

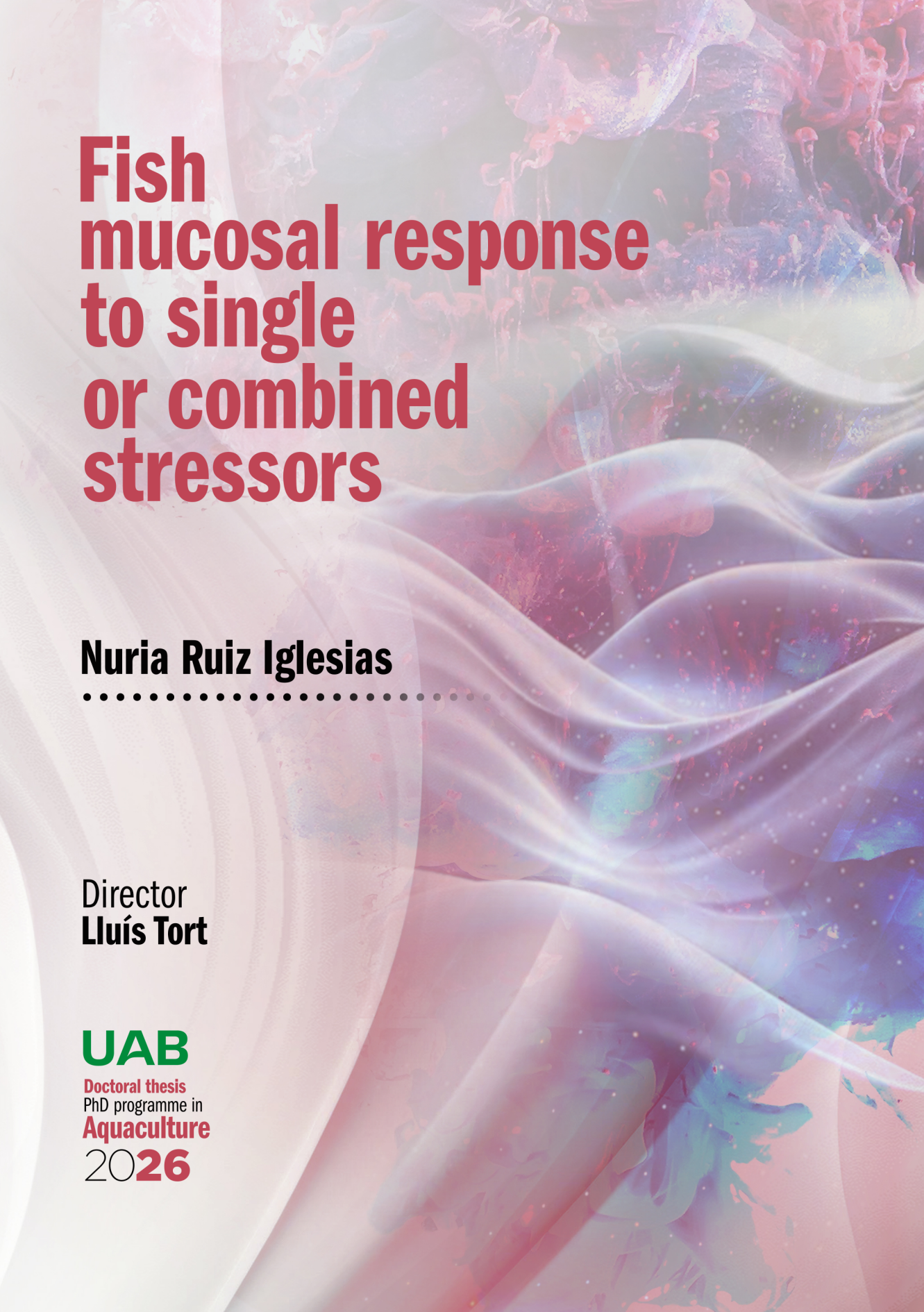
Fish mucosal response to single or combined stressors

Nuria Ruiz Iglesias

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Director
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Doctoral thesis
PhD programme in
Aquaculture

20**26**

“Lo que conocemos es una gota, lo que no conocemos es un oceano.”
— **Isaac Newton**

Abbreviations

18s: Ribosomal protein S18	DEPC: Diethyl pyrocarbonate
3Rs: Replacement, reduction and refinement	Di: Diethyl ether
ABC: ATP-binding cassette	DLS: Dynamic light scattering
AC: Absolute control	DNA: Deoxyribonucleic acid
AChE: Acetylcholinesterase	DO: Dissolved oxygen
ACTH: Adrenocorticotrophic hormone	E: Ethanol
ADA: Adenosine deaminase	eDNA: Environmental deoxyribonucleic acid
ANOVA: Analysis of variance	EIA: Enzyme immunoassay
AQUAB: UAB fish animal facilities	elf-1α: Elongation factor-1 α
ASR: Aquatic surface respiration	ELISA: Enzyme-linked immunosorbent assay
ASW: Artificial salt water	eno: Enolase
ATP: Adenosine triphosphate	eRNA: Ribonucleic acid
BALT: Buccal associated lymphoid tissue	Et: Ethyl acetate
BSC: Brain–sympathetic–chromaffin	FAO: Food and agriculture organization of the United Nations
cAMP: Cyclic adenosine monophosphate	FAWC: Farm animal welfare committee
CAT: Catalase	FBS: Fetal bovine serum
cath1: Cathepsin 1	FW: Forward
cDNA: Complementary DNA	GALT: Gut-associated lymphoid tissue
cfos: c-Fos protein	gadph: Glyceraldehyde-3-phosphate dehydrogenase
CREB: cAMP responsive element binding protein	GIALT: Gill-associated lymphoid tissue
CRH: Corticotropin releasing hormone	GLC: Glucose
CSR: Cellular stress response	gr: Glucocorticoid receptor
CSRS: Cutaneous system of response to stress	gr1: Glucocorticoid receptor 1
CoA: Coenzyme A	gr2: Glucocorticoid receptor 2
COL: Cholesterol	GzLM: Eeneralised linear model
CV: Variation coefficient	H1: Hypoxic shock 1
	H2: Hypoxic shock 2

H3: Hypoxic shock 3	MCHC: Mean corpuscular haemoglobin concentration
HBSS: Hank's balanced salt solution	MCV: Mean corpuscular volume
HGB: Haemoglobin concentration	MHC: Mean haemoglobin concentration
HCT: Haematocrit	MIQE: Minimum information for publication of quantitative real-time PCR experiments
hif: Hypoxia-inducible factor	mr: Mineralocorticoid receptor
hif1α: Hypoxia-inducible factor-1 alpha	MS-222: Tricaine methanesulfonate
hif1β: Hypoxia-inducible factor-1 beta	mRNA: Messenger RNA
HPA: Hypothalamus-pituitary-adrenal	muc18: Mucin 18
HPI: Hypothalamus-pituitary-interrenal	N₂: Nitrogen gas
HRE: Hypoxia response element	NaHCO₃: Sodium bicarbonate
hsp: Heat shock protein	NALT: Nasal-Associated Lymphoid Tissue
hsp70: Heat shock protein 70	NADPH: Nicotinamide Adenine Dinucleotide Phosphate
IEG: Immediate early gene	NF: Nuclear factor
IFN: Interferon	NO: Nitric oxide
igf1: Insulin-like growth factor 1	NP: Nanoplastic
igt: Immunoglobulin T	OWI: Operational welfare indicator
il-6: Interleukin-6	PAMP: Pathogen-associated molecular pattern
il-8: Interleukin-8	PBS: Phosphate buffer saline
il-10: Interleukin-10	PCD: Programmed cell death
il-12: Interleukin-12	PCR: Polymerase chain reaction
il-1β: Interleukin-1 beta	PE: Polyethylene
IU: International unit	PDI: Polydispersity index
JAK / STAT: Janus kinase and signal transducer and activator of transcription	POD: Peroxidase
LABWI: Laboratory welfare indicator	PRR: Pattern recognition receptor
LPS: Lipopolysaccharides	pH: Hydrogen potential
M: Methanol	PhALT: Pharyngeal-associated lymphoid tissue
MALT: Mucosal-associated lymphoid tissue	PLT: Platelet
MC: Manipulated control	ppar: Peroxisome-proliferator-activated receptor

ppar α : Peroxisome-proliferator-activated receptor alpha

PS: Polystyrene

PSNP: Polystyrene nanoplastic

psu: Practical salinity unit

QC: Quality control

qPCR: Real-time quantitative PCR

RAS: Recirculated aquaculture system

RASOPTA: Recirculated aquaculture systems optimisation for production and training in aquaculture

RBC: Red blood cell

RDW: Red blood cells distribution widening

RDW-SD: Red blood cells distribution widening - standard deviation

RNA: Ribonucleic acid

RNAseq: RNA sequencing

rps5: Ribosomal protein S5

rps20: Small ribosomal subunit protein uS10

ROS: Reactive oxygen species

RRBC: Rabbit red blood cell

RTgutGC: Rainbow trout intestinal epithelial cell line

RTS11: Rainbow trout spleen cell line

RV: Reverse

SALT: Skin-associated lymphoid tissue

SDG: United Nation's Sustainable Development Goal

sirt1: Sirtuin 1

socs: Suppressor of cytokines signalling proteins

socs1: Suppressor of cytokine signalling 1

sod: Super-oxide dismutase

SPE: Solid phase extraction

SEM: Standard error of the mean

TG: Triglycerides

tgf β 1: Transforming growth factor beta 1

TLR: Toll like-receptor

tnf α : Tumour necrosis factor alpha

UAB: Autonomous University of Barcelona

W: Water

WBC: White blood cell

Abstract

Fish mucosal tissues form the primary barrier between the animal and the aquatic environment and represent a critical interface where neuroendocrine, immune, and cellular stress pathways converge. Despite increasing attention to mucosal health in aquaculture, important knowledge gaps remain regarding how each mucosa contributes to the overall stress response and how responses are reshaped under repeated or combined stressor exposure, which is common both in natural ecosystems and production facilities.

Within this framework, this thesis examines fish mucosal response in two aquaculture-relevant species: rainbow trout (*Oncorhynchus mykiss*), which is sensitive to low oxygen concentration, and gilthead seabream (*Sparus aurata*), a euryhaline Mediterranean species frequently exposed to variable salinity and husbandry stressors. The overarching aim is to provide an integrated characterisation of how environmental and aquaculture-associated stressors modulate responses at both the functional (haematology and plasma biochemistry) and molecular (mucosal gene expression) levels. The central hypothesis is that mucosal tissues can mount local stress responses that differ across tissues, due to distinct stressors generating specific “signatures”, and the response severity and persistence depend on stressor intensity and duration. To test this, the thesis combines *in vivo* and *in vitro* approaches, assessing endocrine and metabolic markers (plasma cortisol, glucose, lactate; regulation of *gr1 / mr* receptors), haematological indicators (RBC, HGB, WBC and PLT), and hypoxia and cellular stress (*hif1 α* , *hsp70*), and immune antioxidant pathways (*il-1 β* , *il-10*, *sod*, and *cat*), emphasizing in the comparison among mucosae.

In rainbow trout, the effects of repeated acute hypoxia are evaluated using controlled episodes (2 mg O₂/L for 1 h) applied once, twice, or three times, with sampling at 1, 6, and 24 h post-exposure. The results support functional habituation: with repeated exposure and adequate recovery intervals, several haematological and metabolic variables tend to return toward baseline, consistent with improved tolerance. Importantly, responses are tissue specific across mucosae. Gills display distinct modulation of local endocrine sensitivity and hypoxia signalling, including patterns in *hif1 α* and *myoglobin* that differ from those observed in skin and gut, highlighting specialized strategies aligned with gill physiology and oxygen exchange.

In gilthead seabream, the thesis addresses both combined chronic stressors and acute husbandry challenges. A month long co-exposure study tests high salinity (55 psu) and polystyrene nanoplastics (PSNPs), alone and in combination. Responses are strongly mucosa dependent: the intestine emerges as the most responsive tissue, showing coherent activation of endocrine, antioxidant, and immune gene programs across treatments; gills respond prominently to salinity; and skin shows pronounced responses to PSNPs, while the combined condition produces additive or non linear outcomes, including antagonistic regulation in skin. Systemic signals such as changes in WBC, PLT, and ADA activity further suggest that combined exposures can intensify physiological challenge even when circulating metabolites remain largely unchanged. Acute stressor comparisons (anoxia and high density) reveal rapid but mostly transient transcriptional changes that differ by tissue and stressor type. Crowding predominantly affects gills (immediate early and immune markers with delayed dynamics), whereas air exposure primarily activates skin hypoxia and stress regulation pathways, with more limited intestinal involvement and an overall trend toward recovery by 24 h.

Finally, an *in vitro* intestinal epithelial model (RTgutGC) shows that cortisol can modulate LPS induced immune responses, supporting a nuanced regulatory role for stress hormones in mucosal immunity rather than a uniform suppression.

Overall, this thesis demonstrates that mucosal tissues operate under distinct regulatory logics and that systemic recovery can mask persistent or specialized local responses. These findings support the use of multi marker, multi tissue frameworks for stress and welfare assessment and provide evidence relevant to aquaculture management, including the importance of limiting co stressors, ensuring recovery windows, and incorporating non invasive monitoring tools such as water borne cortisol.

Resumen

Las mucosas de los peces constituyen una barrera defensiva entre el ambiente y el medio acuático. Además de ser una barrera defensiva, se integran otras respuestas como la endocrina, o antioxidantes que se activan en la presencia de un factor estresante. A pesar de su importancia, aún existen lagunas relevantes sobre el papel específico de cada mucosa frente a los factores estresantes de distinta naturaleza e intensidad, y especialmente sobre lo que ocurre cuando los peces afrontan estresores de forma repetida o combinada, situación frecuente tanto en ambientes naturales como en sistemas de producción. En este marco, la presente tesis estudia la respuesta mucosal inmuno-endocrina en dos especies clave para la acuicultura: la trucha arcoíris (*Oncorhynchus mykiss*), particularmente sensible a la hipoxia, y la dorada (*Sparus aurata*), especie eurihalina representativa de la producción mediterránea.

El objetivo general de la tesis es caracterizar de manera integradora cómo estresores ambientales y asociados al manejo en acuicultura modulan la respuesta a nivel funcional (hematología y bioquímica plasmática) y molecular (expresión génica en mucosas). La hipótesis de partida plantea que las mucosas pueden generar respuestas locales de estrés con patrones diferentes entre tejidos; relacionados con su función; y que la duración y la intensidad determinan la magnitud y persistencia de la respuesta. Para ello, se combinan aproximaciones *in vivo* e *in vitro*, evaluando biomarcadores endocrinos (cortisol y regulación de receptores *gr1/mr*), metabólicos (glucosa y lactato), hematológicos (RBC/HGB, WBC y PLT), así como marcadores de hipoxia/estrés celular (*hif1 α* y *mioglobina*) y vías inmunes y antioxidantes (interleuquinas, *sod/cat*, *hsp70*) con especial atención a las diferencias entre mucosas.

En trucha arcoíris, se analiza el impacto de episodios repetidos de hipoxia (2 mg O₂/L durante 1 h) aplicados una, dos o tres veces, con muestreos a 1, 6 y 24 h tras la exposición. Los resultados evidencian habituación funcional: tras exposiciones repetidas, varios indicadores hematológicos y metabólicos tienden a normalizarse, sugiriendo tolerancia cuando se dispone de intervalos de recuperación adecuados. A nivel mucosal, se observan respuestas dependientes del tejido: las branquias muestran una regulación particular del eje endocrino local y marcadores de hipoxia en los que la relación entre *hif1 α* y *mioglobina* difiere respecto a piel e intestino, apoyando mecanismos de adaptación especializados según la función fisiológica de cada mucosa.

En dorada, se evalúan tanto estresores combinados como agudos. En un ensayo crónico, se estudia la exposición a salinidad elevada (55 psu) y nanoplásticos de poliestireno (PSNPs), solos o combinados. Los resultados muestran una marcada especificidad por mucosa: el intestino destaca como tejido altamente reactivo, con activación consistente de marcadores endocrinos, antioxidantes e inflamatorios; las branquias responden de manera robusta a la salinidad; y la piel refleja respuestas pronunciadas frente a PSNPs, mientras que la combinación puede generar efectos aditivos o no lineales (incluyendo modulaciones opuestas en piel). Además, algunos cambios sistémicos (p. ej., en WBC / trombocitos y actividad ADA) indican que la coexposición puede intensificar el desafío fisiológico incluso cuando metabolitos circulantes no varían significativamente. Complementariamente, se comparan dos estresores agudos habituales en acuicultura: anoxia por exposición al aire y alta densidad. Se detectan respuestas transitorias y dependientes del tejido: el hacinamiento afecta predominantemente a branquias (marcadores inmediatos e inmunes con respuestas retardadas), mientras que la exposición al aire activa preferentemente la piel (marcadores de hipoxia y regulación de estrés), con implicación menor del intestino y retorno general a las 24 h.

Finalmente, los experimentos *in vitro* con la línea intestinal RTgutGC muestran que el cortisol puede modular la respuesta inducida por LPS, apoyando un papel regulador específico de la inmunidad epitelial bajo estrés.

En conjunto, esta tesis demuestra que las mucosas operan bajo lógicas reguladoras propias y que una aparente recuperación sistémica puede coexistir con respuestas locales persistentes o especializadas. Estos hallazgos refuerzan la necesidad de un enfoque donde se estudien diferentes amrcadores y tejidos para interpretar estrés y bienestar, y aportan conocimiento aplicable a la gestión en acuicultura (p. ej., minimizar co-estresores, asegurar tiempos de recuperación e incorporar métricas no invasivas como el cortisol en agua).

Resum

Les superfícies mucoses dels peixos constitueixen la primera línia de defensa i un punt de contacte permanent amb el medi aquàtic, on es produeix una integració estreta entre la resposta neuroendocrina, la immunitat mucosal i els mecanismes de resposta cel·lular a l'estrès. Malgrat els avenços recents, continua sent limitat el coneixement sobre el paper específic de cada mucosa davant dels diferents factors estressants i com varia la resposta en funció de la naturalesa i intensitat i com a conseqüència la manera com les respostes canvien quan els estressors actuen de forma repetida o combinada, una situació habitual tant a la natura com en sistemes d'aqüicultura.

En aquest context, la tesi explora la resposta mucosal del peixos a diferents factors estresant únics o en combinació en dues espècies d'alta rellevància productiva: la truita arc iris (*Oncorhynchus mykiss*), especialment sensible a la hipòxia, i l'orada (*Sparus aurata*), espècie eurihalina i representativa de la producció mediterrània. L'objectiu general de la tesi ha estat caracteritzar, amb un enfoc integrador, com els estressors ambientals i de manipulació associats a l'aqüicultura modulen la resposta a nivell funcional (hematologia i bioquímica plasmàtica) i molecular (expressió gènica en mucoses). Es parteix de la hipòtesi que les mucoses poden desplegar respostes locals d'estrès que difereixen segons el teixit, que cada estressor produeix signatures específiques, i que la durada i intensitat determinen la severitat i la persistència de la resposta. Per abordar-ho, la tesi combina estudis *in vivo* i *in vitro*, incorporant biomarcadors endocrins (cortisol i modulació de receptors *gr1 / mr*), metabòlics (glucosa i lactat), hematològics (RBC / HGB, WBC i PLT), i marcadors de vies d'hipòxia / estrès cel·lular (p. ex. *hif1 α* , *mioglobina*) i d'inflamació/antioxidants (p. ex. interleuquines, *sod/cat*, *hsp70*), fent una comparativa entre mucoses.

En el primer bloc, s'avalua l'efecte de la hipòxia repetida en truita mitjançant episodis aguts (2 mgO₂/L durant 1 h) aplicats una, dues o tres vegades i mostreig a 1, 6 i 24 h post-exposició. Els resultats indiquen una habituació funcional: després de episodis repetits, diversos paràmetres hematològics i metabòlics tendeixen a normalitzar-se, suggerint tolerància quan hi ha temps de recuperació entre episodis. Paral·lelament, es detecten patrons mucosa-específics: brànquies mostren una regulació particular d'eixos endocrins locals (augment de *gr1* i *mr* en determinats punts) i una modulació de marcadors d'hipòxia on la relació entre *hif1 α* i *mioglobina* difereix de pell i intestí. Això apunta a mecanismes d'adaptació diferenciats entre teixits, en línia amb les seves funcions fisiològiques distintives.

En orada, la tesi aborda la complexitat dels estressors combinats i aguts. D'una banda, s'analitza una coexposició crònica a salinitat elevada (55 psu) i nanoplàstics de poliestirè (PSNPs), sols o combinats. Aquesta aproximació revela respostes clarament dependents de la mucosa: l'intestí emergeix com el teixit més reactiu, amb activació coherent de marcadors endocrins (*gr*, *mr*), antioxidants (*sod*, *cat*) immunes (*il-1 β* , *il-10*); les brànquies responen de forma destacada a la salinitat, i la pell mostra respostes marcades a PSNPs, mentre que la combinació pot induir efectes additius o no lineals (incloent regulacions oposades en pell). A nivell sistèmic, alguns indicadors hematològics i enzimàtics (p. ex. canvis en WBC, PLT i ADA) reforcen que la coexposició pot intensificar el desafiament fisiològic encara que els metabòlits circulants no sempre canviïn. D'altra banda, es comparen dos estressors aguts típics de l'aqüicultura: anòxia per exposició a l'aire (minuts) i una elevada densitat (cultius intensius). Els resultats mostren respostes transitories i molt teixit dependents: l'alta densitat afecta sobretot brànquies (marcadors immediats i immunitaris amb resposta retardada), mentre que l'exposició a l'aire activa preferentment la pell (marcadors d'hipòxia i regulació metabòlica/estrès) i, en menor mesura, l'intestí, amb recuperació general al cap de 24 h.

Finalment, els experiments *in vitro* amb la línia intestinal RTgutGC indiquen que el cortisol pot modular la resposta inflamatòria induïda per LPS, donant suport a una regulació específica (més que una immunosupressió inespecífica) de l'epiteli intestinal sota condicions d'estrès.

En conjunt, la tesi demostra que les mucoses operen amb lògiques reguladores pròpies i que la recuperació sistèmica pot coexistir amb senyals locals persistents o especialitzats. Aquest coneixement reforça la necessitat d'un enfocament multimarcador i multiteixit per avaluar estrès i benestar, i aporta evidència útil per optimitzar el maneig en aqüicultura (p. ex. minimitzar coestressors, garantir períodes de recuperació i incorporar eines no invasives com la mesura de cortisol a l'aigua).

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1

Introduction

1.1 Stressors in aquaculture

1.1.1 Stress

There are different definitions for the "stress" concept since there is no clear definition, and it has changed and refined over the years (Barton, 2002). In the present work, the definition that was adopted is "The physiological cascade of events that occurs when the organism is attempting to resist death or re-establish homeostatic norms in the face of insult", defined by Schreck (2000). Another important concept is "stressor", which is the stimulus that causes this stress response (Schreck and Tort, 2016). Although the stress is often viewed negatively by and linked to harmful outcomes, by the society, it should be understood as a fundamental response mechanism with an inherently adaptive role aimed at supporting survival (Dhabhar, 2014). Exposure to low levels of stress can produce beneficial or positive effects, known as eustress, whereas more intense stressors trigger adaptive responses but may also lead to adverse or maladaptive effects, referred to as distress (Schreck and Tort, 2016).

The stress response unfolds through three sequential phases, which are activated according to the intensity and nature of the stressor, as well as the animal's previous experience (Wendelaar Bonga, 1997). Two principal neuroendocrine axes mediate this response: the Brain–Sympathetic–Chromaffin (BSC) axis and the hypothalamus–pituitary–interrenal (HPI) axis, responsible for the release of catecholamines and corticosteroids, respectively (Dara et al., 2023). The HPI axis is exclusive for fish, but it is functionally equivalent to the hypothalamus–pituitary–adrenal (HPA) axis described in mammals (Harris and Carr, 2016). Following exposure to a stressor, both axes become activated, and since adrenergic and glucocorticoid receptors (GRs) are widely distributed across tissues, these signalling molecules can affect numerous organs, including those involved in immune and defence functions (Tort et al., 2024). A key difference between these axes lies in their activation dynamics: adrenaline is released within seconds and is rapidly cleared from circulation, whereas cortisol levels rise more slowly, over minutes to hours, in response to stress (Vijayan et al., 2010). Consequently, assessing stress is generally more straightforward through the evaluation of HPI axis activity, which explains the focus on this pathway throughout the thesis.

Activation of the HPI axis corresponds to the primary stress response (Bernier and Peter, 2001). This occurs when the central nervous system (CNS) recognises the stressful stimulus, then the hypothalamus synthesises corticotropin-releasing hormone (CRH), which triggers the release of adrenocorticotrophic hormone (ACTH) in the anterior pituitary. This hormone travels through the blood until it reaches the interrenal tissue, where it is recognised by a receptor, stimulates the activation of a signalling cascade that ultimately leads to the synthesis of glucocorticoids such as cortisol in the head kidney.

The primary response involves the activation of brain centres, leading to the release of catecholamines and corticosteroids. In contrast, the secondary response refers to the immediate physiological effects of these hormones at the blood and tissue levels, including metabolic adjustments, increased cardiac output, enhanced oxygen uptake, mobilisation of energy reserves, and disruptions in hydromineral balance. Finally, the tertiary response operates at the organismal and population levels, reflecting changes in overall performance such as reduced growth, altered reproductive function, modulation of the immune system, and a decreased ability to cope with additional or subsequent stressors (Wendelaar Bonga, 1997) (Figure 1.1).

1.1.2 Aquaculture as a production system

Aquaculture, defined as the farming of aquatic organisms, has been the fastest-growing food production sector worldwide since the 1990s (Finegold, 2009). Between 2020 and 2022, it surpassed capture fisheries for the first time, accounting for 59 % of total global fish production (FAO, 2024). Projections indicate that an additional 40 million tonnes of aquatic food will be required by 2030 to sustain current per capita consumption levels (Aich et al., 2020). Aquatic systems are now widely acknowledged as essential contributors to food and nutrition security. Nevertheless, further efforts are needed to meet the demands of an expanding and increasingly urban population. Owing to their diversity and their ability to deliver ecosystem services while supporting nutritious diets, aquatic food systems offer a practical and efficient means to enhance global food security and nutrition, both at present and in the future (FAO, 2024). However, intensive aquaculture practices may lead to reduced environmental quality, including higher stocking densities and an increased incidence of production-related diseases, among other challenges faced by the industry (Martos-Sitcha et al., 2020).

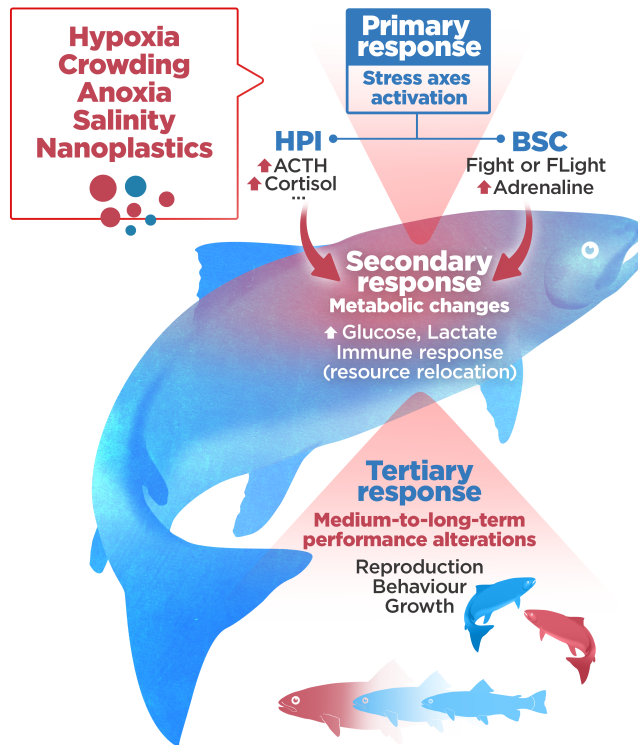


Figure 1.1: Representation of three steps of the stress response (primary, secondary and tertiary) due to the different stressors used in this thesis and the changes related to each response.

Over the past two decades, the global aquaculture sector has continued to expand while progressively meeting key objectives related to environmental, economic, and social sustainability (Boyd et al., 2020). In addition to food production, aquaculture can serve as a bioremediation approach, contributing to the removal of pollutants from aquatic environments and supporting the restocking of wild populations (Domingues et al., 2022). Traditionally, in aquaculture, the most common systems were ponds or flow-through systems, but these systems involved a high environmental impact and are now declining (Bostock et al., 2016).

Moreover, recirculated aquaculture system (RAS) is usually combined during production with other systems, such as cages, often involving RAS in the hatchery and early grow-out phases, with fish being transferred to cages after reaching a certain size for the final grow-out step, until they reach commercial size and are, therefore, ready to be consumed (Bostock et al., 2016).

Today, an increasing number of aquaculture companies use RAS, although some aspects, such as the impact of high stocking densities and the cost of technology, may represent a constraint (Balami, 2021; Aich et al., 2020). On the one hand, RAS present many advantages, including the partial reuse of the water after undergoing proper treatment to remove the fish waste by-products and adding oxygen, thus improving the effluent quality (Ramírez-Godínez et al., 2013). Moreover, these systems are isolated from the external environment, involving a strict control of the environmental parameters to increase productivity (Gonçalves and Gagnon, 2011; Malone, 2013) and reduced possibility of escapes and the transmission of diseases. Finally, in RAS, it is possible to raise large quantities of fish in much smaller volumes of water (Bregnballe, 2022). On the other hand, the main disadvantage of RAS, is the high cost of the equipment (tanks, filtering, system, pumps). Also, since it is an environmentally isolated system, it is necessary to maintain all parameters at an optimal range of the specific cultured species (nitrogenous compounds, oxygen, temperature), otherwise it can be a stressful environment for the fish, leading to mortality events (Aich et al., 2020).

In summary, thanks to the improvements in the aquaculture sector, it is possible to start thinking about a sustainable aquaculture activity to ensure the environmental viability and the welfare of the cultured organisms to reach the United Nation's Sustainable Development Goals (SDG), with the aim of establishing a universal call to action to eradicate poverty, safeguard the planet, and ensure that by 2030 all people can enjoy peace and prosperity. In this context aquaculture can help to reach the SDG2, SDG3, SDG6, SDG8, SDG12, SDG13, SDG14, SDG15 and SDG 17 (Figure 1.2).



Figure 1.2: The United Nations' Sustainable Development Goals (SDGs) provide a shared framework of guidelines agreed upon by all Member States. The content of this publication has not been endorsed by the United Nations and does not necessarily represent the views of the organisation or those of its official Member States.

1.1.3 Types of stressors

Stressors can be classified in different ways, depending on the duration (acute or chronic), the frequency (single, multiple / combined or repeated) and the origin (biotic or abiotic) (Madaro et al., 2015; Schreck and Tort, 2016).

Starting with the duration of the stressor, there are two main groups, namely, acute and chronic stress. Acute stress is a stress that lasts for a period of minutes to hours, while chronic stress can persist for days to months (Dhabhar, 2000). The most common causes of acute stress responses are handling and abrupt environmental changes (Barcellos et al., 1999), while chronic stressors are more related to anthropogenic and husbandry activities and pollution (Tort, 2011). Acute stress is often more intense than chronic stress, although the latter is more persistent (Tort, 2011). Stressors may be specific or occur in isolation, and can arise either as single events or as repeated exposures over time. Fish can also be exposed to multiple stressors simultaneously, which may be either acute or chronic. This situation is common in natural environments, where environmental conditions cannot be controlled. Closely related to multiple stressors are sequential stressors, defined as single stressors that occur repeatedly within a short period (Figure 1.3). Both multiple and sequential stressors appear to induce cumulative effects on the stress response (Moberg and Mench, 2000). In these cases, the interval between stressors is critical, as stressors occurring in close temporal proximity may be perceived by the animal as a single event (Barton et al., 1985). Additionally, fish may experience repeated stressors, consisting of repetitions of the same stressor occurring on consecutive occasions, with either predictable or unpredictable frequency, which can differentially affect the stress response. In some cases, repeated stressors are considered equivalent to a single chronic stressor (Thomson et al., 2020).

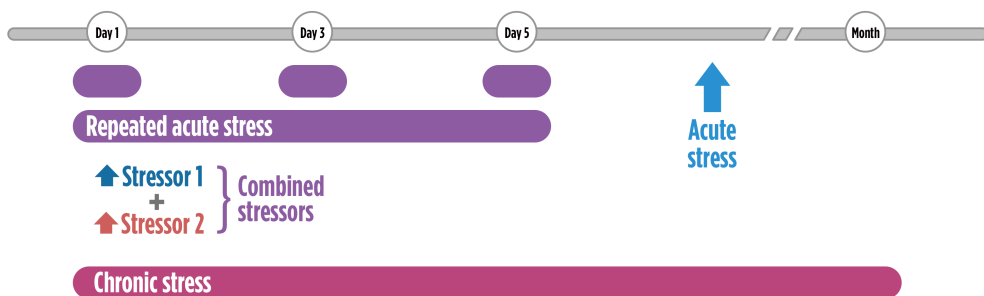


Figure 1.3: Representation in a time manner of the different stressors classified by the duration.

Moreover, stressors can be classified depending on the origin in abiotic or biotic. Abiotic stressors encompass variations in temperature, water quality, hypoxia, the presence of pollutants, as well as changes in osmotic and ionic conditions. In contrast, biotic stressors are primarily related to the impact of potentially pathogenic microorganisms, including viruses, bacteria, and parasites, in addition to pressures arising from predation and both intra- and interspecific competition (Chakraborty and Krishnani, 2022). For instance, social stressors such as hierarchy (dominance), crowding-caused aggressiveness, or even possible perceived threats can have an impact on the homeostasis of the animal (Tort, 2011; Schulte, 2014). Additionally, artificial environments such as those found in aquaculture rearing systems can themselves act as sources of stress. Factors including high stocking densities, social interactions, handling procedures, transport, sampling, and seasonal disease outbreaks can all contribute to allostatic load (Tort et al., 2024).

Despite significant improvement of the aquaculture sector, the industry still faces major challenges from climate change, environmental disasters, water scarcity, pollution, including micro and nanoplastics, biodiversity loss and other anthropogenic impacts (FAO, 2024). Cultured organisms are likely to be exposed to several simultaneous stressors since there is little to no possibility of escape (Madaro et al., 2015). The threat of climate change due to anthropogenic greenhouse gas emissions to the global food system is well recognised and a major concern for food security, sustainability, and resilience of the systems on which humans rely (Froehlich et al., 2022; Blanchard et al., 2017; Thiault et al., 2019).

RAS, being isolated from the natural environment, offers the opportunity to dismiss all the aforementioned environmental stressors if the maintenance and structure of the facility is adequate (Bregnballe, 2022; Pino et al., 2021), but other stressors related to the stocking density, and the soundscape or photoperiod are still present (Radford and Slater, 2019).

1.2 Stress response

The stress response is intended to ensure the survival and maintenance of biological equilibrium of the organisms in front of a challenging situation that can be real or perceived, involving both physiological and biochemical rearrangements and reallocation of energy available for processes involved in the so-called fight, flight, or coping responses (Schreck and Tort, 2016). The magnitude and duration of this response are influenced not only by the severity and persistence of the stressor, but also by the extent to which the challenge can be predicted or controlled (Pottinger, 2008; Schreck and Tort, 2016).

1.2.1 Systemic stress response

As mentioned above, systemic responses can be divided into three categories: the primary response, characterised by the activation of both BSC and HPI axes driven by the neuroendocrine regulatory systems; the secondary response, involving the physiological and biochemical response; and the tertiary response, which is related to the longer-term performance of the animal. The response starts when the stimulus (stressor) is perceived by the limbic system in the brain (Herman et al., 2005). Although both the BSC and HPI axes are activated concurrently, their responses originate in different parts of the body. Upon perception of the stressor, cells in the hypothalamus release corticotropin-releasing hormone (CRH), which is transported to the pituitary gland. There, CRH stimulates adrenocorticotrophic hormone (ACTH)-producing cells to synthesise and secrete ACTH. This hormone then acts on the interrenal cells in the head kidney, promoting the production and release of cortisol into the bloodstream (Schreck and Tort, 2016). Cortisol is generally regarded as the principal hormone mediating the physiological stress response in vertebrates (Martos-Sitcha et al., 2020).

Both CRH and ACTH can exert direct effects on the secondary stress response. ACTH, in particular, acts both centrally at the level of the brain (Clements et al., 2002) and peripherally on target tissues (Schreck and Maule, 2003). The subsequent release of cortisol and catecholamines initiates the secondary response, which comprises a range of physiological adjustments, including cardio-respiratory changes (Figure 1.4). In fish, these responses involve increases in respiratory capacity, heart rate, stroke volume, and blood flow to the gills, and may also be accompanied by the mobilisation of carbohydrate and lipid reserves, caused by the catecholamines. In contrast, cortisol regulates the hydromineral balance and energy metabolism (Wendelaar Bonga, 1997).

Finally, the tertiary stress response is the long-term response that most often involves negative or deleterious effects on performance. For instance, cortisol is known to cause immune suppression, thus preventing autoimmune reactions (Nesse et al., 2007). Similarly, the generally suppressive effects of cortisol on reproductive functions (Schreck et al., 2001) and growth (McCormick et al., 1998) can be interpreted as a strategic reallocation of resources away from non-essential processes until the organism's survival is secured. Alongside these physiological changes, behavioural adjustments also occur, such as heightened alertness and vigilance, the expression of escape or freezing responses, and modifications in cognitive performance (Johnson et al., 1992).

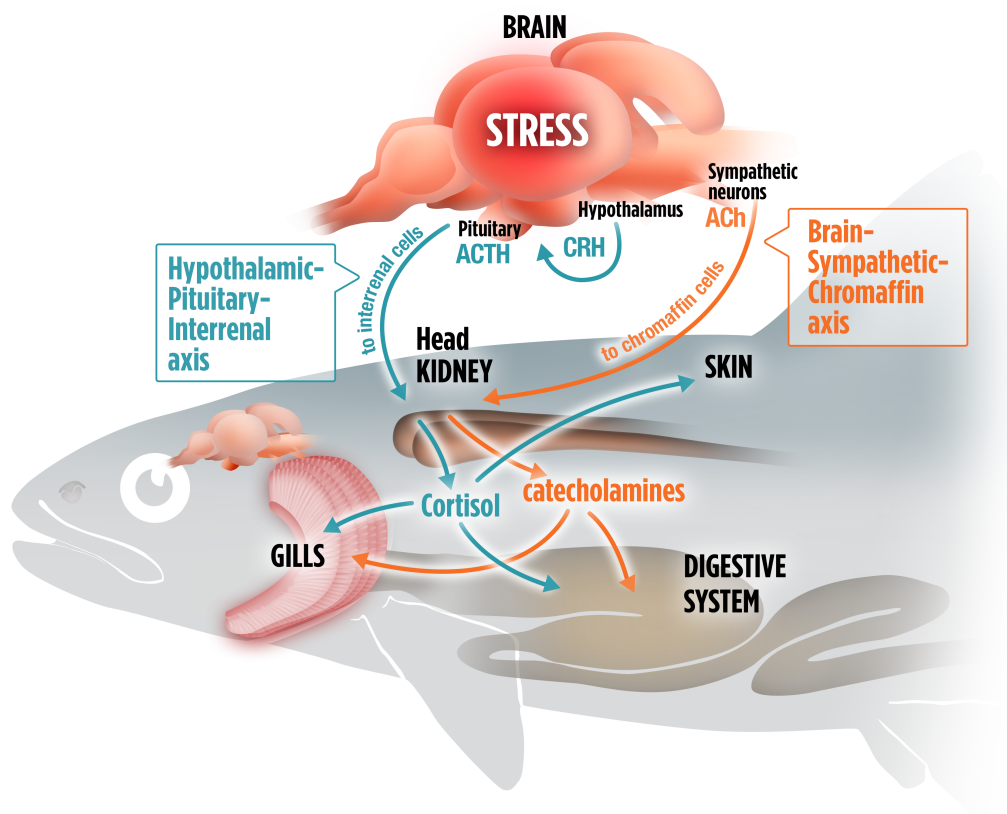


Figure 1.4: Representation of the hormones and tissues involved in the stress response at organism level.

Many of these actions, and particularly the ones associated with hormones such as CRH, ACTH or cortisol, depend on the interaction of the hormone with its specific receptors. With respect to cortisol receptors, two glucocorticoid receptors (GR1 and GR2) and one mineralocorticoid receptor (MR) have been described in fish. These receptors exhibit distinct functional properties, which may explain the tissue-specific responses to cortisol. Studies indicate that GR1 is primarily sensitive to elevated plasma cortisol levels and acts as a key mediator of the stress response following acute stress. In contrast, GR2 shows higher sensitivity to lower cortisol concentrations and is involved in fine-tuning cortisol-mediated immune, metabolic, and energetic processes (Verburg-van Kemenade et al., 2017).

1.2.2 Mucosal response

Fish have three large mucosal surfaces (*i.e.* skin, gut, and gills), and three small mucosal surfaces (*i.e.* olfactory, buccal and pharyngeal organs), and most research has studied the first three, which are the ones this thesis has focused on (Figure 1.5). Although each mucosa shows particular physiological functions, all are well recognised for being the first line of defence, since they provide the interface between the external and internal environment (Cabillon and Lazado, 2019). All mucosae display high levels of plasticity and respond to changes in the aquatic environment when stressors occur. Therefore, mucosae can give information about the organism and the environment at the same time. In recent years, extensive studies have been directed to understand the role of mucosae in the overall health and welfare of fish, aiming to secure optimum conditions in controlled aquaculture environments (Lazado et al., 2015).

In this context, the health status of fish and the impact of stressors should be considered not only at the whole-organism level but also at peripheral sites, particularly the mucosal surfaces. Given that fish remain in constant contact with the surrounding aquatic environment, tissues such as the skin, gills, and gut are continuously exposed to a wide range of microorganisms, and each of these mucosal interfaces is capable of mounting immune responses. Stressors can alter the integrity and function of these mucosal barriers, triggering an initial local response to external challenges that may progress to a systemic response depending on the intensity and/or duration of the stimulus. This process involves the activation of defence mechanisms and the production of various mediator molecules (Tort et al., 2024).

In most fish species, the gills serve as the primary organ for aquatic respiration. This tissue is characterised by an intricate vascular network, enclosed by an extensive epithelial surface that forms a thin barrier facilitating the exchange of gases between the bloodstream and the surrounding water. Beyond their respiratory function, the branchial epithelium plays a central role in a variety of physiological processes, including the regulation of osmotic and ionic balance, the maintenance of acid–base homeostasis (*i.e.* metabolic and respiratory compensation and acid–base excretion), and the elimination of nitrogenous waste products (Shi Dan et al., 2015; Evans et al., 2005).

The intestinal tract is the main site for digestion and nutritional uptake, therefore a key organ in fish nutrition (Kumar et al., 2005; Wang et al., 2018; Zhou et al., 2021). Moreover, intestinal homeostasis is a dynamic balance of the interaction between the intestinal mucosal barrier and intestinal microbiota, which plays an important role in maintaining overall health (Lippert et al., 2017). The intestine maintains homeostasis through selective permeability, allowing the organism to absorb critical nutrients and excrete waste (Leigh et al., 2023).

The fish skin is a multifunctional organ with highly relevant physiological roles. This surface is known for its remarkable mechanical properties: elasticity, resistance to penetration and environmental interactions (Vernerey and Barthelat, 2014). For example, the skin has been demonstrated to play an important structural role in fish locomotion by influencing the propagation of body waves (Long Jr et al., 1996) and by acting as an external tendon (Hebrank and Hebrank, 1986), but also possesses inherent hydrodynamic properties (Sudo et al., 2002) that are crucial for swimming efficiency. Kulczykowska (2019) summarized current understanding highlights about the role of the skin as a cutaneous stress-responsive system, in which cortisol, melatonin, and their derived peptides interact to help protect the organism under adverse conditions (Martos-Sitcha et al., 2020). This cutaneous system of response to stress (CSRS) represents an emerging and valuable source of information for assessing welfare status in fish. This system was defined for mammals and works in a similar way in fish. Under stress (after detection of cortisol) there is a stimulation of melatonin and different kuramines that helps to cope with the stressors (Kulczykowska et al., 2017).

Mucosal structures play a crucial role in immunity, and all of them have lymphoid tissue. Mucosal Associated Lymphoid Tissues (MALT) can be further divided into three principal lymphoid compartments: Skin-Associated Lymphoid Tissue (SALT), Gill-Associated Lymphoid Tissue (GIALT), and Gut-Associated Lymphoid Tissue (GALT). In addition, there are smaller lymphoid structures, including Nasal-Associated Lymphoid Tissue (NALT), Buccal-Associated Lymphoid Tissue (BALT) (Salinas, 2015), and Pharyngeal-Associated Lymphoid Tissue (PhALT) (Figure 1.5). One of the reasons of their important immune function is the existence of the mucus layer.

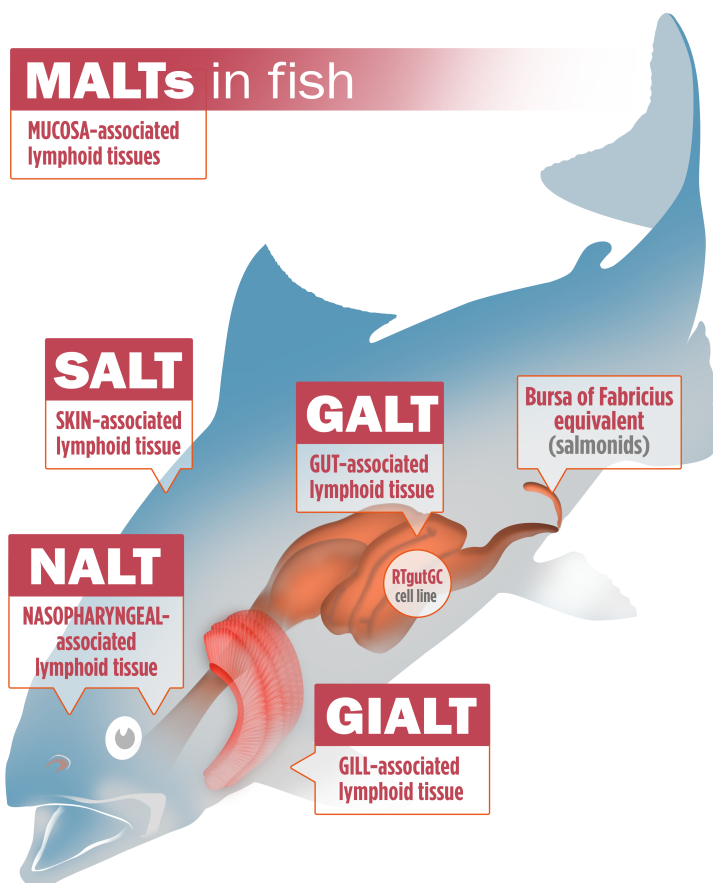


Figure 1.5: Representation of the different mucosal tissues on a fish.

This slimy polymer secreted by mucous and goblet cells contains inhibitory activity against pathogens, with potent molecules present in the matrix, including mucins, lysozymes, complement proteins, lectins, antimicrobial peptides, and immunoglobulins, among many others (Ellis, 2001; Nigam et al., 2012). The composition of the mucus and its properties are crucial for the survival of the organism. It has been observed that endogenous and exogenous factors can affect the composition and production of the mucus, increasing the susceptibility to pathogens. Mucus viscosity will determine the ability to block the entrance of different pathogens. Another function of the mucus is the intra- or interspecific communication. The objective of this communication is to attract cues enabling habitats or partners, alerting to danger, and or shoaling (Reverter et al., 2018).

Mucosal surfaces present similarities as they are all involved in local immune responses (i.e., the microanatomical structure and mucus, both serving as physical and chemical barriers), and each have distinct associated microbial communities (Cabillon and Lazado, 2019). These interactions involve complex recognition and defence mechanisms that enable fish to respond to microorganisms and other challenges linked to environmental factors, such as fluctuations in temperature or salinity, as well as the presence of chemical substances or contaminants. Moreover, the importance of mucosae-related immune function is further highlighted by their potential to start local responses that trigger a systemic response, thus playing a role as secondary lymphoid organs (MALTs) (Tort et al., 2024). Apart from the specific role of each mucosa (above-mentioned). Mucosa can start a local response based on two phases: the first is an inflammatory process by the release of cytokines, and the second is the presentation of the antigen that activates its function, recognising specific molecules. This elicits secondary responses, including processes involved in adaptive immunity. Environmental changes (i.e. oxygen, salinity) impact the overall welfare status, influencing the survival and resistance to pathogens due to the direct connection between the mucosal immune system and the environment (Cabillon and Lazado, 2019).

1.2.3 Cellular stress response

The cellular stress response (CSR) constitutes a highly conserved physiological mechanism that has evolved to enable organisms to cope with severe environmental stressors that cause damage to macromolecules. Key features of the CSR include the protection and repair of macromolecules, arrest of the cell cycle, redistribution of metabolic energy, repairing DNA, maintaining redox balance, and, where the limits of cellular tolerance are exceeded, the initiation of programmed cell death (Evans and Kültz, 2020) to remove the damaged macromolecules and organelles. Stressors can disrupt the structure and function of proteins, lipids, and DNA, ultimately leading to a loss of cellular homeostasis. In this context, the CSR acts as an early response aimed at stabilising, repairing, and safeguarding essential cellular components (Kültz, 2003). These macromolecules function within an intracellular environment that has evolved to differ markedly from external conditions (Somero et al., 2016). Certain aspects of this internal *milieu*, such as the intracellular concentrations of sodium and potassium ions, are tightly regulated to ensure that macromolecules maintain their optimal structure and function efficiently (Somero et al., 2016). Consequently, environmental changes

that alter the concentrations of inorganic ions or other solutes beyond their optimal ranges can induce structural modifications in macromolecules, impairing their function and necessitating compensatory cellular responses.

This difference explains why marine and freshwater fish adopt distinct osmoregulatory strategies. Marine teleosts must eliminate excess salts while absorbing water, whereas freshwater species need to take up salts and actively excrete water (Evans and Claiborne, 2008). Variations in environmental salinity, however, can lead to passive gains or losses of ions and water, thereby altering the chemical composition of both intra- and extracellular fluids. Such changes can disrupt the structure and function of proteins, DNA, and lipids (Evans and Kültz, 2020). Likewise, shifts in pH may modify the charge of amino acid side chains, which in turn can interfere with the non-covalent interactions that are essential for maintaining protein stability (Konermann, 2012).

One mechanism involved in restoring homeostasis is the accumulation of unfolded proteins, which activate components of the CSR that contribute to the re-establishment of protein stability under salinity stress. In addition, molecular chaperones, such as heat shock proteins (HSPs), are able to bind to denatured proteins and facilitate their refolding (Feder and Hofmann, 1999). The expression of these chaperones is regulated by the intracellular levels of misfolded proteins, providing a clear example of how macromolecular damage is linked to an appropriate adaptive response (Evans and Kültz, 2020).

Other molecules that can affect the homeostasis are the reactive oxygen species (ROS) (i.e. H_2O_2 , $\text{O}_2^{\cdot-}$, $\text{HO}^{\cdot-}$), produced during the energy production process in aerobic organisms. ROS can regulate different activities of NADPH, act as a secondary messenger, or alter DNA. In order to maintain this homeostasis, cells have stress response systems that are sensitive to changes in the cytosolic levels of ROS. So, when these changes are detected, changes in the transcription or antioxidant enzymes, such as SOD and CAT.

Nucleic acids, including DNA and RNA, are also susceptible to alterations in structure and function due to changes in intra- and extracellular ion concentrations, largely because of the negatively charged phosphate backbone that gives these molecules strong polyanionic properties (Auffinger et al., 2016). Deviations from the optimal intracellular balance of cations may therefore lead to abnormal interactions with nucleic acids, ultimately affecting the structural integrity and functional capacity of DNA and RNA (Evans and Kültz, 2020).

The CSR is also associated with changes in the abundance of metabolic enzymes that could facilitate such lipid-membrane rearrangements during salinity stress in fish. Long-chain fatty acid ABC transporter, multifunction beta oxidation protein, and long-chain fatty acid CoA ligase are all members of the so-called “minimal stress proteome” (Kültz, 2005). Moreover, stress responses are highly energy-demanding processes in fish and other organisms. Because of this, the organisms need to reallocate energy from the normal cellular response (growth, reproduction, and storage) to CSR-specific functions (macromolecular protection and repair; (Evans and Kültz, 2020)).

Programmed cell death (PCD) is a comprehensive term describing a range of highly conserved CSR mechanisms that function to remove irreversibly damaged cells, thereby reducing the risk of maladaptive genotypes and phenotypes in multicellular organisms. Under conditions of severe stress, several conserved PCD pathways can be activated, including both extrinsic and intrinsic apoptotic pathways, autophagic cell death (Gozuacik and Kimchi, 2007), and cathepsin D-mediated apoptosis (Carew et al., 2011).

Osmoregulatory hormones are thought to play a key role in regulating extrinsic apoptosis. For example, in fish exposed to salinity stress, the balance between apoptosis and cell proliferation in the gastrointestinal epithelium is modulated by hormones such as prolactin and cortisol (Sakamoto and McCormick, 2006). An important implication of these mechanisms is that protective responses which help maintain macromolecular integrity under one type of environmental stress may also enhance the organism’s ability to withstand other stressors. This phenomenon, known as cross-tolerance, is evident in fish subjected to both salinity and temperature challenges, as these stressors activate shared components of the CSR that act to prevent macromolecular damage (Todgham and Stillman, 2013).

Fish cell lines emerged as a new approach for environmental toxicology studies within aquaculture (Segner, 1998). Fish cell lines are *in vitro* culture of cells derived from fish tissues that provide a homogeneous and stable model for biological research (Nguyen et al., 2024). These cell lines retain many genetic and biochemical characteristics of the original tissue, enabling detailed studies on cellular responses to various stimuli (Freshney, 2015). The adaptability of fish cell lines to *in vitro* conditions offers several advantages over whole organism studies, including reduced variability, lower maintenance costs, and the ability to conduct experiments under better controlled conditions (Goswami et al., 2022), allowing

for control of experimentation on specific cellular responses. Moreover, the use of cell lines reduces the need for live animal testing, aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement) to enhance ethical research practices (Clark, 2018). Also, the use of cell lines enhanced reproducibility and the ability to systematically manipulate experimental conditions. The employment of fish cell lines for mechanistic studies in aquaculture toxicology, cell physiology or stress can offer profound insights into the cellular and molecular mechanisms of how stress agents affect aquatic organisms. Cellular responses which involve changes at the level of the entire cell are crucial for understanding the complex interactions between environmental stressors and biological systems (Nguyen et al., 2024).

1.3 Evaluation methodologies

A clear understanding and careful selection of suitable stress indicators are crucial for the early identification of stress and immunocompromising conditions, as well as for assessing welfare and preventing mortality events. While many stress indicators are common across fish species, their sensitivity and threshold values are typically species-specific and may also vary according to sex and life stage (Fanouraki et al., 2011; Samaras et al., 2016). The choice of methodology should therefore be made with consideration of multiple factors, including required equipment, level of technical difficulty, study objectives, and degree of invasiveness. It is also important to recognise that relying on a single indicator is likely to yield incomplete information and may lead to inaccurate interpretations. Consequently, a multi-indicator approach is recommended in order to obtain a more comprehensive evaluation of stress and overall welfare status in fish. In **Figure 1.6** a summary of some of the methodologies that can be performed on fish and their location is observed.

Methodologies can be classified into two different types of analysis: the functional and the molecular ones. On one hand, functional analyses are the ones that measure and reflect the integrated outcome. Some of these analyses include the measurement of hormones (i.e. cortisol) or metabolites (i.e. glucose, lactate, cholesterol), and behavioural tests. On the other hand, molecular analyses mainly consist of the measurement of the abundance of mRNA that can be more general, looking at genes of a particular pathway (i.e. qPCR, PCR) or more precise, measuring the regulation of all the genes of a pathway (i.e. RNAseq). Other

molecular and *omics* techniques can be microbiome metabolomics and proteomics. The relevance of having different approaches to the same question lies in the need for parallel measurements since, despite there being a great correlation between these, molecular changes are not necessarily reflected in functional ones (Blong et al., 2025).

Another key aspect to consider when selecting stress or welfare indicators is the level of invasiveness. Certain measures, referred to as non-invasive indicators, can be evaluated through straightforward visual observation, such as behavioural assessment, or through analyses of the surrounding water. For behaviour, there are different methodologies that are used to check different aspects of the stress and welfare, such as feeding activity, social behaviour, or swimming performance (Noble et al., 2018).

Another non-invasive methodology is the measurement of different parameters from the water, which can provide valuable information. To check if the environmental conditions are optimal for the fish it is possible to measure parameters such as pH, salinity, nitrogenous compounds, dissolved oxygen level, and temperature, among others (Noble et al., 2018). Additionally, to check if there are pathogens in the environment, there is the possibility to measure the environmental DNA / RNA (eDNA, and eRNA, respectively) (Benedicenti et al., 2024). Finally, to check some stress marker there is the possibility to measure cortisol levels in water (Sadoul and Geffroy, 2019).

Another source of non-invasive methodologies is the measurement on faeces content. In the faeces, cortisol (Sadoul and Geffroy, 2019), and microbiome analysis (Silva et al., 2020) are currently considered as valuable and representative options of stress and health status.

The next level is the little-invasive methodologies, which require at least the handling of the animal. This group includes all the parameters that can be measured in different matrices, such as blood, mucus or scales. Data from blood as haematological parameters, indicators of immune response (De Andrade et al., 2004), and genotoxicity (Barreto et al., 2017) are currently assessed. Additionally, from blood it is possible to extract the serum/plasma to measure parameters related to the physiological stress, hepatic health, oxidative stress, and metabolic status (Bertotto et al., 2010; Lulijwa et al., 2022). Other matrices where physiological stress can be measured and are less invasive are the mucus and the scales (Sadoul and Geffroy, 2019). Finally, the overall health status observation (e.g. presence

of wounds, deformities, presence of parasites) and performance (e.g. growth) can also be considered as little invasive assays (Ramírez-Godínez et al., 2013).

Finally, the lethal methodologies imply the euthanasia of the animals, as the extraction of the organs is required. In all the organs, techniques such as gene expression (qPCR, RNAseq), transcriptomics, physiological measurements and microbiome analysis can be done (Lama et al., 2020; Zhang et al., 2018). For endpoint analyses in the fillet quality in the muscle (Sánchez-Muros et al., 2013) can be assessed.

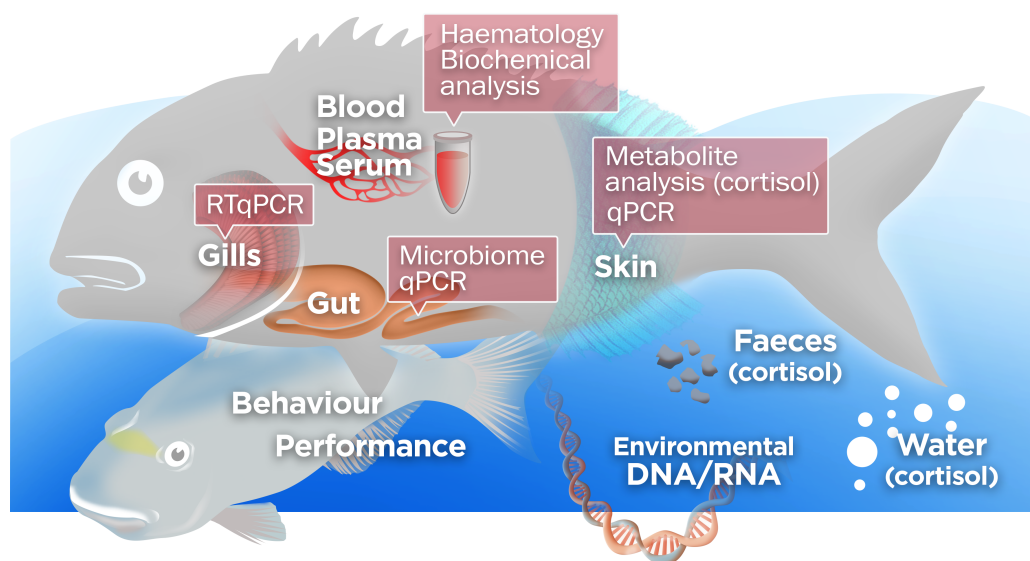


Figure 1.6: Examples of evaluation methodologies depending on the tissues.

1.3.1 Methodologies used during the thesis

1.3.1.1 Haematological analysis

The haematological analysis in chapter 3.1 and chapter 4 was done using the automated flow cytometer blood cell analyser Sysmex XN-1000V for veterinary use (Sysmex Corporation, Kobe, Japan). Internal quality control (QC) was performed daily using three levels of commercially available QC material (Sysmex XN Check level 1 or low range, level 2 or normal range, and level 3 or abnormal high range;

Sysmex Corporation, Kobe, Japan). The system was adapted for fish cells based on the avian model. Once the samples were collected, aliquots containing heparin (1:40) were maintained at 4 °C until the analysis was performed within 2 - 12 h after blood sampling.

1.3.1.2 Biochemical analysis

The biochemical analyses from chapters 3.1 and 3.2 were done in the UAB facilities. Glucose and lactate analyses were performed using colourimetric test kits (POD glucose and POD lactate, SPINREACT, Spain) following the manufacturer's recommendations. Plasma and mucus cortisol was also analysed using a commercial EIA kit (Cortisol ELISA Kit; Neogen ® Corporation, Ayr, United Kingdom) following the manufacturer's instructions.

In chapters 4 and 5, the biochemical parameters were analysed using an automatic analyser (Olympus Diagnostica, GmbH). All the parameters were determined in the plasma of fish using commercially available kits (Olympus Systems Reagents; Olympus Life and Material Science Europe GmbH, Hamburg, Germany) following the manufacturer's guidelines. Intra- and inter-variation coefficient (CV) was below 10 % in all cases.

1.3.1.3 Immunological assays

Immunological assays were only performed in chapter 3.3. The alternative complement was assayed in rabbit blood cells (RRBC) (Delta-clon S.L) at 3 % diluted with gelatine veronal buffer as described in [Sunyer and Tort \(1995\)](#) with modifications ([Milla et al., 2010](#)). Briefly, a serial dilution of plasma was prepared, and the sample was added to a 96-well plate. Then, rabbit red blood cells (RRBC) were added to each well and were incubated at 27 °C for 100 min. Once the time had elapsed, the plate was centrifuged during 2.5 min at room temperature and 100 µL of supernatant was transferred to another 96-well plate and read at 414 nm. Finally, appropriate calculations were used to estimate alternative complement activity ([Sunyer and Tort, 1995](#)).

The lysozyme assay was performed as described in Ellis (1999) with modifications ([Milla et al., 2010](#)). Briefly, 10 µL of plasma were mixed with 200 µL of lyophilized *Micrococcus lysodeikticus* (Sigma, Aldrich) at a concentration of 0.2 mg/mL in phosphate buffer (pH= 6.2) in a 96-well plate. The difference in

absorbance was measured for 20 min every two minutes at 540 nm, and used to calculate the lysozyme activity in U/mL of plasma assuming that 1 U corresponded to a decrease of 0.001 in absorbance per minute.

1.3.1.4 Gene expression analysis

In chapters 3.2, 3.3, and 6, the RNA extraction was done using TRIzol following the manufacturer's instructions, but with some differences between both protocols, which are specified in the corresponding manuscripts. In chapters 3.2 and 3.3, there were tissues, and in chapter 6, there were cells. Moreover, in chapters 4 and 5, the RNA extraction was done using RSC Maxwell Simply RNA tissue.

The cDNA of each sample was synthesised from 1 μ g of RNA using the iScript™ cDNA Synthesis Kit (Bio-rad, USA) following the manufacturer's instructions. Gene expression was analysed using real-time quantitative PCR (qPCR) in a CFX Touch™ Real-Time PCR Detection System (Bio-Rad, USA), according to MIQE guidelines (Bustin et al., 2009).

Prior to the analyses, a dilution curve with a pool of samples was run to determine the appropriate cDNA dilution for each gene, as well as to confirm the absence of primer dimers, and the specificity of the reaction by a single peak in the melting curve for each primer set. Briefly, all the analyses were performed in duplicate wells using 384-well plates with a final volume of 5 μ L (2.5 μ L iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA), 250 nM of forward and reverse primers and 2 μ L of diluted cDNA for each sample). The qPCR program had an initial desaturation step of 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The software Bio-Rad CFX Maestro version 4.1 was used to confirm reference genes stability by the gNorm algorithm, allowing the calculation of the expression level of each target gene relative to the geometric mean of the three most stable genes for each tissue by the Pfaffl method (Pfaffl, 2001).

1.4 Fish welfare

Although the assessment of welfare was not the objective of this thesis, it includes some considerations about welfare in fish, as for the intimate relationship with stress situations and more precisely, their absence.

The concept of “animal welfare” encompasses both the physical condition and the subjective well-being of animals, and it has been increasingly studied over the past fifty years (Dara et al., 2023). Welfare is a multifaceted concept that is not straightforward to define, as it is interpreted differently across disciplines and by individuals with diverse perspectives, leading to a range of definitions over time. In this thesis, the definition adopted is based on the framework of the Five Freedoms, developed in the 1990s (Mellor, 2016). According to this approach, an animal is considered to be in a good state of welfare when it experiences freedom from hunger, thirst, and malnutrition; freedom from discomfort; freedom from pain, injury, and disease; freedom from fear and distress; and the freedom to express normal behaviour.

Nevertheless, the concept of the Five Freedoms has evolved towards a more affective, feelings-based perspective (Korte et al., 2007). This approach recognises that a certain degree of stress and discomfort may be beneficial, and that good welfare can be achieved when animals possess the capacity and resources to adapt to the challenges they encounter (Mellor et al., 2009). In this context, an alternative “Five Domains” model has been proposed, comprising four physical and functional domains (nutrition, environment, health, and behaviour) that together influence the fifth domain, namely the animal’s mental state. Similarly, the Farm Animal Welfare Committee (FAWC) has shifted its focus beyond merely preventing poor welfare and suffering, towards an approach that also addresses animals’ needs and promotes the idea of “a life worth living” from the animal’s perspective (Council et al., 2009; Webster, 2016), although still interpreted by humans. Furthermore, in response to growing public concern, many retailers now require fish producers to comply with specific welfare standards (Richards et al., 2013).

When referring to animal welfare needs, this encompasses the full range of requirements to which animals are inherently linked and which are reflected in their subjective experiences, ultimately regulating their cognitive and emotional control mechanisms. Although the spectrum of welfare needs can be extensive (e.g. (Noble et al., 2018)), for practical purposes these are commonly grouped into five broad categories relevant to farmed fish: adequate nutrition, suitable water quality, good health, the ability to express natural behaviours, and a sense of safety.

1.4.1 Relation between stress and welfare

Efforts to reduce stress are closely linked to improvements in welfare and reductions in mortality among farmed fish, as highlighted in the European Directive 2010/63/EU, with welfare implementation having been assessed by studies such as [Toni et al. \(2019\)](#). Enhancing fish welfare through stress reduction can also lead to increased productivity, making the application of appropriate management practices essential for the economic viability of the aquaculture industry. Furthermore, there is growing recognition, both among the public and within the scientific community, of the importance of understanding the physiological and behavioural foundations of stress responsiveness and welfare in fish ([Castanheira et al., 2017](#); [Martos-Sitcha et al., 2020](#); [Huntingford et al., 2006](#)).

Although the relationship between stress and welfare is influenced by numerous biological and environmental factors, stress responses provide valuable insights, particularly when multiple components of the stress response are affected in a similar manner under conditions, being an indicator of welfare status. Stress represents a major challenge to fish health and welfare across a range of contexts. The ability to exert some degree of control over stressors, or to anticipate and prepare for them, can enhance coping capacity. Providing opportunities for behavioural expression, such as access to shelter or the ability to redirect behaviour, can contribute to environmental enrichment.

Conditioning fish to associate specific cues with the onset of stress may allow them to anticipate challenges and adjust accordingly, which can be advantageous. Similarly, operant conditioning approaches, such as the use of self-feeders, enable fish to regulate their own feeding behaviour and have been shown to benefit overall welfare ([Kristiansen et al., 2020](#)). More generally, the provision of appropriate cognitive stimulation, alongside optimal environmental conditions, suitable feeding strategies, and opportunities for social interaction, appears to play a key role in minimising stress in captive settings where feasible ([Kristiansen et al., 2020](#)).

1.4.2 Welfare assessment

Within this framework, an animal's general health and physical condition are widely regarded as key indicators of welfare, to the extent that some authors equate welfare with a state of good health or optimal biological functioning. Behavioural research plays a central role in welfare science ([Branson, 2008](#)), both

by helping to identify species-specific behavioural requirements and because changes in behaviour often represent an early and readily observable response to unfavourable conditions. Moreover, preference or choice tests, which allow animals to express natural preferences, can be valuable for identifying environmental conditions that either support or compromise welfare (Branson, 2008). For instance, fear-related responses may function as adaptive mechanisms that protect individuals from potential threats (Noble et al., 2018). In general, welfare indicators have been developed to provide insight into the extent to which an animal's welfare needs are met, a concept assumed to be closely linked to its overall welfare state. Such indicators should be scalable, allowing observations or measurements to be categorised into binary outcomes (e.g. presence or absence) or multiple graded levels reflecting progressively more positive or negative welfare states. Welfare indicators are typically classified as either input-based measures, which refer to factors that can influence the extent to which one or more welfare needs are met, or outcome-based measures, which consist of observations or assessments reflecting how an animal responds to changes affecting its welfare state. In addition to these categories, other types of welfare indicators have also been described, including operational welfare indicators (OWI), laboratory-based welfare indicators (LabWI), as well as indicators assessed at either the individual or group level.

1.4.3 Negative and Positive welfare

Animal welfare is shaped by a balance between negative and positive factors. Historically, animal welfare science has focused on alleviating suffering (chronic stress, pain, etc.) and identifying physiological and behavioural signs of negative welfare, such as frequent illness, elevated blood cortisol and stereotypic behaviours (Fraser and Duncan, 1998). However, there is now a growing recognition among animal welfare scientists that pursuing the goal of relieving suffering does not lead to a lack of bad welfare (Burn, 2017; Mellor, 2016), thus indicating that even neutral animal welfare necessarily does not involve positive welfare (Fife-Cook and Franks, 2019), and that indicators of positive welfare should be pursued. Positive welfare means a mental and physical state exceeding what is strictly necessary for short-term survival of the fish (Fife-Cook and Franks, 2019). Hence, it has been described that positive welfare can protect from boredom, distress, and other negative aspects, at the same time that improves the long-term well-being, resilience, and overall health (Fredrickson, 2013).

Fish research has followed a similar trend. In the context of fish specifically, welfare indicators are commonly linked to performance outcomes, key measures of environmental conditions, and the absence of stress-related responses. By contrast, identifying indicators of positive welfare is considerably more challenging, particularly those that demonstrate the animal's capacity to express natural and species-typical behaviours. One way to assess welfare is using OWI's and LabWI's, as both indicators include scores that may indicate a good and a bad state. Low scores would indicate distress (*e.g.* overcrowding, poor water quality, among others) and the consequences of these stressors could include reduction of growth or immune suppression. On the other hand, to reduce the chances of poor welfare, it is necessary to maintain good conditions for the fish and eliminate stress. On the contrary, high scores obtained from these indicators can be used as a proxy for a good welfare status. In order to promote this, additional practices are necessary, such as environmental enrichment and the observation of natural behaviour indicating eustress (*i.e.* improving the living quality of the animals; (Arechavala-Lopez et al., 2022; Sneddon et al., 2016)).

1.5 Species studied

The two species chosen in the present thesis, rainbow trout and gilthead seabream, were selected taking into account the commercial availability, economic importance, and the proximity of the providers to the UAB facilities (AQUAB). Moreover, species characteristics were taken into account to select the studied stressors. In other words, since rainbow trout is sensitive to low oxygen levels, it was selected for the hypoxia experiments, and for the high salinity challenge, a euryhaline species, the gilthead seabream, was chosen. For the rest of the experiments of the thesis, since both species were adequate, we selected the gilthead seabream, as a major production of Mediterranean aquaculture.

1.5.1 *Onchorynchus mykiss*

The rainbow trout (*Oncorhynchus mykiss*; Walbaum, 1792) is a fish species from the *Salmonidae* family native to the Pacific drainages of North America, ranging from Alaska to Mexico (FAO, 2026). Since 1874, it has been introduced to waters of all continents except Antarctica, for recreational angling and aquaculture purposes (Stoumboudi et al., 2017). The requirements for the species include well-oxygenated, cold, and clean freshwater environments, with optimal culture

temperatures generally below 21 °C to support efficient growth, feed conversion rates, and survival. Although the species tolerates a broad thermal range (0–27 °C), sustained exposure to warmer conditions can impair metabolic performance and delay maturation. Rainbow trout thrive in habitats with stable water quality, low pollutant loads, and adequate flow rates to maintain high dissolved oxygen levels that are essential for their aerobic physiology and sensitivity to environmental stressors. Hence, rainbow trout has been widely used in experiments related to hypoxia (Holeton and Randall, 1967; Schurmann et al., 1991), but also with other stressors (Øverli et al., 2005). These ecological requirements shape both natural distribution and aquaculture practices, influencing site selection, system design, and overall production efficiency. In recent years, global aquaculture of rainbow trout has expanded significantly, with production increasing by approximately 47 % between 2011 and 2020, reflecting both technological improvements and a growing market demand. Within this context, Spain contributed around 15,000 tonnes in 2020 of farmed rainbow trout, positioning the country as a relevant European producer and highlighting the importance of maintaining optimal environmental conditions to sustain its industry (EUMOFA, 2023). Regarding reproduction, genetic manipulation produces sterile, triploid females to ensure that introduced/escaped individuals cannot breed. Trout will not spawn naturally in aquaculture systems; eggs are artificially spawned from high-quality brood fish when fully mature (ripe). Although two-year-old trout start spawning, females are seldom used for propagation before they are three or four years old (EUMOFA, 2023).

RTgutGC is a fish cell line that was derived from a small female rainbow trout. After the dissection of the gut, pieces of about 1 mm² were isolated from the distal section and cultured. After 8 weeks, they showed an extensive stroma surrounded by epithelial-like cells. Until recently, the RTgutGC, was the only immortalised cell line available from fish gut tissue (Kawano et al., 2011). This cell line has been extensively characterised since its establishment and has been a valuable tool for studying nutrient uptake, toxicity, functional feed ingredients, gut immune function, and epithelial integrity in fish (Kawano et al., 2011).

1.5.2 *Sparus aurata*

The gilthead seabream (*Sparus aurata*; Linnaeus, 1758) is a teleost species belonging to the *Sparidae* family, native to the eastern Atlantic Ocean and the Mediterranean Sea. It is both euryhaline and eurythermal, capable of tolerat-

ing substantial variations in salinity and temperature, which enables it to inhabit marine as well as brackish environments (Craig et al., 2008). This species is a protandrous hermaphrodite, with individuals initially developing as males before transitioning to females upon reaching sexual maturity (Chaves-Pozo et al., 2008). Gilthead seabream represents one of the most economically significant species in aquaculture, ranking third globally and holding particular importance within the Mediterranean region. It is widely cultivated in sea cages and recirculating aquaculture systems (RAS), with production increasing markedly from fewer than 90.000 tonnes in 2000 to over 280.000 tonnes by 2020 (FAO, 2024). Furthermore, this species has been extensively used as a model organism in experimental studies, including ecotoxicology research (Barbosa et al., 2019), as well as investigations into environmental stressors such as salinity (Bodinier et al., 2010), hypoxia (Samaras et al., 2016), and high stocking density (Ortuno et al., 2001).

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2

Objectives

2.1 Aim and rationale

Mucosal surfaces constitute the first line of defence in fish since they constitute the interface between the organism and the environment. These barriers are able to perform local stress responses and mount an immune response to maintain homeostasis. Nonetheless, the understanding of the specific role of each mucosa in the face of different stressors is still poorly understood.

Aquatic environments are subjected to changes that can be stressful factors for fish, such as climate change, pollution, and factors associated with aquaculture practices. Although some of these stressors have been previously studied individually in each mucosa or in other tissues of fish, the combined effects of simultaneous exposure to different stressors are still severely understudied. Stressors differ in intensity and duration, and their effects in the mucosal tissues is complex and nowadays is still poorly understood.

This thesis aims to address some of these knowledge gaps by examining the mucosal immuno-endocrine response when exposed to different environmental and aquaculture-associated stressors that vary in duration, intensity, and origin. By characterizing changes in gene expression and biochemical parameters in plasma, this thesis seeks to clarify the mechanisms underlying the stress and immune response. Such knowledge can enhance our understanding of fish resilience and contribute to improve fish welfare aquaculture in production systems. For this purpose, two relevant species for human consumption were selected, namely, rainbow trout (*Oncorhynchus mykiss*) and the gilthead seabream (*Sparus aurata*).

On one hand, some of the stressors that have been used in this thesis, namely increased salinity, overcrowding, hypoxia, and nanoplastic contamination, are relevant in a context of heavy anthropogenic pressure on natural ecosystems (e.g. climate change and environmental pollution). On the other hand, other assessed stressors such as crowding and anoxia naturally occur in aquatic environments, and are often associated with aquaculture facilities. Moreover, a relevant and novel aspect of the present thesis is the diversity of stressor types investigated, in terms of intensity and duration (e.g. acute, chronic, repeated, individual and combined), to compare the modulation of the stress, immune, and endocrine responses.

2.2 Objectives

The **general objective** of this thesis was to investigate the fish mucosal response to different environmental and aquaculture-associated stressors with different durations and intensities on the three most important mucosal tissues in freshwater and marine fish species. To this end different **specific objectives** were defined as follows:

1. Evaluate the mucosal stress response to different environmental stressors
2. Assess the modulation of the different stress biomarkers in face of stressors of different duration and intensity
3. Achieve a comprehensive understanding of the activation dynamics of each mucosa under the different stressors
4. Provide a comprehensive framework on the modulation of the immune and endocrine response under different stressors in the different mucosal tissues
5. Compare and identify the stress and immune response on the *in vitro* interaction in the gut mucosa

2.3 Hypothesis

The general hypothesis of this thesis was that fish subjected to aquaculture systems are subjected to a variety of stressors, both endogenous and exogenous to the system, which impact fish welfare, as well as their ability to respond to and cope with subsequent stressors. Furthermore, our hypothesis assumed that mucosal tissues are able to perform a local stress response and that different stressors will have different impacts on the different mucosae. Finally, it was also hypothesised that the duration and the intensity of the stressor will determine the severity of the response.

2.4 Experimental design

All experiments were designed to maximise the representativeness of the available samples, minimising the number of animals used, and avoiding all unnecessary

suffering, as per the 3Rs' principles of ethical animal research. All the stressors used were designed to be similar to real-life aquaculture scenarios. Moreover, in order to increase the representativeness of these controlled, laboratory exposures with respect to real-life scenarios, fish were exposed, in some cases, simultaneously to different stressors. The thesis was based in 3 *in vivo* experiments and 1 *in vitro* experiment:

- **Study 1: Repeated hypoxia experiment.** In this comprehensive experiment, the investigated stressor was hypoxia, which occurs both in aquaculture facilities and in natural environments. In this case the species selected was *O. mykiss* since it is a particularly sensitive species to low oxygen levels. The hypoxia was applied as a repeated acute stressor in order to evaluate the possibility of habituation when fish face the same stressor repeatedly over time.
- **Study 2: Polystyrene nanoplastics and salinity stress.** In this study, *S. aurata* juveniles were subjected to a single or combined chronic stressor (i.e. nanoplastics and high salinity), over 1 month. Thus, this experiment was designed to mimic a stress situation more likely to occur in the natural environment, or in open aquaculture systems (e.g. sea cages) rather than in closed aquaculture facilities, such as RAS. In this experiment the effects of the different stressors were analysed both alone and in combination to evaluate whether any synergistic impact occurred between them.
- **Study 3: Multiple stressors.** In this case, intrinsic stressors associated with aquaculture were selected, namely, anoxia and crowding, and were applied in an acute manner in juveniles of *S. aurata*. The study was designed to compare the response of the same batch of fish to these two different stressors, one being a social one and the other physical.
- **Study 4: Immunomodulation on RTgutGC.** In this *in vitro* study, the intestinal rainbow trout cell line RTgutGC was selected to try to understand the immune response under stressed conditions. A stressful stimulus was mimicked by administering cortisol to the cell culture to simulate a primary stress response, followed by a lipopolysaccharide (LPS) exposure, in order to determine whether the previous cortisol exposure affected the ability of cells to mount an immune response.

3

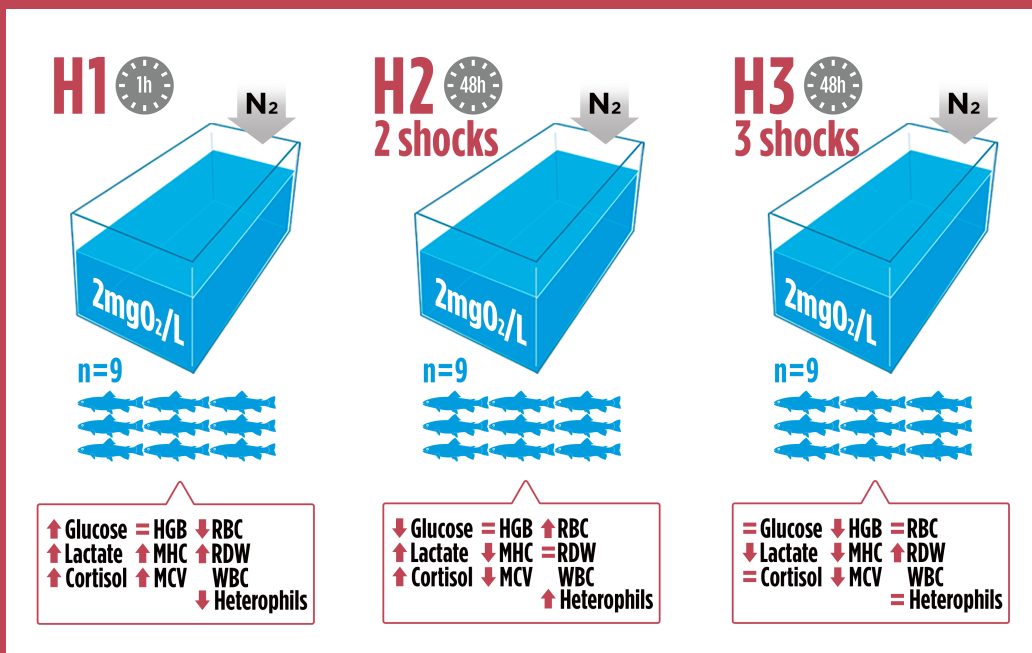
Repeated hypoxia experiment

Repeated hypoxic episodes allow haematological and physiological habituation in rainbow trout

Nuria Ruiz, Irene García-Meilán, Ali Reza Khansari, Mariana Teles, Josep Pastor, and Lluís Tort

3.1

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Abstract

Introduction: Under climate change, the increase in temperature in aquatic environments may induce oxygen depletion. In extreme cases, low oxygen may become a limiting factor for fish, thus generating stress. In addition, consecutive hypoxic episodes may complicate the recovery of individuals and hinder their ability to modulate physiological and biochemical responses to maintain homeostasis. Thus, the aim of this study was to determine the haematological and physiological responses of rainbow trout under a condition of repeated hypoxic and manipulation stress at three different time points.

Methods: Every hypoxic episode consisted of exposing the fish to low dissolved oxygen concentrations (2 mgO₂/L for 1 h). Following the exposure, the fish were allowed to recover for 1 h, after which they were sampled to investigate haematological and physiological parameters.

Results and discussion: The results showed a pattern of habituation reflected by values of haematocrit, haemoglobin, and mean corpuscular volume, indicating a certain ability of rainbow trout to resist this type of repeated hypoxic events, provided that the fish can have some recovery time between the exposures.

3.1.1 Introduction

The current context of climate change is expected to strongly affect fish because of its consequences on water temperature and other tightly linked quality parameters. The expected changes are related to biotic (primary production, pathogens, and food availability) and abiotic (temperature, salinity, pH, and, particularly, hypoxia) factors, affecting aquatic organisms in their distribution, growth, size, and overall health (FAO, 2020). As wild populations may be exposed to more than one of these environmental stressors, much uncertainty is generated about the consequences of these stressors and their potential synergy (e.g. hypoxia and temperature rise) (Petitjean et al., 2019). As fish are aerobic organisms, the concentration of dissolved oxygen (DO) is a limiting factor both in the environment and in production systems, and its availability depends on the amount and solubility. Episodes of repeated hypoxia may become more common in the future. For instance, diel cycling hypoxia, a worldwide phenomenon affecting freshwater and coastal systems due to an increase in partial pressure during daylight hours associated with photosynthetic activity and a subsequent diminution overnight as a consequence of biological demand, is particularly common during spring and summer months (Williams et al., 2019). In natural environments, it can be a result of eutrophication and increasing temperatures. Furthermore, hypoxic phenomena may be amplified in aquaculture settings because of overstocking or overfeeding (Bera et al., 2017).

Hypoxia occurs when oxygen concentrations drop low enough to cause negative physiological, immune, and behavioural effects on fish, affecting their growth and overall performance (Abdel-Tawwab et al., 2019). This can affect acutely or chronically, depending on the duration and recurrence of the impact of temperature change, seasonality, water flow, and/or chemical composition (Xiao, 2015). Most species have a high ability to habituate to fluctuating oxygen levels, modifying O₂ uptake, delivery, and metabolism to prevent hypoxemia and energy exhaustion (Boutilier, 2001). However, if fluctuations are recurrent, they can compromise the overall health and lead to increased mortality. Nevertheless, preconditioning exposure to hypoxia demonstrated the existence of physiological plasticity in fish (Borowiec et al., 2015), even in rainbow trout, which is considered a species particularly sensitive to this stressor (Gamperl et al., 2001). Therefore, to maintain optimal fish performance, growth, and feeding, oxygen levels should be kept near saturation. Ideally, DO levels in aquaculture settings should be above 5 mgO₂/L, which is the accepted baseline for most species (Abdel-Tawwab et al.,

2019). Although under aquaculture conditions, DO levels are generally monitored and controlled, some factors, such as overcrowding or abnormal increases in temperature, may lead to suboptimal rearing conditions, and if the fish are unable to adapt, they may develop a stress response and maladaptation (Magnoni et al., 2019).

The stress response is a very primitive and highly conserved response in extant species since it preserves the organism's homeostasis (Barton, 2002). By activating primary, secondary, and tertiary responses (Schreck and Tort, 2016), it has been shown that animals are able to adapt to different stressors, including hypoxia, by reducing their physiological costs and modifying behavioural responses (Conde-Sieira et al., 2018; Alfonso et al., 2020). However, the capacity for habituation will depend on several factors, namely, the species itself, as well as the type and intensity of stress (Nilsson et al., 2012; Koakoski et al., 2013). Moreover, as the importance of animal welfare and its public awareness in production systems is increasing, the maintenance of suitable conditions for fish husbandry should consider the consequences of these stress episodes.

In the present work, we monitored the haematological and physiological parameters as they can be suitable indicators of the process of oxygen uptake and distribution to tissues (Fazio, 2019). The number of red blood cells (RBCs), haemoglobin (HGB) concentration, haematocrit (HCT), mean corpuscular volume (MCV) of red blood cells, and mean haemoglobin concentration (MHC) are most commonly used to evaluate the haematological status in fish (Pavlidis et al., 2007). However, other less-investigated factors, such as the widening of the distribution of red blood cells (RDW), a parameter related to the pathway of ionic erythropoiesis to measure the size and volume of erythrocytes, have been monitored as additional indicators of health status, as shown in humans (Fish et al., 2019). In addition, the determination of the number of white blood cells (WBCs) provides information on the animal's immune status, as well as the proportion of heterophilic cells (neutrophils, basophils, and eosinophils) and mononuclear cells (lymphocytes and monocytes). Finally, the number of platelets (PLTs) was also assessed (Fazio, 2019). Altogether, the correlation between haematological parameters, as indicators of oxygen uptake and distribution (Abdel-Tawwab et al., 2019), and physiological stress markers, such as cortisol, glucose, and lactate in plasma (Groff and Zinkl, 1999), provides valuable insights on the specific physiological compartments of functional allostasis, thus becoming markers of a range of physiological variations and useful tools to detect, identify, and calibrate specific stressors.

Many studies have addressed the hypoxia effects on fish (Pollock et al., 2007; Xiao, 2015), particularly in rainbow trout (*Oncorhynchus mykiss*) (see, for instance, the work of Pilgaard et al. (1994), Sappal et al. (2016), and Zhang et al. (2018)). The present study focuses on two aspects that have been discussed in previously published work. First, studies on hypoxia have dealt with fish that have been taken out of the water, so, effectively, subjected to anoxia. Furthermore, these fish are simultaneously subjected to the consequent handling stress associated with catching and removing the fish out of the water. However, these two stressors are difficult to separate. Second, studies on repeated hypoxia are scarce, and the ability of fish to respond repeatedly to this stressor is, therefore, less understood. Since hypoxic episodes may become more frequent in the context of climate change, the aim of the present research was to determine the effects of repeated hypoxia and manipulation by subjecting the animals to one, two, or three acute hypoxic shocks and to determine whether there is a cumulative effect, a certain adaptive capacity or, on the contrary, a constant response. In addition, the stress recovery phases, specifically at 1, 6, and 24 h after hypoxia and manipulation, were also investigated.

3.1.2 Materials and methods

3.1.2.1 Fish and rearing conditions

A total of 135 rainbow trout (*Oncorhynchus mykiss*) with a mean weight of 62.89 ± 11.10 g, a mean length of 17.58 ± 1.09 cm, and a condition factor of 1.2 ± 0.1 were obtained from a local fish farm (Molinou, Rialb, Spain) and acclimated to a recirculating aquaculture system (RAS) in the facilities of the Autonomous University of Barcelona (AQUAB) for 2 weeks. The RAS is equipped with water pumps, a recirculating cooling system, a sand filter, a biofilter, and an aeration system for the tanks, allowing for the DO concentrations to be maintained between 7.20 - 8.10 mgO₂/L. The photoperiod was held at 12 L: 12 D, and an average temperature of 14.6 ± 0.3 °C was maintained. Throughout the acclimation and experimental periods, the density was 6.80 ± 0.1 Kg/m³. All experimental procedures involving fish were submitted and authorised by the Ethics and Animal Care Committee of the "Universitat Autònoma de Barcelona" (permit numbers OH4218 4219 and DAMM 11251), in accordance with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

3.1.2.2 Experimental design

Five experimental groups were established, divided into two control groups and three treatment groups, each undergoing a different number of manipulations and hypoxic shocks. Each group had 9 fish per sampling time, with a total of 27 rainbow trout per group. The first group was the absolute control (AC) group, being the only group that was sampled without prior manipulation. Since it is known that the manipulation of individuals is an additional stressor to hypoxia, a manipulated control (MC) group was considered with the aim of determining how manipulation by itself can alter the evaluated parameters without decreasing the DO concentration. The remaining three groups were subjected to hypoxic shocks, all handled in the same manner as the MC group, and subsequently exposed to a decreased concentration of oxygen. The H1 group was exposed to hypoxic conditions once, whereas fish in the H2 group were challenged with two hypoxic shocks, and the H3 group was subjected to low levels of DO for a total of three hypoxic shocks. Thus, the H2 and H3 groups suffered repeated acute hypoxia stress, with 48 h between hypoxia shocks. The MC group is only comparable to the H1 group, as H2 and H3 had more manipulations than the MC group. Since H2 and H3 were only compared with the AC group, we cannot exclude the effect of hypoxia and manipulation in these groups. In the groups where several hypoxic shocks were performed, the fish were returned to the acclimation tank under normal oxygen and water physicochemical levels until the next shock. Once the fish experienced the last shock, they were transferred to the recovery tank until sampling. As the number of fish in the tank decreased after each sampling, the water volume was accordingly decreased with the aim to maintain the density at $6.80 \pm 0.1 \text{ Kg/m}^3$.

3.1.2.3 Hypoxic shock and the sampling procedure

Hypoxic levels were reached by decreasing the DO concentration by adding nitrogen gas (N_2) into the water (i.e., 5 min to reach hypoxic conditions), as in the work of [Schurmann and Steffensen \(1992\)](#), and not removing the fish from the water. Three tanks were used to replicate the treatment procedure by maintaining a density of $6.80 \pm 0.1 \text{ Kg/m}^3$. After the procedure, all fish were placed in the same recovery tank to avoid any tank effect. The shock included catching the fish from the experimental tanks and putting them into the hypoxia tanks, where the DO concentration was decreased from 7.5 ± 0.5 to $2.2 \pm 0.5 \text{ mgO}_2/\text{L}$ by adding nitrogen gas. The fish were introduced once the oxygen level had reached the

desired values and remained there for 1 h. It should be added that during the shock, the DO concentration was continuously monitored to check that the levels were within the study range and to avoid a decrease below 1.5 mgO₂/L, which would prove lethal to this species. Throughout the experiment, the density was constantly maintained at 6.80 ± 0.1 Kg/m³ (**Figure 3.1.1**). Following the hypoxia shock, the fish were transferred to a recovery tank with 7.2 - 8.1 mgO₂/L, which was isolated from the system to avoid any possible circulating cortisol levels affecting the other fish, as a chemical detection by peripheral cortisol receptors that may affect fish response has been previously suggested (**Kolosov and Kelly, 2019**). After the last hypoxia shock, the fish were transferred to a recovery tank where sampling took place. As the number of fish per tank changed, the water volume was adjusted to maintain the same density throughout the experiment. By the time the sampling of the experimental group was finished, the oxygen and water turnover levels were returned to optimal levels. For sampling, a total of 27 fish per group, with 9 fish per time point of the hypoxia and treatment groups (AC, MC, H1, H2, and H3), were anaesthetised with sublethal doses of tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate. All fish were weighed and measured, and blood samples were collected within 3 min. After that, fish were sacrificed by sectioning the spinal cord.

3.1.2.4 Haematological analysis

Blood collection was performed using a heparinised syringe through caudal puncture. The first 500 µL aliquot of blood was added to Eppendorf tubes containing heparin (1:40) for the determination of haematological parameters. A second aliquot was centrifuged at 1500 g for 10 min to collect the plasma, which was subsequently frozen at -20 °C until the physiological analysis. Haematological analyses were performed within 2 - 12 h after blood sampling using the automated flow cytometer blood cell analyser Sysmex XN-1000V for veterinary use (Sysmex Corporation, Kobe, Japan). Internal quality control (QC) was performed daily using three levels of commercially available QC material (Sysmex XN Check level 1 or low range, level 2 or normal range, and level 3 or abnormal high range; Sysmex Corporation, Kobe, Japan).

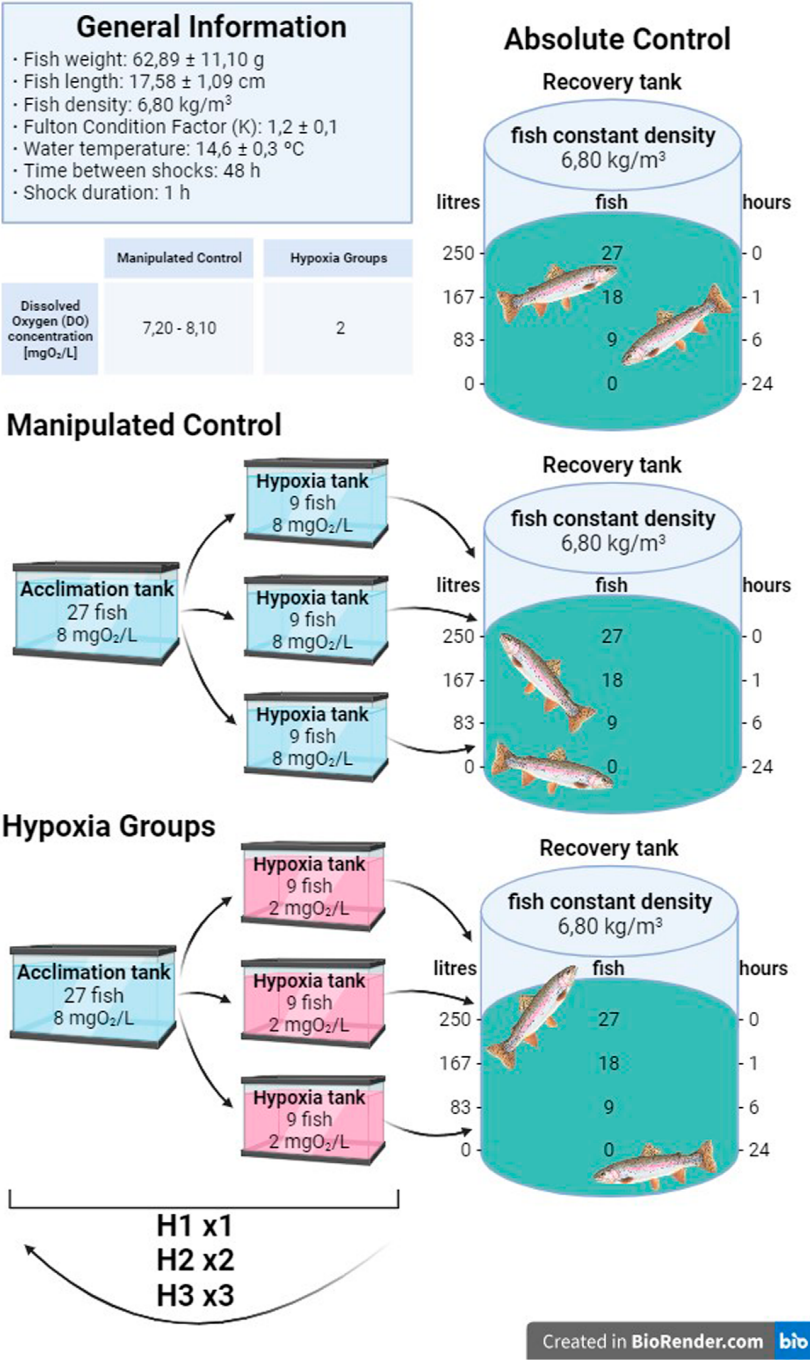


Figure 3.1.1: Graphical abstract of the design of the experiments.

3.1.2.5 Physiological analysis

For cortisol analysis, the plasma was first diluted in the analysis buffer, and the aliquots were frozen at -20 °C for at least 24 h until analysis with an ELISA test using a commercial EIA kit (Cortisol ELISA Kit; Neogen[®] Corporation, Ayr, United Kingdom) following the manufacturer's instructions. This kit has been previously validated for this species and used in past experiments (Carbajal et al., 2019).

Glucose and lactate analyses were performed using colourimetric test kits (POD glucose and POD lactate, SPINREACT, Spain) following the manufacturer's recommendations.

3.1.2.6 Statistical analysis

The obtained data were analysed with a generalized linear model (GzLM) using the Gaussian family for all the variables using RStudio software (<https://www.R-project.org>). Data normality and residual distribution of the model were checked through Shapiro-Wilk test. The factors analysed were hypoxia treatments, time, and the interaction between them. If significant differences (p -value < 0.05) were found, pairwise comparisons by Tukey's correction were applied. Therefore, variables where only the effect of treatment is found are presented first, followed by those with only time effect significance, and finally, all the parameters that had an interaction between the two factors. For the pairwise comparisons, the effects of manipulation and hypoxia were differentiated, and therefore, AC, MC, and H1 were compared on one side and AC, H1, H2, and H3 on the other. Regarding treatment and time effect, it has to be added that only when one factor was significant, all the data were gathered by treatment or time, depending on the case.

3.1.3 Results

In this section, the results are presented according to the significant effect of each factor and the significant interaction between them in the order mentioned above. As can be observed in the figures, the significance is shown for the differences among the factors. For instance, a parameter included only in the treatment effect indicates that significant differences can be found between treatments but not between times or with interacting factors.

3.1.3.1 Treatment effects

The number of RBCs, the amount of HGB and PLTs, and MHC were affected by treatment (**Table 3.1.1**) but not by time or interaction between them. An increase in RBC and HGB levels, likely due to handling stress, was observed in the MC compared to the AC group, whereas one hypoxic shock tended to decrease these values since the values of H1 are lower than MC and, in theory, it had to be higher due to manipulation. In addition, significantly higher RBC values were measured in H2 compared to H1, with H3 displaying intermediate values, similar to those found in AC.

Table 3.1.1: RBC number, HGB, PLT number, MHC, and heterophil cells in percentage in rainbow trout. Data are represented as mean $\pm$ SEM ($n = 27$ fish per treatment). Significant differences between treatments are marked with different letters (p -value < 0.05). AC, absolute control; MC, manipulated control; H1, one hypoxic shock; H2, two hypoxic shocks; and H3, three hypoxic shocks.

Group	RBC ($10^3/\mu\text{L}$)	HGB (g/dL)	PLT ($10^3/\mu\text{L}$)	MHC (pg)	Heterophils (%)
AC	0.961 ± 0.0224^b	5.57 ± 0.108^{ab}	2.34 ± 0.140	50.8 ± 0.770^{ab}	17.5 ± 1.37^b
MC	1.058 ± 0.0229^a	5.78 ± 0.110^a	2.34 ± 0.162	48.3 ± 0.770^{bc}	20.1 ± 1.40^b
H1	0.939 ± 0.0224^b	5.61 ± 0.108^{ab}	2.38 ± 0.149	52.8 ± 0.770^a	22.0 ± 1.49^{ab}
H2	1.031 ± 0.0229^a	5.77 ± 0.123^{ab}	2.28 ± 0.154	49.5 ± 0.846^{bc}	27.3 ± 1.46^a
H3	0.975 ± 0.0224^{ab}	5.35 ± 0.108^b	2.61 ± 0.154	47.6 ± 0.770^c	19.3 ± 1.43^b

PLT count displayed a decreasing trend with an increase in the number of hypoxic shocks and manipulations, although above the threshold for statistical significance (p -value < 0.07 ; **Table 3.1.1**), except for H3, which showed increased PLT values. Finally, the results indicated an increase in MHC in H1 compared to MC, with a return to AC in H2, and a significant reduction in MHC in H3 compared to AC (**Table 3.1.1**). Furthermore, the results suggest that both hypoxia plus manipulation and time independently alter the proportion of heterophilic and mononuclear cells, although no interactive effect of these factors was found. It should be noted that H2 is the treatment group that showed the highest levels of heterophils compared to the AC and H1 groups, regardless of the time after hypoxia exposure and manipulation (**Table 3.1.1**).

3.1.3.2 Time effects

A time effect was observed in both haematocrit values (**Figure 3.1.2**) and the percentage of heterophilic and mononuclear cells (**Figure 3.1.3**). Interestingly, in both parameters, a significant increase was observed at 1 h regardless of the experimental treatment.

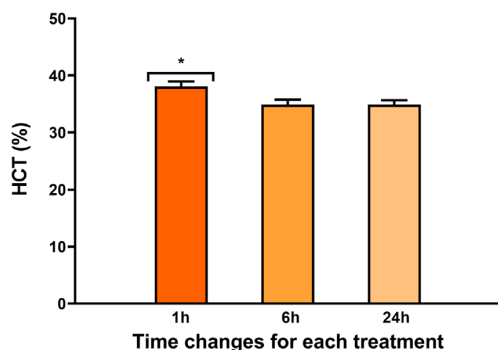


Figure 3.1.2: Percentage of haematocrit in rainbow trout blood 1, 6, and 24 h after treatment. Data are represented as mean $\pm$ SEM ($n = 45$ per sampling time). The significant differences in sampling time are indicated by an asterisk (p -value < 0.05).

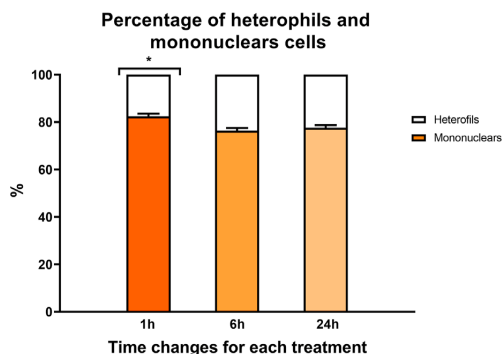


Figure 3.1.3: Percentage of mononuclear cells (colour bars) and heterophils (white bars) in rainbow trout blood 1, 6, and 24 h after treatment. Data are represented as mean $\pm$ SEM ($n = 45$ per sampling time). The significant differences in sampling time are indicated by an asterisk (p -value < 0.05).

3.1.3.3 Interaction effects

A significant interaction between manipulation and exposure to hypoxia and time was found in MCV, RDW, WBC, glucose, lactate, and cortisol (**Figure 3.1.4**). In H1 and MC groups, a gradual reduction of the MCV can be observed over time, $t = 1$ h vs. 24 h. In addition, H1 also presented higher MCV levels at 1 and 6 h compared to both AC and MC and the other groups that underwent manipulations and hypoxic shocks (H2 and H3) (**Figure 3.1.4A**). RDW showed differences between AC, MC, and H1 in all the sampling times and AC with all the groups that underwent manipulation and hypoxic exposures only at the sampling point $t = 6$ h. On one side, looking at $t = 6$ h, it can be observed that H1 and MC are the groups with the highest values, and AC is the group with the lowest values. On the other side, at $t = 6$ h, comparing the groups that underwent manipulations and hypoxia, H1 is the group with the highest values. In addition, the more the manipulations and hypoxias, the lower the RDW values. Thus, H3

shows similar values to AC. The most relevant difference regarding time occurs in the MC and H1 groups, showing lower RDW values at 1 h and 24 h after treatment than at 6 h. This trend was also found when comparing all experimental groups versus AC, being higher in H1 and, therefore, suggesting a greater anisocytosis 6 h after treatment (**Figure 3.1.4B**). Regarding immune cells, the lowest WBC levels occurred 6 h after either manipulation or hypoxic exposure when compared with AC. In MC, these differences are maintained even after 24 h; however, this is not observed in the groups subjected to repeated hypoxia and manipulations (H2 and H3) (**Figure 3.1.4C**). In terms of physiological response, plasma glucose levels significantly increased 6 h after treatment in H1 compared with AC and all the groups that suffer manipulation and hypoxia. In contrast, glucose values were reduced at 24 h, except in the MC and H1 groups (**Figure 3.1.4D**). Lactate levels clearly increased 1 h after exposure and then decreased until recovering basal levels, except for the H2 group, which presented higher levels at 24 h than AC. It should be highlighted that lactate did not peak at 1 h in the H3 group, as observed in H1 and H2 (**Figure 3.1.4E**). Finally, a significant rise in plasma cortisol levels at 1 h post-exposure was observed in rainbow trout that underwent hypoxic shock and manipulation (H1, H2, and H3) compared with the AC group. Similarly, the MC group also had elevated cortisol levels when compared to the AC group. The cortisol levels dropped at 6 h and then, interestingly, rose again at 24 h (**Figure 3.1.4F**). Significant differences were maintained at 24 h post-shock between AC and both MC and H1, but not H2 nor H3, both groups showing intermediate values.

3.1.4 Discussion

In this work, the results are presented in two sets of groups, the first including the control with the manipulated control and one hypoxia exposure group, and the second including the groups experiencing repeated manipulations and hypoxias. In the first set, AC represents the basal levels that are expected to represent the basal resting values. The MC group allows differentiation between the basal and manipulated fish, and finally, hypoxia 1 (H1) allows to differentiate the effects of the manipulated and the hypoxia groups. In the second set, we compare the effect of repeated manipulations and hypoxia exposures related to AC with the basal levels of our animals. Since there is no MC group with two and three manipulations, we excluded the MC group from the analysis of this second set of groups, as they are not directly comparable.

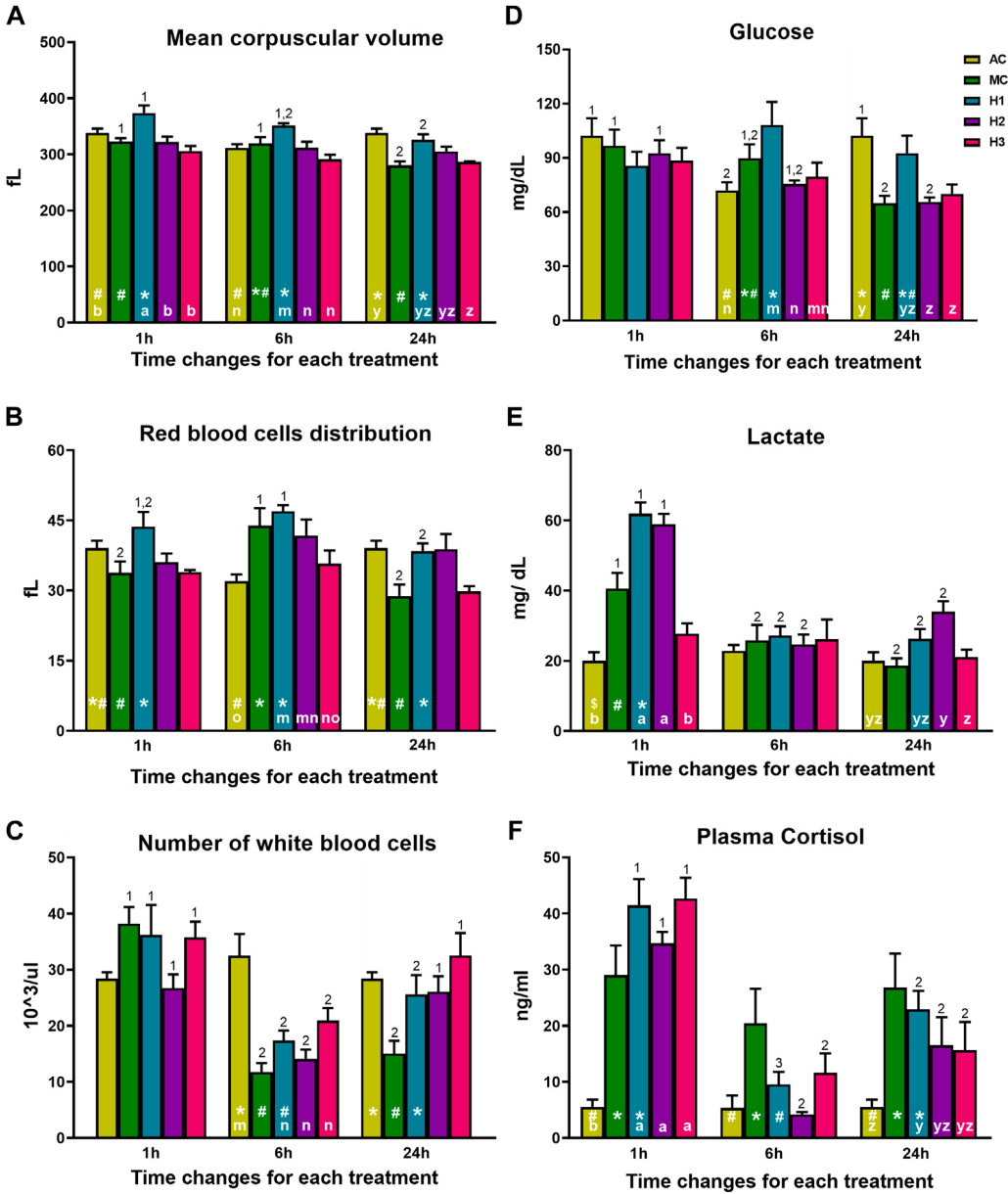


Figure 3.1.4: A) Mean corpuscular volume, B) red blood cells distribution widening - standard deviation, C) number of white blood cells, D) plasma glucose, E) plasma lactate, and F) plasma cortisol in rainbow trout blood 1, 6, and 24 h after treatment. Data are represented as mean $\pm$ SEM ($n = 9$ per sampling time). Significant differences (p -value < 0.05) between treatments at the same sampling time are shown with different letters (a/b at 1 h, m/n at 6 h, and y/z at 24 h after treatment). Significant differences in sampling time among the same treatment are shown by numbers (p -value < 0.05). Differences between AC, MC, and H1 are shown by * and #.

The overall results suggest that rainbow trout are able to cope with repeated manipulation and acute hypoxia (1 h with DO levels down to 2 mgO₂/L) and recover from the shock 24 h after the stressor, and that subjecting the fish again to the same stressor leads to a certain level of habituation. Among the haematological variables, red blood cells and haemoglobin showed an increase in the MC group, presumably due to the handling of the animals, as observed in the work of [Acerete et al. \(2004\)](#). On the contrary, as observed in H1, hypoxia generates a decrease in RBC, probably due to an excessive alteration of the oxygen delivery system, resulting in a reduction in the overall metabolism and activity ([Pichavant et al., 2002](#)). After the second manipulation and shock (H2), the RBC level increased, suggesting a recovery in oxygen content and an improvement in blood transport ([Aboagye and Allen, 2018](#)), and recovered control values in H3. A similar trend was observed in HCT, which tended to diminish as exposures and manipulations increased. Moreover, the increase of HCT 1 h after manipulation and/or treatment could be associated with balancing out the additional requirements for oxygen under hypoxia ([Muusze et al., 1998](#)). All these data suggest an adaptation of the animals, resulting in a lower use of oxygen in habituated fish ([Petersen and Gamperl, 2011](#); [Remen et al., 2012](#)).

Significant differences in MCV were observed both between groups and sampling points. The highest MCV was observed in H1, which indicates that the erythrocyte volume increased. This could be explained by two mechanisms that have been previously described. On one hand, the osmoregulatory changes that occur over time in an attempt to increase the efficiency of oxygen transfer through the cell membrane ([Aboagye and Allen, 2018](#)). On the other hand, the increase in HCT and MCV values, without much change in RBC, may suggest the swelling of erythrocytes ([Aboagye and Allen, 2018](#)). The swelling of the erythrocytes has been observed as a result of catecholamine release after stress, trying to compensate for the efficiency of cell oxygen uptake due to the increased demand of oxygen, in this case caused by the hypoxic stress and manipulations ([Caldwell and Hinshaw, 1994](#)). Interestingly, both HCT and MCV displayed a correlation with MHC. In this case, the groups with higher levels of HCT and MCV presented higher MHC. This could be explained by cellular hemolysis, which may occur during the first hour after the manipulation and hypoxic shock, as previously demonstrated ([Caldwell and Hinshaw, 1994](#); [Muusze et al., 1998](#)). Furthermore, this effect is reduced over time, suggesting recovery. This idea of haemolysis was initially proposed by [Swift and Lloyd \(1974\)](#), describing how HCT and MCV values increase because of cell swelling, which causes haemolysis. Afterwards, differences were also observed

between treatments, and as in the rest of the erythrocyte-related parameters, a certain degree of habituation can be observed in the groups subjected to hypoxia. Therefore, the more the hypoxia shocks accumulate, the lower the response, as previously seen during chronic hypoxia exposure (Caldwell and Hinshaw, 1994).

It has been previously demonstrated that stressors, such as infections by trematode metacercaria, entail a significant decrease in the distribution by the size and volume of the cell (i.e., RDW) (Khan et al., 2017). In other studies associated with nutritional stress, an inverse relationship between RDW and other haematological parameters, such as MCV or HGB, was observed (Goran et al., 2017). In the present study, RDW resulted in higher 6 h post-shock, suggesting greater heterogeneity and, therefore, greater anisocytosis, since some cells are recovering while others are not, thus increasing the heterogeneity levels at this time point. After 24 h, homogeneity is restored, but since the cells are smaller because no swelling is produced, the values at 24 h are lower than those at 1 h. It should be added that this parameter has been little studied in fish; however, in humans, a decrease in RDW levels may reflect anaemia, implying that erythrocytes do not have the capacity to efficiently transport oxygen (Maiolo et al., 1991). As it has been seen with the rest of the parameters, in the case of H3, RDW returns to the levels of the AC at 1 and 24 h, suggesting, once again, habituation.

For white blood cells, all treatments showed a decrease at 6 h with respect to the absolute control group, especially fish subjected to two hypoxic shocks that also presented a significant decrease at 1 h after treatment. These results agree with those found by Sheng et al. (2019) in the face of repeated stress (hypoxia and manipulation) and may be related to cell damage detection, one of the functions of WBCs (Sheng et al., 2019; Missinhoun et al., 2021). Moreover, H3 rainbow trout showed an ability to recover WBC levels, further suggesting habituation.

Among white blood cells, two different cell types can be distinguished depending on their nuclear morphology: heterophils and mononuclear cells. Heterophils can be further classified into neutrophils, eosinophils, and basophils. The majority of heterophils are neutrophils, which do not show relevant changes when there is circulating cortisol, as described by (Mcleay, 1973). On the other hand, in the face of a stressful situation, eosinophils have been shown to decrease, whereas no changes have been observed in the basophil populations. In this sense, the depletion of eosinophils would match with the peak of plasma cortisol (Wojtaszek et al., 2002). Similarly, mononuclear cells can be subdivided into monocytes and

lymphocytes. These authors demonstrated a lack of changes in monocyte levels when facing a stressful situation, whereas lymphocytes displayed a significant reduction in their abundance. The decrease in monocytes observed in the present study could be explained by the combination of the reduction of lymphocytes and the small proportion of eosinophils among this group of cells. Further studies are needed to ascertain the lesser-known consequences of hypoxia and manipulation on white blood cells.

The main function of PLTs is related to clotting, and since no lesions were observed at any point of the experiment, no differences were expected in levels of these particular cells after hypoxia and manipulation. In addition, it has been reported that cortisol levels do not alter thrombocyte levels (Wojtaszek et al., 2002). Although a trend of decrease by repeated hypoxia and manipulation is suggested, differences were not significant.

The variation observed in cortisol levels between treated groups and control ones is commonly found in stress studies and is particularly associated with handling procedures (Acerete et al., 2004; Sadoul and Geffroy, 2019). This may also be the case in our results under hypoxia exposure since, as observed in the work of O'connor et al. (2011), neither acute nor chronic exposure to hypoxia caused significant differences with respect to the control groups. On the other hand, handling is a stressor to which the fish responds with increased cortisol levels, although the potential for habituation has been suggested (Pagès et al., 1995), so manipulation and hypoxia can raise the levels of cortisol, as observed in this study. In addition, it can be observed that the WBC levels seem to mimic the rise in cortisol levels and, therefore, the activated response of the hypothalamic-pituitary-interrenal axis (Pottinger and Carrick, 1999), as cortisol and WBC levels show a high correlation (Figure 3.1.4C, F). Similar results have been observed in other studies, such as in the work of Carbajal et al. (2019), where WBCs were higher in a polluted environment under suboptimal conditions, inducing neutrophilia and/or lymphopenia in fish. Furthermore, the increase in WBCs is related to the migration of these cells from the spleen to the blood (Barcellos et al., 2004). Moreover, regarding cortisol measurements, two different types of response patterns can be observed. On one hand, the groups that were not subjected to hypoxia did not display any significant differences between sampling points, although statistically significant differences arose when comparing the AC and MC groups, as expected. On the other hand, there is a significant reduction in cortisol levels in all groups exposed to hypoxic conditions at $t = 6$ h, with a subsequent significant increase

at $t = 24$ h. This could be partly due to circadian rhythms inherent to the species, as described by [Naderi et al. \(2018\)](#), although the lack of differences within the AC and MC groups suggests there are other factors at play. Therefore, it can be hypothesised that the decrease observed at $t = 6$ h reflects the start of the recovery period, but that the fish have developed, to some extent, a predictable behaviour to this specific stressor, as both shocks occurred at the exact same time 1 day apart. This would explain the significant increase in cortisol levels at $t = 24$ h in the treatment groups. Nonetheless, further research is needed to investigate this possible adaptive behaviour in *O. mykiss*. This hypothesis is supported by the fact that the predictability in fish was demonstrated after a positive stress stimulus (i.e., feeding) or a negative one (i.e., crowding) by changes in plasma cortisol levels ([Galhardo and Oliveira, 2009](#); [Galhardo et al., 2011](#)), and the anticipatory responses are not unusual in animals. This fact allows self-preparation for the upcoming event, giving the fish a certain control capacity on the stressor response, thus optimising the efficacy of the response ([Galhardo et al., 2011](#)). In our work, plasma cortisol levels 24 h after the hypoxic shock were higher in the hypoxic groups versus the AC group, suggesting that there is no lower response than expected. This difference should be attributed to the type of stressor, which, in this case, is hypoxia in water instead of anoxia, like in most of the studies. We must consider that the predictability response depends on the stressor properties (nature, intensity, and frequency), the signalling cascade, and the time elapsed between the signal and the onset of the negative event, as it is currently observed in stress studies ([Galhardo and Oliveira, 2009](#)).

At this point, it is interesting to emphasise that, in the present work, hypoxia was achieved by reducing the oxygen concentration in water, which is rather different from the hypoxia caused by air exposure. The latter involves a concurrent stressor, such as significant manipulation, plus the maintenance of the fish out of its aquatic environment. As observed in the study by [Franco-Martinez et al. \(2022\)](#), where an aerial exposure experiment on trout was performed, cortisol levels were much higher in percentage than in the present case, in which hypoxia was achieved by reducing the levels of oxygen from the water by displacement with nitrogen. It should be noted that the physiological stress response is effectively and quickly detected as plasma cortisol increases as early as 3 min post-stress, although the maximum peak is detected after approximately 1 h post-stress ([Gesto et al., 2013, 2015](#)). Fish that have suffered different types of stress (manipulation and hypoxia shocks) show a progressive decrease in glucose levels since it is used as a substrate for glycolytic activity, leading to the release of cell energy ([Dunn](#)

and Hochachka, 1986). In the present work, this is observed neither in the case of the AC group, which displays a return to initial levels 24 h, nor in H1, which did not display any significant variations in glucose levels throughout the sampling points. Furthermore, in most cases, acute stress induces an increase in plasma glucose, in part because of the energetic needs derived from the stress situation and the mediating effects of cortisol and catecholamines (Abdel-Tawwab et al., 2019). In addition, the absence of significant differences between the different groups at 1 h indicates that the animals have the capacity to replenish their reserves during the 48 h recovery period between different shocks. Moreover, lactate, the product generated by the glycolytic activity (Dunn and Hochachka, 1986), displays significant increases when facing a situation of hypoxia to help maintain cellular energy balance (Dunn and Hochachka, 1986; Richards, 2011), as observed in the present study. This happens because oxygen-independent energetic mechanisms are needed since oxygen-dependent mechanisms are 15 times lower under hypoxic conditions (Richards, 2011). In this sense, the group subjected to one hypoxia is the one with the highest lactate values, so it would be the group that activated anaerobic metabolism the most. It is important to highlight the fact that the group subjected to three exposures and manipulations did not display significant increases in lactate levels, which, again, suggests habituation in the same way as occurs in the case of haematological parameters. This habituation may be facilitated by the mildness of the stressor, which was applied only for short periods of time (Tort et al., 2001).

3.1.5 Conclusion

The overall results show tolerance and a capacity for a certain degree of habituation in an oxygen-sensitive species such as *O. mykiss* as a response to repeated manipulation and hypoxia. Initial differences are observed in haematological parameters between the treated groups and the control, although these differences are overall minimised after three shocks. This return to absolute control levels suggests habituation of some sort, regarding both the correct functioning of red blood cells in transporting and supplying oxygen and white blood cells in host immunity and cell repair. Although cortisol measurements did not return to absolute control levels, they did display a decreasing trend, indicating that, in the long term, fish might be able to recover basal levels, provided that the stressors, hypoxia and manipulation, maintain the same characteristics. The hypothesis of habituation is further supported by the trends observed in glucose and lactate

levels, which display significant alterations associated with changes in aerobic and anaerobic metabolism but return to absolute control levels after the third shock and manipulation. As a general conclusion, it can be stated that rainbow trout are capable of habituating to both these temporary hypoxia episodes and manipulation events without having significant negative consequences at the functional level. However, to firmly affirm this, it would be necessary to analyse how gene and protein expression are modulated, since it may help to understand basic molecular and cellular changes associated with the tolerance of hypoxia and manipulation.

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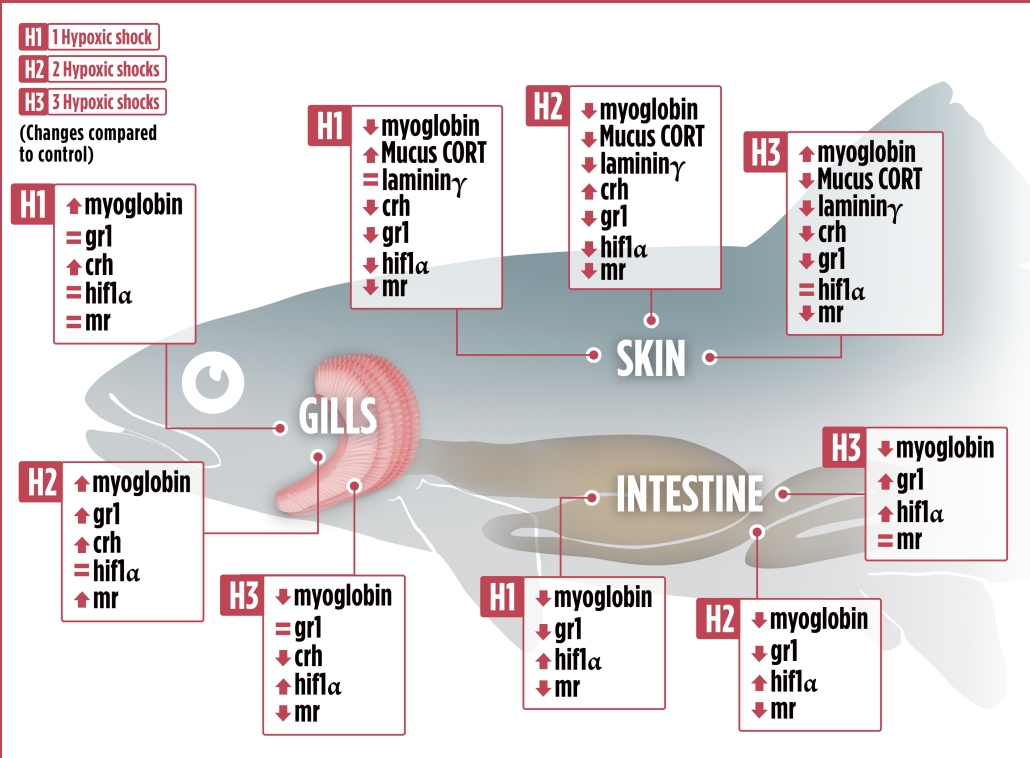
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Response of rainbow trout mucosal tissues under repeated hypoxia episodes

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3.2

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Abstract

Hypoxic events are common in both natural and aquaculture environments due to external factors or anthropogenic activities. To cope with this fluctuating environment, fish activate complex systemic stress responses, but less is known about their mucosal responses. To investigate this, five experimental groups were established: absolute control (AC), manipulated control (MC), and three groups exposed to a single hypoxic shock (H1) or repeated hypoxic shocks (H2 and H3), sampled at 1, 6, and 24 h after hypoxia. Mucus cortisol levels increased 1 h after manipulation and hypoxic shocks, confirming that the stress response was activated. However, cortisol levels decreased as the fish underwent more shocks. A significant up-regulation of the *crh* gene by treatment was observed in the gills, while no expression was detected in the intestine. Additionally, an up-regulation of *gr1* and *mr* gene expression was found in the gills, whereas the skin and intestine showed an opposite response, with gene expression returning to control levels after the third hypoxic shock (H3). *Hif1 α* and myoglobin gene expression showed clear tissue-specific regulation, likely related to their primary functions. In the skin and intestine, as *hif1 α* levels increased, *myoglobin* expression decreased, while in the gills, the opposite pattern was observed, with higher *myoglobin* expression during the reoxygenation phase. Overall, the results suggest that repeated hypoxia promotes tolerance and a certain degree of habituation in rainbow trout, without compromising their survival.

3.2.1 Introduction

Organisms are dependent on oxygen to perform cellular aerobic metabolism (Baik and Jain, 2020), but in aquatic environments, hypoxia, low dissolved oxygen (DO) or even their lack (anoxia), are not uncommon phenomena in both marine and freshwater systems (Breitburg et al., 2018; Wang et al., 2021; Zhao et al., 2024). In nature, fluctuations in DO levels can result from changes in external factors, such as barometric pressure, temperature, depth, salinity (Díaz and Breitburg, 2011) and anthropogenic eutrophication (Breitburg et al., 2018; Riesch et al., 2015). Hypoxic events also occur in aquaculture rearing conditions, due to low water flow, confinement and crowding, developmental stage, and management protocols (e.g., handling, slaughtering, overfeeding, organic waste accumulation due to lack of proper maintenance, disease treatments) (Bera et al., 2017; Magnoni et al., 2019; Miller et al., 2008; Wood, 2018). Furthermore, considering the actual scenario of climate change, the incidence of aquatic hypoxia is expected to increase, also favoured by pollution and other anthropogenic activities (Borowiec et al., 2015; Ficke et al., 2007).

Low DO availability constitutes a serious threat to fish by homeostasis imbalance, compromising oxygen and consequently cellular ATP supplies. This could trigger a stress response that activates primary, secondary, and tertiary responses (Schreck and Tort, 2016). To cope with such common oxygen fluctuations, fish employ several strategies, including behavioural, physiological, and biochemical responses (Farhat et al., 2021; Wang et al., 2016, 2023; Zhao et al., 2024). Those actions contribute to reducing energy consumption by altering metabolic pathways and facilitating O₂ uptake under hypoxic conditions (Richards, 2009; Wang et al., 2023). The effectiveness of the set of strategies will depend on the pattern and/or severity of hypoxia exposure, habitat, fish species and their life stages (Balasch and Tort, 2019; Borowiec et al., 2015; Dan et al., 2014; Farrell and Richards, 2009; Pollock et al., 2007). Thus, knowledge in how aquatic animals cope with this stochastic environment, like repeated hypoxia, is essential to understand how fish physiology mechanisms help to resilience under such changing environments, as has been demonstrated in mammals (Douglas et al., 2007; Farahani et al., 2008; Neubauer, 2001). In this sense, some studies demonstrate that diel-cycling hypoxia and preconditioning improve fish hypoxia tolerance (Borowiec et al., 2015; Brady et al., 2009; Madaro et al., 2016, 2015; Williams et al., 2019; Yang et al., 2013) even in hypoxia-sensitive species like rainbow trout (*Oncorhynchus mykiss*) (Gamperl et al., 2004, 2001). Accordingly, recently published haematological

results related to the present experiment showed a habituation pattern after repeated hypoxia shocks, supported by reductions in plasma cortisol, glucose, and lactate levels as the number of hypoxic shocks increased (Ruiz et al., 2024).

One of the main regulators of the hypoxic stress response is the hypoxia signalling pathway, crucial to maintaining cell oxygen homeostasis, which is evolutionarily conserved in animals (Xiao, 2015). The Hypoxia-inducible factor is a ubiquitous heterodimer constituted by HIF α , an oxygen-labile subunit, and HIF β constitutively expressed. Under normoxia, HIF α is hydroxylated, ubiquitinated and degraded by the proteasome (Kaelin, 2007), whereas under hypoxic conditions heterodimerizes with HIF β subunit, in the nucleus, to regulate the transcription of several genes through binding to hypoxia response elements (HREs) (Dunwoodie, 2009; Majmundar et al., 2010; Semenza, 2014, 2012) contributing to hypoxic survival (Mandic et al., 2021). Despite its regulation seeming to be similar in fish and mammals, a direct link between HIF function and hypoxia tolerance is not found in fish (Richards, 2009). Another relevant molecule involved in the regulation of the hypoxic stress response is *myoglobin*. This molecule is widely believed to be exclusively present in muscle tissue (Ordway and Garry, 2004; Wittenberg and Wittenberg, 2003), but alternative functions conferring protection by the generation of nitric oxide (NO) during ischaemia (Elkholi et al., 2022; Hendgen-Cotta et al., 2008; Rassaf et al., 2007) and scavenging reactive O₂ species (ROS) (Brunori et al., 2001) have been described in mammals. The first studies conducted in fish related to these alternative functions were done in carp and zebrafish (Cossins et al., 2009; Fraser et al., 2006) and demonstrated the presence and upregulated expression of *myoglobin* in non-muscle tissues, such as liver, pancreas, brain, gills, intestine, and kidney after hypoxia treatments.

Recently, mucosal tissues have received more attention as they establish a physical barrier against external insults, being the first line of defence in contact with the aquatic environment. In those epithelial barriers, scattered immune cells are present, as well as their effector molecules that constitute mucosal-associated lymphoid tissues (MALTs). In addition, a mucus layer, secreted by goblet cells, contains several bioactive compounds and the co-habitant microbiota (Cabillon and Lazado, 2019). The plasticity of these mucous barriers is key to detecting and adapting to environmental changes since they could act as environmental sensors. Thus, a local stress detection and production of glucocorticoids may allow fish to cope with this allostatic load without triggering a broad response (Wingfield, 2013), which will confer an adaptive advantage to cope with stressful situations.

With this background, the objectives of the present study were 1) to confirm that a peripheral stress response takes place by mucus cortisol determination, 2) to verify the mucosal tissues stress response following corticotrophin-releasing hormone (*crh*), glucocorticoid receptor (*gr1*) and mineralocorticoid receptor (*mr*) local gene expression and 3) to elucidate if, under repeated hypoxic conditions, there is a regulation of hypoxic signalling pathways and myoglobin expression in different mucosal tissues (gills, intestine and skin) that would confer an improvement in the hypoxia tolerance in rainbow trout.

3.2.2 Material and methods

3.2.2.1 Fish, experimental design, and sampling

Rainbow trout juveniles (*Oncorhynchus mykiss*) (Molinou, Rialb, Spain) were randomly distributed in triplicate tanks ($n = 27$; $6.80 \pm 0.1 \text{ Kg/m}^3$) and acclimated to the recirculating aquaculture system (RAS) facilities of the AQUAB group (Universitat Autònoma de Barcelona). During this period, fish were fed *ad libitum* once a day with a commercial diet, under 12 L: 12 D photoperiod, $14.6 \pm 0.3 \text{ }^\circ\text{C}$ and an oxygen concentration between 7.20 and 8.10 mgO_2/L maintained by air pumps. After the acclimation, the trout, with a final body weight of $62.9 \pm 11.1 \text{ g}$, were divided in five experimental groups by triplicate (**Table 3.2.1**) two of them remained in normoxia, whereas the other 3 groups underwent one, two or three acute hypoxic shocks ($2.2 \pm 0.5 \text{ mgO}_2/\text{L}$) for 1 h, being 48 h the time between shocks.

Table 3.2.1: Description of the different experimental groups and the kind of treatment.

Experimental group	Treatment ($n = 27$ fish, 9 per sampling point)
Absolute control (AC)	Fish in normoxia, always in the same tank
Manipulated control (MC)	Fish in normoxia with manipulation ^a
Hypoxia 1 (H1)	Fish that underwent 1 hypoxia challenge
Hypoxia 2 (H2)	Fish that underwent 2 hypoxia challenges ^b
Hypoxia 3 (H3)	Fish that underwent 3 hypoxia challenges ^b

^aFish were changed from a tank to a new one and moved to a recovery tank after one hour to know the effects of stress manipulation.

^bRepeated acute hypoxia stress every 48 h, between shocks the fish were returned to the acclimation tank under normal rearing conditions.

Briefly, hypoxic levels were reached by reducing the dissolved oxygen (DO) concentration, from $7.5 \pm 0.5 \text{ mgO}_2/\text{L}$ to $2.2 \pm 0.5 \text{ mgO}_2/\text{L}$, by adding nitrogen gas (N_2) to the water and after that, fish were transferred for 1 h. Three tanks were used to replicate the procedure, and fish density was maintained. Continuous oxygen levels monitoring was carried out to ensure that DO did not fall below $1.5 \text{ mgO}_2/\text{L}$, which would be lethal to the species (**Figure 3.2.1**). After the hypoxia shock, rainbow trout were placed in a recovery tank (DO between 7.2 and $8.1 \text{ mgO}_2/\text{L}$) that was isolated from the other tanks to prevent cortisol levels from affecting other fish. In this tank, the water volume was adjusted to maintain a constant density of $6.80 \pm 0.1 \text{ Kg/m}^3$. By the end of the experiment, oxygen levels and water turnover were restored to optimal levels. For a detailed description of the experimental design and how the hypoxic condition in tanks was achieved, see [Ruiz et al. \(2024\)](#).

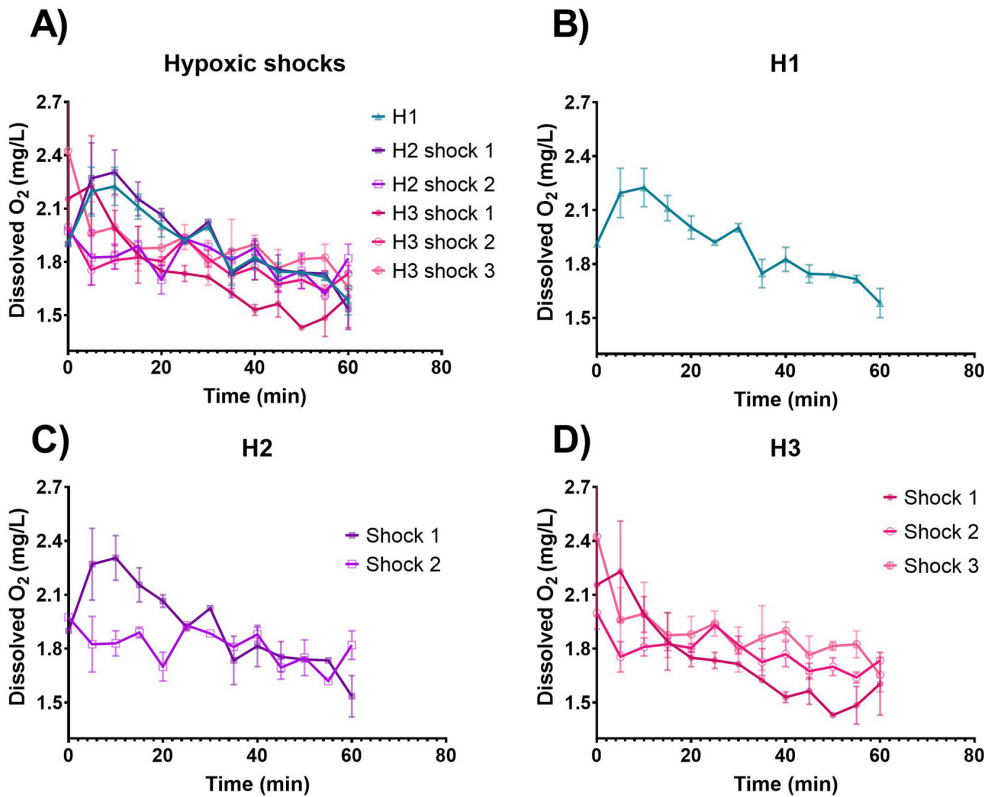


Figure 3.2.1: Dissolved oxygen concentration in tanks during the 60 min under the hypoxic shock. A) Hypoxic shocks from H1, H2 and H3 rainbow trout, B) Hypoxic shock from H1 rainbow trout, C) Hypoxic shocks from H2 rainbow trout and D) Hypoxic shock from H3 rainbow trout. Data are represented as mean $\pm$ SEM from triplicate tanks.

After manipulation or the last shock for each group, fish were transferred to the same recovery tank to avoid the tank effect. This tank was isolated from the system until sampling at different time points after hypoxia shock was finished (1 h, 6 h and 24 h), since cortisol present in water from these fish could affect the rest of the fish in the rearing system. Moreover, this kind of sampling makes it necessary to reduce water levels in the tank to keep fish density constant ($6.80 \pm 0.1 \text{ Kg/m}^3$). For sampling, 9 fish per time point and treatment (AC, MC, H1, H2 and H3), a total of 27 rainbow trout per group, were anaesthetised with tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate, measured, and weighed. Skin mucus samples were quickly and accurately collected with a slide into a Falcon and remained on ice for later processing. After that, fish were sacrificed by sectioning the spinal cord and samples from gills, intestine and skin were collected and frozen in liquid nitrogen and stored at -80°C for gene expression analysis. All animal procedures carried out comply with the guidelines of the Council of the European Union directive (EU 2010/63) and were approved by the Ethics and Animal Care Committee of the Universitat Autònoma de Barcelona (permit number OH4218_4219 and DAMM 11251).

3.2.2.2 Skin mucus cortisol determination

After the sampling, phosphate buffer saline (PBS, Ref: P5493, Sigma Aldrich) was added to fresh skin mucus samples and was homogenised with a syringe and centrifuged (10.000 g, 10 min, 4°C) and the supernatant was stored at -80°C until cortisol analyses. Skin mucus cortisol levels were determined using a commercial EIA kit (Cortisol ELISA KIT; Neogen[®] Corporation, MerAyr, United Kingdom), previously validated for rainbow trout by Carbajal et al. (2019) and following manufacturer's instructions. To make mucus cortisol concentration comparable between treatments, the amount of total protein was determined by the Bradford method, according to Fernández-Alacid et al. (2018).

3.2.2.3 RNA extraction and cDNA synthesis

TRIzol reagent solution[®] (Sigma Aldrich, USA) was used for total RNA isolation, according to the manufacturer's instructions. Briefly, 1 mL of TRIzol was used for tissue homogenization (about 30 mg of gills or intestine; 75-100 mg of skin). Bromochloropropane was added, and after centrifugation, the aqueous phase was extracted and mixed with isopropanol for RNA precipitation. After that, a couple

of washes with 75 % of ethanol have been done to finally resuspend the RNA in DEPC water. Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA) was used to assess RNA purity (A260/A280 ratio) and RNA concentration at 260 nm. The cDNA of each sample was synthesised from 1 μ g of RNA using the iScript[™] cDNA Synthesis Kit (Bio-rad, USA) following the manufacturer's instructions.

3.2.2.4 Real-time quantitative-PCR (qPCR)

Gene expression was analysed using real-time quantitative PCR (qPCR) in a CFX Touch[™] Real-Time PCR Detection System (Bio-Rad, USA), according to MIQE guidelines (Bustin et al., 2009). The genes analysed in gills, intestine and skin include stress-related genes corticotropin-releasing hormone (*crh*), glucocorticoid receptor (*gr1*) and mineralocorticoid receptor (*mr*) and genes related to hypoxia hypoxia-inducible factor 1 alpha (*hif1 α*), *myoglobin*, and *laminin γ* , only analysed in skin since it is a keratinocyte secreted glycoprotein. β -actin (β -*actin*), elongation factor- 1 α (*elf-1 α*) and ribosomal protein S18 (*18s*) were used as reference genes for intestine and gills, whereas ribosomal protein S5 (*rps5*), β -*actin* and *elf-1 α* were used for skin. All pairs of primers had been previously validated in rainbow trout tissues, and their efficiency was tested specifically for each tissue (García-Meilán et al., 2022; Holen et al., 2021; Rebl et al., 2017; Teles et al., 2013, 2011) and their sequence and reference are specified in Table 3.2.2, and their efficiency and GenBank accession number in Table 3.2.3.

Prior to the analyses, a dilution curve with a pool of samples was run to determine the appropriate cDNA dilution for each gene, as well as to confirm the absence of primer dimers, and the specificity of the reaction by a single peak in the melting curve for each primer set. Briefly, all the analyses were performed in duplicate wells using 384-well plates with a final volume of 5 μ L (2.5 μ L iTAq[™] Universal SYBR[®] Green Supermix (Bio-Rad, USA), 250 nM of forward and reverse primers and 1 μ L of diluted cDNA for each sample). The qPCR program had an initial desaturation step of 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The software Bio-Rad CFX Maestro version 4.1 was used to confirm reference genes stability by the gNorm algorithm, *18s*, *elf-1 α* and β -*actin* in gills and intestine; and *rps5*, β -*actin* and *elf-1 α* in skin, allowing to calculate the expression level of each target gene relative to the geometric mean of the three most stable genes for each tissue by the Pfaffl method (Pfaffl, 2001).

Table 3.2.2: Primer sequences used in the experiment. Forward (FW) and reverse (RV) sequence (3'-5') and the reference of where the sequences were obtained.

Gene	Sequence	Reference
<i>gr1</i>	FW: CGCAGCAGAACCAACAGTTG RV: ATGAGGGCGTCCAAGTACAGA	(Teles et al., 2013)
<i>mr</i>	FW: GGCAGCGTTTGAGGAGATGA RV: CATGGCGTCCAGTAGCTTGG	(Teles et al., 2013)
<i>crh</i>	FW: AAGTTGGGAACATCAGCCG RV: ATCTGTCTCGGAGCATGTGGAAC	(García-Meilán et al., 2022)
<i>hif1α</i>	FW: TTCTCTGTGCTCTTCTGTGCG RV: TGAGTAAGGAAGCAGGGCAA	(García-Meilán et al., 2022)
<i>myoglobin</i>	FW: AACAAACACGGAGGACTGGTT RV: CGATGCCTGCGAACTTAGGG	(García-Meilán et al., 2022)
<i>laminin γ</i>	FW: CAGTCTGCAACGACGAGTTCAG RV: CGAATGTGTTGAGCCTGTCCAG	Designed
<i>18s</i>	FW: CGAGCAATAACAGGTCTGTG RV: GGGCAGGGACTTAATCAA	(García-Meilán et al., 2022)
<i>β-actin</i>	FW: GGACTTTGAGCAGGAGATGG RV: ATGATGGAGTTGTAGGTGGTCT	(García-Meilán et al., 2022)
<i>elf-1α</i>	FW: TGCCCCTGGACACAGAGATT RV: CCCACACCACCAGCAACAA	(Holen et al., 2021)
<i>rps5</i>	FW: ATGACATCTCACTGCAGGATTAC RV: ATCAGCTTCTTGCCGTTGTTGC	(Rebl et al., 2017)

Table 3.2.3: Efficiencies and accession number of the selected primers.

Gene	Gills (%)	Skin (%)	Intestine (%)	Accession number
<i>gr1</i>	90.4	91.5	90.6	Z54210.1
<i>mr</i>	99.2	101.3	99.8	AF209873.1
<i>crh</i>	106.8	101.9	-	NM_001124286.1
<i>hif1α</i>	93.3	90.2	94.5	NM_001124288.1
<i>myoglobin</i>	92.9	91.1	101.2	NM_001171862.1
<i>lamininγ</i>	-	97.2	-	XM_036977011.1
<i>18s</i>	92.1	91.5	90.6	AF243428.2
<i>β-actin</i>	95.8	91.3	93.8	XM_036972332.1
<i>elf-1α</i>	92.2	91.2	91.0	NM_001124339
<i>rps5</i>	-	105.9	-	NM_001160519

3.2.2.5 Statistical analysis

The statistics software used was RStudio (<https://www.R-project.org>). Once the outliers were removed, the normality was checked by the Shapiro-Wilk normality test and the homogeneity of the variance by the Bartlett test. Since none of the parameters followed normality and/or homogeneity requirements, a generalised linear model (GzLM) was performed as a non-parametric test. The main factors: treatment (normoxic and hypoxic groups) and recovery time were analysed, also searching for their interaction effects. If the factor analysed had differences between groups (p -value < 0.05), a post-hoc test was performed using Tukey's correction (p -value < 0.05).

3.2.3 Results

Among all the parameters analysed, treatment is the factor that exhibits the most significant differences. For this reason, we decided to present the results grouped by treatment (T), and only in cases where there is a clear effect of time (H) will the corresponding results be analysed. The interaction between both factors was also commented on when significant differences were found. All results regarding statistical analyses are included in a supplementary appendix (**Chapter 9**).

The presence of cortisol in skin mucus was strong and significantly affected by time, treatment and their interaction (p -value < 0.000 for all factors), being time the one with the higher effect ($\eta_p^2 = 0.479$), followed by the interaction ($\eta_p^2 = 0.307$) and finally treatment ($\eta_p^2 = 0.252$) (**Figure 3.2.2**). The highest levels of mucus cortisol were observed in the MC group, followed by H1. The H2 and H3 groups displayed slightly lower levels, although the differences were below the threshold for significance, showing that cortisol levels decreased as more shocks they received. Finally, cortisol levels were significantly lower in the AC group regardless of time post-treatment (p -value < 0.05) (**Figure 3.2.2A**). Regarding time, cortisol levels were significantly higher 1 h after manipulation or hypoxic shock, while they were practically recovered at 6 h, although they presented a rebound at 24 h after the shock (**Figure 3.2.2B**), only significant in MC rainbow trout (p -value < 0.05) (**Figure 3.2.3**). Regarding the interaction effect, it can be observed in (**Figure 3.2.3**) a clear increase of the levels at 1 h in the groups that underwent manipulations and hypoxic shocks that is statistically significant with the values at 6 h and 24 h in all the groups.

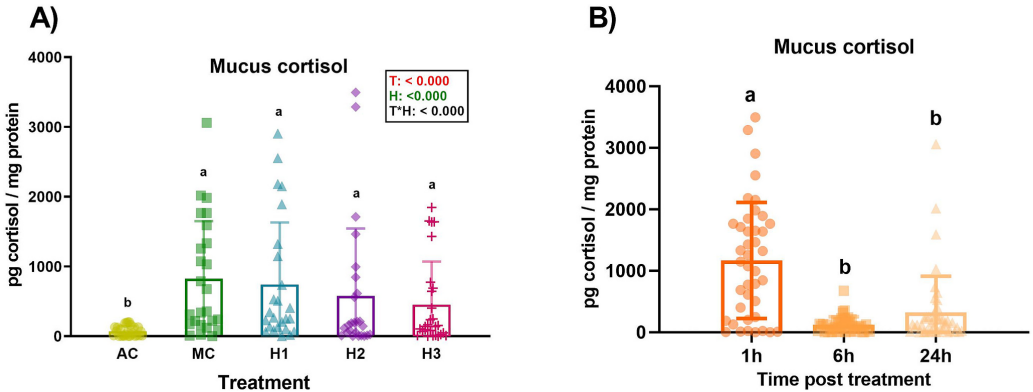


Figure 3.2.2: A) Skin mucus cortisol levels in rainbow trout by treatment. Data are represented as mean $\pm$ SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p -value < 0.05). P -values for T: Treatment effect; H: Time effect and T*H: Interaction effect are included. B) Skin mucus cortisol levels in rainbow trout by time. Data are represented as mean $\pm$ SEM ($n = 45$, 5 treatments and 9 fish per sampling time at 1, 6 and 24 h). Significant differences in sampling time are shown by letters (p -value < 0.05).

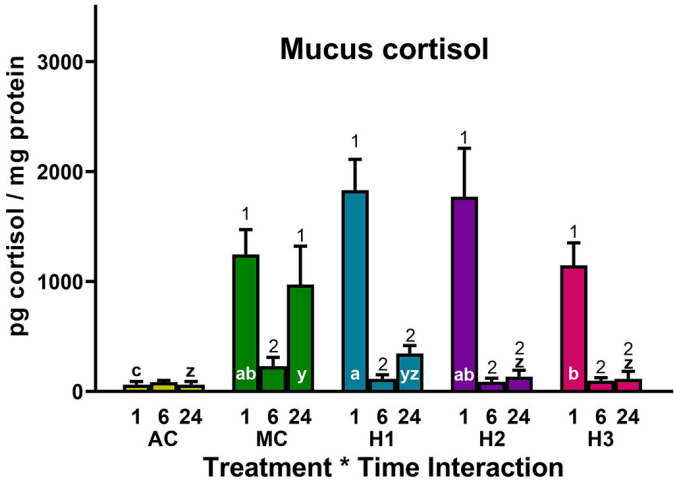


Figure 3.2.3: Mucus cortisol in rainbow trout 1, 6 and 24 h after treatment. Data are represented as mean $\pm$ SEM ($n = 9$ per sampling time). Significant differences in sampling time among the same treatment are shown by numbers (p -value < 0.05). In case of interaction effects, significant differences (p -value < 0.05) between treatments at the same sampling time are shown with different letters (a/c at 1 h, and y/z at 24 h after treatment).

Stress-related gene expression was studied in three different mucosal tissues: gills, directly related to oxygen delivery, skin, a surface area in direct contact with water and finally the intestine, whose response against stress is very little known.

In the skin and intestine, most of the gene expression at the neuroendocrine level was only affected by treatment, being independent of recovery time. However, in gills, relative gene expression was only affected by treatment in both *crh* and *mr* (Figure 3.2.4A and Figure 3.2.6A, respectively), whereas both factors (T: 0.003 and H: 0.003, respectively) and an interaction effect were found for *gr1* (Figure 3.2.5A).

The expression of *crh* in gills increases significantly after manipulation (MC), one, and two hypoxic shocks (H1, and H2) with respect to control (AC) (p -value < 0.05), no differences were found after three shocks (H3) (p -value > 0.05) (Figure 3.2.4A). In skin, significant differences in *crh* expression did not show a clear pattern related with treatment, since significant differences were detected in AC and H2 groups compared to H1 and H3 groups, showing MC intermediate values, all of them independent of time recovery (Figure 3.2.4B). Regarding the expression in the intestine, it is worth noting that *crh* was not detected under these experimental conditions.

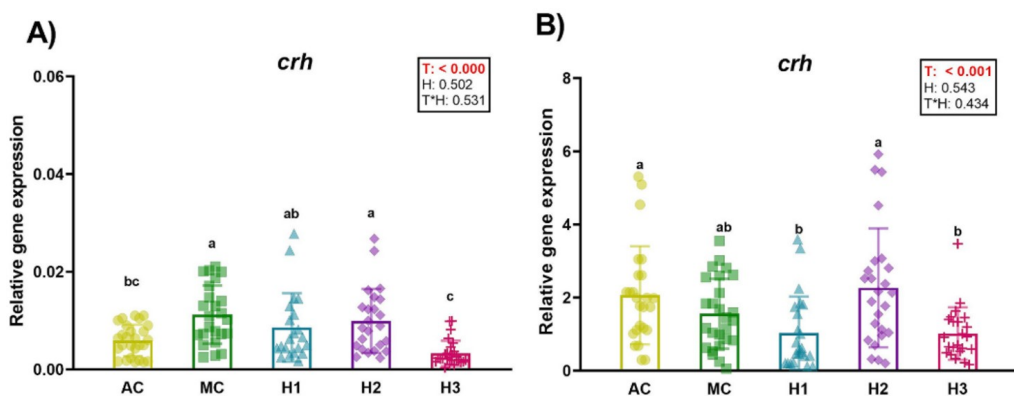


Figure 3.2.4: A) Gills and B) Skin *crh* gene expression in rainbow trout by treatment. Data are represented as mean $\pm$ SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p -value < 0.05). P -value for T: Treatment effect; H: Time effect and T*H: Interaction effect are included.

The glucocorticoid receptor (*gr1*) expression in gills was significantly higher in H2 among treatments (p -value < 0.05) (Figure 3.2.5A). Moreover, *gr1* expression levels increase over time, being at 24 h post hypoxia and or manipulation significantly higher than 1 and 6 h after the shock (p -value < 0.05) (Figure 3.2.7A). H1 and H2 rainbow trout presented the higher expression levels at 24 h, together with H2 at 6 h being significantly different from H3 and AC, respectively (Figure 3.2.7B).

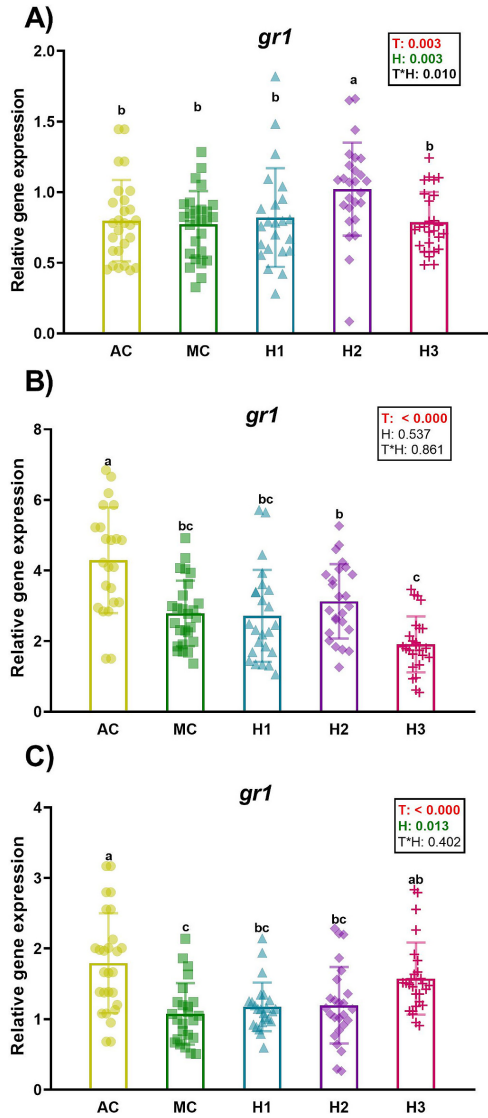


Figure 3.2.5: A) Gills, B) Skin and C) Intestine *gr1* gene expression in rainbow trout by treatment. Data are represented as mean $\pm$ SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p -value < 0.05). P -value for T: Treatment effect; H: Time effect and T*H: Interaction effect are included.

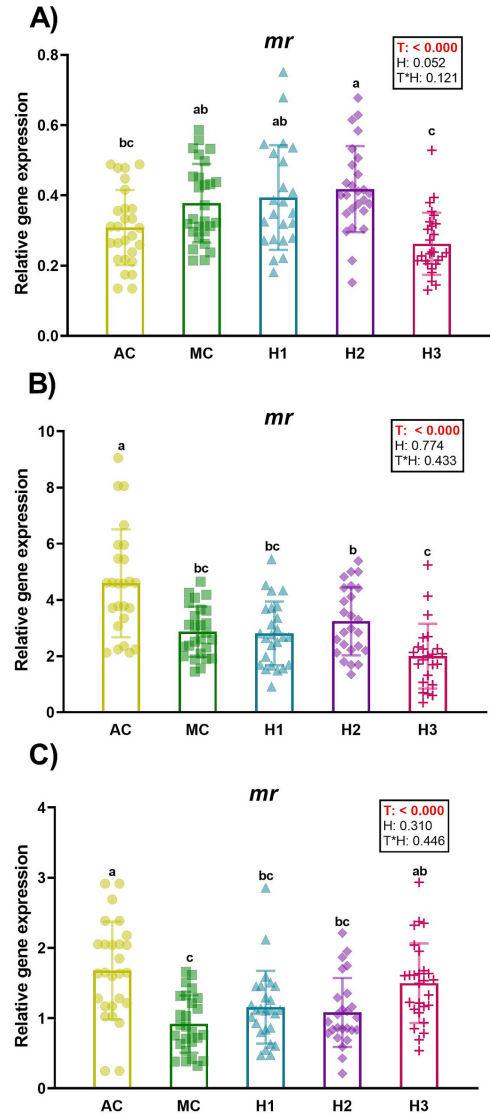


Figure 3.2.6: A) Gills, B) Skin and C) Intestine *mr* gene expression in rainbow trout by treatment. Data are represented as mean $\pm$ SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p -value < 0.05). P -values for T: Treatment effect; H: Time effect and T*H: Interaction effect are included.

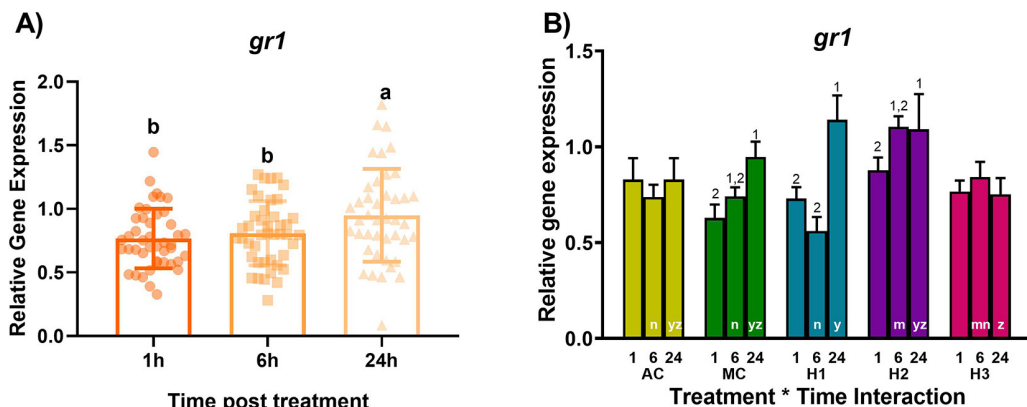


Figure 3.2.7: A) Gills *grl* gene expression in rainbow trout by time. Data are represented as mean $\pm$ SEM ($n = 45$, 5 treatments and 9 fish per sampling time at 1, 6 and 24 h). Significant differences in sampling time are shown by letters (p -value < 0.05). B) Gills *grl* gene expression in rainbow trout 1, 6 and 24 h after treatment. Data are represented as mean $\pm$ SEM ($n = 9$ per sampling time). Significant differences in sampling time among the same treatment are shown by numbers (p -value < 0.05). In case of interaction effects, significant differences (p -value < 0.05) between treatments at the same sampling time are shown with different letters (m/n at 6 h, and y/z at 24 h after treatment).

On the contrary, in skin and intestine a downward regulation in the trout that had been handled (MC), and also those subjected to hypoxia shocks was found (p -value < 0.05); but with a differential response in H3 for the two tissues studied, since it increases the expression in the intestine reaching similar values to those of the control (AC) (Figure 3.2.5C), while in the skin it reaches the lowest values (Figure 3.2.5B). In addition, a time effect in *grl* gene expression was found in the intestine, where a significant increase between 6 and 24 h post hypoxia was found in all groups (p -value < 0.05). Regarding mineralocorticoid receptor (*mr*) relative gene expression, in gills, a significant upward trend was found in H2 rainbow trout versus AC and H3 (p -value < 0.01 and p -value < 0.000 , respectively), showing the latter group the lowest values (Figure 3.2.6A). In skin, the expression of this receptor followed the same profile as those of *grl*, showing a downward regulation as more hypoxic shocks fish received (p -value < 0.01) (Figure 3.2.6); while in intestine manipulation clearly downregulates its *mr* expression compared to AC (p -value < 0.001), whereas trout under hypoxia, firstly downregulated (p -value < 0.05 for H1 and p -value < 0.01 for H2 versus AC), but presented a rebound after 3 hypoxic shocks (Figure 3.2.6C).

The expression of genes related to the hypoxia response, such as *hif1 α* and *myoglobin*, was also studied and seems to present an opposite expression profile, since if the expression of *hif1 α* rose, *myoglobin* expression diminished and vice versa in gills and intestine, whereas in skin a similar expression pattern was measured (**Figure 3.2.8** and **Figure 3.2.9**). In gills and skin, gene expression of *hif1 α* was modulated by the interaction of treatment and recovery time (**Figure 3.2.8A, B**), whereas in the intestine was only affected by treatment (**Figure 3.2.8C**), like *myoglobin* among the three tissues (**Figure 3.2.9A-C**). In gills, (**Figure 3.2.10A**) A time effect was observed with 6 h being the most upregulated group different from 1 h, while 24 h had intermediate values between the other two groups, *hif1 α* was lower than control in groups MC, H1 and H2 (p -value < 0.001, for all treatments), mainly 1 and 6 h after treatment (**Figure 3.2.10B**). However, in H3 a significant upward regulation is observed regardless of the time after the shock compared to AC (p -value < 0.001) (**Figure 3.2.8A**). Regarding skin, no differences in expression were detected in the MC and H3 fish compared to AC, while in the H1 and H2 groups *hif1 α* expression significantly diminished (p -value < 0.01 for both treatments, respectively). It is worth noting that all treatments tended to decrease their expression 1 h after shock compared to AC group, reaching similar levels at 6 h, but only MC and H3 have recovered *hif1 α* gene expression at 24 h (**Figure 3.2.11**). In intestine, *hif1 α* showed significant upward regulation for MC and all groups subjected to hypoxia (p -value < 0.05) (**Figure 3.2.8C**). *Myoglobin* gene expression modulation seems to be tissue-specific (**Figure 3.2.9**). In this sense, in gills *myoglobin* showed an upward regulation respect to control trout, both after handling and hypoxic shock (p -value < 0.01), except for H3 that was similar to AC (**Figure 3.2.9A**); whereas *myoglobin* expression in skin and intestine was reduced compared to AC fish, with no differences between fish only handled or subjected to acute hypoxia shocks (p -value < 0.01 and p -value < 0.01, respectively) (**Figure 3.2.9B, C**).

Finally, *laminin γ* gene expression was assessed in skin due to the absence of a clear *hif1 α* pattern (**Figure 3.2.12**). In this sense, a clear treatment effect was observed (p -value < 0.000), showing all groups subjected to hypoxia shocks a decrease in their expression, having the lowest H3 rainbow trout compared to AC (p -value < 0.001).

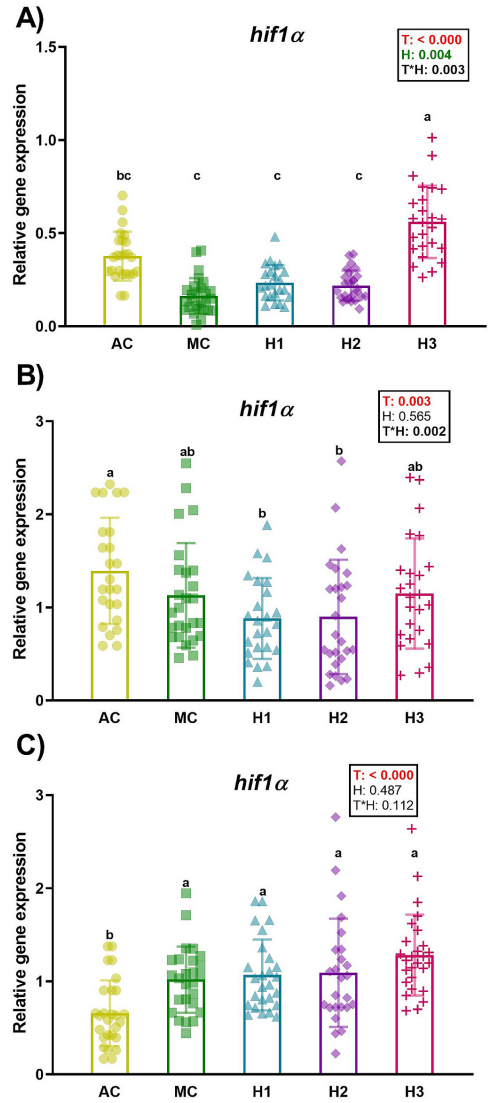


Figure 3.2.8: A) Gills, B) Skin and C) Intestine *hif1α* gene expression in rainbow trout by treatment. Data are represented as mean ± SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p -value < 0.05). P -value for T: Treatment effect; H: Time effect and T*H: Interaction effect are included.

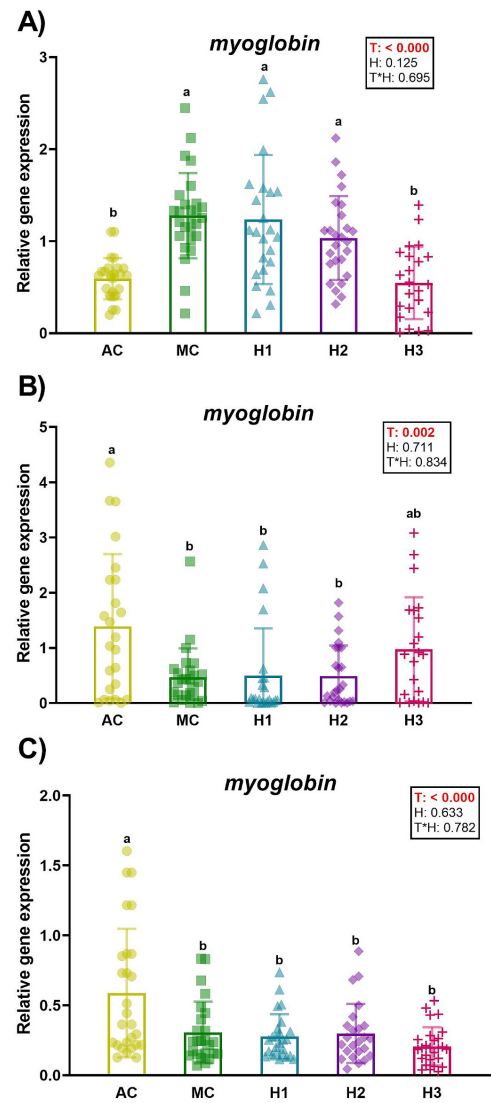


Figure 3.2.9: A) Gills, B) Skin and C) Intestine *myoglobin* gene expression in rainbow trout by treatment. Data are represented as mean ± SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p -value < 0.05). P -value for T: Treatment effect; H: Time effect and T*H: Interaction effect are included.

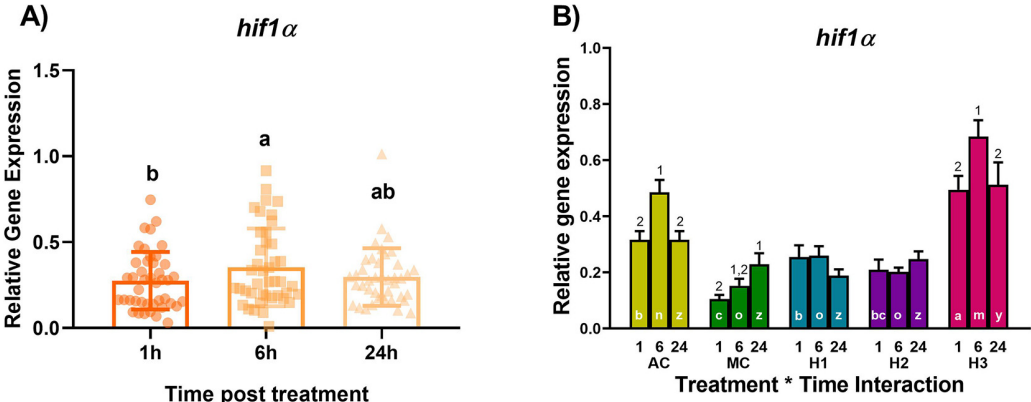


Figure 3.2.10: A) Gills *hif1α* gene expression in rainbow trout by time. Data are represented as mean $\pm$ SEM ($n = 45$, 5 treatments and 9 fish per sampling time at 1, 6 and 24 h). Significant differences in sampling time are shown by letters (p -value < 0.05). B) Gills *hif1α* gene expression in rainbow trout 1, 6 and 24 h after treatment. Data are represented as mean $\pm$ SEM ($n = 9$ per sampling time). Significant differences in sampling time among the same treatment are shown by numbers (p -value < 0.05). In case of interaction effects, significant differences (p -value < 0.05) between treatments at the same sampling time are shown with different letters (a/c at 1 h, m/o at 6 h, and y/z at 24 h after treatment).

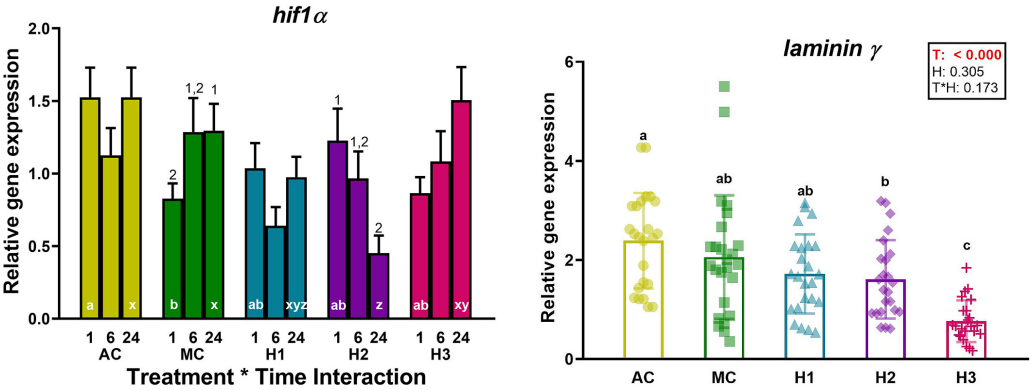


Figure 3.2.11: Skin *hif1α* gene expression in rainbow trout 1, 6 and 24 h after treatment. Data are represented as mean $\pm$ SEM ($n = 9$ per sampling time). Significant differences in sampling time among the same treatment are shown by numbers (p -value < 0.05). In case of interaction effects, significant differences (p -value < 0.05) between treatments at the same sampling time are shown with different letters (a/b at 1 h, and x/z at 24 h after treatment).

Figure 3.2.12: Skin *lamininγ* gene expression in rainbow trout by treatment. Data are represented as mean $\pm$ SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p -value < 0.05). P -value for T: Treatment effect; H: Time effect and T*H: Interaction effect are included.

In **Figure 3.2.13** a normalized gene expression heatmaps were presented allowing to clearly visualize the different gene expression regulation among tissues by treatment. Thus, in gills most of the genes are upregulated regarding the AC group, except for *hif1 α* . Despite this, in most cases after three hypoxic shocks (H3) expression levels tended to achieve control values. On the contrary in skin, most of the genes analysed were downregulated compared to AC rainbow trout (**Figure 3.2.13B**); whereas in intestine those subjected to three hypoxic shocks (H3) partially recovered expression, being in this case *hif1 α* upregulated compared to AC fish (**Figure 3.2.13C**) and *myoglobin* is downregulated, unlike in the gills.

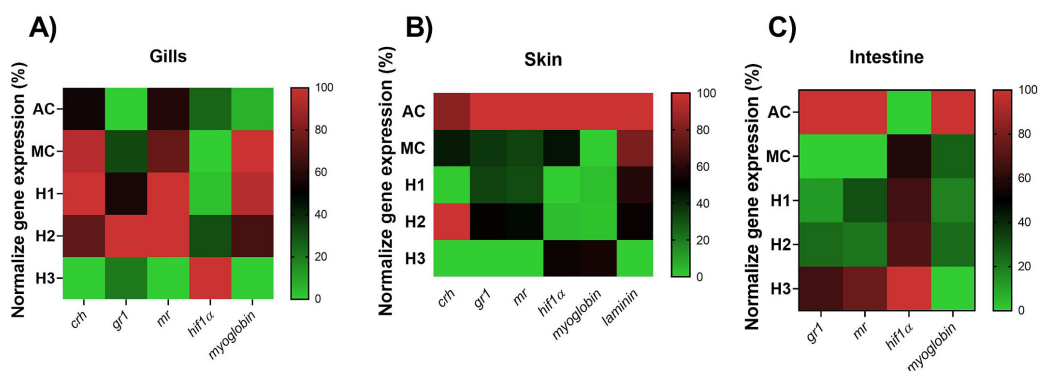


Figure 3.2.13: A) Gills, B) Skin and C) Intestine normalized gene expression heatmap by treatment in rainbow trout.

3.2.4 Discussion

Oxygen homeostasis is essential for animal growth, development, and reproduction, leading animals to evolve responses to hypoxia (Semenza, 2014). This study investigated the mucosal tissues response of rainbow trout to manipulation or acute and repeated hypoxic stress (2.2 ± 0.5 mgO₂/L), during the reoxygenation phase (1, 6, and 24 h post-stress), addressing the limited understanding of repeated hypoxia effects in fish (Borowiec et al., 2015). As expected, mucus cortisol levels, a stress response biomarker, peaked 1 h after stress across experimental groups compared to control, aligning with plasma cortisol trends reported by (Ruiz et al., 2024) in the same experiment.

In both matrices their levels were downregulated 6 h after recovery. A moderate positive Pearson correlation was found between plasma and mucus cortisol levels ($r = 0.76$, p -value < 0.0001) **Figure 3.2.14**, as previously reported in rainbow trout

and meagre by Carbajal et al. (2019) and Fernández-Alacid et al. (2019). Despite this, little differences in cortisol between plasma and mucus in H1 group were found, and could be inherent to the matrix of measurement, as the bloodstream is the place where the hormone is initially released to activate the systemic response to stress (Schmidt et al., 2008; Taves et al., 2011). However, the cortisol in the skin mucus or water is found due to diffusion by its lipophilic nature (Ellis et al., 2007; Gesto et al., 2015) and could be detected by receptors of another fish (Domenici et al., 2017, 2007; Schumann et al., 2023; Wang et al., 2023). Therefore, it was expected that the cortisol levels in mucus would be lower than those in plasma (De Mercado et al., 2018; Guardiola et al., 2016), and differences between groups would be diluted such as in the present study, where all groups at 24 h returned to AC values. However, the stress response could be different since it is species-specific and dependant on the kind of stressor and their intensity (acute or prolonged) (De Mercado et al., 2018). Accordingly, a decrease in skin mucus cortisol compared with the plasma was found by Guardiola et al. (2016) in gilthead sea bream after anoxia by air exposure. In the present study the hypoxia shock was performed by decreasing the DO in the water for 1 h showing high levels of skin mucus cortisol 1 h after the shock, according to the results found by De Mercado et al. (2018) for the same species after a 10 min hypoxic shock also in water.

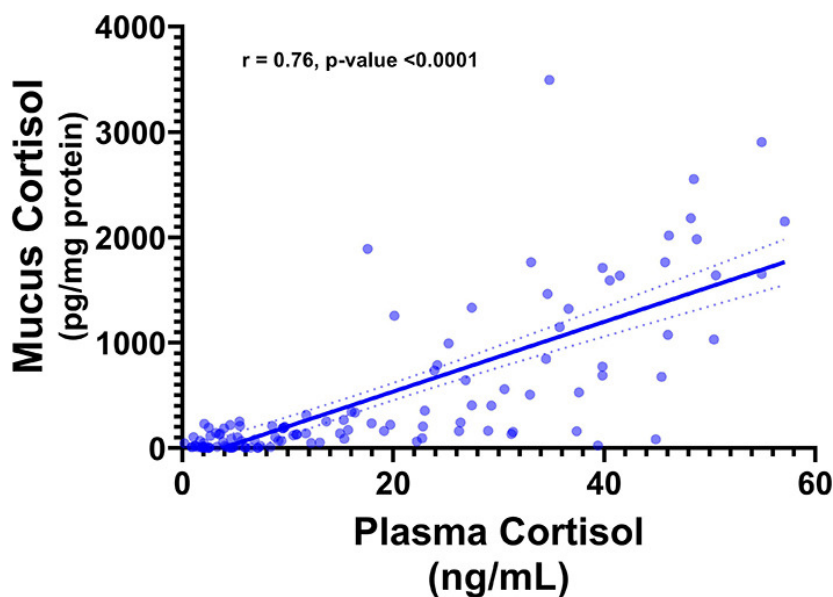


Figure 3.2.14: Moderate positive Pearson correlation between plasma and mucus cortisol levels from all samples, calculated using GraphPad 8.0 (GraphPad Software Inc., San Diego, CA, USA).

Mucosal tissues' gene expression stress response was assessed, and two different tendencies were found that could be related to the physiological roles of each tissue under stress. Thus, in gills, a tendency to upregulate *crh* gene expression was found after handling and acute hypoxic shocks. On the contrary, in skin, a downregulation was found in H1 and H3 groups compared to AC, whereas no *crh* expression was detected in intestinal tissue regardless of treatment or time.

One of the most important properties of skin in mammals, and probably in fish, is the ability to recognise, discriminate and integrate signals from the changing environment and launch appropriate responses to maintain homeostasis (Slominski and Wortsman, 2000). Despite this, several important limitations must be considered related to this analogy, due to differences in structure, function and environment (Akat et al., 2022; Rakers et al., 2010). In this sense, while a cutaneous stress response system (CSRS) is well established in mammals (Slominski et al., 2008), only some studies have been done in fish. Cortisol directly stimulates melatonin secretion and its metabolites as a protective action in a dose-dependent manner in European flounder skin (Kulczykowska et al., 2018) and also in three-spined sticklebacks (Gozdowska et al., 2022). In common carp, Mazon et al. (2006) demonstrated the presence of CRH-positive cells in skin that could be produced by immune cells present within the tissue (Baker, 2003). This points out the potential existence of a similar system in gills and skin, probably at a different scale, since skin plays a general protective role against external insults, whereas the main function of the gills is related to oxygen uptake.

In mammals, two intracellular receptors are involved in corticosteroid hormone recognition: the glucocorticoids by the GR receptor, which mediates stress response and liver glycogen deposition; and the mineralocorticoids by the MR receptor, which regulates the hydromineral balance (Fuller et al., 2000). Unlike mammals, in fish the main mineralocorticoid aldosterone was found in levels that are biologically irrelevant, but despite this, MR receptor is found in teleost fish being 11-deoxycorticosterone a possible ligand that could act as a mineralocorticoid. However, its function in fish has not yet been well established (Sakamoto et al., 2016). Moreover, two different GR receptors (GR1 and GR2) were found in rainbow trout and other teleost fishes (Aedo et al., 2023; Greenwood et al., 2003; Takahashi and Sakamoto, 2013; Tokarz et al., 2015) that showed different cortisol affinities, thus responding to different cortisol levels (Bury et al., 2003; Stolte et al., 2006). GR1 the one with the lower affinity, is activated at higher cortisol concentrations (Vazzana et al., 2010). GR2 and similarly MR present

higher cortisol affinity, making possible the hormone receptor complex that acts as a transcription factor at low cortisol levels (Tokarz et al., 2015). In a previous study conducted in gilthead sea bream stressed by acute air exposure, only *gr1* gene expression was modulated in the mucosal tissues (gills, skin and anterior gut) in a time and tissue-specific manner (Khansari et al., 2018; Vallejos-Vidal et al., 2022). According to these results in the present study in rainbow trout only *gr1* and *mr* expression were determined, also showing tissue-specific responses.

In rainbow trout gills, *gr1* expression was upregulated after two hypoxia shocks and *mr* expression was modulated by both manipulation and hypoxia treatments, but after 3 repeated hypoxia shocks the expression levels achieved similar values to those found in the control group. A similar, but faster response for both receptors was found in sea bream, after 3 min of air-exposure (Vallejos-Vidal et al., 2022). Regarding skin and intestine, both *gr1* and *mr* expression were significantly lower than in AC group, except after 3 repeated hypoxic shocks in the intestine. This suggests that cortisol levels in those tissues were lower than in gills, probably related to a lower vascularisation and/or a different mucosal-specific response in front of the stressors. Such downregulation could be also related to the feedback effect of circulating cortisol in the different levels of the HPI axis after the hypoxic shock (Bernier et al., 2009). Similarly, a downregulation in *gr1* and *mr* gene expression was found in leucocytes 24 h after an acute netting stress, whereas in spleen *gr1* and *gr2* were also downregulated, while no changes were found in glucocorticoid receptors in gills (Yada et al., 2007). This tissue-specific modulation was also found after chronic stress (Bernier et al., 2009; Yada et al., 2019). A recent study in European sea bass also demonstrated a desensitisation in glucocorticoid receptors in gills in chronic stress fish (Samaras et al., 2025).

Overall, it is suggested that further studies are needed to clarify mucosal tissues' desensitisation under predictable or repetitive acute hypoxic shock; this possibility cannot be discarded. In addition, under low DO conditions in water, the downregulation of cortisol receptors in the skin and intestine may contribute to desensitisation to cortisol. This regulatory mechanism would support survival and enhance hypoxia tolerance by preventing excessive metabolic response, thereby reducing oxygen consumption and conserving energy for more immediate physiological needs. In support of this mechanism, a previous experiment of our group showed a relevant tolerance to hypoxia (down to about 2 mgO₂/L) and a drastic decrease of activity, which helped fish to cope with this stressor (Ruiz et al., 2024). This idea was reinforced by the low swimming activity and schooling

behaviour observed in fish during the hypoxic phase, thus counteracting hypoxic environments and enhancing survival (Colson et al., 2019; Schumann et al., 2023); whereas fish were able to recover their normal activity after the shock.

The molecular mechanisms linking hypoxia-inducible factor (HIF) function to oxygen-sensing pathways remain incompletely understood in teleost's (Zhang et al., 2017). Interest in these mechanisms has grown in the context of climate change, particularly as diel-cycling hypoxia has been shown to enhance hypoxia tolerance in fish (Borowiec et al., 2015; Wang et al., 2021; Williams et al., 2019; Yang et al., 2013). In this study, we observed that *hif1 α* expression in rainbow trout gills was consistently downregulated following handling stress, with marked suppression at 6 h post-exposure in both H1 and H2 groups. However, at 24 h (H3), *hif1 α* expression was upregulated, suggesting, among other potential mechanisms, a habituation or adaptive response to repeated hypoxic stress. These expression patterns may be partially attributed to aquatic surface respiration (ASR), a behaviour observed during severe hypoxia in this study and widely reported in fish as a compensatory mechanism to increase oxygen uptake (Kramer, 1983, 1987; Kramer and McClure, 1982). Supporting this, Mandic et al. (2021) demonstrated that ASR capacity modulates hypoxia survival in *hif1 α* -knockout zebrafish (*Danio rerio*), alongside factors such as age and hypoxia severity. In contrast, yellow catfish exhibit upregulation of *hif1 α* /2 α in gills under hypoxia, accompanied by morphological remodelling to enhance respiratory efficiency (Chen et al., 2023). These interspecies differences likely reflect variation in hypoxia sensitivity and adaptive strategies. Further research is needed to elucidate the regulation of HIF1 α in fish, particularly regarding its protein stability, transcriptional activity, and oxygen-dependent degradation pathways.

The study of the presence of myoglobin, an oxygen-binding haemoprotein in non-muscle tissues, specifically in mucosal tissues of fish, is poorly understood. It is known that the myoglobin expression is upregulated in mammals during hypoxia in aerobic muscles (Hoppeler and Vogt, 2001; Noren and Williams, 2000), and later studies on hypoxia-tolerant fish species, demonstrated that there are different isoforms in non-muscle tissues that are hypoxia-inducible (Cossins et al., 2009; Fraser et al., 2006). Recently, its expression was also discovered in the rainbow trout, a non-tolerant hypoxia species (Hasan et al., 2018). Our results of *myoglobin* expression upregulation in gills after stress by manipulation and single hypoxia agrees with previous results found in common carp gills in response to temperature (Fraser et al., 2006), hypoxia treatment (Cossins et al., 2009), and were also found

in zebrafish (Van Der Meer et al., 2005), goldfish (Roesner et al., 2008) and Atlantic cod gills (Hall et al., 2009). This suggests a higher sensitivity of this mucosal tissue to hypoxia, probably related to its physiological function. In this sense, after ischemia-reperfusion, high levels of nitric oxide (NO) will be found in the gills, promoting vasodilation in one way, and increasing ROS production by the other (Brunori et al., 2001; Chen et al., 2023). The results of this study suggest that myoglobin may play a key regulatory role in modulating the stress response to repeated hypoxic exposure in rainbow trout (*Oncorhynchus mykiss*). We hypothesise that the transient upregulation of myoglobin gene expression observed after initial hypoxic shocks contributes to NO homeostasis, thereby attenuating the physiological stress response. Myoglobin is known to bind NO, forming a myoglobin-NO complex (Rayner et al., 2009), which acts as a NO scavenger. This mechanism may facilitate recovery between hypoxic events by preventing excessive NO accumulation, which could otherwise disrupt cellular function (Borowiec et al., 2015).

Interestingly, *myoglobin* expression decreased progressively with repeated hypoxic exposures, eventually reaching control levels after the third shock. This pattern may reflect an adaptive response, where initial upregulation supports NO regulation and stress mitigation, while subsequent downregulation indicates a return to homeostasis and reduced need for NO buffering. Such dynamics could explain the observed reduction in catecholamine release, consistent with previous findings that link increased NO availability to dampened stress responses (Mcneill and Perry, 2006).

Moreover, these findings align with studies in *Salmo salar*, where repeated chasing stress led to decreased cortisol levels, suggesting habituation or desensitisation of the hypothalamic-pituitary-interrenal (HPI) axis (Madaro et al., 2016). In this context, myoglobin may serve as a molecular mediator that enhances resilience to hypoxic stress by regulating NO levels and facilitating faster recovery, as previously proposed (Brunori et al., 2001; Wunderlich et al., 2003).

Taken together, our data support the hypothesis that myoglobin contributes to stress adaptation in fish exposed to repeated hypoxia by modulating NO availability, thereby influencing neuroendocrine responses and promoting physiological recovery.

Similar results were obtained in vascular smooth muscle cells (either in cell culture or intact vessels) treated with accumulative doses of NO that promoted an upregulation of the myoglobin gene expression and even its protein, suggesting a feedback relationship between both (Rayner et al., 2009). These results, together with the recovery of plasma cortisol, glucose and lactate levels and haematology parameters that had been found in this same experiment (results recently published, (Ruiz et al., 2024)) suggest that fish can cope with this kind of stressor.

To the best of our knowledge, this is the first time that fish *myoglobin* gene expression has been determined in three different mucosal tissues in rainbow trout, showing that its regulation is tissue-specific. This fact opens a new area of study regarding *myoglobin* and its physiological function in fish. Less is known about *hif1 α* and *myoglobin* expression in the other mucosal tissues studied, skin and intestine. In both, *myoglobin* expression was downregulated by handling and hypoxia shocks, suggesting that in those tissues the ROS production will probably be lower than in gills. On the contrary, *hif1 α* expression was significantly upregulated in the intestine, as was found by (Hasan et al., 2018), also in rainbow trout.

In mammals, it has been described that the activation of HIF signalling pathway contributes to the enhancement of the intestinal barrier by the regulation of several components of the mucus layer that protect the epithelial surface. This maintains the permeability through tight junctions by the regulation of claudins expression and contribute to keep the polarization of the epithelia by the adherent junctions (Glover et al., 2017; Zheng et al., 2015) suggesting that the intestinal epithelial barrier would be strengthened after hypoxia stress. To our knowledge, no information is available on the expression in skin, and the present study does not show a clear pattern of expression in relation to time or treatment. It could be hypothesised that HIF1 α contributes to the reinforcement of the epithelial barrier in fish under hypoxic conditions, potentially through mechanisms such as increased mucus secretion, as previously reported (De Mercado et al., 2018; Shephard, 1994). This idea is supported by the observed decrease in *laminin γ* expression with increasing hypoxic stress.

Laminin γ chain is a subunit of the glycoprotein laminin, essential in basement membranes, that is an important attachment ligand that inhibits human keratinocytes' motility and its under *hif1 α* control (Fitsialos et al., 2008; O'toole et al., 1997). Notably, in those *in vitro* experiments, similar findings have been

described in human keratinocytes, where hypoxia led to reduced expression of laminin-5 (O'toole et al., 1997). Moreover, HIF1 α has been shown to enhance barrier integrity in the intestinal epithelium by regulating tight junction proteins and extracellular matrix components, suggesting a broader role in epithelial protection across tissues. However, caution is warranted when extrapolating these findings to fish, as structural and functional differences in laminin isoforms between species may influence their specific roles in hypoxia-induced responses. The important role of laminin has been demonstrated, for example, in the formation of zebrafish fins (Webb et al., 2007), where a mutant for laminin 5 cannot form its pectoral fins.

Although the present study is preliminary, our findings suggest that epithelial tissue may exhibit the capacity to respond to hypoxic conditions and may contribute to skin integrity properties. However, given the limited scope of the investigation and the absence of direct mechanistic analyses, such as skin histology, these interpretations should be approached with caution. Further studies employing more comprehensive methodologies and targeted approaches are necessary to confirm and expand upon these initial observations.

3.2.5 Conclusion

The overall results show tolerance and some degree of habituation of an oxygen-sensitive species, such as rainbow trout, to this type of repeated hypoxia, as a clear recovery can be observed in the H3 group that shows progressive adaptation to repeated hypoxic shocks. In gills, our best marker to identify that fish suffered a hypoxic episode was *myoglobin* expression, since it could act as a ROS scavenger after reoxygenation, and also as an oxygen donor in such high vascularized tissue. In skin and intestine, the best marker was *hif1 α* , showing significant expression increases after both manipulation and hypoxia stressors, which would be related to the "flight-fight-freeze" response, specifically "freeze", since the expression of neuroendocrine receptors against stress decreases. All and all and regarding eventual climate change episodes, results indicate that rainbow trout are able to adapt to periods of repeated hypoxia, without showing negative permanent consequences at the functional level, provided that fish have a suitable recovery period to regulate the response to such hypoxic stressor. Furthermore, this study confirms the presence of myoglobin also in a third mucosal tissue, the skin, suggesting that their functions in these tissues are still poorly understood.

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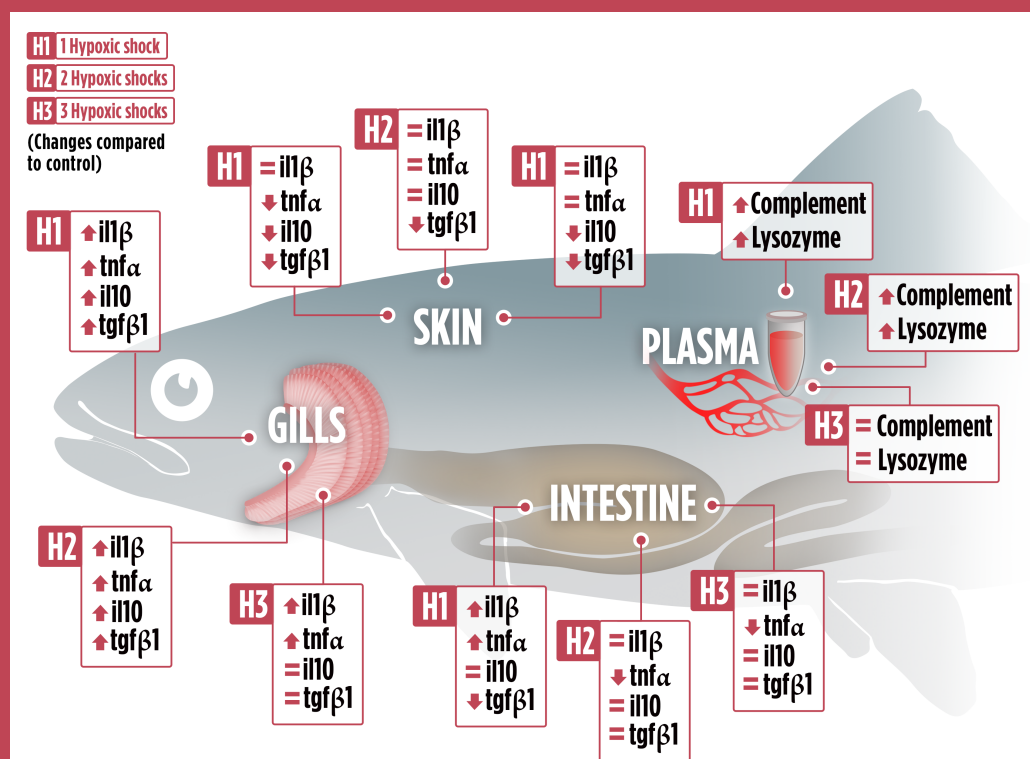
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Early immune response to repeated acute hypoxia in mucosal tissues of rainbow trout (*Oncorhynchus mykiss*)

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3.3

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Abstract

Under climate change, the increase in temperature in aquatic environments may induce oxygen depletion. Reiterated hypoxic episodes may complicate the recovery of individuals and hinder their ability to modulate physiological and biochemical responses to maintain homeostasis. The aim of this study was to determine the early immune response in mucosal tissues (gills, skin and intestine) of rainbow trout (*Oncorhynchus mykiss*) during repeated events of acute hypoxia (2 mgO₂/L). The transcript levels of pro-inflammatory and anti-inflammatory cytokines were measured, as well as the activities of the plasmatic complement and lysozyme. An increase in the expression of *il-1 β* , *tgfb1*, and especially, *tnfa* in the gills of exposed trout, with lesser expression in skin and intestine, together with enhanced activities of complement and lysozyme. The results indicate that the early activation of the immune response in fish mucosal tissues should be considered not only during hypoxia episodes in the wild that may preclude disease outbursts, but also during other events involving immune response, such as in vaccination programs.

3.3.1 Introduction

The amount of dissolved oxygen (DO) constrains the metabolic trade-offs, growth rates and developmental outcomes of fish both in natural and cultured environments. Oxygen concentrations lower than 2 mg/L in water bodies (Diaz and Rosenberg, 2008) may arise temporarily due to increased nocturnal animal and plant respiration, unexpected harsh weather or sudden deposition of organic matter, among other factors (Le Grix et al., 2023). Persistent hypoxia usually follows severe eutrophication processes in natural environments due to temperature-induced changes in the stratification of oxyclines, recurrent pollution, and fertilizers discharges that promote algal blooms that ultimately affect population structure, and local species assemblages (Pezner et al., 2023). Aquaculture practices, including handling, crowding and inadequate water quality control in enclosed ponds or intensive farming, may facilitate acute hypoxic conditions, even in recirculating aquaculture systems (Refaey et al., 2025; Zhan et al., 2023).

Hypoxia, either chronic or transient, stresses the adaptive features of energy allocation in organismal biology, and may reduce the amount of resources dedicated to maintain a robust immune response, and other physiological functions (Budria, 2017). A hypoxic condition can also affect the welfare of the animal since it activates the stress response (Li et al., 2022). Fish respond in a marked species- and context-specific manner to acute hypoxia outbursts (Duskey, 2023), usually arresting growth, altering immune reactivity, promoting oxidative stress, improving tolerance to mild hypoxia and swimming by relying on anaerobic metabolism, glycolysis and lipid catabolism (Ma et al., 2024; Negrete Jr et al., 2024; Wang et al., 2025; Zhan et al., 2023). Several studies have analysed the adaptive metabolic redirection in fish exposed to transient hypoxia (Duskey, 2023; Fang et al., 2025), but few have considered the effects of repeated exposure to low doses of oxygen on the immune response, which may be of concern during unexpected pathogen outbursts in natural or artificial environments, and also during programmed vaccination schedules in cultured fish (Budria, 2017).

In fish, the mucosal surfaces of gills, skin and gastrointestinal tract interface with the physicochemical and biotic variables to maintain a complex interaction between host and pathogen. The mucosa-associated lymphoid tissues (MALT) in gills (gill-associated lymphoid tissue; GIALT), skin (skin-associated lymphoid tissue; SALT), and intestine (gut-associated lymphoid tissue; GALT) contributes to the onset of local inflammatory reactions, regulates the impact of commensal, opportunistic, and pathogen microbial communities on fish mucosal tissues, in-

fluence the disease resistance of fish, and the effectiveness of vaccine programs and prophylaxis treatments (Mistri et al., 2025; Salinas et al., 2022). Due to the multifunctional characteristics of gill epithelia and associated GIALT structures, low oxygen concentrations may induce local inflammation (Li et al., 2022), and affect primarily the remodelling of lamellae and surface area to increase the oxygen uptake, and manage the ion transport and homeostasis (Gilmour and Perry, 2018). The same phenotypic plasticity to cope with low oxygen concentrations maintain nutrient absorption processes and antigen sampling in the gastrointestinal mucosa (Goodrich, 2025), and the defensive capacities of skin (García-Meilán et al., 2022; Vega et al., 2024).

Moreover, the immune system of teleost fish consists of primary (thymus and head kidney) and secondary (spleen, kidney and mucosal) lymphoid organs. Lysozyme and complement are proteins that mediate the immune response. Although lysozyme is involved in the innate response, complement has an intermediary role between innate and adaptive responses. Complement can be activated by three different pathways, showing an intermediate role between innate and adaptive responses and being the most commonly analysed the alternative one (Buchmann and Secombes, 2022).

The dynamics and the recruitment of cellular components of lymphoid organs in stressed fish, relying on the alarm signals emitted in mucosal tissues, are strongly temperature- and species- dependent (Tort and Balasch, 2022), but the immediate effect of hypoxia on the local immune response in gills and other MALTs is still understudied. Here we aim to analyse the influence of low oxygen concentration on mucosal tissues of rainbow trout (*Oncorhynchus mykiss*), considering the responses of pro- and anti-inflammatory cytokine transcripts, together with the plasmatic complement system activity and lysozyme levels, to help define the determinants of early inflammation and acute phase responses during repeated hypoxic events.

3.3.2 Materials and methods

3.3.2.1 Fish husbandry

A total of 135 rainbow trout (*Oncorhynchus mykiss*) with a mean weight of 62.89 ± 11.10 g, a mean length of 17.58 ± 1.09 cm, and a condition factor of 1.2 ± 0.1 were obtained from a local fish farm (Molinou, Rialb, Spain) and acclimated

to a recirculating aquaculture system (RAS) in the facilities of the Autonomous University of Barcelona (AQUAB) for 2 weeks. Regarding the rearing conditions the DO concentrations were maintained between 7.20–8.10 mgO₂/L. The photoperiod was held at 12 L: 12 D, and an average temperature of 14.6 °C ± 0.3 °C was maintained. Throughout the acclimation and experimental periods, the density was 6.80 ± 0.1 Kg/m³. All experimental procedures involving fish were submitted and authorized by the UAB's Ethics and Animal Care Committee (permit numbers OH4218 4219 and DAMM 11251), in accordance with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

3.3.2.2 Experimental design

The treatment includes 3 hypoxia (H) groups (2 mgO₂/L), an absolute control group (AC), sampled without prior manipulation, and a manipulated control (MC) that underwent the same procedure as the H groups but under normoxia (7.5-8 mgO₂/L). Fish in the H groups were exposed to one event (H1), two events (H2), or 3 events (H3) of hypoxia during 1 h, with 48 h between exposures. In the groups H2 and H3, after the exposure to hypoxia the fish were returned to the acclimation tank, isolated from the system with a normal DO and water physicochemical levels. After the last exposure of each group, fish were transferred to a recovery tank with the same characteristics as the acclimation tank. As the number of fish in the tank decreased after each sampling, to maintain a constant density of 6.80 ± 0.1 Kg/m³ water volume was consequently reduced. For each group 3 sampling points (1, 6 and 24 h post hypoxia; 9 fish sampled per group) were arranged.

3.3.2.3 Immunological assays

Alternative complement pathway

The alternative complement was assayed in rabbit blood cells (RRBC) (Deltaclon S.L) at 3 % diluted with gelatine veronal buffer as described in [Sunyer and Tort \(1995\)](#) with modifications ([Milla et al., 2010](#)). Briefly, a serial dilution of plasma was prepared and 40 µL of sample was added in a 96-well plate. Then, 10 µL of rabbit red blood cells (RRBC) were added in each well and were incubated at 27 °C for 100 min. Once the time was elapsed the plate was centrifuged during 2.5 min at room temperature and 100 µL of supernatant was transferred to another 96-well plate and read at 414 nm. The spontaneous haemolysis was obtained by adding 40 µL of gelatine veronal buffer to 10 µL of RRBC, and the total lysis

was obtained by adding MilliQ water instead of gelatine veronal buffer. Finally, appropriate calculations were used to estimate alternative complement activity (Sunyer and Tort, 1995).

Plasma lysozyme assay

The lysozyme assay was performed as described in Ellis (1999) with modifications (Milla et al., 2010). Briefly, 10 μ L of plasma were mixed with 200 μ L of lyophilized *Micrococcus lysodeikticus*, (Sigma, Aldrich) at a concentration on 0.2 mg/mL in phosphate buffer (pH= 6.2) in 96-well plate. The difference in absorbance was measured for 20 min every two minutes at 540 nm, and used to calculate the lysozyme activity in U/mL of plasma assuming that 1 U corresponded to a decrease of 0.001 in absorbance per minute.

3.3.2.4 Gene expression analysis

RNA extraction and cDNA synthesis

The RNA extraction was performed as described in García-Meilán et al. (2022) with some modifications. About 30 mg of gills and intestine tissue, and 80 mg of skin were added in 1 ml TRIzol reagent solution® (Thermo Fisher Scientific, ES). After the RNA isolation, a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA) was used to assess RNA concentration and purity (A260/A280 ratio). The cDNA of each sample was synthesized from 1 μ g of RNA using the iScript™cDNA Synthesis Kit (Biorad, USA) following the manufacturer's instructions.

Real-time quantitative-PCR (qPCR)

Gene expression was analysed using real-time quantitative PCR (qPCR) in a CFX Touch™ Real-Time PCR Detection System (Bio-Rad, USA), according to MIQE guidelines (Johnson et al., 2014). The immune transcripts analysed in gills, intestine and skin were interleukin-1 β ; *il-1 β* , interleukin-10; *il-10*, transforming growth factor 1- β ; *tgf β 1*, and tumoral necrosis factor- α ; *tnf α* . β -actin, elongation factor-1- α ; *elf-1 α* , and *18s* used as reference genes in gills and intestine. For the skin β -actin, *elf-1 α* and *rps5* were used. All pairs of primers had been previously validated in rainbow trout tissues (García-Meilán et al., 2022; Holen et al., 2021; Rebl et al., 2017) and their sequence, and reference are specified in (Table 3.3.1) and their efficiency and GeneBank accession number in (Table ??).

Table 3.3.1: Primer sequences used in the experiment. Forward (FW) and reverse (RV) sequence (3'-5') and the reference from where the sequences were obtained.

Gene	Sequence	Reference
<i>il-1β</i>	FW: CTGAAGCCAGACCTGTAGCC RV: GCAACCTCCTCTAGGTGCAG	(García-Meilán et al., 2022)
<i>il-10</i>	FW: CGACTTTAAATCTCCCATCGAC RV: GCATTGGACGATCTCTTTCTT	(García-Meilán et al., 2022)
<i>tgfβ1</i>	FW: GGGCGACAGCAGACGATACC RV: TTCAGCCATTCCTTGAGGGT	(García-Meilán et al., 2022)
<i>tnfα</i>	FW: CGCTGACACAGTGCAGTGGA RV: TCCCCGATGGAGTCCGAATA	(Teles et al., 2011)
<i>18s</i>	FW: CGAGCAATAACAGGTCTGTG RV: GGGCAGGGACTTAATCAA	(García-Meilán et al., 2022)
<i>β-actin</i>	FW: GGACTTTGAGCAGGAGATGG RV: ATGATGGAGTTGTAGGTGGTCT	(García-Meilán et al., 2022)
<i>elf-1α</i>	FW: TGCCCCTGGACACAGAGATT RV: CCCACACCACCAGCAACAA	(Holen et al., 2021)
<i>rps5</i>	FW: ATGACATCTCACTGCAGGATTAC RV: ATCAGCTTCTTGCCGTTGTTGC	(Rebl et al., 2017)

Table 3.3.2: Efficiencies and accession number of the selected primers.

Gene	Gills (%)	Skin (%)	Intestine (%)	Accession number
<i>il-1β</i>	103.3	108.8	105.9	XM_036979104.1
<i>il-10</i>	103.6	109.5	91.3	NM_00124599.1
<i>tgfβ1</i>	94.0	91.4	93.4	XM_036961610.1
<i>tnfα</i>	92.2	103.5	99.5	AJ401677
<i>18S</i>	92.1	91.5	90.6	AF243428.2
<i>β-actin</i>	95.8	91.3	93.8	XM_036972332.1
<i>elf-1α</i>	92.2	91.2	91.0	NM_001124339
<i>rps5</i>	-	105.9	-	NM_001160519

Prior to analyses, a dilution curve with a pool of samples was run to determine the appropriate cDNA dilution for each gene, as well as to confirm the absence of primer-dimers, and the specificity of the reaction by a single peak in the melting

curve for each primer set. Briefly, all the analyses were performed in triplicate wells using 384-well plates with a final volume of 5 μL (2.5 μL iTAqTMUniversal SYBR[®] Green Supermix (Bio-Rad, USA), 250 μM of forward and reverse primers and 2 μL of diluted cDNA for each sample). The qPCR program had an initial desaturation step of 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The software Bio-Rad CFX Maestro 2.3 was used to confirm reference genes stability by the gNorm algorithm (β -actin, *elf-1 α* and *18s* in gills and intestine; and β -actin, *elf-1 α* and *rps5* in skin), allowing to calculate the expression level of each target gene relative to the geometric mean of the three most stable genes (Pfaffl, 2001).

3.3.2.5 Statistical analysis

The statistics software used to perform the generalised linear model (GzLM) was RStudio. All data were analysed using the Gaussian family for the model and checking for data and residuals normality by the Shapiro-Wilk test. Before the GzLM, normality and homogeneity of variance were checked using the Shapiro-Wilk and Bartlett Test, respectively. The main factors: treatment (normoxic and hypoxic groups) and recovery time were analysed, also searching for interaction effects. Appropriate pair-wise comparisons were applied by Tuckey's correction if significant differences were found (p -value < 0.05).

3.3.3 Results

Gene expression results are explained for the different mucosal tissues since a tissue-specific response was detected. The results of the plasma immunological assay are shown at the end of the section.

3.3.3.1 Gills

An interaction effect was observed in all the transcripts analysed except for *tgf β 1*, which showed a treatment effect (p -value = 6.23×10^{-6}) with upregulation of all the groups compared with AC, except H3 with recovers the control levels (Figure 3.3.1). Regarding *il-10* there was an interaction effect (p -value = 1.016×10^{-8}), with an upregulation at 24 h at 1, and 6 h, and also being significantly different at the same times compared to AC and H3, showing both the lowest levels.

In the case of *tnf α* there was an interaction effect (p -value $< 2.2 \times 10^{-8}$). There was a general upregulation in all treatments at all times compared with AC, and also differences between times within the same experimental group (**Figure 3.3.1**). Regarding time differences between the same group, an upregulation was found at 24 h compared to 1 and 6 h, without differences among them, except MC, which had intermediate values at 6 h and H2, and the values at 1 h are even lower than at 6 h. For the differences among treatments, at 1 h, all the hypoxic groups (H) were the most upregulated ones, compared with the MC, which showed intermediate values with the AC and H1. In contrast, at 6 h, the most upregulated group was H3, followed by H2 and H1, which had intermediate values with the MC, and finally, this group was more upregulated than AC. Lastly, at 24 h, the most upregulated was H2, and H1 followed by H3, which showed intermediate values between H1 and MC, with MC being more upregulated than AC.

Regarding *il-1 β* , there was an interaction between treatment and time (p -value $= 3.14 \times 10^{-10}$). In this case, differences between times in the same group were found for MC, H2, and H3 with different effects. While in MC and H3 the highest values were observed at 6 h, being different from 1 and 24 h, in H2, the most upregulated group was 24 h, and it was different only from 6 h. As for the treatment differences at each sampling time, no differences were found at 1 h, whereas at 6 h the most upregulated group was MC, being different from the rest of the groups, followed by H3, and H1 that had intermediate values between H3 and H2 and AC, and finally H2 that was not different from AC. Finally, at 24 h, the most upregulated group was H2, followed by MC, and H1, which had intermediate values between H2 and H3 and AC, so that they were not different among them (**Figure 3.3.1**).

3.3.3.2 Skin

A time effect was observed for *il-1 β* (**Figure 3.3.1**), with differences between 1 and 6 hours (p -value < 0.001). The manipulations by themselves did not have any type of effect on the fish. A treatment effect was observed for *tnf α* (p -value $= 0.02962$), and *tgf β 1* (p -value $= 6.94 \times 10^{-6}$). For *tnf α* , a downregulation of the group subjected to 1 hypoxia was observed, and the manipulated control and the rest of the hypoxia groups had intermediate values between AC and H1. Regarding *tgf β 1*, a generalised downregulation of all groups occurred compared with the AC. Finally, *il-10* showed a time and treatment effect, without interaction, being an upregulation of the genes at 1 h compared to 6 h and a general downregulation of all the groups, except H2, which had intermediate values (**Figure 3.3.1**).

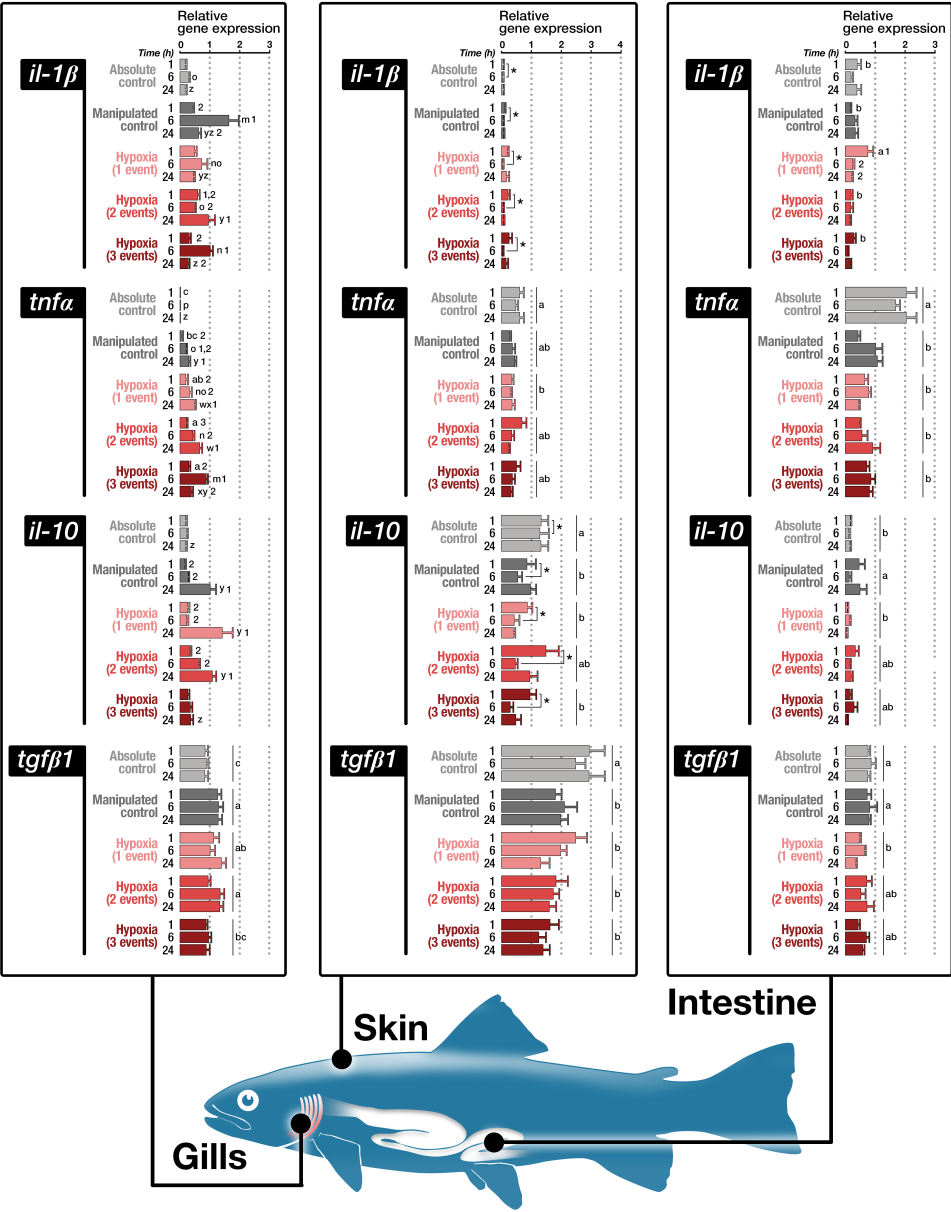


Figure 3.3.1: Results of the gene expression transcripts of *il-1β*, *tnfa*, *il-10*, and *tgfb1*, in gills, skin, and intestine of rainbow trout. Data are represented as mean ± SEM (n = 27, 9 fish per sampling time at 1, 6 and 24 h). Significant differences between treatments are shown with different letters (p-value < 0.05). Differences when there is only time effect are shown with *. When there is and interaction effects, differences at the same time but different treatment are shown with letters, and differences between the same group at different time are shown with numbers.

3.3.3.3 Intestine

For the intestine a treatment effect in all the groups was found except for *il-1 β* that an interaction effect was observed. *Il-1 β* showed a clear upregulation at 1 h in H1 group, being different from 6 and 24 h of H1 and the rest of the group at 1 h. Regarding *tnf α* a clear downregulation was observed in all groups compared with AC. In contrast, for *il-10* the lowest transcription levels were found in AC and H1, while the highest values were found in MC whereas H2, and H3 showed intermediate values (**Figure 3.3.1**). Lastly, *tgf β 1*, AC and MC were the most upregulated groups, followed by H2, and H3 that had intermediate values and between this two and H1, that was the group with lowest transcription levels.

3.3.3.4 Immunological assays

In both parameters analysed, complement and lysozyme activity, an interaction effect was found (p -value = 6.464×10^{-16} , p -value = 8.634×10^{-14} , respectively). Regarding complement activity a time effect was only found in MC and H2, being 1 h MC group the one with highest values, whereas in H2 was 24 h group. Regarding treatment effects, at 1 h the group with highest values was MC being different from the rest of groups, where no differences were found among them. No differences were observed at 6 h, and a similar effect as at 1 h was observed at 24 h, but in this case the group with the highest values that were different to the rest of the groups was the H2.

Regarding lysozyme activity, differences between time in the same group were found in all the groups except for AC, and H3 (**Figure 3.3.2**). Regarding time differences, while in MC the group with the lowest values was at 24 h, in H1 was the one with the highest values. Also, in H2 the group with the lowest values were found at 1 h. The differences between treatments were associated with the differences between time, since at 1 h the group with the highest values is MC followed by H1, which showed intermediate values between MC and the rest of the groups. At 6 h the group with the highest values was MC, and H2 followed by H1 with intermediate values and finally the lowest ones were AC and H3. Finally, at 24 h the highest values were found in H1 group, which was different from the rest, then H2 and H3 had intermediate values with them and both controls (AC and MC).

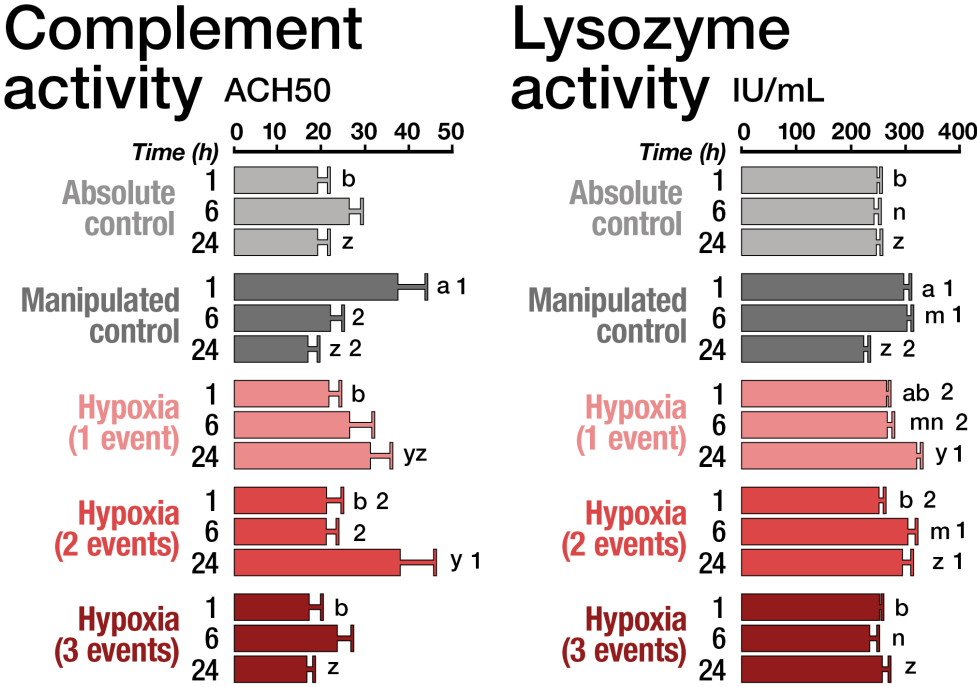


Figure 3.3.2: Results of complement and lysozyme activity in plasma of rainbow trout. Data are represented as mean $\pm$ SEM ($n = 27$, 9 fish per sampling time at 1, 6 and 24 h). Significant differences (p -value < 0.05) when there is an interaction effect are marked as follow. Differences at the same time but different treatment are shown with letters, and differences between the same group at different time are shown with numbers.

3.3.4 Discussion

Oxygen depletion in natural and artificial aquatic environments originate mainly from climate change-driven switch of thermoclines, circadian upwelling of anoxic waters, eutrophication, high stocking densities, elevated content of organic matter, algal blooms and biofouling, among other factors (Budria, 2017). In wild and farmed fish, these factors enhance the impact of hypoxia on the normal physiology in the mucosal lymphoid tissues of gills, skin and intestine, influencing the early immune activation. In this sense, our results indicate that repeated events of hypoxia (2 mgO₂/L) exert an organ-specific changes in the expression of transcripts related to pro-inflammatory (*il-1 β* , *tnf α*) and anti-inflammatory (*il-10*, *tgf β 1*) responses in rainbow trout (*O. mykiss*), being the gills the most affected organ in terms of relative transcript expression changes between hypoxia events. The relative values of transcript expression in the analysed tissues are not high, as

expected both due to the timing of exposure to low concentrations of oxygen, and to the inherent nature of the acute treatment, that differs from chronic treatments that enhance hypoxic stress. It is also worth mentioning that we cannot rule out that the procedures of the manipulation themselves may have an influence on the expression levels of the analysed immune transcripts. The acute phase response, measured through the plasma lysozyme activity and activation of alternative complement system, suggest a mild effect of repeated exposure to hypoxia.

In addition, to the well-known respiratory (coupled with acid-base modulation) and osmo-excretory functions (Evans et al., 2005), fish gills also harbors lymphoid tissues capable to orchestrate inflammatory processes. Our results indicate a mild effect of repeated hypoxia on the relative expression of *il-1 β* and *tgfb1* transcripts in gills, an increased expression of *il-10* and, interestingly, a growing expression of *tnfa* following the repeated exposure to 2mg O₂/L. An increasing expression of classical pro-inflammatory cytokines, such as *il-1 β* and *tnfa*, has already been described in the gills of fish exposed to pathogens (Cui et al., 2023) in turbot (*Scophthalmus maximus*) exposed to *Vibrio anguillarum*, and also to environmental stressors, leading to activation of NF- κ B signaling-, and P53-mitochondria-Caspase-3 pathway- mediated apoptosis and inflammasome expression, enhancing local inflammation (Cao et al., 2023; Jiao et al., 2019).

Similarly, during chronic or recurrent hypoxia, fish respond to hypoxic stress by remodelling the gill's interlamellar and filament growth (Gilmour and Turko, 2024). In *Gymnocypris przewalskii* exposed to 24 h of moderate to severe hypoxia (3 to 0.7 mgO₂/L), Chen et al. (2022) found that the gill remodelling requires the activation of Bcl-2-mediated caspase-3, that promotes apoptosis of the lamellar epithelia. In this sense, the pro-apoptotic activity of TNF α described in fish (Sakai et al., 2021) may be of special concern if recurrent or transient hypoxia events combine with inflammation raised by opportunistic or pathogenic infections. In fish, IL-10 controls inflammation by dampening the activation of T-cell subsets, monocyte/macrophages, and pro-inflammatory cytokines, whereas TGF β acts as an immuosuppressor regulating the production and survival of immune cells in a more pleiotropic way, acting also as an immunomodulator, promoting the viability of leukocytes, and becoming highly expressed after pathogen exposure in several tissues in a marked species-specific manner (Li et al., 2023). Both cytokines respond primarily to pathogen outbursts and act synergistically, but acute hypoxia (1.1 to 2.08 mgO₂/L) has been described to enhance the expression of *tgfb1a* in the blood of *Paralichthys olivaceus* (Li et al., 2025) and in tilapia

(*Oreochromis niloticus*) exposed to 7 % oxygen saturation for 96 h (Choi et al., 2007). Moreover, an increased expression of *tgfb1* transcripts in the gills facilitated the adaptation of (*Gymnocypris dobula*), a fish living in the hypoxic (5.08 mgO₂/L on average) lakes of the Tibetan plateau (Le et al., 2025). These results coincide with the observed upregulation of anti-inflammatory cytokines in the gills *O. mykiss* enduring repeated episodes of hypoxia.

Overall, our results suggest that, even if the early expression of immune transcripts in *O. mykiss* exposed to hypoxia are a mix of pro- and anti-inflammatory regulators, the activity of TNF α and TGF β may predispose the lamellar tissue to further remodelling, and subsequent caspase- and anti-inflammatory regulation of inflammatory processes if the hypoxia events become recurrent or chronic. However, it is worth mentioning that we cannot formulate a generalized model for the early expression and distribution of the cytokine network in fish under hypoxia, due to the highly variable tissue- and species-specific distribution, activity and impact of cytokines and related inflammatory response (Han et al., 2026; Li et al., 2023).

Considering the respiratory-biased multifunctional physiological properties of fish gills, it is not unexpected that the effect of hypoxia on the expression levels of the aforementioned immune transcripts is lessened in the other mucosal tissues analysed. Repeated exposures to 2 mgO₂/L did not elicit remarkable changes in the expression levels of cytokine transcripts in the intestine and skin of *O. mykiss*, except for a peak of intestinal *il-1 β* during the first iteration of hypoxia, which also replicated in the skin during the second and third hypoxia events. Contrary to what we observed in the gills, the expression of *tnfa* transcripts remained low during the repeated events of hypoxia. *Il-1 β* is considered a *bona fide* marker of inflammatory processes in fish, and also participates in the regulation of metabolism (Secombes, 2022), which may partially explain the expression levels observed during the repeated exposure to hypoxia in *O. mykiss* skin and intestine, both metabolically demanding tissues.

Exposure to low concentrations of oxygen in mammals, as in fish, induces the transcription of Hypoxia Inducible factor (HIF1 α) to enhance overall metabolism, angiogenesis, erythrocyte production, mobilization and differentiation of immune cells, and, through the activation of NF- κ B signalling, production of TNF α and other pro-inflammatory cytokines (Palazon et al., 2014). Following sustained acute hypoxia (12 h, 3 mgO₂/L), Guo et al. (2026) described the activation of

complement component c3, chemokine receptors, and C-type lectins in the head kidney of *O. mykiss*, and in *Epinephelus cyanopodus* the combination of hypoxic stress and immune challenge by exposure to *Vibrio harveyi*, LPS and Poly(I:C) enhanced the expression of HIF1 α and immune transcripts in the head kidney and liver (Gao et al., 2025). The authors suggested that hypoxia might act as an early activator of immune response in fish, anticipating regulation in front of subsequent exposure to pathogens. Our results seem to confirm this “preconditioning” of the immune response during hypoxia episodes in the gills, skin and intestine of *O. mykiss*, as demonstrated not only by the early activation of pro-inflammatory cytokines, but also by the observed increase in plasma activity of the complement system and lysozyme during the whole set of hypoxia events.

However, the complexity and tissue-differences of the interaction between pro- and anti-inflammatory cytokines suggest that, even considering the “pre-activation” of the immune response against potential infections, the recurrence of events in which oxygen concentration decreases may be more important for the management of immune homeostasis in fish farms, especially during vaccination periods. Recurrent hypoxia has been described to impair the expression of IL-1 β , but not TNF α , in Atlantic salmon (*Salmo salar*) vaccinated against *V. anguillarum* (Kvamme et al., 2013), and Gallage et al. (2016) found the expression of IgM, IL-1 β , TCR- β , and MHC-II β transcripts suppressed after 2 weeks exposure to moderate ($55 \pm 5\%$ DO) hypoxia in *O. niloticus* vaccinated against *V. anguillarum*, which may increase the susceptibility to infections and opportunistic pathogens in artificial environments.

3.3.5 Conclusions

Repeated episodes of acute hypoxia enhance the expression of pro- and anti-inflammatory cytokine transcripts in the gills, skin and intestine, and of complement and lysozyme activities in the plasma of rainbow trout. The activation of such early mild acute phase response could be considered a “pre-activation” of the immune system for subsequent, and inevitable, exposure to opportunistic or pathogenic infections, and may be of special relevance in aquaculture practices, where high density stockings and occasional changes in the concentration of oxygen induced by contamination, excess of food, and biofouling are not uncommon. However, the response to repeated acute hypoxia remains tissue-dependent; being the gills the most affected structure. This suggests that the remodelling of gill architecture driven by chronic hypoxia could be affected by the early activation of

immune components, potentially compromising the physiological response of fish in hypoxic waters.

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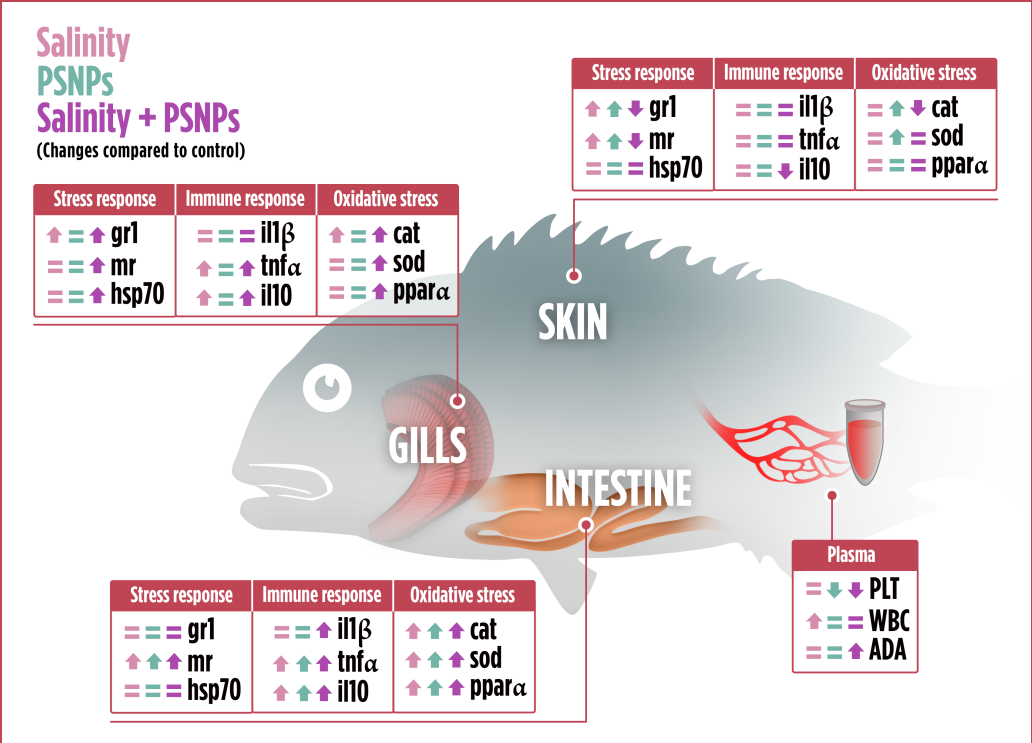
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Immunoendocrine response to individual or combined exposure of polystyrene nanoplastics and elevated salinity on gilthead seabream

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4

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Abstract

The gilthead seabream (*Sparus aurata*) is one of the most important fish species in marine aquaculture, directly affected by different aspects of the “triple planetary crisis” (environmental contamination, loss of biodiversity, and climate change). This study aimed to elucidate the impact of two major components of this crisis, namely, elevated water salinity -as a direct consequence of climate change- and nanoplastics (NPs) pollution. The mucosal and systemic responses of juveniles (*S. aurata*) to exposure to high salinity and polystyrene (PS) NPs (PSNPs) both alone and in combination were assessed by analysing the expression of relevant endocrine and immune genes in mucosal barriers (skin, gills, and intestine), as well as haematological and biochemical parameters in plasma. The results indicated tissue-specific responses to the experimental conditions, with intestine being the most responsive organ. Gills and skin were more heavily affected by exposure to salinity and PSNPs alone, respectively, and, in both cases, the combination of both challenges had a major impact compared with individual stressors. Similarly, significant haematological (white blood cell (WBC) and platelet (PLT) count) and biochemical (adenosine deaminase (ADA)) alterations occurred upon exposure to both stressors combined. Overall, the challenges induced the activation of the stress response of exposed fish, and elicited endocrine and antioxidant responses, particularly when exposed to the combination of high salinity and PSNPs. Altogether, this study highlights the role of mucosal surfaces when dealing with environmental and chemical stressors, and the importance of conducting co-exposure experiments to obtain a deeper, more realistic understanding of what aquatic organisms experience when challenged with several stressors conjointly.

4.1 Introduction

The gilthead seabream (*Sparus aurata*), a protandrous marine species, is one of the most economically important fish in the Mediterranean area, being widely reared in aquaculture facilities throughout the region (Rosati et al., 2025). Given its high adaptability, its euryhaline (reportedly able to survive in salinities ranging from 2 to 60 psu; (Bodinier et al., 2010)) and eurythermal nature (Vargas-Chacoff et al., 2009), and its tolerance to high stocking densities, the production of this species by the aquaculture industry has triplicated since the beginning of the century, making it the third most widely produced marine fish (FAO, 2022). Therefore, the production of *S. aurata* plays a major role in safeguarding regional and global food supply and security.

Marine environments continuously face a variety of challenges arising from anthropogenic activities, with the most relevant (i.e., biodiversity loss, climate change and environmental contamination) being dubbed the “triple planetary crisis” by the United Nations. Evaluating the impact of these threats on natural ecosystems is of utmost importance to understand the response of both wild and cultured organisms, to predict their consequences on the aquaculture industry and food security, and to develop and implement prevention and mitigation strategies. One of the direct consequences of climate change on coastal regions is the change in salinity levels, with regional variations in observed trends and expected fluctuation intensities (Dye et al., 2020). Moreover, one of the primary sources of environmental contamination is plastic pollution (Li et al., 2021). Plastic particles smaller than 1,000 nm (Gigault et al., 2018), or nanoplastics, are widely recognised as ubiquitous in aquatic systems, and are believed to represent the majority of plastic contamination in the ocean (Moon et al., 2024). In some cases, the environmental concentrations of nanoplastics exceed 130 $\mu\text{g/L}$, with polyethylene (PE) and polystyrene (PS) being some of the most commonly detected polymers (Cai et al., 2021; Li et al., 2024; Llorca et al., 2021).

Previous research has investigated the adaptation potential of *S. aurata* to salinities as low as 0.3 psu (Bodinier et al., 2010) and as high as 55 psu (Cuesta et al., 2005; Gregório et al., 2013; Vargas-Chacoff et al., 2009). The impact of either elevated salinity or nanoplastics contamination on fish has already been widely studied on fish (including on *S. aurata*) (see reviews by Agarwal et al. (2024); Dasmahapatra et al. (2025)). On the one hand, *S. aurata* exposed to strong fluctuations in salinity have been reported to display slight changes in the concentration of circulating

metabolites, as well as in the osmotic balance and expression of relevant genes, with the greatest response detected in mucosal surfaces, such as gills and intestine, which are in direct contact with the external environment (Cuesta et al., 2005; Gregório et al., 2013; Vargas-Chacoff et al., 2009; Zarantoniello et al., 2021). Previous research exposing this species to 55 psu water suggests that, even though *S. aurata* exhibits altered immune function under hypersaline conditions, it is able to acclimatise, resulting in little to no effect on individual performance (Cuesta et al., 2005). Moreover, gilthead seabream challenged with hyperosmotic stress reportedly display significant increases in plasmatic metabolites (e.g., cortisol), as well as overexpression of relevant genes (e.g., *mr* and *hsp70*) shortly after exposure (Zarantoniello et al., 2021), and this species is able to recover basal values over time (Vargas-Chacoff et al., 2009).

On the other hand, previous research has demonstrated that waterborne virgin PS nanoplastics (PSNPs) lead to slight changes in haematological parameters, as well as alterations in the intestine microbiome and overall metabolome in this species, causing a downregulation of genes related to antioxidant defences and lipid metabolism (Blonç et al., 2025; Okon et al., 2025).

However, in natural environments, wild animals are unlikely to experience single, isolated stressors but are rather prone to encountering multiple challenges at once. Co-occurring stressors might have additive or synergistic effects, significantly worsening their impact on natural ecosystems. Thus, investigating the combined effects of high salinity and PSNPs is of utmost importance to elucidate the repercussions of these two common environmental stressors (both being directly linked to the triple planetary crisis), allowing for the obtainment of results of greater representativeness of real-life scenarios.

In fish, mucosal surfaces represent the first line of defence against external stressors such as pathogens, fluctuations in environmental parameters, and environmental contaminants, playing a major role in the ability of individuals to cope with physical and chemical threats (Reverter et al., 2018; Wang et al., 2024). To the best of the authors' knowledge, a single study to date has focused on the combined impact of these stressors on fish, using the freshwater species *Oreochromis niloticus* as a model (Iqbal et al., 2025). Presumably, results obtained from challenges to salinity fluctuations and environmental contaminants will vary greatly between marine and freshwater species, considering their intrinsic differences in osmoregulatory mechanisms. Thus, the cited work does not necessarily serve as a basis for the

present study. Instead, salinity levels and PSNPs concentrations were selected by investigating the available literature on research exposing *S. aurata* to these stressors. Therefore, the present study is the first to investigate both the isolated and combined effects of prolonged exposures (i.e., 1 month) to elevated salinity (i.e., 55 psu) and waterborne PSNPs (100 $\mu\text{g/L}$). To this end, variations in haematological parameters, plasma biochemistry [i.e., glucose, cholesterol, triglycerides, and adenosine deaminase (ADA)], and the expression of endocrine genes involved in the primary stress response (i.e., *mr*, *gr1*, and *hsp70*), antioxidant defences (i.e., *cat* and *sod*), and immune responses (i.e., *tnf α* , *il-10*, and *il-1 β*) of different mucosal surfaces (i.e., skin, gills, and intestine) in response to these stressors, both alone and in combination, were assessed. In this regard, it is hypothesised that although the gilthead seabream is expected to efficiently cope with increased salinity levels over time, the presence of environmentally relevant PSNPs concentrations might hinder this ability, triggering a myriad of responses in *S. aurata*, including stress, antioxidant, and immune mechanisms.

4.2 Materials and methodology

4.2.1 Fish and husbandry, bioassay, and sampling

A total of 24 gilthead seabream (*S. aurata*) with a mean weight of 24.1 ± 0.8 g and a mean length of 14.2 ± 0.8 cm were obtained from a nearby aquaculture facility (Avramar, Burriana, Spain) and acclimated to a recirculating aquaculture system (RAS) in the facilities of the Autonomous University of Barcelona (AQUAB) for 1 month. Individuals were randomly placed in the experimental tanks subjected to four different conditions: “Control” (0 $\mu\text{g/L}$ PSNPs, 35 psu), “PSNPs” (100 $\mu\text{g/L}$ PSNPs, 35 psu), “Salinity” (0 $\mu\text{g/L}$ PSNPs, 55 psu) and “Salinity + PSNPs” (100 $\mu\text{g/L}$ PSNPs, 55 psu) (**Figure 1**). For the experiment, 40 nm of PSNPs were obtained from Bangs Laboratories Inc. (Fishers, IN, USA).

Throughout the experimental period, fish were fed until satiation at the same hour every day with a commercial diet (Skretting, Spain). The physicochemical parameters of the water were kept at optimal levels for the studied species. Regarding the procedure of PSNPs exposure, 75 % of water was replaced every 48 h, after which PSNPs were added again in order to ensure the exposure concentration remained constant throughout the challenge, minimising the impact of particle aggregation and internalisation.

Experimental conditions:

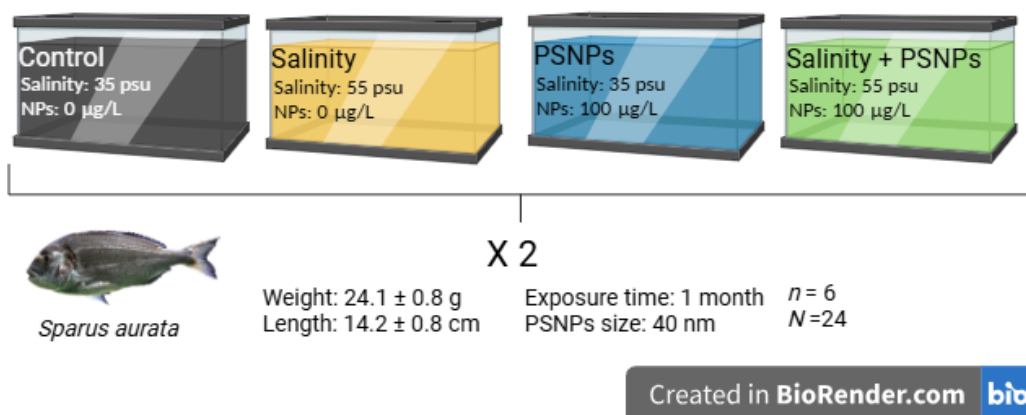


Figure 1: Graphical representation of the experimental design.

At the end of the experimental period, all animals were euthanised by overdose of tricaine methanesulfonate (MS222; 300 mg/L buffered with 600 mg/L NaHCO_3) through immersion in a bath. Each fish was subsequently measured and weighed, and blood was extracted. Gills, skin, and intestine were then extracted from all fish ($n = 6$, $N = 24$) and snap-frozen with liquid nitrogen and stored at -80°C until analysis.

The entirety of the experiment was carried out following the 3 Rs of Animal Experimentation (Replacement, Reduction, and Refinement), under Spanish legislation (law 32/2007 and RD53/ 2013), and in agreement with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

4.2.2 Characterization of nanoplastics

PSNPs of 40 nm (Bangs Laboratories Inc., Fishers, IN, USA) were characterised in different media (i.e., Ultra-pure Milli-Q water and artificial saltwater (ASW) at 35 and 55 psu) using dynamic light scattering (DLS; Zetasizer Pro, Malvern Instruments) to obtain particle size and polydispersity index (PDI). To this end, serial dilutions of the stock solution (100 mg/mL) were performed to obtain the experimental concentration (100 $\mu\text{g/L}$), and measurements were run five times at 25°C . PDI was automatically calculated by the software as the ratio of the weight-average molecular weight to the number-average molecular weight, and values over 0.2 were considered an indicator of particle aggregation.

4.2.3 Haematological analyses

Blood collection was performed using a heparinised syringe through caudal puncture. Blood (500 μL) was placed in Eppendorf tubes with heparin (1:40) for the haematological analysis. After this analysis, the blood was centrifuged at 2.000 g for 10 min at 4 °C and plasma was kept at -20 °C until biochemical analysis.

Haematological analyses were performed within 2-12 h after blood sampling by inserting 90 μL of heparinised blood into the automated flow cytometer blood cell analyser Sysmex XN-1000V (Sysmex Corporation, Kobe, Japan). This analyser was validated for fish and adapted from an earlier version used for avian blood analysis software. Leukocyte and erythrocyte counts (WBC and RBC, respectively), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined.

4.2.4 Biochemical analyses

Cholesterol (COL), triglycerides (TG), and glucose (GLC) were determined in the plasma of fish using commercially available kits (Olympus Systems Reagents; Olympus Life and Material Science Europe GmbH, Hamburg, Germany) following the manufacturer's guidelines. Intra- and inter-variation coefficient (CV) were below 10 % in all cases. ADA activity was determined using commercially available kits (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA) following the manufacturer's instructions. All parameters were measured with an automatic analyser (Olympus Diagnostica, GmbH).

4.2.5 RNA extraction and cDNA synthesis

RNA extraction was performed using the Maxwell[®] RSC simplyRNA Tissue Kit following the manufacturer's instructions with some modifications. In brief, 15 mg of tissue of each sample was added to 400 μL of homogenization solution and homogenised using a tissue lyser (Restch MM-400) with two ceramic balls for 4 min at 25 Hz and immediately stored at -80 °C for at least 24 h. After thawing the samples, 400 μL of lysis buffer and 50 μL of Proteinase K from *Tritirachium album* (20 mg/L, Sigma-Aldrich, P8044) were added. The homogenates were subsequently incubated for 10 min at room temperature and the cartridges were

set into the RNA extraction equipment (Maxwell[®] RSC). Once this process was finished, the assessment of the quantity and quality (A_{260}/A_{280} ratio) of the RNA was performed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). The cDNA of each sample was synthesised from 1 μ g of RNA using the iScript[™] cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer's instructions.

4.2.6 Real Time quantitative PCR (qPCR)

Gene expression was analysed using real-time quantitative PCR (qPCR) in a CFX Touch[™] Real-Time PCR Detection System (Bio-Rad, USA), according to MIQE guidelines (Bustin et al., 2009). The genes analysed in the gills, intestine, and skin were related to stress, immune, and oxidative stress responses. Regarding the stress, glucocorticoid receptor 1 (*gr1*), mineralocorticoid receptor (*mr*), and heat shock protein 70 (*hsp70*) were selected. For the immune and inflammatory responses, interleukin-1- β , (*il-1\beta*), interleukin-10 (*il-10*), and tumoral necrosis factor (*tnf\alpha*) were chosen. In relation to the antioxidant for oxidative stress, catalase (*cat*), superoxide dismutase (*sod*) and peroxisome proliferator-activated receptor alpha (*ppar\alpha*) were the genes analysed. The genes β -actin (β -actin), elongation factor 1 α (*elf-1\alpha*), and glyceraldehyde-3-phosphate dehydrogenase (*gadh*) were employed as reference genes. All pairs of primers had been previously validated in *S. aurata* tissues (Balasch et al., 2019, 2021; Brandts et al., 2021; Vallejos-Vidal et al., 2022), and their sequence and reference are specified in **Table 1**. Primer efficiency displayed tissue-specific values, and these together with the GenBank accession number are specified in **Table 2**.

Prior to the analyses, a dilution curve with a pool of samples was run to determine the appropriate cDNA dilution for each gene, as well as to confirm the absence of primer dimers and the specificity of the reaction by a single peak in the melting curve for each primer set. All the analyses were performed in triplicated wells using 384-well plates with a final volume of 5 μ L [2.5 μ L iTaq[™] Universal SYBR[®] Green Supermix (Bio-Rad, USA), 250 μ M of forward and reverse primers, 250 μ L of RNA-free water, and 2 μ L of diluted cDNA for each sample]. The qPCR program had an initial desaturation step of 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The *GNorm* Software was used to calculate the best combination of two of the three reference genes tested. For the gills and intestine, the genes selected were β -actin and *gadh*, whereas *elf-1\alpha* and β -actin were the most suitable in skin samples. Then, the expression level of each target gene was calculated following the Pfaffl method (Pfaffl, 2001).

Table 1: Primer sequences used in the experiment. Forward (FW) and reverse (RV) sequence (3'-5') and the reference of where the sequences were obtained.

Gene	Sequence	Reference
<i>gadph</i>	FW: TGCCCAGTACGTTGTTGAGTCCAC RV: CAGACCCTCAATGATGCCGAAGTT	(Brandts et al., 2021)
<i>elf-1α</i>	FW: CCCGCCTCTGTTGCCTTCG RV: CAGCAGTGTGGTTCCGTTAGC	(Brandts et al., 2021)
<i>β-actin</i>	FW: TCCTGCGGAATCCATGAGA RV: GACGTCGCACTTCATGATGCT	(Balasch et al., 2019)
<i>gr1</i>	FW: GAAGGATGGAGAGCACGACAAAA RV: GCTTCCAAGTTCATTCCGGC	(Vallejos-Vidal et al., 2022)
<i>mr</i>	FW: CGCCTGGCTGGAAAGCAGATG RV: GAGGTCAGGGGCAAAGTAGAGCAT	(Vallejos-Vidal et al., 2022)
<i>hsp70</i>	FW: AGGTTGGGTCTGAAAGGAAC RV: TGAACCTCTGCGATGAAGTGG	(Vallejos-Vidal et al., 2022)
<i>il-1β</i>	FW: TCAGCACCGCAGAAGAAAAAC RV: TAACACTCTCCACCCTCCAC	(Vallejos-Vidal et al., 2022)
<i>tnfα</i>	FW: TCGTTCAGAGTCTCCTGCAG RV: AAGAATTCTTAAAGTGCAAACACACCAAA	(Balasch et al., 2021)
<i>il-10</i>	FW: AACATCCTGGGCTTCTATCTG RV: TGTCCCTCCGTCTCATCTG	(Balasch et al., 2021)
<i>cat</i>	FW: TGGTCGAGAACTTGAAGGCTGTC RV: AGGACGCAGAAATGGCAGAGG	(Brandts et al., 2021)
<i>sod</i>	FW: CCTGACCTGACCTACGACTATGG RV: AGTGCCTCCTGATATTTCTCCTCTG	(Brandts et al., 2021)
<i>pparα</i>	FW: GCACCTGTGAGTCTTGTGAGTGA RV: CTCCATCAGGTCTCCACACAGC	(Brandts et al., 2021)

Table 2: Efficiencies and GenBank accession number of the selected primers.

Gene	Gills (%)	Skin (%)	Intestine (%)	Accession number
<i>gadph</i>	93.9	94.2	96.6	DQ641630
<i>elf-1α</i>	96.4	96.3	90.7	AF184170
<i>β-actin</i>	91.8	93.2	97.1	X89920
<i>gr1</i>	94.1	100.5	103.5	XM_030437675.1
<i>mr</i>	94.7	94.0	93.1	XM030418022.1
<i>hsp70</i>	95.0	92.7	99.0	EU805481.1
<i>il-1β</i>	103.2	108.9	90.0	AJ277166.2
<i>tnfα</i>	96.4	92.2	92.5	AJ413189.2
<i>il-10</i>	106.1	108.5	98.8	JX976621
<i>cat</i>	95.8	92.5	90.4	JQ308823
<i>sod</i>	90.4	101.9	90.6	JQ308833
<i>pparα</i>	102.2	93.9	108.2	AY590299

4.2.7 Statistical analysis

All data were analysed using the RStudio software (<https://www.R-project.org>). The data and residuals were fitted into the Gaussian family, and normality and homoscedasticity were tested by the Shapiro-Wilk and Bartlett tests, respectively. Data that met parametric assumptions were analysed with analysis of variance (ANOVA) tests, whereas data that did not meet the requirements were analysed with GzLM. Appropriate pairwise comparisons were applied by Tukey's correction if significant differences were found (p -value < 0.05). Results are shown as mean $\pm$ standard errors (SEM, $n = 6$, $N = 24$). Data were represented using the GraphPad Prism 8 software (<https://www.graphpad.com>). In tables, data are shown as the mean $\pm$ standard deviation.

4.3 Results

In this section, the results of the characterisation of PSNPs and the effects on the fish are presented. Plasma results are divided into two groups since there were two different types of analysis. Because of tissue-specific responses, the results are presented separately for each organ.

4.3.1 Characterization of nanoplastics

DLS characterisation of PSNPs revealed clear differences in particle size between ultrapure and artificial salt water (ASW), although no differences emerged between ASW and hypersaline media (**Table 3**). PSNPs in ultrapure Milli-Q water showed less dispersion in size than those in ASW. A PDI value over 0.2, as observed in ASW, indicates that the particles in this medium aggregated, as reflected by the increase in size. Nonetheless, the characterised particles remained, in all cases, in nanoscale size.

Table 3: Characterisation of polystyrene nanoplastics (100 µg/L) in Ultrapure Milli-Q water and artificial salt water at 35 and 55 psu. Measured parameters were size (nm) and Polydispersity Index (PDI), shown as mean ± SD.

Water	Size (nm)	Polydispersity Index (PDI)
Ultrapure Milli-Q water	47.37 ± 1.03	0.06 ± 0.006
Artificial saltwater 35 psu	557 ± 67.07	0.98 ± 0.115
Artificial saltwater 55 psu	516 ± 90.01	0.72 ± 0.205

4.3.2 Haematological analyses

The results obtained from the automated haematological analyser indicated no significant differences in any of the investigated parameters following the chronic waterborne exposure to PSNPs and salinity except in the case of the thrombocytes and white blood cell (WBC) count (**Table 4**). Thrombocytes showed a decrease in the case of PSNPs and salinity + PSNPs compared to the control. In the case of the WBC, an increase in the salinity group compared with the control was observed.

Table 4: Haematological parameters of *Sparus aurata* exposed to polystyrene nanoplastics and salinity, both alone and in combination for a 1 month period (Control: PSNPs = 0 $\mu\text{g/L}$, salinity = 35 psu; PSNPs: PSNPs = 100 $\mu\text{g/L}$, salinity = 35 psu; salinity: PSNPs = 0 $\mu\text{g/L}$, salinity = 55 psu; salinity + PSNPs: PSNPs = 100 $\mu\text{g/L}$, salinity = 55 psu). Expressed as group mean $\pm$ SD. RBC: Erythrocyte count; WBC: Leukocyte count; HGB: Haemoglobin; HCT: Haematocrit; Heterophils %; MCHC: Mean Corpuscular Haemoglobin Concentration; PLT: Thrombocytes count; RDW: red cell distribution width MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin. Letters mean significant differences between groups. Different letters indicate significant differences ($p < 0.05$).

Parameter	Control	Salinity	PSNPs	Salinity + PSNPs
RBC ($10^3/\mu\text{L}$)	2.34 $\pm$ 0.00	2.68 $\pm$ 0.36	2.47 $\pm$ 0.29	2.77 $\pm$ 0.20
WBC ($10^3/\mu\text{L}$)	55.85 $\pm$ 6.28 ^b	72.44 $\pm$ 3.98 ^a	64.01 $\pm$ 14.71 ^{ab}	71.40 $\pm$ 9.33 ^{ab}
HGB (g/dL)	4.82 $\pm$ 0.65	5.75 $\pm$ 1.08	5.55 $\pm$ 0.71	6.06 $\pm$ 0.76
HCT (%)	39.30 $\pm$ 1.20	51.83 $\pm$ 11.19	46.43 $\pm$ 6.49	53.16 $\pm$ 10.02
Heterophils (%)	7.96 $\pm$ 1.53	8.08 $\pm$ 1.33	8.70 $\pm$ 1.42	7.67 $\pm$ 0.59
MCHC (g/dL)	11.34 $\pm$ 1.48	11.17 $\pm$ 1.08	12.00 $\pm$ 0.57	11.52 $\pm$ 1.13
PLT ($10^3/\mu\text{L}$)	4.40 $\pm$ 1.81 ^a	3.00 $\pm$ 1.26 ^{ab}	1.50 $\pm$ 1.05 ^{bc}	1.00 $\pm$ 0.00 ^c
RDW (fL)	14.28 $\pm$ 1.10	14.68 $\pm$ 2.33	12.47 $\pm$ 1.10	15.74 $\pm$ 3.09
MCV (fL)	145.3 $\pm$ 4.45	168.4 $\pm$ 14.54	163.6 $\pm$ 13.03	168.4 $\pm$ 30.56
MCH (pg)	17.46 $\pm$ 2.11	18.77 $\pm$ 1.57	19.60 $\pm$ 1.48	19.18 $\pm$ 2.24

4.3.3 Biochemical analyses

Regarding biochemistry, no differences were found in glucose, triglycerides, and cholesterol (**Table 5**). In the case of the adenosine deaminase, the combination of both stressors produced a significant increase compared to the PSNPs group, whereas each individual stressor and the combination of both did not differ from the control.

Table 5: Biochemical parameters of *Sparus aurata* exposed to polystyrene nanoplastics and salinity, both alone and in combination for a 1 month period (Control: PSNPs = 0 $\mu\text{g/L}$, salinity = 35 psu; PSNPs: PSNPs = 100 $\mu\text{g/L}$, salinity = 35 psu; salinity: PSNPs = 0 $\mu\text{g/L}$, salinity = 55 psu; salinity + PSNPs: PSNPs = 100 $\mu\text{g/L}$, salinity = 55 psu). Expressed as group mean $\pm$ standard deviation. GLC: glucose; TG: Triglycerides; ADA: Adenosine-deaminase; COL: cholesterol. Letters mean significant differences between groups. Different letters indicate significant differences (p -value < 0.05).

Parameter	Control	Salinity	PSNPs	Salinity + PSNPs
GLC (mg/dL)	67.87 $\pm$ 1.62	82.70 $\pm$ 11.30	71.67 $\pm$ 9.38	86.10 $\pm$ 12.78
TG (mg/dL)	176.7 $\pm$ 20.24	232.6 $\pm$ 55.50	269.6 $\pm$ 62.12	198.8 $\pm$ 74.69
ADA (IU/L)	5.18 $\pm$ 1.49 ^{ab}	4.52 $\pm$ 1.03 ^{ab}	3.89 $\pm$ 1.11 ^b	11.58 $\pm$ 8.52 ^a
COL (mg/dL)	321.5 $\pm$ 42.58	359.8 $\pm$ 30.29	345.3 $\pm$ 49.98	38.2 $\pm$ 57.00

4.3.4 Gills gene expression

The expression of the analysed genes displayed similar patterns between genes in an tissue-specific manner. In the case of *grl*, the PSNPs group did not differ from the control group, but overexpression was observed in the salinity and salinity + PSNPs compared to the control, with the group exposed to both stressors displaying the greatest fold change (**Figure 2A**). Similarly, *mr* became upregulated to some extent in all exposed groups. Although only the combination of both stressors yielded results significantly different from the control, these were not significantly different from those observed in groups exposed to individual stressors. In other words, stressors alone showed intermediate values between control and the combined stress (**Figure 2B**). Lastly, the expression of *hsp70* did not show any significant changes between groups exposed to individual stressors and the control. However, and similarly to what was described for *mr*, the combination of both stressors resulted in the upregulation of this gene with a fold change of over 3, compared to all other experimental groups (**Figure 2C**).

As for the oxidative stress response, the expression of the genes followed a similar pattern to those related to the generalised stress response, with the group exposed to combined stressors displaying the highest degrees of upregulation (**Figure 2D-F**). Regarding *cat*, only the salinity and salinity + PSNPs groups were upregulated compared to the control, but they were not significantly different from each other (**Figure 2D**), and no changes were observed between the PSNPs and the control group.

In a similar manner, the expression of *ppar α* in the PSNPs group did not significantly differ from that in the control. As observed with the expression levels of other genes analysed in the present study, the group exposed to the combination of high salinity and PSNPs displayed a significant upregulation compared to the control (**Figure 2E**). Similarly, the mRNA abundance of *sod* only became significantly higher in the group exposed to the combined stressors, with no other significant differences arising (**Figure 2F**).

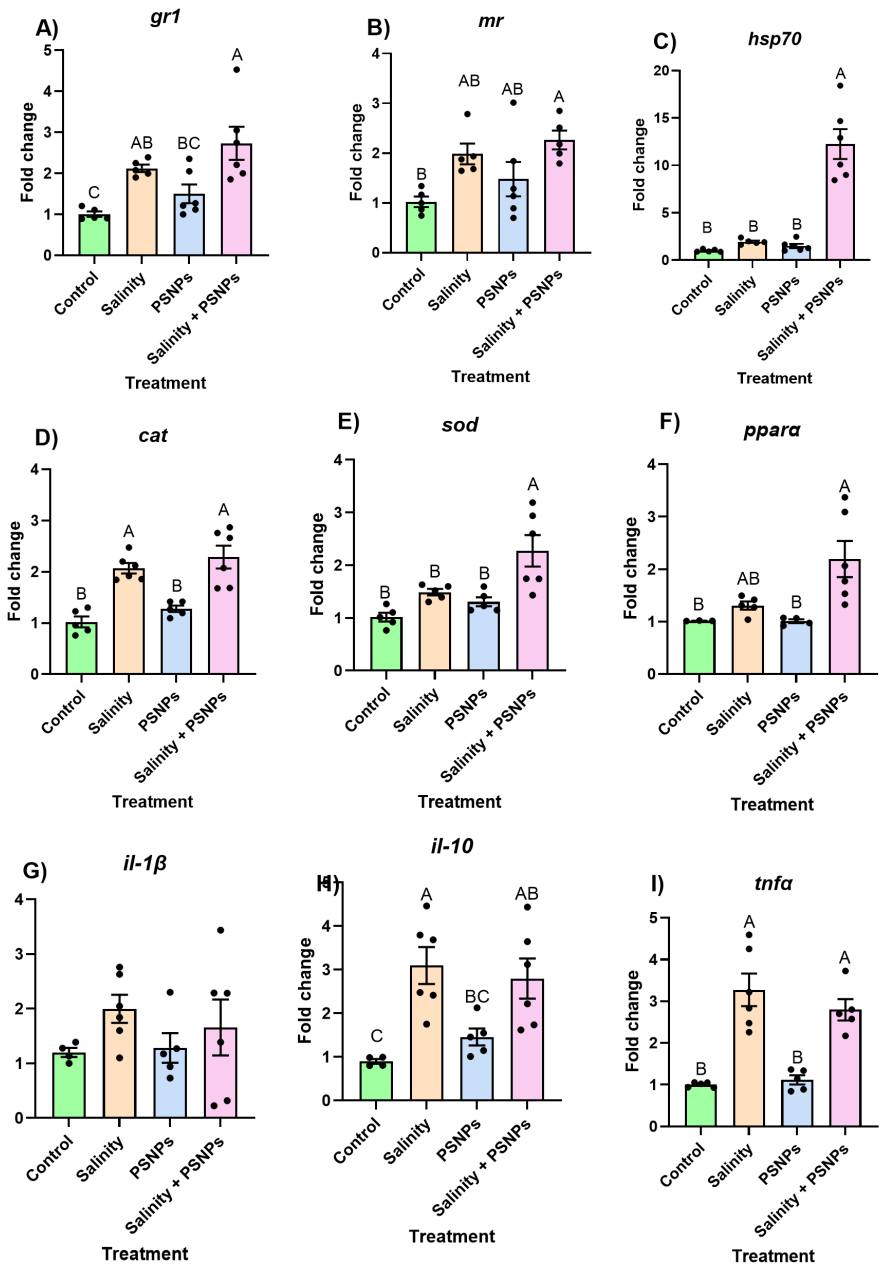


Figure 2: Relative expression of A) *gr1*, B) *mr*, C) *hsp70*, D) *cat*, E) *sod*, F) *ppara*, G) *il-1β*, H) *il-10*, and I) *tnfa* in gills of fish exposed to 1 month waterborne exposure to salinity and nanoplastics. Data are represented as mean ± SEM ($n = 6$ per group). Significant differences (p -value < 0.05) are shown with different letters. Individual values are plotted as dots in the figure.

The response of immune-related genes somewhat differed from the patterns observed in the other gene classes, with salinity causing the highest degrees of regulation. In the case of *tnf α* salinity alone, and in combination with PSNPs, both caused a significant increase in mRNA abundance, whereas PSNPs alone did not have any effect (**Figure 2G**). Similarly, *il-10* was significantly upregulated in the high-salinity group. Interestingly, the expression of this gene did not differ between the PSNPs and the control group, and the combined effect of PSNPs and high salinity appeared less severe than that observed in the group exposed solely to high salinity. Finally, the PSNPs group was a bit upregulated, but it was not different from the control (**Figure 2H**). Lastly, regarding *il-1 β* , no differences were found between groups (**Figure 2I**).

4.3.5 Intestine gene expression

In the intestine, the only gene related to the generalised stress response that became significantly altered by the experimental conditions was *mr* (**Figure 3A-C**), being significantly upregulated in all exposed groups compared to the control, but without any differences between each other (**Figure 3B**).

Regarding the oxidative stress, a generalised upregulation of the stressors alone or in combination compared with the control group was observed (**Figure 3D-F**). In the case of *sod* and *ppar α* , the combination of stressors had a similar impact to individual stressors (**Figure 3E, F**). In the case of *cat*, all exposed groups induced significant increases, although individual stressors resulted in greater upregulation compared to the combined exposure (**Figure 3D**).

Finally, in the case of the immune response, *tnf α* and *il-10* showed an upregulation in the stressed groups compared with the control, and no differences were observed between single or combined stressors (**Figure 3G, H**). The results of *il-1 β* showed that the combination of both stressors led to the upregulation of this gene, with measured values being significantly different from the control. Nevertheless, each stressor alone had a light impact on the expression of this gene, leading to its slight overexpression, resulting in intermediate values between control and the combined exposure (**Figure 3I**).

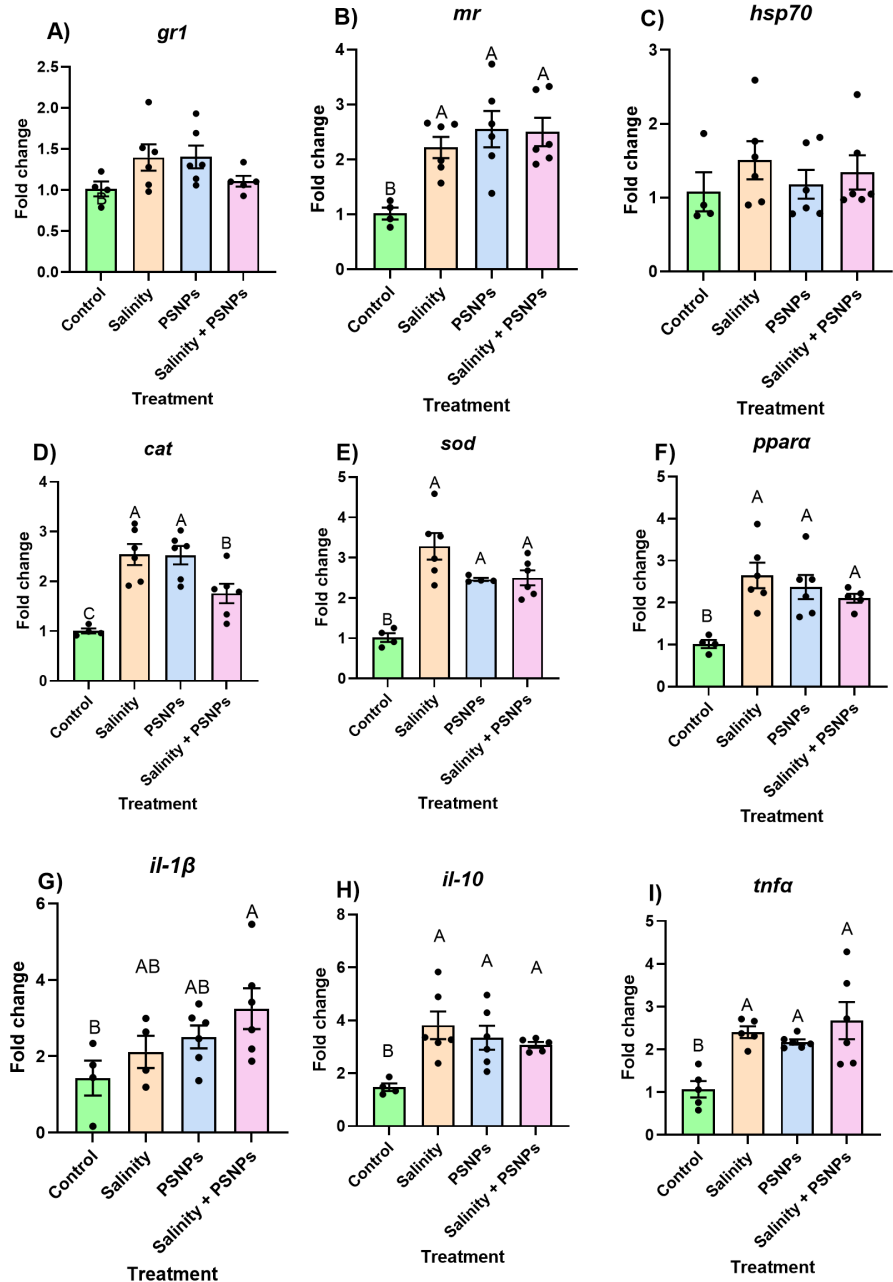


Figure 3: Relative expression of A) *gr1*, B) *mr*, C) *hsp70*, D) *cat*, E) *sod*, F) *pparα*, G) *il-1β*, H) *il-10*, and I) *tnfα* in intestine of fish exposed to 1 month waterborne exposure to salinity and nanoplastics. Data are represented as mean $\pm$ SEM ($n = 6$ per group). Significant differences (p -value < 0.05) are shown with different letters. Individual values are plotted as dots in the figure.

4.3.6 Skin gene expression

The results obtained from the gene expression analyses of stress-related genes in skin samples revealed that, in both *gr1* and *mr*, significant upregulations occurred when exposed to either high salinity or PSNPs alone. Nevertheless, when both stressors are combined, a significant downregulation was observed compared with all other experimental groups, including the control (**Figure 4A, B**). No differences were found in the case of *hsp70*.

A similar pattern occurred when investigating the response of the oxidative stress-related gene *cat*. Indeed, the abundance of this gene was significantly reduced in fish exposed to salinity + PSNPs compared with control values, and even though exposure to salinity alone had little impact on this gene, the sole exposure to PSNPs resulted in a significant upregulation of *cat* compared with all other experimental groups (**Figure 4D**). Regarding *sod*, the combination of both stressors showed downregulation compared with each stressor alone but not with the control. Moreover, the control was not different from salinity stress despite the fact that it was being slightly upregulated, whereas PSNPs displayed the strongest degree of upregulation differing significantly from the control (**Figure 4E**). Regarding *ppar α* , no changes were observed compared to the control. In this case, differences were observed between treatments, with each individual stressor resulting in greater mRNA abundances of *ppar α* in comparison with both stressors together (**Figure 4F**). Finally, the immune genes in the case of *tnf α* were downregulated in the group of salinity + PSNPs compared to the control and each stressor alone (**Figure 4G**). Regarding the *il-10*, no changes were observed compared to the control. As described above for *ppar α* , expression of this gene was upregulated under each single-stressor condition compared to the combined exposure (**Figure 4H**). Finally, no differences were found regarding *il-1 β* (**Figure 4I**).

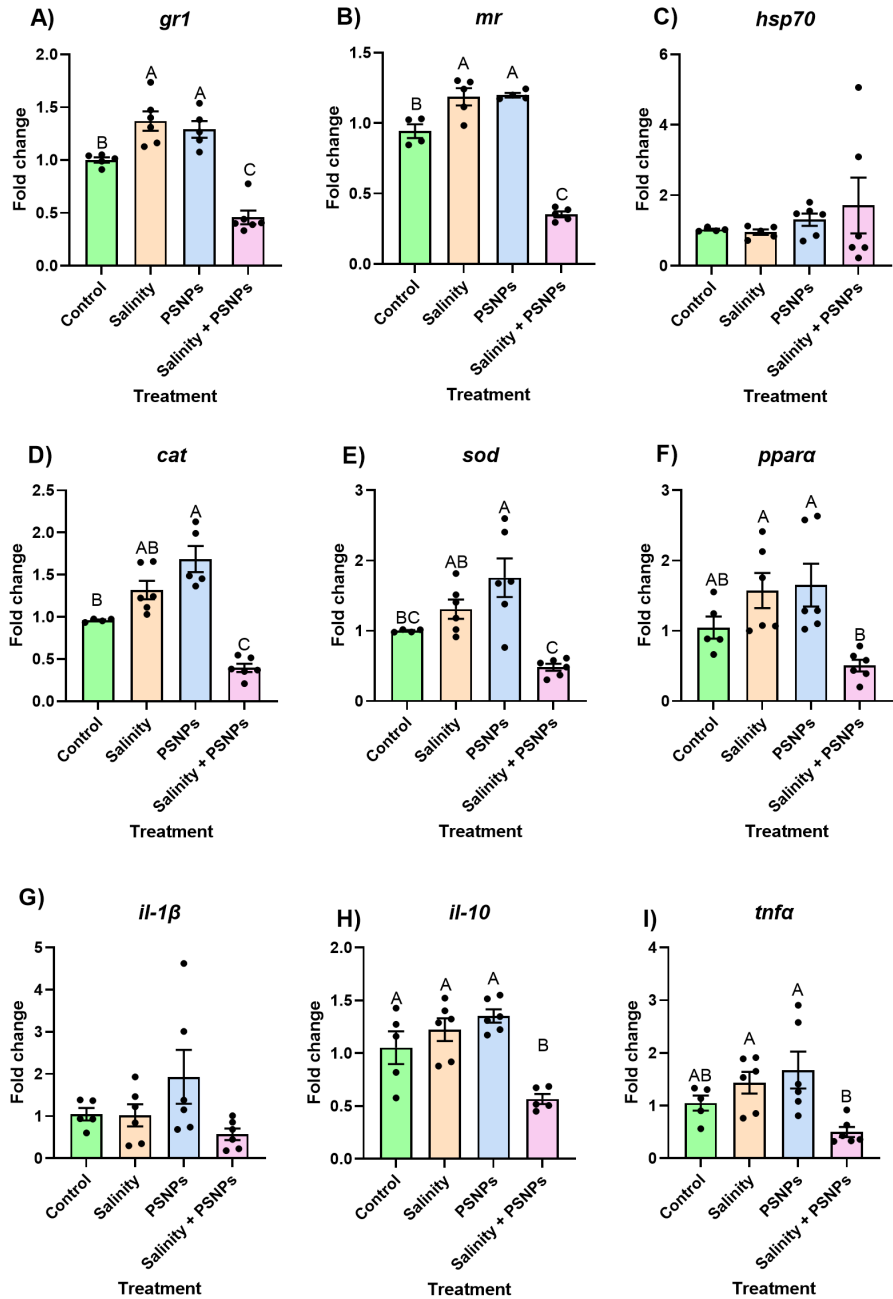


Figure 4: Relative expression of A) *gr1*, B) *mr*, C) *hsp70*, D) *cat*, E) *sod*, F) *pparα*, G) *il-1β*, H) *il-10*, and I) *tnfa* in skin of fish exposed to 1 month waterborne exposure to salinity and nanoplastics. Data are represented as mean $\pm$ SEM ($n = 6$ per group). Significant differences (p -value < 0.05) are shown with different letters. Individual values are plotted as dots in the figure.

4.4 Discussion

The expression of the investigated genes in *S. aurata* juveniles, both alone and in combination, was modulated by the experimental factors, elevated salinity levels and PSNPs, and displayed tissue-specific patterns. The response of each tissue was also dependent on the stressor, showing marked differences between fish exposed to hyperosmotic stress, PSNPs, and the combination of both. Previous research demonstrated that, although different organs might display similarities in their response to the same stressor, tissue-specific responses also arise. The same patterns have been reported in other euryhaline species exposed to changes in salinity (Dawood et al., 2022; Singer et al., 2007; Tipsmark, 2008), and PSNPs (Blonç et al., 2023; Sarasamma et al., 2020).

4.4.1 Impact of increased salinity

Abrupt changes in salinity levels have been described to cause significant changes in the measured levels of circulating metabolites (e.g., glucose and triglycerides), with basal values being recovered approximately 3 days after exposure, indicating correct acclimation (Polakof et al., 2006; Sangiao-Alvarellos et al., 2005). Thus, it is likely that changes in these parameters occurred in the fish hereby challenged during the first days of the experiment but did not persist until the sampling period.

As high salinity may disrupt ion and water balance in fish mucosae, causing cellular oxidative stress and local inflammation, as shown in the gene expression results, pro-inflammatory signals may produce recruitment and proliferation of phagocytes and other leukocytes, contributing to the increased WBC count. In addition, as hyperosmotic exposure activates the hypothalamus-pituitary-interrenal (HPI) axis, elevating cortisol and catecholamines, leukocytes will mobilise from haematopoietic organs (head kidney and spleen) into the bloodstream (Raza et al., 2026).

The exception is that platelets show a decrease after salinity exposure, which can be related to transient endothelial injury activating homeostasis, recruiting and aggregating thrombocytes at damaged sites and reducing them in the bloodstream, as it has been shown in fish after contaminant exposure (Gao et al., 2021).

Although such high salinity levels are not particularly common, they can occur under environments such as shallow waters or estuaries in which seabream can thrive and be cultured, and even more frequently under heat episodes driven by climate change.

In gills, hypersaline water appeared to slightly trigger the primary stress response, and to elicit a mild antioxidant response, as evidenced by the upregulation of *grl* and *cat*. Previous research has shown that, in euryhaline species, including *S. aurata*, exposures to both low and elevated salinity levels lead to the upregulation of glucocorticoid receptor-coding genes (Singer et al., 2007; Zarantoniello et al., 2021). In a similar note, Martos-Sitcha et al. (2014) described that hyperosmotic stress induced the activation of the HPI axis in the gilthead seabream. Similar to what was described by Zarantoniello et al. (2021), the expression of *hsp70* was not altered in juvenile *S. aurata* following a salinity challenge. Likewise, exposure to a hypersaline environment altered the mRNA abundance of genes related to an immune response in the gills of the studied fish. Indeed, genes coding for both pro-inflammatory cytokines *il-1 β* and *tnf α* were significantly upregulated in this tissue. Moreover, the anti-inflammatory *il-10* also became upregulated in this context.

Anti-inflammatory mechanisms were likely delayed relative to proinflammatory responses, acting as a negative feedback loop (Lu et al., 2022). Nonetheless, the immune response elicited by the high salinity appears successfully suppressed, allowing for maintained homeostasis (Zou and Secombes, 2016). However, given that measurements were taken at a single time point, this cannot be verified by the present study. Future research could benefit from investigating the temporal dynamics of gene expression in the gills of gilthead seabream exposed to high salinities.

The gastrointestinal tract has been demonstrated to play a major role in coping with osmotic stress in both marine and freshwater species challenged with fluctuations in water salinity (Hieu et al., 2022; Takei and Hwang, 2016). Previous research investigating the impact of variations in salinity on fish intestine has mostly focused on microbiome alterations, describing species-specific responses (Hieu et al., 2022; Mkulo et al., 2025; Peng et al., 2025). In the analyzed intestine samples, the significant increase in the mRNA abundance of *mr*, *cat*, and *sod* corroborates the hypothesis that exposure to 55 psu induced both stress and antioxidant responses in juvenile *S. aurata*. Moreover, the immune-related genes investigated in this tissue

also became significantly upregulated, indicating a potential pro-inflammatory response to the challenge, followed by negative feedback of anti-inflammatory mechanisms, as observed in the gills. In fish, salinity changes might cause intestine dysbiosis, which has been suggested to lead to inflammatory responses in this organ (Sun et al., 2025). Furthermore, and in accordance with the present results, hyperosmotic stress has been reported to modulate the expression of intestinal genes involved in both pro -and anti-inflammatory processes, and antioxidant defences, as evidenced by transcriptomics analysis (Ouyang et al., 2023). In addition, intestine samples displayed significantly greater levels of *pparα* in the high-salinity group compared to the control, hinting towards a mild disturbance in lipid metabolism pathways, agreeing with previously published reports of hyperosmotic stress altering metabolic processes (Peng et al., 2025). However, the measured levels of circulating triglycerides and cholesterol did not significantly change from one group to another.

Differences in the response to environmental pollutants between marine and freshwater species might be partly explained by the osmoregulatory mechanisms put in place. Indeed, marine fishes, being hypo-osmoregulators, constantly lose water through their skin and gills and are therefore compelled to constantly drink water. In contrast, freshwater fish are hyper-osmoregulators and continually absorb water from the surrounding environment (Evans, 1968; Greenwell et al., 2003). These differential osmoregulatory requirements might translate into differing rates in (nano) plastic absorption and retention, particularly through the gastrointestinal tract (Pratiwi et al., 2025).

In the skin samples, only two genes were significantly upregulated as a result of hyperosmotic stress, namely, *mr* and *gr1*, both closely involved in the primary stress response by modulating the expression of cortisol receptors (Vallejos-Vidal et al., 2022), indicating that this organ also plays an important role in this process.

4.4.2 Impact of waterborne PSNPs

In agreement with previous studies looking into the impact of PSNPs on the haematological profiles of *S. aurata*, no changes were observed in haematological parameters following PSNPs treatment (Blonç et al., 2025; Brandts et al., 2021) or in the investigated plasmatic biochemical assays (Okon et al., 2025). None of the investigated genes in gills became significantly up- or downregulated in the face of waterborne PSNPs alone. Although previous research has shown that waterborne

exposure to PSNPs might induce transcriptional changes in the gills of some fish species (Lu et al., 2018; Vineetha et al., 2024), these are generally observed after exposure to particle concentrations higher than environmentally relevant and, thus, several orders or magnitude greater than the concentration employed here. To the best of the authors' knowledge, no study has investigated the impact of PSNPs on gene expression levels in the gills of *S. aurata*, and differences in published results might, therefore, also be partly explained by species-specific characteristics. Indeed, the impact of PSNPs seems highly dependent, not only on exposure concentration but also on polymer, particle size, and the species studied (Barriá et al., 2020; Dasmahapatra et al., 2025).

In contrast with what was observed in the gills, the exposure to PSNPs elicited a response in the intestine similar to that to salinity, with gene expression values significantly different from the control group, but not from the high-salinity group. In this organ, the mRNA abundance of *pparα* was also significantly greater than values from the control group, potentially hinting, in a similar manner, towards a light disturbance of the lipid metabolism, in accordance with previous reports of PSNPs altering intestinal lipid profiles (Luo et al., 2025). This could be linked to a direct disruption of the expression of peroxisome-proliferator-activated receptor (PPAR)-associated genes (Xu et al., 2025b) or to the indirect impact of shifts in the intestinal microbiome (Zhu et al., 2024), although no evidence to verify this hypothesis has been obtained through the present work. Moreover, as observed in the skin of fish exposed to hyperosmotic stress, the skin samples of the PSNPs-exposed group exhibited a significant upregulation of both *mr* and *gr1* compared to the control group, reaffirming the role of this organ in the primary stress response (Takahashi and Sakamoto, 2013; Vallejos-Vidal et al., 2022). In addition, significantly higher levels of *cat* and *sod* were observed in this organ, indicating that antioxidant processes were also triggered in the skin (Hamed et al., 2017). As observed in the intestine, no statistically significant differences arose in the gene expression levels of the skin between both groups exposed to single stressors.

4.4.3 Combined impact of increased salinity and water-borne PSNPs

Perhaps more interestingly, salinity and PSNPs appear to have had, to some extent, a synergistic effect on the gills of *S. aurata* juveniles. Indeed, only this experimental group displayed significant changes in ADA. The significant increase

in this parameter might reflect the activation of an immune response, given its demonstrated role in the modulation of inflammatory and homeostatic processes in fish following exposure to stressful events (Piato et al., 2011; Zimmermann et al., 2016).

Moreover, out of all studied genes, only five were significantly altered in gills when exposed to 55 psu, and none were altered by the sole exposure to PSNPs. However, eight genes were significantly upregulated by the combination of both stressors, two of which were significantly upregulated compared to individual treatments, namely, *hps70* and *sod*. Therefore, although the upregulation of certain genes observed in the co-exposed group can be solely attributed to the effect of salinity, the combined impact of salinity and PSNPs appears to have triggered a stress and antioxidant response at a greater magnitude in the gills than that observed in single exposures.

In the intestine, the combination of both stressors also appeared to have a slightly different effect than either isolated stressor. Indeed, the co-exposed group displayed a higher and a lower expression of *il-1 β* and of *cat*, respectively, compared to all other groups. As observed in the other tissues, the collective impact of hyperosmotic stress and PSNPs caused a greater level of disturbances in the skin than either stressor on its own.

In this specific case, however, the combination of both challenges had the opposite effect than that observed for single exposures. The expression of stress-related genes, genes coding for antioxidant enzymes, and genes involved in both pro- and anti-inflammatory processes were all significantly lower in this group compared to all three other experimental groups. This might indicate, to some extent, a suppression of the immune function of the skin in juvenile *S. aurata* as a result of the experimental challenge. In this group, this contrasts with the significantly higher circulating levels of ADA, known to modulate inflammatory responses by promoting the production of T cells (Baldissera et al., 2018), compared to that solely exposed to PSNPs. Interactive effects of PSNPs and co-contaminants on aquatic organisms have been widely studied, generally indicating that such co-exposures result in increased toxicity (Chu et al., 2025; Xu et al., 2025a; Zhang et al., 2024). For instance, the only study investigating similar stressors to those employed in the present study in fish demonstrated that high salinity levels exacerbate the impact of PSNPs in the fish, amplifying their effect on the modulation of immune and antioxidant responses (Iqbal et al., 2025).

It is important to note that, given the intricacy of gene roles, interactions and expression modulation, or changes in the mRNA abundance of single genes do not necessarily translate into functional changes at the whole-organism level (Santos et al., 2022). This was observed in the present study, for example, with the somewhat contradictory results obtained from expression analysis of lipid metabolism-related genes and plasmatic levels of circulating lipids, indicating that other factors than those hereby analyzed were at play. Thus, the present results should be considered as preliminary, serving as a basis for future research investigating functional alterations on *S. aurata* exposed to hypersaline media and waterborne PSNPs. Alternatively, it could also prove interesting to look at similar scenarios in hypoosmotic conditions. In either case, future research would benefit from establishing multiple sampling points throughout the exposure, thus assessing the evolution of the response of *S. aurata* to the combination of these stressors over time, to evaluate whether habituation might occur. Similarly, investigating the combined impact of PSNPs or salinity with other environmental or chemical stressors, as well as mechanical stressors linked to the aquaculture industry (e.g., overcrowding, handling, and transportation), would allow to broaden the current knowledge available on the impact of the “triple planetary crisis” on the aquaculture industry.

4.5 Conclusion

The present study provides valuable knowledge on the endocrine, metabolic, and haematological response of mucosal surfaces in a marine fish of major economic importance to two major aspects of the “triple planetary crisis”, namely, increased salinity, as a direct consequence of climate change, and PSNPs, one of the most important components of environmental contamination, both alone and in combination. Obtained data suggest that high salinity and PSNPs had, to some extent, a synergistic impact on the haematological and biochemical profiles of the exposed fish, inducing the activation of the immune system. Moreover, the results indicate marked tissue-dependent differences in the responses to these stressors, with intestine being the most responsive organ to salinity and PSNPs, both alone and in combination, but with little difference between these. On the other hand, gills and skin were more heavily affected by exposure to salinity and PSNPs alone, respectively. In both these cases, the stressors had a major impact combined than individually. The results highlight the differential importance of mucosal surfaces when a marine fish is exposed to environmental and chemical stressors. Moreover,

the present study emphasizes the relevance of investigating combined exposures to different stressors to obtain more reliable results, reflecting to a greater extent what occurs in real-life scenarios. The present study can help future studies as a foundation for research investigating the mucosal response of fish to the combined effect of stressors linked to anthropic activities.

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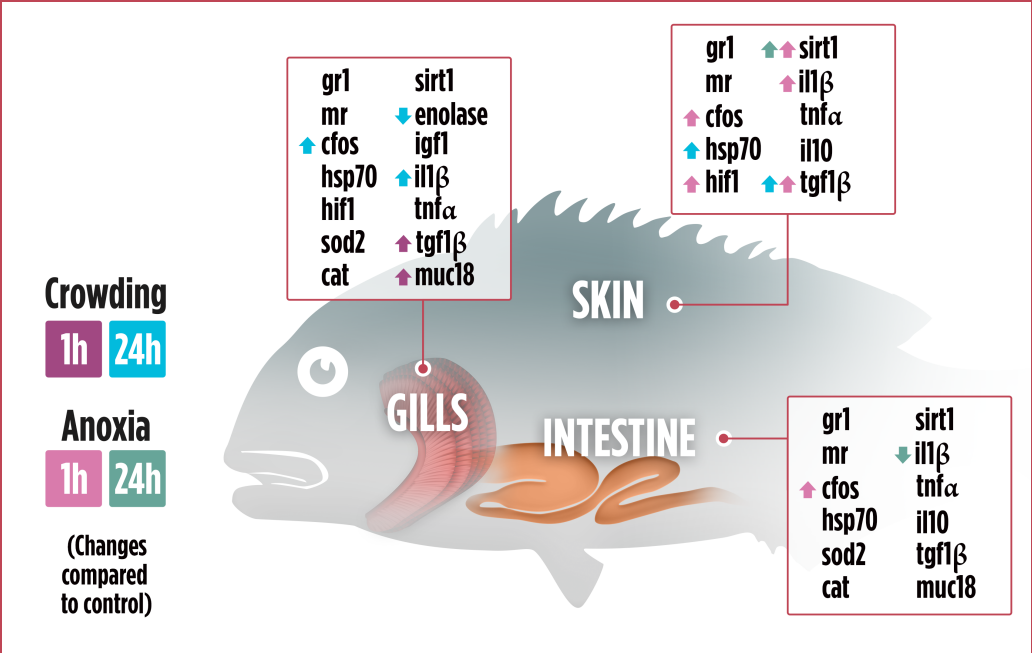
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Mucosal response of the gilt-head seabream (*Sparus aurata*) to anoxia and crowding: a comparison between mucosae

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5

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Abstract

Aquaculture practices expose fish to a variety of acute stressors that can differentially elicit physiological and molecular responses across tissues. The present study investigated the effects of two common aquaculture-related stressors, direct anoxia and crowding, on the stress, immune, antioxidant, and metabolic responses of juvenile gilthead seabream (*Sparus aurata*). Fish were acutely exposed to either 3 min of air exposure or increased stocking density for 30 min and sampled at 1 and 24 h post-exposure. Plasma biochemical parameters were measured, and the transcription of genes related to stress signalling, immune function, oxidative stress, energy metabolism, and mucus production was analysed in the gills, skin, and intestine. No significant changes were detected in plasma glucose or acetylcholinesterase activity, indicating the absence of a sustained secondary stress response. In contrast, clear tissue- and stressor-specific transcriptional responses were observed. Crowding predominantly affected gene expression in the gills, particularly genes associated with stress and immune responses, whereas air exposure elicited stronger responses in the skin and intestine, including the modulation of hypoxia-related, inflammatory, and regulatory genes. Most transcriptional changes were transient and returned to control levels within 24 h, suggesting an effective recovery of homeostasis. Overall, these findings highlight the importance of mucosal tissues as sensitive and stressor-specific indicators of acute stress in fish and underscore their relevance for welfare assessment in aquaculture conditions.

5.1 Introduction

Aquaculture is the fastest growing sector of the food production industry, and, having displayed a considerable increase of 6.6 % between 2020 and 2022, it surpassed the fisheries sector in terms of produced seafood (FAO, 2024). Thus, this industry plays a major role in safeguarding global food security, allowing to meet the growing demand for seafood in a sustainable manner, alleviating anthropogenic pressure on overly exploited wild fish stocks. The gilthead seabream (*Sparus aurata*) is an euryhaline and eurythermal marine fish, being able to tolerate significant fluctuations in temperature and salinity (Craig et al., 2008). Given its high adaptability, this species has become economically the third most important fish farmed, with particular importance in the Mediterranean area, with a production leaping from 90 thousand tonnes in 2000 to over 280 thousand tonnes in 2020 (FAO, 2022).

Aquatic animals cultured in farms encounter a wide variety of challenges of biotic or abiotic nature, intrinsically linked to aquaculture practices, including husbandry (e.g., handling, transportation, and crowding), and chemical stressors (e.g. nitrogenous compounds or dissolved oxygen levels in the water; (Calabrese et al., 2017; Hang et al., 2021)). These stressors can be further classified according to intensity and duration. On one hand, acute stressors are characterised by being short-term and relatively intense, with often positive, or adaptive, consequences on living organisms (Calabrese et al., 2017; Hang et al., 2021). On the other hand, chronic stressors are differentiated by being of lower severity and generally lasting over prolonged periods, having negative, or maladaptive, consequences, and significantly impairing the overall fitness and survival of an organism (Schreck et al., 2001; Yada and Tort, 2016).

Fish, as many vertebrates, encounter a stressor of any kind, which will trigger a well-defined stress response, generally divided into three stages. First, the primary stress responses is put in place, characterised by the activation of two main neuroendocrine axes, namely, the brain-sympathetic-chromaffin (BSC) axis, and the hypothalamus-pituitary-interrenal (HPI) axis; (Schreck and Tort, 2016). When studying the stress response in fish, attention is primarily directed to the HPI axis, given its relatively slower reactivity and higher persistence compared to the BSC axis, thus allowing for more accurate measurements. The activation of the HPI axis consists of the release of the corticotropin-releasing hormone from the brain into the pituitary, inducing the subsequent release of the adrenocorticotrophic

hormone, which in turn promotes the production and release of glucocorticoids from the head kidney into the bloodstream (Wendelaar Bonga, 1997).

If persistent, the increase in circulating levels of glucocorticoids leads to the activation of the secondary stress response, characterised by metabolic changes, including variations in the haematological profile, alterations in the energetic metabolism (e.g. as evidenced by spikes in plasmatic levels of glucose or lactate), and changes in immune function (Balasch and Tort, 2019). If at this stage, the stressor has not been overcome, the tertiary stress response is triggered, leading to long-term effects on the overall performance of an individual, including hindered growth, impaired reproductive ability, altered behaviour, and decreased immune performance (Yada and Tort, 2016). The tertiary stress response is most likely to have deleterious consequences on the performance of fish in the aquaculture industry by directly affecting production efficiency (Noakes and Jones, 2016; Pankhurst, 2016; Sadoul and Vijayan, 2016). Thus, it is of particular interest for managers and operators of aquaculture facilities to understand how different aspects of the most common practices affect the overall stress status of the cultured fish, and to develop early-warning systems to address these issues, in order to prevent the triggering of the tertiary stress response, avoid unnecessary stress to the animals, and prevent economic losses.

For this reason, the present study focused on investigating the impact of two stressors commonly encountered by aquacultured species (i.e. crowding and direct air exposure), expected to trigger the primary, and potentially the secondary, stress responses, in *S. aurata*. To this end, alterations in plasmatic biochemical profiles, in the expression of genes relevant to the primary and secondary stress responses, and in the antioxidant and immune responses were investigated in juvenile gilthead seabream acutely exposed to either one of the aforementioned stressors. The effects were assessed in the three main mucosal barriers of the fish, skin, gills, and gut, which are known to play a major role in the stress response (Cabillon and Lazado, 2019) and the responses were compared among such mucosae.

5.2 Materials and methods

5.2.1 Fish and husbandry, bioassay and sampling

Juvenile gilthead seabream (*Sparus aurata*, $N = 48$) with a mean weight of 30.72 ± 6.66 g and a mean length of 13.06 ± 2.26 cm, were obtained from Avramar (Burriana, Spain). Fish were kept at the AQUAB facilities of the Autonomous University of Barcelona in a recirculated aquaculture system for one month prior to the start of the experiment to recover from the transport stress and acclimatise. Individuals were randomly allocated to one of three experimental conditions (**Figure 1**): “Control” (no manipulation), “Anoxia” (3 min of direct air exposure), and “Crowding” (density change from 12 to 24 kg/m³). Each group was sampled at 1 and 24 h after the exposure (hpe). Throughout the experimental period, fish were fed a commercial diet (Skretting, Spain) until satiation.

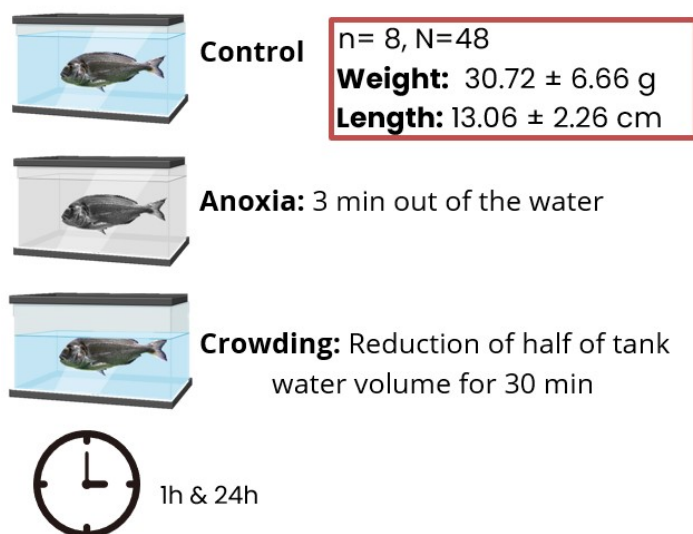


Figure 1: Graphical representation of the design of the experiment.

Following the challenge, all animals were euthanised through immersion in a bath with an overdose of Tricaine methanesulfonate (MS-222; 300 mg/L and 600 mg/L sodium bicarbonate; NaHCO_3), measured and weighed. Blood was immediately collected using heparin-coated syringes through caudal puncture and stored at 4 °C. Gills, skin, and intestine were extracted from all fish ($n = 8, N = 48$), snap-frozen in liquid nitrogen, and stored at -80 °C until analysis.

The whole experiment was performed in accordance with the 3 Rs of Animal Experimentation (Replacement, Reduction, and Refinement), as per Spanish legislation (law 32/2007 and RD53/ 2013), and in agreement with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63). As required by the local regulations, these procedures were further supervised by the regional authority (Generalitat de Catalunya) through the Habilitated Organ (OH-CEEA) competent for the evaluation of animal experimentation procedures (specific code OH-P2-6770).

5.2.2 Biochemical analysis

Plasma was obtained through the centrifugation of the collected blood (2000 g, 10 min). Glucose (GLC) levels were measured with a commercially available kit (Olympus Systems Reagents; Olympus Life and Material Science Europe GmbH, Hamburg, Germany) following the manufacturer's guidelines. Acetylcholinesterase (AChE activity was measured using acetylthiocholine as a substrate (Ellman et al., 1961). The method was adapted from (Tecles et al., 2000). All parameters were determined with an automatic analyser (Olympus Diagnostica, GmbH) (Tecles et al., 2000). In all cases, intra- and inter-variation coefficient (CV) remained below the 10 % acceptance threshold.

5.2.3 RNA extraction and cDNA synthesis

RNA was extracted from individual tissue samples with the Maxwell® RSC simplyRNA Tissue Kit following the manufacturer's instructions with some modifications specified in (Ruiz et al., 2026). Briefly, 15 mg of tissue were added to 400 µl of homogenisation solution and homogenised using a tissue lyser (Restch MM-400) with two ceramic balls (4 min at 25 Hz). Subsequently, the samples were frozen at -80 °C for at least 24 hours. Then, samples were unfrozen, and 400 µl of lysis buffer and 50 µl of Proteinase K from *Tritirachium album* (20 mg/L, Sigma-Aldrich, P8044) were added. This was incubated for 10 minutes at room temperature, after which the RNA extraction cartridges were set up following the manufacturer's instructions. The quantity and quality (A260/A280 ratio) of the obtained RNA were assessed with a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). From 1 µg of each RNA sample, cDNA was synthesised using the iScript™ cDNA Synthesis Kit (Biorad, USA) as per the manufacturer's instructions.

5.2.4 Real-time quantitative-PCR (qPCR)

The relative expression of selected genes was analysed using real-time quantitative PCR (qPCR) in a CFX Touch™ Real-Time PCR Detection System (Bio-Rad, USA), according to MIQE guidelines (Bustin et al., 2009). The genes analysed in the gills, gut and skin mucosal tissues were genes related with stress, immunity, oxidative stress response, energy metabolism, and mucus production. Related to stress, glucocorticoid receptor 1 (*gr1*), mineralocorticoid receptor (*mr*), hypoxia inducible factor 1 α (*hif1* α), c-Fos protein (*cfos*), and heat shock protein 70 (*hsp70*) were selected. For the immune response, interleukin-1 β (*il-1* β), interleukin-10 (*il-10*), transforming growth factor β 1 (*tgfb1*), mucin 18 (*muc18*), and tumor necrosis factor α (*tnfa*). As indicators of the oxidative stress response, catalase (*cat*), and superoxide dismutase (*sod*) were analysed, while enolase (*eno*), sirtuin 1 (*sirt1*) and insulin-like growth factor 1 (*igf1*) were selected as indicators of energy metabolism. Following the user's manual, genes β -actin (β -*actin*), elongation factor 1 α (*elf-1* α), and glyceraldehyde-3-phosphate dehydrogenase (*gadph*) were selected as reference genes.

All pairs of primers used in the present study were previously validated in *Sparus aurata* tissues (Balasch et al., 2019, 2021; Brandts et al., 2021; Vallejos-Vidal et al., 2022) and their sequence, and reference are specified in **Table 1**. Tissue-specific efficiencies and the GeneBank accession number are reported in **Table 2**. The performance of qPCR was done according to Ruiz et al. (2026). Briefly, to determine the optimal cDNA dilution factors for each gene, dilution curves were ran with a pool of all samples, also allowing for the evaluation of the presence of primer-dimers. All the analyses were performed in 384-well plates with a final volume of 5 μ L (2.5 μ L iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA), 250 μ M of forward and reverse primers, 250 μ L of RNA-free water and 2 μ L of diluted cDNA for each sample), with three technical replicates for each sample. The qPCR protocol was as follows: 3 min: initial desaturation step at 95 °C, 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The GNorm Software was utilized to determine the most suitable combination of reference genes for each tissue. The selected genes were β -*actin* and *gadph* for the gills and intestine, and *elf-1* α and β -*actin* for the skin samples. The expression of the target genes, relative to the geometric mean of the selected reference genes, was calculated using the Pfaffl method (Pfaffl, 2001).

Table 1: Primer sequences used in the experiment. Forward (FW) and reverse (RV) sequence (3'-5') and the reference of where the sequences were obtained.

Gene	Sequence	Reference
<i>gadph</i>	FW: TGCCAGTACGTTGTTGAGTCCAC RV: CAGACCCTCAATGATGCCGAAGTT	(Brandts et al., 2021)
<i>elf-1α</i>	FW: CCCGCCTCTGTGCCTTCG RV: CAGCAGTGTGGTTCCGTTAGC	(Brandts et al., 2021)
<i>β-actin</i>	FW: TCCTGCGGAATCCATGAGA RV: GACGTGCGACTTCATGATGCT	(Balasch et al., 2019)
<i>gr1</i>	FW: GAAGGATGGAGAGCAGACAAAA RV: GCTTCCAAGTTCATTCCGGC	(Vallejos-Vidal et al., 2022)
<i>mr</i>	FW: CGCCTGGCTGGAAAGCAGATG RV: GAGGTCAGGGGCAAAGTAGAGCAT	(Vallejos-Vidal et al., 2022)
<i>cfos</i>	FW: GTTGCTGTTGCTTCCTCTCC RV: GTTGCTGTTGCTTCCTCTCC	(Carvalho et al., 2022)
<i>hif1α</i>	FW: CAGATGAGCCTCTAACTTGTGGAC RV: TTAGCAAGAATGGTGGCAAGATGAG	(Pérez-Sánchez et al., 2013)
<i>hsp70</i>	FW: AGGTTGGGTCTGAAAGGAAC RV: TGAACTCTGCGATGAAGTGG	(Vallejos-Vidal et al., 2022)
<i>cat</i>	FW: TGGTCGAGAACTGAAGGCTGTC RV: AGGACGCAGAAATGGCAGAGG	(Brandts et al., 2021)
<i>sod</i>	FW: CCTGACCTGACCTACGACTATGG RV: AGTGCCTCTGATATTCTCCTCTG	(Brandts et al., 2021)
<i>igf1</i>	FW: GCCACACCCTCTCACTACTG RV: AAGCAGCACTCGTCCACA	(Mohammed-Geba et al., 2016)
<i>sirt1</i>	FW: GGTTCTACAGTTTCATCCAGCAGCACATC RV: CCTCAGAATGGTCCTCGGATCGGTCTC	(Simó-Mirabet et al., 2017)
<i>eno</i>	FW: GATCTGACCGTGACCAACCC RV: GACACCATCACACCCCATCC	(Raposo de Magalhães et al., 2021)
<i>il-1β</i>	FW: TCAGCACCGCAGAAGAAAAC RV: TAACACTCTCCACCCTCCAC	(Vallejos-Vidal et al., 2022)
<i>tnfa</i>	FW: TCGTTCAGAGTCTCCTGCAG RV: AAGAATTCTTAAAGTGCAAACACACCAAA	(Balasch et al., 2021)
<i>il-10</i>	FW: AACATCCTGGGCTTCTATCTG RV: TGTCTCCGTCTCATCTG	(Balasch et al., 2021)
<i>tgfb1</i>	FW: GCATGTGGCAGAGATGAAGA RV: TTCAGCATGATACGGCAGAG	(Khansari et al., 2018)
<i>muc18</i>	FW: ATGGAGGACAGAGTGGAGG RV: CGACACCTTCAGCCGATG	(Perez-Sanchez et al., 2013)

Table 2: Efficiencies and accession number of the selected primers.

Gene	Gills (%)	Skin (%)	Intestine (%)	Accession number
<i>gadph</i>	93.5	96.6	91.7	DQ641630
<i>elf-1α</i>	107.3	97.0	101.4	AF184170
<i>β-actin</i>	99.6	100.4	97.8	X89920
<i>gr1</i>	91.8	102.7	98.7	XM_030437675.1
<i>mr</i>	96.7	95.2	101.1	XM030418022.1
<i>cfos</i>	101.1	96.1	107.0	XM_030405977.1
<i>hif1α</i>	101.0	99.1	97.7	JQ308830
<i>hsp70</i>	109.4	96.1	100.4	EU805481.1
<i>cat</i>	95.8	92.5	-	JQ308823
<i>sod</i>	90.4	101.9	-	JQ308833
<i>igf1</i>	95.1	-	-	AY996779
<i>sirt1</i>	100.8	97.8	99.4	KF018666
<i>eno</i>	96.8	-	-	AY263379.1
<i>il-1β</i>	103.2	108.9	90.0	AJ277166.2
<i>tnfα</i>	111.9	111.4	108.3	AJ413189.2
<i>il-10</i>	-	112.0	112.1	JX976621
<i>tgfβ1</i>	101.5	95.7	102.4	AF424703.1
<i>muc18</i>	95.0	-	106.8	JQ277714

5.2.5 Statistical analysis

All data were analysed using the RStudio software (<https://www.R-project.org>). A Gaussian family model was employed, and Shapiro-Wilk tests were used to check for data and residuals normality. After checking the normality, the homogeneity of the variance was checked using a Bartlett test. If the data followed the parametric standards, a two-way ANOVA test was applied, but if the data did not follow the standards, a generalised linear model test was applied (GzLM). When there were significant differences, appropriate pair-wise comparisons were applied using Tuckey's correction (p -value < 0.05). Results are shown as the mean $\pm$ the standard error (SEM, $n = 8$, $N = 48$). Data were visually represented using the GraphPad Prism 8 Software. In the case of the results displayed in tables, data is shown as the mean $\pm$ the standard deviation.

5.3 Results

5.3.1 Biochemical parameters

Regarding biochemical analysis, no differences were found in glucose and acetylcholinesterase (**Table 3**).

Table 3: Biochemical parameters of *Sparus aurata* exposed to anoxia or density sampled at 1 or 24 hours post stress. Expressed as group mean $\pm$ SD. GLC: glucose; Ache: Acetylcholinesterase.

Group	GLC (mg/dL)	Ache (μ mol/ml·min)
Control 1h	66.100 $\pm$ 14.261	0.418 $\pm$ 0.002
Control 24h	66.138 $\pm$ 8.222	0.417 $\pm$ 0.011
Anoxia 1h	69.450 $\pm$ 12.830	0.411 $\pm$ 0.023
Anoxia 24h	58.500 $\pm$ 6.369	0.393 $\pm$ 0.008
Density 1h	55.875 $\pm$ 10.311	0.415 $\pm$ 0.031
Density 24h	66.638 $\pm$ 9.179	0.404 $\pm$ 0.013

5.3.2 Gills gene expression

In gill homogenates, the genes related to the stress response displayed very little variation in expression. The expression of *mr* did not display any significant differences between groups (**Figure 2B**), whereas the expression of *gr1* resulted in significantly different values only in the high-density group between 1 and 24 hours (**Figure 2A**). On the other hand, *cfos* was significantly upregulated at 24 h after the crowding stress, being significantly different from both the same group at 1 h and from the two other experimental conditions at 24 h (**Figure 2C**). No significant differences were observed in the expression of *hsp70* or *hif1 α* (**Figure 2D,E**).

A series of genes related to oxidative stress were also analysed (i.e. *cat*, *sod* and *sirt1*), although no differences were found in the gills (**Figure 2F-H**). Despite the lack of differences in the oxidative stress-related genes, some differences were found in those involved in energy metabolism. *Enolase* showed a downregulation in the overcrowded group 24 hours following exposure, both compared with the control group and with the overcrowded group sampled at 1 h post-exposure.

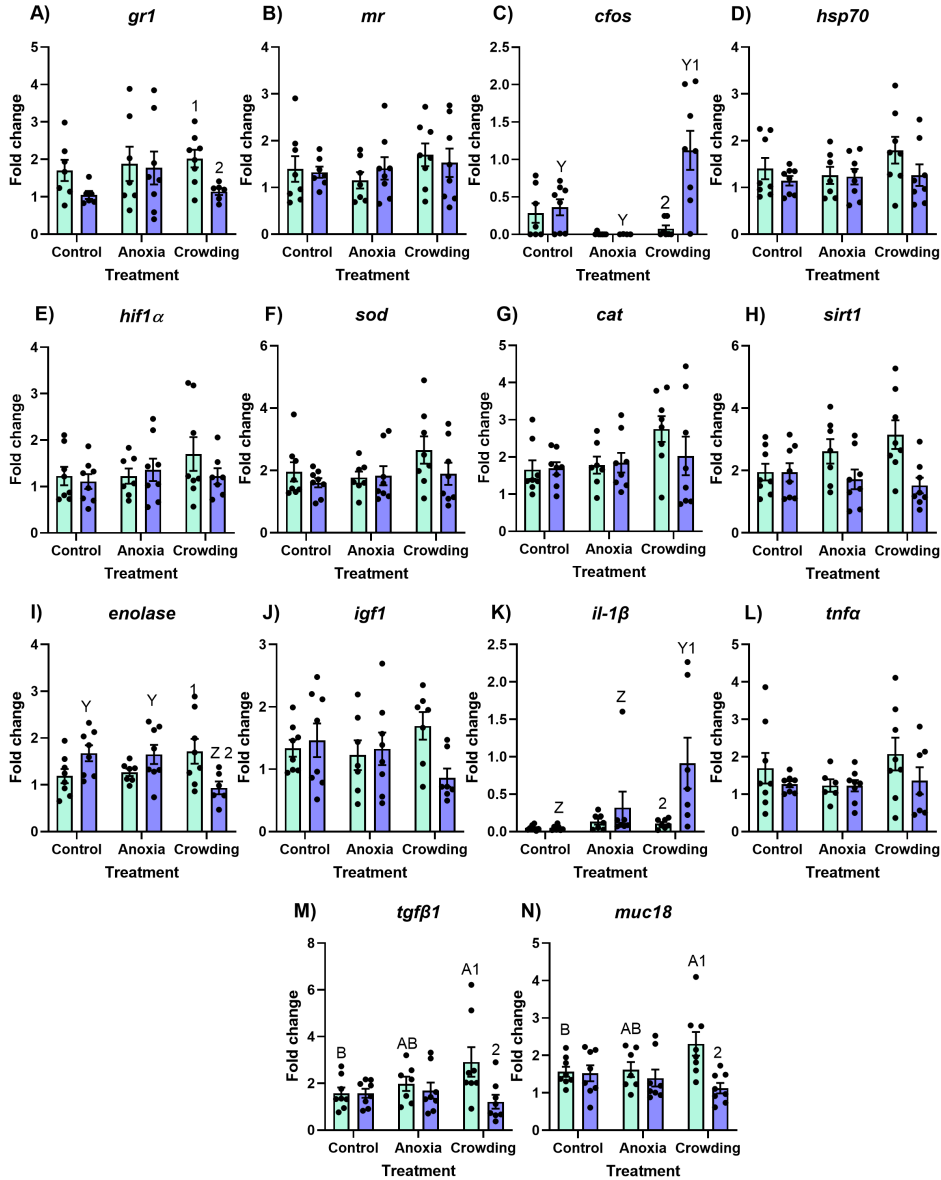


Figure 2: A) *gr1*, B) *mr*, C) *cfos*, D) *hsp70*, E) *hif1α*, F) *sod*, G) *cat*, H) *sirt1*, I) *enolase*, J) *igf1*, K) *il-1β*, L) *tnfa*, M) *tgfb1*, and N) *muc18* in gills of fish exposed to density or anoxia stress at 1 and 24 hours post stress. Data are represented as mean ± SEM (n = 6 per group). Significant differences (p-value < 0.05) are shown with different letters. Differences between the same group at different times are shown with numbers.

Interestingly, no changes were observed in the anoxia group (**Figure 2I**). No differences were detected in the growth-related gene *igf1* in gills (**Figure 2J**).

Finally, regarding the immune response, pro- and anti-inflammatory cytokine-coding genes were analysed. The pro-inflammatory response (*il-1 β* and *tnf α*) was slightly activated, with only *il-1 β* displaying significant differences (**Figure 2K, L**). Indeed, this gene was upregulated in the overcrowded group at 24 h post-exposure. Similarly, the two other immune-related genes, namely *tgf β 1* and *muc18*, resulted significantly upregulated in the density group at 1 h, returning to basal values at 24 h (**Figure 2M, N**).

5.3.3 Skin gene expression

The glucocorticoid receptors showed different responses in the skin. While *gr1* did not display any differences, *mr* showed a slight upregulation in the 1 h anoxia group, being significantly different from crowding but not from control (**Figure 3B**). Moreover, the expression of this gene in the anoxia group was significantly lower at 24 h compared to 1 h. *Cfos*, which is also involved in the stress and immediate early responses, showed an increase in anoxia 1 h, being different from the control and density groups, and returning to basal values at 24-hours (**Figure 3C**). As opposed to the gills, both genes related to the cellular stress response (i.e. *hsp70* and *hif1 α*) displayed significant alterations in skin samples. In the case of *hsp70*, the observed differences occurred at 24 h, showing an upregulation in the density group (**Figure 3D**). In contrast, *hif1 α* was only upregulated in anoxia 1 h, returning to values similar to those of the control after 24 h (**Figure 3E**). In the case of *sirt1*, no time effect was found, with an upregulation observed in the anoxia group compared with the control and density groups at both sampling points (**Figure 3F**).

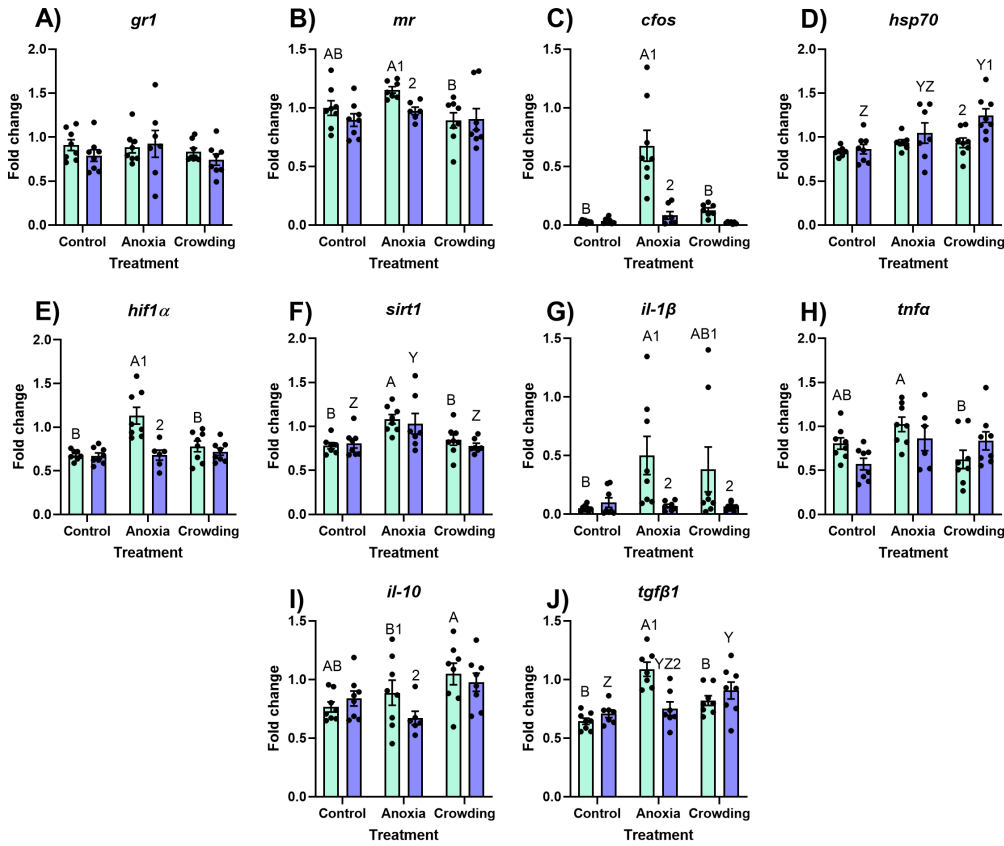


Figure 3: A) *gr1*, B) *mr*, C) *cfos*, D) *hsp70*, E) *hif1α*, F) *sirt1*, G) *il-1β*, H) *tnfa*, I) *il-10*, and J) *tgfb1* in skin of fish exposed to density or anoxia stress at 1 and 24 h post stress. Data are represented as mean ± SEM ($n = 6$ per group). Significant differences (p -value < 0.05) are shown with different letters. Differences between the same group at different times are shown with numbers.

Regarding the immune response, all studied genes were significantly altered in this organ (**Figure 3G-J**). The expression of *il-1 β* was significantly greater at 1 h in both stress groups compared to the control, and in both cases, basal values recovered at 24 h (**Figure 3G**). For *tnf α* , no significant differences were observed between the exposed groups and the control, although the expression of this gene in the skin of fish exposed to high stocking density resulted significantly lower than in the anoxia group at 1 h (**Figure 3H**). Interestingly, a similar but opposing trend was observed in the anti-inflammatory-related gene *il-10*. In this case, neither group showed significant differences compared to the control group, but the values measured in the density group were significantly greater than in the anoxia-exposed fish, and its expression was significantly downregulated in the anoxia group at 24 h (**Figure 3I**). Lastly, the mRNA abundance of *tgf β 1* significantly increased 1 h after air exposure, returning to control values at 24 h. On the contrary, *tgf β 1* was significantly upregulated at 24 h in the density group (**Figure 3J**).

5.3.4 Intestine gene expression

In this organ, very few differences were found. A slight, but significant downregulation was observed in the anoxia group 24 h in *gr1* (**Figure 4A**). In contrast, *cfos* was significantly upregulated 1h after air exposure, with values returning to control levels after 24h (**Figure 4C**). No differences in the expression of genes related to the cellular stress response, oxidative stress response, or energy metabolism were observed. Finally, regarding the immune response, differences only arose in the expression of *il-1 β* with a significant downregulation of the anoxia group compared to the control at 24 h (**Figure 4H**).

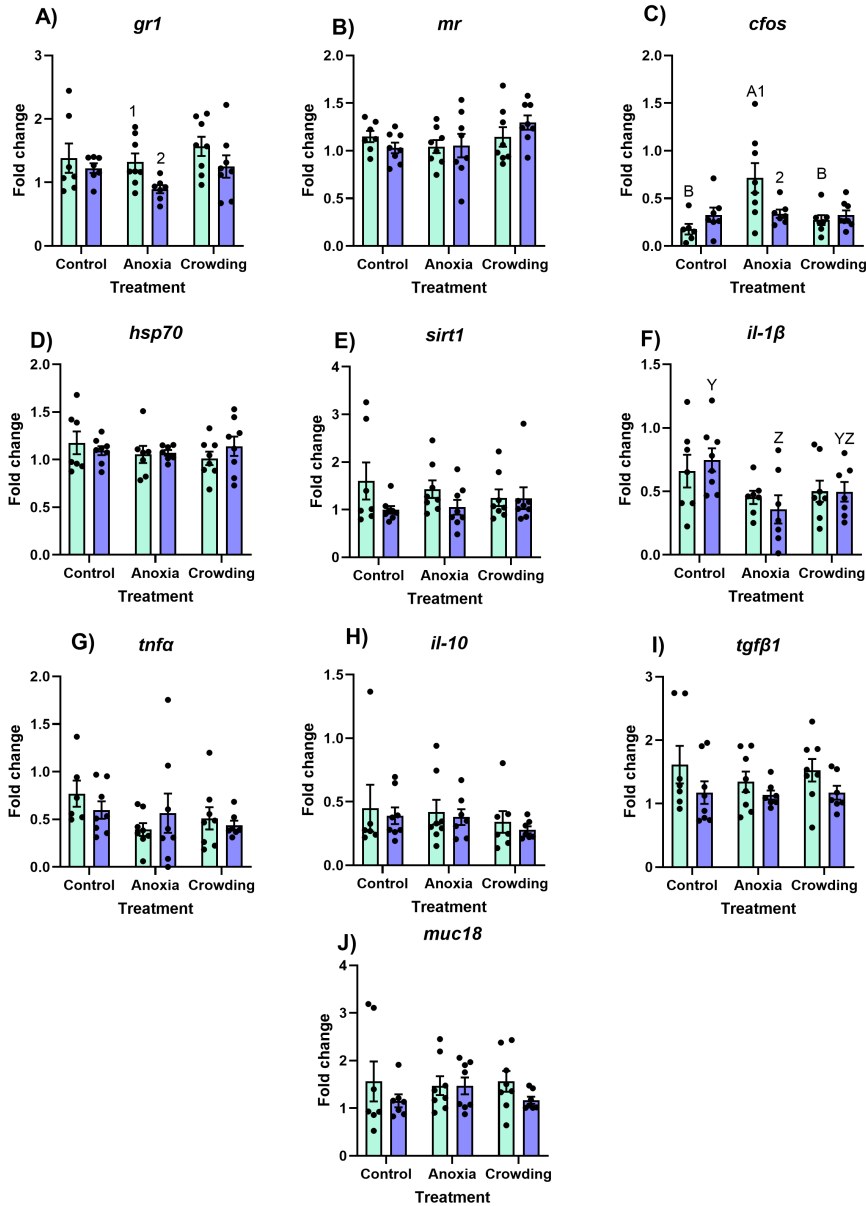


Figure 4: A) *gr1*, B) *mr*, C) *cfos*, D) *hsp70*, E) *sirt1*, F) *il-1β*, G) *tnfa*, H) *il-10*, I) *tgfb1*, and J) *muc18* in intestine of fish exposed to density or anoxia stress at 1 and 24 h post stress. Data are represented as mean ± SEM ($n = 6$ per group). Significant differences (p -value < 0.05) are shown with different letters. Differences between the same group at different times are shown with numbers.

5.4 Discussion

This study investigated the independent impact of either direct air exposure or crowding stress in the transcription of relevant stress, immune and energy genes in one of the most economically important aquaculture species in the Mediterranean area, the gilthead seabream.

No differences were found in circulating levels of glucose amongst experimental groups and sampling points. This indicates that, under the present experimental conditions, *S. aurata* juveniles directly exposed to air or to increased stocking densities for reduced periods did not trigger their secondary stress response. Although the experimental challenge was expected to induce the activation of both primary and secondary stress responses, as evidenced by previous similar studies (Vera et al., 2014; Khansari et al., 2018), other studies have reported a lack of alterations in glucose levels following exposure to high densities or hypoxic conditions (Araújo-Luna et al., 2018; Sánchez-Muros et al., 2017). It is possible that hormonal levels in the particular stress conditions of this experiment were not sufficiently activated as to elicit hyperglycemia, or that increases in glucose are masked by the considerable variability observed in measurements of this parameter (Araújo-Luna et al., 2018). Moreover, the potential differences in glucose levels might have been partly cloaked by the previously described daily rhythms in plasmatic metabolite concentrations (Vera et al., 2014).

The transcriptional response of *S. aurata* under the current experimental conditions displayed clear tissue- and stressor-specific patterns, supporting previously published results providing evidence that, in various fish species, including gilthead seabream, different tissues of the same animal respond differently to the same stressor (Khansari et al., 2018). Interestingly, crowding stress seems to have induced stronger gene expression modulation in the gills, compared to the other two studied organs. Similar patterns have been described in *S. salar* exposed to crowding, with a more pronounced transcriptomics response from the gills than from skin (Lazado et al., 2021). In contrast, direct air exposure had a greater impact on the expression of the studied genes in gut and skin samples, indicating potential differential roles of these tissues in the coping of different stressors, as previously described in this species (Khansari et al., 2018). Given the role of gills in osmoregulatory and respiration processes, it could have been expected that this organ would react more intensively to direct air exposure than the other two mucosal surfaces hereby investigated.

The stress-related gene whose expression resulted in the most significant regulation in the present study across tissues is *cfos*. This immediate early gene (IEG) is widely used as an indicator of neuronal activity and stress in vertebrates as its mRNA abundance is often significantly altered following exposure to different stimuli (Duman et al., 2005; Chatterjee et al., 2015; Salierno et al., 2006). More interestingly to the present study, previously published data on the response of this gene in the face of crowding stress indicates a significant upregulation of *cfos* in the gills of *Salmo salar* exposed to above-optimal density levels (Ellison et al., 2020).

Regarding the expression of *gr*, it was significantly reduced in the gills and gut of fish challenged with crowding and air exposure, respectively, 24 h post-exposure. This possibly reflects the differential roles of these mucosal surfaces in coping with distinct stressors. Similar patterns of decreases in *gr* mRNA abundance have been reported in the gills of this species when exposed to an acute stressor (Vallejos-Vidal et al., 2022). Similarly, the expression of *mr* was significantly downregulated in the skin of air-exposed fish, further agreeing with previous studies (Vallejos-Vidal et al., 2022). In rainbow trout (*Oncorhynchus mykiss*), cortisol implants, mimicking the increase in circulating cortisol levels raised when fish faced a stressful situation, caused a significant downregulation of glucocorticoid receptor-coding genes in mucosal surfaces, particularly gills and skin. A similar trend was observed in skin tissue, although differences remain above the threshold for significance (Teles et al., 2013). The specific response dynamics of fish to similar stressors are species- and tissue-specific, but general stress response mechanisms are, anyway, well conserved amongst vertebrates (Balasch and Tort, 2019). Thus, it is reasonable to speculate that, in the present study, the significant decrease in the mRNA abundance of glucocorticoid receptor-related genes observed in mucosal surfaces might serve as a proxy for increased circulating cortisol levels.

The stress-related *hsp70* was only significantly altered in the skin of fish exposed to high stocking densities 24 h following exposure. These results are in agreement with previous studies (Lazado et al., 2021) and suggest that the skin may play a particular role in coping with mechanical stressors, as crowding is likely to increase awareness of environmental obstacles or conspecifics. To the best of the authors' knowledge, no study has investigated the response of enolase-coding genes in the gills of fish subjected to crowding stress or direct air exposure. However, previous evidence suggests that *enolase* is significantly downregulated in the liver of fish exposed to repeated handling stress, as observed in the fish exposed to

high stocking densities, indicating the potential relocation of energetic resources (Cordeiro et al., 2012).

The transcription of genes involved in the immune response was particularly impacted in the gut and skin of fish exposed to air, whereas greater differences were found in the gills of those challenged with crowding. Direct air exposure led to the significant downregulation of the pro-inflammatory cytokine *il-1 β* in the intestine. This too, could reflect an increase in circulating cortisol levels, which has been described as suppressing the expression of pro-inflammatory interleukins (Sternberg, 2006). However, this gene was significantly upregulated in skin samples in fish exposed to the same stressor, indicating the possible reallocation of energetic resources towards an area more exposed to the perceived danger and potentially more susceptible to damage, hinting towards the existence of compensatory mechanisms between mucosal surfaces (Schreck, 2010). In contrast, the transcription of the anti-inflammatory cytokine *il-10* was significantly decreased in the skin of these fish 24 h, once the transcription levels of *il-1 β* returned to normal, hinting towards the suppression of a negative feedback loop of the inflammatory processes (Ruiz et al., 2026). Moreover, the expression of *il-1 β* was significantly increased in both skin and gills of fish after crowding stress, indicating that crowding leads to the activation of immune defences in peripheral tissues, potentially as a response to increased physical contact and friction between conspecifics. This is in accordance with previous studies reporting the modulation of pro- and anti-inflammatory cytokines, and other immune-related compounds, in fish mucosal surfaces in face of different densities (Cordero et al., 2016; Yarahmadi et al., 2016).

Another clear example of stressor-specific response in the present study is the regulation of *tgfb1*. Indeed, this gene was significantly upregulated in the gills and skin 1 h and 24 h after crowding, respectively, whereas its expression was significantly reduced in the air-exposed group. An increase in the expression of this gene indicates a possible occurrence of cellular stress, as it is deeply involved in controlling cellular processes, such as proliferation and differentiation (Lin et al., 2026; Schiller et al., 2004). As expected, the expression of *hif1 α* was significantly upregulated shortly after direct air exposure. This gene is directly related to the response of fish to hypoxic or anoxic conditions, with similar patterns observed across species, and often triggers a series of subsequent responses, modulating the expression of other relevant genes (Abdel-Tawwab et al., 2019; Murphy and Rees, 2024; Terova et al., 2009). Similarly, *sirt1* was upregulated in air-exposed

fish throughout the experimental period, further agreeing with previous reports on the responsive role of this gene in a range of living organisms, including fish, to hypoxic or anoxic conditions, particularly in mucosal surfaces (Lobo, 2025; Meng et al., 2017; Wang et al., 2021). Moreover, the overexpression of *sirt1* in mice has been described to inhibit pro-inflammatory mechanisms (Yoshizaki et al., 2010). Thus, the upregulation of this sirtuin-coding gene might be involved in negative feedback mechanisms aiming to modulate an immune response to the stimulus. Moreover, the expression of this gene is intricately connected with that of other genes related to antioxidant mechanisms, and stress and immune responses (Simó-Mirabet et al., 2018). It is therefore possible that *sirt1*, in conjunction with *hif1 α* , played a major role in the modulation of the other altered genes observed in the skin of fish in the air exposure group.

The mechanisms modulating the transcription of mucin-coding genes is generally poorly understood in fish, but it has been described that membrane-bound mucins, such as *muc18*, influencing the viscosity of mucus, play a major role in the fish ability to cope with a variety of endogenous stressors, including pathogen infection (Adamek et al., 2017; Riera-Ferrer et al., 2024) and transfer at sea, involving handling and, more relevant to the present work, acute crowding stress (Hoem and Tveten, 2023). Thus, the observed short-term increase in *muc18* mRNA abundance in skin samples of the high-density group agrees with previous reports and could have contributed to the activation of the observed immune response (Radhakrishnan et al., 2023).

5.5 Conclusions

The present study aimed to investigate how molecular endpoints in different mucosal barriers respond to two stressors commonly encountered by farmed fish, namely crowding and direct air exposure. The results indicate somewhat tissue-dependent responses to these challenges, with gills displaying a more severe reaction to crowding, and direct air exposure having an increased impact on markers measured in skin and intestine. Overall, the two experimental conditions triggered primary stress responses, as well as antioxidant and immune mechanisms, although most parameters returned to control values at 24 h, indicating a successful recovery of homeostasis, making potential maladaptive repercussions highly unlikely. This study also presents some evidence suggesting both the occurrence of negative feedback loops and complementary mechanisms within and

between tissues. It would be of interest to investigate the combined effect of these stressors, as wild and farmed animals are likely to encounter multiple concurrent stimuli, in addition to isolated ones.

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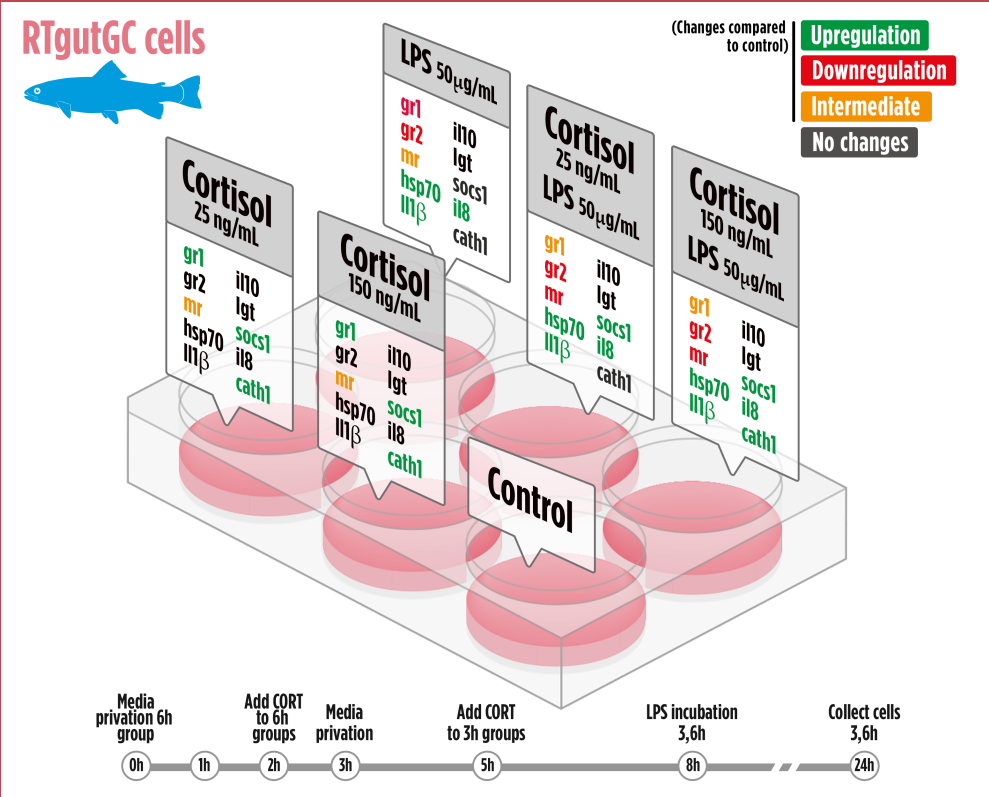
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Cortisol Modulates Lipopolysaccharide-Induced Immune Responses in a Rainbow Trout Intestinal Epithelial Cell Line (RTgutGC)

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6

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Abstract

The fish intestinal epithelium constitutes a critical barrier at the interface between the host and the environment, integrating nutrient absorption, stress responses and immune defence. Stress-induced cortisol release is known to modulate immune function; however, its specific effects on intestinal epithelial cells remain poorly understood. In this study, we investigated the immunomodulatory effects of physiologically relevant concentrations of cortisol on lipopolysaccharide (LPS)-induced immune responses using the rainbow trout (*Oncorhynchus mykiss*) intestinal epithelial cell line RTgutGC. Cells were exposed to basal and stress-related cortisol levels (25 and 150 ng/mL) for 3 or 6 hours, followed by 16 h LPS stimulation (50 μ g/mL). Gene expression of glucocorticoid and mineralocorticoid receptors (*gr1*, *gr2*, and *mr*), stress markers (*hsp70*), pro- and anti-inflammatory cytokines (*il-1 β* , *il-8*, *il-10*), as well as antimicrobial and immune regulatory genes (*cath1*, *socs1*, *igt*) was analysed by qPCR. LPS exposure elicited a clear pro-inflammatory response, characterised by increased *il-1 β* and *il-8* expression, whereas cortisol alone did not significantly induce the expression of inflammatory markers. Pre-exposure to cortisol attenuated LPS-induced *il-1 β* expression at later time points, indicating an immunosuppressive effect. Cortisol exposure differentially regulated corticosteroid receptors, with *gr1* upregulated, and *gr2* conjointly with *mr* downregulated, suggesting receptor-specific feedback mechanisms. Moreover, cortisol strongly induced *socs1* expression, indicating interference with cytokine regulatory signalling pathways. The antimicrobial peptide *cath1* was upregulated in response to both LPS and cortisol, whereas no significant modulation of *il-10* or *igt* was observed. These results demonstrate that cortisol modulates intestinal epithelial immune responses by selectively regulating inflammatory mediators, stress-associated genes and immune signalling pathways. This study highlights the importance of epithelial-specific mechanisms in stress-immune interactions and supports the utility of RTgutGC cells as a mechanistic *in vitro* model for investigating fish mucosal immunology.

6.1 Introduction

The intestinal epithelium in fish, like other external mucosae, is a key interface constantly interacting with the environment, which implies the need for maintaining homeostasis due to the impacts of both environmental and/or microbial challenges. To this end, the functions of the gut involve nutrient uptake, osmoregulation and protection from infectious diseases and environmental stressors (Fosse et al., 2025; Schug et al., 2019). Moreover, the intestinal barrier possesses the capacity to effectively clear infections, primarily through the function of the intestinal epithelium (Salinas and Parra, 2015). Research on the molecular and biochemical processes in specialised intestinal cells fosters fundamental knowledge and drives advances in improving fish health, either in aquaculture, biodiversity protection, or environmental risk assessment (Minghetti et al., 2017).

As part of the innate immune system, the intestinal epithelial cells express pattern recognition receptors (PRR) (Tao et al., 2012). PRRs identify pathogens by their pathogen-associated molecular patterns (PAMPs). In fish, most of the research on the PRRs has focused on the toll-like receptors (TLRs), which are crucial for the activation of the innate and the adaptive immune systems (Tao et al., 2012). Among different PAMPs, lipopolysaccharides (LPS), as important components of the outer membrane of gram-negative bacteria, are the most widely used model to mimic bacterial infection (Zhang et al., 2014).

In any antigenic interaction, such as after LPS treatment or after pathogenic contact, cytokine signalling is the essential mechanism for intercellular communication in the immune system, mediating and controlling immune and inflammatory processes and coordinating the activities of many cell types, ultimately leading to both innate and adaptive immune responses. After antigenic stimulation, the increase in cytokine levels mediates many processes such as inflammation, B- and T-cell growth and differentiation, and the activation of effector cells (Rogatsky and Ivashkiv, 2006). Complex interactions exist between cytokines, inflammation, and the adaptive immune responses for maintaining homeostasis, and most of these processes depend on the cytokine-induced activation of transcription factors that modulate the expression of target genes (Refojo et al., 2001). As part of this process, during an inflammatory reaction, the release of cytokines, such as tumour necrosis factor-alpha (TNF α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-12 (IL-12), also results in the activation of the stress response mechanisms (Calcagni and Elenkov, 2006). Evidence has revealed that stress hormones influence the immune response in several ways.

In teleosts, the main active glucocorticoid is cortisol (Aluru and Vijayan, 2009; Jiang et al., 1998) and is thus regarded as the most important transducer of the endocrine stress response in fish. Glucocorticoids (GCs) produce their effects on responsive cells by acting through the glucocorticoid receptors (GRs), which regulate the transcription of target genes (Castro et al., 2011). The relationship between the immune system of fish and the Hypothalamus-Pituitary-Interrenal axis (HPI) axis, responsible for the release of cortisol, indicates that glucocorticoids are important modulators of this system (Calduch-Giner et al., 1997; Harris and Bird, 2000). Most of the studies performed in fish have focused on the suppressive effects of cortisol on the immune system during stress responses. On the other hand, and as described in mammals, cytokines are also known to influence the endocrine control of the stress response in fish (Yada and Nakanishi, 2002; Engelsma et al., 2002).

Therefore, the functional interaction between cytokines and GCs involves crosstalk between the activated GR in the nucleus with different proinflammatory transcription factors that control cytokine synthesis, and action molecules, such as nuclear factor (NF)- κ B, activator protein (AP)-1, cAMP responsive element binding protein (CREB), and NF of activated T cells (Barnes, 2006). The activation of the factor (NF)- κ B triggers the production of proinflammatory cytokines (e.g. TNF α or IL-1 β), antiviral agents (e.g. interferons, IFN), as well as antimicrobial components and chemotactic cytokines (Tort et al., 2024).

Several GR-related genes have been described in teleost fish (Greenwood et al., 2003; Bury and Sturm, 2007; Stolte et al., 2008b), including two GRs (GR1 and GR2) and a mineralocorticoid receptor (MR), each exhibiting different functional properties. These receptor-specific characteristics might help explain the tissue-specific responses to cortisol. Research suggests that GR1 is sensitive to heightened levels of plasma cortisol, acting as the mediator for acute stressors. On the other hand, GR2 appears sensitive to lower levels of cortisol and is responsible for fine-tuning the modulation of cortisol-driven immune, metabolic, and energetic effects (Verburg-van Kemenade et al., 2017). GCs affect nearly every cell type in the immune system, but there is evidence for cell type-specific mechanisms (Amsterdam et al., 2002). For example, in monocytes, GCs modulate apoptosis, adherence, cellular motility, chemotaxis, phagocytosis, and reactive oxygen metabolism (Ehrchen et al., 2007), and can affect their differentiation into specialised macrophage subpopulations (Yona and Gordon, 2007). Macrophage activation is a complex process controlled by multiple inflammatory mediators,

including both exogenous factors such as pathogen-associated molecules and endogenous mediators such as cytokines and chemokines. These macrophage cells participate in almost every event of an immune reaction, as they consist of a heterogeneous population with distinct phenotypes involved in a wide range of pro- and anti-inflammatory activities (Anderson and Mosser, 2002; Gordon, 2003; Mosser, 2003).

Many of the studies mentioned above have been performed in *in vivo* models looking at the mucosal response after subjecting experimental fish to different types of environmental or husbandry stressors. Nonetheless, *in vitro* models, such as intestinal epithelial cell lines, are invaluable for targeted research regarding the epithelial immune response and barrier function of the fish gut (Fosse et al., 2025). Moreover, allowing to investigate the cell response isolated from the systemic control helps to understand the mechanisms behind the overall physiological response. In addition, these cell line models not only align with the 3Rs principles by reducing the need for experimental animals, but also offer a faster, more cost-effective, and less labour-intensive screening tool for the compounds of interest. Nevertheless, few cell lines have been available for fish cell research, and only recently, the RTgutGC cell line, derived from the distal intestine of a small female rainbow trout (*Oncorhynchus mykiss*), was the only immortalised cell line available from fish gut tissue (Kawano et al., 2011). This cell line has been extensively characterised since its establishment and has been a valuable tool for studying nutrient uptake, toxicity, functional feed ingredients, gut immune function, and epithelial integrity in fish (Kawano et al., 2011).

Research on the responses of fish intestinal cells to stress and immune stimuli can provide valuable fundamental knowledge about fish immune mechanisms with potential applications for fish health in aquaculture. Therefore, the present study aimed to advance mechanistic knowledge about immunomodulation. To this end, a fish intestinal barrier preparation, built on the epithelial rainbow trout cell line, RTgutGC, was first exposed to cortisol to simulate a stress situation, and later immunostimulated with LPS to simulate a bacterial infection, to determine the interactive immunomodulatory response.

6.2 Materials and methods

6.2.1 Cell culture

RTgutGC cells were obtained from Dr Carolina Tafalla's lab (Kawano et al., 2011). Routine cultivation followed the protocol described by Kawano et al. (2011). In short, cells were cultured at 20 °C, without CO₂ in L-15 medium with GlutaMAX and supplemented with 10 % FBS and 1 % penicillin–streptomycin in 75 cm³, and the media were changed every 48 h. Sub-confluent cultures (at an approximate density of 80 %) were detached by adding TrypLE Express enzyme (Gibco, cat.12605010) and left to incubate for 10 min. Detached cells were split up into new flasks at a 1:2 seeding ratio. Experiments for LPS and cortisol were performed at 80 % confluence.

6.2.2 Incubation of cells with cortisol and LPS

Physiologically relevant cortisol levels in unstressed salmonid fish range from 0 to 82 ng/ml and the presence of an acute stressor typically elevates plasma cortisol to levels of 40 to 250 ng/ml (Skinner et al., 2010). Considering this, 4 different doses (2 low and 2 high) of cortisol (H0888, Sigma) were selected: 25, 50, 150 and 300 ng/ml, and cells were exposed for 3 h, 6 h and 16 h. In addition, a similar dose response was done with the LPS from baker's yeast (L4391, Sigma) using different doses of 50, and 75 µg/ml at 1 h, 6 h and 16 h of exposure. Following this, a final experiment consisting of a first 3 h or 6 h incubation with cortisol at 50 or 150 ng/ml and then a 16 h LPS incubation at 50 µg/ml was designed. For the exposure, the media was changed from a complete to a deprived medium for 2 h, and the corresponding amount of each compound was subsequently added to each well.

6.2.3 RNA extraction and cDNA synthesis

Once the exposure time had elapsed, the media was removed, and 1 ml of HBSS was added to wash the wells. After 1 min, the HBSS was removed, and 500 µl of TRIzol were added to each well, incubated for 5 min at room temperature, transferred to 1.5 ml Eppendorf tubes and frozen at -80 °C. For the extraction, the samples were unfrozen and 100 µL of chloroform was added in each well. Samples were subsequently vortexed for 15 s and incubated at room temperature

for 15 min. After the incubation, the samples were centrifuged at 13.000 g for 15 min. Next, the supernatant was transferred to a new Eppendorf, and 250 μ L of isopropanol and 0.5 μ L of glycogen were added, after which, the resulting samples were stored for at least 1 h at -80 °C. The samples were then unfrozen, incubated at room temperature for 10 min, and centrifuged for 10 min at 13.000 g and the supernatant was discarded. To wash the pellet, 500 μ L of 75 % ethanol was added to each tube and centrifuged for 8 min at 13.000 g and the supernatant was discarded. This step was repeated twice. Finally, the pellet was air dried for 10 min and 11 μ L and quality (A260/A280 ratio) of the purified RNA were assessed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). The cDNA of each sample was synthesised from 1 μ g of RNA using the iScriptTMcDNA Synthesis Kit (Biorad, USA) following the manufacturer's instructions.

6.2.4 Real-time quantitative-PCR (qPCR)

Gene expression was analysed using real-time quantitative PCR (qPCR) in a CFX TouchTMReal-Time PCR Detection System (Bio-Rad, USA), according to MIQE guidelines (Bustin et al., 2009). The genes analysed were different depending on the experiment. In the case of the LPS dose-response, only two immune genes were analysed to check the activation of the immune response, namely interleukin-1 β , (*il-1 β*), and interleukin-8 *il-8*. For the cortisol dose-response experiments, only both glucocorticoid receptors were evaluated, glucocorticoid receptor 1 (*gr1*), glucocorticoid receptor 2 (*gr2*), and mineralocorticoid receptor (*mr*). For the experiment with the combination of LPS and cortisol the selected genes were: cathepsin 1 (*cath1*), *gr1*, *gr2*, *mr*, *il-1 β* , *il-8*, interleukin-10 (*il-10*), heat shock protein 70 (*hsp70*), suppressor of cytokine signalling 1 (*socs1*), and immunoglobulin T (*igt*).

The genes ribosomal Protein S16 (*s16*), Small ribosomal subunit protein uS10 (*rps20*), and glyceraldehyde-3-phosphate dehydrogenase (*gadph*) were employed as reference genes. All pairs of primers had been previously validated in *O. mykiss* tissues (Shekh et al., 2017; Sappal et al., 2016; Marandel et al., 2015; Teles et al., 2013; García-Meilán et al., 2022; Gioacchini et al., 2008; Castro et al., 2011; Mackenzie et al., 2004; Wang and Secombes, 2008; Han et al., 2023; Tongsri et al., 2020) and their sequence, and GeneBank accession number are specified in (Table 1).

Table 1: Primer sequences used in the experiment. Forward (FW) and reverse (RV) sequence (3'-5'), accession number and the reference of where the sequence were obtained.

Gene	Sequence	Accession number	Reference
<i>gadph</i>	FW: ACGAAGACAAGTTCGACCCC RV: GGATGATGTTCTGGTGGGCA	NM_001124246.1	(Shekh et al., 2017)
<i>rps20</i>	FW: GCAGACCTTATCCGTGGAGCTA RV: GTGGTGATGCGCAGAGTCTTG	BG936672	(Sappal et al., 2016)
<i>16s</i>	FW: TTTCAGGTGGCGAAACATGC RV: GGGGTCTGCCATTACCTTG	XM_021603090.2	(Marandel et al., 2015)
<i>gr1</i>	FW: CGCAGCAGAAACCAACAGTTG RV: ATGAGGGCGTCCAAGTACAGA	Z54210.1	(Teles et al., 2013)
<i>gr2</i>	FW: GGCAGCGTTTGAGGAGATGA RV: CATGGCGTCCAGTAGCTTGG	AY495372.1	(Teles et al., 2013)
<i>mr</i>	FW: CGCTTGCTGGAAAGCAGATG RV: GAGGTCAGGGGCAAAGTAGAGCAT	XM030418022.1	(Teles et al., 2013)
<i>hsp70</i>	FW: ATTCTGAACGTAGCAGCGGT RV: GCCATCTTCTCCCTCTGTGC	NM_001124228.1	(García-Meilán et al., 2022)
<i>il-1β</i>	FW: GGAGAGGTAAAGGGTGGCGA RV: TGCCGACTCCAACCTCCAACA	AJ223954	(Gioacchini et al., 2008)
<i>il-8</i>	FW: CACTGAGATCATTTGCCACTCTGA RV: ATGACCCTCTTGACCCACGG	AJ279069	(Gioacchini et al., 2008)
<i>il-10</i>	FW: CGACTTTAAATCTCCCATCGAC RV: GCATTGGACGATCTCTTTCTT	AB118099.1	(Castro et al., 2011)
<i>socs1</i>	FW: GATTAATACCGCTGGGATTCTGTG RV: CTCTCCCATCGCTACACAGTTCC	AM748721	(Wang and Secombes, 2008)
<i>cath1</i>	FW: TCACAGTGATGGAAATCGC RV: CATTTCTTTGATAGATGGGTTCT	NM_001124480.1	(Han et al., 2023)
<i>igt</i>	FW: TGTCCGTCCACATTCTC RV: TTGCCTTTCTTGTTT	AY870265.1	(Tongsri et al., 2020)

Prior to the analyses, a dilution curve with a pool of samples was run to determine the appropriate cDNA dilution for each gene, as well as to confirm the absence of primer-dimers, and the specificity of the reaction by a single peak in the melting curve for each primer set. All the analyses were performed in triplicate wells using 384-well plates with a final volume of 5 μ L (2.5 μ L iTAq™Universal SYBR® Green Supermix (Bio-Rad, USA), 250 μ M of forward and reverse primers, 250 μ L of RNA-free water and 2 μ L of diluted cDNA for each sample). The qPCR program had an initial desaturation step of 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The *GNorm* Software was used to calculate

the best combination of two of the three reference genes tested. The genes selected were *rps20* and *16s*. Then, the expression level of each target gene was calculated following the Pfaffl method (Pfaffl, 2001).

6.2.5 Statistical analysis

All data were analysed using RStudio software. A Gaussian family model was employed, and Shapiro-Wilk tests were used to check for data and residuals normality. After checking the normality, the homogeneity of the variance was checked using a Bartlett test. If the data followed the parametric standards, a one-way ANOVA test was applied, but if the data did not follow the standards, a generalised linear model (GzLM) test was applied. Appropriate pair-wise comparisons were applied by Tuckey's correction if significant differences were found (p -value < 0.05). To indicate the differences between groups at the same time, different letters were used. In the dose response at 6 h, M and N, and at 24 h, Y and Z, were used. In the final experiment at 3 h, A, B and C, and for 6 h, V, W, X, Y, and Z were used. Different letters indicate differences between groups. Results are shown as the mean $\pm$ the standard error (SEM, $n = 6$, $N = 144$) in the final experiment. Data were visually represented using violin plots with the GraphPad Prism 10 Software.

6.3 Results

6.3.1 Dose-response curves

Before the experiment, dose-response assays for LPS and cortisol were performed to confirm that the exposure concentrations were non-toxic for the cells and effectively elicited their respective responses. For the LPS treatment, the selected doses were 50 and 75 $\mu\text{g/ml}$ at 3 different exposure times. The interleukin-coding genes *il-1 β* and *il-8* were selected to analyse the output of the LPS treatment. The results showed that there was no effect of LPS at 1 h in either of the investigated genes. At 6 h, there was an upregulation of *il-1 β* compared with the control and PBS at 75 $\mu\text{g/ml}$ but not at 50 $\mu\text{g/ml}$, and no effects in *il-8* gene transcription were found (Figure 1). Finally, with the 16 h incubation, there was a clear upregulation of *il-8* at both doses, although this trend was only observed in *il-1 β* at 50 $\mu\text{g/ml}$ of LPS.

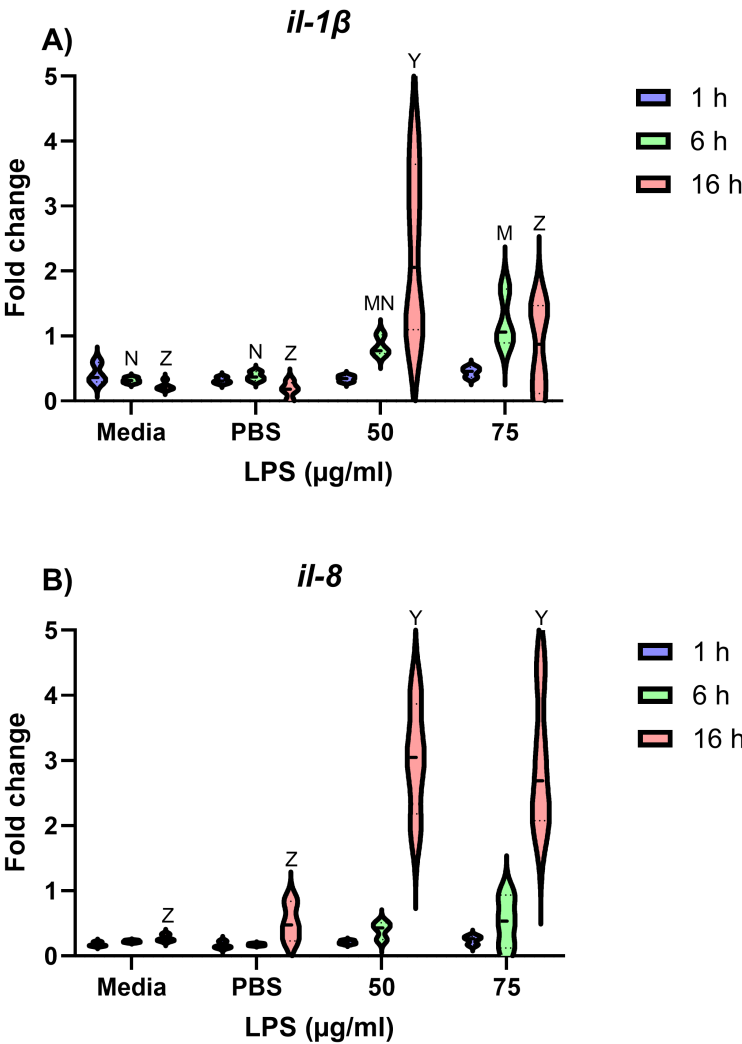


Figure 1: Relative expression of A) *il-1β*, and B) *il-8* in RTgutGC cell line under 3 different exposure times, 1 h (blue), 6 h (green), and 16 h (red), exposed to different doses of LPS. Data are represented as mean $\pm$ SEM ($n = 6$ per group). Significant differences (p -value < 0.05) are shown with different letters.

In the case of cortisol, no differences in the dose-response were observed across 3, 6 h and 16 h of incubation for either the glucocorticoid receptors or the mineralocorticoid receptor (**Figure 2**). Nevertheless, based on the literature 3 and 6 h were selected as the incubation periods.

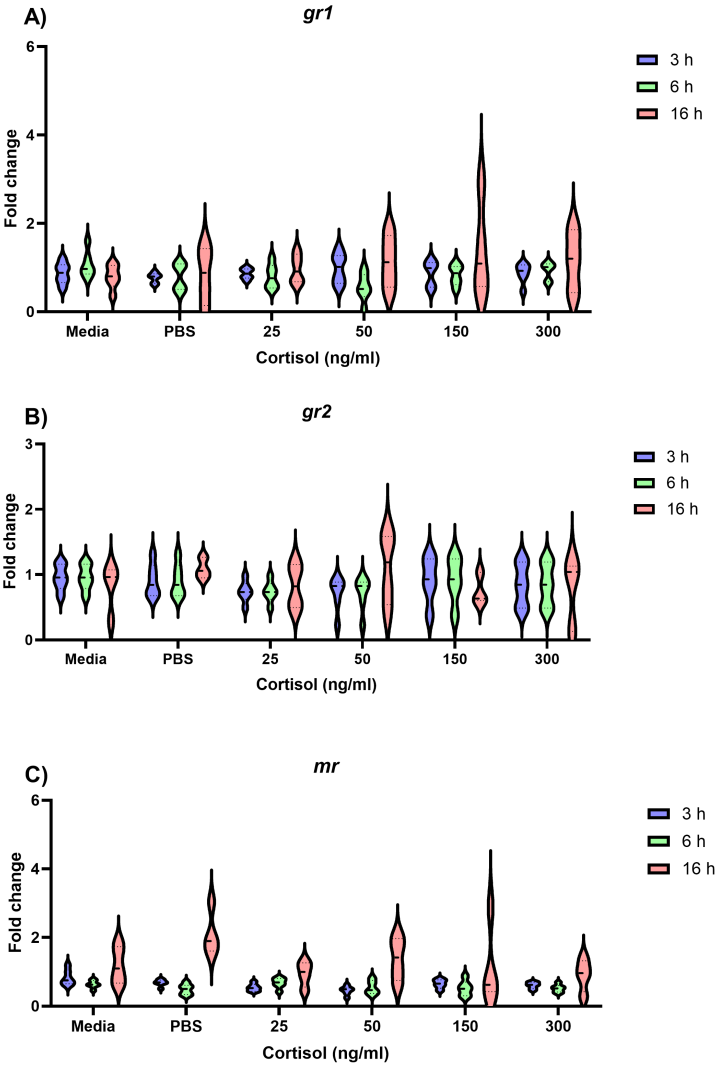


Figure 2: Relative expression of A) *gr1*, B) *gr2*, and C) *mr* in RTgutGC cell line after an incubation of 3 h (blue), 6 h (green), and 16 h (red) of different doses of cortisol. Data are represented as mean $\pm$ SEM ($n = 8$ per group). Significant differences (p -value < 0.05) are shown with different letters.

In conclusion, the result of both dose–response experiments supported the selection of cortisol incubations at 3 and 6 h basal (25 ng/mL) and stress-related (150 ng/mL) concentrations to simulate a stress condition, followed by an 16 h incubation with 50 μ g/ml LPS to induce immunostimulation.

6.3.2 Cell stress response

To evaluate the stress response, the expression of *hsp70*, a crucial molecular chaperone protein assisting in the proper folding of new proteins was measured in addition to the 3 endocrine receptors described in the dose-response, **(Figure 3)**. In the case of the glucocorticoid receptors (*gr1* and *gr2*) no differences were found at 3 h cortisol incubation. Nevertheless, at 6 h there was an upregulation of *gr1* in the groups exposed only to cortisol compared with the LPS alone **(Figure 3A)**, and no significant differences between the controls and the co-exposed groups were found. In contrast, at 6 h *gr2* showed a downregulation in the groups exposed to LPS alone and in combination with cortisol compared with the control media 1, and no significant differences were observed in the groups exposed only to cortisol **(Figure 3B)**. Moreover, no differences were observed in *gr2* at 3 h.

In the case of the mineralocorticoid receptor, similar differences were observed at 3 and 6 h cortisol exposure. At 3 h, a clear downregulation of the co-exposure and high cortisol dose groups was observed in comparison with the control media, PBS, and LPS. The lowest cortisol dose alone had intermediate values between the co-exposed groups, LPS and PBS groups. Nevertheless, despite the same trend being observed at 6 h, the LPS group was not different from cortisol alone or in combination with the LPS **(Figure 3C)**. Finally, *hsp70*, also showed similar results at 3 and 6 h of incubation. In this case, all groups exposed to LPS showed an upregulation at 6 h, while at 3 h, only the high cortisol concentration with LPS showed the upregulation, and no more differences were found between groups **(Figure 3D)**.

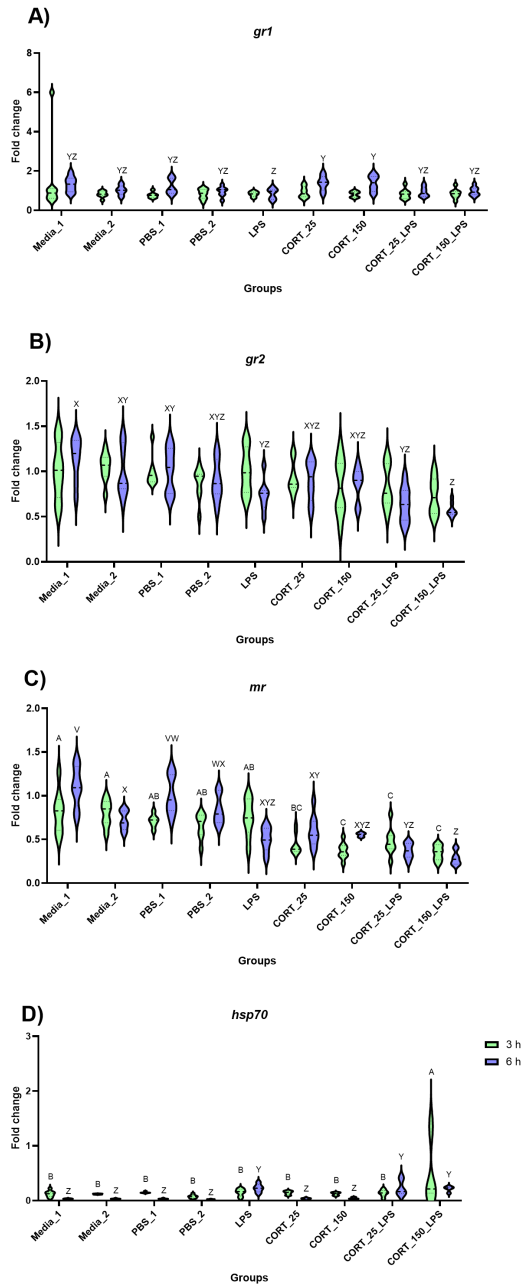


Figure 3: Relative expression of A) *gr1*, B) *gr2*, C) *mr*, and D) *hsp70* in RTgutGC cell line after an incubation of 3 h (green), and 6 h (blue) of cortisol and a following 16 h exposure to LPS. Data are represented as mean $\pm$ SEM ($n = 8$ per group). Significant differences (p -value < 0.05) are shown with different letters.

6.3.3 Cell immune response

For the evaluation of the immune response 3 different interleukins were chosen (**Figure 4**), as well as other genes that mediate the immune response such as *socs1*, *igt*, and *cath1* (**Figure 5**).

In the case of *il-1 β* , a clear upregulation in the groups exposed to LPS alone or in combination at 6 h was observed compared with the controls. Moreover, the upregulation in the groups exposed to cortisol and LPS was lower than the group exposed to LPS alone. In contrast, at 3 h, the only group that showed differences was the highest cortisol dose and LPS, being upregulated compared with the rest of the groups (**Figure 4A**). For *il-8*, the highest value was found in the group exposed to the highest cortisol dose and LPS at 3 and 6 h. At 3 h this group was different from the rest and LPS alone had intermediate values between the controls and the low cortisol in combination with LPS, while the groups exposed to cortisol alone had the lowest values being significantly different from the LPS, but not from the controls and the low cortisol dose + LPS. In contrast, at 6 h, despite the high cortisol dose + LPS having the highest values, these were not different from the other co-exposure group. At the same time the co-exposed group of the low cortisol concentration was not different from any group. In addition, the LPS group was upregulated compared to some media, PBS, and cortisol groups, having both cortisol groups the lowest expression (**Figure 4B**). Finally, no differences were found in *il-10* (**Figure 4C**).

No differences were observed after 3 h incubation in immune-related genes, other than interleukins. In addition, no changes were detected in *igt* whereas some differences were observed in *socs1* and *cath1* (**Figure 5**). Regarding *cath1*, a general upregulation was observed in groups exposed to LPS and / or cortisol, with the lowest expression levels in the control group, except for PBS_1. However, most of the groups showed intermediate values and no clear patterns were found for this gene (**Figure 5A**). Finally, regarding *socs1*, the highest upregulation was observed in the high dose cortisol group (150 ng/mL). The remaining groups exposed to LPS and/or cortisol also showed increased expression compared to the control and PBS groups, but lower than cortisol 150 (ng/mL). These groups displayed intermediate values between the high dose of cortisol and controls, so they were not different to any group (**Figure 5C**).

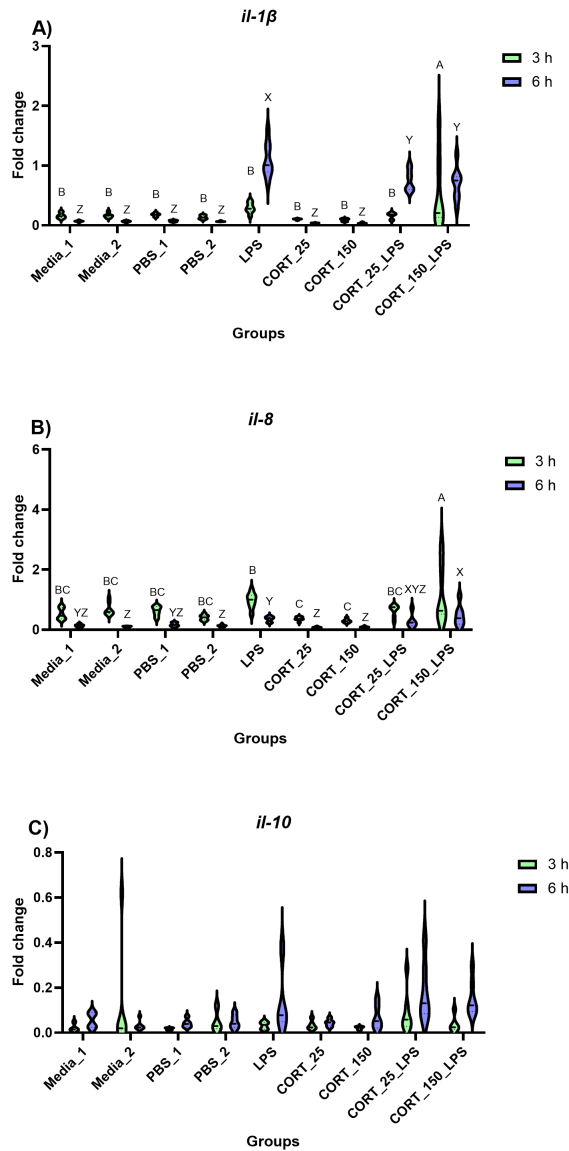


Figure 4: Relative expression of A) *il-1β*, B) *il-8*, and *il-10* in RTgutGC cell line after an incubation of 3 h (green), and 6 h (blue) of cortisol and a following 16 h exposure to LPS. Data are represented as mean $\pm$ SEM ($n = 8$ per group). Significant differences(p -value < 0.05) are shown with different letters.

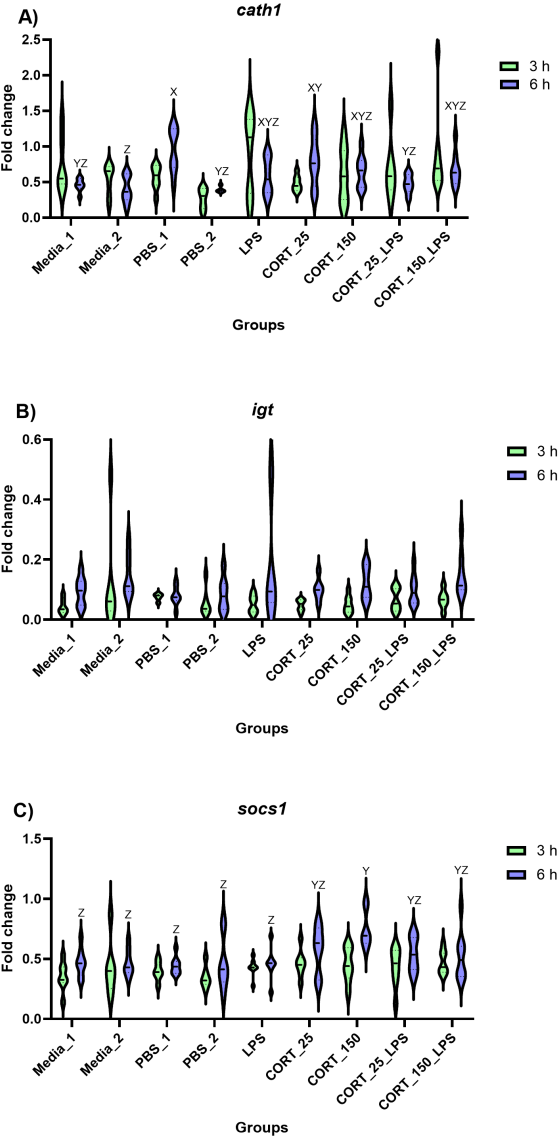


Figure 5: Relative expression of and A) *cath1*, B) *igt*, and C) *socs1*, in RTgutGC cell line after an incubation of 3 h (green), and 6 h (blue) of cortisol and a following 16 h exposure to LPS. Data are represented as mean $\pm$ SEM ($n = 8$ per group). Significant differences(p -value < 0.05) are shown with different letters.

6.4 Discussion

Glucocorticoids (GCs) are immunosuppressive molecules used most widely as immunosuppressive drugs, but their effects on specific immune cell types is not well defined in fish, with few studies describing GCs actions on cells of the innate immune system (Castro et al., 2011). It is also well known that stress can lead to changes in the immune response (Tort et al., 2024). In this study, a comparative stress and immune response was assessed under different physiological cortisol levels and/or LPS immunostimulation using the cell line RTgutGC.

IL-1 β is a pro-inflammatory cytokine with an important role in mediating the activation of cytokines such as *il-8*, whose function is to promote the recruitment of neutrophils and other cell types (Montero et al., 2008). Macrophages activation is regulated through feedback inhibition mechanisms (Hu et al., 2008) dictated by monocytes and macrophages using mediators such as *tgfb1* and *il-10* (Wells et al., 2005). IL-10 is a potent inhibitor of chemokines and cytokines of the inflammatory response produced by a wide variety of cell types (Moore et al., 2001) as in the oral tolerance. Oral tolerance in fish has been described as the state of systemic unresponsiveness that is the default response to food antigens in the gastrointestinal tract (Tordesillas and Berin, 2018). Complementarily, there are other cytokine suppressors as the suppressors of cytokines signalling proteins (SOCS). There are different isoforms of SOCS but, their functions are not very clear (Philip et al., 2012). For this study, *socs1* was selected, since it inhibits the JAK/STAT pathway.

As noted above, cortisol acts as an immunosuppressive agent. Accordingly, groups exposed solely to cortisol showed *il-1 β* and *il-8* levels comparable to those of the control group, but lower than those observed in the LPS-treated group, particularly for *il-1 β* after 6 h of cortisol exposure. This indicates that, although the immune response was activated, its magnitude was reduced in the presence of cortisol (Castro et al., 2011). In contrast, after 3 h of incubation, the highest interleukin levels were detected in groups exposed to both cortisol and LPS. It is noteworthy that a reduction in *il-1 β* expression has been reported in seabream head kidney cell cultures treated with cortisol (Castillo et al., 2009). Together, these findings suggest that the immunomodulatory effects of cortisol may differ depending on whether it is administered to a single cell type or to a heterogeneous cell population. Additionally, these differences may be partly explained by species-specific factors, as trout is a freshwater species, whereas gilthead seabream lives primarily in marine environments.

Glucocorticoids activate GRs and MR in cells, resulting in the modulation of targeted gene expression (Peterson and Laniel, 2004). Once the glucocorticoid binds to the GRs, the complex is transported to the nucleus, and this interaction leads to changes in gene transcription (Castro et al., 2011). Glucocorticoids tend to suppress the inflammatory response (Barnes, 2006), through different mechanisms (Refojo et al., 2001) as observed in this study. The role of the microbial component (LPS) as a proinflammatory agent has been widely described and has led to its widespread use as enhancer of the immune system (Goetz et al., 2004; DeWitte-Orr et al., 2007). Inflammatory responses are subjected to a negative regulation by other pathways, such as the anti-inflammatory response, with an increase of *il-10* or *tgfb1*, for example (Medzhitov and Horng, 2009). In this study, no differences were found in *il-10*, possibly due to the time or the intensity, since the inflammatory response is not excessive, as evidenced by the fold changes of *il-1 β* and *il-8* not being very high, potentially indicating that it was not necessary to activate the anti-inflammatory processes. This could be attributed to the sampling or incubation times because, even though no differences were detected at 3 and 6 h, there is a trend of higher fold changes in the 6 h groups.

To the best of the authors' knowledge there is no information available on the effects on the glucocorticoid receptors (*gr1*, *gr2* and *mr*) in RTgutGC and other cell lines other than observing relevant effects of cortisol on the overall immune response (Parra et al., 2015). Cortisol is recognised by the mineralocorticoid receptor (*mr*) and glucocorticoid receptors (*gr1* and *gr2*). Both gr receptors (Bury et al., 2003) have different functions. *Gr1* is responsible for recognizing cortisol under higher concentrations, while *gr2* acts at low cortisol levels (Vazzana et al., 2010). In this study, at 6 h, an upregulation of *gr1* can be observed in the cortisol exposed groups and a downregulation in the LPS group, while the rest have intermediate values. Meanwhile, in *gr2*, the groups exposed to LPS and/or cortisol display the lowest levels. This opposite behaviour of the GRs could be related to a putative negative feedback to avoid overexpression and restore homeostasis. Thus, it is known that GRs have an important role in this feedback regulation since GR knockouts are not able to regulate cortisol levels (Faught and Vijayan, 2019).

It has described that *mr* acts similarly to *gr2* (Vazzana et al., 2010), as observed in this study. The difference between the GRs and MR is that, in *mr*, the downregulation can also be observed at 3 h and that this downregulation is stronger, perhaps related to the fact that *mr* is also involved in other functions, such as ion transport or water permeability (Takahashi and Sakamoto, 2013). This gene is

also subjected to regulation through feedback mechanisms (Faught and Vijayan, 2018). After an infection, a strong pro-inflammatory response is activated, and the stress response is responsible for attenuating it.

Apart from the cell stress response of the GRs, other molecules and proteins, such as heat shock proteins (HSP) are involved in such cell regulation. For instance, *hsp70* assists in the folding of nascent polypeptides and acts as a molecular chaperone whose expression is induced after stress (Liberek et al., 2008). In fish, this regulation was demonstrated to be performed by a combined response of the GRs and *hsp70* (Basu et al., 2003). Since in fish there are two GRs, *hsp70* can be modulated by either a single GR or the combination of both receptors (Stolte et al., 2008a). In our study, there was an upregulation of *hsp70* in the groups exposed to LPS and/or cortisol, in agreement with previous reports, although with less marked differences (Stolte et al., 2009). These changes could be attributed to a tissue-specific response, since our cells belong to the gut (mucosal), whereas the Stolte et al. (2009) study was performed on head kidney (a tissue with systemic and coordination roles of the response). However, in both studies, the expression of *hsp70* was more similar to that of *gr1* than to *gr2*.

GRs are also related to SOCS, with both being important regulators of the JAK/STAT pathway (Haffner et al., 2008), both in fish and other vertebrates. When a cytokine or growth factor binds to a cell-surface receptor, the receptor-associated-JAK kinases are activated, and STAT proteins are phosphorylated. Then, STATs move to the nucleus and switch specific genes of the stress and the immune response on or off (Liongue et al., 2012). Our results showed an upregulation of the expression of the *socs1* gene under cortisol exposure in agreement with what was observed in Philip et al. (2012), and with the combination of cortisol and LPS, but not with LPS alone, only in the groups exposed to cortisol at 6 hours. This activation could be due to an adaptative response that restricts the activation of cytokine signalling (Philip et al., 2012). The authors suggested that the isoforms upregulated by cortisol were *socs1* and *socs2*, but not *socs3*. Although the function of each one is not very clear, they would work in a similar way as in mammals, inhibiting the JAK/STAT pathway (Crocker et al., 2008). Moreover, their role appears related to the energy substrate partitioning, relocating energy away from growth to cope with an increase in energy demand associated with stress (Philip et al., 2012), a trade-off commonly observed following stress and infection situations. As for the LPS effect, in mammals and in the cell line (rainbow trout spleen RTS11) it has been shown that LPS upregulates *socs1* (Jin et al., 2008). However, this was not

observed in this study, in agreement with Philip et al. (2012). These differences could be explained by the possible existence of a tissue-specific response, but further investigation is required to confirm the details of these mechanisms.

Igt is an exclusive immunoglobulin of bony fish that was discovered in 2005 (Salinas et al., 2005). *Igt* has been reported to be specialized in mucosal responses since it is more abundant in these surfaces (Zhang et al., 2010). Under some parasitic infections *igt* is upregulated, and, although it is thought that it plays an intermediate role between innate and adaptative response, its functionality in the adaptative immune response has not yet been fully established (Salinas et al., 2022). In our work, despite no significant differences being observed amongst groups, a greater upregulation was observed at 6 h than at 3 h, suggesting the upregulation of this gene. Discrepancies in the fold changes have also been observed by other authors (Contente et al., 2023), that could be attributed to differences in incubation time, as our results are focused on the first 6 hours whereas other authors measured the effects after 24 hours. Other possible explanations for the discrepancies are the different isoforms used and the fact that most of the studies have been performed in vivo rather than in vitro (Buchmann, 2020). In this study, the sequence used belongs to isoform 1 which is present in the mucosal and lymphoid organs (Zhang et al., 2010).

Cathelidicin 1 is an antimicrobial peptide with important functions in both the innate immunity, representing the first line of defence, and the adaptive immunity, participating in inflammatory processes (Shabir et al., 2018; Martín-Martín et al., 2020). It has been observed that *cath1* is upregulated with the activation of the NF- κ B, as with *il-1 β* , triggering the response of *il-8*. All these genes show correlated responses at 6 h since there is a signalling cascade that needs more time to be activated than *il-1 β* alone (Schug et al., 2019).

6.5 Conclusions

This study provides novel insights into the interaction between stress and immune responses at the level of the fish intestinal epithelium tissue. Using the RTgutGC cell line, we demonstrate that LPS induces a clear pro-inflammatory response characterised by increased expression of *il-1 β* and *il-8*, confirming the immune competence of these epithelial cells. Cortisol exposure alone did not activate inflammatory cytokine expression; however, pre-exposure to cortisol modulated

the LPS-induced immune response in a time- and concentration-dependent manner, supporting its immunosuppressive role.

Cortisol differentially regulated corticosteroid receptors, with *gr1* showing increased expression under prolonged cortisol exposure, while *gr2* and *mr* were downregulated, suggesting the involvement of receptor-specific feedback mechanisms to maintain cellular homeostasis. The upregulation of *hsp70* in response to both stress and immune stimulation further supports the coordinated role of stress response pathways in modulating epithelial immunity. Importantly, the marked induction of *socs1* by cortisol indicates a potential inhibitory effect of *socs1* on cytokine signalling through the JAK/STAT pathway, providing a mechanistic explanation for the observed attenuation of pro-inflammatory responses. In contrast, the absence of significant modulation of *il-10* and *igt* suggests that anti-inflammatory and adaptive immune pathways were not strongly activated under the experimental conditions used.

Overall, these findings demonstrate that cortisol modulates intestinal immune responses by fine-tuning inflammatory signalling rather than completely suppressing immune activation. This work emphasises the relevance of epithelial cell-specific responses in stress–immune interactions and reinforces the value of in vitro intestinal cell models for advancing mechanistic understanding of fish mucosal immunity, with implications for aquaculture health management and stress mitigation strategies.

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7

Discussion and Conclusions

7.1 Discussion

Stressors always impact fish, inducing adaptive responses to cope with the allostatic load and maintain homeostasis. Nonetheless, as the present results clearly show, the specific features of the stressor (i.e., intensity, time, predictability and controllability) modify the characteristics of the response, and the anatomical pathway through which the stressor is perceived, (i.e., centrally or peripherally through different mucosae) also characterises the response. Moreover, during this thesis, a tissue-specific response of each mucosal tissue, linked to the stressor's characteristics, was confirmed. Following the results described in each previous chapter, the discussion is hereby organised depending on the response to each stress model, further trying to clarify the observed tissue-specific patterns. To finalise the discussion, I find it appropriate to discuss the limitations of the thesis and how future research can complement it.

7.1.1 Compared response to repeated hypoxia or acute anoxia

Hypoxia is a pervasive stressor in both natural and aquaculture environments, and its impacts span from whole animal physiology to tissue-specific molecular responses. In this thesis, repeated hypoxic challenges were used as a unifying experimental framework to investigate how rainbow trout integrate haematological, metabolic, endocrine, and mucosal immune responses. By combining classical physiological indicators with gene expression analyses in key barrier tissues, this work addresses an important knowledge gap regarding how systemic stress responses translate into local mucosal adjustments, and whether habituation observed at the organismal level is conserved at tissue-specific immune and endocrine levels.

The applied stressors do activate the stress response. The haematological and biochemical results clearly demonstrate the activation of the general stress response following acute hypoxia, including changes in RBC indices, elevated cortisol, and increased lactate levels. These responses are consistent with previous studies showing that hypoxia rapidly activates the HPI axis and induces compensatory mechanisms aimed at preserving oxygen transport and metabolic homeostasis (Pichavant et al., 2002; Aboagye and Allen, 2018; O'connor et al., 2011). Importantly, repeated hypoxic exposure resulted in progressive attenuation of these

responses, with normalisation of RBC, HGB, MCV, and lactate, supporting the presence of physiological habituation. This concept of habituation to hypoxia has been observed under chronic exposures (Caldwell and Hinshaw, 1994). However, Pagès et al. (1995) suggested that fish also have a potential ability to habituate to repeated stressors, such as handling, by reducing metabolic disruption and endocrine overactivation (Pagès et al., 1995). The blunted lactate response in repeatedly stressed fish suggests improved aerobic efficiency or altered energy allocation (Richards, 2011), while partial normalisation of cortisol levels indicates adaptive regulation rather than exhaustion of the stress axis (O'Connor et al., 2011; Galhardo et al., 2011). Together, these data provide a solid systemic framework within which mucosal immune and endocrine responses can be interpreted.

Mucosal tissues represent the first line of interaction with the environment and must balance respiration, barrier integrity, and immune defence. The immune gene expression data reveal a clear tissue-specific response to repeated hypoxia, with the gills being the most responsive mucosa, while skin and intestine showed limited or inconsistent changes. This pattern mirrors earlier studies demonstrating that gills act as both a respiratory and an immunologically active tissue, highly sensitive to environmental stressors such as hypoxia, pathogens, and pollutants (Khansari et al., 2018; Vallejos-Vidal et al., 2022). The increased expression of pro-inflammatory cytokines (e.g., *il-1 β* , *tnf α*) in the gills, particularly after repeated hypoxic events, suggests that habituation at the systemic level does not equate to immune quiescence at the mucosal surface. Instead, repeated stress appears to enhance or prolong local inflammatory signalling. Similar dissociations between systemic stress indicators and localised immune activation have been reported in hypoxia-exposed fish, where cortisol levels decline during recovery, but inflammatory markers remain elevated in barrier tissues (Khansari et al., 2018; Vallejos-Vidal et al., 2022). This sustained gills-specific immune activation likely reflects the unique vulnerability of this tissue: hypoxia directly compromises gill function, increases epithelial permeability, and may promote pathogen entry or tissue damage (Evans et al., 2005). The minimal responses observed in skin and intestine may indicate either lower hypoxic exposure, stronger local regulatory mechanisms, or delayed responses beyond the sampling window. Importantly, these findings highlight that immune habituation is not uniform across tissues and may be absent or incomplete at critical mucosal interfaces.

The endocrine gene expression data further support the notion of mucosal-specific regulation under repeated hypoxia. Although plasma cortisol showed the expected acute increase and partial habituation, glucocorticoid receptor 1 (*gr1*) and mineralocorticoid receptor (*mr*) expression exhibited contrasting patterns between gills and other mucosal tissues. The upregulation of *gr1* and *mr* in gills, combined with their downregulation in skin and intestine, suggests that local sensitivity to cortisol is actively modulated rather than passively reflecting circulating hormone levels (Bernier et al., 2009). Also, Bernier et al. (2009) and Yada et al. (2019) found the same response under chronic stress, which they explained by desensitisation of the *gr* in gills. Another plausible explanation is the occurrence of the previously described tissue-specific regulation of cortisol signalling, which is thought to fine-tune local stress responses and avoid excessive immunosuppression in non-critical tissues (Yada and Tort, 2016). The delayed upregulation of *gr1* at 24 h further supports the idea of a secondary, regulatory phase of endocrine signalling following acute stress, potentially associated with either recovery or anticipation of predictable stressors as signalled by the increase of the circulating cortisol levels (Galhardo et al., 2011). The inverse correlation between *hif1 α* and *myoglobin* expression adds another layer of complexity. In gills, the downregulation of *hif1 α* alongside with increased *myoglobin* suggests a shift toward oxygen buffering and nitric oxide (NO) regulation, mechanisms known to facilitate recovery and vascular control under fluctuating oxygen availability (Borowiec et al., 2015; McNeill and Perry, 2006). In contrast, the opposite pattern in skin and intestine implies a hypoxia-sensing response without parallel enhancement of oxygen handling capacity, reinforcing the functional specialisation of the gills.

A key question arising from this thesis is whether habituation observed in haematological and metabolic parameters is confirmed at immune and endocrine levels, and the answer appears to be nuanced. While systemic physiological indicators clearly show habituation, mucosal tissues, particularly gills, exhibit persistent or even amplified responses with repeated hypoxia. Rather than a contradiction, this likely reflects adaptive prioritisation: maintaining heightened vigilance at the primary site of oxygen exchange may confer protection under recurring hypoxic conditions, even as the whole body stress costs are minimised. Therefore, habituation should not be viewed as a uniform dampening of all responses, but as strategic modulation across tissues and physiological systems.

Air exposure and aquatic hypoxia experiments showed both overlaps and differences in the response. Both stressors activate hypoxia-sensing pathways and the HPI axis, but aquatic hypoxia allows continued ventilation and induces more sustained haematological and metabolic adjustments (Aboagye and Allen, 2018). In rainbow trout, repeated hypoxia resulted in clear habituation, evidenced by normalisation of haematological parameters, attenuated lactate responses, and improved immune recovery (Pichavant et al., 2002; Aboagye and Allen, 2018; Richards, 2011; O’connor et al., 2011). In contrast, air exposure combines anoxia with handling and mechanical stress, producing rapid but largely transient responses with no evidence of habituation since repetitions were not performed. Species-specific physiological traits likely contribute to these differences. Gilthead seabream, for instance, are more tolerant to salinity fluctuations and short-term hypoxia than rainbow trout, which are among the most oxygen-sensitive salmonids (Schurmann et al., 1991). Such differences likely modulate tissue sensitivity and recovery capacity across stressors.

Immune indicators showed high sensitivity to stress type and duration. Pro-inflammatory cytokines (*il-1 β* , *tnf α*) were consistently activated under stress but differed in magnitude and persistence. For instance, repeated hypoxia induced sustained gill-specific inflammation despite systemic habituation, highlighting a decoupling between systemic stress recovery and local immune vigilance (Khansari et al., 2018; Vallejos-Vidal et al., 2022). In contrast, immune activation under air exposure was rapid and tightly regulated, particularly in skin, showing early pro-inflammatory signals followed by resolution within 24 h (Schreck, 2010).

7.1.2 Integrative responses to multiple environmental stressors across mucosal tissues

As aquaculture-relevant stressors rarely occur in isolation, fish are often exposed to combinations of physicochemical and husbandry-related challenges that interact at multiple physiological levels. Therefore, in this thesis, multiple stressors such as nanoplastics (NPs), salinity, overcrowding, hypoxia, and air exposure (anoxia) were investigated as distinct but partially overlapping stressors (i.e. salinity and PSNPs), allowing a direct comparison of their impact on systemic indicators and mucosal tissues. By integrating transcriptional, endocrine, immune, and physiological data, this work provides a comprehensive framework to understand stressor-specific mechanisms, tissue susceptibility, and the potential for habituation or maladaptation.

Salinity variations, air exposure, and overcrowding reflected activation of the primary response by the modulation of glucocorticoid and mineralocorticoid receptors expression (*gr1*, *mr*) in mucosal tissues. Similar activation of the HPI axis under diverse stressors has been widely reported in teleost fish (Schreck and Tort, 2016). However, the downstream immune, antioxidant, and metabolic consequences differed markedly between stressors and tissues. Elevated salinity elicited robust transcriptional responses in gills and intestine, consistent with their fundamental roles in osmoregulation and ion transport (Evans, 1968; Greenwell et al., 2003). In both tissues, hyperosmotic stress induced immune and antioxidant gene expression followed by delayed anti-inflammatory feedback, a pattern previously described under salinity challenges in gilthead seabream and other euryhaline species (Lu et al., 2022). The strong intestinal response corroborates evidence that the gastrointestinal tract is a major site of osmotic adjustment and metabolic regulation in fish (Hieu et al., 2022; Takei and Hwang, 2016), specifically in marine fish, since, due to osmoregulation characteristics, they need to drink water constantly, resulting in a higher rate of retention and absorption (Pratiwi et al., 2025). In contrast, polystyrene nanoplastics alone did not produce transcriptional effects, particularly in the gills. Since, only few studies have been conducted in this regard, but with considerably greater concentrations of the contaminant as the one performed for this thesis (Lu et al., 2022; Vineetha et al., 2024), the differences can be related to the concentration used or the species selected. Nonetheless, skin and intestine exhibited activation of stress (Takahashi and Sakamoto, 2013), and antioxidant response pathways (Hamed et al., 2017). Moreover, an alteration in the intestinal lipid metabolism was observed with the upregulation of *pparα* (Luo et al., 2025), consistent with their role as primary interfaces for particle contact and uptake.

Combined exposure to salinity and NPs resulted in synergistic responses in some of the genes, since most of them were a consequence of the salinity stress. In the intestine, it is important to note that, under both individual stressors, genes were generally upregulated compared with the control, whereas under the combined stress, downregulations occurred. This interaction supports growing evidence that co-exposure to contaminants and osmotic stress increases toxicity and immune dysregulation in fish (Chu et al., 2025; Xu et al., 2025). Overcrowding produced a distinct response profile dominated by gill sensitivity as reported by Lazado et al. (2021). Gills showed delayed activation of the immediate early gene *cfos*, modulation of cortisol signalling (*gr1* downregulation) and neuronal activity (Duman et al., 2005), suppression of metabolic genes, and transient immune activation.

Similar patterns have been associated with density-related stress responses, where increased conspecific interactions (physical contact and friction) and mechanical irritation may play a major role (Cordero et al., 2016; Yarahmadi et al., 2016). These mechanisms differ fundamentally from the osmotic pathways activated by salinity or the chemical / particle-driven effects of PSNPs, highlighting how stressors can converge on shared endocrine axes but diverge in effector pathways.

A central finding across all experimental chapters is the strong tissue-specificity of stress responses. Gills showed consistency among the most responsive tissues to salinity, overcrowding, and hypoxia, reflecting their multifunctional role in respiration, osmoregulation, and immune surveillance (Martos-Sitcha et al., 2014; Khansari et al., 2018; Lazado et al., 2021; Vallejos-Vidal et al., 2022). Skin responses were particularly pronounced under PSNPs exposure and air exposure, supporting its role as a frontline mucosal barrier directly exposed to physical and particulate stressors (Lazado et al., 2021). The intestine showed strong sensitivity to salinity, PSNPs, and combined stressors but comparatively weaker to air exposure, and muted responses to short-term overcrowding, which could be due to a conservative regulatory strategy aimed at preserving barrier integrity. These findings reinforce the concept that mucosal tissues operate under distinct specific regulatory logics, shaped by their primary physiological functions and exposure routes (Lazado and Caipang, 2014).

7.1.3 Influence of stress type and intensity on physiological response patterns and biomarker reliability

From an aquaculture perspective, hypoxia and overcrowding are common across production systems, particularly during transport and high-density rearing, whereas anoxia is more likely during grading, handling or slaughtering (Khansari et al., 2018; Vallejos-Vidal et al., 2022). Salinity stress is especially relevant in semi-intensive systems, and, in future, maybe also in cages associated with climate change, while nanoplastics represent a chronic, low-intensity background stressor in natural environments, open systems, and in RAS. Specifically, it has been demonstrated that, in RAS, the aquaculture activity increases the amount and the variety on NPs present in the water and internalised in fish tissues due to the materials found in the biofilters, tanks, and other facility components (Blonç et al., 2023). Habituation has been clearly demonstrated in fish under repeated hypoxia, consistent with hypoxic preconditioning (Pagès et al., 1995). Evidence

for habituation to overcrowding or salinity is less conclusive, with some studies reporting partial acclimation at the cost of immune or metabolic performance (Polakof et al., 2006).

Across this thesis, fish were exposed to a range of stressors differing in nature (e.g. chemical, physical, environmental), intensity, duration, and predictability, either under individual or combined stressor scenarios. Differences on the responses to this array of stressors strongly influenced the balance between immune, endocrine, antioxidant, metabolic, and physiological counteractions, contributing to variability among studies. While this variability may be viewed as a limitation, it also reflects the complexity of real aquaculture conditions and provides a broader, more ecologically relevant understanding of how fish integrate stress signals at multiple biological levels (Schreck and Tort, 2016; Tort et al., 2024).

Although primary responses showed consistency, downstream responses showed relevant differences among tissues. A common feature across all stressors was the activation of the primary stress response in most of the tissues, primarily mediated through the HPI axis, as reflected by changes in cortisol levels or modulation of corticosteroid receptors (*gr1*, *gr2*, and *mr*) (Vazzana et al., 2010; Martos-Sitcha et al., 2014; Khansari et al., 2018; Vallejos-Vidal et al., 2022). This response was evident under acute (air exposure), repeated (hypoxia), chronic (salinity), and combined stress scenarios (salinity + NPs), supporting the role of endocrine markers as general stress indicators. However, downstream responses diverged substantially depending on stress intensity and duration. Acute stressors, such as air exposure, triggered rapid but transient transcriptional activation, particularly of immediate early genes (*cfos*) (Duman et al., 2005) and hypoxia-related pathways (*hif1 α*) (Abdel-Tawwab et al., 2019), with limited systemic metabolic disruption. In contrast, repeated hypoxia led to progressive attenuation of haematological, metabolic, immune, and endocrine responses, indicating functional habituation and physiological plasticity (Pichavant et al., 2002; Aboagye and Allen, 2018; Richards, 2011; O'connor et al., 2011). Combined stressors (salinity + NPs) tended to induce stronger and more persistent immune and antioxidant responses than single stressors, particularly in gills, supporting the idea that stressor interaction amplifies allostatic load (more toxicity) and biological cost (Chu et al., 2025; Xu et al., 2025). Overcrowding, although relatively mild at the systemic level, produced delayed but sustained mucosal modulation, especially in gills, likely driven by potential continuous physical and / or social stress (Lazado et al., 2021).

The intestine displayed a pronounced immune sensitivity and a reactive response to salinity and combined salinity plus PSNPs exposure, but immunosuppression under acute air exposure, suggesting cortisol-driven anti-inflammatory regulation (Sternberg, 2006). These findings illustrate that immune markers are highly informative but strongly context dependent, making them powerful indicators of specific stressor impacts rather than universal stress metrics, since the same stressor in different tissue can elicit opposite regulation mechanisms.

Endocrine markers, particularly corticosteroid receptor expression (*gr1*, *mr*), were among the most consistent indicators across stress types (Table 1). Modulation of these receptors occurred under hypoxia, salinity, overcrowding, NPs exposure, and air exposure, albeit with tissue-specific patterns (Vazzana et al., 2010; Martos-Sitcha et al., 2014; Khansari et al., 2018; Vallejos-Vidal et al., 2022). Their repeated involvement suggests that receptor level regulation may be more informative than circulating cortisol alone, especially when systemic hormone levels show limited variation. Thus, the relationship between *gr* and *mr* is possibly regulated by the level of stress since *mr* followed the pattern of *gr2* in the *in vitro* experiment, as in Vazzana et al. (2010), while it followed *gr1* in the *in vivo* as in Vallejos-Vidal et al. (2022). Nonetheless, endocrine markers were relatively less discriminatory between stressors, as similar receptor changes were observed under different challenges. Thus, while reliable for general stress detection, they are less suitable for identifying stress sources or mechanisms when used in isolation.

Table 1: Relation between bioindicator and the use for detecting stress.

Biomarker	Function
WBC, <i>gr1</i> , <i>mr</i> , <i>cfos</i>	General welfare assessment and stress indicator
Pro-inflammatory cytokines and antioxidant markers (<i>sod</i> , <i>cat</i>)	Consistent stress indicators but context dependent
<i>hif1α</i> , <i>myoglobin</i> , lactate, cortisol	Hypoxia, air exposure stress marker
(<i>il-10</i>)	Salinity stress marker
(<i>il-1β</i> , <i>tgfβ1</i>)	High density acute stress

Antioxidant markers (*cat*, *sod*) showed marked variability depending on stressor complexity. They were weakly affected by acute stress (air exposure), moderately activated under salinity and PSNPs exposure, and heavily dysregulated under combined stressors, particularly in skin and intestine (Lu et al., 2018; Santos et al., 2022). The reduction of antioxidant expression under combined stressors exposure in skin suggests functional exhaustion, positioning antioxidant genes as sensitive

indicators of chronic or combined stress, but unreliable for detecting low intensity acute challenges.

Other markers such as *cfos*, haematological indices, and plasma lactate were consistently responsive to stress intensity, particularly under acute scenarios (Aboagye and Allen, 2018; Richards, 2011; Duman et al., 2005). However, these indicators largely reflected stress magnitude rather than stress identity, making them robust but stressor unspecific. For example, lactate effectively indicated anaerobic metabolism under hypoxia but was not informative under overcrowding or air exposure, where metabolic disruption was limited.

In the hypoxia experiments, elevated cortisol coincided with transient leukopenia and suppressed WBC counts, supporting its established role in immune cell redistribution rather than generalized immunosuppression (Sheng et al., 2019). Importantly, repeated hypoxia led to partial normalization of immune parameters despite sustained cortisol signalling, indicating adaptive recalibration rather than endocrine exhaustion (Pagès et al., 1995). At tissue level, differential regulation of *gr* and *mr* expression across gills, skin, and intestine served as a proxy for local cortisol sensitivity. Upregulation of these receptors in gills under hypoxia and salinity stress contrasted sharply with downregulation in skin and intestine, suggesting tissue specific feedback mechanisms aimed at preventing excessive corticosteroid signalling (Bernier et al., 2009).

7.1.4 Synthesis of tissue-specific stress responses, welfare implications, and relevance for aquaculture

A central result emerging from this thesis is that fish stress responses are highly tissue-specific and stressor-dependent, rather than being uniform systemic reactions. Across all experimental designs including hypoxia, air exposure, overcrowding, salinity, nanoplastics (NPs), and combined stressors the three main mucosal tissues (gills, skin, intestine) displayed distinct sensitivity profiles consistent with their physiological functions (Khansari et al., 2018; Lazado et al., 2021; Vallejos-Vidal et al., 2022). The gills were consistently the most responsive tissue to stressors that directly impaired respiration or osmotic balance, such as hypoxia, salinity, overcrowding, or combined salinity + PSNPs exposure. This reflects their central role in gas exchange, ion regulation, and immune surveillance, but also highlights their vulnerability to inflammatory dysregulation under repeated or combined stress (Khansari et al., 2018; Vallejos-Vidal et al., 2022). The skin

functioned as a highly sensitive frontline barrier, particularly responsive to air exposure and PSNPs exposure, but showed signs of functional suppression when multiple stressors co-occurred, suggesting a risk of barrier compromise under complex environmental scenarios (Lazado et al., 2021). The intestine emerged as a key integrative organ against osmotic and contaminant stress, mounting strong immune and antioxidant responses to salinity and PSNPs exposure, while showing comparatively conservative responses to short-term physical stressors such as air exposure and overcrowding (Sternberg, 2006). These consistent tissue-specific response patterns reinforce the conclusion that mucosal surfaces must be considered independently when assessing fish welfare, and that reliance on systemic indicators alone may overlook localised but biologically meaningful stress effects.

Air exposure emerged as a qualitatively different stressor compared with salinity, NPs, overcrowding, and aquatic hypoxia. Although systemic secondary stress indicators such as plasma glucose remained stable, air exposure induced rapid and tissue-specific transcriptional responses. Skin was the most responsive tissue, with early upregulation of *cfos* and hypoxia-related genes such as *hif1 α* , consistent with previous reports of acute air exposure triggering rapid hypoxia signalling and inflammatory responses at the epithelial surface (Schreck, 2010). In contrast, gills showed relatively limited transcriptional responses to short-term air exposure, likely due to the brief duration of anoxia and rapid return to normoxic conditions. The intestine exhibited transient stress perception followed by downregulation of immune markers at later time points, consistent with cortisol-mediated anti-inflammatory regulation described in vertebrates under acute stress (Sternberg, 2006). These patterns differ from overcrowding, which induced slower but more sustained gill responses, driven by prolonged physical and social stress rather than abrupt oxygen deprivation (Lazado et al., 2021).

Among the stressors evaluated, the most complex ones, like repeated hypoxia or combined stressors (salinity + NPs), appear to be the most likely to exert stronger, long-lasting, negative impacts on fish health and welfare. Understanding how these stressors interact at mucosal surfaces is, therefore, critical for improving welfare-based management strategies. One of them, repeated hypoxia induced systemic habituation, accompanied by persistent or enhanced immune activation in gills, suggesting that physiological acclimation may occur at the cost of sustained local inflammation and potential respiratory impairment (Cao et al., 2023). Such chronic gill activation may increase susceptibility to pathogens, compromise vaccination efficacy, and reduce growth or feed efficiency, particularly in juveniles.

Combined salinity and PSNPs exposure produced synergistic effects in several tissues, notably gills and skin, where antioxidant and immune pathways were either overactivated or suppressed, respectively. These findings are consistent with growing evidence that co-exposure to environmental contaminants amplifies stress toxicity beyond additive expectations (Chu et al., 2025; Xu et al., 2025). From a production standpoint, such interactions are of particular concern because NPs represent a chronic background stressor that may exacerbate otherwise manageable environmental challenges. In contrast, short-term air exposure and overcrowding, as investigated in this thesis, primarily triggered acute and reversible responses without major secondary stress activation. Although these stressors are undeniably relevant to fish welfare, their impact appears strongly dependent on duration, repetition, and husbandry practices (Lazado et al., 2021). The health and welfare control in aquaculture should therefore incorporate the management of combined stressors.

The stressors investigated closely correspond to critical phases in aquaculture production; hypoxia and overcrowding are likely found during transport, high-density rearing, and periods of poor water quality (Khansari et al., 2018). Similarly to hypoxia, anoxia (i.e. direct air exposure) commonly occurs during grading, harvesting, vaccination, and slaughter. Moreover, salinity fluctuations affect fish in coastal farms and transitional environments. Finally, NPs represent an emerging, diffuse stressor associated with feed, water sources, and equipment degradation (Blonç et al., 2023).

Overall, one common physiological-metabolic aspect appearing across experimental results is the energetic trade-off component of the response. Any significant physiological or biological response comes with the cost of supporting the allostatic load associated with overcoming the stressor effect. Moreover, the compensatory mechanisms may involve the reduction of energetic resources for other biological mechanisms that may involve consequent challenges for health and welfare.

7.1.5 Linking *in vivo* and *in vitro* findings

The RTgutGC cell line experiment provided direct mechanistic support for the *in vivo* observations, confirming that cortisol acts primarily as a regulatory hormone, shaping immune magnitude and controlling immunosuppression, rather than causing blanket suppression. Cortisol alone did not induce pro-inflammatory responses but fine-tuned immune activation triggered by LPS, attenuating cytokine expression through receptor-specific regulation and induction of inhibitory pathways such as *socs1* (Philip et al., 2012). The induction of *hsp70* and antimicrobial peptides such as *cath1* further supports the concept that epithelial tissues possess intrinsic stress–immune feedback loops designed to preserve barrier function under challenge (Basu et al., 2003; Shabir et al., 2018). While cortisol dynamics were most comprehensively analysed in the hypoxia and RTgutGC studies, the expression of *gr* and *mr* across other chapters provides strong inferential support for cortisol’s central role across stress contexts. Although the lack of simultaneous cortisol measurement in all experiments represents a limitation of the present thesis, receptor modulation and downstream immune patterns consistently align with expected cortisol regulatory action (Schreck and Tort, 2016).

7.1.6 Limitations and recommendations for future research and management

A main limitation of laboratory *in vivo* studies is their controlled, short-term nature, which may not fully capture the cumulative and fluctuating stress experienced under complex environments such as commercial aquaculture conditions. Additionally, transcriptional responses do not always translate directly into functional outcomes (Santos et al., 2022). In this regard, a major limitation was the lack of a complete set of results of cortisol levels throughout all experiments, since it is traditionally the main hormone to measure the stress. However, since the relation between cortisol and its receptors is well studied, it is possible to assume that an upregulation of the receptors indicate modulation of circulating cortisol levels. However, the strength of this approach lies in its mechanistic clarity and capacity for controlled stressor comparison. Moreover, the use of stressors with different intensities, durations, and combinations introduces an unavoidable variability that limits direct quantitative comparison across studies. However, this diversity mirrors real world aquaculture conditions, where fish experience fluctuating and overlapping challenges rather than uniform experimental stress. As such, this approach strengthens the ecological and applied relevance of the findings, and highlights the importance of multimarker and multitissue strategies for accurate stress and welfare assessment.

Future research, should, on one side, incorporate longer, chronic exposure periods and recovery cycles, and include additional sampling points associated to specific parameters with different optimal times of activation. Alternatively, focusing on a single stressor, and modulating the intensity and / or, duration, allowing to understand the importance of each characteristic of the stressor. Moreover, it would be highly beneficial to incorporate additional sampling events since, each response (endocrine, immune, energetic) displays distinct temporal dynamics, and their consideration is essential to obtain the whole picture, especially regarding chronic stress. Hence, under chronic stress situations, it would be convenient to incorporate different sampling points at the first stage of the experiment, and additional periodical sampling points to understand the evolution of the responses. Regarding the analytical techniques, it is remarked that more correlation between functional and molecular data needs to be done. Moreover, incorporating additional approaches such as the study of the mucus will be interesting since a unique characteristic of all the mucosal tissues is the presence of the mucus layer. In a lot of studies, the regulation observed in the genes might reflect alterations in the microbiome, and investigating intestinal mucus would be crucial in future studies. Thus, combining molecular, histological, and performance-based endpoints, with other high throughput techniques, such as the study of microbiome or metabolome in the case of the mucosal tissues, can provide useful complementary information on how this response is mediated by different components, as interleukines, mucins, hormones, and proteins among others; that can be modulated depending on the physiological status of fish. Additionally, it would be desirable to investigate different life stages on the same organism, to elucidate potential transgenerational, as well as different coping strategies in both intra and interspecific perspectives. Finally, and related with the optimization of welfare assessment through the prioritization of novel non-invasive techniques, the assessment of water-based parameters, such as water cortisol, environmental DNA (eDNA), (eRNA), as well as functional analyses, including behaviour, should be incorporated in future studies, in accordance with the 3R's principle.

7.2 Conclusions

1. Repeated hypoxia triggers both a clear systemic (haematology, cortisol, lactate) and local (gene transcription) stress response that is progressively attenuated through repetition, supporting hypoxic preconditioning, but this habituation is not uniform across biological levels.
2. Mucosal responses are strongly tissue-specific: gills are consistently the most sensitive and responsive interface, showing sustained (or amplified) immune and endocrine activation under repeated hypoxia and across several other stressors, even when systemic indicators normalize. This sensitivity is related to the intrinsic functions of the tissue.
3. Different stressors converge on the primary endocrine axis (HPI/cortisol signalling) but diverge in downstream pathways, with salinity strongly affecting gills/intestine, nanoplastics showing modest standalone effects but stronger impacts in combination, overcrowding producing delayed gill-dominated modulation, and air exposure driving rapid, mainly skin-centred transcriptional responses.
4. Stress responses in fish are not only stressor-specific but also shaped by intensity, repetition, and interaction. Endocrine markers provide reliable general stress indicators, whereas immune and antioxidant responses offer sensitivity to cumulative and localized challenges, and specialized molecular markers permit discrimination among stress types. The integration of these indicators across tissues provides a nuanced framework for interpreting fish stress responses and optimizing biomarker selection for aquaculture welfare monitoring.
5. Cortisol acts as an integrative, tissue-regulated modulator of stress-immune interactions rather than only a blanket immunosuppressor or metabolic driver, with local glucocorticoid/mineralocorticoid receptor regulation shaping mucosal sensitivity and inflammatory resolution; *in vitro* results support this regulatory role.
6. Comparing the *in vitro* and *in vivo* experiments glucocorticoid receptors show different modulation, possibly due to the intensity of the stressors, or maybe because the *in vivo* experiments involve different cell types and tissues when triggering the response, while the *in vitro* experiment focuses on a particular cell type.

7. The biomarker performance seems to follow a functional hierarchy: endocrine receptors and general stress markers are reliable for overall stress detection but less indicative for stressor specificity, while immune and antioxidant markers better capture localized/cumulative burden, and improve stressor discrimination as specialized markers (e.g., hypoxia genes).
8. In terms of aquaculture implication welfare assessment must involve multi-marker and multi-tissue approaches, because systemic recovery can mask persistent mucosal costs (notably chronic gill changes), and combined stressors (e.g., salinity + nanoplastics), and may generate synergistic impacts relevant to real farming conditions.
9. For future research emphasis should be put on investigating the link between functional and molecular techniques, while focusing on the study of the whole pathways, and trying to implement as many non-invasive techniques as possible is a must considering the increasing relevance of fish welfare issues.

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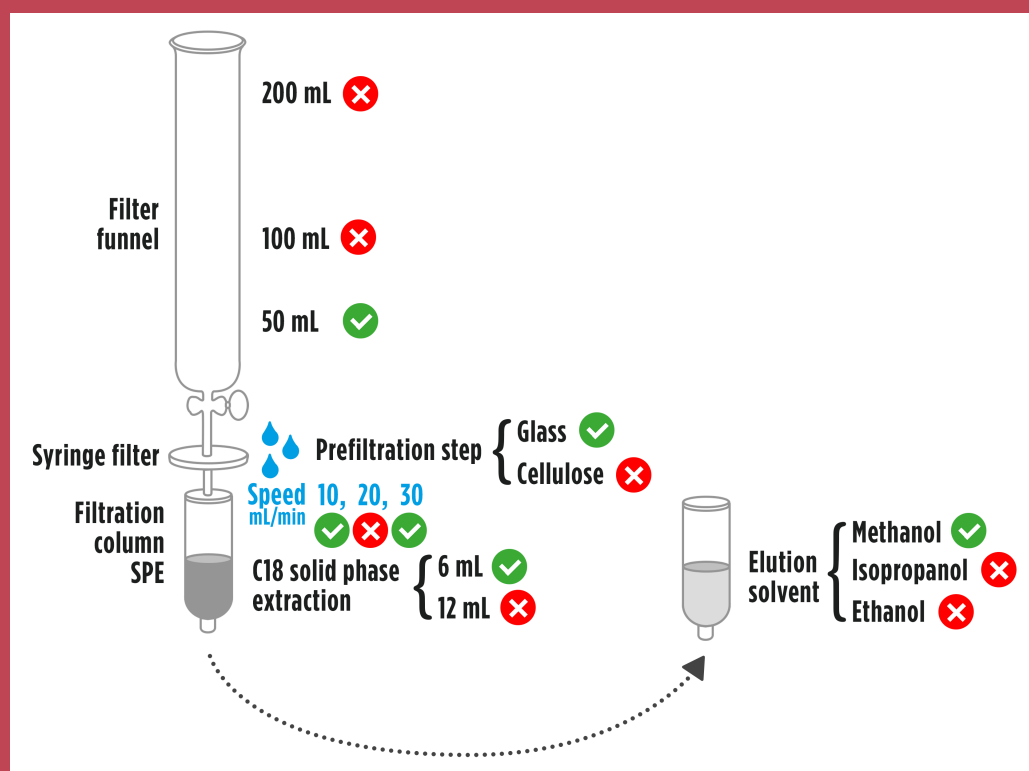
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Appendix I: New method for water cortisol extraction

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8



8.1 Introduction

Water-borne cortisol measurement is a methodology that allows checking the release of the hormone cortisol from fish to the surrounding water, thus giving a proxy of an eventual stress episode to fish contained in a water body. Water cortisol was first explored in the goldfish *Carassius auratus* (Sørensen and Scott, 1994) and later proposed as a non-invasive stress assay by Scott et al. (2001), with validation in rainbow trout by Ellis et al. (2004). Compared with plasma sampling, measuring cortisol in water offers some major advantages: it avoids the fish stress caused by capture and blood collection, enables repeated sampling from the same group, and allows studies on fish too small to bleed (Scott et al., 2001). Nonetheless, it is a measurement devoted to assess a group stress response, not the individual one, unless fish is isolated.

Although the extraction process is more time-consuming and costly than plasma analysis, waterborne cortisol has been shown to accurately reflect plasma concentrations. Cortisol is stable in water, exhibiting a half-life of 16 h at 12 °C (Ellis et al., 2004), and accumulates sufficiently for reliable quantification even under water-renewal conditions. Release rates can be estimated using the equation provided by Ellis et al. (2004), which incorporates water volume, cortisol concentrations, dilution rate, fish mass, and sampling interval. Fish mass should be considered when normalising data due to metabolic scaling effects (Killen et al., 2010).

However, there are differences in the methodology of the extraction of water cortisol (**Table 1**) as observed in the current scientific literature. For this reason, the aim of this study was to simulate the most complete protocols by filtering water with a known cortisol concentration and compare the rate of obtention of cortisol to try to standardize the methodology taking into consideration the following factors: duration of the extraction, easiness of use, precision, accuracy, variability reduction, and cost.

Table 1: Summary table of factors that changed in the literature. M: Methanol, E: Ethanol, W: Water, Di: Diethyl ether, Et: Ethyl acetate.

Method	Flow (mL/min)	Elution rate	Volume (mL)	Preservation (°C)	Reconstitution solvent	Evaporation temp. (°C)	Reference
Vacuum	-	M + W	800	-80	E + EIA buffer	37	(Archard et al., 2012)
Vacuum	-	M + W	500	-20	E + RIA buffer	42	(Boulton et al., 2015)
Vacuum	-	M + W	250	-20	M + EIA buffer	37	(Contreras et al., 2014; Forsburg et al., 2019)
Peristaltic	25	M + W	500	-20	Dy + RIA buffer	45	(Ellis et al., 2004; Geffroy et al., 2018; Sadoul et al., 2015, 2017; Zuberi et al., 2014)
Peristaltic	10	M + W	1000	-20	Dy + RIA buffer	45	(Fanouraki et al., 2008)
Vacuum	-	E + W	500	-20	E + EIA buffer	Centrifuge Speed vacuum +	(Félix et al., 2013)
Vacuum	-	M + W	200	-80	E + EIA buffer	35	(Friesen et al., 2012; Gabor and Contreras, 2012; Contreras et al., 2014)
Vacuum	-	W	300	Frozen	Et	37	(Ligocki et al., 2015)
Vacuum	-	M + W	100	-	M + W + EIA buffer	40	(Lorenzi et al., 2008; Ruane and Komen, 2003; Solomon-Lane and Grober, 2012)
Peristaltic	-	-	500	-20	Et	45	(Pavlidis et al., 2013)
Vacuum	2	M + W	2000	-20	Et	-	(Pounder et al., 2018)
Peristaltic	1	M + W	50	-20	Et	55	(Reemeyer et al., 2019; Sebire et al., 2009; Takahara et al., 2011)
Vacuum	-	M + W	300	-20	Et + M + EIA buffer	37	(Wong et al., 2008)

8.2 Materials and methods

8.2.1 Prototype standarization

To enable the standardisation, speed up the filtration step, and achieve better repeatability, a novel designed machine was developed that automates the filtration. The prototype also combines the benefits of the different designs mentioned in the literature and their improvement. Similarly to vacuum-driven designs, this machine consists of a chamber where water with cortisol from a fish tank or, as in this work, a synthetic solution, a filter system and a pressure-controlled device. Moreover, there are key differences that have been implemented compared to vacuum-driven designs. First, the chamber with the water cortisol solution is positively pressurized to allow for a greater differential pressure (Δp) over the filtration stages, enabling higher flow rates. Second, the flow rate of the filtration stages is controlled in a closed loop enabling better precision between samples.

Compared to other designs in the literature, such as the ones using peristaltic pumps, the designed machine differs from them because it offers better cost scalability using parallel channels that share the same central compressor, making the addition of supplementary channels effortless. Also, by using a flow rate closed loop, the actual flow rate is known and more accurate. Finally, the large and fast pressure spikes derived from the nature of peristaltic pumps are not present.

8.2.2 Sample extraction

The prototype allowed the automatization of the filtration step in the extraction of cortisol from water in different channels at the same time. The water cortisol sample is first placed inside the chamber under ambient pressure. Once loaded, the chamber is closed and pressurized to the operational level of up to 1.2 bar. The selected filters are then installed in the machine in a two-stage configuration, consisting of a cellulose filter for organic matter followed in series by a C18 Solid Phase Extraction (SPE) filter. Using the machine's control panel, the desired flow rate and total volume to be filtered are selected. The filtration process is then initiated from the control panel, and it automatically stops once the specified volume has passed through the system or when the sample in the chamber is depleted. After filtration stops, the chamber can either be depressurized to allow loading of a new sample or kept closed and pressurized to continue using any remaining volume of the previous sample. Finally, the filters are removed from the machine for further downstream processing.

The extraction protocol consisted in the activation of the solid phase extraction cartridge C18 with 5 mL of the selected solvent and then 5 mL of distilled water. The next step was to set up the cartridge in the machine with the pre-filtration step also and then filter the water. After the filtration, the cartridge was eluted with 5 mL of distilled water that was discarded, followed by 5 mL of the selected solvent that was put in a glass vial and frozen at -20 °C until the evaporation step. During evaporation, sample were put in the thermoblock at 50 °C until water was completely evaporated. The remaining residue was reconstituted with 200 μ L of EIA buffer.

8.2.3 Standardization of the procedure

The following factors were selected as variable regarding the results obtained by previous authors in the scientific references: After the literature review (**Table 1**) it was observed that factors as the size of the filters (SPE C18, and pre-filter), speed, elution buffer, preservation temperature, reconstitution solvent, and the evaporation temperature were different. For this reason, once the prototype was deemed repeatable, different experiments were designed to test and understand the effect of each factor.

- **Experiment 1: C18 solid phase cartridge comparison.** For this, two different cartridges of 6 and 12 mg were used.
- **Experiment 2: Prefiltration step comparison.** The objective was to test the pre-filtration step. For this, two different matrices, fiber glass and cellulose were used.
- **Experiment 3. Filtration speed** The idea was to analyse if the filtration speed was relevant, since in the majority of the papers, there is no information about it. Additionally, they rely on unregulated vacuum filtration where the range goes from 1-25 mL/min. For this, 3 different flow rates were chosen (10, 20, and 30 mL/min) to see if filtration speed is related with the ability of cortisol to adhere to the filter.
- **Experiment 4. Elution solvent.** The idea was to try the 3 different elution solvent used in the literature to see which one is more efficient.
- **Experiment 5. Filtration volume.** For this 3 different volumes were selected to try to optimise the extraction and to see the effect of the volume

8.3 Results and discussion

The first filter in the filtration step is the pre-filtration filter. In this case the type of the microfibre used is important, since glass fibre allows higher accumulation of cortisol than cellulose (**Figure 1A**). Such higher cortisol recovery was obtained when using a fiberglass filter compared to cellulose because cellulose can actively retain cortisol before it reaches the C18 cartridge, which would involve loss of recovered cortisol for analysis. Cellulose filters are rich in hydroxyl groups and highly hydrophilic, which promotes hydrogen-bond interactions with steroid hormones such as cortisol, leading to partial analyte adsorption and loss during pre-filtration. In contrast, fiberglass is chemically inert and non-polar, showing minimal affinity for cortisol and allowing it to pass through efficiently. As a result, more cortisol is delivered to the C18 sorbent when fiberglass is used, enhancing overall retention and recovery in the extraction workflow.

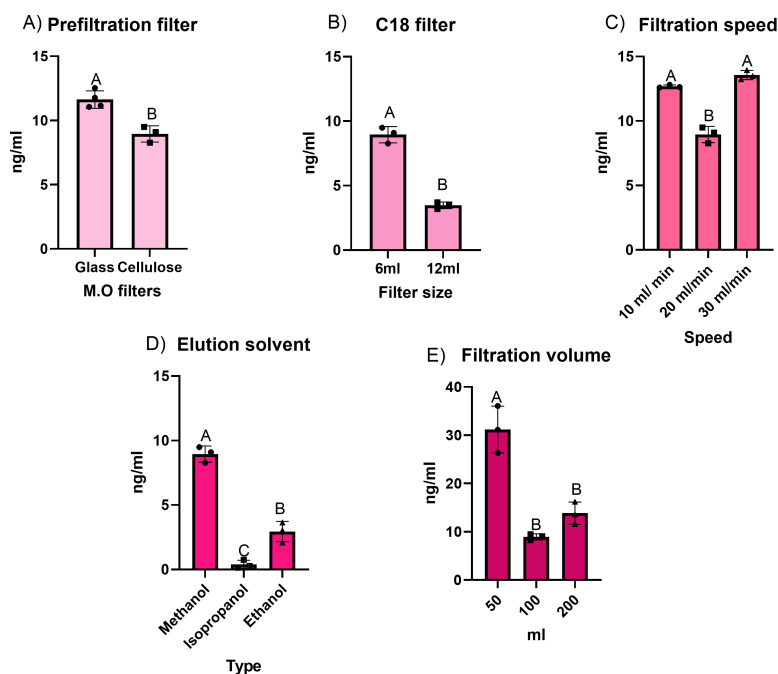


Figure 1: Results of the different experiments, A) Prefiltration step filter, B) C18 solid phase extraction, C) speed filtration, D) Elution solvent, and E) filtration volume. Significant differences (p -value < 0.05) are shown with different letters. The colours used were for illustrative purposes, and each group is specified in the x axis.

The next step involved the use of a C18 solid-phase filter. In this case, two different cartridge sizes were tested. The material and pore size were identical; only the sorbent mass differed (6 and 12 mg). The results showed two two times higher cortisol retention with the 6 mg filter compared to 12 mg (**Figure 1B**). The higher retention observed with the 6 mg C18 cartridge compared to the 12 mg one can be explained by differences in mass-transfer efficiency and solvent-to-sorbent ratio rather than sorbent capacity. In the smaller cartridge, the elution solvent interacts more effectively with the entire sorbent bed, maintaining a higher local analyte concentration and stronger hydrophobic interactions between cortisol and the C18 phase. In contrast, the larger 12 mg cartridge increases bed volume and pore diffusion distances, which can promote channelling, incomplete wetting, and analyte dispersion. These effects reduce effective contact and facilitate partial breakthrough or inefficient elution. For moderately retained compounds such as cortisol, excess sorbent does not necessarily improve retention and may instead decrease recovery due to kinetic limitations, making lower-mass C18 cartridges more efficient under fixed volume and flow conditions.

Regarding flow rate, three different speeds were evaluated. The lowest and highest flow rates (10 and 30 mL/min, respectively) resulted in higher retention of cortisol **Figure 1C**. Although the elution volume was kept constant, flow rate strongly affected cortisol recovery due to kinetic and hydrodynamic effects. At low flow (10 mL/min), sufficient contact time allows near-equilibrium interactions between the solvent, sorbent, and cortisol, resulting in efficient elution. At intermediate flow (20 mL/min), contact time is reduced, but convective forces are still insufficient, which may promote incomplete desorption and partial re-adsorption within the sorbent, leading to lower apparent recovery. At high flow (30 mL/min), convective transport dominates, effectively sweeping cortisol off the sorbent before re-interaction occurs, thereby increasing recovery again. This non-linear behavior is typical for moderately retained analytes and reflects the balance between equilibrium and kinetic control during elution.

The effect of the elution solvent was also evaluated using three solvents: methanol, isopropanol, and ethanol. Methanol provided the best results, with a substantial improvement compared to the other two (**Figure 1D**), being two or three times higher than the other two solvents. This can be explained by several factors. First, cortisol is a relatively polar molecule with some hydrophobic regions. Methanol is more polar than ethanol and isopropanol, which aligns with the observed results. Additionally, methanol is more effective at disrupting polar interactions

and hydrogen bonding, making it more efficient for elution in aqueous conditions using a C18 cartridge. Furthermore, ethanol and isopropanol are more likely to co-elute lipids and proteins along with cortisol, which can interfere with ELISA quantification. In addition, ethanol and isopropanol may interact more strongly with the antibodies used in the assay, potentially affecting measurement accuracy.

Finally, the effect of filtration volume was assessed using three volumes: 50, 100, and 200 mL. The highest cortisol retention was observed at 50 mL, being three times higher compared with the other groups (**Figure 1E**). This result is likely related to matrix load and sorbent saturation effects. At lower volumes, fewer particulates and co-extracted compounds reach the C18 cartridge, minimising clogging and competition for binding sites, and allowing more efficient interaction between cortisol and the sorbent. As the volume increases, matrix components accumulate, which can partially block pores or occupy hydrophobic sites, reducing effective retention. This leads to an early plateau in recovery, explaining why no major differences are observed between 100 and 200 mL. The slightly higher retention at 200 mL may reflect a mass balance effect, where the increased total cortisol load partially compensates for reduced efficiency, although the system is likely operating near its retention limit.

8.4 Conclusion

Overall, the optimized conditions for cortisol extraction were achieved by balancing efficient analyte–sorbent interactions with minimal matrix interference across all tested parameters. Methanol proved to be the most effective elution solvent due to its polarity and compatibility with cortisol. Regarding flow rate, both low and high values outperformed the intermediate condition, with low flow favoring equilibrium-controlled desorption and high flow enhancing convective removal.

The use of a fiberglass pre-filter was preferred over cellulose, as it minimized analyte loss prior to extraction and improved cortisol transfer to the C18 sorbent. Additionally, a lower sorbent mass (6 mg C18) resulted in higher retention compared to 12 mg, likely due to improved mass transfer efficiency and reduced kinetic limitations. Finally, lower sample volumes (50 mL) yielded the highest recovery, probably by limiting cartridge clogging and reducing competition for binding sites. Increasing the volume did not produce substantial gains, suggesting an early saturation of retention efficiency. Based on these findings, future studies should

employ the following conditions: pre-filtration using fiberglass filters, followed by a 6 mg C18 cartridge; filtration at a flow rate of 30 mL/min; a sample volume of 50 mL; and methanol as the elution solvent.

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9

Appendix II: Supplementary material for Section 3.2.3 Results

Glossary

Datos Refers to the dataframe of the experiment.

Shock Refers to the amount of shocks received by each group, 0 for controls (AC and CM) and 1, 2, 3 for the other groups respectively.

Hora Refers to the sampling time (1h, 6h, 24h).

Methodology of the statistical analysis

Once the outliers were removed, the normality was checked by *Shapiro-Wilk normality test* and homogeneity of the variance by *Bartlett test*. Since none of the parameters followed normality and/or homogeneity requirements, a generalized linear model (GzLM) was performed as a non-parametric test. If the parameter had differences between groups, a post-hoc test was performed using *Tukey's* correction. Also, to check the size of the effect of the differences, *eta's squared* was done. This test is used when data follow normal distribution, however, since we did not find the equivalent test in non-parametric form, we preferred to do this test following your suggestions.

R output

```
#### GENE EXPRESSION ####
```

```
> datos<-data.frame(Expresion_relativa_articulo_def)
```

```
> #### SKIN ####
```

```
> #GR1
```

```
> shapiro.test(datos$GR1)
```

```
Shapiro-Wilk normality test
```

```

data:  datos$GR1
W = 0.95627, p-value = 0.0005368

> bartlett.test(datos$GR1~datos$Subset)

Bartlett test of homogeneity of variances

data:  datos$GR1 by datos$Subset
Bartlett's K-squared = 23.719, df = 14, p-value = 0.04953

> gr1_glm <-glm(datos$GR1~datos$Shock*datos$Hora, family = gaussian)
> Anova(gr1_glm)
Analysis of Deviance Table (Type II tests)

Response: datos$GR1
              LR Chisq Df Pr(>Chisq)
datos$Shock      52.732  4 9.695e-11 ***
datos$Hora         1.245  2    0.5365
datos$Shock:datos$Hora  3.955  8    0.8611
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> etaSquared(gr1_glm)
              eta.sq eta.sq.part
datos$Shock      0.316893492  0.32807544
datos$Hora         0.007483999  0.01139972
datos$Shock:datos$Hora 0.023768809  0.03532862
> emmeans(gr1_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
  Shock emmean    SE  df lower.CL upper.CL
AC      4.28 0.243 108     3.79     4.76
CM      2.78 0.227 108     2.33     3.23
H1      2.72 0.238 108     2.25     3.19
H2      3.13 0.232 108     2.67     3.59
H3      1.90 0.232 108     1.44     2.36

Results are averaged over the levels of: Hora
Confidence level used: 0.95

```

```
$contrasts
```

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	1.4973	0.333	108	4.496	0.0002
AC - H1	1.5547	0.340	108	4.572	0.0001
AC - H2	1.1407	0.336	108	3.394	0.0084
AC - H3	2.3712	0.336	108	7.055	<.0001
CM - H1	0.0574	0.329	108	0.175	0.9998
CM - H2	-0.3566	0.325	108	-1.098	0.8074
CM - H3	0.8739	0.325	108	2.690	0.0621
H1 - H2	-0.4141	0.332	108	-1.247	0.7240
H1 - H3	0.8164	0.332	108	2.458	0.1081
H2 - H3	1.2305	0.328	108	3.751	0.0026

Results are averaged over the levels of: Hora

P value adjustment: tukey method for comparing a family of 5 estimates

```
> #HIF1
```

```
> shapiro.test(datos$HIF1)
```

Shapiro-Wilk normality test

```
data: datos$HIF1
```

```
W = 0.94945, p-value = 0.0001419
```

```
> bartlett.test(datos$HIF1~datos$Subset)
```

Bartlett test of homogeneity of variances

```
data: datos$HIF1 by datos$Subset
```

```
Bartlett's K-squared = 13.571, df = 14, p-value = 0.4821
```

```
> hif1_glm <-glm(datos$HIF1~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(hif1_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$HIF1

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	15.6866	4	0.003470	**
datos\$Hora	1.1408	2	0.565293	
datos\$Shock:datos\$Hora	24.7245	8	0.001731	**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> etaSquared(hif1_glm)

	eta.sq	eta.sq.part
datos\$Shock	0.103539607	0.12480731
datos\$Hora	0.007530011	0.01026466
datos\$Shock:datos\$Hora	0.163194533	0.18351917

> emmeans(hif1_glm, pairwise~Hora|Shock, adjust="Tukey")

\$emmeans

Shock = AC:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1.526	0.186	110	1.1572	1.894
24hR	1.526	0.186	110	1.1572	1.894
6hR	1.126	0.186	110	0.7571	1.494

Shock = CM:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.826	0.175	110	0.4790	1.174
24hR	1.295	0.186	110	0.9265	1.664
6hR	1.286	0.175	110	0.9389	1.634

Shock = H1:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1.037	0.199	110	0.6430	1.431
24hR	0.975	0.175	110	0.6275	1.322
6hR	0.640	0.186	110	0.2711	1.008

Shock = H2:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1.227	0.175	110	0.8792	1.574
24hR	0.451	0.186	110	0.0822	0.819
6hR	0.966	0.175	110	0.6189	1.314

Shock = H3:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.865	0.186	110	0.4960	1.233
24hR	1.506	0.186	110	1.1374	1.874
6hR	1.083	0.175	110	0.7359	1.431

Confidence level used: 0.95

\$contrasts

Shock = AC:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.00000	0.263	110	0.000	1.0000
1hR - 6hR	0.40008	0.263	110	1.521	0.2850
24hR - 6hR	0.40008	0.263	110	1.521	0.2850

Shock = CM:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.46857	0.256	110	-1.833	0.1636
1hR - 6hR	-0.45991	0.248	110	-1.855	0.1569
24hR - 6hR	0.00866	0.256	110	0.034	0.9994

Shock = H1:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.06199	0.265	110	0.234	0.9703
1hR - 6hR	0.39731	0.272	110	1.460	0.3144
24hR - 6hR	0.33532	0.256	110	1.312	0.3916

Shock = H2:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.77589	0.256	110	3.036	0.0084
1hR - 6hR	0.26036	0.248	110	1.050	0.5471
24hR - 6hR	-0.51553	0.256	110	-2.017	0.1129

Shock = H3:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.64133	0.263	110	-2.439	0.0429
1hR - 6hR	-0.21876	0.256	110	-0.856	0.6691
24hR - 6hR	0.42257	0.256	110	1.653	0.2279

P value adjustment: tukey method for comparing a family of 3 estimates

```
> emmeans(hif1_glm, pairwise~Shock|Hora, adjust="Tukey")
```

```
$emmeans
```

```
Hora = 1hR:
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	1.526	0.186	110	1.1572	1.894
CM	0.826	0.175	110	0.4790	1.174
H1	1.037	0.199	110	0.6430	1.431
H2	1.227	0.175	110	0.8792	1.574
H3	0.865	0.186	110	0.4960	1.233

```
Hora = 24hR:
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	1.526	0.186	110	1.1572	1.894
CM	1.295	0.186	110	0.9265	1.664
H1	0.975	0.175	110	0.6275	1.322
H2	0.451	0.186	110	0.0822	0.819
H3	1.506	0.186	110	1.1374	1.874

```
Hora = 6hR:
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	1.126	0.186	110	0.7571	1.494
CM	1.286	0.175	110	0.9389	1.634
H1	0.640	0.186	110	0.2711	1.008
H2	0.966	0.175	110	0.6189	1.314
H3	1.083	0.175	110	0.7359	1.431

Confidence level used: 0.95

```
$contrasts
```

```
Hora = 1hR:
```

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.6992	0.256	110	2.736	0.0551
AC - H1	0.4888	0.272	110	1.795	0.3815
AC - H2	0.2990	0.256	110	1.170	0.7684
AC - H3	0.6611	0.263	110	2.514	0.0949

CM - H1	-0.2105	0.265	110	-0.794	0.9318
CM - H2	-0.4002	0.248	110	-1.614	0.4917
CM - H3	-0.0381	0.256	110	-0.149	0.9999
H1 - H2	-0.1897	0.265	110	-0.716	0.9525
H1 - H3	0.1724	0.272	110	0.633	0.9694
H2 - H3	0.3621	0.256	110	1.417	0.6183

Hora = 24hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.2307	0.263	110	0.877	0.9048
AC - H1	0.5507	0.256	110	2.155	0.2049
AC - H2	1.0749	0.263	110	4.087	0.0008
AC - H3	0.0198	0.263	110	0.075	1.0000
CM - H1	0.3201	0.256	110	1.252	0.7207
CM - H2	0.8443	0.263	110	3.210	0.0147
CM - H3	-0.2109	0.263	110	-0.802	0.9295
H1 - H2	0.5242	0.256	110	2.051	0.2491
H1 - H3	-0.5309	0.256	110	-2.077	0.2373
H2 - H3	-1.0551	0.263	110	-4.012	0.0010

Hora = 6hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-0.1608	0.256	110	-0.629	0.9701
AC - H1	0.4860	0.263	110	1.848	0.3518
AC - H2	0.1593	0.256	110	0.623	0.9710
AC - H3	0.0423	0.256	110	0.165	0.9998
CM - H1	0.6467	0.256	110	2.530	0.0913
CM - H2	0.3201	0.248	110	1.291	0.6974
CM - H3	0.2030	0.248	110	0.819	0.9243
H1 - H2	-0.3267	0.256	110	-1.278	0.7051
H1 - H3	-0.4437	0.256	110	-1.736	0.4164
H2 - H3	-0.1170	0.248	110	-0.472	0.9897

P value adjustment: tukey method for comparing a family of 5 estimates

```
> emmeans(hif1_glm, pairwise~Shock, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

```
$emmeans
```

Shock	emmean	SE	df	lower.CL	upper.CL
-------	--------	----	----	----------	----------

AC	1.392	0.107	110	1.180	1.61
CM	1.136	0.103	110	0.931	1.34
H1	0.884	0.108	110	0.670	1.10
H2	0.881	0.103	110	0.677	1.09
H3	1.151	0.105	110	0.942	1.36

Results are averaged over the levels of: Hora
 Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.2564	0.149	110	1.721	0.4257
AC - H1	0.5085	0.152	110	3.340	0.0099
AC - H2	0.5111	0.149	110	3.430	0.0074
AC - H3	0.2411	0.150	110	1.603	0.4990
CM - H1	0.2521	0.149	110	1.687	0.4459
CM - H2	0.2547	0.146	110	1.743	0.4121
CM - H3	-0.0153	0.148	110	-0.104	1.0000
H1 - H2	0.0026	0.149	110	0.017	1.0000
H1 - H3	-0.2674	0.151	110	-1.773	0.3945
H2 - H3	-0.2700	0.148	110	-1.830	0.3618

Results are averaged over the levels of: Hora
 P value adjustment: tukey method for comparing a family of 5 estimates

```
> emmeans(hif1_glm, pairwise~Hora, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

\$emmeans

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1.10	0.0825	110	0.933	1.26
24hR	1.15	0.0822	110	0.988	1.31
6hR	1.02	0.0803	110	0.861	1.18

Results are averaged over the levels of: Shock
 Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
----------	----------	----	----	---------	---------

```

1hR - 24hR  -0.0544 0.116 110  -0.467  0.8869
1hR - 6hR   0.0758 0.115 110   0.658  0.7880
24hR - 6hR   0.1302 0.115 110   1.133  0.4961

```

Results are averaged over the levels of: Shock

P value adjustment: tukey method for comparing a family of 3 estimates

```
> #MYO
```

```
> shapiro.test(datos$MYO)
```

Shapiro-Wilk normality test

```
data:  datos$MYO
```

```
W = 0.74708, p-value = 3.741e-13
```

```
> bartlett.test(datos$MYO~datos$Subset)
```

Bartlett test of homogeneity of variances

```
data:  datos$MYO by datos$Subset
```

```
Bartlett's K-squared = 74.116, df = 14, p-value = 3.436e-10
```

```
> myo_glm <-glm(datos$MYO~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(myo_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$MYO

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	16.7853	4	0.002128	**
datos\$Hora	0.6821	2	0.711029	
datos\$Shock:datos\$Hora	4.2492	8	0.833969	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(myo_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.131408537	0.136704460
datos\$Hora	0.005339899	0.006393623

```

datos$Shock:datos$Hora 0.033266109 0.038541802
> emmeans(myo_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
Shock emmean      SE  df lower.CL upper.CL
AC      1.384 0.199 106   0.9882   1.779
CM      0.468 0.192 106   0.0881   0.849
H1      0.516 0.200 106   0.1190   0.914
H2      0.492 0.196 106   0.1036   0.879
H3      0.975 0.209 106   0.5610   1.388

```

Results are averaged over the levels of: Hora
 Confidence level used: 0.95

```

$contrasts
contrast estimate      SE  df t.ratio p.value
AC - CM      0.9151 0.277 106   3.307  0.0110
AC - H1      0.8672 0.283 106   3.067  0.0225
AC - H2      0.8920 0.279 106   3.193  0.0156
AC - H3      0.4088 0.289 106   1.417  0.6184
CM - H1     -0.0479 0.277 106  -0.173  0.9998
CM - H2     -0.0231 0.274 106  -0.084  1.0000
CM - H3     -0.5062 0.283 106  -1.786  0.3871
H1 - H2      0.0248 0.280 106   0.088  1.0000
H1 - H3     -0.4583 0.289 106  -1.584  0.5108
H2 - H3     -0.4831 0.286 106  -1.689  0.4451

```

Results are averaged over the levels of: Hora
 P value adjustment: tukey method for comparing a family of 5 estimates

```
> #MR
```

```
> shapiro.test(datos$MR)
```

Shapiro-Wilk normality test

```
data:  datos$MR
```

W = 0.93485, p-value = 1.444e-05

```
> bartlett.test(datos$MR~datos$Subset)
```

Bartlett test of homogeneity of variances

data: datos\$MR by datos\$Subset

Bartlett's K-squared = 25.584, df = 14, p-value = 0.02922

```
> mr_glm <-glm(datos$MR~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(mr_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$MR

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	50.405	4	2.972e-10	***
datos\$Hora	0.513	2	0.7736	
datos\$Shock:datos\$Hora	8.005	8	0.4330	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(mr_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.30019382	0.31620574
datos\$Hora	0.00305797	0.00468851
datos\$Shock:datos\$Hora	0.04767386	0.06841413

```
> emmeans(mr_glm, pairwise~Shock, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

\$emmeans

Shock	emmean	SE	df	lower.CL	upper.CL
AC	4.58	0.264	109	4.05	5.10
CM	2.86	0.259	109	2.35	3.37
H1	2.83	0.264	109	2.31	3.36
H2	3.27	0.264	109	2.75	3.79
H3	2.00	0.275	109	1.45	2.54

Results are averaged over the levels of: Hora

Confidence level used: 0.95


```
$contrasts
  contrast estimate      SE df t.ratio p.value
AC - CM      1.7137 0.369 109   4.639 0.0001
AC - H1      1.7408 0.373 109   4.667 0.0001
AC - H2      1.3059 0.373 109   3.501 0.0060
AC - H3      2.5782 0.381 109   6.764 <.0001
CM - H1      0.0271 0.369 109   0.073 1.0000
CM - H2     -0.4078 0.369 109  -1.104 0.8042
CM - H3      0.8645 0.378 109   2.289 0.1562
H1 - H2     -0.4349 0.373 109  -1.166 0.7706
H1 - H3      0.8374 0.381 109   2.197 0.1886
H2 - H3      1.2723 0.381 109   3.338 0.0100
```

Results are averaged over the levels of: Hora

P value adjustment: tukey method for comparing a family of 5 estimates

```
> #CRH
```

```
> shapiro.test(datos$CRH)
```

Shapiro-Wilk normality test

data: datos\$CRH

W = 0.88309, p-value = 2.398e-08

```
> bartlett.test(datos$CRH~datos$Subset)
```

Bartlett test of homogeneity of variances

data: datos\$CRH by datos\$Subset

Bartlett's K-squared = 37.837, df = 14, p-value = 0.0005503

```
> crh_glm <-glm(datos$CRH~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(crh_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$CRH

```

              LR Chisq Df Pr(>Chisq)
datos$Shock      22.0543  4  0.0001955 ***
datos$Hora        1.2183  2  0.5438264
datos$Shock:datos$Hora  7.9940  8  0.4340537
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> etaSquared(crh_glm)
              eta.sq eta.sq.part
datos$Shock      0.158764353  0.17089133
datos$Hora        0.008769953  0.01125735
datos$Shock:datos$Hora 0.057547482  0.06951690
> emmeans(crh_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
  Shock emmean    SE  df lower.CL upper.CL
AC      2.03 0.237 107    1.564    2.50
CM      1.57 0.233 107    1.105    2.03
H1      1.03 0.251 107    0.535    1.53
H2      2.27 0.239 107    1.800    2.75
H3      1.01 0.247 107    0.521    1.50

Results are averaged over the levels of: Hora
Confidence level used: 0.95

$contrasts
  contrast estimate    SE  df t.ratio p.value
AC - CM      0.4677 0.332 107    1.408  0.6242
AC - H1      1.0025 0.345 107    2.906  0.0353
AC - H2     -0.2390 0.336 107   -0.710  0.9537
AC - H3      1.0220 0.343 107    2.981  0.0287
CM - H1      0.5349 0.342 107    1.564  0.5233
CM - H2     -0.7066 0.333 107   -2.121  0.2189
CM - H3      0.5543 0.340 107    1.632  0.4804
H1 - H2     -1.2415 0.346 107   -3.589  0.0045
H1 - H3      0.0194 0.352 107    0.055  1.0000
H2 - H3      1.2609 0.344 107    3.669  0.0034

```

Results are averaged over the levels of: Hora

P value adjustment: tukey method for comparing a family of 5 estimates

```
> #LAM
```

```
> shapiro.test(datos$LAM)
```

Shapiro-Wilk normality test

data: datos\$LAM

W = 0.9348, p-value = 1.942e-05

```
> bartlett.test(datos$LAM~datos$Subset)
```

Bartlett test of homogeneity of variances

data: datos\$LAM by datos\$Subset

Bartlett's K-squared = 50.431, df = 14, p-value = 5.17e-06

```
> lam_glm <-glm(datos$LAM~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(lam_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$LAM

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	44.786	4	4.405e-09	***
datos\$Hora	2.372	2	0.3054	
datos\$Shock:datos\$Hora	11.546	8	0.1726	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(lam_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.27255907	0.29899971
datos\$Hora	0.01443559	0.02209147
datos\$Shock:datos\$Hora	0.07026683	0.09906819

```
> emmeans(lam_glm, pairwise~Shock, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

```
$emmeans
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	2.392	0.179	105	2.036	2.75
CM	2.081	0.177	105	1.731	2.43
H1	1.719	0.179	105	1.364	2.07
H2	1.590	0.180	105	1.233	1.95
H3	0.766	0.183	105	0.402	1.13

Results are averaged over the levels of: Hora
 Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.310	0.252	105	1.232	0.7327
AC - H1	0.672	0.253	105	2.652	0.0684
AC - H2	0.801	0.254	105	3.153	0.0176
AC - H3	1.626	0.256	105	6.339	<.0001
CM - H1	0.362	0.252	105	1.438	0.6048
CM - H2	0.491	0.252	105	1.946	0.3001
CM - H3	1.315	0.255	105	5.163	<.0001
H1 - H2	0.129	0.254	105	0.508	0.9864
H1 - H3	0.953	0.256	105	3.718	0.0029
H2 - H3	0.824	0.257	105	3.206	0.0150

Results are averaged over the levels of: Hora
 P value adjustment: tukey method for comparing a family of 5 estimates

```
> ##### MUCUS CORTISOL #####
```

```
> datos<-data.frame(Expresion_relativa_articulo_def)
> shapiro.test(datos$Mucus.cortisol..Out)
```

Shapiro-Wilk normality test

```
data:  datos$Mucus.cortisol..Out
W = 0.69716, p-value = 5.722e-15
```

```
> bartlett.test(datos$Mucus.cortisol..Out~datos$Subset)
```

Bartlett test of homogeneity of variances

```
data:  datos$Mucus.cortisol..Out by datos$Subset
Bartlett's K-squared = 187.5, df = 14, p-value < 2.2e-16
```

```
> cortisol_glm <-glm(datos$Mucus.cortisol..Out~datos$Shock*datos$Hora, f
> Anova(cortisol_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$Mucus.cortisol..Out

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	38.364	4	9.427e-08	***
datos\$Hora	104.823	2	< 2.2e-16	***
datos\$Shock:datos\$Hora	50.536	8	3.224e-08	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(cortisol_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.1254598	0.2517902
datos\$Hora	0.3428003	0.4790311
datos\$Shock:datos\$Hora	0.1652657	0.3071413

```
> emmeans(cortisol_glm, pairwise~Shock|Hora, adjust="Tukey")
```

\$emmeans

Hora = 1hR:

Shock	emmean	SE	df	lower.CL	upper.CL
AC	63.3	167	114	-267.89	395
CM	1247.9	177	114	896.60	1599
H1	1832.2	177	114	1480.93	2184
H2	1772.5	190	114	1396.92	2148
H3	1146.7	167	114	815.52	1478

Hora = 24hR:

Shock	emmean	SE	df	lower.CL	upper.CL
AC	63.3	167	114	-267.89	395
CM	973.0	167	114	641.76	1304
H1	347.4	177	114	-3.88	699
H2	135.7	167	114	-195.50	467

H3	115.5	167	114	-215.66	447
----	-------	-----	-----	---------	-----

Hora = 6hR:

Shock	emmean	SE	df	lower.CL	upper.CL
AC	85.3	167	114	-245.91	416
CM	232.0	177	114	-119.31	583
H1	117.0	167	114	-214.21	448
H2	90.3	167	114	-240.89	422
H3	99.4	167	114	-231.79	431

Confidence level used: 0.95

\$contrasts

Hora = 1hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-1184.58	244	114	-4.860	<.0001
AC - H1	-1768.92	244	114	-7.258	<.0001
AC - H2	-1709.16	253	114	-6.762	<.0001
AC - H3	-1083.42	236	114	-4.582	0.0001
CM - H1	-584.33	251	114	-2.330	0.1430
CM - H2	-524.58	260	114	-2.021	0.2628
CM - H3	101.17	244	114	0.415	0.9937
H1 - H2	59.76	260	114	0.230	0.9994
H1 - H3	685.50	244	114	2.813	0.0449
H2 - H3	625.74	253	114	2.476	0.1035

Hora = 24hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-909.65	236	114	-3.847	0.0018
AC - H1	-284.10	244	114	-1.166	0.7708
AC - H2	-72.39	236	114	-0.306	0.9981
AC - H3	-52.23	236	114	-0.221	0.9995
CM - H1	625.55	244	114	2.567	0.0835
CM - H2	837.26	236	114	3.541	0.0051
CM - H3	857.42	236	114	3.626	0.0039
H1 - H2	211.71	244	114	0.869	0.9078
H1 - H3	231.87	244	114	0.951	0.8760
H2 - H3	20.16	236	114	0.085	1.0000

Hora = 6hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-146.69	244	114	-0.602	0.9745
AC - H1	-31.70	236	114	-0.134	0.9999
AC - H2	-5.02	236	114	-0.021	1.0000
AC - H3	-14.12	236	114	-0.060	1.0000
CM - H1	114.99	244	114	0.472	0.9897
CM - H2	141.67	244	114	0.581	0.9776
CM - H3	132.57	244	114	0.544	0.9825
H1 - H2	26.68	236	114	0.113	1.0000
H1 - H3	17.59	236	114	0.074	1.0000
H2 - H3	-9.10	236	114	-0.038	1.0000

P value adjustment: tukey method for comparing a family of 5 estimates

```
> emmeans(cortisol_glm, pairwise~Hora|Shock, adjust="Tukey")
```

\$emmeans

Shock = AC:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	63.3	167	114	-267.89	395
24hR	63.3	167	114	-267.89	395
6hR	85.3	167	114	-245.91	416

Shock = CM:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1247.9	177	114	896.60	1599
24hR	973.0	167	114	641.76	1304
6hR	232.0	177	114	-119.31	583

Shock = H1:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1832.2	177	114	1480.93	2184
24hR	347.4	177	114	-3.88	699
6hR	117.0	167	114	-214.21	448

Shock = H2:

Hora	emmean	SE	df	lower.CL	upper.CL
------	--------	----	----	----------	----------

1hR	1772.5	190	114	1396.92	2148
24hR	135.7	167	114	-195.50	467
6hR	90.3	167	114	-240.89	422

Shock = H3:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1146.7	167	114	815.52	1478
24hR	115.5	167	114	-215.66	447
6hR	99.4	167	114	-231.79	431

Confidence level used: 0.95

\$contrasts

Shock = AC:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.0	236	114	0.000	1.0000
1hR - 6hR	-22.0	236	114	-0.093	0.9952
24hR - 6hR	-22.0	236	114	-0.093	0.9952

Shock = CM:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	274.9	244	114	1.128	0.4989
1hR - 6hR	1015.9	251	114	4.051	0.0003
24hR - 6hR	741.0	244	114	3.040	0.0082

Shock = H1:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	1484.8	251	114	5.921	<.0001
1hR - 6hR	1715.2	244	114	7.038	<.0001
24hR - 6hR	230.4	244	114	0.945	0.6128

Shock = H2:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	1636.8	253	114	6.475	<.0001
1hR - 6hR	1682.2	253	114	6.655	<.0001
24hR - 6hR	45.4	236	114	0.192	0.9799

Shock = H3:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	1031.2	236	114	4.361	0.0001
1hR - 6hR	1047.3	236	114	4.429	0.0001
24hR - 6hR	16.1	236	114	0.068	0.9974

P value adjustment: tukey method for comparing a family of 3 estimates

```
> emmeans(cortisol_glm, pairwise~Shock, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

\$emmeans

Shock	emmean	SE	df	lower.CL	upper.CL
AC	70.6	96.5	114	-121	262
CM	817.6	100.5	114	619	1017
H1	765.5	100.5	114	567	965
H2	666.2	101.0	114	466	866
H3	453.9	96.5	114	263	645

Results are averaged over the levels of: Hora

Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-747.0	139	114	-5.361	<.0001
AC - H1	-694.9	139	114	-4.988	<.0001
AC - H2	-595.5	140	114	-4.262	0.0004
AC - H3	-383.3	137	114	-2.808	0.0455
CM - H1	52.1	142	114	0.366	0.9961
CM - H2	151.5	142	114	1.063	0.8250
CM - H3	363.7	139	114	2.611	0.0751
H1 - H2	99.4	142	114	0.698	0.9566
H1 - H3	311.7	139	114	2.237	0.1738
H2 - H3	212.3	140	114	1.519	0.5522

Results are averaged over the levels of: Hora

P value adjustment: tukey method for comparing a family of 5 estimates

```
> emmeans(cortisol_glm, pairwise~Hora, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

```
$emmeans
```

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1213	78.7	114	1056.7	1368
24hR	327	75.7	114	177.0	477
6hR	125	75.7	114	-25.2	275

Results are averaged over the levels of: Shock
Confidence level used: 0.95

```
$contrasts
```

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	886	109	114	8.111	<.0001
1hR - 6hR	1088	109	114	9.963	<.0001
24hR - 6hR	202	107	114	1.889	0.1466

Results are averaged over the levels of: Shock
P value adjustment: tukey method for comparing a family of 3 estimates

```
> ##### INTESTINE #####
```

```
> #GR1
```

```
> shapiro.test(datos$GR1)
```

Shapiro-Wilk normality test

```
data: datos$GR1
```

```
W = 0.93855, p-value = 2.051e-05
```

```
> bartlett.test(datos$GR1~datos$Subset)
```

Bartlett test of homogeneity of variances

```
data: datos$GR1 by datos$Subset
```

Bartlett's K-squared = 41.774, df = 14, p-value = 0.0001342

```
> gr1_glm <- glm(datos$GR1~datos$Shock*datos$Hora, family = gaussian)
> Anova(gr1_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$GR1

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	35.131	4	4.367e-07	***
datos\$Hora	8.690	2	0.01297	*
datos\$Shock:datos\$Hora	8.326	8	0.40233	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(gr1_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.2166573	0.23877270
datos\$Hora	0.0535955	0.07200627
datos\$Shock:datos\$Hora	0.0513452	0.06919215

```
> emmeans(gr1_glm, pairwise~Hora, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

\$emmeans

	Hora	emmean	SE	df	lower.CL	upper.CL
1hR	1.35	0.0795	112	1.19	1.50	
24hR	1.52	0.0874	112	1.35	1.69	
6hR	1.22	0.0782	112	1.07	1.38	

Results are averaged over the levels of: Shock

Confidence level used: 0.95

\$contrasts

	contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.176	0.118	112	-1.487	0.3009	
1hR - 6hR	0.121	0.112	112	1.088	0.5234	
24hR - 6hR	0.297	0.117	112	2.534	0.0336	

Results are averaged over the levels of: Shock

P value adjustment: tukey method for comparing a family of 3 estimates

```
> emmeans(gr1_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	1.79	0.0999	112	1.595	1.99
CM	1.07	0.1060	112	0.862	1.28
H1	1.20	0.1050	112	0.988	1.40
H2	1.23	0.0999	112	1.036	1.43
H3	1.53	0.1160	112	1.295	1.76

Results are averaged over the levels of: Hora
Confidence level used: 0.95

```
$contrasts
```

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.7200	0.146	112	4.932	<.0001
AC - H1	0.5982	0.145	112	4.138	0.0006
AC - H2	0.5598	0.141	112	3.963	0.0012
AC - H3	0.2682	0.153	112	1.750	0.4082
CM - H1	-0.1218	0.149	112	-0.817	0.9251
CM - H2	-0.1603	0.146	112	-1.098	0.8074
CM - H3	-0.4518	0.158	112	-2.865	0.0392
H1 - H2	-0.0384	0.145	112	-0.266	0.9989
H1 - H3	-0.3300	0.156	112	-2.110	0.2231
H2 - H3	-0.2915	0.153	112	-1.902	0.3225

Results are averaged over the levels of: Hora
P value adjustment: tukey method for comparing a family of 5 estimates

```
> #HIF1
```

```
> shapiro.test(datos$HIF1)
```

Shapiro-Wilk normality test

```
data: datos$HIF1
```

```
W = 0.95456, p-value = 0.0002433
```

```
> bartlett.test(datos$HIF1~datos$Subset)
```

Bartlett test of homogeneity of variances

```
data:  datos$HIF1 by datos$Subset
```

```
Bartlett's K-squared = 26.681, df = 14, p-value = 0.02118
```

```
> hif1_glm <-glm(datos$HIF1~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(hif1_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$HIF1

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	29.3170	4	6.74e-06	***
datos\$Hora	1.4378	2	0.4873	
datos\$Shock:datos\$Hora	13.0067	8	0.1116	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(hif1_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.182297969	0.20174534
datos\$Hora	0.008940352	0.01224292
datos\$Shock:datos\$Hora	0.080877745	0.10082192

```
> emmeans(hif1_glm, pairwise~Shock, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions
\$emmeans

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.656	0.0813	116	0.495	0.817
CM	1.021	0.0829	116	0.857	1.186
H1	1.057	0.0813	116	0.896	1.218
H2	1.144	0.0813	116	0.983	1.305
H3	1.184	0.0946	116	0.997	1.372

Results are averaged over the levels of: Hora

Confidence level used: 0.95

```
$contrasts
```

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-0.3650	0.116	116	-3.143	0.0177
AC - H1	-0.4002	0.115	116	-3.482	0.0062
AC - H2	-0.4876	0.115	116	-4.243	0.0004
AC - H3	-0.5280	0.125	116	-4.233	0.0004
CM - H1	-0.0352	0.116	116	-0.303	0.9981
CM - H2	-0.1226	0.116	116	-1.056	0.8285
CM - H3	-0.1630	0.126	116	-1.296	0.6945
H1 - H2	-0.0874	0.115	116	-0.760	0.9413
H1 - H3	-0.1278	0.125	116	-1.025	0.8435
H2 - H3	-0.0404	0.125	116	-0.324	0.9976

Results are averaged over the levels of: Hora

P value adjustment: tukey method for comparing a family of 5 estimates

```
> #MYO
```

```
> shapiro.test(datos$MYO)
```

Shapiro-Wilk normality test

data: datos\$MYO

W = 0.76345, p-value = 4.382e-13

```
> bartlett.test(datos$MYO~datos$Subset)
```

Bartlett test of homogeneity of variances

data: datos\$MYO by datos\$Subset

Bartlett's K-squared = 63.875, df = 14, p-value = 2.426e-08

```
> myo_glm <-glm(datos$MYO~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(myo_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$MYO

LR	Chisq	Df	Pr(>Chisq)
----	-------	----	------------

```

datos$Shock          30.7500  4  3.443e-06 ***
datos$Hora           0.9154  2    0.6327
datos$Shock:datos$Hora 4.7669  8    0.7822
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> etaSquared(myo_glm)

              eta.sq eta.sq.part
datos$Shock    0.206510858 0.213913022
datos$Hora     0.006147802 0.008035992
datos$Shock:datos$Hora 0.032013397 0.040477229
> emmeans(myo_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
  Shock emmean      SE  df lower.CL upper.CL
AC      0.587 0.0525 113    0.483    0.691
CM      0.299 0.0550 113    0.190    0.408
H1      0.276 0.0536 113    0.169    0.382
H2      0.285 0.0536 113    0.179    0.391
H3      0.228 0.0612 113    0.107    0.350

Results are averaged over the levels of: Hora
Confidence level used: 0.95

$contrasts
  contrast estimate      SE  df t.ratio p.value
AC - CM    0.2878 0.0760 113    3.787  0.0023
AC - H1    0.3115 0.0750 113    4.152  0.0006
AC - H2    0.3020 0.0750 113    4.025  0.0010
AC - H3    0.3588 0.0806 113    4.451  0.0002
CM - H1    0.0237 0.0768 113    0.309  0.9980
CM - H2    0.0142 0.0768 113    0.185  0.9997
CM - H3    0.0710 0.0822 113    0.863  0.9097
H1 - H2   -0.0095 0.0758 113   -0.125  0.9999
H1 - H3    0.0473 0.0813 113    0.581  0.9776
H2 - H3    0.0568 0.0813 113    0.698  0.9565

```

Results are averaged over the levels of: Hora
P value adjustment: tukey method for comparing a family of 5 estimates

```

> #MR

> shapiro.test(datos$MR)

Shapiro-Wilk normality test

data:  datos$MR
W = 0.9601, p-value = 0.0006971

> bartlett.test(datos$MR~datos$Subset)

Bartlett test of homogeneity of variances

data:  datos$MR by datos$Subset
Bartlett's K-squared = 21.927, df = 14, p-value = 0.08013

> mr_glm <-glm(datos$MR~datos$Shock*datos$Hora, family = gaussian)
> Anova(mr_glm)
Analysis of Deviance Table (Type II tests)

Response: datos$MR

              LR Chisq Df Pr(>Chisq)
datos$Shock      33.085  4  1.148e-06 ***
datos$Hora        2.344  2    0.3098
datos$Shock:datos$Hora  7.872  8    0.4460
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> etaSquared(mr_glm)

              eta.sq eta.sq.part
datos$Shock      0.20855448  0.22191839
datos$Hora        0.01477503  0.01980565
datos$Shock:datos$Hora 0.04962447  0.06355186
> emmeans(mr_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
  Shock emmean      SE df lower.CL upper.CL

```

AC	1.679	0.106	116	1.469	1.89
CM	0.925	0.108	116	0.711	1.14
H1	1.185	0.106	116	0.976	1.39
H2	1.105	0.106	116	0.896	1.31
H3	1.459	0.123	116	1.215	1.70

Results are averaged over the levels of: Hora
 Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.7536	0.151	116	4.982	<.0001
AC - H1	0.4933	0.150	116	3.295	0.0112
AC - H2	0.5733	0.150	116	3.830	0.0019
AC - H3	0.2197	0.162	116	1.352	0.6592
CM - H1	-0.2604	0.151	116	-1.721	0.4251
CM - H2	-0.1803	0.151	116	-1.192	0.7560
CM - H3	-0.5339	0.164	116	-3.257	0.0126
H1 - H2	0.0801	0.150	116	0.535	0.9836
H1 - H3	-0.2736	0.162	116	-1.684	0.4481
H2 - H3	-0.3536	0.162	116	-2.176	0.1961

Results are averaged over the levels of: Hora
 P value adjustment: tukey method for comparing a family of 5 estimates

```
> ##### GILLS #####
```

```
> #GR1
```

```
> shapiro.test(datos$GR1)
```

Shapiro-Wilk normality test

```

data:  datos$GR1
W = 0.97481, p-value = 0.01664

> bartlett.test(datos$GR1~datos$Subset)

Bartlett test of homogeneity of variances

data:  datos$GR1 by datos$Subset
Bartlett's K-squared = 28.906, df = 14, p-value = 0.01076

> gr1_glm <-glm(datos$GR1~datos$Shock*datos$Hora, family = gaussian)
> Anova(gr1_glm)
Analysis of Deviance Table (Type II tests)

Response: datos$GR1
              LR Chisq Df Pr(>Chisq)
datos$Shock      16.345  4  0.002589 **
datos$Hora       11.581  2  0.003057 **
datos$Shock:datos$Hora 20.093  8  0.009989 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> etaSquared(gr1_glm)
              eta.sq eta.sq.part
datos$Shock      0.10115672  0.12540042
datos$Hora       0.07166908  0.09221658
datos$Shock:datos$Hora 0.12435163  0.14984568
> emmeans(gr1_glm, pairwise~Hora|Shock, adjust="Tukey")
$emmeans
Shock = AC:
  Hora emmean      SE  df lower.CL upper.CL
1hR   0.829 0.0879 114    0.654    1.003
24hR  0.829 0.0879 114    0.654    1.003
6hR   0.738 0.0879 114    0.564    0.912

Shock = CM:
  Hora emmean      SE  df lower.CL upper.CL
1hR   0.629 0.0879 114    0.454    0.803
24hR  0.946 0.0879 114    0.772    1.120

```

```
6hR    0.741 0.0879 114    0.567    0.916
```

Shock = H1:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.730	0.0933	114	0.545	0.914
24hR	1.140	0.0933	114	0.956	1.325
6hR	0.560	0.0997	114	0.362	0.757

Shock = H2:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.877	0.0879	114	0.702	1.051
24hR	1.091	0.0933	114	0.907	1.276
6hR	1.105	0.0879	114	0.930	1.279

Shock = H3:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.766	0.0879	114	0.592	0.941
24hR	0.753	0.0933	114	0.568	0.938
6hR	0.842	0.0879	114	0.667	1.016

Confidence level used: 0.95

\$contrasts

Shock = AC:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.0000	0.124	114	0.000	1.0000
1hR - 6hR	0.0902	0.124	114	0.726	0.7489
24hR - 6hR	0.0902	0.124	114	0.726	0.7489

Shock = CM:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.3174	0.124	114	-2.553	0.0320
1hR - 6hR	-0.1129	0.124	114	-0.908	0.6367
24hR - 6hR	0.2046	0.124	114	1.645	0.2311

Shock = H1:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.4106	0.132	114	-3.113	0.0066

1hR - 6hR	0.1698	0.137	114	1.244	0.4301
24hR - 6hR	0.5804	0.137	114	4.251	0.0001

Shock = H2:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.2146	0.128	114	-1.674	0.2194
1hR - 6hR	-0.2279	0.124	114	-1.833	0.1636
24hR - 6hR	-0.0133	0.128	114	-0.104	0.9941

Shock = H3:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.0136	0.128	114	0.106	0.9938
1hR - 6hR	-0.0753	0.124	114	-0.605	0.8175
24hR - 6hR	-0.0888	0.128	114	-0.693	0.7680

P value adjustment: tukey method for comparing a family of 3 estimates

```
> emmeans(gr1_glm, pairwise~Shock|Hora, adjust="Tukey")
```

\$emmeans

Hora = 1hR:

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.829	0.0879	114	0.654	1.003
CM	0.629	0.0879	114	0.454	0.803
H1	0.730	0.0933	114	0.545	0.914
H2	0.877	0.0879	114	0.702	1.051
H3	0.766	0.0879	114	0.592	0.941

Hora = 24hR:

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.829	0.0879	114	0.654	1.003
CM	0.946	0.0879	114	0.772	1.120
H1	1.140	0.0933	114	0.956	1.325
H2	1.091	0.0933	114	0.907	1.276
H3	0.753	0.0933	114	0.568	0.938

Hora = 6hR:

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.738	0.0879	114	0.564	0.912

CM	0.741	0.0879	114	0.567	0.916
H1	0.560	0.0997	114	0.362	0.757
H2	1.105	0.0879	114	0.930	1.279
H3	0.842	0.0879	114	0.667	1.016

Confidence level used: 0.95

\$contrasts

Hora = 1hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.1999	0.124	114	1.607	0.4958
AC - H1	0.0988	0.128	114	0.771	0.9385
AC - H2	-0.0482	0.124	114	-0.387	0.9952
AC - H3	0.0622	0.124	114	0.500	0.9872
CM - H1	-0.1011	0.128	114	-0.789	0.9334
CM - H2	-0.2481	0.124	114	-1.995	0.2751
CM - H3	-0.1377	0.124	114	-1.107	0.8024
H1 - H2	-0.1470	0.128	114	-1.147	0.7814
H1 - H3	-0.0366	0.128	114	-0.285	0.9985
H2 - H3	0.1104	0.124	114	0.888	0.9009

Hora = 24hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-0.1175	0.124	114	-0.945	0.8786
AC - H1	-0.3118	0.128	114	-2.432	0.1142
AC - H2	-0.2628	0.128	114	-2.050	0.2491
AC - H3	0.0758	0.128	114	0.591	0.9762
CM - H1	-0.1943	0.128	114	-1.516	0.5546
CM - H2	-0.1453	0.128	114	-1.133	0.7885
CM - H3	0.1933	0.128	114	1.508	0.5595
H1 - H2	0.0490	0.132	114	0.371	0.9959
H1 - H3	0.3876	0.132	114	2.938	0.0320
H2 - H3	0.3386	0.132	114	2.567	0.0835

Hora = 6hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-0.0032	0.124	114	-0.026	1.0000
AC - H1	0.1784	0.133	114	1.342	0.6659

AC - H2	-0.3663	0.124	114	-2.946	0.0313
AC - H3	-0.1033	0.124	114	-0.831	0.9206
CM - H1	0.1816	0.133	114	1.366	0.6508
CM - H2	-0.3632	0.124	114	-2.920	0.0336
CM - H3	-0.1001	0.124	114	-0.805	0.9286
H1 - H2	-0.5447	0.133	114	-4.097	0.0007
H1 - H3	-0.2817	0.133	114	-2.119	0.2194
H2 - H3	0.2630	0.124	114	2.115	0.2209

P value adjustment: tukey method for comparing a family of 5 estimates

```
> emmeans(gr1_glm, pairwise~Hora, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

```
$emmeans
```

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.766	0.0398	114	0.687	0.845
24hR	0.952	0.0408	114	0.871	1.033
6hR	0.797	0.0404	114	0.717	0.877

Results are averaged over the levels of: Shock

Confidence level used: 0.95

```
$contrasts
```

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.1858	0.0570	114	-3.261	0.0042
1hR - 6hR	-0.0312	0.0567	114	-0.550	0.8468
24hR - 6hR	0.1546	0.0574	114	2.693	0.0221

Results are averaged over the levels of: Shock

P value adjustment: tukey method for comparing a family of 3 estimates

```
> emmeans(gr1_glm, pairwise~Shock, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

```
$emmeans
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.798	0.0508	114	0.698	0.899
CM	0.772	0.0508	114	0.671	0.873
H1	0.810	0.0551	114	0.701	0.919
H2	1.024	0.0518	114	0.922	1.127

H3 0.787 0.0518 114 0.684 0.890

Results are averaged over the levels of: Hora

Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.0264	0.0718	114	0.368	0.9961
AC - H1	-0.0116	0.0749	114	-0.154	0.9999
AC - H2	-0.2258	0.0725	114	-3.112	0.0195
AC - H3	0.0116	0.0725	114	0.159	0.9999
CM - H1	-0.0379	0.0749	114	-0.506	0.9866
CM - H2	-0.2522	0.0725	114	-3.476	0.0064
CM - H3	-0.0148	0.0725	114	-0.204	0.9996
H1 - H2	-0.2142	0.0757	114	-2.832	0.0427
H1 - H3	0.0231	0.0757	114	0.306	0.9981
H2 - H3	0.2373	0.0733	114	3.239	0.0134

Results are averaged over the levels of: Hora

P value adjustment: tukey method for comparing a family of 5 estimates

```
> #HIF1
```

```
> shapiro.test(datos$HIF1)
```

Shapiro-Wilk normality test

data: datos\$HIF1

W = 0.90959, p-value = 3.669e-07

```
> bartlett.test(datos$HIF1~datos$Subset)
```

Bartlett test of homogeneity of variances

data: datos\$HIF1 by datos\$Subset

Bartlett's K-squared = 38.96, df = 14, p-value = 0.00037

```
> hif1_glm <- glm(datos$HIF1 ~ datos$Shock * datos$Hora, family = gaussian)
> Anova(hif1_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$HIF1

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	198.583	4	< 2.2e-16	***
datos\$Hora	10.814	2	0.004484	**
datos\$Shock:datos\$Hora	23.141	8	0.003188	**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(hif1_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.57886966	0.64145277
datos\$Hora	0.03152398	0.08877751
datos\$Shock:datos\$Hora	0.06745688	0.17251402

```
> emmeans(hif1_glm, pairwise~Hora|Shock, adjust="Tukey")
```

\$emmeans

Shock = AC:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.316	0.0407	111	0.2353	0.397
24hR	0.316	0.0407	111	0.2353	0.397
6hR	0.485	0.0384	111	0.4093	0.561

Shock = CM:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.105	0.0384	111	0.0293	0.181
24hR	0.229	0.0384	111	0.1528	0.305
6hR	0.152	0.0384	111	0.0763	0.228

Shock = H1:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.255	0.0407	111	0.1746	0.336
24hR	0.188	0.0407	111	0.1075	0.269
6hR	0.260	0.0435	111	0.1735	0.346

Shock = H2:

Hora	emmean	SE	df	lower.CL	upper.CL
------	--------	----	----	----------	----------

1hR	0.210	0.0384	111	0.1335	0.286
24hR	0.247	0.0407	111	0.1666	0.328
6hR	0.202	0.0384	111	0.1259	0.278

Shock = H3:

Hora	emmean	SE	df	lower.CL	upper.CL
1hR	0.494	0.0384	111	0.4184	0.571
24hR	0.512	0.0407	111	0.4315	0.593
6hR	0.684	0.0407	111	0.6035	0.765

Confidence level used: 0.95

\$contrasts

Shock = AC:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.00000	0.0576	111	0.000	1.0000
1hR - 6hR	-0.16939	0.0560	111	-3.027	0.0086
24hR - 6hR	-0.16939	0.0560	111	-3.027	0.0086

Shock = CM:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.12353	0.0543	111	-2.275	0.0635
1hR - 6hR	-0.04696	0.0543	111	-0.865	0.6634
24hR - 6hR	0.07656	0.0543	111	1.410	0.3391

Shock = H1:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	0.06710	0.0576	111	1.165	0.4764
1hR - 6hR	-0.00438	0.0596	111	-0.074	0.9970
24hR - 6hR	-0.07148	0.0596	111	-1.199	0.4561

Shock = H2:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.03774	0.0560	111	-0.674	0.7788
1hR - 6hR	0.00764	0.0543	111	0.141	0.9891
24hR - 6hR	0.04538	0.0560	111	0.811	0.6971

Shock = H3:

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.01774	0.0560	111	-0.317	0.9462
1hR - 6hR	-0.18969	0.0560	111	-3.390	0.0028
24hR - 6hR	-0.17195	0.0576	111	-2.986	0.0097

P value adjustment: tukey method for comparing a family of 3 estimates

```
> emmeans(hif1_glm, pairwise~Shock|Hora, adjust="Tukey")
```

```
$emmeans
```

```
Hora = 1hR:
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.316	0.0407	111	0.2353	0.397
CM	0.105	0.0384	111	0.0293	0.181
H1	0.255	0.0407	111	0.1746	0.336
H2	0.210	0.0384	111	0.1335	0.286
H3	0.494	0.0384	111	0.4184	0.571

```
Hora = 24hR:
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.316	0.0407	111	0.2353	0.397
CM	0.229	0.0384	111	0.1528	0.305
H1	0.188	0.0407	111	0.1075	0.269
H2	0.247	0.0407	111	0.1666	0.328
H3	0.512	0.0407	111	0.4315	0.593

```
Hora = 6hR:
```

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.485	0.0384	111	0.4093	0.561
CM	0.152	0.0384	111	0.0763	0.228
H1	0.260	0.0435	111	0.1735	0.346
H2	0.202	0.0384	111	0.1259	0.278
H3	0.684	0.0407	111	0.6035	0.765

Confidence level used: 0.95

```
$contrasts
```

```
Hora = 1hR:
```

contrast	estimate	SE	df	t.ratio	p.value
----------	----------	----	----	---------	---------

AC - CM	0.2106	0.0560	111	3.763	0.0025
AC - H1	0.0606	0.0576	111	1.053	0.8299
AC - H2	0.1064	0.0560	111	1.901	0.3229
AC - H3	-0.1785	0.0560	111	-3.190	0.0156
CM - H1	-0.1499	0.0560	111	-2.680	0.0635
CM - H2	-0.1042	0.0543	111	-1.919	0.3133
CM - H3	-0.3891	0.0543	111	-7.167	<.0001
H1 - H2	0.0458	0.0560	111	0.818	0.9247
H1 - H3	-0.2391	0.0560	111	-4.273	0.0004
H2 - H3	-0.2849	0.0543	111	-5.248	<.0001

Hora = 24hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.0870	0.0560	111	1.556	0.5289
AC - H1	0.1277	0.0576	111	2.218	0.1807
AC - H2	0.0686	0.0576	111	1.192	0.7559
AC - H3	-0.1962	0.0576	111	-3.408	0.0080
CM - H1	0.0407	0.0560	111	0.727	0.9498
CM - H2	-0.0184	0.0560	111	-0.329	0.9974
CM - H3	-0.2833	0.0560	111	-5.063	<.0001
H1 - H2	-0.0591	0.0576	111	-1.026	0.8428
H1 - H3	-0.3240	0.0576	111	-5.626	<.0001
H2 - H3	-0.2649	0.0576	111	-4.600	0.0001

Hora = 6hR:

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	0.3330	0.0543	111	6.134	<.0001
AC - H1	0.2256	0.0580	111	3.888	0.0016
AC - H2	0.2834	0.0543	111	5.220	<.0001
AC - H3	-0.1988	0.0560	111	-3.553	0.0050
CM - H1	-0.1074	0.0580	111	-1.850	0.3505
CM - H2	-0.0496	0.0543	111	-0.914	0.8911
CM - H3	-0.5318	0.0560	111	-9.504	<.0001
H1 - H2	0.0578	0.0580	111	0.995	0.8569
H1 - H3	-0.4244	0.0596	111	-7.121	<.0001
H2 - H3	-0.4822	0.0560	111	-8.617	<.0001

P value adjustment: tukey method for comparing a family of 5 estimates

```
> emmeans(hif1_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
```

	Shock	emmean	SE	df	lower.CL	upper.CL
AC		0.372	0.0231	111	0.327	0.418
CM		0.162	0.0222	111	0.118	0.206
H1		0.234	0.0241	111	0.187	0.282
H2		0.220	0.0226	111	0.175	0.264
H3		0.564	0.0231	111	0.518	0.609

Results are averaged over the levels of: Hora
Confidence level used: 0.95

```
$contrasts
```

	contrast	estimate	SE	df	t.ratio	p.value
AC - CM		0.2102	0.0320	111	6.571	<.0001
AC - H1		0.1380	0.0333	111	4.140	0.0006
AC - H2		0.1528	0.0323	111	4.730	0.0001
AC - H3		-0.1912	0.0326	111	-5.861	<.0001
CM - H1		-0.0722	0.0327	111	-2.208	0.1846
CM - H2		-0.0574	0.0317	111	-1.813	0.3716
CM - H3		-0.4014	0.0320	111	-12.548	<.0001
H1 - H2		0.0148	0.0330	111	0.449	0.9915
H1 - H3		-0.3292	0.0333	111	-9.876	<.0001
H2 - H3		-0.3440	0.0323	111	-10.648	<.0001

Results are averaged over the levels of: Hora
P value adjustment: tukey method for comparing a family of 5 estimates

```
> emmeans(hif1_glm, pairwise~Hora, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
```

	Hora	emmean	SE	df	lower.CL	upper.CL
1hR		0.276	0.0176	111	0.241	0.311
24hR		0.299	0.0180	111	0.263	0.334
6hR		0.357	0.0179	111	0.321	0.392

Results are averaged over the levels of: Shock

Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
1hR - 24hR	-0.0224	0.0252	111	-0.889	0.6483
1hR - 6hR	-0.0806	0.0251	111	-3.214	0.0048
24hR - 6hR	-0.0582	0.0254	111	-2.294	0.0608

Results are averaged over the levels of: Shock

P value adjustment: tukey method for comparing a family of 3 estimates

```
> #MYO
```

```
> shapiro.test(datos$MYO)
```

Shapiro-Wilk normality test

data: datos\$MYO

W = 0.95116, p-value = 0.0001793

```
> bartlett.test(datos$MYO~datos$Subset)
```

Bartlett test of homogeneity of variances

data: datos\$MYO by datos\$Subset

Bartlett's K-squared = 37.283, df = 14, p-value = 0.0006684

```
> myo_glm <-glm(datos$MYO~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(myo_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$MYO

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	55.421	4	2.651e-11	***
datos\$Hora	4.152	2	0.1255	
datos\$Shock:datos\$Hora	5.575	8	0.6947	

```

Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> etaSquared(myo_glm)

              eta.sq eta.sq.part
datos$Shock      0.31584707  0.33301851
datos$Hora        0.02366037  0.03605389
datos$Shock:datos$Hora 0.03177074  0.04782152
> emmeans(myo_glm, pairwise~Shock, adjust="Tukey")
NOTE: Results may be misleading due to involvement in interactions
$emmeans
  Shock emmean      SE   df lower.CL upper.CL
AC      0.590 0.0924  111    0.407    0.773
CM      1.278 0.0906  111    1.099    1.458
H1      1.243 0.0966  111    1.051    1.434
H2      1.032 0.0924  111    0.849    1.215
H3      0.542 0.0988  111    0.346    0.738

Results are averaged over the levels of: Hora
Confidence level used: 0.95

$contrasts
  contrast estimate      SE   df t.ratio p.value
AC - CM    -0.6882 0.129  111   -5.318 <.0001
AC - H1    -0.6526 0.134  111   -4.882 <.0001
AC - H2    -0.4419 0.131  111   -3.381 0.0087
AC - H3     0.0480 0.135  111    0.354 0.9966
CM - H1     0.0356 0.132  111    0.269 0.9988
CM - H2     0.2463 0.129  111    1.903 0.3217
CM - H3     0.7361 0.134  111    5.492 <.0001
H1 - H2     0.2107 0.134  111    1.576 0.5156
H1 - H3     0.7006 0.138  111    5.071 <.0001
H2 - H3     0.4899 0.135  111    3.621 0.0040

```

Results are averaged over the levels of: Hora
P value adjustment: tukey method for comparing a family of 5 estimates

```
> #MR
```

```
> shapiro.test(datos$MR)
```

```
Shapiro-Wilk normality test
```

```
data:  datos$MR
```

```
W = 0.96862, p-value = 0.004157
```

```
> bartlett.test(datos$MR~datos$Subset)
```

```
Bartlett test of homogeneity of variances
```

```
data:  datos$MR by datos$Subset
```

```
Bartlett's K-squared = 19.557, df = 14, p-value = 0.1447
```

```
> mr_glm <-glm(datos$MR~datos$Shock*datos$Hora, family = gaussian)
```

```
> Anova(mr_glm)
```

```
Analysis of Deviance Table (Type II tests)
```

```
Response: datos$MR
```

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	35.173	4	4.28e-07	***
datos\$Hora	5.876	2	0.05298	.
datos\$Shock:datos\$Hora	12.748	8	0.12083	

```
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
> etaSquared(mr_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.20871920	0.23421614
datos\$Hora	0.03486726	0.04860989
datos\$Shock:datos\$Hora	0.07564638	0.09978859

```
> emmeans(mr_glm, pairwise~Shock, adjust="Tukey")
```

```
NOTE: Results may be misleading due to involvement in interactions
```

```
$emmeans
```

	Shock	emmean	SE	df	lower.CL	upper.CL
AC		0.308	0.0216	115	0.265	0.351
CM		0.378	0.0216	115	0.335	0.420
H1		0.393	0.0235	115	0.346	0.439
H2		0.419	0.0221	115	0.376	0.463

H3 0.262 0.0216 115 0.219 0.305

Results are averaged over the levels of: Hora
Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-0.0695	0.0306	115	-2.271	0.1620
AC - H1	-0.0845	0.0319	115	-2.645	0.0689
AC - H2	-0.1112	0.0309	115	-3.595	0.0043
AC - H3	0.0461	0.0306	115	1.505	0.5614
CM - H1	-0.0150	0.0319	115	-0.470	0.9899
CM - H2	-0.0417	0.0309	115	-1.348	0.6621
CM - H3	0.1156	0.0306	115	3.776	0.0023
H1 - H2	-0.0267	0.0322	115	-0.827	0.9217
H1 - H3	0.1306	0.0319	115	4.087	0.0008
H2 - H3	0.1572	0.0309	115	5.085	<.0001

Results are averaged over the levels of: Hora
P value adjustment: tukey method for comparing a family of 5 estimates

> #CRH

> shapiro.test(datos\$CRH)

Shapiro-Wilk normality test

data: datos\$CRH

W = 0.8787, p-value = 1.5e-08

> bartlett.test(datos\$CRH~datos\$Subset)

Bartlett test of homogeneity of variances

data: datos\$CRH by datos\$Subset

Bartlett's K-squared = 61.125, df = 14, p-value = 7.442e-08


```
> crh_glm <-glm(datos$CRH~datos$Shock*datos$Hora, family = gaussian)
> Anova(crh_glm)
```

Analysis of Deviance Table (Type II tests)

Response: datos\$CRH

	LR	Chisq	Df	Pr(>Chisq)
datos\$Shock	41.101	4	2.561e-08	***
datos\$Hora	5.985	2	0.05017	.
datos\$Shock:datos\$Hora	15.326	8	0.05311	.

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> etaSquared(crh_glm)
```

	eta.sq	eta.sq.part
datos\$Shock	0.24316437	0.27752141
datos\$Hora	0.03540767	0.05297031
datos\$Shock:datos\$Hora	0.09067093	0.12528680

```
> emmeans(crh_glm, pairwise~Shock, adjust="Tukey")
```

NOTE: Results may be misleading due to involvement in interactions

\$emmeans

Shock	emmean	SE	df	lower.CL	upper.CL
AC	0.00592	0.000964	107	0.00401	0.00783
CM	0.01125	0.001030	107	0.00922	0.01329
H1	0.00902	0.001100	107	0.00684	0.01121
H2	0.01005	0.001030	107	0.00801	0.01209
H3	0.00326	0.000984	107	0.00131	0.00521

Results are averaged over the levels of: Hora

Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
AC - CM	-0.00534	0.00141	107	-3.784	0.0023
AC - H1	-0.00310	0.00146	107	-2.121	0.2188
AC - H2	-0.00413	0.00141	107	-2.931	0.0330
AC - H3	0.00266	0.00138	107	1.930	0.3081
CM - H1	0.00223	0.00151	107	1.480	0.5773
CM - H2	0.00120	0.00145	107	0.828	0.9215
CM - H3	0.00799	0.00142	107	5.616	<.0001

H1 - H2	-0.00103	0.00151	107	-0.682	0.9600
H1 - H3	0.00576	0.00148	107	3.903	0.0015
H2 - H3	0.00679	0.00142	107	4.771	0.0001

Results are averaged over the levels of: Hora

P value adjustment: tukey method for comparing a family of 5 estimates

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Acknowledgments

Acknowledgments

Welcome the non-scientific section of the thesis. Before starting I would like to thank you to all the people that I met all along this period, because all the people that I met had teach me something, maybe not in the better way but in the end it has been an important lesson in my life. Sometimes, most of the PhD think in a thanklessness section, but at the end of the day we need to thanks all this people because they teach us something. In the second place this section will be a mix of different languages, since I think that all the people in this sections deserves his place.

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Fish mucosal response to single and combined stressors

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Fish mucosal surfaces (gills, skin, and gut) represent the first line of defence and a key interface between the organisms and the environment, where stress and immune processes are integrated in response to environmental, and aquaculture-related stressors.

We analyzed the mucosal response in two commercial species, rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*), exposed to stressors that are increasingly relevant under climate and production scenarios: repeated hypoxia, elevated salinity, polystyrene nanoplastics, crowding, and anoxia/air exposure.

- In rainbow trout, repeated hypoxic episodes reveal functional habituation, with progressive recovery of haematological and metabolic parameters and modulation of the neuroendocrine response.
- In gilthead seabream, combined exposure to high salinity and nanoplastics elicits mucosa-dependent responses and can amplify stress, immune, and antioxidant activation.
- Acute stressor comparisons (anoxia vs. crowding) demonstrate transient, tissue- and stressor-specific transcriptional signatures, often returning toward baseline within 24 h.
- In all the *in vivo* experiments a tissue-specific response was observed with gills being the most responsive tissue under hypoxia, salinity and crowding, whereas intestine was a very responsive tissues under nanoplastics and gills under anoxia stress.
- Finally, *in vitro* intestinal epithelial assays support that cortisol can modulate inflammatory/immune responsiveness under challenge conditions.

Overall, the thesis supports a multi-marker, multi-tissue framework to better interpret stress and welfare status and provides evidence relevant for improving management strategies aimed at enhancing fish resilience and welfare in aquaculture.