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TESIS:
“Estilos de afrontamiento al estrés en juveniles de lisa (Mugil cephalus): Efecto en el comportamiento, parámetros fisiológicos y transcriptómica”

Que para obtener el grado de
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PRESENTA:
M.C. Joel Fitzgerald Linares Cordova

DIRECTOR DE TESIS:
Dr. Gustavo Alejandro Rodríguez Montes de Oca

CO-DIRECTOR DE TESIS:
Dr. Zohar Ibarra Zatarain

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DEDICATORIA

A Dios, *por guiarme en el camino correcto*

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A mis padres, *Luz María Córdova Chincha y Joel Linares Cruz*

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"Por mala que la vida pueda parecer, siempre hay algo por hacerse, y tener éxito en ello. Mientras hay vida, hay esperanza"
Stephen Hawking

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RESUMEN

Estilos de afrontamiento al estrés en juveniles de lisa (*Mugil cephalus*): Efecto en el comportamiento, parámetros fisiológicos y transcriptómica

Joel Fitzgerald Linares Cordova

En una población existen diferencias de comportamiento entre los individuos que la constituyen al ser sometidos a eventos de estrés. Esta diferenciación conductual es consistente a nivel intra e interindividual entre contextos y a lo largo del tiempo y se acompaña de diferencias a nivel fisiológico, las cuales se reconocen como una variación adaptativa y, por lo tanto, como un factor relevante para la evolución. Dicha variación individual se ha conservado evolutivamente y está presente en todos los taxones de vertebrados, incluidos los peces. En los animales de granja, el interés sobre el desarrollo de estudios enfocados a la variación adaptativa, reconocida como Estilos de Afrontamiento al Estrés (EAE), ha aumentado significativamente en los últimos años y se ha demostrado vínculos con rasgos de rendimiento productivo, reproducción, bienestar animal y resistencia a enfermedades. Este aspecto también ha resultado importante en la piscicultura, ya que el entendimiento de esta adaptación promueve mejoras en la producción, incluida la cría selectiva. Los EAE se caracterizan típicamente por dos comportamientos extremos definidos como proactivos y reactivos. Se ha reconocido que organismos proactivos tienen una mayor actividad en nuevos entornos, son mas impulsivos en la toma de decisiones, asumen mayor riesgo cuando se enfrentan a un peligro potencial, y muestran niveles más bajos de glucocorticoides que organismos reactivos. Estudios recientes aplican un enfoque de personalidad para conocer la variación entre aspectos fisiológicos y mecanismos moleculares sugiriendo un vínculo genotipo-fenotipo, entre la regulación del transcriptoma y el comportamiento. Ante ello, la presente tesis tuvo como objetivos: **i)** Conocer el estatus del bienestar de peces en Latinoamérica, mediante un análisis cienciométrico (Capítulo II), **ii)** Caracterizar y determinar la consistencia de los EAE a lo largo del tiempo y entre contextos, identificados mediante pruebas conductuales grupal e individuales en juveniles de lisa (*Mugil cephalus*) (Capítulo III) y **iii)** Evaluar la sincronización conductual-transcriptómica del cerebro en juveniles de *M. cephalus* utilizando RNA-Seq para facilitar futuros estudios sobre la interaccion entre la expresión génica y la conducta en respuesta al estres (Capítulo IV). Los resultados obtenidos son la primera evidencia de la relación entre la variación individual conductual y la expresión de genes en respuesta al estrés en juveniles de lisa, brindando información valiosa a la industria para el manejo de esta especie acuícola y la mejora de su bienestar en condiciones productivas.

Palabras claves: *Mugil cephalus*, estilos de afrontamiento al estrés, transcriptómica, bienestar de peces.

ABSTRACT

Stress coping styles in flathead grey mullet (*Mugil cephalus*) juveniles: Effects on behaviour, physiological parameters and transcriptomic

Joel Fitzgerald Linares Cordova

In a population, there are differences in behaviour among the individuals that constitute it when subjected to stress events. This behavioural differentiation is consistent at the intra- and inter-individual level between contexts and over time and is accompanied by differences at the physiological levels, which are recognized as an adaptive variation, and therefore as a relevant factor for evolution. Such individual variation has been evolutionarily conserved and is present in all vertebrate taxa, including fish. In farm animals, interest in the development of studies focused on adaptative variation, recognized as stress coping styles (SCS), has increased significantly in recent years and links have demonstrated links with traits of productive performance, reproduction, welfare and disease resistance. This aspect has also been important in fish farming since he understanding of this adaptation promotes improvements in production, including, selective breeding. SCSs are typically characterized by two extreme behaviours define as proactive and reactive. It has been recognized that proactive fish have greater activity in new environments, are more impulsive in decision making, take greater risk when faced with potential danger and show lower levels of glucocorticoids than reactive fish. Recent studies apply a personality approach to understand the variation between physiological and molecular mechanisms, suggesting a genotype-phenotype link between the regulation of the transcriptome and behaviour. Given this, the objectives of this study were i) to know the status of fish welfare in LatinAmerica, through a scientometric analysis (Chapter II), ii) to characterize and determine the consistency of the SCSs over time and between contexts, identified through group and individual behavioural tests in *Mugil cephalus* juveniles (Chapter III) and iii) to evaluate the behavioural-transcriptomic synchronization of the brain in juveniles *M. cephalus* using RNA-Seq to facilitate future studies on the interaction between the expression genetics and behaviour in response to stress (Chapter IV). The results obtained are the first evidence of the relationship between individual behavioral variation and gene expression in response to stress in juvenile mullet, providing valuable information to the industry for the management of this aquaculture species and the improvement of its welfare in productive conditions.

Keywords: mullet juveniles, stress coping styles, brain transcriptomics, fish welfare.


"Estilos de Afrontamiento al estrés"
Lisa
• Cultivo de Peces
• Pruebas de Comportamiento
• Cortisol/Glucosa
• Cerebro
Consistencia

CAPÍTULO 1. INTRODUCCIÓN Y REVISIÓN DE LITERATURA

1.1. INTRODUCCIÓN

Con la tendencia al incremento de la población mundial y al mismo tiempo del número de personas con propensión a la desnutrición; la acuicultura, se presenta como la actividad agropecuaria que podría favorecer el incremento en la producción de alimentos para compensar la demanda de proteína animal de los aproximadamente 10 mil millones de habitantes que se prevé para el año 2050 (FAO, 2020).

De las actividades agropecuarias, la acuicultura es la única que ha mantenido una tasa de crecimiento global promedio de 6,7% anual (1990-2020). Además, genera altos ingresos y, de acuerdo a la FAO (2022), esta actividad en el 2020 logró una producción récord de pescado con 122,6 millones de toneladas de peso vivo, que correspondieron a aproximadamente una derrama económica cerca de USD $281 mil millones y contribuyó a la generación de casi 58,53 millones de empleos en el mundo, confirmando así no solo el impacto de la actividad sobre la producción de alimentos, sino también en el sector económico a nivel mundial. Sin embargo, el continuo crecimiento de la actividad, conlleva a generar un mayor estrés en los organismos bajo cultivo (Martos-Sitcha *et al.*, 2020), causado por las maniobras de captura y manejo frecuente de los peces, transporte, altas densidades de cultivo, variaciones en la calidad del agua, exposición a nuevos ambientes de cautiverio, e incidencia de enfermedades (Naylor *et al.,* 2005; Bianca, 2009; Zaki *et al.*, 2020). Esto deriva en una concientización general en cuanto al bienestar de los peces en cultivo, no solo por la percepción pública, sino también por los productores acuícolas, debido a que el bienestar animal impacta sobre la eficiencia de producción en cuanto a supervivencia y costo de producción y la cantidad y calidad del producto (Broom, 2011; Mustapha*,* 2014).

De acuerdo con Shreck (2010) y Ciji y Akhtar (2021), la comprensión de los factores del estrés es un objetivo fundamental en la investigación en acuicultura para mejorar el crecimiento y lograr una producción exitosa, debido a que el estrés altera la capacidad de los peces a mantener o restablecer la homeostasis y desencadena respuestas fisiológicas y neuroendocrinas que pueden impactar negativamente en la asimilación de nutrientes, el crecimiento y la salud de los organismos bajo cultivo (Figura 1.1). Estas respuestas fisiológicas al estrés ocurren específicamente mediante un sistema coordinado del eje Hipotálamo-Hipófisario-Interrenal (HHI o Hypothalamic-Pituitary-Interrenal Axis, HPI en ingles), homólogo al eje Hipotálamo-Hipófisario-Suprarrenal (HHS o Hypothalamic-Pituitary-Adrenal Axis, HPA en ingles) en mamíferos, y del eje Hipotálamo Simpático Cromafín (HSC), que regulan los sistemas nervioso y endocrino como medida de control (Clements y Schreck, 2004; Gesto *et al.*, 2013).

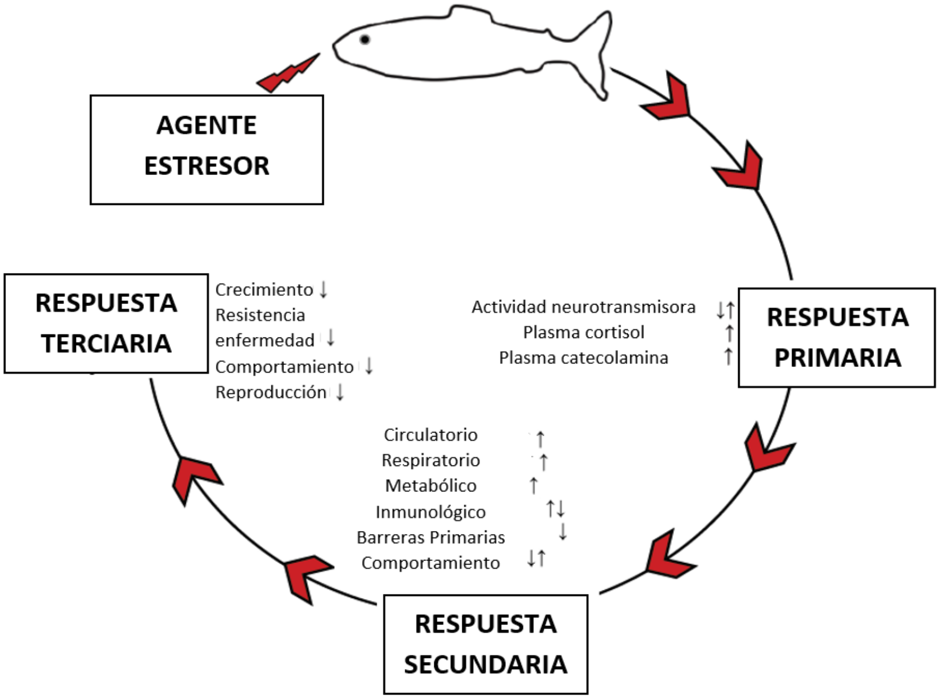

Figura 1.1. Esquematización de Respuestas neuroendocrinas al estrés (Tomado y traducido de Tesis Doctoral en Ciencias Naturales de Malin Rosengren, 2017, Universidad de Gothenburg, Suecia).

En la actualidad, diversos estudios han demostrado que diferentes indicadores fisiológicos (respuestas metabólicas e inmunológicas; Vargas *et al.*, 2018), bioquímicos (alteración de niveles sanguíneos de glucosa, lactato y cortisol; Fiori *et al.,* 2019; Gesto, 2019), reproductivos (índice gonadosomático y hepatosomático; Argumedo *et al*., 2021) y conductuales (actividad de exploración; Ibarra-Zatarain *et al.*, 2016) son eficientes para evaluar las estrategias que exhiben los organismos para responder al estrés y que se diferencian individualmente en sus estilos de afrontamiento al estrés (EAE) (Boulton *et al.,* 2015; Castanheira *et al.,* 2016; Colchen *et al.,* 2017).

El concepto de estilos de afrontamiento al estrés (EAE), que será utilizado en el presente estudio, cobró impulso a fines de la década de los años 1970, con el trabajo publicado por Henry y Stephens (1977) en ganado vacuno. Posteriormente, Koolhaas *et al.* (1999) definieron a los EAE como estrategias de comportamiento y respuestas fisiológicas que exhiben individuos, de una misma población, y que son consistentes al tiempo y entre contextos. Asimismo, sugirieron los términos de respuesta al estrés de tipos proactivo y reactivo. De manera general, los organismos proactivos son relativamente más exploratorios, audaces y agresivos que sus homólogos reactivos (Koolhaas *et al.,* 1999). Los EAE se han confirmado para especies de diferentes taxones como seria: **i)** Mamíferos: ratones *Mus musculus domesticus* (Benus *et al.,* 1991), cerdos *Sus scrofa domesticus* (Hessing *et al.,* 1994), perro galgo inglés *Canis lupus familiaris* (Wormald *et al*., 2016); **ii)** Aves: pollo *Gallus gallus domesticus* (Blokhuis y Metz 1992), pájaro *Parus major* (Carere *et al*., 2001); **iii)** Reptiles: camaleón *Anolis carolinensis* (Greenberg, 2002), entre otras especies. En peces, los estudios de EAE iniciaron con un trabajo donde evaluaron respuestas de comportamiento de delimitación territorial a diferentes depredadores en el pez espinoso (*Gasterosteus aculeatus*) (Huntingford, 1976). Desde entonces, se han desarrollado estudios que abordan diferentes peces de cultivo; como en la carpa común *Cyprinus carpio* (MacKenzie *et al*. 2009; Huntingford *et al*. 2010), la tilapia del Nilo *Oreochromis niloticus* (Barreto y Volpato 2011; Martins *et al*. 2011bd), el salmón del Atlántico *Salmo salar* (Vaz-Serrano *et al*. 2011; Kittilsen *et al*. 2012;), el halibut del Atlántico *Hippoglossus hippoglossus* (Kristiansen y Fernö 2007), la trucha arco iris *Oncorhynchus mykiss* (Øverli *et al*. 2006ab; Ruiz-Gomez *et al*., 2011), la lubina europea *Dicentrarchus labrax* (Millot *et al*. 2009 ab), la dorada *Sparus aurata*

(Castanheira *et al*. 2013ab; Herrera *et al*. 2014), el lenguado senegalés *Solea senegalensis* (Ibarra-Zatarín *et al*., 2016) y la lisa *Mugil cephalus* (Jiménez-Rivera *et al*., 2023).

Adicionalmente, las pruebas para caracterizar los EAE han ido incrementando en número y adaptándose a las especies para ampliar el entendimiento sobre la interpretación de los comportamientos de respuesta al estrés. En este sentido, las pruebas que más comúnmente se utilizan son pruebas grupales e individuales. Las pruebas grupales incluyen tests como: **i)** prueba de toma de riesgo, en donde se evalúa la capacidad que tiene el individuo para tomar riesgo al cruzar de una zona segura a una zona de riesgo (Ferrari *et al*., 2015; Alfonso *et al*., 2019; Carbonara *et al*., 2019), **ii)** prueba de hipoxia, que mide el comportamiento de escape desde el compartimiento de ensayo, con condiciones de hipoxia hacia el de normoxia (Castanheira *et al*., 2013a; Ferrari *et al*., 2015; Vindas *et al*., 2017), y **iii)** prueba de reacción a un objeto nuevo, donde se evalúa la capacidad que tiene el individuo para pasar o mantenerse encima del objeto nuevo (Ibarra-Zatarain *et al*., 2016). Relativo a las pruebas individuales, las mas utilizados son: **i)** prueba de restricción, la cual consiste en mantener a cada individuo en una red emergida por un tiempo limitado dependiendo de la especie (Castanheira *et al*., 2016; Hoglund *et al*., 2020), **ii)** prueba de nuevo ambiente, donde se transfiere a cada pez individualmente a otro tanque que simule un nuevo ambiente (Castanheira *et al*., 2013a, Ibarra-Zatarain *et al*., 2016; Fatsini *et al*., 2020), **iii)** prueba de confinamiento, en la cual se colocan cada pez individualmente en otro tanque de menores dimensiones y con menor volumen de agua, para simular un área confinada (Barreto y Volpato, 2011; Ibarra-Zatarain *et al*., 2020), **iv)** prueba de respuesta al depredador, que consiste en transferir a cada pez individualmente a otro tanque, el cual contiene un pez de plástico, simulando un depredador, que debe ser aproximadamente un 50 % más grande que los peces de estudio, lo que representa un riesgo significativo para los peces (Solomon-Lane y Hofmann, 2019), y **v)** prueba de primera alimentación después de un evento de captura, que se basa en capturar individualmente con una red a cada organismo y en regresarle a su tanque de origen después de unos segundos para evaluar a partir de cuando retoma una actitud de búsqueda e ingestión de comida (Silva *et al*., 2010; Castanheira *et al*., 2013a; Ferrari *et al*., 2015).

Aunado a lo anterior, estudios recientes han documentado una posible influencia del genoma sobre la respuesta al estrés en varias especies de peces (Balasch y Tort, 2019; Fatsini *et al.,* 2020; Thörnqvist *et al.,* 2015). Por ejemplo, Sadoul *et al*. (2022) observaron que las diferencias conductuales interindividuales se correlacionaron con los cambios transcriptómicos en muestras de glandula pituitaria (o hipófisis) y riñón, principalmente en la expresión de genes relacionados con el sistema inmunológico resaltando la importancia de investigar las diferencias interindividuales en el comportamiento, la fisiología y la ecología de las especies, ya que probablemente explican la dispersión de la variabilidad conductual a escala poblacional. Asimismo, Vu *et al*. (2020) demostraron que los comportamientos de lucha y la expresión de genes cerebrales del pez beta *Betta splendens* dentro de una pareja de lucha están altamente sincronizados, proporcionando un marco para comprender que esta sincronización ocurre durante una competencia o colaboración a través de interacciones sociales y tiene por origen mecanismos moleclares subyacentes a la conducta. Estudiar la variación de la expresión de genes asociados a diferentes EAE permite confirmar si las características comportamentales y fisiológicas de organismos proactivos y reactivos corresponden también a patrones distintivos de expresión de genes involucrados en la respuesta al estrés. Por tanto, la generacion de conocimiento acerca a la interaccion del genoma con la conducta representa una ventaja para el sector acuícola, ya que permite la selección de animales con base a su respuesta al estrés, la cual está asociada a características de éxito reproductivo, crecimiento, resistencia al manejo y a enfermedades (King *et al*., 2013; Ibarra-Zatarain *et al*., 2020) y otros rasgos de interés para la productividad a escala comercial; como, por ejemplo, densidad de cultivo (Carbonara *et al*., 2019), protocolos de alimentación (Huntingford y Adams 2005), entre otros.

La lisa (*Mugil cephalus*) es una especie marina que se cultiva desde décadas en diversos países del mundo (Egipto, Túnez, China e India, entre otros), con métodos tradicionales de acuicultura, como serían estanquerias rústicas y modelos semi-intensivos (Barman *et al.,* 2005; Chang *et al.,* 2000; Kheriji *et al.,* 2003; Magdy *et al.,* 2016). La producción mundial de mugílidos en 2015 fue de 164 937 toneladas, mientras que la producción de lisa fue de 15 000 toneladas (FAO, 2015), siendo Asia quien contribuye con más del 90% de la producción acuícola de esta especie. La mayor parte de este aporte se debió a la producción de Taiwán, que con 2,182 toneladas es el mayor productor, seguido de Israel (2,147 toneladas), China (984 toneladas), Singapur (500 toneladas), Grecia (251 tonelada), Túnez (247 toneladas) y Arabia Saudita (60 toneladas) (FAO - FIGIS, 2021). Sin embargo, la expansión futura de la producción de lisa está limitada porque depende de la disponibilidad de alevines silvestres (Saleh, 2022), ya que pocos países lograron dominar su reproducción y cerrar su ciclo biológico en cautiverio. La importancia económica de la lisa se estima con base en la demanda y su valor en el mercado, pudiendo variar de un país a otro y dependiendo del tipo de producto comercializado: pez entero, filete u ovas (caviar o botarga); esta última tiene un valor agregado que puede alcanzar los 65 euros/ Kg en el mercado europeo (Crosetti, 2016). En México, la lisa es un recurso pesquero de importante valor económico, ya que su producción proviene principalmente de la captura. Para el año 2019, la producción nacional fue de 12,487 toneladas (CONAPESCA, 2020). Los estados donde se consume habitualmente esta especie son Tamaulipas, Nayarit, Sinaloa, Guerrero y Baja California Sur, quienes aportan el 85% de la producción total en México (CONAPESCA, 2020).

La lisa es una especie con potencial acuícola en México que puede favorecer al crecimiento económico y diversificación del sector acuícola, ya que se adapta fácilmente al cautiverio y al manejo, tolera variaciones en sus condiciones de cultivo, tiene hábitos alimenticios omnívoros y acepta dietas inertes (Saleh, 2008; Prakoso *et al.*, 2019). Adicionalmente, la lisa es una especie de carácter social y ausencia de habitos agresivos o dominio a sus congéneres, lo que valida su potencial para acuicultura (Jiménez-Rivera *et al*., 2022). No obstante, a pesar de las características mencionadas anteriormente, los avances de investigación en temas como fisiología, comportamiento, nutrición, reproducción, entre otros, aún son escasos en México, contrario a los avances realizados por países asiáticos principalmente. (Aldana, 2015; Prakoso *et al*., 2019). Por ejemplo, realizar trabajos relacionados a EAE contribuirán a la comprensión de las condiciones de cultivo, manipulación y domesticación mas adecuadas para esta especie y que influyen directamente en su crecimiento y bienestar (Toni *et al*., 2019). Por lo anterior, conocer la conducta, fisiología y genes vinculados a los EAE en juveniles de lisa permitirán entender las diferencias individuales en términos de adaptabilidad y capacidad de respuesta al estrés en condiciones de cautiverio, lo que apoyará al sector acuícola en la selección de animales con características de interés para su producción exitosa, en base a su comportamiento en cautiverio. En este contexto, el objetivo general del presente trabajo de investigación fue caracterizar los EAE tipo proactivo y reactivo en juveniles de lisa (*Mugil cephalus*) extraídos del medio silvestre y adaptados al cautiverio, mediante pruebas conductuales (individuales y grupales), cuantificación de parámetros fisiológicos (cortisol y glucosa) y medición de la expresión de genes asociados a la respuesta al estrés, así como determinar el grado de consistencia de los comportamientos a lo largo del tiempo y entre contextos.

1.1.1. Planteamiento del Problema

Considerando el rápido crecimiento y expansión de la acuicultura, así como sus prácticas comunes de cultivo en altas densidades, condiciones medioambientales variables y manipulación constante, el bienestar de los animales bajo cultivo está recibiendo atención por parte de comités de ética y organizaciones de cuidado animal. Ante ello, los EAE se presentan como una herramienta científica que permite reconocer como los animales afrontan una situación estresante, lo cual es relevante para explicar diferencias individuales en términos de adaptabilidad y capacidad de respuesta al estrés en cautiverio (Koolhaas *et al*., 1999; Øverli *et al*., 2007). Además de los estudios de comportamiento para la detección de los EAE en especies acuicolas, diferentes investigaciones han reportado que los EAE deben ser incluidos como una variable adicional en los estudios de expresión génica ya que reducen significativamente la variación dentro de la población (MacKenzie *et al*., 2009; Rey *et al*., 2013). La aplicación de pruebas conductuales para identificar de manera confiable a individuos con rasgos de personalidad contrastantes facilita la exploración de la regulación molecular y fisiológica subyacente, lo que proporciona un juego de datos complementarias a diferentes escalas bioologicas, facilitando la comprensión de la adaptación y evolución de los rasgos de comportamiento (Robinson *et al*., 2008). Esto ha permitido lograr un avance significativo en la comprensión de la variación intra-individual y entre los fenotipos de comportamiento proactivo o reactivo y su relación con la regulación transcripcional. Empero lo anterior, un desafío pendiente es documentar cómo el entorno interactúa con las vías moleculares para dar forma a la variación en los comportamientos individuales (Maruska y Fernald, 2014; Benowitz *et al*., 2017; Berens *et al*., 2015). Los estudios evolutivos que utilizan la secuenciación de ARN (RNA-Seq) para abordar la brecha fenotipo-genotipo han sugerido que muchos genes están vinculados transcripcionalmente a un fenotipo determinado (Mackay et al., 2009), como por ejemplo: genes involucrados en la plasticidad neuronal y la neurogénesis (*pcna, bdnf, neurod*), en donde una mayor expresión génica se observa en individuos reactivos (Johansen *et al*., 1992; Alfonso *et al*., 2019), respaldando su mayor flexibilidad conductual ante situaciones estresante.

Actualmente, las plataformas de secuenciación de nueva generación (NGS, por sus siglas en inglés) han transformado drásticamente la forma de estudiar el genoma, ya que en periodos cortos de tiempo, son capaces de procesar una gran cantidad de información genómica (desde megabases a gigabases), generando datos masivos, inclusive de organismos no modelos; como es el caso de *Mugil cephalus*, o con pobres anotaciones de genes (Fang *et al*., 2012; Zhao et al., 2014; Huang *et al*., 2016), siendo la tecnología resultante denominada secuenciación de RNA (RNA-seq). Esta tecnología permite no sólo a mapear y anotar el transcriptoma (genómica estructural), sino también comprender muchos procesos biológicos (genómica funcional), como el desarrollo, respuesta inmune, neurogénesis, respuesta al estrés, entre otros, en peces teleósteos (Qian *et al*., 2014). Es por ello, que los resultados del presente trabajo sugieren un potencial para explicar los mecanismos que controlan la pleiotropía conductual que permitirán fortalecer la comprensión del contexto de regulación en la expresión de genes para la variación adaptativa entre individuos de una misma población en esta especie.

El presente trabajo de investigación aportará conocimiento básico ya que al realizar un analisis transcriptomico comparativo del cerebro de juveniles de lisa (*Mugil cephalus*) entre peces identificados con EAE proactivos o reactivos mediantes de pruebas conductuales y fisiológicas, se generarán datos novedosos sobre la regulación molecular de procesos fisiológicos asociados la respuesta al estrés en juveniles de esta especie. Por consiguiente, se podría confirmar el papel de estos genes en la regulación individual del estrés, lo cual sería útil para la industria acuícola de esta especie,para mejorar practicas de manejo y promover metodoloigas de bienestar en condiciones de cautiverio, con la finalidad apoyar e incentivar a su producción, como ya se ha hecho para mejorar la producción de la tilapia del Nilo *Oreochromis niloticus* (Vera Cruz y Brown, 2007), de la carpa común *Cyprinus carpio* (MacKenzie *et al.*, 2009; Rey *et al.*, 2016), o de la trucha arcoíris *Oncorhynchus mykiss* (Thomson *et al.*, 2011).

1.1.2. Hipótesis

Ho: Los estilos de afrontamiento al estrés (EAE) en juveniles de lisa *Mugil cephalus* presentan diferentes tipos de respuesta proactiva o reactiva, caracterizadas mediante el empleo de pruebas conductuales de respuesta al estrés, que a su vez se espera que la variabilidad interindividual se correlacione con los niveles de producción de cortisol y glucosa, así también con los patrones transcriptómicos de cerebro para cada perfil de comportamiento.

Ha: Los estilos de afrontamiento al estrés (EAE) en juveniles de lisa *Mugil cephalus* no presentan diferentes tipos de respuesta proactiva o reactiva, al emplear pruebas conductuales de respuesta al estrés, sin evidenciar que la variabilidad interindividual se correlaciona con los niveles de producción de cortisol y glucosa ni con los patrones transcriptómicos de cerebro para cada perfil de comportamiento.

1.1.3. Objetivo General

Caracterizar la variabilidad interindividual de la respuesta al estrés entre patrones consistentes de EAE de tipo proactivo y reactivo en juveniles de lisa (*Mugil cephalus*) capturadas del medio silvestre y adaptadas al cautiverio, a nivel conductual, fisiológico y molecular, mediante pruebas conductuales, cuantificación de niveles plasmáticos de cortisol y glucosa y análisis transcriptomico comparativo de la expresión génica en muestras de cerebro.

1.2. REVISIÓN DE LITERATURA

1.2.1. Lisa común (*Mugil cephalus*)

La lisa pertenece al orden Mugiliformes, familia Mugilidae, que son teleósteos actinopterigios. Dentro de la familia Mugiliade, existen 26 géneros y 80 especies (Eschmeyer y Fong, 2021), de las cuáles sólo tres especies son de importancia para la acuicultura (Crosetti, 2016). siendo la lisa común la especie más comúnmente cultivada. La lisa *Mugil cephalus* (Linnaeus, 1758) es una especie nerítica catádroma que se encuentra en todos los océanos del mundo (Whitfield *et al.*, 2012) y es considerada una candidata potencial para la diversificación de la acuicultura marina latinoamericana debido a las siguientes características: se adapta rapidemente al cautiverio en diferentes sistemas acuícolas (Nordlie, 2016), tolera variaciones amplias de salinidad y temperatura (Crosetti, 2016), siendo omnívora, acepta alimentos libres de harina de pescado mas sustentables (Gisbert *et al.*, 2016; Koven *et al.*, 2020), tiene una eficiente conversión del alimento (1.25:1, Waldrop *et al.*, 2019) en masa corporal (EUMOFA, 2020) y presenta un crecimiento relativamente interesante para una producción rentable (~ 1 kg por año) (FAO, 2019),

La lisa es un pescado comercialmente atractivo, posee una fuente proteica de alta calidad y su carne tiene buenas propiedades organolépticas (Khemis *et al.*, 2019). Además, la hueva de lisa en botargas (salada y seca de hembras grávidas) alcanza un valor considerable) en el sur del Mediterráneo y Asia, con precios que superan los 100 €/kg (Biswas *et al*., 2012; EUMOFA, 2020). En Nayarit, la lisa es una especie apreciada y consumida frito, al horno, guisado, en sopas a lo largo del estado. Particularmente, la carne y las gónadas son los productos más atractivos de esta especie en la región ya que alcanzan a duplicar o triplicar su valor, como seria: hueva de lisa ($85.00 a $110.00/kg), molleja ($40.00 a $90.00/kg), filete ($20.00 a $48.00/Kg) y frescos ($38.00 a $55.90/kg) (Ramos *et al.*, 2010; INAPESCA, 2014; PROFECO, 2022). Además de Nayarit, la lisa se consume habitualmente en los estados de Sinaloa, Nayarit, Colima, Jalisco, Oaxaca y Tamaulipas en la misma presentación (Whitefield *et al.,* 2012; CONAPESCA, 2020).

1.2.1.1. Biología y características generales

La lisa se caracteriza por habitar aguas costeras de la mayoría de las regiones tropicales y subtropicales del mundo; su distribución abarca desde el Atlántico occidental donde se le encuentra en Nueva Escocia, Canadá, hasta Brasil, incluyendo el Golfo de México. En el Atlántico oriental, habita desde la Bahía de Biscaya (Francia) hasta Sudáfrica, incluyendo el Mar Mediterráneo y el Mar Negro, por último, en el Pacífico oriental abarca desde el sur de California hasta Chile (Figura 1.2). Aunque se considera una especie marina, las lisas son eurihalinas y se pueden encontrar en un amplio rango de salinidades estuarinas, (0 hasta 75 PSU) (Nordlie, 2016).

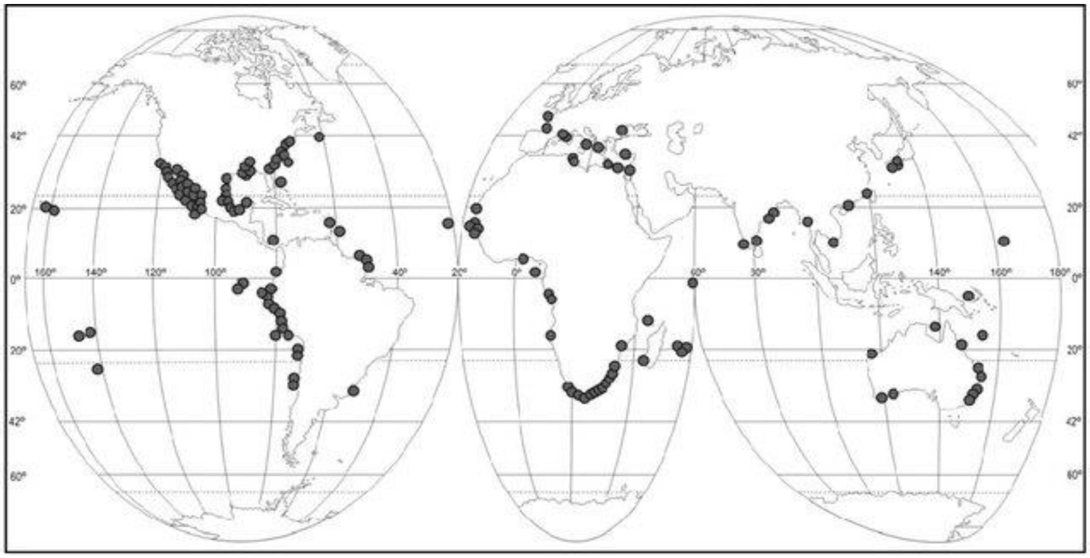

Figura 1.2. Distribución mundial de lisa *Mugil cephalus*

Morfológicamente, la lisa *Mugil cephalus* presenta un cuerpo cilíndrico en su parte anterior y algo comprimida en su extremo posterior, un lado dorsal superior de color gris azulado o verdoso que se vuelve plateado a lo largo de los laterales de su cuerpo y una línea lateral ausente (Reséndez, 1973) (Figura 1.3). La especie puede alcanzar una longitud de hasta 120 cm, lo que la convierte en la especie de lisa más grande (González-Castro y Ghasemzadeh, 2016). Es una especie gonocórica, con sexos separados, pero no presenta dimorfismo sexual externo aparente (González-Castro y Minos, 2016).

Figura 1.3. Juvenil de lisa *Mugil cephalus* (Crédito: Joel Fitzgerald Linares, 2020)

La especie se caracteriza por su cabeza ancha que está aplanada dorsalmente y su párpado adiposo grueso, suave y transparente que parece ser el más desarrollado dentro de las especies de mugilidos. El párpado adiposo cubre la mayor parte del ojo y tiene una abertura elíptica vertical. Esta especie tiene dos aletas dorsales separadas, la primera tiene cuatro espinas y la segunda tiene una espina y ocho radios ramificados. Las aletas pélvicas son sub-abdominales con una espina y cinco radios ramificados. La aleta anal tiene tres espinas y ocho radios ramificados. Sin embargo, los recuentos de espinas y radios no se pueden utilizar para diferenciarla con otras especies de la familia. Las escamas son de tipo percomorfo típico y son cicloides en los juveniles tempranos y luego se vuelven ctenoides (González-Castro y Ghasemzadeh, 2016). La especie posee un mecanismo de alimentación por filtración oral y branquial con branquiespinas y un órgano faringobranquial denticulado utilizado para la filtración del material ingerido (Cardona, 2016).

La lisa es principalmente diurna. En cuanto a sus hábitos alimenticios, las etapas larvarias son zooplanctívoras; mientras que, los juveniles y adultos son principalmente detritívoros alimentándose de microalgas bénticas, ingierendo y filtrando materia orgánica, aunque también pueden alimentarse de invertebrados y planctón (Cardona, 2016). La talla de madurez sexual registrada para esta especie es de 25 a 30 y de 27 a 35 cm de longitud estándar (LS) para los machos y las hembras, respectivamente, lo que equivaldría a 3 años aproximadamente. Sin embargo, la época de desove difiere según las zonas geográficas evitando temperaturas extremas del agua, por debajo de los 17 ºC y por encima de los 28 ºC.

Las lisas adultas migran en grandes cardúmenes desde las aguas costeras y los estuarios hasta el mar para desovar en un solo desove durante la temporada de otoño e invierno. La fecundidad total es alta y Ramos-Júdez *et al*. (2021) reportó una media de 855,800 huevos por hembra (1 ± 0,2 Kg); el cuál está dentro del rango estimado por González-Castro y Minos (2016) entre los 500,000 a 3,000.000 huevos por hembra dependiendo del tamaño del adulto, lo que correspondería a 1,473.488 huevos en individuos de 1 kg. Es una especie ovípara y los huevos pelágicos se liberan en el agua y se fertilizan. Cuando las post-larvas alcanzan los 16 - 20 mm, regresan a los ríos y estuarios (Whitfield *et al.*, 2012).

A nivel biológico, la lisa ha sido considerada como una excelente especie centinela debido a que puede tolerar ambientes con altos índices de contaminación, así como una eficiente especie para temas de biorremediación, ya que comúnmente se ha observado que consume alimento sobrante en cultivos intensivos de crustáceos y de peces marinos en jaulas flotantes (Lupatsch *et al.*, 2003). A nivel comercial, se ha establecido que las pesquerías comerciales y recreativas de lisa han ido en aumento durante la última década, en el caso de México, esta especie ha visto incrementado sus capturas y los precios han incrementado considerablemente (CONAPESCA, 2020).

1.2.1.2. Estado del cultivo

La lisa tiene una larga historia de producción y ha sido de gran importancia en varios países. Por ejemplo, los jeroglíficos del Antiguo Egipto muestran a los lugareños cultivando lisas, o también conocidos como salmonetes, hace unos 4300 años (Crosetti, 2016). Los primeros registros de datos de producción de esta especie fueron publicados en 1950 con 1,040 toneladas; el 90 % de la cual se produjo en Asia (con mas de la mitad de la produccion en China) y el reto en Europa (FAO-FIGIS, 2021). Sin embargo, los mayores niveles de producción se alcanzaron a finales de los años 90’s con la intensificación de los cultivos (Crosetti, 2016) con un máximo de 9,585 toneladas en 1997 (FAO-FIGIS, 2021). A la fecha, la producción de lisa no ha alcanzado ese nivel de forma consistente. En 2019 la producción mundial de lisa fue de 6,124 toneladas, con un crecimiento medio anual del 0,05% en la última década (2009 - 2019). Asia ostentaba más del 90 % de la producción acuícola mundial de lisa en el 2019 y los países que destacán con una mayor producción son: Taiwán (2,182 toneladas), Israel (2,147 toneladas), China (984 toneladas), Singapur (500 toneladas), Túnez (247 toneladas) y Arabia Saudita (60 toneladas) (FAO-FIGIS, 2021).

Actualmente, la producción de lisa sigue dependiendo en gran medida de las capturas silvestres (Aizen *et al.*, 2005; Yousif *et al.*, 2010; Cardona, 2016). La experiencia con la recolección de juveniles silvestres y los altos costos de producción de su cultivo han obstaculizado el desarrollo de criaderos comerciales de esta espécie. Adicionalmente, el suministro de huevos para los criaderos se obtiene principalmente de reproductores silvestres capturados en etapas avanzadas de vitelogénesis y son inducidos a desovar (Das *et al.*, 2014; Vazirzadeh y Ezhdehakoshpour, 2014; Karim *et al.*, 2016). La captura de ejemplares silvestres y de la actividad pesquera son prácticas poco confiables e impredecibles en su regularidad de obtención de animales, ya que los organismos silvestres están disponibles estacionalmente. Ante ello, la inducción artificial a la puesta y la fecundación asistida representa la solución la mas sustentable para obtener un suministro constante de alevines (Crosseti *et al.*, 2016a; Ramos-Júdez *et al*., 2020). Por ejemplo, en India, esfuerzos en investigación y desarrollo (I+D) relacionado al cultivo de lisa en cautiverio ha permitido un avance exitoso en la cría por parte del Instituto Central de Acuicultura de Aguas Salobres (CIBA, por sus siglas en inglés) durante el periodo 2016-2020, permitiendo la entrega de juveniles producidos en criaderos a potenciales productores emprendedores de los estados de Andhra Pradesh, Tennessee y Kerala, con el objetivo de lograr la expansión del cultivo de *M. cephalus* (Ashok, 2021)

En términos de sistema de cultivo, la producción de lisa se realiza habitualmente en sistemas extensivos de lagunas costeras (131 - 350 kg. ha^{-1}), estanques de tierra semi-intensivos (1-8 ton. ha^{-1}) o jaulas flotantes (3 kg.m^{-3}). Adicionalmente, se ha reportado que policultivos con especies como tilapias (*Oreochromis niloticus*), camarones (*Litopennaeus vannamei*) o carpas (*Cyprinus carpio*) es viable para esta especie (Sadek, 2015; Saleh, 2008).

1.2.2. Estrés en peces

El estrés se define como "*una condición en la cual la homeostasis de los organismos se ve alterada como resultado de las acciones de estímulos* *intrínsecos o extrínsecos y que se restablece por una secuencia de respuestas adaptativas de los organismos"* (Moberg, 2000; Shreck, 2010). Los estudios relacionados al estrés se basaron inicialmente en trabajos realizados en mamíferos, pero su relevancia para los peces fue establecida por Mazeaud *et al*. (1977), concluyendo que los principales mecanismos de control neuroendocrino de la respuesta al estrés son comparables a los de los mamíferos y otros animales terrestres y, por lo tanto, se ajustan a un patrón general de vertebrados (Donaldson, 1981; Pickering, 1981).

Los peces se enfrentan con frecuencia a situaciones de estrés tanto en la naturaleza como en condiciones controladas, como es la acuicultura. La percepción sensorial de un agente adverso es un prerrequisito para provocar una respuesta fisiologica y endocrina, tanto en peces como en otros vertebrados (Schreck, 2010). Los posibles factores de estrés en la naturaleza incluyen la amenaza por depredación, la escasez o dificultad para obtener alimentos, los cambios rápidos e impredecibles en el medio ambiente y el establecimiento y mantenimiento de jerarquías sociales. En la acuicultura, los factores estresantes comunes a los que se enfrentan los peces incluyen redes, manipulación, tratamientos veterinarios, infecciones parasitarias, mala calidad del agua, así como hacinamiento y métodos de transporte (Martos-Sitcha *et al*., 2020). Estos factores de estrés pueden definirse como agudos (a corto plazo), causada principalmente por la captura y el manejo de los peces, biometrías, transporte o crónicos (prolongados), causada por agentes persistentes, como son las altas densidades de cultivo, variación en la calidad del agua, la exposición a nuevos ambientes, la dominancia social de los peces. En consecuencia, la capacidad de lidiar con el estrés puede ser crucial para volverse socialmente dominante o para mantener o recuperar el rango social (Dingemanse y de Goede, 2004).

1.2.2.1. Rol del cerebro en respuesta al estrés

El cerebro es el órgano central de los organismos y tiene la capacidad de interpretar las experiencias como adecuadas y riesgosas en los organismos.

Asimismo, está involucrado en la respuesta y adaptación al estrés (McEwen, 2009; Vernier, 2017). Entre otras funciones, el cerebro es el encargado de procesar constantemente la información, clasificar las entradas sensoriales relevantes e iniciar las reacciones a situaciones de estrés. Estas reacciones incluyen la activación del sistema neuroendocrino, autónomo e inmune, que es vital para recuperar la homeostasis (Johnson et al., 1992), ya que las áreas cognitivas superiores del cerebro, como por ejemplo el telencéfalo, juega un papel importante en la memoria y la toma de decisiones; mientras que los lóbulos ópticos son áreas del cerebro que procesan la información visual (McEwen, 2007). Por tanto, el cerebro puede brindar estabilidad a través del cambio, o alostasis, refiriéndose a los ajustes conductuales, fisiológicos y bioquímicos consecutivos de un organismo para mantener la homeostasis y así lograr la supervivencia (McEwen, 2007). Los principales mediadores para este aspecto son las hormonas que incluyen: corticosteroides, catecolaminas y citoquinas. Cuando los organismos estan sometidos a un estímulo de estrés, se activan varias redes neuronales; entre ellas, el sistema serotoninérgico y dopaminérgico, para liberar o producir los metabolitos principales de la serotonina (5-HIAA, por sus siglas en inglés) y dopamina (DA, por sus siglas en inglés) (Gesto *et al*., 2015). En el caso de la serotonina, al estimular los receptores postsinápticos va a activar una respuesta de la hormona adrenocorticótropa (ACTH), del cortisol y de la prolactina a través del eje HPI permitiendo una regulación de las respuestas osmorreguladores, hematológicas, de comportamiento y neuroendocrina al estrés (Backström y Winberg, 2017); mientras que la presencia de dopamina en el hipotálamo de los peces induce una regulación positiva de la expresión de la horomona liberadora de corticotropina (CRH), lo que sugiere que el efecto de la activación del sistema dopaminérgico en condiciones de estrés podría estar mediado por las neuronas del CRF hipotalámico (Conde-Sieira *et al*., 2018).

En situaciones de estrés crónico, o prolongado, el cerebro se somete a una sobrecarga alostática, es decir, a un sobrefuncionamiento en la forma en que este órgano y los sistemas neuroendócrinos responden al estrés adicional (McEwen, 2007; Vargas *et al*., 2018). Un aumento en la carga alostática generalmente es seguido por un aumento en las concentraciones de glucocorticoides, que posteriormente pueden desencadenar procesos fisiológicos y de comportamiento que ayudan al organismo a disminuir la carga alostática, permitiendo que cada uno evite o resista el peligro potencial del estrés crónico (Goymann y Wingfield 2004).

Este incremento de hormonas, en particular la secreción de cortisol en peces teleósteos es dominado por el eje hipotálamo-hipofisario-interrenal (HHI), homólogo al eje hipotálamo-hipofisario-adrenal (HHA) en mamíferos, pero los peces teleosteos no poseen una glándula adrenal compacta como los mamíferos, sino un conjunto difuso de células interrenales en la porción anterior del riñón cefálico (Barandica y Tort, 2008). Subsecuente al eje HHI, se encuentra un segundo eje fisiológico implicado en el conjunto de la respuesta a los factores estresantes, que es el eje Hipotálamo-Simpático-Cromafín (HSC), donde se produce la síntesis y liberación de catecolaminas, que son la adrenalina y la noradrenalina, y que están estimuladas por las fibras colinérgicas del sistema simpático. Las catecolaminas son sintetizadas en la pared de la vena cardinal posterior en la región del riñón anterior (Reid *et al.*, 1998) y activan respuestas de tipo cardiovascular, respiratoria y metabólica, dirigidas a mitigar los efectos de factores estresantes agudos. Este sistema de respuesta es inmediato tras percibirse el estímulo, mientras que la liberación de las hormonas glucocorticoides toma mas tiempo, regularmente entre 1 a 3 minutos después de la percepción de la situacion de estrés agudo (Wendelaar Bonga, 1997; Perry *et al*., 2004). La hormona liberadora de la corticotropina (CRH, por sus siglas en inglés) es la hormona iniciadora de la activación del eje HHI, siendo liberada por neuronas hipotalámicas de la región pre-óptica. En peces se ha encontrado junto a otra hormona, la hormona liberadora de la tiroides (TRH, por su sigla en inglés). Ambas hormonas estimulan la liberación de la hormona adenocorticotropa (ACTH, por su sigla en inglés) por la parte anterior de la glandula pituitaria o hipófisis en el torrente sanguineo, hasta inducir la producción y liberación del cortisol en la sangre por las células interranales (Pepels *et al*., 2002). Esta cascada neuroendocrina se conoce como respuesta primaria al estrés (Figura 1.4).

Durante el inicio del estrés, el cortisol induce varios procesos catabólicos que proporcionan energía a las diferentes acciones fisiológicas que ocurren para restaurar la homeostasis (Mommsen *et al*., 1999). El cortisol liberado al torrente sanguíneo junto a otras hormonas del estrés moviliza y elevan la producción de glucosa en los peces a través de las vías de glucogénesis y glucogenólisis (Iwama *et al*., 1999) para hacer frente a la demanda de energía producida por el factor estresante para la reacción de "lucha-escape". Esta producción de glucosa es principalmente mediada por la acción del cortisol que estimula la gluconeogénesis hepática y también detiene la captación periférica de azúcar (Wedemeyer *et al*., 1990). Luego, la glucosa se libera (desde el hígado y los músculos) hacia la circulación sanguínea y entra en las células a través de la acción de la insulina (Nelson y Cox, 2005). Toda esta acción metabólica del cortisol en los peces está mediada por los receptores de glucocorticoides (GR), que en la mayoría de los peces teleósteos presentan dos isoformas (GR1 y GR2) que se localizan en una gran cantidad de tejidos centrales y periféricos (Teitsma *et al*., 1997). Estas respuestas, que son de tipo secundarias, incluyen las respuestas cardiovasculares y respiratorias (Rodnick y Planas, 2016), que aumentan la distribución de oxígeno y los sustratos de energía que se liberan en la circulación como resultado de la respuesta al estrés. Otras respuestas secundarias que acompañan se mencionan en la Figura 1.4.

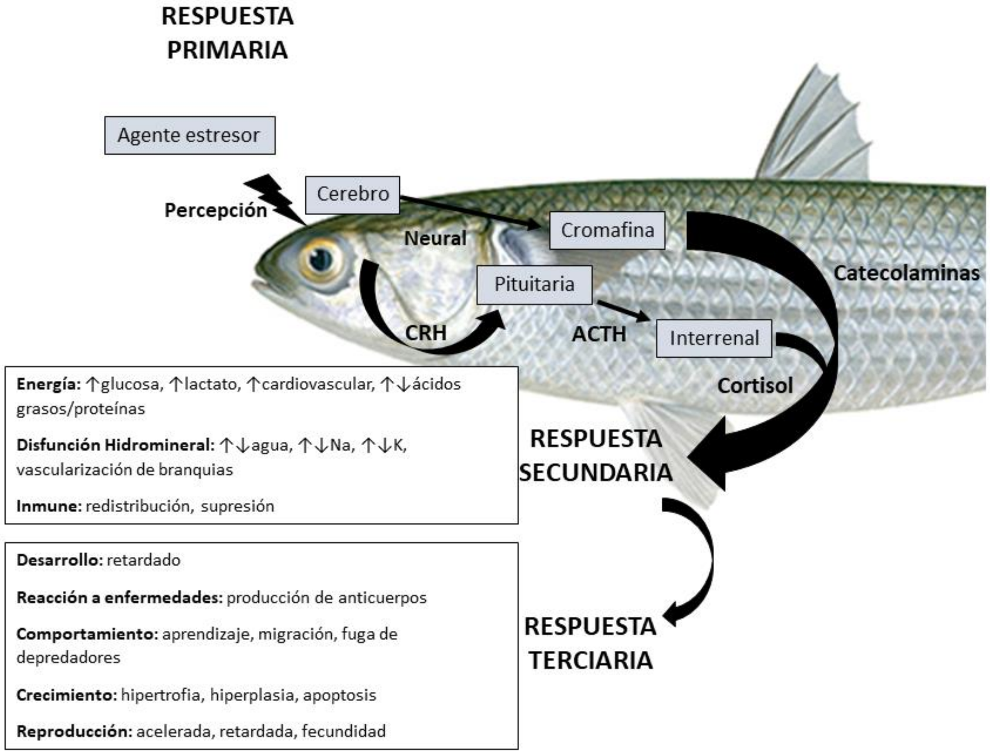

Figura 1.4. Rol del Cerebro en la activación de respuestas neuroendocrinas al estrés (Adaptado de Schreck y Tort, 2016).

Adicionalmente a un agente estresor, las concentraciones de cortisol varían en un organismo en función a su ciclo circadiano (Brüning *et al*., 2015). En condiciones sin estrés, los glucocorticoides exhiben una secreción temporal en los mamíferos y peces teleósteos, con un ritmo circadiano que se superpone a un ritmo ultradiano (Walker *et al*., 2010; Spiga *et al*., 2014). La secreción de cortisol fluctúa con el ciclo luz-oscuridad y está claramente relacionada con el patrón de comportamiento diario, observándose un aumento al comienzo de la fase de la actividad diaria. El sistema nervioso simpático y el núcleo supraquiasmático central (SNC, por sus siglas en inglés) controlan tales variaciones diarias a través del eje HHI (Haus, 2007; Tonsfeldt y Chappell, 2012). Sin embargo, cada vez más la evidencia ha revelado que múltiples componentes del sistema circadiano, y particularmente el reloj suprarrenal/interrenal, están involucrados en la regulación del ritmo de los glucocorticoides en los mamíferos principalmente (Son *et al*., 2011; Spiga *et al*., 2014); mientras que en peces no es evidente, informándose una amplia variedad de patrones diarios de cortisol plasmático según la especie, el fotoperíodo, la estación y los patrones de alimentación y actividad (Isorna *et al*., 2017). Es importante mencionar que el cortisol también controla una variedad de respuestas bioquímicas, fisiológicas y conductuales, como el metabolismo energético, la regulación osmótica de iones, la inmunidad y el crecimiento (Mommsen *et al*., 1999, Ellis *et al*., 2012).

Consecuentemente, si un animal puede hacer frente eficazmente a un factor estresante mediante la adaptación o si se trata de un estres agudo de corta duración, la homeostasis puede restablecerse sin consecuencias graves para el animal y sin afectar su salud, bienestar, crecimiento o supervivencia. Por el contrario, si el factor estresante tiene una naturaleza crónica, y la adaptación al factor estresante no es eficiente o incluso ausente, la respuesta al estrés de tipo terciario podrá afectar a la ingesta alimentaria, a la resistencia a enfermedades, al crecimiento, a la reproducción o a la supervivencia de los animales y como consecuencia impactar parámetros productivos de manera negativa y generar perdidas económicas para granjas acuicolas (Wendelaar Bonga, 2011).

Respeto a la regulación fisiológica de estos ejes HHA y HHI, estudios de fisiología comparativa del estrés sugieren que el factor liberador de la corticotropina (*crf*, por sus siglas en inglés) en el hipotálamo juega un papel clave en la regulación e integración de los sistemas neuroendocrino, autónomo, inmune y del comportamiento como respuesta a estímulos estresantes (Barandica y Tort, 2008) y está mediada por al menos dos receptores (*crfr1* y *crfr2*) que se encuentran en diferentes regiones del cerebro. Se ha informado que el receptor *crfr1* regula la activación del eje HHA/HHI, mientras que el receptor *crfr2* participa en la expresión de varias reacciones conductuales y fisiológicas en respuesta al estrés, como la estimulación de las vías noradrenérgicas (Backström y Winberg, 2013; Flik *et al.*, 2006). Además, la proteína de unión a CRF (*crf-bp*) puede desempeñar un papel regulador en el eje de HPI al modular la biodisponibilidad de CRF y péptidos relacionados (Manuel *et al*., 2014; Seasholtz *et al*., 2002). Asimismo, de manera similar a los mamíferos, el receptor serotoninérgico 5-HT en peces teleósteos afecta la liberación de CRF del hipotalámo, donde el receptor 5-HT tipo 1A (5HT1A) también desempeña un papel central en la regulación del eje HHA/HHI (Höglund *et al.*, 2005; Medeiros *et al.*, 2010). Además, el eje HHI está bajo control de retroalimentación por cortisol, a través del receptor de mineralocorticoides (MR) y del receptor de glucocorticoides (GR) en el hipotálamo y la hipofisis (Bury *et al.*, 2003; Sturm *et al.*, 2005). Asimismo, el aumento de la CRF provoca la liberación de otras hormonas como endorfinas y los péptidos derivados de la hormona pro-opiomelanocortina (POMC, por sus siglas en inglés), cuya función es codificar para un precursor multipeptídico que a través de un complejo procesamiento postraduccional específico da lugar a una variedad de hormonas y neuropéptidos entre los cuales se encuentra la hormona adenoconicotrófica (ACTH, por sus siglas en inglés), la hormona estimulante de los melanocitos (MSH), β-lípotrofina (β-LPH), las formas opioides y no opioides de β-endorfina (Civelli et al., 1982). ACTH y MSH son los principales candidatos para el control hipofisario de la liberación de cortisol (Hoglund *et al*., 2000) y se producen en diferentes células de la hipófisis, pero todos comparten la POMC como molécula precursora (Wendelaar Bonga 1997), tal como se menciona anteriormente.

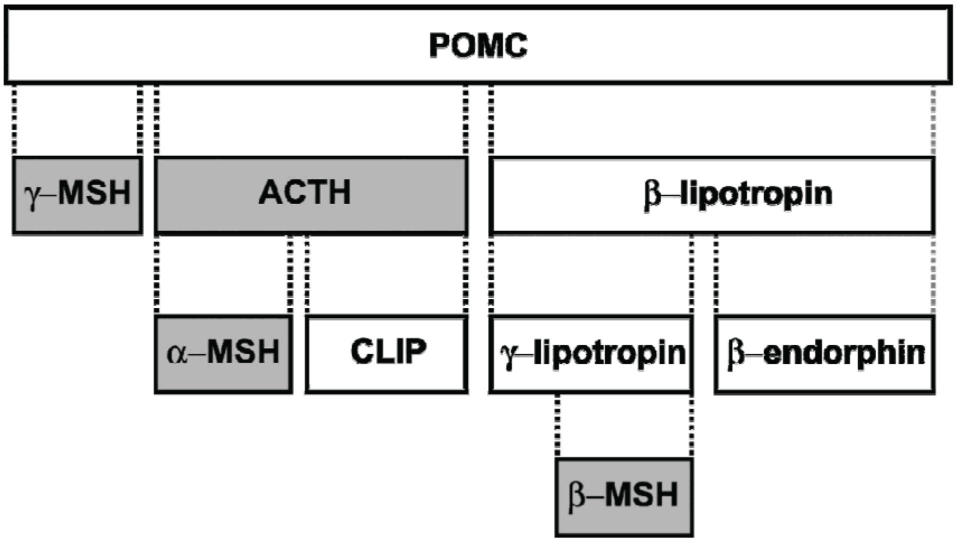

Figura 1.5. Hormonas y neuropéptidos opioides derivados de la escisión de proopiomelancortina (POMC) (Tomado de Tesis Doctoral en Acuicultura de Martin Iversen, 2013, Universidad de Nordland, Noruega).

1.2.3. Estilos de afrontamiento al estrés (EAE)

Los Estilos de Afrontamiento al Estrés (EAE) se definen como las estrategias de comportamiento y respuestas fisiológicas que exhiben individuos, de una misma población, a situaciones de estrés y que son consistentes a lo largo del tiempo y entre contextos (Koolhaas *et al*., 1999; Øverli *et al.,* 2007). Koolhaas *et al*. (1999) han reconocido dos tipos de respuestas al estrés: proactivo y reactivo, cuyas diferencias se detallan en el cuadro 1.1. La evaluación de los EAE cobró impulso a fines de la década de 1970 en animales de granja, con el trabajo publicado por Henry y Stephens (1977), donde aplicaron el concepto de EAE al ganado doméstico (*Bos taurus*) y comenzaron a relacionar los distintos rasgos individuales con el afrontamiento al estrés bajo condiciones de cría con la finalidad de evaluar los diferentes acontecimientos que ocurren durante la interacción social que pudieran provocar una conducta de afrontamiento adaptativa.

Asimismo, Huntingford (1976) realizó uno de los primeros estudios que documentaron la variación individual en el comportamiento en peces teleosteos, en el que estudió las diferencias en la agresividad en el pez espinoso (*Gasterosteus aculeatus*). Los autores reportaron, en dicho estudio, que los individuos proactivos eran más agresivos con sus conespecíficos (relación consistente en la pertenencia a una misma especie) y con los heteroespecíficos (relación consistente en la pertenencia entre individuos de diferentes especies). Aunque el término "estilo de afrontamiento al estrés" nunca se aplicó a lo largo del artículo, el concepto aflora en este estudio, donde se observó consistencia en el comportamiento agresivo y proactivo de ciertos peces a lo largo del de la temporada de reproducción, así como consistencia en el comportamiento agresivo de peces con sus conespecificos y con los heteroespecíficos, en acuerdo con la definición de la estrategia de EAE proactiva posteriormente dada por Koolhaas *et al*. (1999).

Cuadro 1.1. Diferencias entre peces proactivos y reactivos (adaptado de la revisión Estilos de Afrontamiento al Estrés en peces en cultivo: Consecuencias para la acuicultura de Castanheira *et al.*, 2016)

A) Características Comportamentales	***Proactivo***	***Reactivo***	***Referencias Bibliográficas***
Eficiencia de Alimentación	Alto	Bajo	Martins *et al.,* 2005a, b, c ; 2006a, b ; Van de Nieuwegiessen *et al.,* 2008.
Agresividad	Alto	Bajo	Øverli *et al.,* 2004a, 2005 ; Castanheira *et al.*, 2013a.
Influencia Social	Bajo	Alto	Magnhagen, 2007; Magnhagen y Staffan, 2005; Magnhagen y Bunnegeld, 2009.
Zona de riesgo y Exploración	Alto	Bajo	Huntingford *et al.,* 2010 ; Øverli *et al.,* 2006a, b ; MacKenzie *et al.,* 2009 ; Millot *et al.*, 2009a, b.
Escape activo al estresor	Alto	Bajo	Silva *et al.,* 2010 ; Martins *et al.*, 2011c ; Breling *et al.*, 2005 ; Laursen *et al.,* 2011.
Plasticidad/Flexibilidad/Formación rutina	Bajo	Alto	Chapman *et al.,* 2010 ; Ruíz-Gomez *et al.,* 2011.
B) Características fisiológicas			
Reactividad HPI	Bajo	Alto	Castanheira *et al.,* 2013a ; Øverli *et al.,* 2006a, b.
Respuesta al estrés por cortisol	Bajo	Alto	Overli *et al.*, 2004; Kittilsen *et al.*, 2013; Backstrom *et al.*, 2014
Inmunidad	Alto	Bajo	Kittilsen *et al.,* 2012.
Consumo de Oxígeno	Alto	Bajo	Herrera *et al.*, 2014 ; Killen *et al.,* 2011 ; Marton *et al.,* 2011d.
Reactividad Simpática	Alto	Bajo	Schjolder *et al.,* 2006; Verbeek *et al.,* 2008; Barreto y Volpato 2011.

Plasticidad Neuronal	Bajo	Alto	Johansen *et al.,* 2012.
Reactividad Parasimpática	Bajo	Alto	Verbeek *et al.,* 2008; Barreto y Volpato 2011.

Actualmente, la caracterización de EAE se ha realizado en diferentes especies de interés acuícola, como en el caso de la dorada (*Sparus aurata*) (Castanheira *et al*., 2013a, b; Herrera *et al*., 2016), lubina europea (*Dicentrarchus labrax*) (Millot *et al.,* 2009), trucha arcoíris (*Oncorhynchus mykiss*) (Øverli *et al.*, 2006; Ruiz-Gómez *et al.*, 2011; Laursen *et al.*, 2011), carpa común (*Cyprinus carpio*) (MacKenzie *et al.*, 2009; Huntingford *et al.*,2010), tilapia del Nilo (*Oreochromis niloticus*) (Barreto y Volpato, 2011; Martins *et al.*, 2011a), salmón del Atlántico (*Salmo salar*) (Kittilsen *et al.*, 2012), lenguado senegalés (*Solea senegalensis*) (Ibarra-Zatarín *et al.*, 2015, Fatsini *et al.*, 2017) y lisa común (*Mugil cephalus*) (Jiménez-Rivera *et al.*, 2023).

Referente a las metodologías o pruebas que se han adaptádo y aplicado a los peces para caracterizar conductualmente sus EAE, existen: 1) pruebas individuales, como pruebas de restricción; pruebas de objeto nuevo (Basic *et al.*, 2012); pruebas de exploración (Killen *et al.*, 2011); pruebas de amenazas externas (Øverli *et al*., 2002; Brelin *et al*., 2005) y 2) pruebas grupales, como prueba de hipoxia (Laursen et al., 2011) y prueba de riesgo o exploración (Millot *et al*., 2009). Adicionalmente, junto con las respuestas conductuales, se miden las respuestas fisiológicas, como la produccion de cortisol, la frecuencia cardíaca (Korte *et al*., 2005), la ulceración gástrica o la vocalización (Van Erp-van der Kooij *et al*., 2003; Van Reenen *et al*., 2005; Spake *et al*., 2012). Además, se están desarrollando nuevos estudios como herramientas para caracterizar EAE y rasgos de personalidad en peces basados en características morfológicas y moleculares, como el patrón de pigmentación (Kulczykowska, 2019) y marcadores genéticos de estrés (Rexroad *et al*., 2012).

Comprender las consecuencias de los EAE en peces resulta importante para la acuicultura, ya que se ha demostrado relaciones entre tipos proactivos y reactivos de respuesta al estrés y factores de interés comercial acuícola. Por ejemplo, se ha observado en trucha arco iris *O. mykiss* y tilapia *O. niloticus* (Øverli *et al.,* 2002a; Martins *et al.,* 2011a, b), que los individuos proactivos son los que presentan una rápida reanudación de ingesta de alimentos y una mayor tasa de crecimiento que en individuos reactivos de una misma población, después de una transferencia a un nuevo ambiente. Asimismo, Vargas et al. (2018) observaron una propensión mas elevada en ejemplares de dorada con perfil proactivo a la inducción de respuestas proinflamatorias en los tejidos branquiales en las etapas iniciales de una vacunación, permitiendo reducir el impacto de la vibriosis entre la población, tanto en entornos naturales como artificiales.

Entre otras investigaciones, Castanheira *et al*. (2016) encontraron que la calidad espermática evaluada a través del valor porcentual de la motilidad espermática en reproductores proactivos de dorada (*Sparus aurata*) era mayor que en los reactivos. Finalmente, Ibarra-Zatarain *et al*. (2020) reportaron en lenguado senegalés (*Solea senegalensis*) que los juveniles proactivos de esta especie iniciaron la pubertad y maduraron sexualmente en menor tiempo que los juveniles reactivos. Por tanto, aplicar técnicas de cultivo basadas en respuestas conductuales como los EAE, permitirá reducir los efectos causados por los sistemas de cultivo e impulsará la mejora de los métodos de domesticación a partir de características conductuales y fisiológicas.

1.2.3.1. Consistencia en EAE

La consistencia de las diferencias individuales es un elemento clave para identificar los EAE en los peces; y deben ser: **i)** a lo largo del tiempo, es decir que el comportamiento individual en un contexto determinado y en en un momento dado se correlacionacon el comportamiento en el mismo contexto en un momento posterior; y, **ii)** entre contextos, es decir que la conducta individual expresada en un contexto y un momento dado se correlaciona con la conducta expresada en otros contextos en el mismo momento distintos, en la medida que el comportamiento en todos los contextos se mide a la misma etapa de desarrollo (Stamps y Groothuis, 2010).

El estudio de estas asociaciones de rasgos consistentes en el comportamiento y las respuestas fisiológicas a los desafíos estresantes están asociados con la variación individual adaptativa (Koolhaas *et al*., 1999; Korte *et al*., 2005; Réale *et al*., 2007). La comprensión de esta variación interindividual es esencial para mejorar nuestro conocimiento del valor adaptativo del comportamiento y de los rasgos fisiológicos (Koolhaas et al. 1999). En la literatura científica, estas diferencias individuales consistentes se denominan bajo diferentes términos, entre los que se encuentran: personalidad (Gosling, 2001), temperamento (Réale *et al*., 2007), síndromes de comportamiento (Sih *et al*., 2004) o EAE (Koolhaas *et al*., 1999). Sin embargo, esto no excluye la posibilidad de que los individuos modifiquen dichas diferencias (Balasch y Tort, 2019). De hecho, la plasticidad individual, resulta muy importante ya que permite a los individuos ajustar su comportamiento a entornos novedosos o inestables (Castanheira *et al*., 2017).

Pocos estudios han evaluado la consistencia vinculada a organismos con perfil proactivos y/o reactivos. Asimismo, la contribución de la consistencia de los EAE en la industria acuícola es muy amplia desde el hecho que los individuos dentro de una población a menudo difieren en la manera con la que responden, fisiológica y conductualmente, a condiciones de estrés. El hecho de que peces bajo condiciones de cultivo no lleguen a adaptarse puede generar problemas relacionados con la producción (por ejemplo, crecimiento y resistencia a enfermedades). Bajo esta premisa, Réale *et al*. (2007) sugirieron que existe una escasez de información acerca de la existencia de comportamientos individuales especificos en diversas especies acuicolas y a su consistencia a lo largo del tiempo y entre contextos. Por ejemplo, Castanheira *et al.* (2016) investigaron la consistencia del mismo comportamiento de escape en una prueba de restricción en la dorada (*S. aurata*), en diferentes contextos sociales, donde se observó un rasgo de personalidad consistente en los grupos de EAE intermedios y grupos controles (consistieron en grupos mixtos con 1/3 de cada estilo de afrontamiento); mientras que no se encontró correlación en los grupos proactivos y reactivos, demostrándose que los contextos sociales en los que se crían peces influyen significativamente en los rasgos de personalidad de los individuos y que pueden modificarse por la influencia de otros miembros del grupo. Sin embargo, en un trabajo realizado por Ibarra-Zatarain *et al*. (2020), demostraron que el lenguado senegalés (*Solea senegalensis*) presentó un alto grado de consistencia entre contextos en su respuesta a situaciones de estrés. Asimismo, observaron que los ejemplares tanto proactivos como reactivos tienen oportunidades similares de reproducción, es decir, de llegar a desovar, y, por lo tanto, sugirieron que la conservación de ambos EAE probablemente concede a esta especie ventajas especificas tanto para proactivos como reactivos para maximizar sus oportunidades de reproducción, adaptación y subsistencia de su futura progenie a diferentes situaciones ambientales.

1.2.3.2. EAE y Expresión génica

A medida que los animales responden a los agentes estresores de manera fisiológica y comportamental, estudiar los cambios en la expresión de genes asociados a la respuesta fisologica y conductual a un agente estresor génica permite elucidar los mecanismos moleculares subyacentes al enfrentamiento al estrés y su variabilidad entre individuos. Por ejemplo, Mackenzie *et al*. (2009) evaluaron los efectos sobre la expresión génica ante un desafío inflamatorio con lipopolisacárido bacteriano (LPS, por sus siglas en inglés) intraperitoneal en la carpa común (*Cyprinus carpio*) ya que este desafío causa una reorganización significativa del sistema inmunológico y la remodelación transcriptómica posterior en subconjuntos relevantes de células, considerándola como uno de los eventos de reorganización más fuertes en los sistemas fisiológicos. Los peces fueron colocados en grupos de 70 en acuarios de 35 L conectados a un sistema de recirculación. Después de acondicionados durante 1 semana, se restringió la alimentación durante al menos 12 h y se procedió a la realización de la prueba grupal de toma de riesgo. Para esto, se fueron seleccionando a los peces aleatoriamente en grupos de 10 y transfiriéndolos a un nuevo tanque el cuál estaba dividido en 2 áreas (aclimatación y recolección), las cuáles estaban unidas en la base con un tubo de salida que contenía una compuerta para abrir/cerrar (diámetro 10 cm). Se dejó a los peces en el área de aclimatación durante 5 min para posteriormente abrir la compuerta en 2 fases. Las primeras 3 carpas que cruzaron o despúes de un periodo de 10 min fueron clasificados como proactivos; repitiendólo nuevamente y clasificando a los peces que cruzaron en esta segunda fase como intermedios, y finalmente los peces que no cruzaron fueron clasificadas como reactivos; descartándose al grupo de intermedios. Posteriormente, los peces clasificados se mantuvieron en grupos de 20 (10 reactivos y 10 proactivos) en tanques de 35 L. Después de 6 semanas, 6 peces proactivos y 6 peces reactivos fueron expuestos al agente inflamatorio LPS intraperitoneal. A otros 6 peces de cada EAE se les administró una inyección simulada (NaCl al 0,9%). Después de ≈ 20 horas se anestesiaron los peces, se sacrificaron y se recolectaron muestras de branquias, cerebro, riñón e hígado para el análisis de ciertos genes, como la gliceraldehido 3 fosfato deshidrogenasa o *gapdh*, la fosfopiruvato hidratasa o enolasa, el receptor de cortisol, el factor de necrosis tumoral alfa o *tnf-α* o la interleucina 1 beta *il-1β*. En respuesta a este desafío, los dos grupos de peces caracterizados mostraron respuestas opuestas para el 80% de los genes investigados, siendo por ejemplo, que los resultados mostraron aumentos en la expresión de *tnf-α* en las carpas proactivas. Estos datos contribuyen directamente a comprender la biología de las citocinas proinflamatorias en peces, lo que sugiere que existen diferencias fundamentales en la regulación de las citocinas en peces con diferentes EAE.

Rey *et al*. (2013) reportaron que el comportamiento podría estar relacionado con la expresión génica a través de la modulación de funciones biológicas y fisiológicas explicando el fenotipo conductual. Diversos trabajos han documentado la expresión de genes relacionados con los mecanismos neuroendocrinos de la respuesta al estrés y se han asociado al dominio social en algunas especies de peces (Øverli *et al*., 1999; Winberg *et al*., 2001; Øverli *et al*., 2004b; Teles *et al*., 2013). Por ejemplo, en reproductores de pez cebra *Danio rerio*, se reportó una expresión diferencial de varios genes (dopamina ß-hidroxilasa, dbh; DOPA descarboxilasa, ddc y arginina vasotocina, avt) en el cerebro entre machos dominantes y machos subordinados y estas diferencias pueden reflejar el rango social, lo que podría resultar en ventajas biológicas, como la oportunidad para fecundar hembras y el éxito reproductivo (Pavlidis *et al*., 2011). Además, otros genes, como el factor neurotrófico derivado del cerebro (*bdnf*) y los factores de transcripción (*c-fos*) y el de diferenciación neurogénica (*neuroD*) se han asociado con la regeneración y plasticidad neuronal y el estado social en pez cebra (Teles *et al*., 2016). Asimismo, los genes codificando por el receptor de la serotonina (5Hydroxytryptamine; *5HT*), el triptófano hidroxilasa 1 (*tph1*) y el miembro 13 de la familia de portadores de solutos 6 (*slc6a13*) también han sido asociados con el control de varios aspectos conductuales, como la agresión y la impulsividad (Ho *et al*., 1998; Øverli *et al*., 1999; Lesch y Merschdorf, 2000; Koolhaas *et al*., 2007; Silva *et al*., 2014).

Fatsini *et al*. (2017) reportaron que juveniles de lenguado senegalés (*Solea senegalensis*) mostraron una expresión diferencial de ciertos genes (*nrd2* y *c-fos*) entre peces caracterizados con diferentes perfiles de EAE (proactivos, reactivos e intermedios), lo que coincide con estudios previos sobre la variabilidad entre EAE en aspectos de sociabilidad y dominio. Asimismo, Johansen *et al*. (2011) reportaron por primera vez una elevación de la expresión de receptores de glucocorticoides (*gr*, por su sigla en inglés) y receptores mineralocorticoides (*mr*, por su sigla en inglés) en diferentes regiones cerebrales de trucha arco iris (*Oncorhynchus mykiss*) después de unestrés, inducido a través de la prueba individual de hacinamiento. La actividad locomotora en esta prueba conductutal permitió caracterizar los EAE entre líneas de selección de respuesta baja (LR, por sus siglas en inglés) y respuesta alta (HR, por sus siglas en inglés) (Overli *et al*., 2006; Kittilsen *et al*., 2009). Los peces LR se caracterizan por un perfil de comportamiento más proactivo (agresivo y audaz) y menos flexible que los peces HR (Moreira *et al*., 2004; Schjolden *et al*., 2006; Ruiz-Gómez *et al*., 2011).

Concluyeron que los altos niveles de cortisol post-estrés se asociaban a una expresión génica de *gr* y *mr* alterada negativamente en peces salmónidos. Asimismo, observaron una expresión diferencial de *mr* entre líneas LR y HR y lo relacionaron con diferencias en la retención de la memoria observados entre las líneas LR y HR).

Además de los genes anteriormente citados, se conocen otros genes involucrados en el metabolismo lipídico es el proliferador de peroxisomas (*pparβ*), el cuál está implicado en las funciones esqueléticas, cerebrales y de la piel en mamíferos (Lee *et al*., 2003; Giaginis *et al*., 2007). Además, tiene funciones en el transporte de ácidos grasos y vitamina A mediante lipoproteínas, en la regulación del metabolismo de lípidos, ha sido asociado con el comportamiento de alimentación y su expresión está influenciada por la nutrición en peces como la dorada (Fernández *et al*., 2011) y la lubina (Vagner *et al*., 2009).

1.2.4. Transcriptoma

El transcriptoma es el contenido total de ARN de la célula en un momento dado, correspondiente a la expresión génica instantánea (*snapshot* en inglés). La transcriptómica es la secuenciación del transcriptoma, que la convierte en una opción ideal para la interpretación rápida y rentable del elemento funcional del genoma y los genes de función conocida en las especies no modelo que carecen de un genoma de referencia (Garg *et al.*, 2011). Los objetivos clave de la transcriptómica son: catalogar todos los productos de la transcripción del ADN, lo que incluye a los ARNm, los ARN no codificantes y los ARN cortos para: 1) determinar la estructura transcripcional de los genes, patrones de empalme y otras modificaciones pos-transcripcionales (genómica estructural); 2) cuantificar los niveles de expresión de cada gen durante el desarrollo de cualquier ser vivo y en diferentes condiciones (genómica funcional) (Wang *et al.*, 2009).

En estos últimos años, se han desarrollado diversas tecnologías para cuantificar el transcriptoma, incluyendo los enfoques en hibridación de ácidos nucleicos y los de secuenciación del genoma o transcriptoma. Entre los métodos que permite una aproximación al perfil transcriptómico es la secuenciación del ARN (RNA sequencing or RNASeq en ingles). El RNA-seq es una herramienta transcriptómica, cuyo flujo de trabajo consta de 3 pasos, las cuales incluyen la construcción de una biblioteca, la secuenciación en una plataforma de alto rendimiento (generalmente Illumina) y el análisis bioinformático (Figura 1.6).

Dentro de las aplicaciones y ventajas que tiene esta tecnología está que da una cobertura completa de transcriptos, genera información tanto de la secuencia como de la estructura de exones y sitos de splicing alternativo (Lister *et al*., 2009; Gulledge *et al*., 2012). Asimismo, los datos arrojados por RNA-seq tienen una alta precisión con respecto a los niveles de expresión génica que se obtienen a través de PCR (del inglés polimerase chain reaction) cuantitativa (qPCR) (Wang *et al*., 2009; Ward *et al*., 2012). Además, presenta una mejor relación calidad/precio de los experimentos y datos (Soto Sedano y López Carrascal, 2012) y se necesita una muestra más reducida, medida en ng, lo que permite una evaluación mucho más fina de las estructuras celulares, hasta el nivel una unica célula cuando se mezcla con la amplificación lineal del ADNc (Hashimshony *et al*., 2012), permitiendo obtener resultados altamente reproducibles (Wang *et al*., 2009).

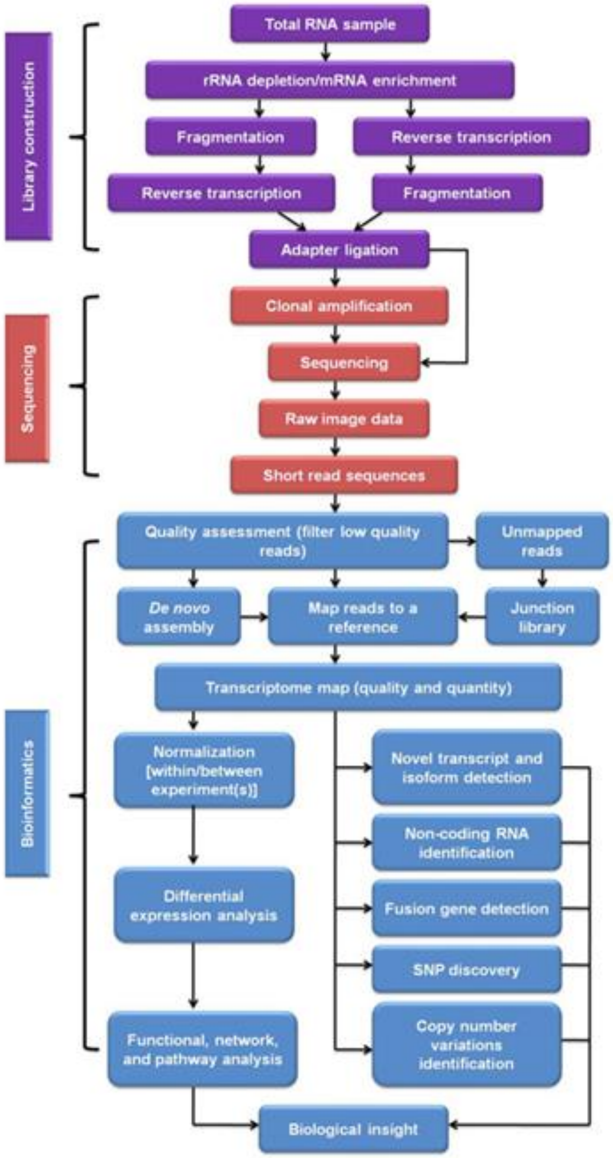

Figura 1.6. Experimento típico de RNA-Seq (Tomado del artículo de Qian *et al*., 2014)

1.2.4.1. Aplicaciones de Transcriptómica en cerebros de peces y otros ejemplos

En este camino, la tecnología RNA-Seq se ha convertido en el método de elección para el estudio y caracterización de transcriptomas dinámicos, y tanto el transcriptoma *de novo* como la pseudoalineación del transcriptoma proporcionan una potente herramienta de investigación para determinar los genes y las rutas moleculares implicadas en procesos asociados a rasgos de rendimiento como la inmunidad, el crecimiento, la reproducción, comportamiento, entre otros. En los últimos años, los estudios transcriptómicos en peces teleósteos han aumentado, lo que ha generado una enorme reserva de conocimiento genético. Sin embargo, hay poco conocimiento sobre el uso de un transcriptoma cerebral para correlacionarlo con perfiles de comportamiento proactivos o reactivos con el fin de identificar la variación en transcripciones de ARNm cerebrales específicas. Por ejemplo, Vu *et al*. (2020), evaluaron los cambios de comportamiento y transcriptómicos cerebrales entre dos oponentes machos del pez luchador *Betta splendens* durante un período de lucha de 20 y 60 minutos. Encontraron que durante la pelea de 20 minutos pudieron capturar genes tempranos inmediatos (IEG), conocidos como marcadores genómicos de la actividad cerebral y experiencias marcadamente nuevas para los peces; mientras que durante la pelea de 60 minutos permitió evaluar las respuestas transcriptómicas cerebrales para toda la gama de comportamiento volitivo en respuesta a un oponente durante el transcurso del encuentro. Primero, el análisis DEG mostró una participación de IEG en ambos grupos de lucha. Es importante mencionar que algunos IEG exhibieron una fuerte regulación positiva en el grupo 60 min, como *cfos, bdnf, nr4a3* y el miembro 9 del transportador de neurotransmisores glicina (*slc6a9*), las cuales están involucrados en la formación de la memoria a largo plazo. Por tanto, un *B. splendens* parece activar vías moleculares implicadas en la formación de la memoria durante la lucha. Asimismo, encontraron que dos subunidades adicionales del receptor de serotonina, *htr7a y htr7c*, que pertenecen a la vía del ligando neuroactivo-receptor, estaban altamente expresadas y sincronizadas en cuatro pares de lucha en ambos periodos de tiempo, lo que sugiere la posible participación de estos genes en la persistencia de la motivación en este sistema de lucha. De esta manera demostraron que los comportamientos de lucha y la expresión de genes cerebrales en esta especie estaban altamente sincronizados y proporciona un marco para comprender la sincronización a nivel conductual y molecular que ocurre durante una competencia o colaboración en peces a través de interacciones sociales.

Sadoul *et al*. (2022), demostraron que las expresiones genéticas en el cerebro fueron significativamente diferentes entre individuos proactivos y reactivos, siendo los peces proactivos los que mostraron una mayor expresión de genes relacionados con la conducta social, de exploración y memoria. Por ejemplo, el gen *dlg4* estaba regulado positivamente en los proactivos y se ha demostrado que la eliminación de este gen conduce a una reducción del comportamiento social y a un aumento del comportamiento de ansiedad (Feyder *et al*., 2010); mientras que, los individuos reactivos tenían una expresión reducida del gen *jph3*, el cual codifica una proteína de la familia de las junctofilinas, que tiene un papel activo en el comportamiento exploratorio (Moriguchi *et al*., 2006).

Asimismo, Yang *et al*. (2018) a través del análisis del transcriptoma hepático en juveniles de mero de Hong Kong *Epinephelus akaara* alimentado con cantidades bajas, medias y altas de carbohidratos, observaron un crecimiento deficiente en peces alimentados con una dieta con alto nivel de carbohidratos. Los resultados transcriptómicos revelaron una sobreexpresión de genes relacionados con el sistema inmunológico, como *l8, tlr9, cxcr4, ccl4* y el inhibidor alfa de *nfkb*, que a su vez suprimieron el crecimiento los organismos. Finalmente, la transcriptómica también juega un papel vital en comprender los mecanismos moleculares de la inmunidad en respuesta a cualquier organismo patógeno, ya que ayuda a identificar y cuantificar los genes expresados diferencialmente entre organismos sanos y enfermos, permitiendo desarrollar estrategias terapéuticas y de manejo adecuadas. Estudios recientes realizados en lisa *M. cephalus*, mostraron una clara activación de vías moleculares a través de la expresión diferencial de un gran número de genes relacionados con el sistema inmunológico (*cxcl12, 257 cxcr5*, interleukin receptor beta *cxc14*, *il-10*, *il-15*, *il-1β*) en el riñón y el bazo tras ser desafiados con la bacteria *Lactococcus garvieae* (Byadgi *et al*., 2016).

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CAPÍTULO 2

FARMED FISH WELFARE RESEARCH STATUS IN LATIN-AMERICA: A REVIEW

Artículo sometido a la revista *Reviews in Fish Biology and Fisheries*.

2.1. ABSTRACT

Latin America (LATAM) plays an important role in the world´s production of aquatic animals, being the second most productive region in the world. Chile, Brazil, Mexico, Colombia and Ecuador contribute with 87% of LATAM aquaculture production. The welfare of fish in aquaculture is of increasing public concern around the world and LATAM is no exception, growing in importance for fish farmers, authorities and scientists. Although the topic is somewhat controversial, the welfare status of farmed fish has direct implications for their production and the sustainability of the industry. Therefore, this study analyzes scientific papers on animal welfare in farmed fish, from the six countries with the most aquaculture production in Latin America. The main objectives were to quantify the number of papers published between 2000 and 2023 on fish welfare by using scientific databases. A total of 285 papers were found for the period analyzed. The country with the largest number of publications was Brazil (75.79%), followed by Chile (13.33%), Mexico (7.02%), Perú (1.75%), Ecuador and Colombia (1.05%). Nile tilapia was the most studies species appearing in 30.18% of the publications, with most of the studies mainly dealing with nutrition (32.28%). With aquaculture growth outpacing welfare knowledge, immediate efforts are needed to safeguard the welfare of high-production, understudied species in order to create policies that minimize welfare risks. Given this, the insights generated by this review could be a useful addition to approaches investigating the trends and concepts of fish welfare in LATAM.

Keywords: fish farming, fish well-being, scientific publications, aquaculture

2.2. INTRODUCTION

2.2.1. Aquaculture status and trends

Considering the exponential growth of population worldwide and the subsequent incidence of nutritional deficiencies, aquaculture has long been considered an important activity that could aid to sustain the rising demand of animal protein by increasing food production for the approximately 10 thousand million inhabitants expected for the year 2050 (FAO, 2020). Global aquaculture has registered a constant growth rate of 6.7% per year during the last decade, and production reached 122.6 million tons of live weight in 2020, corresponding to USD $281 billion, this also contributed with approximately 58.5 million of direct and indirect jobs (FAO, 2022), becoming also an important economic activity. Similarly, 56% of total fish and shellfish consumption in 2020 came from aquaculture (FAO, 2022) with Asia contributing with almost 91.61% of total aquaculture production, followed by America (3.59%), Europe (2.69%), Africa (1.92%) and Oceania (0.19%). Regarding farming systems, the most common are water-based systems (cages and pens, inshore/offshore), land-based systems (rainfed ponds, irrigated or flow-through systems, tanks and raceways) and recycling systems (recirculating aquaculture system or biofloc system) (Verdegem et al., 2023). The most utilized production methods to increment production, improve nutrition and food security while reducing space requirements are semi-intensive and intensive aquaculture production method (Ghamkar et al., 2021). Nevertheless, important challenges remain, such as sustainable production costs, market prices, trade barriers, access to disease-free broodstock or larval quality, among others, to move towards intensification of fish production (Oddsson, 2020).

With the growth of aquaculture, concerns about fish welfare have also increased during last years (Barreto et al., 2021). Routine aquaculture practices, such as: constant handling, high stocking densities, management protocols for water quality and disease prevention and treatment, or sacrifice techniques, have been considered as major concerns for fish welfare, since an inadequate control of procedures might impact production efficiency, health and quality of produced organisms (Martos-Sitcha et al., 2020; Zaki et al., 2020; Daskalova, 2019; Saraiva et al., 2019). Several studies have demonstrated that the levels of stress, inherent to such practices are associated to growth deficit (*Nile tilapia*, Waheed et al., 2023), disease vulnerability (*Sparus aurata*, Salati et al., 2016), inhibited reproduction (*Danio rerio*, Faught & Vijayan, 2018), among other disadvantages in aquaculture.

The aquaculture trends for maintaining constant growth should be focused in the enhancement of biotechnologies that promotes not only the health of fish, but also productivity and sustainability of environment. In this sense, aquaculture productive sector has started to invest in developing vaccines, as preventive measures in fish farming, with broad spectrum against bacterial, viral, fungal or parasitic infectious diseases. Currently, vaccines developed using advanced molecular techniques are considered as effective vectors to treat pathogens that cause disease in aquatic organisms. For example, mucosal vaccines against pathogenic infections are gaining wide attention in aquaculture due to the longer period of immunity of vaccinated fish (Dadar et al. 2016), as they have the potential of eliciting protective responses on mucosal surfaces by blocking pathogens at the initial site of replication (Muñoz-Atienza et al. 2021). Furthermore, artificial intelligence (AI) has become an increasingly relevant tool in aquaculture research and production in recent years, optimizing fish farming operations, improving productivity and improving fish welfare, while minimizing environmental impacts, as it allows for real-time monitoring, early disease detection, precise feeding strategies and proactive interventions to ensure optimal fish health and growth (Mandal & Ghosh, 2023). For example, Tschirren et al. (2021) developed a software named MyFishCheck. This software, which have been tested in rainbow trout and pikeperch, evaluate different indicators of welfare on this two fish species: farm management, water quality, fish behaviour and fish external and internal appearance, with welfare levels from critical to good status, providing a basis for a better appraisal of fish welfare in aquaculture farming. Lastly, the generation and use of sexual recombinant gonadotropin-releasing hormone (rGnRH) is a novel biotechnology that could allow producers, in a near future, to successfully induce spawning in fish species that exhibit reproductive dysfunctions in captivity (Molés et al., 2020). In this context, Ramos-Judez et al. (2022) demonstrated the efficacy of administering recombinant luteinizing (rLH) and follicle stimulating (rFSH) hormones in the flathead grey mullet (*M. cephalus*), a fish species that exhibits reproductive dysfunctions in captivity conditions. Moreover, Jéhannet et al. (2023) in the European eel (*Anguilla anguilla*) demonstrated that eel-specific recombinant gonadotropins (rGTHs) successfully induced to complete gametogenesis and spawning in this fish species, which according literature do not breed naturally because of this inhibition of the development of their reproductive organs.

2.2.2. Latin-American aquaculture

Latin America contributed with 4400.5 thousand tons to world aquaculture production in 2020, and it is expected to expand up 28.5% by 2030 (FAO, 2022). 87% of the total production is attributable to Chile, Brazil, Ecuador, Mexico, Peru and Colombia (Souto Cavalli et al., 2021). Inland aquaculture is the most practiced method and freshwater fish the most exploited group in the region. In terms of volume and value, Chile is the main producer, with 1.2 million tons (40%) of LATAM aquaculture production, being the main species Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and Chilean mussel (*Mytilus platensis*). Chile alone produced (FAO, 2020b). The second aquaculture producer in LATAM is Brazil who produces mainly freshwater fish and shrimp, being Nile tilapia (*Oreochromis niloticus*) and tambaqui (*Colossoma macropomum*) the most produced. Ecuador, the third largest producer has focused on shrimp (*Litopenaeus vannamei*) and Nile tilapia (*O. niloticus*), and in smaller scale, rainbow trout (*Oncorhynchus mykiss*) (FAO, 2020b). Mexico´s production is mainly shrimp (L. vannamei), Nile tilapia (*O. niloticus*), Pacific oyster (*Crassostrea gigas*) and rainbow trout (*O. mykiss*) (FAO, 2022), while Peru´s aquaculture is based on scallops (*Argopecten purpuratus*), rainbow trout (*O. mykiss*), shrimp (*L. vannamei*) and Nile tilapia (*O. niloticus*) (FAO, 2017). Lastly, Colombia´s aquaculture is based on pirapitinga and sabalo (*Prochilodus brachypomus* and *P. magdalenae,* respectively), Nile tilapia (*O. niloticus*) and rainbow trout (*O. mykiss*).

Ensuring optimal welfare of farmed fish is a main concern to guarantee rearing processes of quality and developing protection laws and local research and applying animal welfare standards in aquaculture industry have been recommended (Gallo & Tadich, 2017). However, no information on the application of welfare standards in LATAM farmed fish and no quantitative and qualitative assessment of new studies published could be found until now. Therefore, in order to highlight trends of regional research in the area of farmed fish welfare and to get an overview of the scientific productivity on this topic, the objective of this review was to determine the number of publications produced by the main aquaculture producers countries in LATAM from 2000 to 2023, about farmed fish welfare, in an attempt to find trends and gaps for this area of research.

2.2.3. Aquaculture and welfare

Aquaculture provides more stable environmental conditions than in nature, because of the safe and continuous food supply and the absence of predators (Macaulay et al., 2020). However, captivity also induces fish with stressful and cognitively challenging situations, as described above. These stressful situations activates the hypothalamic-pituitary-interrenal axis (HPI), analog to the hypothalamic-pituitary-adrenal axis in mammals (HPA), which is responsible for the regulation of the degree of severity of the response to perceived stress, reflected by biochemical, physiological, nutritional and behavioural alterations that impact fish welfare (Carbonara et al., 2019; Vieira Madureira et al., 2019; Shreck & Tort, 2016).

Another consideration in the welfare studies, and have been object of debate over the last decades in the field of fish welfare, is whether fish experience pain or not (Brown & Dorey, 2019; Sneddon, 2015). This controversy has risen many concerns about fish welfare and legislation to protect it. According to the accepted definition of the International Association for the Study of Pain (IASP, 1979), pain comprises both sensory and negative affective aspects, implying that it is a conscious experience. On one hand, Key (2015) stated that fish lack essential brain regions, including the neural basis of pain awareness and perception, and they concluded that fear awareness and conscious experiences are impossible for fish. On the other hand, Woodruff (2017) argued that fish are sentient to pain, based on the complexity of brainstem circuitry and on the flexibility of the behaviour. Sneddon (2019) has suggested that fish experience pain and that pain-related behavioural changes are similar to that reported in mammals. This implies that fish may express suffering and this factor should be considered in the aquaculture industry. Therefore, the welfare of farmed fish should be considered during the management process (Sneddon & Roques, 2023). In this sense, legislation on fish welfare is fundamental to promote protocols to warranty that fish are free of pain, suffering, injury and disease and decide to what extent aquaculture producers and researchers should go, in order to respect the law.

Many factors threaten farmed fish welfare in farming conditions at their different production stages, since they often cause suppression of growth and increased mortality (Sampaio & Freire, 2016), susceptibility to diseases (Mateus et al., 2017), and impact on the spawning success (Murugananthkumar & Sudhakumari, 2022). Therefore, ensuring fish welfare in aquaculture farms is a complex issue, due to the large number of rearing procedures and species-specific behavioural, ecological, biological and physiological aspects that need to be considered (Flores-García et al., 2022). As well, overcrowding has been found to induce stress in fish, since it increases competition for space, food, oxygen and reduces feed intake, physiological response, growth performance and, consequently, productivity (Seo et al., 2023). Water quality is another parameter that affect physiology and growth rate and, in severe cases, cause mortality. For instance, Shin et al. (2016) showed that rockfish *S. schlegelii* exposed to high ammonia levels affected the growth performance (daily length gain, daily weight gain, condition factor, and hepatosomatic index), changed hematological parameters (hemoglobin, hematocrit) and induced to significant alterations in serum components (total protein, glucose).

Furthermore, usual slaughtering methods in aquaculture at the end of the production cycle still consist in stunning fish by CO_2 narcosis, asphyxiation in air or ice, *in vivo* chilling, use of carbon dioxide, bleeding without prior stunning, among others, which are not acceptable as they infringe animal welfare due to the avoidable pain and suffering inflicted to the organisms (Mercogliano & Dongo, 2023; Gräns et al 2016). However, other slaughtering protocols, perceived as much more humane and inflicting less suffering, have been employed, such as: percussions for carps and salmonids, spiking or coring for tuna fish and electrical stunning for carps, rainbow trout, eels and salmonids. These methods have showed that both severity and duration of fish suffering were decreased and they appeared to be among the least aversive methods for slaughtering fish, when appropriately applied (Clemente et al., 2023; WOAH, 2022). Nevertheless, stunning processes require careful attention and the operational personnel need to be trained in using the stunning devices and in recognizing indicators of consciousness (Jung-Schroers et al., 2020). Although fish are subject to different factors such as those mentioned above, their welfare is a topic that is not discussed or considered very often, therefore, generating debates in the legal community about the welfare of fish is important to avoid the suffering of animals and offer them an optimal environmental condition during their complete production cycle.

Although scientific investigation works and findings on fish welfare have increased and even though management plans and laws have been developed in several regions to avoid unnecessary suffering of farmed animals during rearing and slaughter, only a few countries have implemented these as welfare requirements for farmed fish; as is the case of Norway, Switzerland and New Zealand (Mood et al., 2023). For example, in Switzerland, farmed fish and other farmed animals are legally protected by the same laws, where legislature states that fish, both farmed and wild, must be stunned before killing (Swiss Federal Council 2018). However, this list of permitted stunning methods includes cervical dislocation, which is not a method recommended by the guidelines of the World Organization for Animal Health, founded as OIE (2022a). As for LATAM countries, there is still a long way to go to raise producers and consumers awareness about why and how to reach optimal welfare conditions for farmed fish.

2.3. MATHERIALS AND METHODS

The methodology used in this study considered the following steps:

2.3.1. Definition of literature search strategies

A wide bibliographic search was first performed to gain insight into academic studies performed on fish welfare in aquaculture farming in LATAM. Search Keywords included combinations of the following farmed fish AND/OR behaviour AND/OR welfare AND/OR cultivo de peces AND/OR comportamiento AND/OR bienestar AND/OR cultivo de peixes AND/OR comportamento AND/OR bem-estar were performed using Science Direct (https://www.sciencedirect.com/search), Scielo (http://www.scielo.org/php/index.php) and Scopus (https://www.elsevier.com/es-mx/solutions/scopus) databases, which covers >12,000 scholarly journals, including all major science, medicine, and technology journals. Although the 3 databases presented shortcomings (e.g., non-English language journals are underrepresented), they are considered to provide a satisfactory representation of international mainstream scientific research (De Castilhos & De Oliveira, 2016; Moed, 2005). These databases were chosen because Science Direct and Scopus had previously been used in similar reviews on animal welfare publications (Barreto et al., 2021) and Scielo includes more indexed journals that accept papers in Spanish or Portuguese which are the official languages in the area covered by this review.

2.3.2. Paper selection criteria

Original papers and bibliographic reviews published from 2000 to 2023 were included in the analysis, with language filter, considering all journals in the areas of veterinary sciences, animal sciences and aquaculture in all databases. The resulting papers were manually categorized and analysed in Excel program, according the following variables of interest:

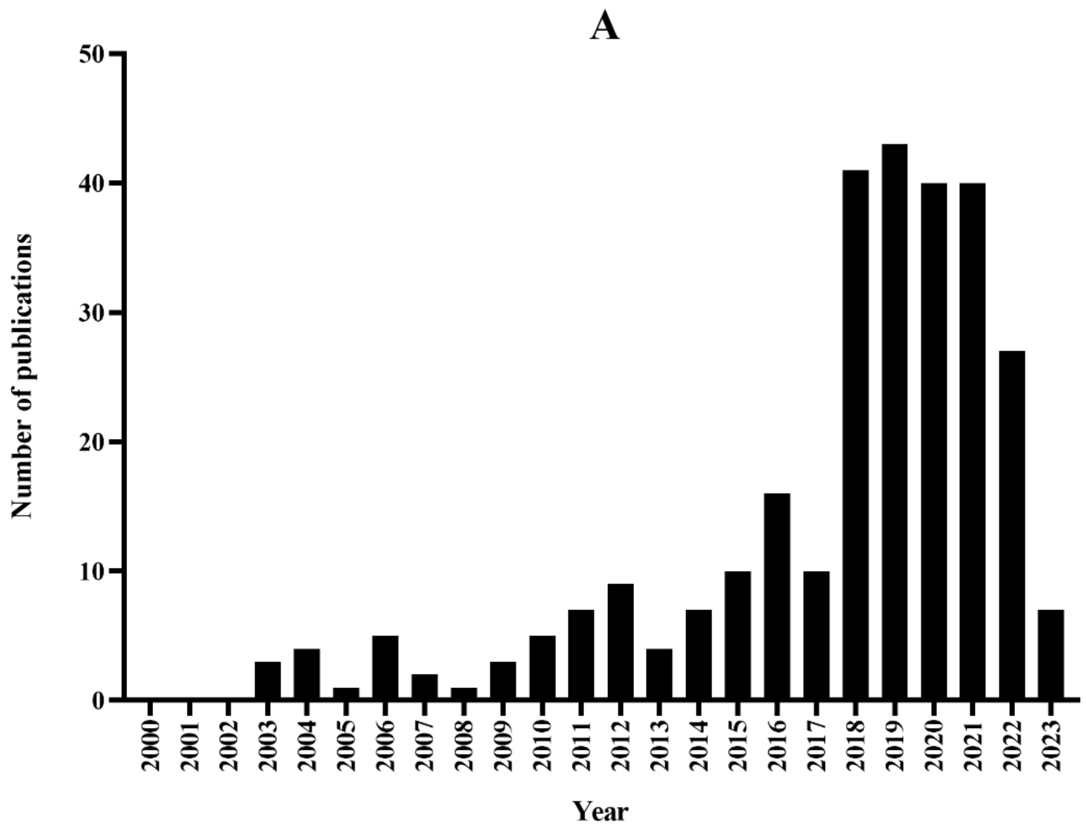
- Country of origin (Brazil, Chile, Mexico, Peru, Ecuador and Colombia): Papers were selected on the basis of the location of the institution of the first author or where the research was undertaken.

- Species: Papers were classified according to the main species produced, and less conventional farmed fish species were classified as “other species”

- Topic: Papers were categorized into 7 common topics in aquaculture: water quality, feeding/nutrition, anaesthesia, pathology/health, stocking density, behaviour and toxicology, based on the paper's keywords and title of each study. When a search returned one or more articles for each topic, the content was looked further based on the paper's abstract. Papers studying fish behaviour or welfare in other fields, such as fisheries for instance, were excluded.

2.4. RESULTS

A total of 285 published papers on fish welfare were found for the six LATAM countries selected according to their contribution to aquaculture (Souto-Cavalli et al., 2021), being: Brazil, Chile, México, Ecuador, Peru and Colombia, between the period of 2000 and 2023. The number of publications related to fish welfare considerably incremented from the first eleven years period (2000-2011) to the second twelve years period (2011-2023), from 31 studies (10.88%) to 254 studies (89.12%), respectively (Figure 2.1A).


A
Number of publications
50
40
30
20
10
0
2000
2001
2002
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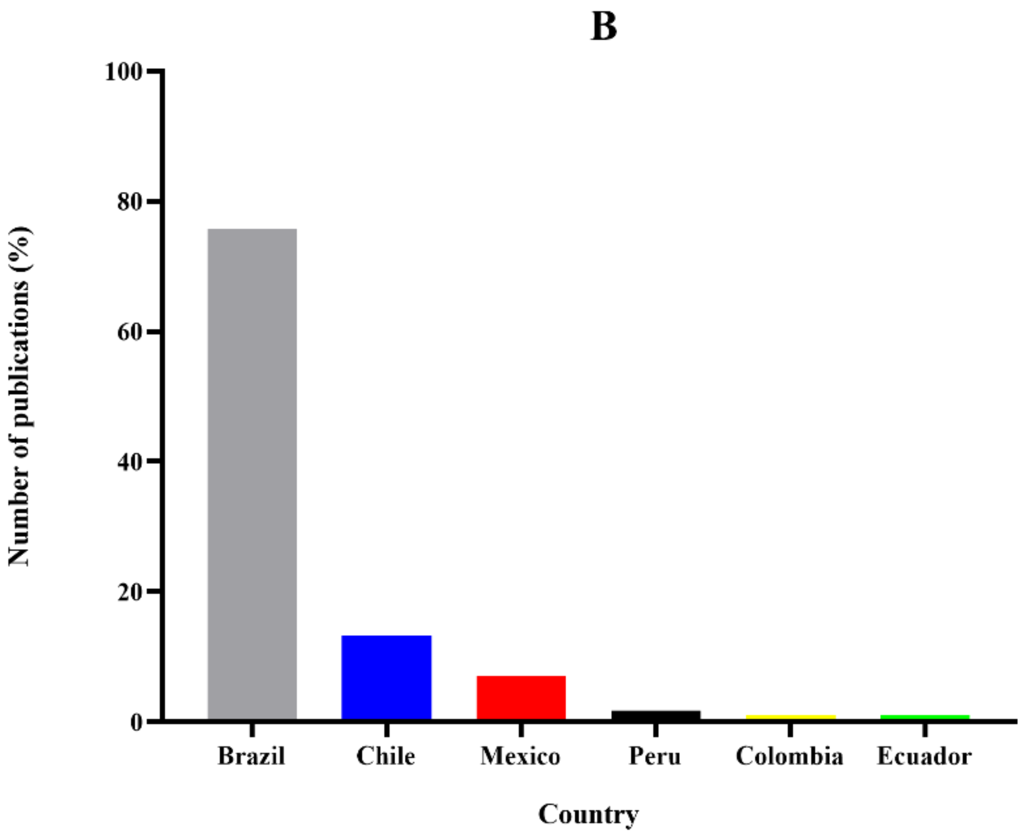
B
Number of publications (%)
100
80
60
40
20
0
Brazil
Chile
Mexico
Peru
Colombia
Ecuador
Country

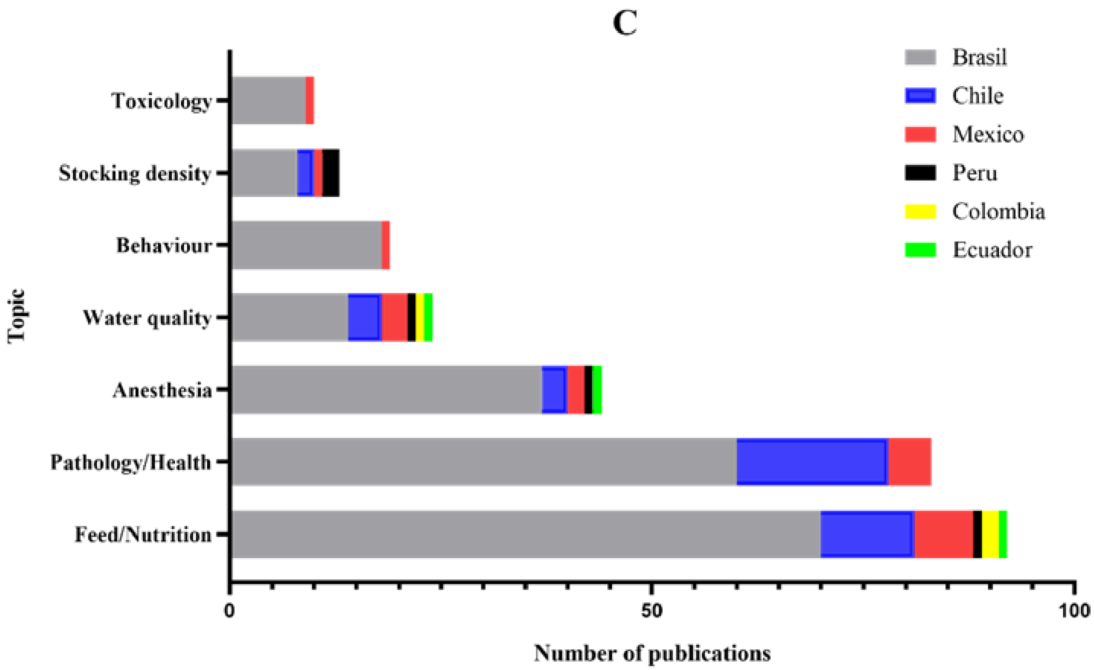

Figure 2.1. Distribution of publications on farmed fish welfare: A: by year; B: by country; C: by associated topics (n = 285).

Considering the total number of papers found (285), the country with the most publications on welfare and behaviour in farmed fish during the analyzed period was Brazil (75.79%), followed by Chile (13.33%), México (7.02%), Peru (1.75%), Colombia and Ecuador (1.05%) (Figure 2.1B). Table 2.1 showed that publications on fish welfare in LATAM dealt mainly with Nile tilapia *Oreochromis niloticus* (30.18 %) and silver catfish *Rhamdia quelen* (22.46 %) in Brazil. Studies with grass carp *Ctenopharyngodon Idella* (3.51 %), jatuarana *Brycon amazonicus* (2.46 %) and totoaba *Totoaba macdonaldi* (1.75 %) were less common. Among less conventional farmed fish species, classified as “other species”, represented less than 8% and corresponded to Pacific red snapper (*Lutjanus peru*), longfin yellowtail (*Seriola rivoliana*), flathead grey mullet (*Mugil cephalus*) and Pac-man catfish (*Lophiosilurus alexandri*), among others.

When categorizing publications on farmed fish welfare by topic associated, feed/nutrition topic and pathology/health issues were the most represented, covering 32.28% and 29.12% of total papers, respectively (Figure 2.1C). Then, papers on fish welfare dealing with anaesthesia techniques (15.44%) covered mainly issues related to transport condition and handling/moving animals. Finally, papers concerning water quality (8.42%), behaviour (6.67%), stocking density (4.56%) and toxicology (3.51%) were less common. Brazil contributed the most to publications on fish welfare for all associated topics, followed by Chile and Mexico, while the contribution of other LATAM countries to publications on farmed fish welfare and any other topic was smaller.

Table 2.1. Principal Fish species cited in each publication and the country that contributes the most.

Fish under study	**Principal country**	**N° of publications (N=285)**
Oreochromis niloticus	Brazil	86 (30.18%)
Rhamdia quelen	Brazil	64 (22.46%)
Colossoma macropomum	Brazil	27 (9.47%)
Salmo salar	Chile	21 (7.37%)
Piaractus mesopotamicus	Brazil	20 (7.02%)
Oncorhynchus mykiss	Chile	13 (4.56%)
Arapaima gigas	Brazil	11 (3.86%)
Ctenopharyngodon idella	Brazil	10 (3.51%)
Brycon amazonicus	Brazil	7 (2.46%)
Totoaba macdonaldi	Mexico	5 (1.75%)
Others	Mexico	21 (7.37%)

Concerning animal protection laws, specifically related to fish welfare, a lack of information on the legislation in the countries under study was evidenced, being Chile the only LATAM country that has a law related to fish welfare (Table 2.2).

Table 2.2. Countries maintaining animal protection laws and year of publication

Country	Law /regulation issue	Year
Brazil	Decree 16590, public entertainment houses, prohibiting animal abuse	1924
	Decree 24645, for animal protection	1934
	Law 9605, for environmental crimes	1998
	Normative Instruction 3, for humane slaughter of animals destined for consumption	2000
	Law 11.794, for animal manipulation and welfare	2008
	Normative Instruction 56, for good animal welfare practices for production animals during breeding and transport	2008
	Normative Instruction 22, Brazilian guide for production, maintenance and use of animals in teaching activities or scientific research	2015
	General Aquaculture Law, the term “welfare” is absent	**2009**
Colombia	Law 5, on animal protection groups	1972
	Law 84, for the protection of animals	1989
	Law 1774, for animal cruelty	2016
	Decree 1835, promotion of Fisheries and Aquaculture, the term “welfare” is absent	**2021**
Chile	Law 20.380, for the protection of animals	2009
	Decree 28, for the protection of animals that provide meat, fur, feathers, and other products at the moment of slaughter at industrialized establishment	2013
	Decree 29, for protection of animals during breeding, commercialization, and at other places where animals are maintained.	2013
	Law 20.434 - Modifies the General Law on Fisheries and Aquaculture	**2010**

	in terms of aquaculture, includes the new paragraph 5 on animal welfare: aquaculture must contemplate regulations that safeguard animal welfare and procedures that avoid unnecessary suffering.	
	Decree 30, for the protection of animals during transport	2013
Ecuador	Organic Code of the Environment, for animals used in entertainment and experimentation	2017
	Organic Law of Agricultural Health, for apply welfare conditions for handling and transporting animals by farmers	2017
	Organic Law for the development of aquaculture and fisheries, the term “welfare” is absent	**2020**
México	Federal Animal Health Law, for protect animals used in farming	2007
	NOM-033-ZOO, for humanitarian care and animal protection during slaughter operations	1995
	NOM-033-SAG/ZOO, for methods to slaughter domestic and wild animals	2014
	NOM-045-ZOO, for operations of establishments where animals gather for fairs	1995
	General Law of Sustainable Fisheries and Aquaculture and the last DOF Reform (12/07/22), the term “welfare” is absent	**2007**
Perú	Protection Act 27265- pets and wild animals kept in captivity	2000
	Legislative Act 635, Criminal Code	2004
	Law 30407, for animal welfare and protection act	2015
	Legislative Decree 1195 - General Aquaculture Law, the term “welfare” is absent	**2015**

2.5. DISCUSSION

This overview of scientific papers published on farmed fish welfare in LATAM countries over 23 years (between 2000 and 2023) quantified and analysed research publications, according to their year of publication, top aquaculture production countries, animal species studied and topics associated to farmed fish welfare in the studies, which is discussed in the next sections.

2.5.1. Number of papers published from 2000 to 2023

The productivity of scientific papers on fish welfare in LATAM countries (n = 285) is lower than that in countries from North America and Europe (n = 768). The review of De Castilhos and De Oliveira (2016) actually collected worldwide data (1979 - 2016) from the ISI-Web of Science and Scielo databases, including all languages and all types of documents and found 851 publications on fish welfare, but authors did not provide any numbers for publications originating specifically in LATAM countries, excepting Brazil (53 papers) and Chile (≈10 papers).

Moreover, the number of studies on fish welfare published by LATAM authors is lower than the number of studies focusing on terrestrial animal welfare, according to data provided by Gallo et al. (2022). These authors searched for publications on animal welfare, using ISI-Web of Science and CAB Abstracts database and found 663 published papers on terrestrial livestock farm animals, excluding aquatic animals, wild and zoo animals, sport horses, pets and laboratory animals.

An exponential increase in the number of publications on fish welfare in LATAM was observed throughout the time period analysed, which coincided with the results of Freire and Nicol (2019) for animal welfare publications, since the 1990s (< 500 papers) and the results of De Castilhos and De Oliveira (2016) on fish welfare for worldwide publications, whom showed a greater increase since 2001 (≈ 10 papers/year) compared to previous years. However, for LATAM countries, the increment of papers related to fish welfare was observed more recently, with an increase from the first period (2000-2011) to the second period (2011-2023) of 238%.

The interest in farmed fish welfare in LATAM started in the 2010s, ten years after the growing interest generated in Europe and North America (Gallo et al., 2022; Freire & Nicol 2019), probably coming within the scope of a worldwide trend observed toward animal welfare issues (Sloman et al., 2019). This included a shift in consumers perception on animal suffering and social pressure in general (Rossi-Borges et al., 2019; Lama et al., 2017). Brazil and Colombia were the first countries in implementing laws regarding animal protection, such as the Decree 24645 and the Law 84, respectively. These laws provided a detailed definition of acts of cruelty to animals and established a duty of care for animal owners. However, since 2008, different LATAM countries began to implement animal welfare laws, due to the work of the World Organization for Animal Health (WOAH), who published animal welfare standards for the first time in 2005 and promoted their implementation in member countries, including Brazil, Chile, Mexico, Peru, Ecuador and Colombia (WOAH 2020; Galindo et al., 2016). The need to include animal welfare as a major concern within LATAM countries and international trade was important, since many worldwide fish producers and exporters are located in LATAM countries (Muñiz et al., 2022). Another reason for LATAM countries to invest in more research and publications on fish welfare, as on terrestrial animals this last decade, could be related to educational and regulation politics of the WOAH and in the LATAM member countries. Animal welfare standards were implemented by member countries of the WOAH since 2009, when a survey was applied to 172 member countries (Stafford & Mellor 2009).

In recent years, much more attention has been paid to the welfare of farmed fish (Toni et al., 2019) and this has led to legislation and guidance in some countries like Chile, which was the only country of the six main aquaculture producers in LATAM that considered animal welfare within its aquaculture laws, probably because it is the second largest producer and international exporter of the most consumed marine fish in the world, salmon *S. salar*. However, protecting fish welfare has been officially delayed since the implementation of laws involves multiple trade-offs and complexities, which raise particular ethical issues requiring specific consideration in each country (Pavlidis, 2022). For instance, fish welfare and environmental sustainability are closely related objectives. An example could be what was stated by Bergh (2007): "there is a fundamental conflict of interest between concern for the environment and concern for animal welfare", arguing that the strict legislation on the use of antibacterial products in Atlantic salmon (*S. salar*) aquaculture in Norway prioritized the reduction of environmental impacts over fish welfare, thus complicating decision-making.

2.5.2. Main countries of origin in publications on fish welfare

Brazil was the main country of origin of the scientific papers on fish welfare, in agreement with the findings of De Castilhos and De Oliveira (2016) in their search for worldwide scientific publications on fish welfare. In addition, Freire and Nicol (2019) and Gallo et al. (2022) also mentioned Brazil as the only visible LATAM country in their study on scientific publications on animal welfare worldwide. The large geographical extent and relevant ichthyodiversity of Brazil within LATAM may explain its leadership in research and number of publications related to fish farmed welfare. Chile, with all research effort focused on salmon, was the second country with the highest scientific publication on fish welfare, in relation to scientific challenges imposed by environmental problems (Quiñones et al., 2019). Mexico was the third country with the highest scientific publications, probably because aquaculture industry is the primary sector with the greatest growth in the last 10 years, with an average growth of 15%, highlighting the cultivation of more than 70 species, including tilapia, carp and trout (Cortés et al., 2021). On the other hand, only a few publications on fish welfare (appearing since 2014) were found in Perú, Colombia and Ecuador, likely because sea products economy in these countries was based on different activities from fish farming, such as fisheries in Peru (Christensen et al., 2014) and shrimp farming in Ecuador (FAO, 2020b).

2.5.3. Main species in publications on fish welfare

Most of publications on fish welfare dealt with Nile tilapia (*O. niloticus*), the most reared fish species in most LATAM countries, explaining the interest in researching and studying welfare in this fish species (Paredes-Trujillo et al., 2022; Yoshida et al., 2022), and representing as well one the most considered species in fish welfare studies worldwide. These results differed from De Castilhos and De Oliveira (2016), who reported that *S. salar* was the species with the most contribution worldwide to scientific production on fish welfare (139 of 851 papers, 16.33%), however, it is necessary to consider that Norway is the main producer of this species and also one of the most advanced country in fish welfare research (Afewerki et al., 2022; Stien et al., 2020). Next most represented species in scientific publications on fish welfare were *Rhamdia quelen* and *Colossoma macropomum,* two freshwater species and mostly in Brazilian papers, where inland aquaculture represents the highest proportion of fish production and those fishes are of the most exploited groups (FAO, 2022).

2.5.4. Main topics in publications on fish welfare

Feeding/nutrition, health/pathology and anaesthesia topics associated with farmed fish welfare topic contributed the most to the total of publications and those papers were published by Brazilian authors mainly. Similar topics had been previously identified when investigating terrestrial animal welfare (Calderón-Amor et al., 2020; Gallo et al., 2022). Interest in the topics mentioned above reflected their importance to provide fish optimal rearing conditions, in order to guarantee their welfare and to limit the impact of high stress in captivity and poor welfare on productivity parameters (Barreto et al., 2021; Lembo et al., 2019).

Regarding, feeding/nutrition topic, the use of feed additives is one of the most important aspect related to fish stress, health and susceptibility to diseases and their control is essential to ensure optimal rearing conditions and welfare in farmed fish (Yadav et al., 2021). For example, Brazilian researchers investigated the impact on growth, metabolic processes, blood parameters and oxidative stress biomarkers, of adding *Lippia alba* essential oil (EO) into the diet of juvenile silver catfish (*Rhamdia quelen*) and recommended it, because it decreased lipid peroxidation (LPO), increased glycogen and lactate reserves (Saccol et al., 2013), therefore, understanding nutritional factor might enhance the antioxidant status in order to increase productivity and improve fish health (Gao et al., 2012).

Relative to the health/pathology topic, the second topic in the present study, a link between fish health and welfare was evidenced. Nonetheless, fish health and fish welfare are not synonymous terms (Broom 2007), since a health-based approach to fish welfare represents a reductionist approach, as it does not take into account all components of welfare. Actually, while an optimal physical health is essential to reach complete welfare (Ashley 2007), the fact that an animal is healthy does not necessarily mean that it presents an absolute welfare status. Therefore, fish welfare is a broader and more encompassing concept than the concept of fish health (Dara et al., 2023). In this sense, González et al. (2015) evaluated how increasing concentrations of attached and mobile stages of sea lice *Caligus rogercresseyi,* the main ectoparasite of farmed *S. salar*, affected fish physiological response and they identified the threshold concentration at which parasite populations caused negligible physiological effects on the host. The welfare of *S. salar* weighing 80 g on average was compromised when fish was infested with six or more adult parasites and fish physiology was found to be altered. The development of *C. rogercresseyi* from copepodite to adult caused a chronic energy demand in the host fish, being the advanced stages is the most detrimental. Alteration in metabolic variables above the determined threshold suggested that salmons tried to return to homeostatic welfare status through a metabolic reorganization when parasites reached the adult stage. These findings were of great importance to implement surveillance and control programs.

Finally, anaesthetics are commonly administrated to fishes in research laboratories or in farms for a wide variety of purposes, in order to reach sedation, general anaesthesia or an euthanasic overdose. Synthetic and natural (plant-based) agents are the two main types of commercial anesthetics widely used to reduce the deleterious effects of stress on fish in captivity (Purbosari et al., 2019). Synthetic anesthetics such as tricaine methanesulfonate (MS-222), benzocaine, phenoxyethanol, etomidate, and quinaldine are commonly used in aquaculture research and fish farming (Priborsky & Velisek, 2018). Natural agents principally include essential oils derived from the leaves, flowers, buds, stems, and roots of terrestrial plant, such as lemon verbena *Aloysia triphylla*, eucalyptus *Eucalyptus* sp., basil-clove *Ocimum gratissimum*, among others (Souza et al., 2019). Essential oils were also reported as a low-cost, abundant, low-toxic, and environmentally friendly product that alleviates handling stress for some fish species in captivity (Aydın et al., 2019). In this sense, a study in Brazil led by Boaventura et al. (2021), evaluated the effect of essential oil of *Ocimum gratissimum* L. (EOOG) on water quality during transport of *Lophiosilurus alexandri*. The use of 10 mg/L EOOG during transport promoted a sedative effect, with reduced oxygen consumption, ammonia excretion and biochemical changes and enhanced protection against oxidative damage.

Toxicology, stocking density, behaviour and water quality topics associated to farmed fish welfare were less present than the other 3 topics detailed above.

2.6. CONCLUSION

Literature on animal welfare is still dominated by topics focused in farmed terrestrial animals, but research on fish welfare in aquaculture is emerging progressively, gaining ground in LATAM and bringing new opportunities for the generation of novel information and the publication of scientific papers. But a lot of work is still need and different challenges are continuously arising. In this context, it is necessary to continue implementing sustainable biotechnologies for the fish rearing, such as biofloc or integrated multi trophic aquaculture systems, since several studies have been demonstrated the positive effects of this rearing technologies on fish growth rate, water quality, gut microbial community, immune system and antioxidant capacity, resulting in improved fish welfare by increasing high nutritional value products in their environment

Including protocols on fish welfare is of vital importance for LATAM countries to improve fish productivity parameters, such as growth performance, survival or disease resistance, but also to guarantee quality standards for the commercialization and the employment generation in the productive sector, as is actually done in Europe, USA and Canada. To reach this goal, universities, research institutions and the government needs to work together in order to improve knowledge, laws and regulations on fish welfare and ultimately transfer it to the productive sector. Besides, establishing training courses and qualifying staff could ensure the fish welfare.

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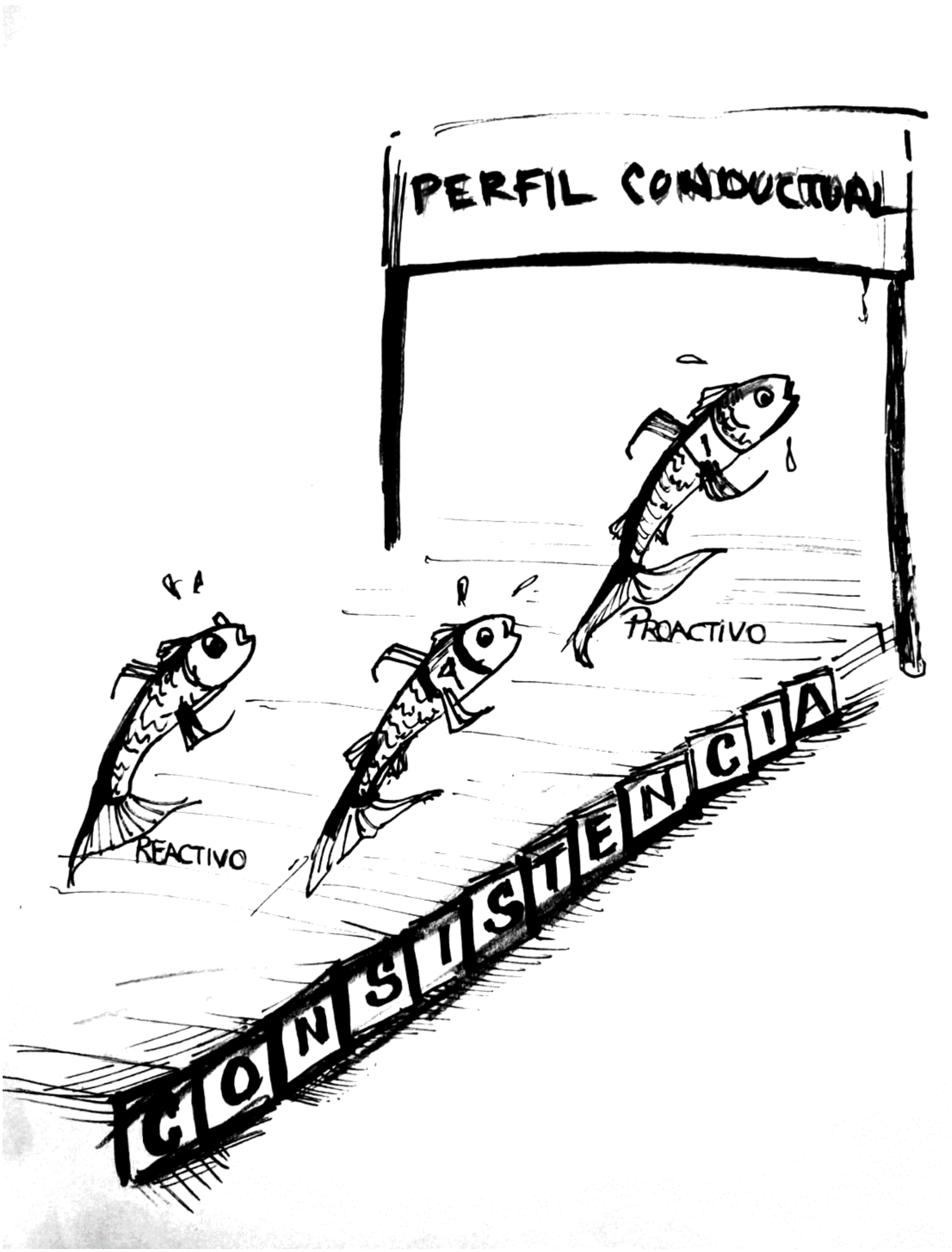
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CAPÍTULO 3

FLATHEAD GREY MULLET (*MUGIL CEPHALUS*) JUVENILES EXHIBIT CONSISTENT PROACTIVE AND REACTIVE STRESS COPING STYLES

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3.1. ABSTRACT

Stress coping styles (SCS) are defined as a coherent set of individual physiological and behavioural differences in stress responses that are consistent across context and over time. Nowadays, an increased attention has been paid to fish welfare in aquaculture production and to consistent behavioural response to stress in captivity, since these aspects present direct implications on fish health, quality and farming productivity. Therefore, the present study aimed to evaluate the differences between proactive and reactive stress coping styles and their consistency over time and across contexts in flathead grey mullet *Mugil cephalus* juveniles reared in captivity. Fish were submitted to two runs of a set of behavioural tests, with a four-months interval: one group test (risk-taking test) and three individual tests (restraining, predator and confinement tests), followed by the quantification of blood cortisol and glucose concentrations (control and post-stress). Results confirmed the existence of proactive and reactive SCS in mullet juveniles and demostrated that behavioural responses in most of the tests were consistent over time (between runs) and across contexts (among tests). Fish that crossed from the safe to the risky area in the risk-taking test also displayed higher activity and escape attempts and lower basal levels of cortisol and glucose than fish than did not cross. This work reported for the first time the consistency of stress coping styles over time and across contexts in *M. cephalus* juveniles. Due to the increased interest in understanding adaptive mechanisms underlying consistent individual behaviours in response to stressful situations, are of interest for the aquaculture industry to improve productive performance of this fish species. Further studies are required to investigate how different selective pressures might influence the expression and consistency of SCS over time and across contexts.

Keywords: *Mugil cephalus*, intra-individual variation, consistency, stress coping style.

3.2. INTRODUCTION

Stress coping styles have been defined by Koolhaas et al. (1999) as "a coherent set of individual physiological and behavioural differences in stress response consistent across context and over time". Overall, proactive organisms are considered to be more active, and aggressive toward conspecifics, with a faster growth performance and higher mating success, but tend to present less adaptability to environmental variations, than reactive organisms (Koolhaas et al., 1999; Höglund et al., 2020). Moreover, proactive individuals are associated with a lower hypothalamic–pituitary-interrenal (HPI) axis activity, equivalent to the hypothalamic–pituitary–adrenal (HPA) axis in mammals, and higher brain-sympathetic-chromaffin cell (BSC) axis reactivity, through lower levels of blood cortisol than reactive organisms (Rosengren et al., 2018; Ferrari et al., 2020). The activation of HPI and BSC axis stimulates the central nervous system (CNS), which in turn promotes the corticotropin releasing factor (CRF) that enhances the synthesis and release of adrenocorticotropic hormone (ACTH) in the bloodstream. ACTH reaches the steroidogenic cells in the interrenal tissue and stimulates the release of cortisol, considered as the main hormone for homeostasis regulation in chronical stress situations (Conde-Sieira et al., 2018; Culbert et al., 2022), by controlling the activity of hormones and enzymes involved in gluconeogenesis and glycogenolysis metabolic pathways, allowing an increase in glucose mobilization for fish being able to fight, escape or hide in response to perceived stress (Huntingford et al., 2006).

Different group and individual behavioural tests have been used to characterize SCS in fishes. The most commonly used group tests are: food intake assessment

(Gesto, 2019), hypoxia test (Vindas et al., 2017) and risk-taking test in new environments (Alfonso et al., 2020). Regarding individual tests, the most commonly used are: confinement test (Ferrari et al., 2020), predator test (Solomon-Lane and Hofmann, 2019), restraining test (Höglund et al., 2020), new environment test (Ibarra-Zatarain et al., 2016) and novel object test (Skov et al., 2019). Both group and individual tests have been recommended as a complementary assessment to evaluate SCS response, since individual behaviours may vary when considering animals into a group, due to hierarchical social levels of organization and gregarious/isolated inter-relationships depending on the species (Champneys et al., 2018; Höglund et al., 2020).

In addition, some behavioural traits can be related to SCS and viceversa where animals tested under different stress situations and recording their responses can be effective (Royauté et al., 2020). Thus, five traits have been recognized: boldness-shyness, exploration-avoidance, activity, aggressiveness and social tolerance (Réale et al., 2007; Cote et al., 2011). The trait of boldness and shyness is a measure of the individual reaction to a situation perceived as threatening (Réale et al., 2007). Exploration-avoidance trait includes behaviours involving the individual willingness to investigate new environments, looking for food or objects in the absence of predators or other threatening stimuli (Ferrari et al., 2015). Activity trait refers to animal general activity level and is measured in terms of spontaneous movements in either familiar or unfamiliar areas (Lothian and Lucas, 2021). Aggressiveness is often assimilated to the defense of a territory or to the competition for food resources (Martorell-Barceló et al., 2021). Sociability traits refers to the reaction of an individual towards its conspecifics, which does not correspond to an aggression (Hirsch et al., 2017).

As previously quoted in the definition of Koolhaas et al. (1999), individual physiological and behavioural responses to stress have been proved to present a consistent pattern over time and across contexts in several studies on animal behaviour. This consistency has been demonstrated as between individuals (repeatability) as within individuals (reliability), where individual response to stress in a specific context often predicted its responses in other contexts and over time, being called individual animal personality (Sih et al., 2015). While some authors have suggested that individual variation of SCS consistency may rely on genetic bases and lead to adaptive behavioural evolution (Wong et al., 2015; White and Wilson, 2019), others proposed that a prior experience can influence the behaviour of an individual in the short and long term (Ibarra-Zataraín et al., 2016; Castanheira et al., 2017) by submitting fish to different tests repeatedly. For example, Toms and Echevarria (2014) demonstrated that boldness, aggression, fear and exploration, measured in zebrafish *Danio rerio* in five different contexts, were consistent over a 7-day period. Likewise, Basic et al. (2012) showed consistent traits (first feeding after transferring to a new environment, responses to a new object and to confinement, aggressiveness) over a 7-day period, in rainbow trout (*O. mykiss*) lines selected for high- or low-responsiveness of plasma cortisol to an acute confinement stressor, although no differences were observed between lines.

Nevertheless, studies investigating the individual consistency over long periods, months or even a year, are scarce, but few studies have shown a long-term consistency in SCS, such as Rey et al. (2013), whom showed a high consistency of the positive correlation of boldness, measured through a risk-taking test, with aggressive behaviour in zebrafish *D. rerio* over 10 months. Moreover, Ibarra-Zatarain et al. (2020) demonstrated a long time intra and inter-individual consistency in Senegalese sole (*S. senegalensis*) for juveniles and breeders submitted to different SCS tests over three- and two-year period, respectively.

The present study aimed to determine whether proactive and reactive stress coping styles were significantly consistent over long time period (four months) and across contexts, in flathead grey mullet (*Mugil cephalus*) juveniles. According to Jiménez-Rivera et al. (2022) this fish species exhibits diurnal and non-aggressive habits and presents social-cooperative structures. Moreover, mullet is considered as a fish species with a high potential for aquaculture due to its euryhaline and eurytherm characteristics and acceptance of fish meal-free diets (Nordlie, 2016; Madrid et al., 2022). Hence, the approach and results of the study may be of interest for the development of rearing protocols for the production of flathead grey mullet in the aquaculture industry.

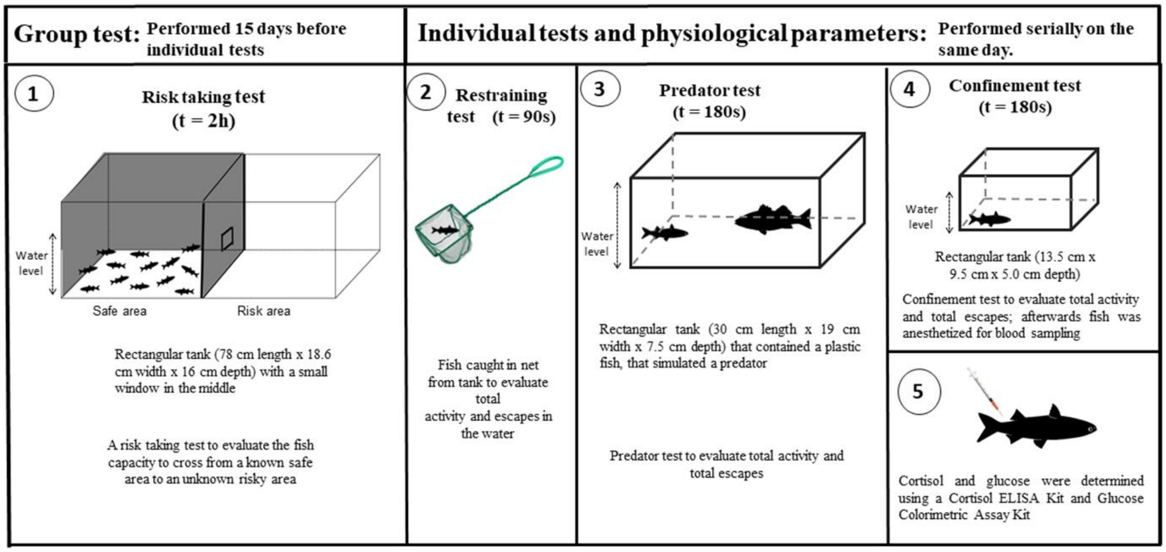
3.3. MATERIALS AND METHODS

3.3.1. Fish handling

A total of 300 wild juveniles of flathead grey mullet were captured in Mazatlán, México and acclimated in two cylindrical 6,000 L tanks during four weeks, with a prophylactic treatment (oxytetracycline baths, ≈ 120 mg.L^{-1} for 1h) upon collection and transport to reduce the incidence of bacterial infectious diseases (Kondera et al., 2020). Thereafter, fish were transported to the aquaculture management and innovation unit at the Nayarit Centre for Innovation and Technological Transference (CENITT-UAN) in Tepic, México. One month later, sixty-six grey mullet juveniles, with an average weight of 10.37 ± 0.95 g and length of 12.10 ± 0.23 cm were randomly selected, anaesthetised with eugenol (50 ppm) (Park et al., 2018), marked with nano PIT-tags (FRD-LF-GT8-64B LE Glass Tag Ø1.4 x 8mm EM4305 125KHz, China) for identification and distributed into three 120 L fiberglass tanks (n = 22 fish per tank; ~2 Kg.m^{-3}) connected to a recirculation aquaculture system and water parameters were controlled as follow: salinity 35 ± 0.50 PSU, temperature 23 ± 1°C and oxygen 6 ± 1.5 mg.L$^{-1.}$ Fish were fed with a commercial diet (Skretting Nutra 160; 38% protein, 8% lipids) at 4% of biomass, following recommendations of Madrid et al. (2022) for this fish species. Uneaten food and faeces were siphoned daily, one hour after feeding fish, to maintain a correct water quality. Ammonia, nitrites, and nitrates concentrations (mg.L^{-1}) were monitored every week with an aquaculture photometer (HANNA; HI83303, USA) to keep values under 0.1, 0.3 and 10.0 mg.L^{-1}, respectively. Photoperiod was adjusted to follow the natural seasonal cycle (Light: Dark, 10: 14 hrs), by means of an automated external dimmer (MyTouchSmart, General Electric®) equipped with white led lights (OSRAM 85 Watts). Fish were not handled during a month prior the experimental trials (Pounder et al., 2016). Animal care and experimental procedures were approved by the Bioethics Commission of the State of Nayarit, Mexico (Number: 96/CEBN/2017). The experimental protocol for handling and use of animals that formed part of this study was in accordance with the guidelines of the national centre for replacement, refinement and reduction of animals in research (NC3Rs, U.K.).

3.3.2. Behavioural tests

One behavioural group test, three behavioural individual tests and blood sample extraction were performed serially in two different occasions, called runs, with an interval of four months between them, according to previous studies evaluating long time consistency of SCS in sticklebacks (*Gasterosteus aculeatus*) (Bell, 2005), gilthead sea bream (*S. aurata*) (Castanheira et al., 2013a), zebrafish (*D. rerio*) (Tudorache et al., 2013) and Senegalese sole (*S. senegalensis*) (Ibarra-Zatarain et al., 2016). Besides, individual tests were performed serially on the same day (i- restraining test; ii- predator test; iii- confinement test) 15 days after the group test to allow the fish to recover homeosthasis (Huntingford et al., 2010; Fatsini et al., 2020) and blood extraction was performed immediately after individual tests in both stress runs (Figure 1).



Rectangular tank (78 cm length x 18.6 cm width x 16 cm depth) with a small window in the middle

A risk taking test to evaluate the fish capacity to cross from a known safe area to an unknown risky area

Fish caught in net from tank to evaluate total activity and escapes in the water

Rectangular tank (30 cm length x 19 cm width x 7.5 cm depth) that contained a plastic fish, that simulated a predator

Predator test to evaluate total activity and total escapes

Rectangular tank (13.5 cm x 9.5 cm x 5.0 cm depth)

Confinement test to evaluate total activity and total escapes; afterwards fish was anesthetized for blood sampling

Cortisol and glucose were determined using a Cortisol ELISA Kit and Glucose Colorimetric Assay Kit

Figure 3.1. Methodological outline for SCS characterization: one groupal test (risk-taking), three individual tests (restraining, predator and confinement) and blood sampling for plasma cortisol and glucose concentrations applied serially on flathead grey mullet juveniles at each run.

Group test

Risk taking test (RTT) evaluated the fish capacity to cross from a known safe area to an unknown risky area, which has been considered as a standardized assay to characterize SCS in fish (van Oers et al., 2004; Huntingford et al., 2010). The safe area was isolated from light (~ 2 lux at the surface), while the risky area was constantly illuminated (~ 45 lux at the surface). The test was performed in a 170 L fiberglass tank (78 cm length, x 18.6 cm width x 16 cm depth), divided into two equal areas with a fiberglass barrier, including a window in its middle (5 cm high x 5 cm width), allowing fish to cross from an area to another. Two video cameras (Swann/2K Series-1080p) were set up inside each area of the tank. Constant water flow and aeration were provided during the test to maintain optimal water conditions. Flathead grey mullet juveniles were initially introduced in the safe zone for 1h of acclimation, then the window was opened for two hours (Alfonso et al., 2019). Juveniles were tested in groups of 13 individuals to reduce the impact of high density on stress (de las Heras et al., 2015). The number of fish that crossed (defined as proactive SCS) and did not cross (defined as reactive SCS) was registered on video recording, identifying individuals from both groups by reading their tag number (Tudorache et al., 2013; Ferrari et al., 2015; Alfonso et al., 2020; Jiménez-Rivera et al., 2023).

Individual tests

Restraining test

Restraining test (RT) consisted in capturing each individual with a nylon net and two behavioural variables were measured by maintaining fish in the net inside the water for 90 seconds: i) total activity time (RTAct), defined as total time (s) that fish spent swimming with constant forward or backward movement in the net and ii) number of escape attempts (RTEsc), defined as the number of body contorsions or elevations performed by fish to free itself from the net. The previous methodology was adapted from Castanheira et al. (2016); Zeng et al. (2019); Höglund et al. (2020) and Jiménez-Rivera et al. (2023).

Predator test

Predator test (PT) was performed in a rectangular tank (30 cm length x 19 cm width x 7.5 cm depth) containing a plastic fish, that simulated a predator (approximately greater than 20% of the bodyweight of mullet juveniles). Plastic fish was hidden by a removable dark wall in a corner of the tank, which was removed once tested fish was placed into the tank. Then, plastic fish was moved until the center of the tank, representing a significant risk for fish (Solomon-Lane and Hofmann, 2019). Two behavioural parameters were evaluated for 180 seconds: i) total activity time (PTAct), defined as the total time (s) that fish spent swimming with forward movement and ii) number of escape attempts (PTEsc) defined as the total number of tries to jump or escape from the area (Bensky et al., 2017; Romano et al., 2017 and Jiménez-Rivera et al., 2023).

Confinement test

Confinement test (CT) was performed in a rectangular plastic tank (13.5 cm length x 9.5 cm width x 5.0 cm depth) with reduced space and water simulating a confinement situation. Two behavioural parameters were measured for 180 seconds: i) total activity time (CTAct), being the total time that fish spent swimming with forward movement (s) and ii) number of escape attempts (CTEsc), defined as the total number of attempts performed by fish to escape from the confinement area. The methodology was adapted from Øverli et al. (2006), Ibarra-Zatarain et al. (2016) and Jiménez-Rivera et al. (2023).

3.3.3. Cortisol and glucose blood concentrations

A blood sample (~ 0.5 mL) was extracted from the caudal vein of previously anesthetized fish with eugenol (100 ppm) (Park et al., 2018) from unstressed (control, prior to the experiment, n = 9) and stressed fish (immediately after finalizing the confinement test for both runs 1 and 2, n = 9 for both proactive and reactive). Syringes (1 mL) and needles (23G x 25mm) were coated with heparin (5000 IU/mL Inhephar, PiSA®) and eppendorf´s tubes were provisted with 10 µL of heparin to avoid blood coagulation. Blood samples were centrifuged (GYROZEN, 1580 R) at 3000 G for 15 minutes at 4 °C and plasma supernatant was collected and stored in triplicates at -80 °C until analysis (Ibarra-Zatarain et al., 2016). Cortisol (Cort) was quantified in triplicate with a Cortisol ELISA Kit (Cayman Chemical, USA), based on the competition between cortisol from samples and enzyme-linked to acetylcholinesterase cortisol from the kit (standard range: 6.6 to 4 000 pg/mL; precision intra- and inter- assay coefficients of variation (CV): 8.2% and 19.1%, respectively). Glucose concentration (Gluc) was quantified in triplicate with a colorimetric method (Glucose Colorimetric Assay Kit, Cayman Chemical, USA). Absorbances were read at 420 nm and 520 nm wavelength, for cortisol and glucose, respectively, by means of a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek), according to Ibarra-Zataraín et al. (2019) and Jiménez-Rivera et al. (2023)

3.3.4. Statistical analyses

Statistical analyses were performed using SPSS Statistics 24.0 software (IBM®). Results were presented as mean ± standard error of the mean (S.E.M). All data were checked for normality and homocedasticity by means of Kolmogorov-Smirnov and Levene tests. The level of significance was established for $P < 0.05$ for all analysis. Firstly, a Chi-square test was performed on data from the risk-taking test to determine significant differences between proportions of fish that crossed and did not cross between runs 1 and 2. Secondly, a Multivariate Analysis of Variance (MANOVA) were performed on behavioural variables from the three individual tests, on morphology (weight-We, length-Le and specific growth rate-SGR), and physiological parameters (cortisol and glucose), between proactive and reactive individuals, for runs 1 and 2. Additionally, a Tukey's post-hoc test was carried out when significant differences were detected among the groups. Thirdly, a General Linear Model with a multivariate repeated measures analyses of variance (GLM-RM MANOVA), with a Wilk´s lambda criterion and Fisher´s exact test, followed by post hoc comparisons with the Tukey test were performed on data from runs 1 and 2 to validate the inter-individual consistency. Repeatability was defined as the intraclass correlation coefficient (ICC), being ICC ≤ 0.2 a low repeatability; 0.2<

ICC < 0.4 a moderate repeatability and ICC ≥ 0.4 a high repeatability (Bell et al., 2009; Wolak et al., 2012). Reliability of exploratory behaviour over time was analized using Alpha Cronbach´s (α) and Fisher tests. Values of $\alpha > 0.7$ were considered as highly reliable and indicated that individuals maintained an intra-individual consistency for the four months of the experiment (Ibarra-Zatarain et al., 2020).

Finally, behavioural traits were highlighted between the three individual tests. To do that, a Principal Component Analysis (PCA), with a Kaiser-Meyer adequacy and Oblimin rotation, was realized to identify those behavioural variables with the highest correlation among them, considering for this the highest communality of each variable from each axis, following criteria from Jiménez-Rivera et al. (2023).

Then, a across-context consistency analysis were performed, with Pearson correlations between variables of the three tests to determine whether behavioural traits exist between tests.

3.4. RESULTS

3.4.1. Coping styles responses

Overall, flathead grey mullet juveniles exhibited a high degree of variability in their individual behavioural responses to the stress tests performed, with coefficients of variation showing high bimodality with individuals ranging from 2.7 to 109.3% (Table 3.1).

Group test

A similar trend in flathead grey mullet SCS was observed for the risk-taking test between runs 1 and 2 (Chi-square test: $\chi^2 = 54.725$, $P = 0.014$), supporting high intra-individual consistency in the proportion of juveniles that successfully crossed for this species over time. Fish that crossed in the risk-taking test were classified as proactive and fish that did not cross as reactive. In the first run, 26 of 66 fish (39.39 %) crossed from the safe to the risky area and 40 fish (60.61%) that did not cross. Seven juveniles died in run 1 (n = 66), from which 5 (19.2%) crossed in the risk-taking test (proactive) and 2 (5%) did not cross (reactive). In the second run, 21 of 49 fish (42.86%) crossed and 28 fish (57.14%) that did not cross. Ten juveniles died in run 2 (n = 49), from which 7 (33.3%) crossed in the risk-taking test (proactive) and 3 (10.7%) did not cross (reactive). Sixteen fish crossed and 24 did not cross in both runs of the risk-taking test, representing a total number of 40 fish of 49 alive at the end of the trial (81.6%).

Table 3.1. Individual behavioural variables, growth and blood parameters in flathead grey mullet *Mugil cephalus* juveniles (n = 49), as mean ± SEM, minimum (Min.), maximum (Max.) and coefficient of variance (CV). Inter-individual variation is represented by the coefficient of variation in flathead grey mullet *Mugil cephalus* juveniles. Superscripts letters indicated significant differences among runs ($p < 0.05$).

		RUN 1				**RUN 2**			
Coping style tests	**Behaviour within context**	**Mean ± SEM**	**Min**	**Max**	**CV (%)**	**Mean ± SEM**	**Min**	**Max**	**CV (%)**
Restraining	RTEsc	41.1 ± 3.8	6	109	65.4	32.8 ± 3.3	6	88	72.8
	RTAct (s)	42.6 ± 2.6	11	88	43.2	37.8 ± 2.4	11	69	44.5
Predator	PTEsc	6.5 ± 0.8	0	19	85.5	6.5 ± 0.6	0	15	63.9
	PTAct (s)	32.0 ± 3.8	3	109	82.8	27.2 ± 2.8	6	79	71.0
Confinement	CTEsc	8.2 ± 0.9	0	43	83.4	7.8 ± 1.2	1	59	109.3
	CTAct (s)	21.0 ± 2.1	2	80	68.7	19.2 ± 1.7	4	58	61.7
Morphology variables	Weight (g)	12.7 ± 0.3	10.16	16.82	14.2	13.9 ± 0.3	10.7	18.4	14.4
	Length (cm)	10.8 ± 0.1	9.5	12.2	6.6	11.4 ± 0.1	10	13.6	7.9
Blood parameters	Cortisol	77.8 ±16.7^{b}	26.23	322.4	91.1	226.3 ± 14.2^{a}	43.96	521.3	68.9
	Glucose	37.6 ± 3.2	20.13	53.63	25.7	41.7 ± 3.0	26.36	68.43	16.5

Individual tests

Significant differences were detected between proactive and reactive fish (identified in the risk taking test) as in run 1 (MANOVA: $F_{6,42}$ = 14.73, *P* = 0.01) as in run 2 (MANOVA: $F_{6,42}$ = 10.04, *P* = 0.01), for all behavioural parameters, morphology and physiological variables (Table 3.2). Fish identified as proactive in the risk-taking test presented a significantly higher activity time and number of escape attempts in the three individual tests than reactive fish, as well as higher weight, SGR, length and lower cortisol and glucose blood concentrations ($P < 0.05$) (Table 2). Basal levels of blood cortisol and glucose in unstressed flathead grey mullet were of 17.86 ± 2.88 $ng.mL^{-1}$ and 31.99 ± 1.61 $mg.dL^{-1}$, respectively.

3.4.2. Consistency of behavioural responses over time

Flathead grey mullets' juveniles showed a high intra-individual consistency (α = 0.90 ± 0.11) for all behavioural parameters measured (Table 3). Nevertheless, cortisol and glucose blood concentrations were not significantly consistent over time (Table 3), although proactive and reactive fish displayed significant differences in both runs 1 and 2 (Table 2). Physiological variables were not significantly correlated with behavioural variables, neither in proactive (Pearson rank correlation: r = 0.023, N = 18, *P* = 0.69) nor in reactive (Pearson rank correlation: r = 0.15, N = 18, *P* = 0.303).

Likewise, they were not significantly correlated with weight and length, neither in proactive (Pearson rank correlation: r = 0.25, N = 21, *P* = 0.06) nor in reactive (Pearson rank correlation: r = 0.0081, N = 28, *P* = 0.706) for this species.

Table 3.2. Behavioural and physiological differences between flathead grey mullet juveniles that crossed (proactive, n = 29) versus those that did not cross (reactive, n = 20) in the risk-taking test, in run 1 and 2 (mean ± S.E.M., MANOVAs and Tukey's HSD post-hocs). Superscripts letters a and b, x and y indicated significant differences between proactive and reactive fish in run 1 and run 2, respectively (p < 0.05)

		RUN 1					RUN 2				
		Proactive	**Reactive**	**MANOVA test**			**Proactive**	**Reactive**	**MANOVA test**		
Coping style tests	**Behaviour within context**	**Mean ± SEM**	**Mean ± SEM**	**df**	**F**	**P**	**Mean ± SEM**	**Mean ± SEM**	**df**	**F**	**P**
Restraining	RTEsc	57.5 ± 4.2^{a}	17.3 ± 1.6^{b}	1	57.95	**0.00**	45.6 ± 4.1^{x}	14.3 ± 1.4^{y}	1	37.58	**0.00**
	RTAct (s)	54.0 ± 2.8^{a}	26.2 ± 1.6^{b}	1	14.18	**0.00**	47.7 ± 2.6^{x}	23.5 ± 1.9^{y}	1	49.09	**0.00**
Predator	PTEsc	9.7 ± 0.9^{a}	1.9 ± 0.6^{a}	1	33.29	**0.01**	8.4 ± 0.7^{x}	3.6 ± 0.6^{y}	1	22.81	**0.01**
	PTAct (s)	47.4 ± 4.5^{a}	9.8 ± 1.2^{b}	1	40.78	**0.01**	38.5 ± 3.2^{x}	10.6 ± 0.9^{y}	1	50.01	**0.02**
Confinement	CTEsc	10.9 ± 1.37^{a}	4.3 ± 0.8^{b}	1	13.99	**0.02**	10.0 ± 1.9^{x}	4.7 ± 0.5^{y}	1	4.85	**0.01**
	CTAct (s)	27.9 ± 2.77^{a}	11.1 ± 1.1^{b}	1	23.07	**0.00**	24.4 ± 2.4^{x}	11.6 ±0.8^{y}	1	17.83	**0.00**
Morphology parameters	Weight (g)	11.7 ± 0.4^{a}	10.8 ± 0.2^{b}	1	9.87	**0.00**	14.8 ± 0.4^{x}	11.9 ± 0.3^{y}	1	19.42	**0.00**
	Length (cm)	13.8 ± 0.1^{a}	12.7 ± 0.1^{b}	1	17.5	**0.00**	16.1 ± 0.2^{x}	13.7 ± 0.1^{y}	1	23.63	**0.00**
	SGR (%)	2.9 ± 0.2^{a}	0.9 ± 0.2^{b}	1	37.18	**0.00**	2.6 ± 0.3^{x}	1.5 ± 0.2^{y}	1	7.29	**0.01**
Blood parameters	Cortisol	45.2 ± 4.9^{b}	110.4 ± 16.0^{a}	1	4.62	**0.03**	120.9 ± 14.2^{y}	331.6 ± 24.8^{x}	1	14.92	**0.01**
	Glucose	29.5 ± 3.6^{b}	46.7 ± 5.8^{a}	1	49.64	**0.00**	30.6 ± 1.0^{y}	52.9 ± 2.7^{x}	1	59.98	**0.00**

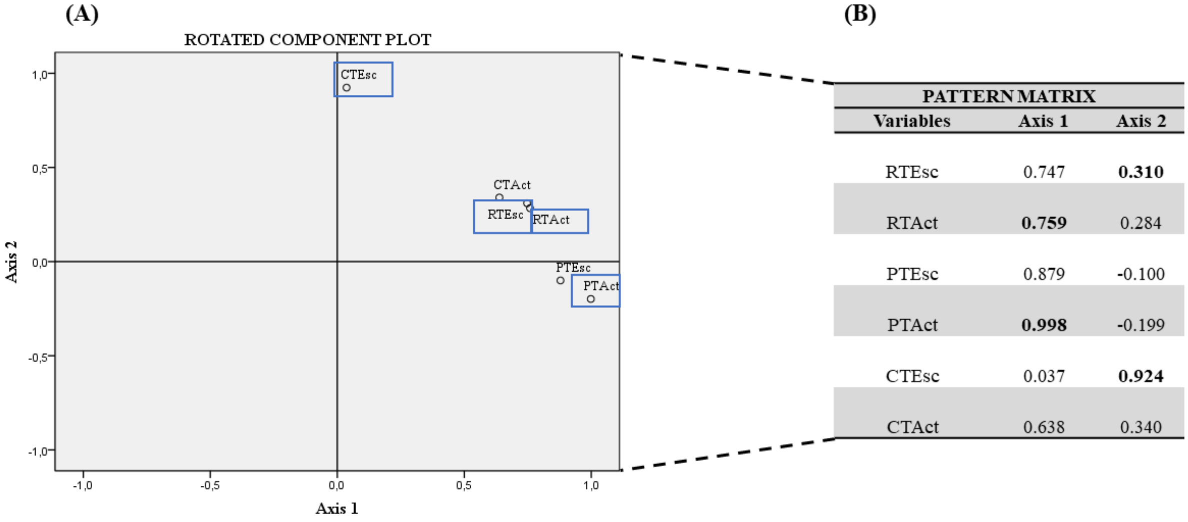
Table 3.3. Intra and inter-individual long-term consistency of SCS of flathead grey mullet juveniles over time (GLM-MANOVA between run 1 and 2). **λ** = Wilk´s lambda value, α = Alpha Cronbach´s value, ICC = intraclass correlation, **d.f.** = degrees of freedom, **F** = Fisher value, **P** = significance level. P-values > 0.05 and P-values < 0.05 in bold indicated significant intra-and inter-individual repeatability for Wilk´s lambda and Alpha Cronbach´s value, respectively.

		Inter-individual consistency (Wilk´s lambda test)				Intra-individual consistency Alpha Cronbach´s test				
Coping style tests	**Behaviour within context**	**λ**	**d.f**	**F**	**P**	**α**	**ICC**	**d.f**	**F**	**P**
Restraining	RTEsc	**0.762**	**1,48**	**14.96**	**0.33**	**0.894**	**0.808**	**48,48**	**9.43**	**0.00**
	RTAct	**0.775**	**1,48**	**13.92**	**0.10**	**0.934**	**0.875**	**48,48**	**15.06**	**0.00**

Predator	PTEsc	**1.00**	**1,48**	**0.005**	**0.95**	**0.789**	**0.653**	**48,48**	**4.73**	**0.00**
	PTAct	**0.919**	**1,48**	**4.21**	**0.46**	**0.852**	**0.743**	**48,48**	**6.77**	**0.00**
Confinement	CTEsc	**0.994**	**1,48**	**0.31**	**0.58**	**0.890**	**0.802**	**48,48**	**9.08**	**0.00**
	CTAct	**0.966**	**1,48**	**1.67**	**0.20**	**0.843**	**0.728**	**48,48**	**6.36**	**0.00**
Blood parameters	Cortisol	**0.999**	**1,17**	**0.20**	**0.89**	0.127	0.068	17,17	1.15	0.39
	Glucose	0.583	1,17	12.17	0.03	0.369	0.226	17,17	1.59	0.18

3.4.3. Characterization of behavioural traits and across-context analysis

The efficacy of the PCA was confirmed by the significant results of the Kaiser-Meyer-Olkin and Barlett´s test of sphericity (KMO = 0.846; $\chi 2$ = 425.735, gl = 15, P < 0.001). The PCA analysis resulted in two principal component (PC) axis, which explained 80% of total variance (first axis = 68.44%, second axis = 12.54%). The behavioural variables with the highest communalities, or correlation, in PC1 were PTAct (0.998) and RTAct (0.759) and for PC2 were CTEsc (0.924) and RTEsc (0.310) (Figure 2).



PATTERN MATRIX		
Variables	**Axis 1**	**Axis 2**
RTEsc	0.747	**0.310**
RTAct	**0.759**	0.284
PTEsc	0.879	-0.100
PTAct	**0.998**	-0.199
CTEsc	0.037	**0.924**
CTAct	0.638	0.340

Figure 3.2. Behavioural variables selected for flathead grey mullet through a PCA analysis: (A) Rotated component plot of behavioural variables selected (in blue square), showing the correlation between variables selected from Pattern matrix (B) (variables with the highest communalities in bold for each axis).

Concerning consistency of behavioural response across context, there was consistency between the restraining and the predator tests (Pearson rank correlation: r = 0.70, N = 49, *P* = 0.001), between the restraining and the confinement tests (Pearson rank correlation: r = 0.44, N = 49, *P* = 0.046), and between the predator and confinement tests (Pearson rank correlation: r = 0.65, N = 49, *P* = 0.0038).

3.5. DISCUSSION

A wide variation in stress coping abilities was observed in flathead grey mullet juveniles, submitted to different group and individual SCS tests. In this sense, fish showed a high degree of inter-individual variability in their behavioural responses, allowing to classify them into proactive and reactive traits. These differences may be linked to the plasticity in the response to induced stressors within individual of a same species (Killen et al., 2016). Plastic changes fluctuate depending on individual intrinsic characteristics and individuals differ in the slope of their reaction norms in changing contexts (Killen et al., 2016), where reaction norms describe differential responses of individuals to environmental changes, *e.g.* perceived risk of predation (Dingemanse et al., 2012). Individuals can differ in stress coping plasticity due to a wide variety of environmental factors or simply as a result of changes in traits over time as animals grow or learn about their environment. This individual phenotypic plasticity implies that individual variation will become context dependent, and trait consistency can be greatly affected by environmental changes (Dingemanse et al., 2010; Mitchell and Houslay, 2021). Thus, it was possible that the intra-individual consistency in flathead grey mullet is replicable between separed traits measured in the same behavioural test and, to some extent, traits in different behavioural tests, in spite of the similarity in contexts.

An animal's tendency to explore unpredictable or risky situations is indicative of different types of stress coping styles (Baker et al., 2018). In the present study, a group of juveniles were more prone to take risk than another group of fish, being in line with other studies that reported similar tendencies in species such as European seabass *D. labrax* (Alfonso et al., 2019), Senegalese sole *S. senegalensis* (Fatsini et al., 2020) and common carp *C. carpio* (Huntingford et al., 2010). Testing fish in group trials has been considered as a good predictor of response to stress, since social context strongly influences individual personality (Crane et al., 2018; Culbert et al., 2019). Social context is involved in the regulation of numerous characteristic behaviours such as social facilitation (Harpaz et al., 2017), social familiarity (Nadler et al., 2021), social dominance (Rodríguez-Santiago et al., 2020), social plasticity (Maruska et al., 2019) and social learning (Webster and Laland, 2017). The presence of conspecifics can push individuals to reinforce or reduce threat-sensitive behavioural traits, such as activity, exploration, risk-taking, feeding rate and courtship opportunities (Ward et al., 2020; Sommer-Trembo et al., 2022). Additionally, individuals with extreme personality types may be affected differently. As flathead grey mullet juveniles exhibit a social behaviour, with the specificity to form small groups and absence of aggressiveness (Jiménez-Rivera et al., 2022), some of the individuals of this fish species were expected to be more sensitive to isolation and to try to follow the behaviour of other members of the shoal, suggesting that social context significantly influence individual personality. Nevertheless, the degree of change in individual responses, induced by the presence of conspecifics, compared to isolated responses, is also influenced by individual initial responsiveness (Magnhagen, 2007; Magnhagen and Bunnefeld, 2009).

In the individual behavioural tests, fish that had been classified as proactive had high activity time and number of escape attempts, while fish classified as reactive had low activity time and number of escape attempts, indicating fish with both stress coping styles behaviours (Farwell and McLaughlin, 2009; Tudorache et al., 2013). These observations were in agreement with Ferrari et al. (2015), Gesto (2019) and Ibarra-Zatarain et al. (2020), whom reported that proactive individuals showed higher activity time and exploratory behaviour than reactive fish, in European seabass *D. labrax*, rainbow trout *O. mykiss* and Senegalese sole *S. senegalensis*, respectively. The restraining test, which measured the ability of fish to respond to invasive and aversive imposed situations, induced two divergent behavioural reactions related to SCS in juveniles, characterized by a group of individuals with high values for activity time and number of escape attempts and another group with low values for both parameters, suggesting a boldness-avoidance trait (Castanheira et al., 2016). Similarly, confinement test, which measured fish explorative reaction to a new confined environment with a reduced freedom in their movements, resulted into two groups that differed in total activity time and number of escapes attempts, being consistent with the stress coping styles concept and representing the exploration-fearfulness trait (Jiménez-Rivera et al., 2023). In addition, fish with proactive profiles were characterized by a lower HPI axis activity than fish with reactive styles, after the stress tests. This was in aggrement with studies by Schjolden et al. (2005), Ibarra-Zatarain et al. (2016) and Alfonso et al. (2020) for rainbow trout *O. mykiss*, Senegalese sole *S. senegalensis*, zebrafish *D. rerio* and gilthead seabream *S. auratus*, respectively. The variation in the regulation of HPI axis between proactive and reactive SCS has been reported by Øverli et al. (2007) as an evolutionary adaptive mechanism process in which proactive individuals were more prepared to unpredictable stressful situations than reactive specimens. Another interpretation is that the reactive profile may have a faster release rate relative to the proactive profile, which may lead to a shorter time to reach peak levels. However, it should be noted that our study design does not allow us to directly assess differences between lines in time to reach peak cortisol levels. Likewise, faster post-stressor corticosterone release has also been observed in zebrafish *D. rerio* and Senegalese sole *S. senegalensis* reactive (Wong et al., 2019; Ibarra-Zataraín et al., 2020). This suggests that glucocorticoid release rates in response to a stressor may be conserved in proactive and reactive stress coping styles among diverse species.

In addition, cortisol also increases the release of catecholamines, which further rises the activity of several enzymes from the glycogenolysis process and modulate cardiovascular and respiratory function (Janssen, 2022). Glycemia is mostly mediated by the action of cortisol which stimulates liver gluconeogenesis and limits peripheral sugar uptake (Marik and Bellomo, 2013), for glucose to be used in metabolic processes involved the response to stress. In the present study, variations in glucose blood concentrations between proactive and reactive fish followed the same pattern than plasma cortisol levels, being significantly lower in proactive than in reactive individuals, as obtained in common carp *C. carpio* (Rey et al., 2016) and gilthead sea bream *S. aurata* (Vargas et al., 2018).

Interestingly, proactive individuals displayed higher SGR, and also showed higher mortality rate than reactive individuals, after four months of experiment. According to Réale et al. (2010) and Damsgård et al. (2019), this trend could be related to the pace-of-life syndrome (POLS) hypothesis, which predicts that proactive profiles tend to present a higher mortality than reactive traits, an ecological strategy helping to compensate the advantage of organisms with higher growth and development rates, and reproduction success. Furthermore, mortality rate of proactive individuals is also suggested to succeeded due organisms are more likely to be predated (Øverli et al., 2007). Even if literature associating growth and personality types is available, information on how proactive and reactive traits are related to developmental rate is scarce (Stamps, 2016). In this sense, differences in energy demand and utilization have been proposed as a likely explanation for the higher growth rates observed in proactive specimens than in reactive ones (Killen et al., 2016). In particular, resting metabolic rate (RMR) has been significantly associated with individual behavioural differences (Careau and Garland, 2012; Niemelä and Dingemanse, 2018), underlying that RMR would be the "inactivity cost" of the metabolic machinery necessary to maintain a given lifestyle (Mueller and Diamond 2001). Careau et al. (2008) even developed a model on metabolic rate performance, predicting that animals with high metabolic rates are able to collect, process and invest nutritional energy faster than other conspecifics and thus, to achieve higher growth rates and higher activity levels (Biro and Stamps, 2010; Damsgård et al., 2019). These studies suggested that high metabolic costs might be associated to proactive behavioural profile, as proactive fish have been characterized as individual which forager actively and achieve higher growth rates than reactive ones, even when resources were limited, as documented in brown trout *S. trutta*, rainbow trout *O. mykiss* and guppies *Poecilia reticulata* (Skov et al., 2019; Godin et al., 2022). Nonetheless, more studies focusing on the link between metabolic rate, growth, activity and SCS are required in flathead grey mullet to demonstrate that these previous assumptions could be true for this fish species.

Characterizing SCS in reared animals and particularly, evaluating individual behavioural consistency over time is of great interest in animal farming, for predicting and organizing their management in captivity. Considering that predictability may rely on a consistent attribute of animal behaviour raises substantially the interest in studying animal personality and particularly, behavioural plasticity (Adriaenssens and Johnsson, 2013). The present study evidenced a correlation between individual and group tests in flathead grey mullet personality consistency. Most of studies on fish personality have been based on individual tests, such as rainbow trout *O. mykiss* (Øverli et al., 2006; Gesto, 2019), Atlantic salmon *S. salar* (Kittilsen et al., 2009), Nile tilapia *O. niloticus* (Barreto and Volpato, 2011) and gilthead seabream *S. aurata* (Castanheira et al., 2013a). Some other studies have been using group tests to characterize SCS in European seabass *D. labrax* (Alfonso et al., 2019), zebrafish *D. rerio* (Alfonso et al., 2020) or Senegalese sole *S. senegalensis* (Fatsini et al., 2020). However, up to now, few studies have used both approaches to assess personality in fish, such as in Senegalese sole *S. senegalensis* (Ibarra-Zatarin et al., 2016), pikeperch *Sander lucioperca* (Colchen et al., 2017) and flathead grey mullet *M. cephalus* (Jiménez-Rivera et al., 2023). One of the main limits of using exclusively individual assessment of the response to stress is that modification of behaviours related to social interactions happening in group is not considered. On one hand, different personalities could exhibit a different degree of sensitization to isolation. On the other hand, group testing may drive individuals to modulate their behaviour according to conspecifics behaviour (Huntingford et al., 2010; Shams et al., 2018). In this sense, statistical analyses performed in the present study supported the fact that flathead grey mullet juveniles showed a significantly high degree of intra-individual consistency across contexts: restraining, predator and confinement tests, and over time: between runs 1 and 2. In other words, fish presenting the highest total activity and highest number of escape attempts in the three individual tests in run 1 maintained constant their behavioural responses to stress in run 2, as well as for individuals displaying the lowest total activity and lowest number of escape attempts, demonstrating behavioural consistency of responses to stress over a long period of time (four months) and being in agreement with other studies on stickleback *G. aculeatus* (Bell, 2005), zebrafish *D. rerio* (Rey et al., 2013), gilthead seabream *Sparus aurata* (Castanheira et al., 2013a) and European seabass *D. labrax* (Ferrari et al., 2015). Results from PCA suggests that PC1 represented the fish boldness-activity behaviour due to the high correlation in total activity between predator and restraining tests, while PC2 characterized flathead grey mullet avoidance-fearfulness behaviour, represented by the escape attempts in restraining and confinement tests. The boldness-activity behaviour can influence the outcome of everyday stressful challenges, such as competition for food (Dingemanse et al., 2007), the time to emerge from shelter into a familiar environment (Brown et al., 2005) and response to predator stimulus (Wilson and Stevens, 2005). Consequently, boldness-activity behaviour can influence standard metabolic rate, stressed locomotor activity, reproduction and survival (Koolhaas et al., 1999; Fu et al., 2021). Likewise, the avoidance-fearfulness behaviour encompasses behaviours that involve individual willingness to not investigate novel environments, food items or objectsovel environments and novel objects allow (Reader and Laland, 2003; Réale, 2007). Finally, the interpretation of components reflecting behavioural traits or syndromes, as in this study, have been previously described in others fish species, such as brown trout (Lothian and Lucas 2021); Senegalese sole (Ibarra-Zataraín et al., 2016) and flathead grey mullet (Jiménez-Rivera et al., 2023), by means of similar tests and statistical analysis to those used in this study.

Lastly, the low repeatability, consistency and correlation of cortisol and glucose blood concentrations over time might be attributed to physical manipulation, fish age and size, to the nutritional status of individuals or to individual behavioural differences. The regulation of glucocorticoids production and their release into the blood circulation have been showed to depend on the interaction of these factors in several fish species such as rainbow trout *O. mykiss* (Øverli et al., 2007; Ruiz-Gomez et al., 2011), Senegalese sole *S. senegalensis* (Ibarra-Zatarain et al., 2020). In agreement with the present study, several other studies have documented a lack of correlation between plasma cortisol levels obtained after stress and behavioural responses to stress, such as in rainbow trout *O. mykiss* and in gilthead seabream *S. aurata* (Silva et al., 2010; Castanheira et al., 2013b), where the quality of the response to a challenging situation (coping styles) was suggested to be independent from the quantity of that response (stress reactivity).

Understanding how different personality types can coexist within a population is complex and beyond the scope of this paper. However, relying on well-established concepts such as the pace-of-life syndrome (POLS) hypothesis (Réale et al., 2010) is helpful to understand why groups of conducts were correlated at the intra-individual level. This hypothesis suggested the existence of a trade-off between maximizing growth rate at the expense of survival (fast strategy) and maximizing survival at the expense of growth rate (slow strategy). As individuals sit on an axis between slow and fast strategy, their expression of behavioural responses to stress reflects their strategy and is therefore correlated to behavioural traits at the intra-individual level. Results of the present study could fit into this model, as bold individuals were predicted to develop faster and die younger, and might have adopted a fast strategy, than shyer individuals, which might have adopted a slow strategy (Réale et al., 2010). Knowledge of SCS is now well recognized in farmed fish, and it has a wide implication for aquaculture, as it helps to improve the sustainability of production by establishing more refined farming strategies, adapting optimal rearing conditions to the different proactive and reactive SCS. In this sense, as each SCS is related to a particular physiology and behaviour, considering SCS in captivity may be useful to improve feed conversions and minimize feed waste and adjusting handling protocols would improve domestication process and adaptation to rearing conditions for this fish species (Castanheira et al., 2017).

3.6. CONCLUSION

In conclusion, the present study evidenced SCS in *Mugil cephalus* juveniles, characterized into two extremes of personality, defined as proactive and reactive, and validated the behavioural and physiological differences between them. Furthermore, a high between and within individual consistency was demonstrated over time and across contexts, in a four months period, for this fish species. Overall, the results of the present study covered new insights in behavioural and physiological traits of flathead grey mullet under rearing conditions that may be of interest for optimizing aquaculture protocols and development of genetic selection programmes for this fish species in order to improve their production. Finally, more studies should be performed in order to increase the knowledge on flathead grey mullet coping styles in relation to food conversion, disease resistance, reproductive success and fitness

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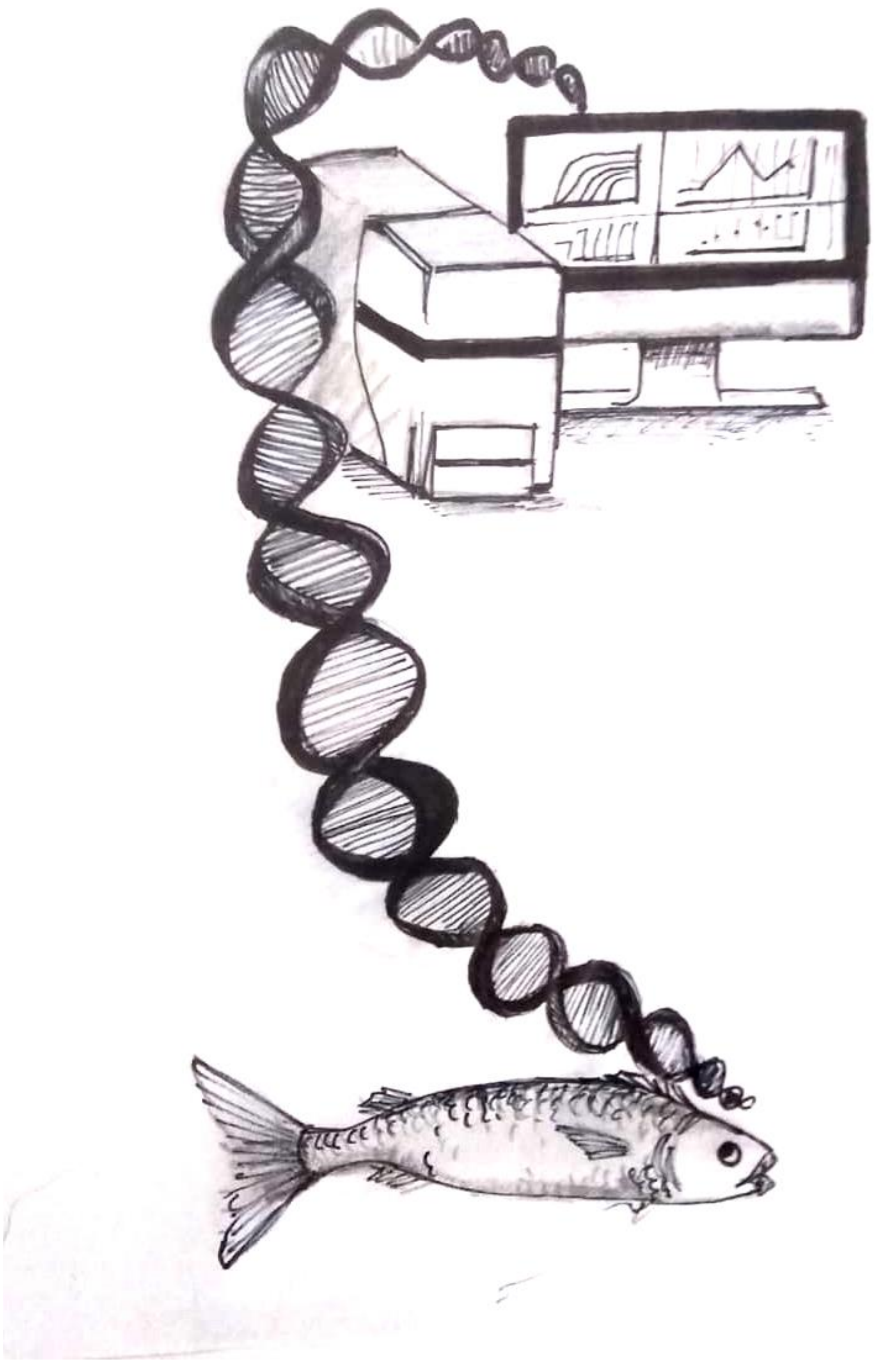


CAPÍTULO 4

BEHAVIOURAL AND CHARACTERIZATION OF THE BRAIN TRANSCRIPTOME OF FLATHEAD GREY MULLETS (*Mugil Cephalus*) JUVENILES BETWEEN PROACTIVE AND REACTIVE COPING STYLES

Artículo elaborado para su envío a la revista *Current Biology*

4.1. ABSTRACT

Animals experience stress in many contexts and often successfully cope. In fish, individuals exhibiting the proactive and reactive stress coping styles display qualitatively different behavioral and variation in molecular expression data to stressors. Stress coping styles have been previously described in flathead grey mullet by validating for the species the use of standard behavioural screening tests. The predisposition to exhibiting a particular coping style is due to genetic and environmental factors. In this study we explore the brain-transcriptomic and gene network biases that are associated with differences between proactive and reactive coping styles, rearing in a common environment. Using RNA-sequencing we quantified the transcriptomes from the brains of flathead grey mullet juveniles selectively bred to exhibit the proactive or reactive stress coping style. We identified 485 genes that differed in gene expression levels. Gene ontology analyses reveal that many of these core genes are implicated in stress responses, exploration behaviours and neural differentiation. This work reported for the first time that proactive and reactive stress coping individuals display distinct neurotranscriptomic states in M. cephalus juveniles, showing that differences in expression of select genes or regulation of specific gene modules are linked to the magnitude of the behavioural response and the display of a coping style, respectively. As such, our results help to depict state-behaviour feedback mechanisms, previously proposed as key in shaping animal personality in this species.

4.2. INTRODUCTION

The family Mugilidae (smooth or mugilids) is a family of fish widely distributed in tropical and subtropical waters around the world, particularly in coastal and estuarine areas where they play an important ecological role and provide biomass to support fisheries (Whitfield et al., 2012). Of the 26 genera and 80 species belonging to the Mugilidae family (Eschmeyer and Fong, 2021), only 3 species are important for aquaculture (Crosetti, 2016). The most cultivated mugil species, the common mullet, is considered a potential candidate for the diversification of Latin-American marine aquaculture due to the following characteristics; it can be cultured in different aquatic environments and different aquaculture systems (Nordlie, 2016), exhibits fast growth (~1 kg per year) (FAO, 2019) and has efficient conversion of feed to body mass (EUMOFA, 2020).

Stress coping styles (SCS) are individual behavioural and physiological trait strategies that are consistent over time and context (Koolhaas et al., 1999; Castanheira et al., 2016). Behaviuoral responses may vary individually over time in proactive-reactive. Proactive animals are considered more active, aggressive, grow faster, and have better mating options, but show less flexibility to variations in the natural environment; while reactive individuals present a more flexible adaptive response to changing and unpredictable environments and only adopt risky behaviors when necessary (Koolhaas et al., 2010; Höglund et al., 2020; Linares-Cordova et al., 2024).

The study of individual differences in animal behaviour is recognized as an important field in social studies related to the ecology and evolution of animals (Morgan and Dall, 2015). Knowledge of coping styles in fish is of great importance, not only from an evolutionary perspective but also for aquaculture, in order to increase productivity and select animals based on their response to stress, helping to improve growth, survival, resistance to handling and diseases (Castanheira et al., 2017; Ibarra-Zatarain et al., 2020). Different SCS classifications (proactive, reactive) related to behavioural and physiological parameters have been defined in a wide range of fish species, such as European seabass (*Dicentrarchus labrax*) (Alfonso et al., 2019), rainbow trout (*Oncorhynchus mykiss*) (Gesto, 2019), Atlantic salmon (*Salmo salar*) (Damsgård et al., 2019), gilthead sea bream (*Sparus aurata*) (Höglund et al., 2020), flathead grey mullet (*Mugil cephalus*) (Linares-Cordova et al., 2024), among others. In addition to behavioural studies for the a priori detection of SCS, different research have reported that SCSs significantly reduce variation within the population in gene expression studies, which allows the observation of different gene expression profiles in proactive and reactive (Wong et al., 2015; Rey et al., 2021).

The brain plays a fundamental role in regulating processes related to managing and adapting to stress in organisms (McEwen, 2009; Vernier, 2017). Likewise, the higher cognitive areas of the brain play an important role in memory, anxiety and decision making (Verburg-van Kemenade et al., 2017; Cerqueira et al., 2020; Fernö et al., 2020). Understanding how species and behaviours are coordinated between and within individuals is of great interest to neuroscience as behavioural variation corresponds to variation within the brain (Jolles et al., 2020). Developing tools to reliably identify individuals with different behavioural profiles facilitates the exploration of the underlying molecular and physiological regulation that in turn facilitates efforts to understand adaptation and the evolution of personality traits (van Oers and Mueller, 2010). Differentes studies are making it possible to understand how individual variation within and between behavioural phenotypes is related with transcriptional regulation; however, establishing how the environment interacts with molecular pathways to shape variation in individual behaviours remains a outstanding challenge (Benowitz et al., 2017; Zhang et al., 2021). Evolutionary studies using RNA sequencing (RNA-Seq) helps to clarify the genetic and environmental effects on phenotypes and their connection. Furthermore, the existence of phenotype-genotype relationships has suggested that many genes are transcriptionally linked to a certain phenotype (Orgogozo et al., 2015). In fish, studies applying a stress coping style approach have been used to resolve variation in physiological and molecular data suggesting a linkage, genotype-phenotype, between behaviour and transcriptome regulation (Wong et al., 2019; Rey et al., 2021; Sadoul et al., 2022). Such studies provide the background to show that trait convergence is often achieved through genetic changes to shared pathways, genes or even molecular sites, suggesting that estimate the degree of genetic convergence expected for specific types of convergent traits (Sackton and Clark, 2019). Therefore, gene expression patterns offer new opportunities to decipher the molecular mechanisms of complex behaviours affected by domestication (Rodríguez-Barreto et al., 2019). Transcriptomics provides the ideal platform to approaches will be most powerful when they are combined into integrative studies that link transcriptional variation with neural activity, physiology and genetic variation in order to revolutionize behavioural studies (Fischer et al., 2021)

Recent studies carried out on mullet, *Mugil cephalus*, showed a clear activation of pathways through the differential expression of a large number inmune related genes to the immune system in the kidney and spleen after being challenged with the bacterium *Lactococcus garvieae* (Byadgi et al., 2016). Furthermore, Ramos-Júdez et al. (2022) evaluated the ovarian transcriptome development under recombinant gonatropin hormones (rGths) induction in the flathead grey mullet. They described enriched paths with genes being differentially expressed in the ovary as induced vitellogenesis progressed. On the other hand, in *Mugil incilis,* Bertel-Sevilla et al. (2020), characterized the liver transcriptome and showed genes related to environmental information, metabolism and organismal system pathways. However, the analysis of fish brain transcriptomes has been limited to only a few species such as *Betta splendens* (Vu et al., 2020), *Dicentrarchus labrax* (Rey et al., 2021; Sadoul et al., 2022) and *Salmo salar* (Rey et al., 2021); and the best for our study is no studies on the brain transcriptome of *M. cephalus* or other mugilid species have been reported. The purpose of this study was to characterize the brain transcriptome of *M. cephalus* using RNA-Seq based on proactive-reactive behaviour profiles to facilitate future studies on gene expression related to the effects of any other stressors on behaviour and rearing in this species. Also, we found the synchronization of the brain transcriptomes between gene sets by analyzing DEGs, gene ontology and gene pathway enrichment to understand what kinds of brain activity might underlie the transcriptomic synchronization during stressful situations.

4.3. MATHERIAL AND METHODS

4.3.1. Experimental Animals

Flathead grey mullet (*M. cephalus*) juveniles used in this experiment were obtained from the estuary el Yugo in Mazatlán, Sinaloa (México). Fish were kept in two cylindrical 6,000 L tanks and acclimated during four weeks, with a prophylactic treatment (oxytetracycline baths, ≈ 120 $mg.L^{-1}$ for 1h) to reduce the incidence of diseases (U.S Fish & Wildlife Service, 2020).

4.3.2. Housing and Husbandry

Animals were housed in one 1700L circular tank connected to a recirculation aquaculture system (RAS). After three weeks, fifty flathead grey mullet juveniles (7.25 ± 4.07 g) were randomly distributed in two 120 L fiberglass tanks (n = 25 fish per tank; ~ 2 Kg/m3), and housed at 24.71 ± 1.20 °C with a 13-h dark and 11-h light photoperiod to conduct the behavioural tests. They were individually tagged, using a PIT tagged (Passive Integrated Transponder: FRD-LF-GT8-64B LE Glass Tag Ø1.4 x 8mm EM4305 125KHz, China), to ensure individual identification. Fish were fed three times a day with a commercial diet (Skretting Nutra 160; 38% protein, 8% lipids). Uneaten food and faeces were siphoned daily, one hour after feeding fish, to maintain a correct water quality.

4.3.3. Behavioural tests

All organisms were submitted to one group test (“Risk-taking”) and three individual behavioural tests (“Restraining”, “New environment” and “Confinement”), since they are the most representative to explain the individual variation (Fatsini et al., 2020; Jiménez-Rivera et al., 2023). Individual tests were applied serially on the same day, one week after the group test in order to allow fish fully recovering their homeostasis (Huntingford et al., 2010; Ibarra-Zatarain et al., 2016). The four tests were performed in two runs (run 1 and run 2) based on previous studies that classify fish according to their proactive and reactive behavioural traits, with fifteen days of interval between both stress runs.

4.3.3.1. Group test

Risk taking test (RTT)

The objective of this test was to determine the fish capacity to cross from a known "safe" area to an unknown "risk" area. This has been established as a standardized test to characterize SCS in fish (Huntingford et al., 2010; Alfonso et al., 2020). The known sheltered area simulated natural conditions for the species, the area was isolated from light (~ 2 lux on the surface). On the other hand, the risk area had more illumination (~ 45 lux on the surface). The test was realized in a 170 L fiberglass tank (78 cm length, x 18.6 cm width x 16 cm depth), divided into two equal dimensions with a fiberglass barrier, including a window in its middle (5 cm high x 5 cm width), allowing fish to cross from an area to another. Besides, two video cameras (Swann/2K Series-1080p) were set up inside each area of the tank. Before beginning the test, the fish were acclimated for 1h in the safe zone keeping the window closed until the beginning of the test. The duration of the test was two hours and the RTT test was video recorded to validate the results registered. Juveniles were test by groups of 13 individuals in order to reduce the impact high density on stress (Linares-Cordova et al., 2024). Individuals that successfully crossed were defined as "proactive", while those that did not cross were defined as "reactive" (Alfonso et al., 2020; Linares-Cordova et al., 2024).

4.3.3.2. Individual tests

Restraining test

Restraining test (RT) was evaluated by capturing each individual fish with a nylon net inside the water for 90 seconds (s). The variables registered in this test were: (a) the total activity time (RTAct), defined as the total time (s) that fish was moving inside the net and (b) the number of escape attempts (RTEsc), defined as the number of body torsions or elevations performed by fish to free itself from the net. The previous methodology were adapted from Höglund et al. (2020), Jiménez-Rivera et al. (2023) and Linares-Cordova et al. (2024)

New environment

New environment test (NT) was performed in a rectangular plastic tank (25 cm length x 14 cm width x 7.5 cm depth) that simulated a new environment and two parameter were measured during 180 seconds: (a) total activity time (NTAct), defined as the total locomotion time (s) and (b) it was also registered the breathing rate, by counting the opercula, (NTObr), movements during the first minute (Ibarra-Zataraín et al., 2016; Fatsini et al., 2020; Jiménez-Rivera et al., 2023).

Confinement test

Confinement test (CT) was performed in a rectangular plastic tank (13.5 cm length x 9.5 cm width x 5.0 cm depth) with reduced space and water simulating a confinement situation. Two behavioural parameters were measured for 180 seconds: (a) total activity time (CTAct), defined as the total time that fish spent swimming with forward movement (in seconds); (b) number of escape attempts (CTEsc), defined as the total number of attempts performed by fish to escape from the confinement area. The methodology was adapted from Ibarra-Zatarain et al. (2016) and Linares-Cordova et al. (2024). After 2 days of finishing the individual tests in the run 2, sixteen fish were euthanized with an overdose of eugenol (100mg/L) (Davis et al. 2015) and were used to generate RNA-seq data for transcriptomic analysis.

4.3.4. Tissue processing and RNA extraction

Brains were rapidly dissected from the two groups (8 proactive and 8 reactive) and were individually collected in 1.5-ml tubes containing 1000 µl RNAlater® (Sigma-Aldrich, Spain) for further processing. Total RNA from each brain (≈ 0.04 g/brain) was extracted using the Total RNA II Omega Biotek E.Z.N.A.® kit and following the manufacturer's protocol. The amount of isolated RNA was measured with a

Nanodrop 2000c spectrophotometer (Thermo Scientific Inc., Waltham, MA). Integrity was assessed using the Agilent 2200 TapeStation instrument (Agilent Technologies, Santa Clara, CA). The RNA quality integrity (RIN) values of the samples ranged from 7.4 to 9.4. Total extracted RNA was kept at –80˚C until processing.

4.3.5. Library preparation and Stranded RNA sequencing

RNA-seq libraries were constructed using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, Inc., San Diego, CA) according to the manufacturer´s protocol. The 16 RNA libraries were sequenced on the Illumina HiSeq X10 system at Omega Bioservices (Norcross, GA), using paired-end (2x150 bp) sequencing. The raw sequence data of this study have been deposited in the NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) under the BioProject ID

4.3.6. Bioinformatic analysis of RNA-Seq data

The bioinformatic analysis were conducted in the supercomputer OOREAM of IPN, CIIDIR Sinaloa. The quality of reads was examined with FastQC v0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before and after the trimming process. Raw reads were filtered with Trimmomatic 0.36 (Bolger et al. 2014) to eliminate adapters, low-quality reads ($Q < 30$), and short reads (< 50 bp). Trimmed reads were pseudo-aligned with Kallisto v0.46.2 (Bray et al. 2016) to the reference transcriptome of *Mugil cephalus* v1.1 (GenBank accession: GCA_022458985.1). The transcript abundance files were imported into R v4.2.2 with the package tximport v1.26.1, then the differential expression analysis was conducted with the package DESeq2 v1.38.3 (Love et al., 2014). Pairwise comparisons were performed between behavioral profiles (proactive vs reactive) and differentially expressed genes (DEGs) were defined as those genes with adjusted P-value < 0.05 and Log2 fold change ± 1. Gene Ontology (GO) annotations were obtained by blastx of *M. cephalus* transcriptome against the swissprot database. The classification of DEGs into GO categories was visualized with WEGO v2.0 (https://wego.genomics.cn/) and the over-represented GO categories from DEGs were identified with R/Goseq v1.44.0 using the Wallenius method (Young et al., 2010). Heatmaps and hierarchical clustering were performed using the gplots and hclust functions in R (Warnes et al., 2019)

4.3.7. Selection of target and reference genes

Validation of gene expression quantification in brain tissue of 16 *M. cephalus* specimens (8 proactive and 8 reactive) was conducted using real time quantitative PCR (qPCR) for nine genes associated with stress responses/detoxifcation (*ef1*, *tnf*-*α*, MHC II), gluconeogenesis/immune signaling (*rpl6*), stress and anxiety-related behaviour (*gabrb*, *crfbp*, *pomcb*), differentiation and neuronal function (*pcdh*, *neurod*). Primers for each target gene were designed from well-conserved regions of an alignment of *Mugil cephalus* sequences deposited in NCBI (accession no. GCA_022458985.1), using the software Primer3 v.0.4.0 (https://bioinfo.ut.ee/primer3-0.4.0/). Oligonucleotides for target are shown in Table 1. In order to identify the most stable reference genes for the experimental condition, Cts were evaluated using the RefFinder tool (http://www.ciidirsinaloa.com.mx/RefFinder-master/). The most stable reference gene found for flathead grey mullet juveniles' brain in the present work was alpha tubulin (tub-α) and the second one was TMM42. The geometric mean of these two most stable reference genes was used to obtain the normalization factor (nf) for each analysis.

Table 4.1. Primers sequences for real-time PCR

Gene names	Gene abbreviation	Annealing temperature (°C)	Forward (5’ to 3’)	Reverse (5’ to 3’)	Amplicon size (pb)
Stress responses/detoxification					
Elongation Factor-1 alpha	*ef1*	61	ATAGCTTCAACGCCCAGGTC	ACGAGCTCCTTGAACTTGCA	115
Major Histocompatibility Complex, Class II, alpha chain	*MHC II*		GGTCTGCAGCGTCTACAAGT	GTAGTACCAGTCCCCGTCCT	121
Tumor necrosis factor alpha induced protein 3	*tnf-α*	66	CCGGTTCCCATCCTGCTGAT	AAACGCCCCAAAGAAGCTGC	186
Gluconeogenesis/immune signaling					
Ribosomal protein large 6	*rpl6*	62	CAAGATGCCCCGCTACTACC	TTCAGGAACACGACACGCTT	168
Stress and anxiety-related behaviour					
Corticotropin releasing hormone binding protein	*crfbp*	66	CATCGACACGTCCAAGCTGC	GACACCATCCTCACCACGGT	105
Gamma-aminobutyric acid receptor subunit beta	*gbrb*	63	CCCGTCTTTATGGCTGTGGT	TTCATTCCCACTCCAACCGG	155
Pro-opiomelanocortin	*pomcb*	66	TGTGGTTAGTGGTGGTGGCG	TCAGGGGCGACTCATTGGTG	153
Differentiation and neuronal function					
Protocadherin 1a	*pcdh*	66	CAAACAGCATCCCCATCGGC	TTCCTCCAAGCCGCTGTCAT	103
Neuronal differentiation	*neurod*	64	CTCCGCCTCGCCAAGAACTA	GGCAAGAAGGTCCGAGGGTT	164
Housekeeping					
Alpha-tubulin	*α-tub*	60	GGCACTACACAATCGGCAAAGAGA	TCAGCAGGGAGGTAAAGCCAGAGC	144
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	65	AGTCATCCCCGAGCTCAACG	CCATGTGCGGCCTTCTTGAC	147
Transmembrane protein 42	*tmm42*	66	TGGGGAGAGCAGCGGAAATT	AGTTGGAGGCAGTGGTGGTC	190
Ubiquitin	*ubi*	60	ACGCACACTGTCTGACTAC	TGTCGCAGTTGTATTTCTGG	

4.3.8. RNA extraction, reverse transcription and PCR assays

Total RNA was extracted from each brain, which were removed from the RNAlater®, and homogenized in 800 µl of TriPure Isolation Reagent (Roche Diagnostics) following the manufacturer's guidelines. RNA integrity was confirmed on a TBE 1% agarose gel with 1× GelRed (Biotium Inc.), visualized in a Gel DocXR+ (Bio-Rad), and was quantified spectrophotometrically (NanoDrop 1000, Thermo Fisher Scientific®). The RNA obtained was treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. The absence of genomic DNA contamination was confirmed by performing a PCR non-amplification using elongation factor 1 alpha (*ef1*) oligonucleotides (0.4 mM each), 0.45 U GoTaq Flexi DNA Polymerase (Promega), 2.0 mM MgCl2, 1× Go Taq Flexi Buffer, 0.20 mM dNTP Mix (Promega) and 1 µl de RNA treatment, in a final volume of 8 µl. The PCR was performed in a C1000 Touch thermos cycler (Bio-Rad Laboratories) using the following conditions: initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 s, 60°C for 30 s and a final extension at 72°C for 10 min. PCR non-amplifications were checked on a TBE 1% agarose gel, as mentioned above. One microgram of RNA was reverse-transcribed using Improm-II Reverse Transcriptase (Promega) according to the manufacturer's instructions, with RNAsin Plus RNase inhibitor (Promega) and a mix of oligo (dT)15 synthesized (T4Oligo), and random primers (Promega) in a volume of 40 µl. The resulting complementary DNA (cDNA) was kept at −20°C. The oligonucleotides and reference genes of this study were first tested using end-point PCR amplification with a cDNA from brain of proactive and reactive organisms. The PCR conditions were as mentioned above, using 1 µl of each cDNA and 0.32 µM each primer, in a final volume of 8 µl (the concentration of oligonucleotides was modified depending on the presence of primer dimers).

4.3.9. Real-time quantitative PCR analysis

The expression of all selected genes of *M. cephalus* in this study was determined using real-time PCR (qPCR) in each sample. qPCR reactions were performed in a total volume of 15 µl with a mix 0.45 U of GoTaq Flexi DNA (Promega), 2.5 mM

MgCl2, 1× Go Taq Flexi Buffer, 0.2 mM dNTP Mix (Promega), 1× EvaGreen fluorescent dye (Biotium), 0.10–0.48 µM each primer and 5 µl cDNA (50 ng/µl) as reported in Morelos-Castro et al. (2019). Each individual sample was measured in triplicate, and a no-template control was added as a negative control. The real-time PCR conditions were as follows: denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s, the annealing temperature from Table 1 for each oligonucleotides pair for 20 s. and the extension at 72°C for 20 s. At the end of each PCR reaction, a melting curve analysis was performed to verify the specificity of the PCR products, confirming that only one PCR product was present. PCR amplification was conducted using a CFX96 Real-Time PCR Detection System (Bio-Rad) with 96-well clear low-profile PCR microplates (Neptune) and ultraclear sealing film (Axygen Scientific).

The stability of reference genes was analysed independently. Standard curves were made for each gene with six different dilutions (factor 1:5) in triplicate of the cDNA pool samples, amplification efficiencies (E) were obtained from the slopes of the log-linear function of the dilution factor versus fluoresce, and these were analysed according to Morelos-Castro et al. (2019)

4.3.10. Statistical analysis

Behaviour. Statistical analyses were performed using SPSS Statistics 24.0 software (IBM®). Results were presented as mean ± standard error of the mean (S.E.M). All data were checked for normatility and homocedasticity by means of Kolmogorov-Smirnov and Levene tests. The level of significance was established for $P < 0.05$ for all analysis. A hierarchical clustering algorithm using the Euclidean distance matrix and complete linkage method was run to classify the 50-flathead grey mullet into proactive and reactive profile according to the total activity time (in seconds) of all the individual behavioural tests conducted (Ibarra-Zatarain et al. 2016; Fatsini et al., 2020). Finally, a Multivariate Analysis of Variance (MANOVA) were performed on behavioural variables from the three individual tests, on growth (weight (We) and length (Le)) and between proactive and reactive individuals.

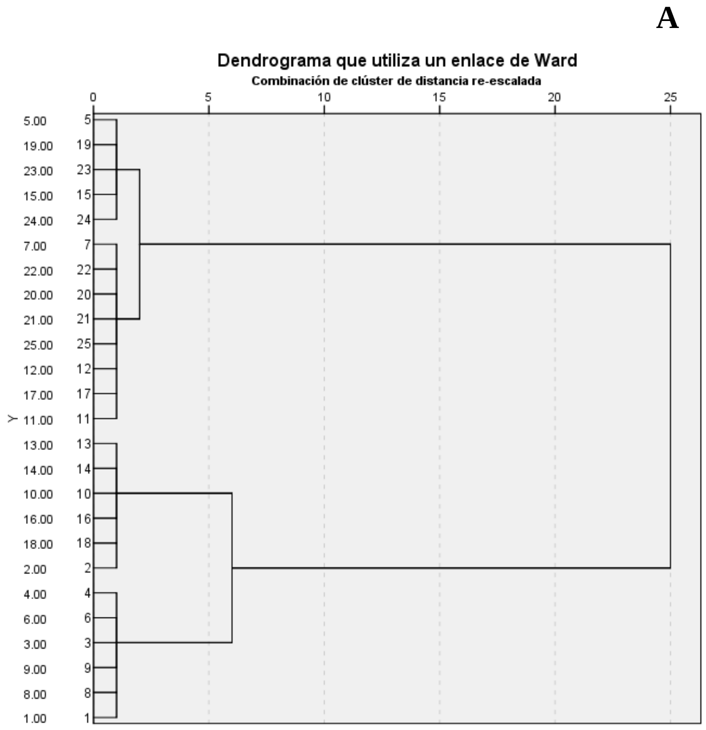
Additionally, a Tukey's post-hoc test was carried out when significant differences were detected among the groups.

q-PCR. Proactive and reactive expression differences in the brain were analysed using one-way analysis of variance after transforming the data to natural logarithms, followed by Tukey's post hoc test (Morelos-Castro et al., 2019), which was used to make pairwise comparisons between SCS. Data are presented as the means ± standard error of the mean (S.E.M). Statistical analyses were performed using SPSS Statistics 24.0 (IBM®). A p-value < 0.05 was considered significant. The results are presented back-transformed.

4.4. RESULTS

4.4.1. Behavioural assays

The hierarchical cluster divided the population in two different clusters grouping similar stress responses in terms on total activity (Figure 1) from the individual tests "RT", "NT" and "CT" in each tank. Therefore, the final classification of the hierarchical cluster was proactive and reactive animals according to the total activity displayed in every individual behavioural test.



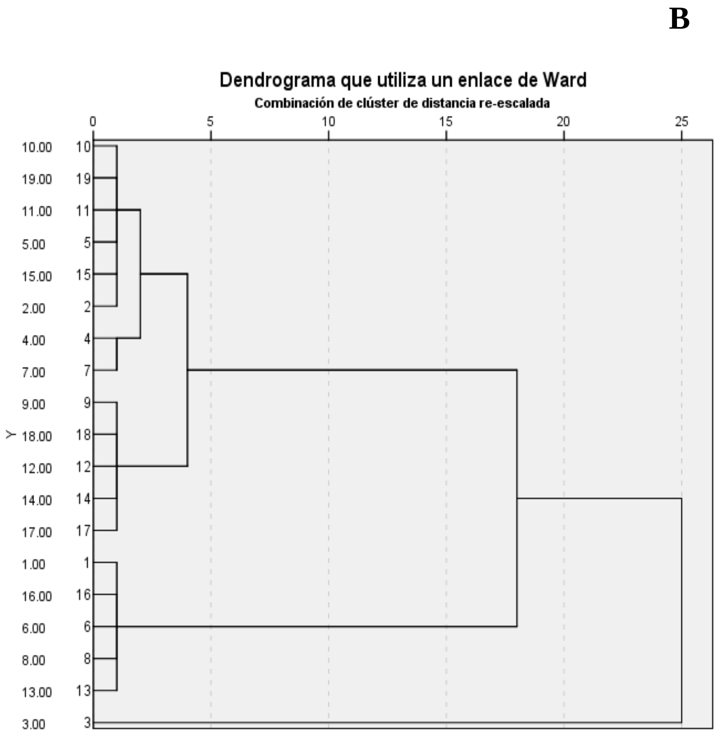


Figure 4.1. Hierarchical cluster to classify the 50-flathead grey mullet into proactive and reactive profile according to the total activity time. a) tank 1 and b) tank 2

Overall, flathead grey mullet juveniles exhibited a high degree of variability in their individual behavioural responses to the stress tests performed, with coeficients of variation ranging from 4.8 to 62.54%, that resembled proactive and reactive traits. (Table 2). Analysing the group-test, the risk-taking test, 28 of fifty flathead grey mullet juveniles (56%) passed from the known zone to the unknown zone and 22 did not cross (44%). Significant diferences were detected between proactive and reactive fish (MANOVA: F = 16.860, *P* = 0.01), for all behavioural parameters and growth variables (Table 2). The classification of the animals into two groups of stress coping styles allowed the selection of 8 proactive and 8 reactive individuals for transcriptomic analysis.

Table 4.2. Behavioural differences between flathead grey mullet juveniles that crossed (proactive, n = 21) versus those that did not cross (reactive, n = 19) in the risk-taking test, in run 1 and 2 (mean ± S.E.M., MANOVAs and Tukey's HSD post-hocs). Superscripts

letters a and b indicated significant differences between proactive and reactive at the end of the experiment.

Coping style test	Variable	Proactive (MEAN ± S.E.M) (%)	CV	Reactive (MEAN ± S.E.M) (%)	CV	MANOVA TEST F	P
Restraining	RTEsc	63.00 ± 4.25^{a}	30.97	15.79 ± 2.01^{b}	55.96	94.24	0.00
	RTAct (s)	55.14 ± 3.84^{a}	32.07	20.84 ± 2.94^{b}	62.54	48.71	0.00
New Environment	NTOBR	112.09 ± 1.70^{b}	6.96	118.37 ± 1.29^{a}	4.84	8.37	0.01
	NTAct (s)	30.10 ± 2.31^{a}	35.12	12.31 ± 0.87^{b}	31.13	47.89	0.00
Confinement	CEsc	12.86 ± 1.14^{a}	41.26	5.31 ± 0.39^{b}	35.22	35.77	0.00
	CAct (s)	32.24 ± 3.18^{a}	45.46	18.31 ± 1.18^{b}	28.47	15.57	0.00
Growth parameters	Weight (g)	8.41 ± 0.19^{a}	10.84	7.67 ± 0.15^{b}	9.31	9.12	0.01
	Length (cm)	9.40 ± 0.15	7.51	8.95 ± 0.17	8.10	4.05	0.51

4.4.2. RNA-Sequencing and analysis

Sixteen cDNA libraries were sequenced using the Illumina Hiseq X10 platform, which generated a total of 445 million raw paired reads, of which 353.9 million reads (75.9%) were kept after quality trimming (Q>30), and 256 million clean reads (72.3%) were pseudo-aligned to the *M. cephalus* reference transcriptome. The summary of RNA-Seq results is showed in Table 3.

Table 4.3. Overview of RNA-Seq reads

Samples	Total raw paired reads (M. Seqs)	Total trimmed pair reads (M. Seqs)	Mean of trimmed reads (%)	Total aligned reads	Mean of aligned reads (%)
Proactive 1	26.7	21.3	79.8	17.5	82.1
Proactive 2	30.3	24.2	79.9	17.0	70.1
Proactive 3	27.9	22.2	79.6	18.0	81.0

Proactive 4	31.1	24.8	79.2	20.2	81.5
Proactive 5	29.5	23.6	80.0	16.4	69.4
Proactive 6	28.7	23.0	80.1	15.7	68.3
Proactive 7	27.0	21.5	79.6	15.7	73.2
Proactive 8	26.6	21.1	79.3	14.8	70.2
Reactive 1	26.2	20.9	79.8	14.6	69.8
Reactive 2	26.8	21.3	79.5	15.1	70.9
Reactive 3	28.2	22.4	79.4	15.8	70.6
Reactive 4	29.9	23.5	78.6	16.4	69.9
Reactive 5	26.0	20.6	79.2	14.5	70.3
Reactive 6	26.9	21.4	79.6	14.9	69.4
Reactive 7	26.9	21.2	78.8	14.8	69.9
Reactive 8	26.3	20.9	79.5	14.6	70.0
Total	**445.0**	**353.9**	**79.5%**	**256.0**	**72.3%**

4.4.3. Differential expression analysis

Principal Component Analysis (PCA) from log2 normalized counts of the 16 samples showed the presence of three atypical replicates (two from proactive samples and one from reactive samples). After the removal of atypical replicates, the remaining samples tended to group together in the PCA according to the behavioural profile (Figure 2), with a wider scattering of the proactive samples compared to the reactive samples. This dispersion could occur because of the six individuals characterized by the RTT as proactive, two of them crossed in a mean time (between the two races) greater than 3000 s; while the others were averaging 1500 s. In this general overview, component 1 of the PCA explained 23% of the variance, while component 2 explained 18% of the variance.

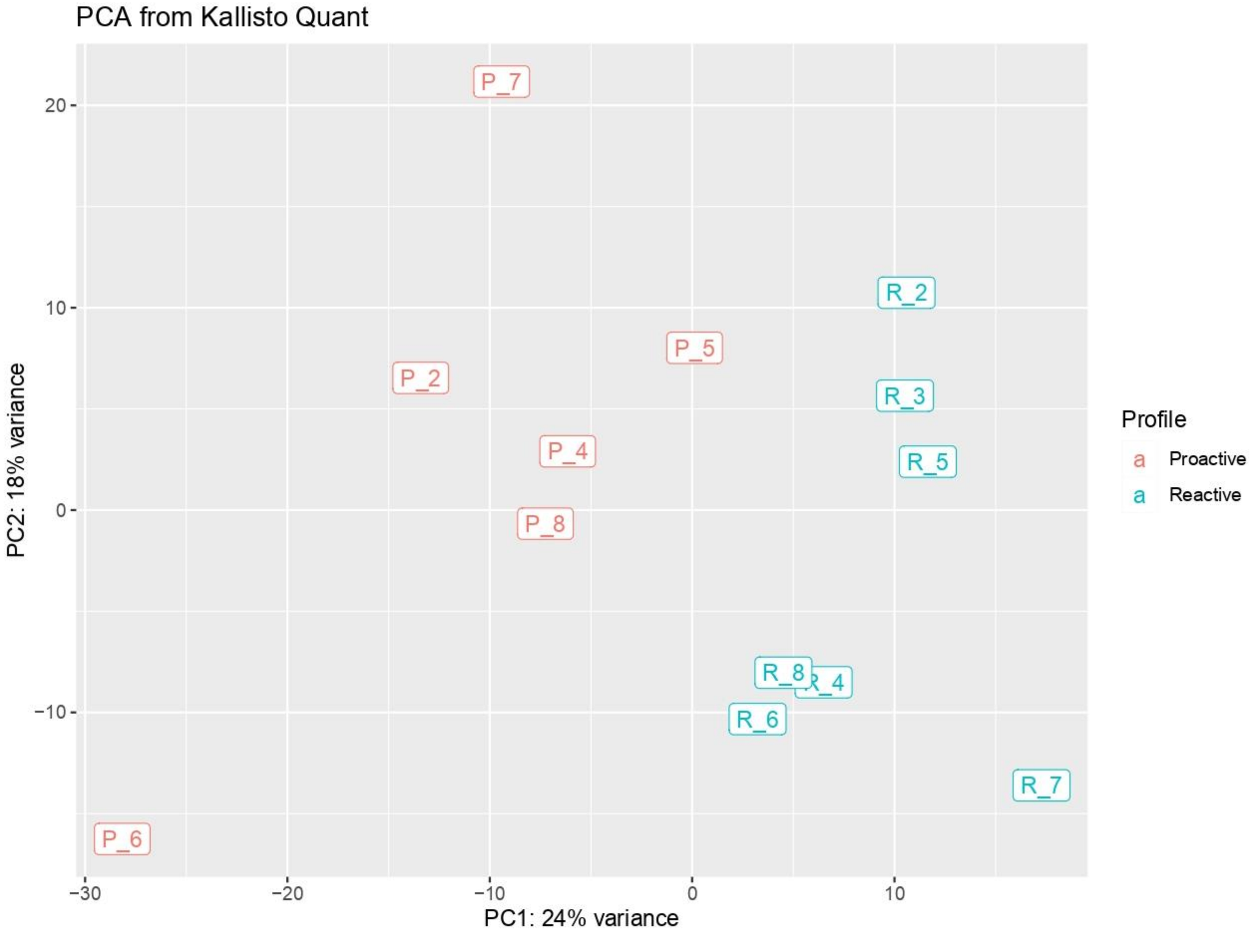


Figure 4.2. Clustering of all 16 brain samples using a Principal component analysis (PCA); blue, reactive profile (B); red, proactive profile

To compare the brain transcriptomic profiles, we tested for DEGs. The differential expression analysis between proactive and reactive profiles revealed a total of 485 DEGs (p-adj < 0.05, |log2FC| > 1), with a greater number of down-regulated genes (309) than up-regulated genes (176) as visualized in the volcano plot (Figure 3).

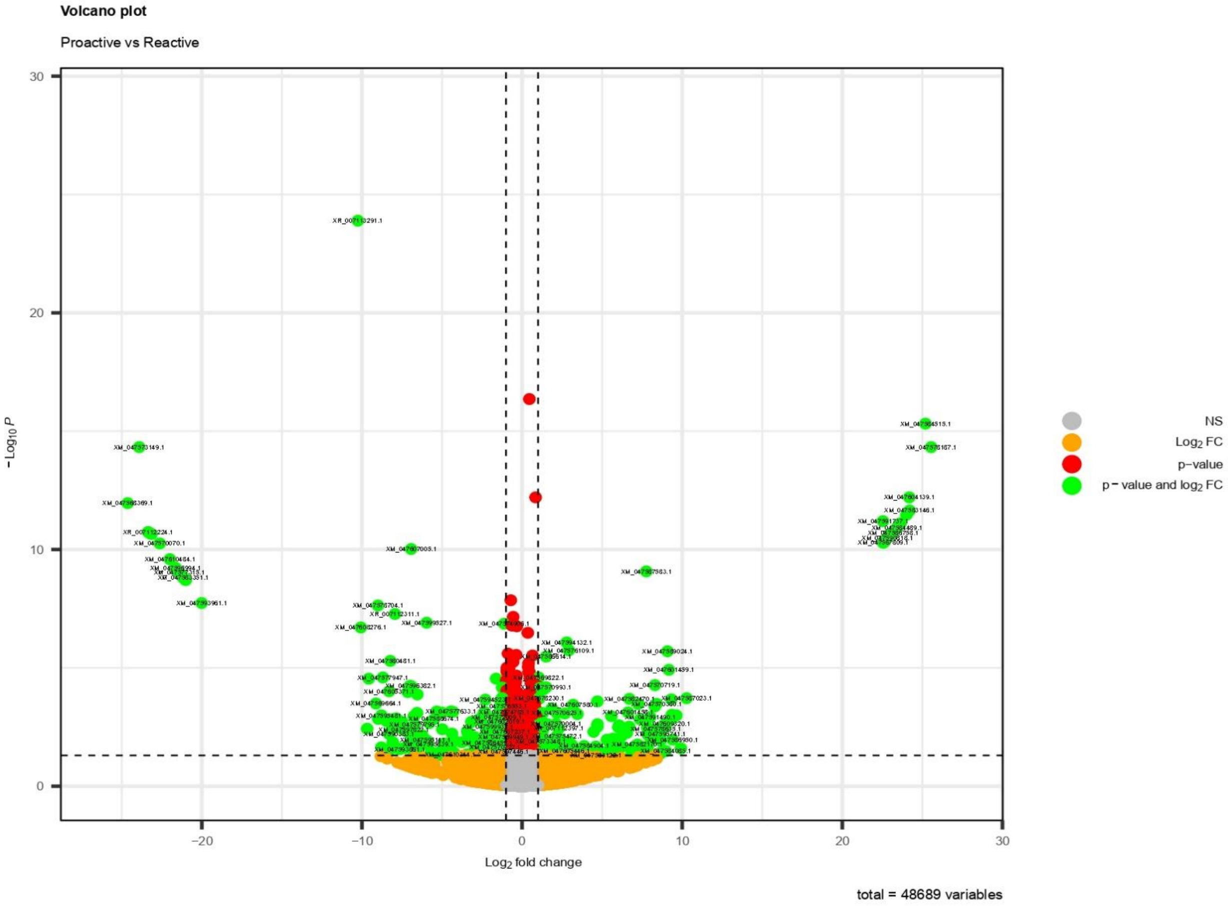


Figure 4.3. Volcano plot of the DEGs obtained from the proactive vs. reactive comparison. Vertical lines indicate the threshold for a relative expression fold change (FC) of 2 or −2. The horizontal line represents the threshold of a 0.05 FDR value. The green points were significantly upregulated or downregulated.

Furthermore, the heatmap generated from all 485 DEGs illustrated two main patterns of gene expression that were distinguishable among the behavioural profiles (proactive and reactive) (Figure 4).

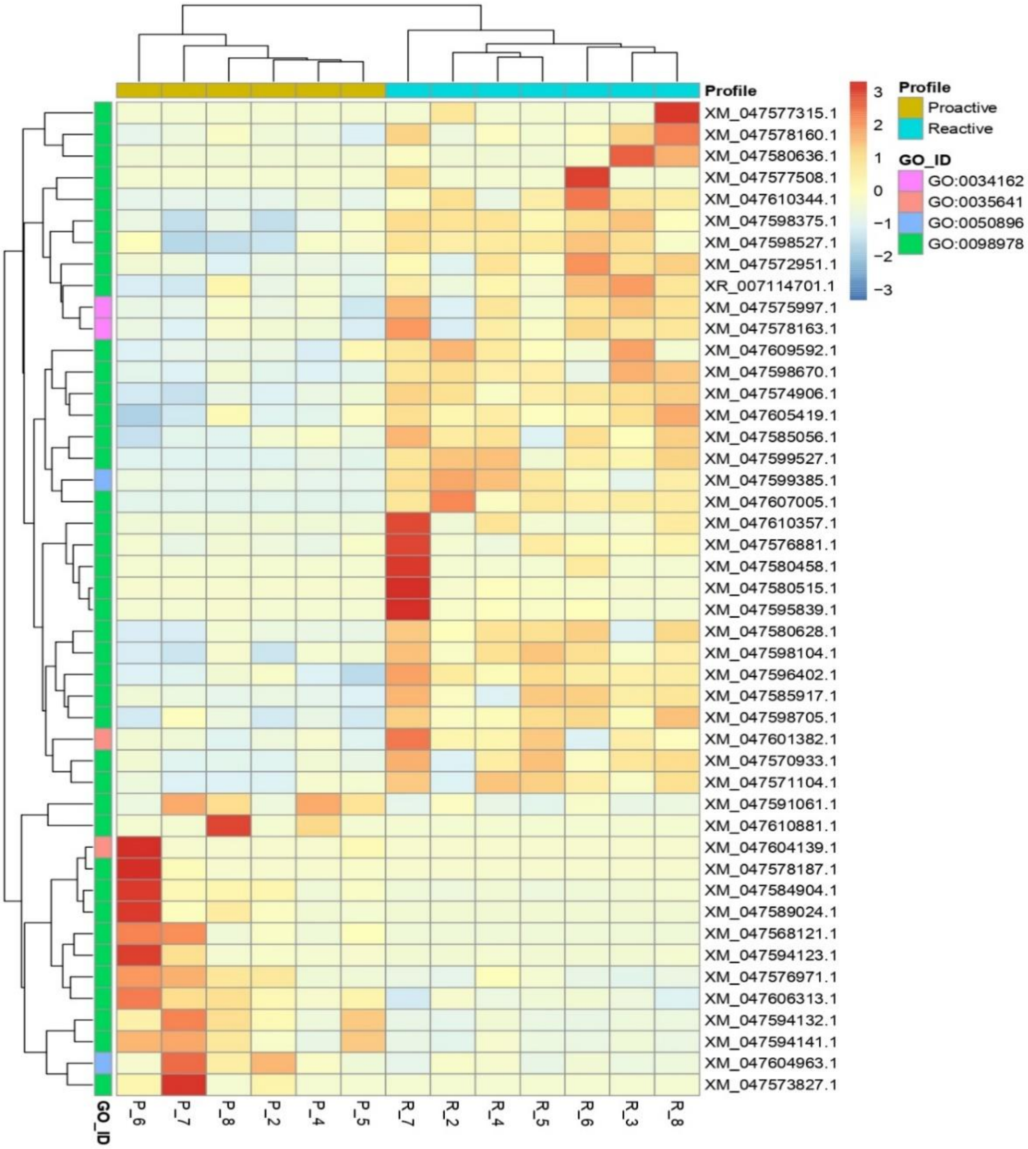


Figure 4.4. Heatmap of all genes DE in the GO 'toll-like receptor 9 signaling', 'locomotory exploration behavior', 'response to stimulus' and 'glutamatergic synapse' in the brain of proactive and reactive flatheadgrey mullet. Intensity of color indicates expression level (dark orange, high expression; sky blue, low expression).

4.4.4. Enrichment of functional categories

From the 485 DEGs between proactive and reactive profiles, 449 genes were annotated to 1,272 GO terms, wich were classified into the categories of biological process (BP) with 416 GO terms, cellular compontent (CC) with 444 GO terms, and molecular function (MF) with 412 GO terms (Figure 5).

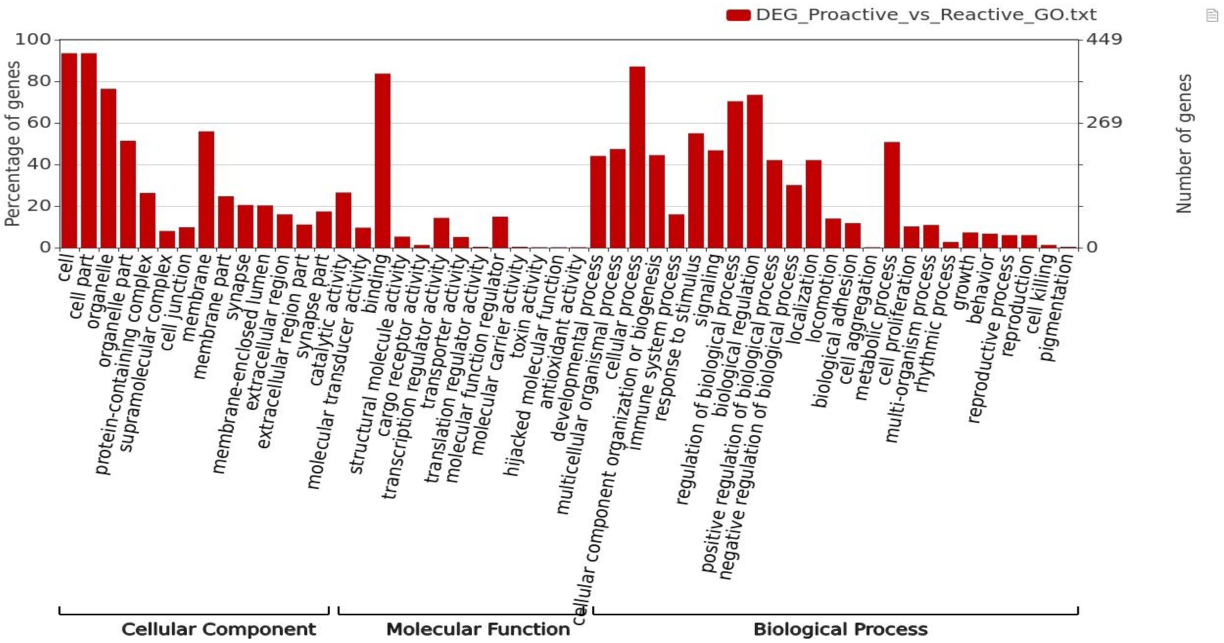


Figure 4.5. Gene ontology (GO) classification of all DEGs. The results are summarized in three main GO categories (cellular component, molecular function, and biological process). The x-axis represents GO subcategories. The left y-axis indicates the percentage of genes. The right y-axis indicates the number of DEGs.

Next, the package goseq was used to identify over-represented GO terms from the DEGs, and several terms associated with stress responses were identified. Some of the significant GO terms (Figure 6) related to BP were *intestinal epithelial cell differentiation (GO:0060575)*, *phosphate-containing compound metabolic process (GO:0006796)*, *regulation of small GTPase mediated signal transduction (GO:0051056)*, *receptor-mediated endocytosis (GO:0006898)*, *protein localization to motile cilium (GO:0120229)* and *toll-like receptor 3 signaling pathway (GO:0034138)*. In the CC category, the over-represented terms included *external side of plasma membrane (GO:0009897)*, *postsynaptic density membrane (GO:0098839)* and *glutamatergic synapse (GO:0098978)*, and in the category of

MF, the over-represented terms were *GTPase activator activity (GO:0005096)*, among others.

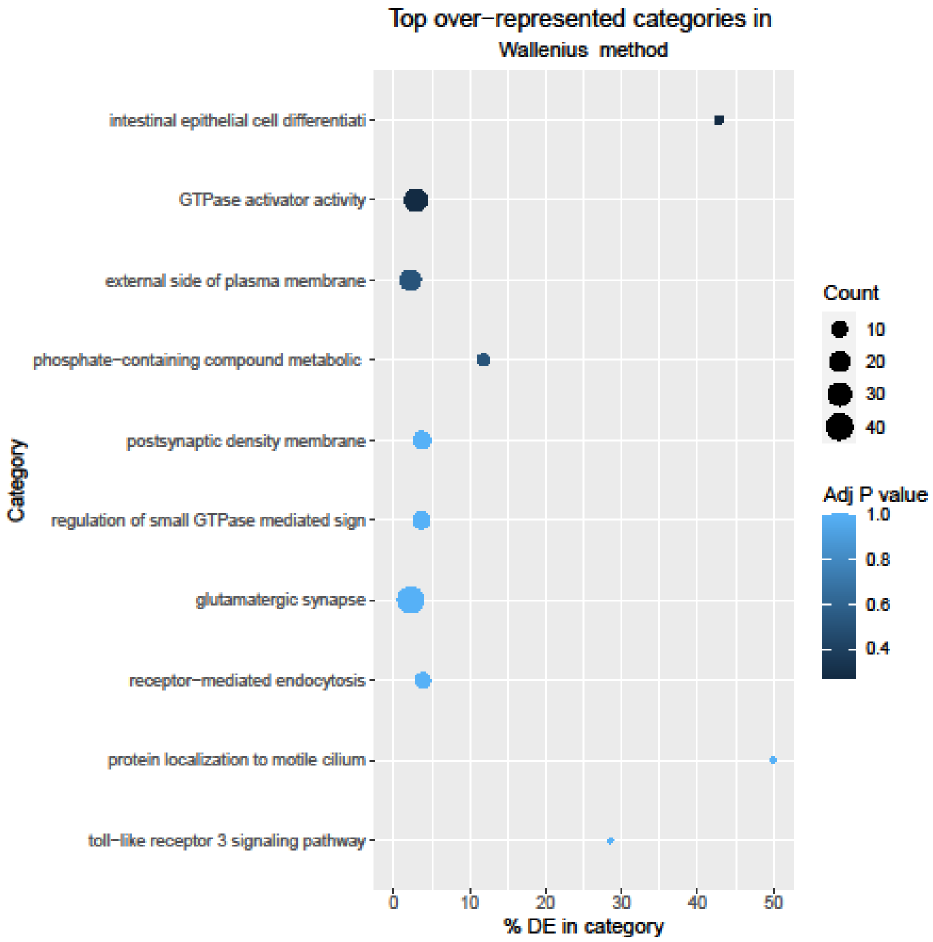


Figure 4.6. Dot plot showing some relevant significantly enriched Gene Ontology biological processes, cellular components and molecular functions from DEGs between proactive and reactive profiles. The x-axis represents the ratio of the differentially expressed gene number to the total gene number in a certain GO term. The y-axis indicates the over-represented categories. The colour and size of the dots represent the range of the p-value and the number of DEGs mapped to the indicated GO term, respectively.

4.4.5. Validation of primers for real-time qPCR assays.

PCR primers of a set of known target genes related to stress responses and behaviour were generated from the transcript's sequences. Nine primers pairs were analyzed, including gene markers of gluconeogenesis, stress responses, anxiety-related behaviour. Accession numbers and primer sequences for the target genes are listed in Table 1. The C_T (Treshold cycle) is the cycle number at which the fuorescence generated within a reaction crosses the threshold line. When C_T values were used to generate a log-linear regression plot, the standard curve for the target genes showed a strong relationship (R^2 ranging from 0.986 to 0.999; PCR efciency ranging from 90.7 to 112.2%). A correlation coeficient greater than 0.99 shows good primer eficiency, and indicates a successful real-time PCR experiment. A melting curve analysis showed no indication of primer-dimerization for the tested candidate oligonucleotides. PCR eficiencies for each primer pair are shown in Table 4. Furthermore, the primers specifcity for target genes was confirmed by gel electrophoresis of the PCR products. All the primer sets amplifed one single band of the expected fragment, and most of them showed specificity for targets from *M. cephalus*.

Table 4.4. PCR primer efficiencies used in Real-time qPCR assays

Genes and pathways	**Amplification efficiency (%)**	**R^2**
Stress responses/detoxification		
ef1	100.2	0.997
MHC-II	90.7	0.999
tnf-α	101.6	0.995
Gluconeogenesis/immune signaling		
rpl6	95.2	0.999
Stress and anxiety-related behaviour		
crfbp	92.1	0.996
gbrb	97.9	0.998
pomcb	107.6	0.986
Differentiation and neuronal function		
neurod	105.2	0.998
pcdh	105.8	0.994
Housekeeping		
α-tub	95.2	0.989
gapdh	101.7	0.996
Tmm42	95.7	0.996
ubi	112.2	0.995

The expressions of 9 candidate genes mentioned above for this study, in brain mullet were examined using qPCR. All the 9 genes showed differential expressions in the comparison between proactive and reactive organisms (Table 5). For example, the expression level of the *ef1* gene was much higher in proactive than reactive (1.620 ± 1.07 vs. 0.62 ± 1.08, $P < 0.001$), while the expression level of the *crfbp* gene was higher in proactive than in reactive (1.49 ± 1.037 vs. 0.67 ± 1.13, $P < 0.05$).

Table 4.5. One-way ANOVA results for the relative expression of nine genes measured in *Mugil cephalus* juveniles (mean ± S.E.M., ANOVAs and Tukey's HSD post-hocs). Superscripts letters a and b indicated significant differences between proactive and reactive at the end of the experiment

Genes	**Proactive (MEAN ± S.E.M)**	**Reactive (MEAN ± S.E.M)**	**ANOVA TEST**	
			F	**P**
ef1	1.62 ± 1.07^{a}	0.62 ± 1.08^{b}	10.16	0.007
MHC II	1.81 ± 1.10^{a}	0.55 ± 1.18^{b}	5.00	0.042
tnf-α	2.77 ± 1.26^{a}	0.36 ± 1.17^{b}	6.67	0.022
rpl6	0.76 ± 1.06^{b}	1.32 ± 1.03^{a}	5.86	0.030
crfbp	1.49 ± 1.03^{a}	0.67 ± 1.13^{b}	5.06	0.041
gbrb	1.81 ± 1.06^{a}	0.55 ± 1.12^{b}	10.03	0.007
pomcb	0.55 ± 1.16^{b}	1.80 ± 1.10^{a}	5.70	0.031
pcdh	0.63 ± 1.09^{b}	1.59 ± 1.08^{a}	7.87	0.014
neurod	3.33 ± 1.14^{b}	9.26 ± 1.09^{a}	5.48	0.035

4.5. DISCUSSION

In the present study natural variation in brain transcriptome was described in flathead grey mullet juveniles and whether stress coping traits were associated with these transcriptional differences. Based on previous studies differences in mRNA brain abundance were expected in relation to the behavioural traits (Fatsini et al., 2020; Vu et al., 2020; Sadoul et al., 2022). The accelerated development of high-throughput sequencing technology has revolutionized our way of studying genetic diversity and gene expression. In this road, the RNA-Seq technology has become the method of choice for the study and characterization of dynamic transcriptomes, and both the *de novo transcriptome* and the transcriptome pseudoalignment provides a powerful research tool for characterise underlying gene networks or signatures that can be ascribed to particular phenotypic traits aiming to further our understanding of animal behaviour (Patro et al., 2017; Bengston et al., 2018; Liu et al., 2022). In the past several years, transcriptomic studies in teleost fish have increased, leading to a tremendous pool of genetic knowledge. However, there is little knowledge on using a brain transcriptome to correlate with a proactive or reactive behavioural profile in order to identify the variation in specific brain mRNA transcripts (Wong et al., 2015; Vu et al., 2020; Rey et al., 2021)

In terms of the behavioural study, previous studies have demonstrated that the same behavioural tests conducted in this study (restraining, new environment and confinement) classify animals according to their behavioural traits (proactive and reactive) in diverse fish species, such as Senegalese sole *S. senegalensis* (Fatsini et al., 2020), flathead grey mullet *Mugil cephalus* (Jiménez-Rivera et al., 2023). In the present study, fish showed a high variability in their behavioural indivivual responses, allowing to classify them in two SCS categories (proactive and reactive) using a hierarchical cluster analysis based in exploratory activity. Exploratory activity in stressful environments is a distinguishing characteristic between proactive and reactive profiles (Koolhaas et al., 2010; Fatsini et al., 2020). This model, with proactive and reactive behavioural profiles has been observed in a wide variety of animal species, including fish such as European seabass *D. labrax* (Alfonso et al., 2019), rainbow trout *O. mykiss* (Gesto, 2019) and Senegalese sole *S. senegalensis* (Ibarra-Zataraín et al., 2016), among others. Hence, in the present study, fish that had been classified as proactive presented higher activity and number of escape attempts, and lower breathing rate than reactive, indicating higher explorative behaviour and different responses to stressful circumstances, which is consistent with previous studies from this laboratory in this species (Jiménez-Rivera et al., 2023; Linares-Cordova et al., 2024).

Proactive and reactive individuals showed differences in the expression of genes in the whole brain with 485 DEGs. This results are similar to those obtained by Rey et al. (2021), with a previous study highlighting 481 DEGs for *Salmo salar*, 292 DEGs for *Dicentrarchus labrax* and 215 DEGs for Danio rerio (Wong et al., 2015), but contrast with the results obtained by Sadoul et al. (2022), whom showed 6 DEGs between behavioural profiles in the whole brain. Nevertheless, in all the studies mentioned above, authors just used a groupal behavioural test (risk-taking or open field) to characterize SCS but in Rey et al. (2021) this test involved an hypoxia condition. These differences may be linked to the plasticity in the response to induced stressors on individual intrinsic characteristics in changing contexts (Killen et al., 2016). Gene expressions in the brain were significantly different between proactive and reactive individuals. In fish, brain undergoes changes in its chemistry and structure in response to changes in the environment and is composed of many endocrine-involved cell types under the control of the pituitary gland (Leng and MacGregor, 2018). They release in the circulatory system hormones playing key roles in many biological processes, such as stress response, behaviour or growth (McMillan 2011). Interestingly, multiple genes related to the “neural plasticity” and "stress response" were significantly different between individuals categorized as proactive or reactive.

In the present study, a significant enrichment for ‘locomotory exploration behavior’ category (*GO:0035641*), such as glutamate decarboxylase 1 – *gd1*, which was down-regulated in proactive individuals. Glutamate decarboxylase 1 gene contributes to cell and neural plasticity (Martin et al., 2000; Samborska et al., 2021) and is involved in the regulation of mammalian brain excitability through the synthesis of the main inhibitory neurotransmitter GABA (Tapia and Meza-Ruíz, 1976). Furthermore, is also expressed in juveniles and adults, which indicates important roles in neuronal function and developmental processes (Katarova et al., 2000; Cocco et al., 2017). Different studies showed an increased expression in other genes related to neural plasticity in reactive individuals (Johansen et al., 2012).

Furthermore, 'glutamatergic synapse' category (*GO: 0098978*), which is essential for learning and memory capacities (Niciu et al., 2012), such as neural cell adhesion molecule (NCAM) and protein shisa-6, were generally downregulated in proactive individuals. Dougherty and Guillette (2019), found evidence that bold/proactive animals are faster learner. On the other hand, it can also be argued that reactive individuals are more efficient in reverse learning and display more elaborated memory processes (Sih and del Giudice, 2012). However, the direction of the relationship between cognition and animal personality are still unclear (Baker and Wong, 2019; Dougherty and Guillette, 2019).

Another significant enrichment category found in this study was 'toll-like receptor 9 signaling pathway' (*GO: 0034162*). Toll-like receptors (TLRs) have been established to play an essential role in the activation of innate immunity by recognizing specific patterns of microbial components (Palti, 2011). TLR9 has a protective role in the early stages of development under pathogenically hostile environments (Byadgi et al., 2014). It is important to mention that this category is found in pathological challenges; for example, Meijer et al. (2004) showed an increased expression after in vivo challenge with *Mycobacterium marinum* in *Danio rerio*. In our study, a up-regulated expression was observed in reactive mullets that contrasts with research in which immunity is higher in proactive organisms (Kittilsen et al., 2012). These differences could be due to the difference in the experimental design (without a bacterial challenge response) and the type of tissue sampled (mostly kidney studies). Although the link between behaviour and immune system is well documented in mammals, this link was investigated in only few fish studies reporting distinct immune capacities between behavioural phenotypes (Kirsten et al., 2018; Vargas et al., 2018).

In this study, nine candidate genes in qPCR were *ef1*, *tnf-α*, *MHC II*, *rpl6*, *gabrb*, *crfbp*, *pomcb*, *pcdh*, *neurod*. Although there is some information about their functions in model organisms and humans, not much information about their role in proactive and reactive fishes is available. Therefore, it is essential to study their functions in the relationship between SCSs classification and gene expression, since it might reveal a specific outline presenting altogether a specific profile, phenotype and genotype (Fatsini et al., 2020). In this study, we selected *gabrb*, *tnf-α*, *crfbp* and *neurod* genes from the 9 candidate genes to study some of its functions. Gamma- aminobutyric acid (*gabaa*) is a crucial inhibitory neurotransmitter of the brain and is the primary neurotransmitter of at least one-third of all central nervous system neurons (Bloom and Iversen, 1971). The β GABAA subunit receptor is a multiple subunit chloride channel receptor that mediates the action of GABA, a fast inhibitor of synaptic transmission in the Central Nervous System (CNS) (Marinho et al., 2018). The receptor is expressed by the *gbrb* gene, which plays a role in modulating synapses and maintains the excitation-inhibition balance in the brain (Terhune et al., 2014) producing a calming effect in controlling nerve cell hyperactivity associated with anxiety, stress and fear (Kalueff et al., 2016; Goodman and Wong, 2020). Our results are consistent with previous studies documenting the involvement of GABA neurotransmitter (Jacobson et al., 2007) and molecular pathways in physiological and behavioural responses to stress (Sarkar et al., 2011; Wong et al., 2015; Goodman and Wong, 2020). It should be emphasized that proactive profile show significant differential expression than reactive, suggesting that higher expression of this subunit in proactive individuals may allow for greater sensitivity of GABAA ligands leading to a greater anxiolytic response, which is similar with the results obtained by Goodman and Wong (2020) and Wong et al. (2015) in *Danio rerio*.

Tumor necrosis factor alpha (*tnf-α*) is produced in response to infection or immunological injury and effects multiple responses, which extend beyond its well characterised proinflammatory properties (Vassalli, 1992) to include diverse signals for cellular differentiation, proliferation and death (Vandenabeele et al., 1995).

There is a consensus that when the stressor is acute and short-term, the response pattern is somehow stimulatory and the fish immune response shows an activating phase that especially enhances innate responses (Sahoo et al., 2021), including the expression of pro-inflammatory genes like *tnf* (Hoseini et al., 2019; Su et al., 2020). In the present study proactive individuals showed a significance difference expression than reactive individuals, this would be because proactive animals have a higher risk of wounding and inflammation in stressful/challenging situations (Korte et al., 2005). Furthermore, thre results obtained in this study is similar with the results reported by MacKenzie et al. (2009) in *Cyprinus carpio* and Vargas et al. (2018) in *Sparus aurata*, whom described as fide clues to infammatory onset in response to pathogen infection and environmental stressors in behavioural-selected fish.

Corticotropin releasing hormone binding protein (*crhbp*) is dispersed in discrete brain regions (Kemp et al., 1998; Slater et al., 2016) where it regulates the bioavailability of corticotropin releasing hormones (*crh*) and reducing CRH receptor activation (Behan et al., 1995; Ketchesin et al., 2017). In this study, a higher expression of *crhbp* gene was observed in proactive individuals than in reactive ones. This result was similar with those obtained by Chen and Fernald (2011) in African cichlids (*Astatotilapia burtoni*), whom found a higher expression of crf-BP (brain) in dominant (proactive) organisms using visual signals. Furthermore, our results support the idea that *crhbp* is a negative regulator in *crh* signaling (Karolyi et al., 1999). On the other hand, Vindas et al. (2017) and Fatsini et al. (2020) showed no differences on *crhbp* expression between proactive and reactive profiles, but there was a tendency for proactive individuals to have higher expression of *crhbp* compared with reactive fish after stress in *Salmo salar* and *Solea senegalensis*, respectively.

Finally, neurogenic differentiation factor (*neurod*) is a basic helix-loop-helix (bHLH) transcription factor involved in the later stages of neurogenesis and is required for determination, differentiation and survival of neural precursor cells (Cho and Tsai, 2004). In particular, in the present study, the increased mRNA expression levels of *neurod* in reactive flathead grey mullet might indicate a larger pool of newborn neurons and different degree of neurogenesis compared to proactive fish. Interestingly, a study on rainbow trout (*Oncorhynchus mykiss*) has shown that *neurod* gen are more abundant in brains of individuals from a high-stress response line than in individuals from a low-stress response line (Johansen et al., 2012), characterised by a confinement stress, suggesting that these differences were caused by variations in coping styles that were at the base of divergent behavioural properties among different profiles. On the other hand, Alfonso et al. (2019) and Vindas et al. (2017) found that relative levels of *neurod* mRNA showed no significant differences between proactive and reactive in *Dicentrarchus labrax* and *Salmo salar*; however, there is a trend towards an increase in reactive fish.

4.6. CONCLUSION

In conclusion, our results indicate a possible species-specific regulation of genes involved in neurogenesis, immune signaling and stress and anxiety-related behaviour in response to different environmental conditions. Furthermore, an individual´s behavioural response can be influenced by its genetic profile and underlying expression of neurotransmitters, and can contribute to the alternative behavioural stress-response between stress coping styles. In addition, the brain transcriptome of *Mugil cephalus* based in inter-individual differences in behaviour opens a large window of opportunities to develop different studies with this organism in order to improve their production.

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Discusión

CAPÍTULO 5. DISCUSIÓN GENERAL

Estudiar el comportamiento de los peces en respuesta al estrés, además de ser desafiante dado el gran repertorio de factores estresantes y de respuestas conductuales, se convierte en un elemento clave para evaluar el bienestar de los peces. En el capítulo II, se realizó una revisión cienciométrica sobre el estado de la investigación en bienestar de peces cultivados en América Latina, y se observó que durante los últimos años hubo un incremento en el número de publicaciones hacia un cuidado mas elevado del bienestar de los organismos. En el capítulo III, se demostró la relación significativa que existe entre los parámetros conductuales y los fisiológicos en juveniles de lisa y, por consecuencia, se amplió la comprensión de la variabilidad intra e inter individual respecto a las estrategias comportementales y fisiológicas de la lisa, lo que es de gran importancia para comprender y mantener el bienestar de los peces en el sector acuicola (Conrad *et al*., 2011; Castanheira *et al*., 2016). En el capítulo IV, se llevo a cabo un transcriptoma del cerebro de lisa para evaluar las diferencias entre individuos caracterizados conductualmente como proactivos y reactivos en juveniles de lisa, validando los resultados mediante la técnica de reacción en cadena de la polimerasa cuantitativa en tiempo real (qPCR, por sus siglas en inglés).

5.1. Análisis cienciométrico de investigaciones sobre bienestar de peces en Latinoamérica (LATAM)

El análisis cienciométrico demostró que el número de artículos científicos sobre bienestar en peces en países latinoamericanos (n = 285) es significamente inferior a la producción científica, sobre el mismo tema, de Estados Unidos, Canadá y Europa, que juntó 851 articulos en las bases de datos ISI-Web of Science y Scielo durante el periodo 1976 - 2016, de acuerdo con De Castilhos y de Oliveira (2016). Estos autores mencionaron que Noruega fue el líder en publicaciones entre un total de 55 países registrados con el 23,5% de los artículos, seguido de Inglaterra y Estados Unidos con el 11,97% y 11,85%, respectivamente. Sin embargo, en dicho estudio no proporcionaron números para las publicaciones originadas específicamente en países latinoamericanos, con excepción de Brasil (53 artículos) y Chile (≈10 artículos).

Es importante señalar que Chile, Brasil, Ecuador, México, Perú y Colombia, países que fueron seleccionados para esta revisión cienciometrica, representan en conjunto el 87% de la producción acuícola latinoamericana (Souto Cavalli e*t al*., 2021). Asimismo, en términos generales, el número de estudios relacionados con el bienestar de los peces en LATAM, continúa siendo bajo en comparación con el número de estudios centrados en el bienestar de animales terrestres, según datos proporcionados por Gallo *et al*. (2022), quienes utilizando la base de datos ISI-Web of Science y CAB Abstracts encontraron 663 artículos publicados sobre animales de granja de ganado terrestre, excluyendo animales acuáticos, salvajes y de zoológico, caballos deportivos, mascotas y animales de laboratorio.

En el presente trabajo de investigación, se documentó un aumento exponencial del número de publicaciones sobre bienestar en peces en LATAM entre 2000 y 2023, lo que coincide con los resultados de Freire y Nicol (2019) y de De Castilhos y de Oliveira (2016), en países como Brasil y Chile en temas relacionados a los métodos de anestesia y aturdimiento, principalmente. Sin embargo, este aumento es más reciente en LATAM que en otros países occidentales, documentando en el presente estudio que el 89.1% de todos los artículos publicados corresponden al periodo del 2011 al 2023. El creciente interés por el bienestar de los peces cultivados en LATAM podría deberse a una tendencia mundial observada hacia cuestiones de bienestar animal (Sloman *et al*., 2019), influenciado por la presión social en general, y el surgimiento del movimiento vegano en particular (Rossi-Borges *et al*., 2019; Lama *et al*., 2017). Brasil y Colombia fueron los primeros países en implementar leyes en materia de protección animal, como el Decreto 24645 y la Ley 84, respectivamente. Estas leyes proporcionaron una definición detallada de los actos de crueldad hacia los animales y establecieron el deber de cuidado de sus animales por los dueños. Sin embargo, a partir de 2008, distintos países de LATAM comenzaron a implementar leyes de bienestar animal, debido al trabajo de la Organización Mundial de Sanidad Animal (WOAH), quien publicó por primera vez normas de bienestar animal en 2005 y promovió su implementación en los países miembros, entre ellos Brasil, Chile, México, Perú, Ecuador y Colombia (WOAH 2020; Galindo *et al*., 2016). La necesidad de incluir el bienestar animal como una preocupación central en los países de LATAM era indispensable, ya que muchos productores de pescado a nivel mundial están ubicados en países de LATAM y las exportaciones internacionales debian de encajar con normas mas estrictas entorno al bienestar animal (Muñiz *et al*., 2022). Otra razón para que los países de LATAM inviertan en más investigaciones y publicaciones sobre el bienestar de los peces, así como de los animales terrestres en la última década, podría estar relacionada con las políticas educativas y regulatorias de la WOAH y de los países miembros de LATAM. Los países miembros de la WOAH implementaron estándares de bienestar animal desde 2009, cuando se aplicó una encuesta a 172 países miembros (Stafford y Mellor 2009), en el cuál se consultó sobre el estado de la legislación sobre bienestar animal. La mayoría de los países encuestados indicaron que tenían legislación que cubría el transporte de animales (83%), el sacrificio de animales para consumo humano (91%), el sacrificio de animales para el control de enfermedades (86%) y el control de perros (61%).

Brasil es el país de LATAM que más ha incursionado con el tema de bienestar acuícola, ya que el 75.8% de los artículos científicos encontrados correspondieron a este país con especies como tilapia del Nilo, bagre negro y cachama negra, por citar algunas. Estos resultados también concuerdan con lo reportado por De Castilhos y de Oliveira (2016) en su búsqueda de artículos sobre bienestar de peces a nivel mundial. El liderazgo de Brasil en investigaciones y publicaciones relacionadas con la piscicultura, se debe probablemente a su relevante ictiodiversidad dentro de LATAM. Chile es el segundo país con mayor número de publicaciones científicas (13.3%) en bienestar animal acuícola en LATAM y la mayor cantidad de su producción científica se enfoca al salmón, debido a los beneficios productivos que conllevan un nivel elevado de bienestar y condiciones optimas de cultivo para un crecimiento máximo, para su exportación al internacional (Quiñones *et al*., 2019). México contribuyó a las publicaciones sobre bienestar en organismos acuaticos de cultivo con el 7.0% de estudios en LATAM, y esto puede deberse a que la industria acuícola en el país es el sector primario con mayor crecimiento en los últimos 10 años, con un crecimiento promedio del 15% (Cortés *et al*., 2021). Por otro lado, Peru, Colombia y Ecador solo produjeron el 3.9% de las publicaciones, las cuales aparecieron a partir del 2014. Esto podría explicarse por el hecho de que varios de estos países basan sus economías en actividades distintas a la piscicultura, como la pesca en Perú (Chriestensen *et al*., 2014) o el cultivo de camaron en Ecuador (FAO, 2020b).

5.2. Caracterización y consistencia de EAE en juveniles de lisa

Se ha demostrado que los EAE desempeñan un papel importante en la ecología de la población y se correlacionen con factores de importancia para la produccion acuícola, como el crecimiento, la resistencia a enfermedades, el bienestar y la reproducción (Vargas *et al*., 2018; Ibarra-Zatarain *et al*., 2020). En el capítulo 3 se caracterizaron los EAE en juveniles de lisa mediante tres pruebas individuales (restricción, predador y confinamiento) y una prueba grupal (toma de riesgos), adaptadas de trabajos realizados en diferentes especies de peces marinos: toma de riesgo (adaptado de Tudorache *et al*., 2013; Ferrari *et al*., 2015; Alfonso *et al*., 2020; Jiménez-Rivera *et al*., 2023), restricción (adaptado de Castanheira *et al*., 2016; Höglund *et al*., 2020; Jiménez-Rivera *et al*., 2023), predador (adaptado de Bensky *et al*., 2017; Romano *et al*., 2017; Jiménez-Rivera *et al*., 2023) y confinamiento (adaptado de Øverli *et al*., 2006, Ibarra-Zatarain *et al*., 2016; Jiménez-Rivera *et al*., 2023). En esta caracterización, se observó una amplia variabilidad conductual inter-individual en las capacidades de afrontamiento del estrés en juveniles de lisa, lo que permitió clasificarlos en perfiles proactivos y reactivos. Estas diferencias pueden estar relacionadas con la plasticidad en la respuesta a factores estresantes inducidos dentro de individuos de una misma especie (Killen *et al*., 2016). Estos cambios difieren por la forma de reacción de los organismos en contextos cambiantes, lo que significa que una situación puede ser percibida como riesgosa por un organismo, mientras su conespecifico la percibirá como segura (Dingemanse *et al*., 2012). Asimismo, los individuos pueden diferir en su plasticidad conductual y fisiológica interna para afrontar el estrés debido a una amplia variedad de estimulos estresantes o como resultado de cambios en los rasgos a lo largo del tiempo y a medida que los animales crecen o aprenden sobre su entorno (Dingemanse *et al*., 2010; Mitchell y Houslay, 2021).

La prueba grupal de toma de riesgo evaluó la tendencia de un animal a explorar situaciones impredecibles o riesgosas, siendo un indicador comúnmente utilizado en etología de peces para la caracterización de diferentes tipos de EAE (Koolhaas *et al*., 1999; Baker *et al*., 2018). Esta prueba permitio identificar un grupo de juveniles que eran más propensos a cruzar de una área segura a una área de riesgo y entonces a correr riesgos y ser mas atrevidos (proactivos) que otro grupo de peces que no cruzaron y fueron caracterizados como más timidos (o reactivos), en acuerdo con otros estudios que reportaron tendencias similares en especies como la lubina europea *D. labrax* (Alfonso *et al*., 2019), el lenguado senegalés *S. senegalensis* (Fatsini *et al*., 2020) y la carpa común *C. carpio* (Huntingford *et al*., 2010). Emplear pruebas grupales se ha considerado un buen predictor de la respuesta al estrés, ya que el contexto social influye fuertemente en la personalidad de los individuos (Crane et al., 2018; Culbert et al., 2019). Este contexto social interviene en la regulación de numerosos comportamientos característicos como la sociabilización (Harpaz et al., 2017), la familiaridad social (Nadler et al., 2021), la dominancia (Rodríguez-Santiago et al., 2020), la plasticidad (Maruska et al., 2019) y el aprendizaje (Webster y Laland, 2017). Asimismo, la presencia de congéneres puede inducir los individuos a reforzar o modificar rasgos de comportamiento sensibles a las amenazas, como la actividad, la exploración, la asunción de riesgos, la tasa de alimentación y las oportunidades de cortejo (Ward et al., 2020; Sommer-Trembo et al., 2022). Dado que los juveniles de lisa presentan un comportamiento social, con especificidad de formar pequeños grupos y sin rasgos de agresión (Jiménez-Rivera et al., 2022), se esperaba que algunos de los individuos de esta especie de pez fueran más sensibles al aislamiento, por lo tanto, también se realizaron pruebas individuales para caracterizar la respuesta individual al estrés, omitiendo para entonces, el contexto social.

En este sentido, los peces que habían sido caracterizados como proactivos en la prueba grupal de toma de riesgo tuvieron un tiempo de actividad y un número de intentos de escape mas alto que los peces identificados como reactivos, lo que indica la existencia de una variabilidad en cuanto a la estrategia de respuesta al estrés o EAE (Farwell y McLaughlin, 2009; Tudorache *et al*., 2013). Estas observaciones estuvieron de acuerdo con estudios en otras especies, como en lubina europea *D. labrax* (Ferrari *et al*., 2015), trucha arco iris *O. mykiss* (Gesto, 2019) y lenguado senegalés *S. senegalensis* (Ibarra-Zatarain *et al*., 2020), donde se reporto que los individuos proactivos mostraron un mayor tiempo de actividad y comportamiento exploratorio que los peces reactivos. Además, en este estudio, los peces con perfiles proactivos se caracterizaron por unas concentraciones plasamaticas de cortisol mas reducidas y una menor actividad del eje HPI que los peces con EAE reactivos. Este resultado coincidió con los estudios de Schjolden *et al*. (2005), Ibarra-Zatarain *et al*. (2016) y Alfonso *et al*. (2020) para la trucha arco iris *O. mykiss*, el lenguado senegalés *S. senegalensis* y la dorada *S. auratus*, respectivamente. Øverli *et al*. (2007) han interpretado la variación en la regulación del eje HPI entre EAE proactivo y reactivo como un mecanismo adaptativo evolutivo en el que los individuos proactivos están más preparados para situaciones estresantes impredecibles que los individuos reactivos. Otra interpretación que se podría dar, es que el perfil reactivo puede tener una tasa de liberación de cortisol más rápida en relación con el perfil proactivo, lo que puede llevar a un tiempo más corto para alcanzar los niveles máximos, como ha sido descrito en pez cebra *D. rerio* y en lenguado senegalés *S. senegalensis*, donde los autores encontraron una liberación más rápida de corticosterona post-estrés en peces reactivos que en peces proactivos (Wong *et al*., 2019; Ibarra-Zataraín *et al*., 2020). Sin embargo, cabe señalar que el diseño del presente estudio no nos permite evaluar directamente las diferencias entre perfiles de comportamiento en el tiempo para alcanzar los niveles máximos de cortisol.

En este estudio, se demostró, por primera ocasión, que la lisa exhibe comportamientos de respuesta al estrés consistentes a lo largo del tiempo (en un plazo de 4 meses) y entre contextos (diferentes pruebas y estimulus agudos estresantes aplicados), sugiriendo que algunos individuos son consistentemente más exploratorios y con mayor capacidad de escape que otros (Ferrari *et al*., 2015; Long *et al*., 2021). En la presente tesis se demostró que los juveniles de lisa muestran una consistencia similar a las observadas en otras especies, como lubina europea *D. labrax* (Ferrari *et al*., 2015), lenguado senegalés *S. senegalensis* (Ibarra-Zataraín *et al*., 2016). Esta consistencia en el comportamiento puede explicarse por la influencia de las condiciones controladas de cultivo en el comportamiento de los peces, que indujeron altos niveles de acondicionamiento durante el tiempo de estudio. Sin embargo, esta suposición debería confirmarse mediante la realización de pruebas adicionales que favorezcan nuevos desafíos para ciertos aspectos del comportamiento de esta especie. Asimismo, la mayoría de los estudios conductuales que evalúan la consistencia de los EAE a lo largo del tiempo se basan en el uso de diferentes pruebas durante un período relativamente corto (entre 7 a 30 días) (Basic *et al*., 2012; Jiménez-Rivera *et al*. 2023) y pocos estudios han investigado la consistencia de los EAE en intervalos de tiempo mas largos, como meses y hasta años (Rey *et al*. 2013; Ferrrari *et al*. 2015; Ibarra-Zataraín *et al*., 2020). La preferencia por realizar estudios por intervarlos cortos, es que diversos factores podrían afectar la consistencia en el comportamiento; como, por ejemplo, la edad, el proceso de maduración sexual y/o jerarquias, las experiencias de la historia de vida (Bell *et al*., 2009; Castanheira *et al*., 2016), el proceso de aprendizaje/cognición (Baker y Wong, 2019); destacándose que los EAE no son fijos a lo largo de la vida y que distintas estrategias comportamentales pueden ser empleadas para hacer frente a las mismas situaciones estresantes en diferentes etapas de desarrollo de una misma especie (Biro y Stamps 2008). Adriaenssens y Johnsson (2009) sugirieron que las correlaciones entre los rasgos de comportamiento y la historia de vida a menudo pueden variar entre entornos. Eso significa que a menudo que el animal crezca, se pueden modificar sus caracteristicas ecológicas y requerimientos nutricionales, por tanto, también su conducta se puede ver cambiada. Eso coincide con lo expuesto por Magnhagen y Borcherding (2008) que mencionan que la complejidad del hábitat (Hojesjo *et al*., 2004) y la disponibilidad de alimentos (Boon *et a*l., 2007; Biro y Post 2008) también podrían afectar los rasgos de comportamiento de los individuos al experimentar una variedad de condiciones ambientales contrastantes a lo largo de su vida. Adicionalmente, también se logró validar la existencia de consistencia entre contextos, reflejado por el hecho que aquellos individuos que tomaron menos tiempo en tomar el riesgo al cruzar de la zona segura a la zona de riesgo en la prueba grupal también exhibieron mayor número de intentos de escape y mayor actividad de natación en las respectivas pruebas individuales. Eso indica que existen estrategias conductuales de respuesta ante un agente estresor (proactivo vs reactivo) consistentes entre las diferentes pruebas/contextos que estimulan distintos aspectos del comportamiento (toma de riesgo, exploración, audacia, evitación) en juveniles de lisa (Sih *et al*., 2004). Los resultados obtenidos están de acuerdo con estudios previos en lenguado senegalés (Ibarra-Zataraín *et al*., 2016) y en tilapia del Nilo (Martins *et al*., 2011).

Para determinar los ejes de personalidad en el presente estudio se realizó un Análisis de Componentes Principales (ACP) que permitió identificar aquellas variables conductuales con mayor correlación entre ellas (Jiménez-Rivera *et al*., 2023). Los resultados de PCA sugirieron 2 ejes de personalidad, categorizadas como audacia-actividad y evitación-miedo. El eje de actividad-audacia puede influir en el resultado de los desafíos estresantes cotidianos, como la competencia por la comida (Dingemanse *et al*., 2007), el momento para salir del refugio a un entorno familiar (Brown *et al*., 2005) y la respuesta a los depredadores (Wilson y Stevens, 2005), interpretándose este comportamiento de alta actividad y conducta exploratoria como una reacción proactiva ante situaciones nuevas, que se asemeja mucho a la curiosidad, impulsividad o reactividad ante la presencia-ausencia de congéneres-predadores y la sociabilidad. Asimismo, el eje de evitación-miedo abarca conductas que implican la voluntad individual de no investigar entornos, alimentos u objetos novedosos (Reader y Laland, 2003; Réale, 2007), interpretándose este comportamiento mas pasivo como una reacción reactiva de los peces en relación con una situación aversiva que se asemeja al miedo, la estimulación y la ansiedad. Finalmente, los rasgos o síndromes de comportamiento encontrados en este estudio habian sido descritos previamente en otras especies de peces, como la trucha marrón (Lothian y Lucas 2021); lenguado senegalés (Ibarra-Zataraín *et al*., 2016) y lisa (Jiménez-Rivera *et al*., 2023), mediante pruebas y análisis estadísticos similares a los utilizados en este estudio.

5.3. EAE y transcriptoma cerebral en juveniles de lisa

Esta tesis presenta la primera biblioteca de secuencias obtenidas por un análisis transcriptomico comparativo en cerebro de 8 juveniles de lisa caracterizadas como proactivos y 8 como reactivos. El empleo de herramientas como RNA-Seq ofrecen grandes ventajas comparado a metodologías de análisis de un numero definido de genes (como la PCR o los microarreglos), dada la cantidad de genes sobre- o intra-expresados que pueden ser identificados entre varias condiciones ambientales o experimentales, sin necesidad de conocer previamente su secuencia mediante un genoma de referencia, aunque ambas técnicas son complementadas para la validación de los marcadores identificados (Wang *et a*l., 2009). En los últimos años, los estudios transcriptómicos en peces teleósteos han aumentado, lo que ha dado lugar a una enorme reserva de conocimiento genético. Sin embargo, hay poco conocimiento sobre la relación entre las variaciones del transcriptoma cerebral y los perfiles de comportamiento proactivo-reactivo (Wong et al., 2015; Vu et al., 2020; Rey et al., 2021)

Los juveniles de lisa mostraron dos categorías de EAE utilizando diferentes parametros como la actividad o número de intentos de escape en diferentes pruebas (grupal: toma de riesgo e individuales: confinamiento, restricción y nuevo ambiente) y factores fisiológicos (cortisol y glucosa) relacionados con el estrés

(Ibarra-Zatarain *et al*., 2016; Jiménez-Rivera *et al*., 2023): EAE proactivos y reactivos. Los juveniles proactivos de lisa exhibíeron tasas de crecimiento más altas y concentraciones de cortisol plasmático menores a los reactivos. Los peces en cultivo deben implementar una serie de mecanismos fisiológicos y conductuales para adaptarse al cautiverio y a los continuos cambios que se presentan en el ambiente, que sea de parámetros bióticos (densidad, competencia por el alimento) o abióticos (temperatura, flujo de agua). Por tanto, el estudio de marcadores moleculares y su regulación, permite relacionar el componente genómico a los mecanismos fisiológicos y estrategias conductuales para comprender como esta especie es capaz de adaptarse y enfrentarse a diferentes situaciones de estrés (Mackenzie, 2009). Esta información resultade gran utilidad para un futuro programa de mejora de cría y de selección de individuos con rasgos deseados y para así optimizar el cultivo de esta especie

La secuenciación de extremos pareados de Illumina HiSeq X10 generó un total de 445 milliones de pares de lecturas de cerebro de ambos perfiles conductuales, de las cuales 353.9 millones de pares de lecturas permanecieron después del recorte (79.5%), para generar un alineamiento de 256 millones de lecturas, con un tamaño promedio de 1036 pares de base (pb por sus siglas en inglés) y un N50 de 2494 pb. El número y tamaño de los contigs son similares a los reportados para otros transcriptomas generados utilizando la misma plataforma Illumina, en otras especies piscícolas no-modelo como pez gato (Li *et al*., 2012), el robalo europeo (Sarropoulou *et al*., 2012) o la mona amarilla (Schunter *et al*., 2014). El análisis diferencial de genes realizado en este trabajo nos entrega información cualitativa y cuantitativa, puesto que pudo identificar la secuencia de los transcritos y conocer además su tasa de cambio de pliegue o magnitud de la variación de su expresión, para ambos perfiles de comportamiento.

En el presente estudio, los individuos proactivos y reactivos mostraron diferencias significativas en la expresión de 485 genes (o genes expresados diferencialmente, DEGs, por sus siglas en inglés). Diversos estudios han relacionado diferencias en el transcriptoma cerebral con EAE extremos proactivos y reactivos en otras especies de peces, como Rey *et al*. (2021), quienes identificaron 481 DEGs entre salmones *S. salar* proactivos y reactivos y 292 DEGs entre lubinas *D. labrax* proactivas y reactivos, como Wong *et al*. (2015), quienes determinaron 215 DEGs entre ambos extremos del pez cebra *D. rerio*. Sin embargo, estos resultados contrastaron con los de Sadoul *et al*. (2022), quienes encontraron solo 6 DEGs entre perfiles de comportamiento proactivos y reactivos en el cerebro de la lubina *D. labrax*. Sin embargo, en todos los estudios mencionados anteriormente, solo se utilizo una prueba de comportamiento grupal (asunción de riesgos o campo abierto) para caracterizar el EAE, menos Rey *et al*. (2021), quienes agregaron una condición de hipoxia a esta prueba grupal. Estas diferencias en la cantidad de genes expresados diferencialmente pueden estar relacionadas con características propias a la especie de la plasticidad del cerebro de los peces en respuesta a factores estresantes en contextos cambiantes (Killen et al., 2016). El cerebro de los peces, principal tejido actuando en respuesta al estrés, sufre cambios en su estructura química y fisiológica en respuesta a los cambios ambientales y está compuesto por muchos tipos de células endocrinas bajo el control de varias glandulas, incluida la glándula pituitaria o hipofisis (Leng y MacGregor, 2018). En el sistema circulatorio se liberan hormonas que desempeñan funciones clave en muchos procesos biológicos, como la respuesta conducutal y fisiológica al estrés el crecimiento (McMillan, 2011). En los resultados del presente estudio, múltiples genes relacionados con la "plasticidad neuronal" y la "respuesta al estrés" fueron identificados con una expresión significativamente diferente entre los individuos clasificados como proactivos y reactivos, entre ellos *pcdh, neuroD, crf-BP*.

En esta tesis, se encontró a la categoría de “comportamiento locomotor de exploración” (GO:0035641) como una de las categorías funcionales las mas representadas entre los genes diferencialmente expresados entre peces proactivos y reactivos. En particular, destacó el gen glutamato descarboxilasa 1 - *gd1*, sobre-expresado en individuos proactivos comparado con reactivos. El gen *gd1* contribuye a la plasticidad celular y neuronal (Martin *et al*., 2000; Samborska *et al*., 2021) y participa en la regulación de la excitabilidad cerebral de los vertebrados, incluido los peces, mediante la síntesis del principal neurotransmisor inhibidor del ácido gamma-aminobutírico, GABA (Tapia y Meza-Ruíz, 1976; Grone y Maruska, 2016), jugando un papel importante en el comportamiento, la cognición y la respuesta del frente al estrés, ya que diversas investigaciones sugieren que el GABA ayuda a controlar el miedo y la ansiedad cuando las neuronas se sobreexcitan (Goodman y Wong, 2020).

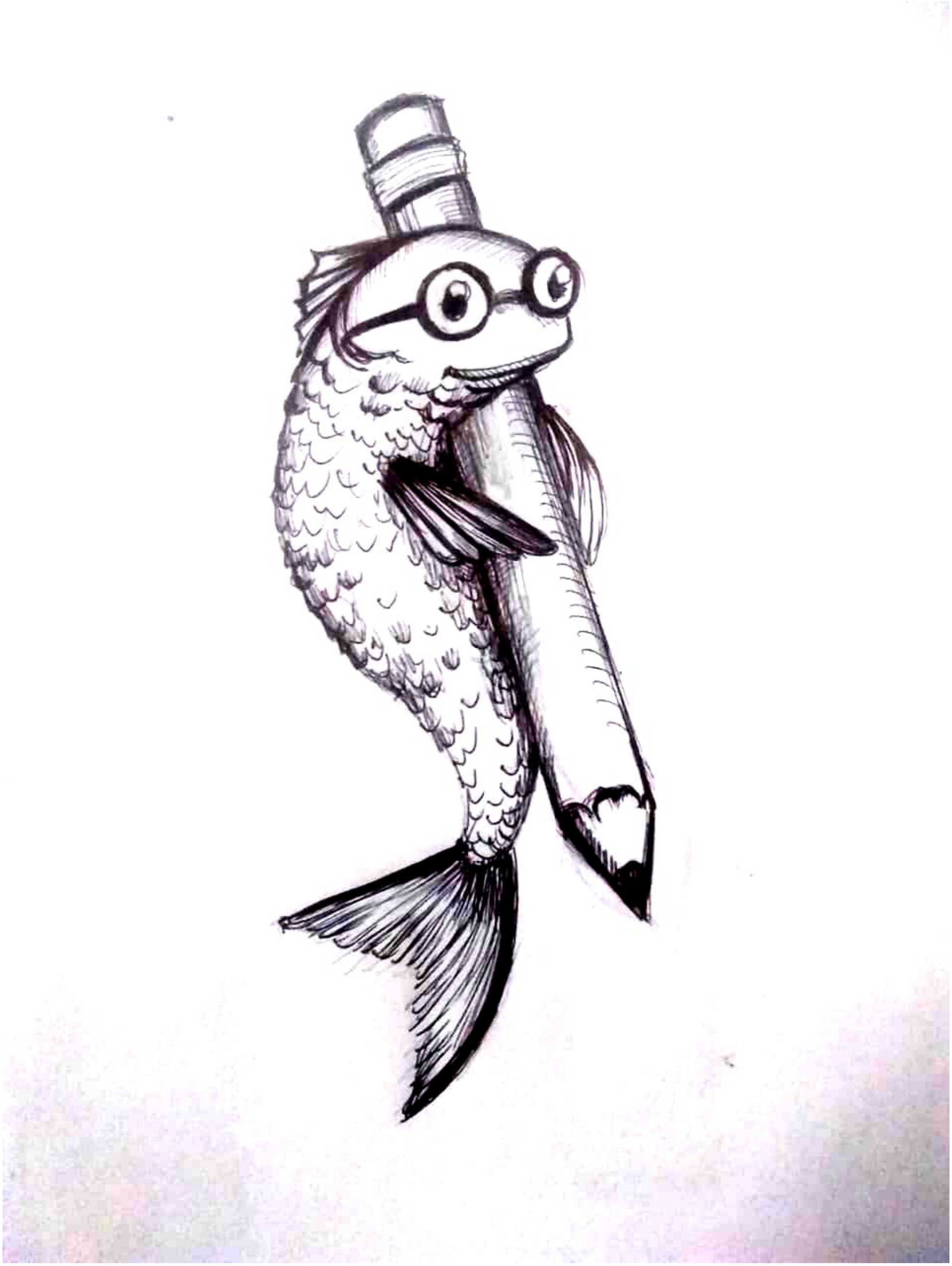
Otra categoría funcional que se encontró tener una mayoría de genes diferencialmente expresados entre individuos proactivos y reactivos fue la de la “via de señalización del receptor tipo toll 9, TLR9" (GO: 0034162). Se ha establecido que los receptores tipo toll (toll-like receptors, TLR, por sus siglas en inglés) desempeñan un papel esencial en la activación de la inmunidad innata al reconocer componentes microbianos (Palti, 2011). Los TLR9 tienen un papel protector en las primeras etapas de desarrollo en ambientes patogénicamente hostiles (Byadgi *et al*., 2014). Es importante mencionar que esta categoría a menudo se expresa de manera diferente en peces sometidos a pruebas de desafío patológicos en comparación con peces no desafiados. Por ejemplo, Meijer *et al*. (2004) mostraron un incremento de la expresión de *tlr9* después de un desafío *in vivo* con *Mycobacterium marinum* en *Danio rerio*. En nuestro estudio, se observó una sobre-expresión de *tlr9* en individuos reactivos comparado con los proactivos, al contrario de otra investigación con salmones *S. salar* demostrando una inmunidad mas estimulada en riñones de organismos proactivos que reactivos (Kittilsen *et al*., 2012). Sin embargo, el presente estudio no presentó ninguna respuesta de desafío bacteriano en su diseño experimental y el tipo de tejido muestreado fue diferente. Aunque el vínculo entre el comportamiento y el sistema inmunológico está bien documentado en mamíferos, se ha investigado poco las diferencias en capacidades inmunes entre fenotipos conductuales en peces (Kirsten *et al*., 2018; Vargas *et al.*, 2018).

Asimismo, validar la expresión diferencial de ciertos DEGs identificados en el análisis transcriptomico entre peces proactivos y reactivos mediante la técnica de RT-qPCR es de crucial importancia. Los resultados de este trabajo muestran, por ejemplo, una mayor expresión del gen factor de necrosis tumoral alfa (*tnf-α*) en individuos proactivos que reactivos. El gen *tnf-α* se produce en respuesta a una infección o a una lesión inmunológica y produce múltiples respuestas, que se extienden más allá de sus propiedades proinflamatorias (Vassalli, 1992) e incluyen diversas señales para la diferenciación, proliferación y muerte celular (Vandenabeele *et al*., 1995). Existe consenso sobre el hecho de que, ante un estresor agudo y de corto plazo, el patrón de respuesta es estimulante para el sistema inmune y la respuesta inmune de los peces muestra una fase activadora que potencia especialmente las respuestas innatas (Sahoo et al., 2021), incluida la expresión de genes pro-inflamatorios como *tnf-α* (Hoseini et al., 2019; Su et al., 2020). En el presente estudio, la expresión diferencial de *tnf-α* entre individuos proactivos y reactivos esta en relación con un mayor riesgo de heridas e inflamación en situaciones desafiantes en animales proactivos que en los reactivos (Korte et al., 2005), de manera similar a los resultados obtenidos por MacKenzie *et al*. (2009) en *Cyprinus carpio* y Vargas *et al*. (2018) en *Sparus aurata*, quienes describieron diferencias en la aparición de la inflamación en respuesta a la infección por patógenos y factores ambientales estresantes entre peces categorizados por su comportamiento.

Otro ejemplo permitiendo validar el análisis transcriptomico comparativo mediante qPCR fue el gen factor de diferenciación neurogénica (*neurod*), el cuál es un factor de transcripción básico involucrado en las últimas etapas de la neurogénesis y que es necesario para la determinación, diferenciación y supervivencia de las células precursoras neurales (Cho y Tsai, 2004). En particular, en el presente estudio, los niveles más altos de expresión de *neurod* en individuos reactivos que en los peces proactivos podrían indicar la mayor formación de nuevas neuronas y un grado mas elevado en el proceso de neurogénesis en peces proactivos comparación con proactivos. Similarmente al presente estudio, un estudio en trucha arco iris *O.*

mykiss ha demostrado que los transcritos del gen *neurod* eran más abundantes en el cerebro de individuos de una línea de respuesta de alto estrés (reactivos) que en individuos de una línea de respuesta de bajo estrés (proactivos) (Johansen *et al*., 2012). Esto sugiere que estas diferencias en la expresión génica estan asociadas a la variabilidad interindividual en los EAE entre ambas líneas de respuesta. Por otro lado, Alfonso *et al*. (2019) y Vindas *et al*. (2017) no encontraron diferencias significativas en los niveles relativos de transcritos de *neurod* entre individuos proactivos y reactivos de lubina *D. labrax* y salmon *S. salar*, aunque observaron una tendencia a una ligera mayor expresión en peces reactivos que en proactivos, como se observó en el presente estudio.

En síntesis, la información suministrada en este estudio es relevante para ampliar el conocimiento en cuanto al manejo de *M. cephalus* ya que se se estableció una relación entre los estilos de afrontamiento y la expresión de genes cerebrales, mostrando que la variación individual en el comportamiento estaba relacionada con la expresión de varios genes con importantes funciones biológicas, que podría aplicarse en la acuicultura como una herramienta potente para un futuro programa de selección en lisa.



CAPÍTULO 6. CONCLUSIONES GENERALES

✓ El análisis cienciometrico confirmó un aumento significativo en la producción de artículos científicos sobre bienestar en peces durante los últimos 10 años del periodo 2000 – 2023; sobre todo en países como Brasil, Chile y México que son los principales productores y exportadores de pescado en esta región, siendo las principales especies la tilapia, gamitana y el salmón. Asimismo, los temas de nutrición/alimentación y salud/patología relacionados al bienestar de peces fueron los más representativos.

✓ Se confirmó la existencia de EAE de tipo proactivo y reactivo en juveniles de lisa, identificados mediante pruebas conductuales grupal e individuales y análisis de la concentración plasmática de cortisol y glucosa. Los organismos proactivos presentaron mayor predisposición para tomar riesgos, mayores tiempos de actividad e intentos de escape en pruebas de comportamiento y niveles de cortisol y glucosa mas bajos que los peces reactivos, lo cual representa una menor susceptibilidad al estrés en términos de cultivo.

✓ Los EAE en juveniles de lisa presentaron un alto grado de consistencia, ya que sus respuestas conductuales, observadas a través de diferentes pruebas (contexto) y a lo largo del tiempo, fueron significativa y altamente repetibles, consistentes y correlacionadas. Estos resultados mostraron por primera vez una consistencia a largo plazo (4 meses) para esta especie.

✓ Se confirmó, mediante el análisis bioinformático, que existen diferencias en el transcriptoma de cerebro de juveniles de lisa *Mugil cephalus* entre individuos caracterizados con perfil conductual proactivo y reactivo mediante pruebas conductuales y análisis de parámetros fisiologicos.

✓ Se determinó la presencia de 485 genes diferencialmente expresados mediante el análisis bioinformático comparativo entre el perfil proactivo y el perfil reactivo, de los cuales 309 fueron genes infra-expresados de proactivos comparados con reactivos (downregulated) y 176 genes sobre-expresados (upregulated).

- ✓ Se asignaron términos de ontología de genes (GO) a 1272 secuencias únicas, clasificándolas en 3 categorías funcionales: proceso biológico (BP) con 416 términos GO (32.7%), componente celular (CC) con 444 términos GO (34.9%) y función molecular (MF) con 412 términos GO (32.4%)

- ✓ Se validó la expresión diferencial entre peces proactivos y reactivos de 9 genes (*ef1*, *tnf-α*, MHC II, *gbrb*, *crfbp*, *pomcb, rpl6, pcdh, neurod)* seleccionados del transcriptoma por la técnica de qPCR, lo que confirma la asociación de la variabilidad de patrón de comportamiento con la variabilidad de perfil de expresión transcriptomico y permitirá plantear futuros estudios de expresión génica en esta especie de una manera confiable.

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