ± 16 %). The calibration was performed over a concentration range of 0.01 to 0.5 µg/ml. Coefficients of variation obtained for all compounds was < 15 % indicating good reproducibility.

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GENERAL DISCUSSION

PROTOCOL AND BIOCHEMICAL VALIDATION

Measurement of steroid hormones in samples from a variety of origins has become a trend in several research fields (Baker et al., 2013; Sheriff et al., 2011). As a consequence, differing methods for sample preparation and steroid quantification between laboratories may be used, which has proven to make data interpretation and reproducibility of results challenging (Berk et al., 2016; Cook, 2012; Hayward et al., 2010). The characteristics and the components of every matrix may differ from each other and across species, as well as the polarities and the structure of the steroid content (Buchanan and Goldsmith, 2004; Palme et al., 2013). Validation and standardization of each new method should be, therefore, the first step to warrant the biological relevance of results (Brown et al., 2004). Accordingly, our first aim was to develop and validate a methodology for the detection of cortisol in fish scales. One of our main concerns was the presence of cortisol on the exterior of the scale, presumably coming from the skin mucus (Bertotto et al., 2010) and how best to remove it. The exclusive chemical properties have made isopropanol the wash solvent of choice to remove external sources of cortisol, particularly in mammal hair (Burnard et al., 2017; Davenport et al., 2006; Tallo-Parra et al., 2015), whereas in fish, the only published study to date used ultrapure water, and no proper validation tests were presented (Aerts et al., 2015). We then explored the appropriateness of water and isopropanol as solvents to clean the scale sample without influencing the hormone contents deposited inside the matrix. After applying three consecutive washings with one or the other solvent, cortisol content in samples washed with isopropanol, unlike scales washed with water, remained constant regardless of the number of washings. The measurement of cortisol levels in the supernatant revealed that each consecutive washing with either water or isopropanol was removing cortisol from the first to the third wash. However, no solvent-induced differences were identified, probably masked by the high levels of cortisol present in the skin mucus (Bertotto et al., 2010; Guardiola et al., 2016a). An incomplete decontamination by isopropanol washings was also considered.

Nevertheless, to support this hypothesis higher amounts of cortisol coming from the skin mucus should have been detected in the scale cortisol analysis. Overall, results indicate that water is probably penetrating into the scale and extracting intrinsic cortisol present therein, as previously observed in the hair shaft (Hamel et al., 2011). Further, the study reveals that isopropanol is effective in removing cortisol coming from external sources without leaching the tightly bound fraction from the interior of the matrix.

Once assured that scale samples have been properly cleaned, next step involves sample grinding and hormone extraction. The protocol applied for cortisol extraction from scales was based on the methanol-based technique originally described for steroid extraction from hair and feathers (Bortolotti et al., 2008; Davenport et al., 2006), and eventually for fish scales (Aerts et al., 2015), but we incorporated crucial modifications. After scales were washed with isopropanol and air-dried, samples were mechanically ground with a ball mill. While Aerts and colleagues (2016) were handy cutting samples using scissors, which can potentially result in a significant source of variability, our protocol assured homogeneity of the powdered particles among samples. Besides that, ground samples have shown to present greater extraction efficiencies compared to samples minced with scissors (Davenport et al., 2006), which could allow the analysis of samples with less cortisol content. In addition, the presented protocol improves the assay by means of its speed and efficiency. Mechanical pulverization eliminates potential inconsistencies due to operator inequalities, allowing several persons to process the samples, plus two samples can be ground per grinding cycle. In addition, smaller particles have a larger surface to volume area, thus less methanol volume is needed for steroid extraction.

In the present thesis, blood and skin mucus samples were collected and processed following methods previously described in the literature (Bertotto et al., 2010; Guardiola et al., 2016a). However, the analytical technique was specifically validated for each matrix and species following the recommendations of Buchanan and Goldsmith (2004). Blood, skin mucus and scale cortisol levels were determined by a competitive enzyme immunoassay (EIA) prepared for cortisol quantification (Neogen® Corporation, Ayr, UK), while taking advantage of the multiple inherent benefits of this technique compared to other assay methods (Sink et al., 2008; Young et al., 2004). Overall, validation tests demonstrated that cortisol levels either in blood, skin mucus and scales processed with the aforementioned methodologies can be reliably quantified by using an EIA kit not specifically designed for the target matrix and species. The test battery executed in this thesis is probably the most extensive validation performed until now for cortisol detection in fish skin mucus and scales. These encouraging results obtained in goldfish, rainbow trout and Catalan chub strongly suggest that the methodologies presented could be applied to other teleost species, especially those from the Cyprinidae and Salmonidae families, which are among the most studied fish groups (Colin et al., 2016; Janz, 2000).

MEASUREMENT OF CORTISOL IN FISH SKIN MUCUS: BIOLOGICAL VALIDATION

Besides performing the methodological validation, we should demonstrate that the novel media for glucocorticoid (GC) measurement is biologically relevant (Palme, 2005). This can be accomplished with the so-called biological or physiological validation by exploring: 1) the relationship between cortisol levels in the new media and those in matched blood samples and 2) the relationship of cortisol levels in the sample to the experience of stress in the animal, also referred to as cause-and-effect link (Cook, 2012; Mormède et al., 2007). In the present thesis, the first condition was evaluated in both laboratory and wild fish, while the second one was studied in experimental animals under controlled settings.

RELATIONSHIP TO BLOOD LEVELS

Correlation between plasma cortisol concentrations (PCC) and skin mucus cortisol concentrations (MCC) was strong and evident when laboratory fish were acutely responding to the stressor with an intense activation of the HPI axis (Chapter II). Whereas control groups and fish that had been subjected to stressful conditions over a prolonged time period did not show correlation in cortisol levels (Chapter II). When such relationship was evaluated in free-ranging fish, correspondence between both metrics was also high and significant (Chapter III). Note that in both field studies performed in this thesis (Chapter III and IV), fish were caught and kept in tanks to trigger a stress response by capture and confinement. We followed this methodology since stressed-induced cortisol concentrations are known to provide a better understanding of the overall stress response (Romero, 2004). Although we could not execute a specific study to evaluate the time course elevation of cortisol in the target species (Catalan chub, *Squalius laietanus*), there is substantial evidence that increases of cortisol can be detected as short as 1 - 2.5 min following exposure to stressors (Mommsen et al., 1999; Pankhurst, 2011). Accordingly, the relationship between matrices in wild fish was probably detected by using stress-induced levels of the hormone, similarly to the single significant correlation identified in laboratory animals. While previous reports have successfully measured MCC in farm fish held under controlled settings, our findings demonstrate that this tool could also be applied to assess the short-term HPI axis activity in wild freshwater fish within its natural environment.

To date, none of the published studies have specifically evaluated the relationship between PCC and MCC by considering pre-stress and stress-induced levels separately. This thesis is, therefore, the first to reveal that cortisol may diffuse to the skin mucus in proportion to the amount of circulating hormone when fish are acutely responding to a stressor, or in other

words, when high amounts of cortisol are released into the bloodstream. Interestingly, a similar connection has actually been demonstrated in mammals between cortisol values in blood and saliva or milk (Cook, 2012; Mormède et al., 2007). Saliva and milk cortisol concentrations present a time lag of few-minutes in relation to circulating levels, a delay comparable to that detected between blood and skin mucus in rainbow trout (De Mercado et al., 2018). Noteworthy is the correlation between matrices since cortisol in saliva and milk better correlate to circulating levels following stimulation of the stress axis compared to resting periods, likewise we have observed in fish MCC. Comparison of our results to data obtained from other vertebrate species further supports the distinct association detected between both cortisol measurements and the HPI axis activation.

RELATIONSHIP TO BIOLOGICAL STRESS

In order to examine the existing link between applying a stressor and its effects on MCC, we studied the consequences of exposing fish to a chronic continuous stress on MCC at specific time points (Chapter III): before applying stress, after 1, 6 and 24 hours, and after 14 and 30 days under stressful conditions. Levels of MCC increased significantly after placing fish into the confinement nets. In addition, the pattern of response detected in MCC in the short-term was comparable to that observed by the analysis of PCC. These observations further validate that increases in skin mucus cortisol levels relate to the individual short-term experience of stress. Accordingly, cortisol measurement in skin mucus constitutes an innovative, non-invasive approach to assess acute stress responses in fish.

After long-term exposure to stress, the patterns of significances in MCC were inconsistent in relation to those detected in PCC. As described, different stressors can alter the structure and cellular composition of the epidermis (Tacchi et al., 2015; Vatsos et al., 2010), which in turn, can lead to physical and biological changes of the skin mucus (Elliott, 2000; Pérez-Sánchez et al., 2017; Shephard, 1994). Accordingly, we interpreted those discrepancies as being likely to arise from an alteration in the skin mucus production. Previous studies assessing the effect of short-term stressors in rainbow trout detected an increase of 2 to 4 times in PCC between control and stress groups (De Mercado et al., 2018; Gesto et al., 2013; López-Patiño et al., 2014), whereas in our study, peak circulating cortisol values were about 11 times higher than at pre-stress levels. It is possible that, triggering an intense initial stress response could have compromised even more the skin mucus production. We, therefore, suggest caution when applying this method for long-term stress assessments, particularly in experimental models subjected to continuous and severe stressful conditions.

Few studies have evaluated the usefulness of MCC as an acute indicator of the HPI axis activity (Bertotto et al., 2010; De Mercado et al., 2018; Guardiola et al., 2016b; Simontacchi et al., 2008), and nothing is known about its applicability far from these short-term

evaluations or after applying a single acute stressor. Our findings provide new evidence that, if one wishes to evaluate long-term stress, MCC may not be a good indicator in fish subjected to severe stress for extended periods of time. Importantly, the fact that we assessed baseline levels of cortisol in chronically stressed fish, rather than stress-induced levels, may not have allowed differentiation between a potential habituation from exhaustion or desensitization. We suggest that further studies including an additional acute stressor at the end of the study would largely help understanding the potential of this approach to be applied in chronically stressed animals. We also suggest future research in cortisol stress responses within physiologically relevant limits, which may help better understand the usefulness of this indicator in naturally occurring situations.

MEASUREMENT OF CORTISOL IN FISH SCALES: BIOLOGICAL VALIDATION

Long-term integrated measures of GC have demonstrated to be especially powerful in mammal and avian endocrine studies (Romero and Fairhurst, 2016; Stalder and Kirschbaum, 2012). Despite the clear importance of developing these methodologies, such source material was lacking in fish until the very recent. In consequence, there are a large number of unresolved concerns associated with the method that need special consideration. Following the same guidelines to as the subsection above, we evaluated whether the quantification of cortisol in scales represents a long-term integrated measure of fish HPI axis activity by 1) studying the relationship of scale cortisol concentrations (SCC) to other sample matrices in wild and laboratory fish, and 2) evaluating the relationship of SCC to a biological stress.

RELATIONSHIP TO OTHER SAMPLE MATRICES

Scales Vs. Blood

Under controlled laboratory conditions (Chapter II), SCC from rainbow trout were highly correlated to PCC, but only in fish that had been subjected to chronic continuous stress. When this connection was assessed in wildlife (Chapter IV), Catalan chub inhabiting the reference site displayed correlation between SCC and PCC. These results show for the first time that SCC correlates to natural and endogenous circulating cortisol levels in both laboratory and wild fish. Note that, SCC only correlated to PCC in chronically stressed fish (lab evidence) and when individuals were potentially responding to an acute stressor (field evidence). Similar to our findings, correlations have been successfully determined between

corticosterone levels in blood and feathers, probably the bird cumulative matrix analogous to fish scales. Interestingly, the correspondence was evident by using stress-induced blood corticosterone levels (Bortolotti et al., 2008) or when blood hormone values were the highest of the overall studied period (Fairhurst et al., 2013a). Blood and scale cortisol concentrations predominantly differ in the time frames that are reflected by the measurements. While PCC offers an instantaneous snapshot view of the HPI axis activity, SCC are hypothesized to provide an integrated measure. Our results, therefore, are unlikely to indicate that a single blood cortisol value reflects the total SCC, but rather that scales may integrate, on an individual basis, the magnitude and duration of the cortisol secretion.

Scales Vs. Skin mucus

The relation between SCC and MCC presented switched results compared to that detected between SCC and PCC in laboratory conditions (Chapter II). While cortisol levels were highly correlated in control groups, after experiencing chronic stress such correlation disappeared. This later result reinforces our hypothesis about the possible alteration of the skin mucus production in long-term stressed fish. The high correlation observed in control fish is nonetheless remarkable. As previously mentioned, in rainbow trout the time-lag between secretion and excretion of cortisol in skin mucus seems to take less than 10 minutes (De Mercado et al., 2018). Accordingly, we expected to find a correspondence between SCC and MCC similar to that detected between SCC and PCC. The mechanism of cortisol secretion in skin mucus has not yet been addressed, neither the way cortisol is incorporated in scales nor the source of scale cortisol. When these matters have been addressed in hair, some researchers suggested that sweat, sebum and external sources of cortisol can also contribute to the total hair cortisol content (Burnard et al., 2017; Henderson, 1993; Stalder and Kirschbaum, 2012). There is evidence that the local production of cortisol could also contribute (Skobowiat et al., 2011; Taves et al., 2011). Although this thesis was not specifically designed to address these issues, our results highlight the need to further conduct experimental work to clarify those knowledge gaps that challenge hormone interpretations. Radiolabelled studies have been successfully applied to determine the steroid secretion and excretion (Meyer and Novak, 2012; Palme et al., 2005; Touma et al., 2003). Thus we encourage future research in this area to better understand whether and how cortisol diffuses from the bloodstream into both fish skin mucus and scales.

RELATIONSHIP TO BIOLOGICAL STRESS

In order to relate the SCC to the experience of stress in the animal, a group of fish was exposed to a chronic continuous stressful condition, and after 14 and 30 days, we analysed the cortisol content deposited in scales (Chapter II). At the end of the study, both 14 and 30-day

stressed fish presented significantly higher SCC than control fish. This result strongly supports the hypothesis that scales are able to provide long-term and retrospective measures of cumulative cortisol secretion in fish. Nonetheless, SCC from stressed fish significantly decreased from day 14th to day 30th. Contrasting results were found by Aerts and colleagues (2015), who identified an increase in SCC from treatment day 21 to 42 in stressed individuals. In the latter study, fish were daily subjected to an acute stressful event throughout the treatment period, whereas we generated a continuous stressful scenario by subjecting fish to a prolonged high-density confinement. Previous studies described that under prolonged physiological stress or extreme environmental conditions, minerals from the scales can be reabsorbed modifying the content previously deposited in the matrix (Loewen et al., 2016; Metz et al., 2014; Mugiya and Watabe, 1977). Taken together, these findings suggest that cortisol in scales could not be metabolically inert, especially when fish are continuously subjected to too demanding conditions. By identifying sings of growth arrest on scales' growth line, we probably would have been able to evaluate whether the scale was resorbed or not (Witten and Huysseune, 2009). We, therefore, suggest caution when assuming that scales can give insight into long-term hormonal levels, particularly in fish potentially exposed to severe stressful conditions. As observed here, we could deduce that SCC reflect the HPI axis activity up to 14 days, but the consequences on scale cortisol deposition in fish subjected to longer periods of stress need further clarification. Like suggested earlier, achieving cortisol elevations within ecologically and physiologically relevant ranges would be particularly revealing. Also, we are aware that studying how chronically stressed fish respond to additional stressors would probably provide new insights about the ability of scale cortisol to reflect the long-term HPI axis activity and the stability of the hormone wherein this matrix.

USEFULNESS AS BIOINDICATORS OF HABITAT QUALITY

We have demonstrated that cortisol in skin mucus and scales are reliably measured by EIA, and afterwards, that cortisol fluctuations in both sample types reflect biological events of interest. But, are both measures equally valid as potential bioindicators of habitat quality? To address this question, MCC and SCC measured in fish from polluted habitats were compared to those from a reference site. Two separate studies were performed, one for each matrix type, using Catalan chub captured in the same river system (Chapter III and IV).

Supported by an accumulating body of evidence, we assumed that the environmental pollution would act as a long-term stressor in wild living fish (Harvey, 2016; Schreck et al., 2016; Wingfield et al., 2011). Studies successfully reporting the impacts of pollutants on fish physiology have commonly used single-point matrices to evaluate the cortisol response to an additional stressor (e.g. King et al., 2016; Mehdi et al., 2017; Pottinger et al., 2013; Quinn et al., 2010). Similar to these studies, the analysis of MCC suggested that fish from the polluted site were probably responding to an acute stress with higher cortisol levels than those from the reference habitat (Chapter III). In addition, MCC correlated well to the frequency of abnormal erythrocytes and to the N:L ratio (relative proportion of neutrophils to lymphocytes), two well-established indices of health status that have been reliably related to high pollution levels (Davis et al., 2008; Houston, 1997; Witeska, 2005). These results, therefore, suggest that the measurement of cortisol concentrations in skin mucus could be potentially used as a bioindicator of habitat quality in fish residing polluted streams. As above-mentioned, in both field studies, samples were collected following an acute stressor with the aim to evaluate the stress-induced levels of cortisol. It should be noted, though, that in several occasions baseline cortisol levels can also denote altered habitat conditions (Mehdi et al., 2017; Pottinger et al., 2013). Accordingly, understanding whether baseline MCC fluctuate in relation to habitat quality would also be advantageous to verify the effectiveness and applicability of this method in wild environments.

On the contrary, SCC remained unchanged between habitats of differing pollution gradient (Chapter IV), although fish from the most polluted site probably presented an impaired activation of the HPI axis (further discussed below). Cumulative matrices, such as feather, hair and shed skin, are hypothesized to be more influenced by basal levels of the hormone rather than by acute and non-recurrent stress responses (Ashley et al., 2011; Berkvens et al., 2013; Fairhurst et al., 2013a; Tallo-Parra et al., 2017). In line with these studies, the lack of differences in SCC between habitats could indicate that fish from the polluted site kept baseline cortisol levels unaltered, a scenario previously described in other freshwater fish species (Belanger et al., 2016; Blevins et al., 2013, 2012; King et al., 2016; Marentette et al., 2012). To further understand the potential of scale cortisol as a measure of long-term HPI axis activity is essential to discern whether acute and non-recurrent elevations of cortisol influence SCC. While these findings suggest that the contribution of single acute stressors to scale cortisol is probably small, results should be interpreted with caution. As previously discussed, the significant correlation detected between SCC and PCC in laboratory and field studies, indicated that scales could be integrating the magnitude and the duration of the stimulated cortisol response.

Furthermore, though no differences in SCC were observed between habitats, SCC varied as the season progressed. This novel evidence that SCC could vary seasonally is in agreement with a growing body of literature, regardless of the species and biological sample employed

(Baker et al., 2013; Cockrem, 2013; Romero et al., 2015; Wingfield and Romero, 2015). Because seasonal GC variation can make hormone interpretations more complex (Romero, 2004), it seems crucial to control for this source of variability when studying free-ranging animals. Interestingly, some authors reported that in order to detect hormonal changes in cumulative matrices, a more intense and/or prolonged activation of the HPI axis is needed (Fairhurst et al., 2013a, 2013b; Lattin et al., 2011). The temporal rise in SCC concurring with a potentially stressful period for the species evaluated strongly supports the latter assumption. We argued that the increase in SCC could be influenced by either the costs related to the reproductive investment (Romero, 2002; Wingfield and Sapolsky, 2003), or to subtle weather and climate changes, in particular drought (Baker et al., 2013; Sloman et al., 2001; Wingfield and Romero, 2015). Importantly, despite the sharp rise in SCC, breeding appeared to continue, as denoted by the increase in the gonadosomatic index, a broadly used indicator of reproductive periods (Brewer et al., 2008). This phenomenon, recently named the "Cort adaptation hypothesis", assumes that elevated GC levels during energetically expensive life-history stages, such as breeding, may represent an adaptive response to facilitate reproduction and survival (Bonier et al., 2009; Crossin et al., 2016; Romero, 2002). Taken together, these findings could indicate that the deposition of cortisol in scales is likely to be more influenced by transient energetically demanding periods rather than by long-term stressors. The controversy remains as to whether and to what extent SCC are influenced by acute and short-term stress responses, highlighting the need for further research, preferably under controlled laboratory conditions.

In an attempt to compare Catalan chub' PCC between studies, contrasting results were observed. While in Chapter III individuals from the polluted habitat presented higher PCC than those inhabiting the reference site, in Chapter IV results displayed the opposite. There are several plausible explanations for the inconsistencies between studies that should be considered. First, there is strong evidence that some aquatic pollutants can inhibit the cortisol secretion at different levels (Jorgensen et al., 2017; Leblond et al., 2001), or delay the stress response (Marentette et al., 2012; Norris et al., 1999), but also that certain contaminants can amplify the cortisol response to acute stressors (Aluru et al., 2010; Gesto et al., 2008; Pottinger et al., 2016). Since the toxic mechanism of action changes depending on the type of pollutant (Matthiessen et al., 2018; Oliveira et al., 2011), the contrasting patterns of response observed between studies could be due to a different contamination profile between polluted areas. The second one is related to the fact that blood samples were collected at different times following exposure to an additional stressor (15 min - Chapter III; 1 hour - Chapter IV). Consequently, each report may reflect a different time-point of the HPI axis activation, challenging even more the comparison between studies. In addition, as discussed earlier in this section, GC levels can vary seasonally (Belanger et al., 2016; Romero, 2002). Considering

that this variation is especially important during the breeding season (Bonier et al., 2011; Romero, 2002), the fact that samples were not collected exactly at the same stage of reproduction could be another source of irregularity between studies. Besides that, also the differing year of sampling should be considered (year 2016 - Chapter III; year 2014 - Chapter IV), since across-year differences in a variety of bioindicators have been described in several fish species (Broeg and Lehtonen, 2006; Katsiadaki et al., 2012), including those measured in Catalan chub (Colin et al., 2017).

Overall, these findings highlight that the impact of habitat quality on circulating cortisol levels is likely to be variable, likewise the ability of the methods to potentially indicate environmental quality, and most notably, that fluctuations on cortisol levels measured in fish scales are probably context-dependent.

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CONCLUSIONS

The studies performed in the present thesis were conducted with the aim to validate the measurement of cortisol in fish skin mucus and scales and evaluate the applicability of these methods as potential tools to assess habitat quality.

The following conclusions can be drawn accordingly:

Specific objective 1

To validate a protocol for the extraction of cortisol from fish scales and skin mucus and the quantification of cortisol concentrations in these matrices by enzyme immunoassay

CONCLUSION 1.1

External sources of cortisol from the surface of the scale sample can be efficiently removed with isopropanol without leaching the tightly bound fraction from the interior of the matrix.

CONCLUSION 1.2

Cortisol levels in skin mucus and scales from different freshwater fish species can be reliably quantified by enzyme immunoassay through the presented protocol.

Specific objective 2

To evaluate whether the quantification of cortisol in skin mucus represents a short-term measure of fish hypothalamic-pituitary-interrenal axis activity

CONCLUSION 2.1

Cortisol diffuses into the skin mucus in proportion to the amount of circulating hormone when the hypothalamic-pituitary-interrenal axis is acutely responding to a stressor, rather than when the axis has been stimulated for a prolonged period.

CONCLUSION 2.2

The measurement of cortisol in skin mucus could constitute an alternative, non-invasive approach to assess the short-term hypothalamic-pituitary-interrenal axis activity in wild freshwater fish within its natural environment.

Specific objective 3

To evaluate whether the quantification of cortisol in scales represents a long-term integrated measure of fish hypothalamic-pituitary-interrenal axis activity

CONCLUSION 3.1

Scale cortisol concentrations correlated to those detected in matched plasma samples suggesting that scales could be integrating, on an individual basis, the magnitude and duration of the cortisol secretion.

CONCLUSION 3.2

The measurement of cortisol in scales offers a retrospective, long-term, integrated measure of the hypothalamic-pituitary-interrenal axis activity in fish subjected to chronic continuous stress.

Specific objective 4

To evaluate the potential use of skin mucus and scale cortisol as bioindicators of habitat quality in a wild freshwater fish

CONCLUSION 4.1

Skin mucus cortisol concentrations differed between habitats of different pollution gradient revealing that this metric could be potentially used as a bioindicator of habitat quality in fish residing contaminated streams.

CONCLUSION 4.2

Scale cortisol levels remained unchanged between habitats of different pollution gradient, suggesting that cortisol deposition in scales may not be influenced by long-term environmental pollution.

CONCLUSION 4.3

The increase in scale cortisol concentrations concurring with a temporarily stressful period for the species suggests that transient energetically demanding periods could influence the deposition of cortisol in fish scales.