
# UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Study of host defense peptides in gilthead seabream (*Sparus aurata*)

Estudio de péptidos de defensa del huésped en dorada (*Sparus aurata*)

**D. John Alberto Serna Duque**

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Autor: D. John Alberto Serna Duque

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UNIVERSIDAD DE MURCIA

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doctorando del Programa de Doctorado en

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# SCIENTIFIC PRODUCTION

**Scientific articles:**

Campos-Sánchez, J.C., **Serna-Duque, J.A.**, Alburquerque, C., Guardiola, F.A., Esteban, M.Á., 2024. Participation of hepcidins in the inflammatory response triggered by λ-carrageenin in gilthead seabream (*Sparus aurata*). Mar Biotechnol (NY) 26, 261–275. https://doi.org/10.1007/s10126-024-10293-0

**Serna-Duque, J.A.**, Espinosa-Ruiz, C., Esteban, M.Á., 2023. Hepcidin and piscidin modulation and antibacterial response in gilthead seabream (*Sparus aurata*) infected with *Vibrio harveyi*. Fish Shellfish Immunol 139, 108899. https://doi.org/10.1016/j.fsi.2023.108899

**Serna-Duque, J.A.**, Espinosa Ruiz, C., Martínez Lopez, S., Sánchez-Ferrer, Á., Esteban, M.Á., 2022. Immunometabolic involvement of hepcidin genes in iron homeostasis, storage, and regulation in gilthead seabream (*Sparus aurata*). Front Mar Sci 9. https://doi.org/10.3389/fmars.2022.1073060

**Serna-Duque, J.A.**, Cuesta, A., Sánchez-Ferrer, Á., Esteban, M.Á., 2022. Two duplicated piscidin genes from gilthead seabream (*Sparus aurata*) with different roles in vitro and in vivo. Fish Shellfish Immunol 127, 730–739. https://doi.org/10.1016/j.fsi.2022.07.013

**Serna-Duque, J.A.**, Cuesta, A., Esteban, M.Á., 2022. Massive gene expansion of hepcidin, a host defense peptide, in gilthead seabream (*Sparus aurata*). Fish & Shellfish Immunology 124, 563–571. https://doi.org/10.1016/j.fsi.2022.04.032

**Serna-Duque, J.A.**, Esteban, M.Á., 2020. Effects of inflammation and/or infection on the neuroendocrine control of fish intestinal motility: A review. Fish Shellfish Immunol 103, 342–356. https://doi.org/10.1016/j.fsi.2020.05.018

**Articles under review and in preparation**

Afef Braiek, **John Alberto Serna-Duque**, Adel Nefzi, Sonia Aroui and María Ángeles Esteban. Potential therapeutic use of dermaseptin S4 from the frog *Phyllomedusa sauvagii* and its derivatives against bacterial pathogens in fish. Submitted on 09-05-2024 to the Journal of Applied Microbiology.

M. Ferez-Puche, **Jhon A. Serna-Duque**, Alberto Cuesta, Álvaro Sánchez-Ferrer, María Ángeles Esteban. Identification of a novel β-defensin gene in gilthead seabream (*Sparus aurata*). Revised on 29-05-2024 for Marine Biotechnology.

**Works submitted to conferences or congresses**

**Serna-Duque J.A.**, Ferez-Puche M., Cuesta A., Sánchez-Ferrer, Á., Esteban M.A. Exploring the role of type 2 β-defensin: a host defense peptide in gilthead seabream (*Sparus aurata*). Latin American & Caribbean Aquaculture 2024. World Aquaculture Society. 2024. Medellín, Colombia. Accepted.

**Serna-Duque J.A.,** Espinosa C., Sánchez-Ferrer, Á., Esteban M.A. Peptidomic of skin mucus from gilthead seabream (*Sparus aurata*) reveals novel antimicrobial peptides in the early stages of *Vibrio harveyi* infection. Mucosal Health in Aquaculture 2024. CIIMAR. Porto, Portugal. Accepted.

**Serna-Duque J.A.,** García-Navarro L., Marin-Parra C., Cuesta A., Esteban M.A. Dual functionality of novel hepcidin genes in the gilthead seabream (*Sparus aurata*): Iron regulation and antibacterial response. 37th European Peptide Symposium and 14th International Peptide Symposium. University of Florence. 2024. Florence, Italy. Accepted.

Marin-Parra C., **Serna-Duque J.A.**, Albaladejo-Riad N., Espinosa C., Esteban M.A. Novel insights into piscidin 1, a host defence peptide of gilthead seabream (*Sparus aurata).* AQUA 2024. European Aquaculture Society and the World Aquaculture Society. 2024. Copenhagen, Denmark. Accepted.

**Serna-Duque J.A.**, C. Leal Ines, CR Cardoso João, Cuesta A., M. Power Deborah, Esteban M.A. Selective antibacterial role of Piscidin and Hepcidin peptides from gilthead seabream (*Sparus aurata*) against distinctive species of *Vibrio*. 21st International Conference on Diseases of Fish and Shellfish. European Association of Fish Pathologists. 2023. Aberdeen, UK. Poster communication.

**Serna-Duque J.A.**, Espinosa C., Esteban M.A. Peptidómica endógena de la mucosa de la piel dorada (*Sparus aurata*) durante la fase temprana de una vibriosis. XVIII Congreso Nacional de Acuicultura. Sociedad Española de Acuicultura. 2022. Cádiz, Spain. Oral communication.

**Serna-Duque J.A.**, Espinosa C., Esteban M.A. Alteration of mucosal skin and antimicrobial host-response of hepcidin, piscidin and defensin genes in iron-overload fish (*Sparus aurata*). Mucosal Health in Aquaculture 2022 (MHA2022). CISA-INIA-CSIC. 2022. Madrid, Spain. Poster communication.

**Serna-Duque J.A.**, Espinosa C., Albaladejo-Riad N., Esteban M.A. Alteration of gilthead seabream (Sparus aurata) hepatocyte morphology induced in the early stage of Vibrio harveyi infection. XXI Congreso de la Sociedad Española de Histología e Ingeniería Tisular. Sociedad Española de Histología e Ingeniería Tisular. 2022. Granada, Spain. Poster communication.

**Serna-Duque J.A.**, Campos-Sánchez, J.C., Espinosa C., Martínez Lopez, S., Esteban M.A. Changes in morphology of hepatocytes and tissue-biodistribution in iron-overload gilthead seabream (*Sparus aurata*). IX Iberian Congress of Ichthyology (SIBIC2022). Sociedad ibérica de Ictiología. 2022. Porto, Portugal. Poster communication.

**Serna-Duque J.A.**, Cuesta A., Esteban M.A. Diversity of pleurocidin-like antimicrobial peptides in a marine bony fish (*Sparus aurata*) by re-identification in ESTs. Galaxy Community Conference (GCC2021). Galaxy Community. 2021. Online. Poster communication.

**Serna-Duque J.A.**, Cuesta A., Esteban M.A. Evolutive and genomic history of a piscine host defence peptide: A new world for hepcidins in teleost fish. The Biology of Genomes. Cold Spring Harbor Laboratory. 2021. Online. Poster communication.
**Serna-Duque J.A.**, Cuesta A., Esteban M.A. A Massive Gene Duplication of Hepcidin Family in a Mediterranean Bony Fish (*Sparus aurata*). 3rd International Biotechnology & Research Conference. Madridge Conferences. 2021. Online. Oral communication.

**International stay**

During the Ph.D. program, a three-month international stay was undertaken from March 1, 2023, to May 31, 2023, at the Faculty of Science and Technology, University of Algarve, Faro, Portugal. This stay was under the supervision of Full Professor Dr.

Deborah Power, principal investigator of the "Comparative Endocrinology and Integrative Biology" group.

# INDEX

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **APD** | Antimicrobial Peptide Database |
| **AMPs** | Antimicrobial peptides |
| **HDPs** | Host defense peptides |
| ***hamp*** | Hepcidin gene |
| **HMMs** | Hidden Markov Models |
| **MAR bacteria** | Multi-antibiotic resistant bacteria |
| ***pisc*** | Piscidin gene |
| **VER** | Viral Encephalopathy and Retinopathy |
| **VNN** | Viral Nervous Necrosis |

# ABSTRACT

The gilthead seabream (*Sparus aurata*) is a species of great commercial interest in Mediterranean aquaculture, an industry that has experienced an exponential growth since the 1980s. However, this sector faces substantial challenges from infectious diseases, particularly vibriosis caused by *Vibrio harveyi*. These diseases threaten both trade and the health of the fish, creating significant economic losses. Climate change further exacerbates these issues by increasing the virulence of pathogens and the incidence of diseases. This Doctoral Thesis investigates the crucial role of host defense peptides (HDPs), specifically hepcidins and piscidins, in the immune and antimicrobial response of gilthead seabream. The research presented in this thesis aims to address these challenges by exploring the genetic and functional roles of HDPs in gilthead seabream. The primary objectives include identifying and screening novel HDP genes in the seabream genome, understanding their evolutionary history, examining their basal transcription under standard physiological conditions, and investigating their regulation in response to pathogens and non-pathogenic *stimuli*.

The first paper delves into the expansion of hepcidin genes in gilthead seabream, identifying fifteen distinct hepcidin genes on chromosome 17. This marks the largest number of hepcidin genes found in any vertebrate. This study used genomic analysis techniques, including Hidden Markov Models (HMM), to uncover this gene cluster. Specific tissue expression profiles and transcriptional responses to bacterial challenges were found, highlighting sophisticated regulatory mechanisms. The presence of multiple hepcidin genes with diverse functions underscores the complexity and adaptability of the seabream's immune system.

The second paper explores two duplicated piscidin genes, *pisc1* and *pisc2*, revealing their distinct roles *in vitro* and *in vivo*. The *pisc1* gene exhibits high expression in thymus, indicating a specialized function in adaptive immunity, while *pisc2* shows variable expression in response to pathogens, emphasizing its role in innate defense. This differential expression suggests that piscidins have specialized and potentially synergistic roles in combating microbial threats. The study highlights the importance of piscidins in the seabream's immune response.

The third paper investigates the immunometabolic role of hepcidin genes in iron homeostasis and pathogen defense. By inducing iron overload, the study observed significant impacts on immune responses and liver morphology, including increased vacuolization and disrupted cellular architecture. The upregulation of the *hamp1* gene and the downregulation of *hamp2.4* and *hamp2.5* highlight the balance between iron regulation and antimicrobial defense.

The fourth paper examines the modulation of hepcidin and piscidin genes in response to *V. harveyi* infection. Significant changes in liver morphology and differential gene expression across tissues were documented, demonstrating a dynamic immune response.

The study found reduced bactericidal activity in skin mucus while maintaining serum activity, suggesting distinct immune strategies. These findings provide a comprehensive understanding of the seabream's immune response to bacterial infections, highlighting the coordinated role of hepcidins and piscidins in immune defense.

In conclusion, this Doctoral Thesis provides comprehensive insights into the genetic and functional diversity of HDPs in gilthead seabream. The expansion and specialized roles of hepcidin and piscidin genes underscore their importance in immune defense and iron regulation. These discoveries offer potential biomarkers for early disease detection and pathways for developing selective breeding programs and HDP-based treatments, promoting sustainable aquaculture practices. Understanding HDPs contributes to develop more effective disease management strategies. The integration of these findings into practical applications could enhance the sustainability of gilthead seabream farming and prevent antimicrobial resistance.

# RESUMEN EN CASTELLANO

La dorada (*Sparus aurata*) es una especie de gran interés comercial en la acuicultura mediterránea, una industria que ha experimentado un crecimiento exponencial desde la década de 1980. Este crecimiento ha sido impulsado por la demanda de pescado y los avances tecnológicos en las prácticas de acuicultura. Sin embargo, el sector enfrenta desafíos sustanciales debido a las enfermedades infecciosas que afectan a los peces, en particular la vibriosis causada por *Vibrio harveyi*. Estas enfermedades no solo amenazan la salud de los peces, sino que también provocan pérdidas económicas significativas, afectando a la sostenibilidad y rentabilidad de la industria acuícola. El cambio climático agrava estos problemas al aumentar la virulencia de los patógenos y la incidencia de enfermedades. El calentamiento global y la acidificación de los océanos crean condiciones ambientales que pueden favorecer la proliferación de bacterias patógenas y debilitar el sistema inmunológico de los peces. En este contexto, los péptidos de defensa del huésped (HDPs) se presentan como componentes cruciales del sistema inmunológico de los vertebrados, incluidos los peces. Estos péptidos son moléculas pequeñas que juegan un papel vital en la primera línea de defensa contra infecciones, actuando de manera rápida y efectiva para neutralizar patógenos. En la dorada, dos tipos principales de HDPs, las hepcidinas y las piscidinas, desempeñan roles esenciales en la respuesta inmunitaria y la defensa antimicrobiana. Las hepcidinas son péptidos antimicrobianos que también regulan la homeostasis del hierro, mientras que las piscidinas son conocidas por su amplia actividad antimicrobiana. Esta tesis doctoral se centra en investigar los aspectos genéticos y funcionales de estos HDPs en la dorada, con el objetivo de mejorar la salud y la resistencia a enfermedades en la acuicultura. La identificación y caracterización de estos péptidos podrían proporcionar nuevas herramientas para manejar enfermedades en la acuicultura, reducir la dependencia de antibióticos y mejorar la sostenibilidad de las prácticas acuícolas.

El objetivo principal de esta tesis es explorar los roles genéticos y funcionales de los HDPs en la dorada, con un enfoque particular en las hepcidinas y las piscidinas. Los objetivos específicos incluyen identificar y caracterizar nuevos genes HDP en el genoma de la dorada, comprender la historia evolutiva de estos genes, examinar la transcripción basal de estos genes en condiciones fisiológicas estándar e investigar la regulación de estos genes en respuesta a patógenos y estímulos no patogénicos. Para lograr estos objetivos, se utilizaron diversas técnicas de biología molecular, genómica y bioinformática.

El primer capítulo de esta tesis se centra en la identificación y caracterización de los genes de hepcidina en la dorada. Utilizando técnicas avanzadas de análisis genómico, incluidos los Modelos Ocultos de Markov, se identificaron quince genes distintos de hepcidina en el cromosoma 17 de la dorada, lo que constituye el mayor número de genes de hepcidina encontrado en cualquier vertebrado hasta la fecha. Este descubrimiento se logró mediante la construcción de un perfil de HMM basado en secuencias de transcritos de hepcidina encontradas en teleósteos, permitiendo generar un modelo evolutivo de esta familia de péptidos. Los análisis revelaron perfiles específicos de expresión tisular y respuestas

transcripcionales a desafíos bacterianos, sugiriendo la existencia de mecanismos regulatorios sofisticados. Los genes de hepcidina mostraron una expresión diferencial en distintos tejidos como el hígado, gónadas, piel, riñón cefálico y timo, mientras que otros tejidos carecían de la transcripción de uno o más de estos genes. En particular, los genes *hamp2.3, hamp2.4 y hamp2.5* se expresaron significativamente en el riñón cefálico y en leucocitos, destacando su papel predominante en estos tejidos. Los estudios sobre la regulación transcripcional indicaron la presencia de varios sitios de unión a factores de transcripción (TFBS) en las regiones promotoras de los genes de hepcidina, incluyendo familias de factores como SMAD, STAT y NF-κB, que están involucrados en la regulación del metabolismo del hierro y la respuesta inmune. La distribución de estos TFBS sugiere una regulación compleja y diferenciada de los genes de hepcidina dependiendo del tipo y del tejido en que se expresan. Por ejemplo, los genes del clado C (*hamp2.3*, *hamp2.4* y *hamp2.5*) presentaron una falta de patrones comunes de NF-κB y STAT3 en comparación con los genes de los clados A y B, lo que podría indicar roles específicos en estados basales antes de la infección. La identificación de estos genes y su diversificación en el genoma de la dorada subraya la adaptabilidad y complejidad del sistema inmunológico de esta especie. Las múltiples copias de hepcidina permiten diversificar sus funciones y mejorar la capacidad del pez para responder a diferentes infecciones y cambios en el entorno. Esto incluye la regulación del hierro, un mineral esencial para muchos procesos biológicos pero también crucial para la proliferación de patógenos. Al controlar la disponibilidad de hierro, las hepcidinas ayudan a limitar el crecimiento bacteriano durante las infecciones. Los resultados también confirmaron una regulación diferencial de la expresión de los genes de hepcidina en respuesta a *Vibrio*. Este comportamiento sugiere que diferentes genes de hepcidina pueden tener roles específicos y temporales durante la respuesta inmune, con algunos actuando como "genes escudo" predominantemente expresados en estados basales, y otros como "genes asesinos específicos" inducidos durante la infección. La identificación y caracterización de quince genes de hepcidina en la dorada proporciona una comprensión profunda de la diversidad y complejidad del sistema inmunológico de este pez.

El segundo capítulo de esta tesis examina dos genes duplicados de piscidina, denominados *pisc1* y *pisc2*, además de sus roles tanto *in vitro* como *in vivo*. Estos genes se encontraron en el cromosoma 22, dentro de una región larga intrónica del gen similar a exostosin-1. Los resultados mostraron que *pisc1* tiene una alta expresión en el timo y el intestino, mientras que *pisc2* se expresa predominantemente en el riñón cefálico, bazo, sangre y branquias. La expresión diferenciada de estos genes en distintos tejidos resalta la especialización funcional de cada piscidina. En el caso de *pisc1*, su alta expresión en el timo, un órgano central en el desarrollo de células inmunitarias adaptativas sugiere que este péptido puede estar involucrado en la maduración y funcionamiento de estas células, potenciando la respuesta inmunitaria específica y de memoria a los patógenos. Por otro lado, la expresión variable de *pisc2* en respuesta a patógenos sugiere que puede ser inducida rápidamente en presencia de infecciones, proporcionando una defensa inmediata. En estudios *in vitro* con leucocitos de riñón cefálico, se observó que la expresión de *pisc2* disminuyó significativamente tras la incubación con *Vibrio*

*anguillarum*, nodavirus y poly I:C, mientras que la expresión de *pisc1* no mostró cambios significativos con ningún estímulo probado. Esto refuerza la idea de que *pisc2* desempeña un papel en la respuesta inmune innata, siendo modulada rápidamente para enfrentar infecciones agudas. En estudios *in vivo*, la expresión de ambos genes de piscidina se analizó tras la estimulación con *Vibrio anguillarum*. Se encontró una regulación a la baja de *pisc1* y *pisc2* en el riñón cefálico y el bazo a las 4 h post-inyección, lo que podría estar relacionado con la movilización de leucocitos desde estos órganos hacia otros tejidos. A las 72 h post-inyección, ambos genes mostraron una regulación al alza significativa en el hígado, particularmente *pisc1*, que incrementó su expresión 2.6 veces, sugiriendo que el hígado actúa como una fábrica de péptidos antimicrobianos en respuesta a infecciones bacterianas. Adicionalmente, la expresión de *pisc1* y *pisc2* se evaluó tras la infección con nodavirus. Se observó que solo *pisc1* mostró cambios significativos en su expresión, siendo regulado a la baja en el riñón cefálico a los 7 días post-inyección y en el cerebro a los 15 días, lo que indica un papel específico en la respuesta antiviral y posiblemente en la resolución de la infección. Aparte de los estudios de expresión génica, los análisis estructurales de las proteínas codificadas por estos genes revelaron diferencias significativas en su composición de aminoácidos y propiedades fisicoquímicas. Pisc1 y Pisc2, aunque similares en longitud y estructura secundaria, presentan variaciones notables en sus regiones N-terminal y C-terminal, lo que podría influir en su actividad antimicrobiana y su papel en la modulación inmunitaria. Esta diferenciación en la expresión y función de las piscidinas sugiere que estos péptidos desempeñan roles especializados y posiblemente sinérgicos en la protección contra amenazas microbianas, reforzando la capacidad inmunitaria de la dorada.

El tercer capítulo investiga el papel inmuno-metabólico de los genes de hepcidina en la regulación de la homeostasis del hierro y la defensa contra patógenos en la dorada (*Sparus aurata*). Se indujo una sobrecarga de hierro en los peces mediante la inyección intraperitoneal de dextrano de hierro para observar los efectos en sus respuestas inmunitarias y la morfología celular del hígado. El hígado, siendo el principal órgano de almacenamiento de hierro, mostró cambios morfológicos significativos bajo condiciones de sobrecarga de hierro. Los estudios histológicos revelaron que los hepatocitos en los peces con sobrecarga de hierro presentaban desplazamiento de los núcleos hacia la periferia de las células y una gran vacuolización en el citoplasma. Además, se observó una congestión de los vasos sanguíneos y depósitos refractarios de hemosiderina, que indicaban una acumulación masiva de hierro en el hígado. La vacuolización observada en las células hepáticas puede ser una indicación de estrés celular y daño potencial. En este contexto, la regulación diferencial de los genes de hepcidina destaca cómo el sistema inmunológico y metabólico trabajan juntos para mantener la homeostasis del hierro y combatir las infecciones. El gen *hamp1*, al ser regulado al alza (14 veces), puede aumentar la degradación de la ferroportina y así reducir la disponibilidad del hierro para los patógenos, mientras que la regulación a la baja de *hamp2.4* y *hamp2.5* puede moderar la respuesta inmune para prevenir el daño por exceso de hierro. Mientras, en el riñón cefálico, que es el principal órgano hematopoyético en la dorada, no se detectaron cambios significativos en la expresión de estos genes tras la inyección de hierro. Esto

sugiere que el hígado juega un papel central en la respuesta al exceso de hierro, actuando como un modulador clave en la regulación del metabolismo del hierro y la respuesta inmune. Por otro lado, la determinación de hierro en varios tejidos y órganos mediante espectroscopia de absorción atómica mostró que el hierro se acumulaba predominantemente en el hígado (61%) y el bazo (36%) en los peces con sobrecarga de hierro, mientras que, en los peces control, el hierro se distribuía principalmente en el intestino anterior, sangre y branquias. Esto refuerza la idea de que el hígado y el bazo son los principales órganos de almacenamiento de hierro en condiciones de sobrecarga. Además, la actividad bactericida del moco de la piel también se vio afectada por la sobrecarga de hierro. Se observó una disminución significativa en la actividad bactericida contra *Vibrio anguillarum* en los peces con altos niveles de hierro, lo que sugiere que la sobrecarga de hierro puede comprometer la capacidad del moco de la piel para actuar como una barrera efectiva contra patógenos. Por último, el análisis filogenético de proteínas reveló que la dorada produce la mayor variedad de hepcidinas maduras tipo II dentro de Eupercaria, con hasta 12 péptidos distintos. Estas hepcidinas tipo II se dividen en varios subclados y muestran una gran variabilidad en sus propiedades fisicoquímicas, indicando una amplia gama de funciones antimicrobianas. Esta diversidad permite a la dorada adaptarse a una variedad de patógenos, siendo la más alta registrada en Eupercaria hasta la fecha. Estos hallazgos proporcionan una comprensión más profunda de la interacción entre el metabolismo del hierro y la respuesta inmune en peces.

El cuarto capítulo de esta tesis se centra en la modulación de los genes de hepcidina y piscidina en respuesta a la infección por *Vibrio harveyi* en la dorada (*Sparus aurata*). Este estudio tiene como objetivo comprender cómo estos péptidos antimicrobianos se regulan y contribuyen a la defensa inmunológica del pez frente a la infección. Se inyectó *Vibrio harveyi* dentro del peritoneo de doradas y se analizaron muestras de moco de piel, suero, piel, riñón cefálico, hígado y bazo a las 4 h post-inyección. Los resultados mostraron alteraciones notables en la morfología del hígado, como un aumento significativo de la vacuolización en los hepatocitos y una desorganización del parénquima hepático. Los hepatocitos infectados presentaban núcleos desplazados hacia la periferia y una gran vacuolización citoplasmática, lo que indica estrés celular y potencial daño hepático. La actividad bactericida se evaluó tanto en el moco cutáneo como en el suero. Se observó una reducción significativa de la actividad bactericida en el moco cutáneo de los peces infectados contra *V. harveyi*, pasando del 33.6% en el grupo no infectado al 12.26% en el grupo infectado. Sin embargo, no hubo diferencias significativas en la actividad bactericida del suero entre los grupos infectados y no infectados. En contraste, la actividad bactericida contra *Vibrio anguillarum* se mantuvo en el moco cutáneo pero se perdió completamente en el suero de los peces infectados, lo que sugiere una especialización de la respuesta inmune en función del patógeno y del tejido. En cuanto a la expresión génica, se realizó un análisis exhaustivo de la expresión de los genes de hepcidina y piscidina en diferentes tejidos. La expresión de la interleuquina-1β (*il1b*) se utilizó como marcador de activación de la respuesta inmune, mostrando una fuerte regulación al alza en hígado, bazo, riñón cefálico y piel, pero no en la sangre. La expresión de los genes de hepcidina mostró dos patrones principales: un grupo de genes, incluidos *hamp1* y

*hamp2.1/7/8/9/10/12/13/14*, presentaron una regulación al alza significativa en respuesta a la infección, especialmente en el hígado y el riñón cefálico, mientras que los genes *hamp2.2/3/4/5/6* y los genes piscidinas mostraron cambios mínimos. La modulación de estos genes parece estar determinada tanto por el tipo de gen como por el tejido específico. Por ejemplo, *hamp1* y *hamp2.7* se regularon al alza significativamente en el hígado, mientras que otros genes como *hamp2.4* y *hamp2.5* mostraron una regulación diferencial dependiendo del tejido. En el caso de los genes piscidinas, se observó una regulación al alza en el bazo pero una disminución en la piel, lo que podría explicar la disminución de la actividad bactericida en el moco cutáneo. Estos hallazgos proporcionan una comprensión integral de la respuesta inmune de la dorada ante las infecciones bacterianas, destacando el papel coordinado de las hepcidinas y las piscidinas en la defensa inmunológica.

En conclusión, esta Tesis Doctoral proporciona conocimientos exhaustivos sobre la diversidad genética y funcional de los HDPs en la dorada. La expansión de los genes y los roles especializados de hepcidina y piscidina subrayan su importancia en la defensa inmunológica y la regulación del hierro. Estos descubrimientos ofrecen biomarcadores potenciales para la detección temprana de enfermedades y vías para desarrollar programas de cría selectiva, y tratamientos basados en HDPs, que promuevan prácticas acuícolas sostenibles. Comprender los HDPs contribuye a desarrollar estrategias de manejo de enfermedades más efectivas, y la integración de estos hallazgos en aplicaciones prácticas podría mejorar la sostenibilidad de la acuicultura de la dorada y prevenir la resistencia antimicrobiana.

# LIST OF PAPERS

## Paper I-Chapter I

**Massive gene expansion of hepcidin, a host defense peptide, in gilthead seabream (*Sparus aurata*).** Serna-Duque, J.A., Cuesta, A., Esteban, M.Á., 2022. Fish & Shellfish Immunology 124, 563–571.

## Paper II- Chapter II

**Two duplicated piscidin genes from gilthead seabream (*Sparus aurata)* with different roles in vitro and in vivo.** Serna-Duque, J.A., Cuesta, A., Sánchez-Ferrer, Á., Esteban, M.Á., 2022. Fish Shellfish Immunol 127, 730–739.

## Paper III- Chapter III

**Immunometabolic involvement of hepcidin genes in iron homeostasis, storage, and regulation in gilthead seabream (*Sparus aurata*).** Serna-Duque, J.A., Espinosa Ruiz, C., Martínez Lopez, S., Sánchez-Ferrer, Á., Esteban, M.Á., 2022. Frontiers in Marine Science 9.

## Paper IV- Chapter IV

**Hepcidin and piscidin modulation and antibacterial response in gilthead seabream (*Sparus aurata*) infected with *Vibrio harveyi*.** Serna-Duque, J.A., Espinosa-Ruiz, C., Esteban, M.Á., 2023. Fish Shellfish Immunol 139, 108899.

# INTRODUCTION

## 1. Gilthead seabream farming and the vibriosis challenge

Mediterranean aquaculture is a sector that took off in the second half of the 1980s, experienced exponential growth throughout the 1990s, and has been expanding persistently over the following decades. Within this industry of aquatic food production, the intensive farming of European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) stands out. The 90% of the aquaculture production of both species is concentrated in just 6 countries: Turkey, Greece, Egypt, Spain, Tunisia, and Italy. These are often cultivated on the same farms and are interchangeable in the market. Specifically, the total aquaculture production of gilthead seabream in Europe and the rest of the Mediterranean was estimated at 320,630 tons in 2022, representing an increase of 1.8% compared to 2021. At present, 96% of the gilthead seabream available on the market comes from aquaculture (Muniesa *et al*., 2020; *APROMAR*, 2023).

In this context, infectious diseases represent one of the main factors hindering the trade of aquaculture products and live fish and restrict the global expansion of aquaculture. Added to this spreading of foreign and emerging diseases as a consequence of the globalization of the market, as well as appearance of pathogens with greater virulence as a result of global warming are also key factors to consider (Lafferty *et al*., 2015). Climate change, specifically the increase in temperatures, significantly affects gilthead seabream farming due to the sharp rise expected to occur in the Mediterranean Sea (between 1.7 and 3°C). This temperature increase can exacerbate the effects of marine heatwaves, causing additional stress to marine animals, increasing mortality, and decreasing aquaculture performance (Fig. 1) (Cascarano *et al*., 2021).

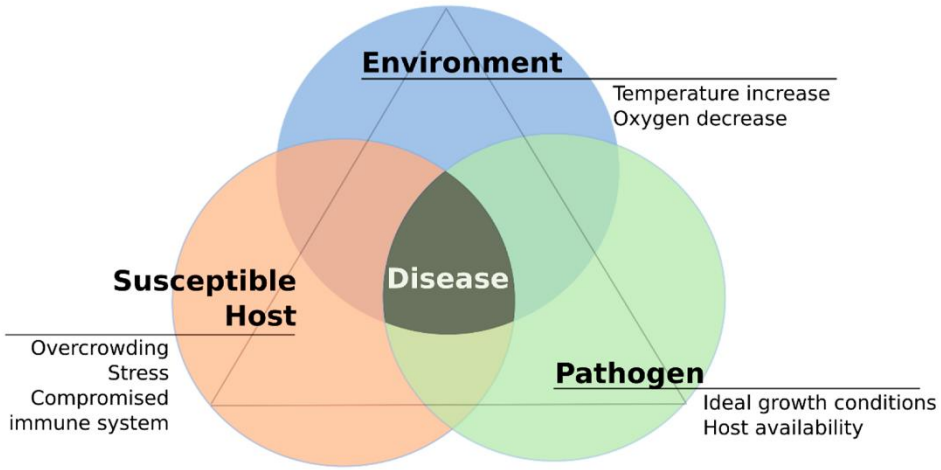


**Figure 1.** Infectious diseases of fish in a changing climate world (Cascarano *et al*., 2021).

The infectious diseases that most affect gilthead seabream are caused by bacteria, viruses, or parasites (Cascarano et al., 2021; Muniesa et al., 2022a, 2022a, 2020). Among the diseases with the highest incidence in the Mediterranean farming of gilthead seabream, the following stand out:

- Bacterial origin: Vibriosis, caused by members of the *Vibrionaceae* family, is of great importance in this Doctoral Thesis. Tenacibaculosis, attributed to *Tenacibaculum* spp. bacteria. Photobacteriosis, resulting from infection by *Photobacterium damselae*.
- Viral origin: Diseases linked to the *Nodaviridae* family, such as Viral Encephalopathy and Retinopathy (VER) and Viral Nervous Necrosis (VNN).
- Parasitic origin: Sparicotylosis, primarily caused by the parasite *Sparicotyle chrysophrii*.

Bacteria belonging to the *Vibrionaceae* family are found in both saline and freshwater environments, participating in interactions with their hosts that can be symbiotic or pathogenic (Ina-Salwany *et al*., 2019). These microorganisms are known for their opportunistic behavior, a characteristic that becomes evident under conditions such as high density and stress, which are very common in aquaculture farms and can lead to outbreaks of infectious diseases (Elias *et al*., 2023). Within the field of aquaculture, several species of *Vibrio* are recognized as pathogens or opportunistic pathogens, affecting a variety of aquatic species such as fish, crustaceans, and mollusks (Helmi *et al*., 2020). Vibriosis in fish can manifest with a broad spectrum of clinical symptoms, which vary depending on the fish's immune status, the affected species, environmental conditions, and the virulence of the bacterial strain. The most common symptoms of vibriosis in fish include: hemorrhagic septicemia with extensive external skin lesions (hemorrhagic fin and ulcers), focal necrosis of some internal organs (liver, spleen, kidney), paleness in kidney and gills, dark pigmentation on the skin, exophthalmic eyes, splenomegaly, complete erosion of the tail, loss of appetite, and lethargy (Ina-Salwany *et al*., 2019; Sanches-Fernandes *et al*., 2022). In particular, in populations of gilthead seabream, both farmed and wild, that have been affected in the Mediterranean Sea, *V. alginolyticus* has been identified as the most commonly isolated *Vibrio* species, followed by *V. harveyi*, *V. splendidus*, *V. anguillarum*, *V. parahaemolyticus*, and *V. tubiashii* (Sanches-Fernandes *et al*., 2022). Vibriosis usually presents acutely in the larval (hatcheries) and juvenile stages of gilthead seabream, spreading rapidly after infection and resulting in high mortality rates, even in those fish that do not show evident clinical symptoms (Muniesa *et al*., 2020).

For the treatment of vibriosis, some authorized antibiotics have been used, such as oxytetracycline, tetracycline, quinolones, nitrofurans, potentiated sulfonamides, trimethoprim, sarafloxacin, flumequine, and oxolinic acid (Mohamad *et al*., 2019). However, in Spain, only the use of oxytetracycline as an effective antibiotic against vibriosis in marine fish farms, such as gilthead seabream, is allowed (Sanches-Fernandes *et al*., 2022; *VPMs for fish*, 2023). This drug is subject to strict regulations and is administered only in specific cases of disease outbreaks. Unlike Spain, in other European Union countries, a greater variety of antibiotics is allowed: enrofloxacin, florfenicol, flumequine, sulfadiazine, trimethoprim, chlortetracycline (*VPMs for fish*, 2023). Thus, expanding the therapeutic options available to combat this disease in aquaculture is needed.

Complementing antibiotic therapy, vaccination emerges as a far-reaching prophylactic strategy within Spanish aquaculture, standing out for its effectiveness against a wide spectrum of *Vibrio* pathogenic species. Currently, the technology behind these vaccines for gilthead seabream and sea bass is based on the use of inactivated strains of *Vibrio anguillarum*. However, the effectiveness of these vaccines could be compromised in outbreaks caused by other *Vibrio* species or by the emergence of new strains, a situation that could potentially lead to high mortality rates in fish farms (*HIPRA*, 2024; *VPMs for fish*, 2023; Triga *et al*., 2023). This vaccination in gilthead seabream is especially administered (62%) in the pre-growing phase, before reaching the on-growing farms (Muniesa *et al*., 2022b).

Additionally, alternative strategies for the management of vibriosis have been explored, including the use of probiotics and plant extracts incorporated into gilthead seabream feeds. These compounds act as immunostimulants, strengthening the fish's immune system and offering a sustainable and effective alternative for the prevention of this disease (Dawood *et al*., 2018; García Beltrán and Esteban, 2022; Sanches-Fernandes *et al*., 2022). These strategies, along with vaccination and regulated use of antibiotics, allow for a comprehensive approach to the management of vibriosis in aquaculture. However, the importance of innovation and continuous research for the development of solutions that are effective against pathogens and environmentally friendly must be emphasized. It is widely recognized that diseases caused by microorganisms still represent a significant challenge for the aquaculture of this important species, hence, research and development of effective methods for their prevention and treatment must be explored and developed.

## 2. Beyond Antimicrobial peptides: Host defense peptides

The use of antibiotics in aquaculture is widespread and regulated to treat infectious diseases. However, this practice can lead to the dispersion of antibiotic residues in the marine environment and exert selective pressure that favors the natural selection of multi-antibiotic resistant bacteria (MAR bacteria). This increases the rates of antibiotic resistance in aquatic bacteria and, critically, transfers that resistance to terrestrial pathogens that ultimately affect humans ("The FAO Action Plan on Antimicrobial Resistance 2016-2020," 2016; Reverter *et al*., 2020; Pepi and Focardi, 2021). Likewise, climate change increases the horizontal transference of genetic material between bacteria due to the rise in temperature of seawater, accelerating the resistance alarmingly in both marine and terrestrial pathogens. In fact, there is a strong correlation between antimicrobial resistance that emerges from aquaculture practices and the resistance observed in human clinical cases (Lettieri *et al*., 2018; Reverter *et al*., 2020).

The exponential growth of antibiotic resistance in recent decades has encouraged to find alternatives that reduce the use of antibiotics in human medicine and veterinary science. In this context, antimicrobial peptides (AMPs) could be a promising and sustainable alternative in the fight against MAR bacteria. Natural AMPs are peptide molecules encoded by genes, conserved and diverse evolutionarily, with structural and functional variety. They all share a wide range of activities against different pathogens in various

organisms. AMPs act on the front line of defense against microbial attacks in Eukaryotes (Mookherjee *et al*., 2020; Moretta *et al*., 2021).

To date, around 3,146 natural antimicrobial peptides have been included, according to the 2023 data version, in the Antimicrobial Peptide Database (APD), described in organisms from the six kingdoms of life: 383 bacteriocins/peptide antibiotics from bacteria, 5 from archaea, 8 from protists, 29 from fungi, 250 from plants, and 2,463 from animals (Fig. 2) (Wang, 2023; Wang *et al*., 2016). Among the natural AMPs, manually curated, there have known antimicrobial activity: 373 from mammals (144 from humans), 1,079 active peptides from amphibians, 146 peptides from fish, 52 from reptiles, 47 from birds, 619 from arthropods, 54 from mollusks, and 6 AMPs from protozoa (December 2023).

Despite fish being the most widespread and diverse group of vertebrates on the planet, the fish AMPs found only represent 6% of the total peptides annotated with antimicrobial function. Most of these 146 AMPs belong to two major families: the hepcidins, which are conserved in all vertebrates except birds, and the piscidins, a group of AMPs exclusive to fish. In addition to these two main families, there are also the cathelicidins, defensins, NK-lysins, and peptides derived from lipoproteins or histones (Valero *et al*., 2018; Wang *et al*., 2016).

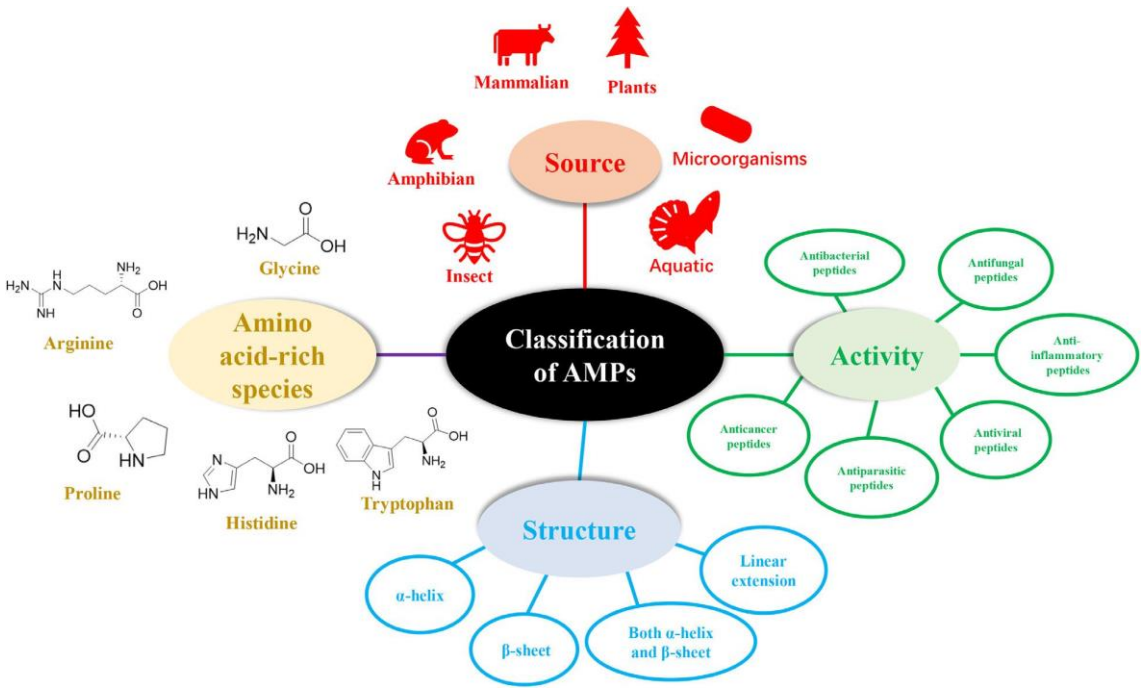


**Figure 2.** Classification of AMPs based on source, activity, structure, and enriched residues (Huan *et al*., 2020).

**Properties and structures**

Despite exhibiting considerable diversity in their physicochemical and structural properties, origins, and mechanisms of action, antimicrobial peptides share some common characteristics:

1. Typically short molecules (20-50 residues).
2. Predominantly net positive charge (+2 to +11), although anionic (-1 to -7) and neutral AMPs have also been identified.
3. Containing a significant proportion of hydrophobic residues (40-50%). They present an amphipathic structure, as contain both hydrophobic and hydrophilic regions, allowing them to be soluble in aqueous environments (Boparai and Sharma, 2020; Wang, 2023).

The amphipathic nature of most AMPs directly determines their structural conformation. Commonly, AMPs are classified based on their secondary structure into four categories (Fig. 2, 3) (Huang and Li, 2023; Wang, 2023):

a. α-helical peptides (α) such as cathelicidins or piscidins.
b. β-sheet peptides (β) with variable presence of disulfide bonds such as defensins or hepcidins.
c. Linear extended structure peptides (non-αβ) like indolicidin.
d. Peptides with both α-helix and β-sheet structures (αβ) like plectasin.

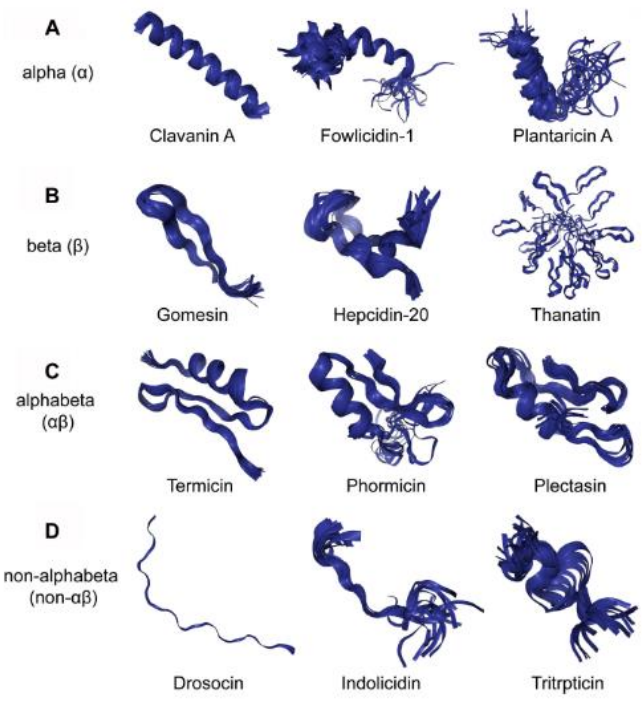


**Figure 3.** Structural classification of AMPs (Huang and Li, 2023).

**Functions and Applications**

Initial research in the field of AMPs focused on exploring their antimicrobial capabilities, with the aspiration of discovering new antibiotic therapies derived from natural peptides. However, their limited microbicidal efficacy under standard physiological conditions, especially at the low concentrations found naturally *in vivo*, did not fully justify their role in the host's defense against pathogens. This initial puzzle suggested that AMPs might have additional functions in the host's immune response (Steiner *et al*., 1981; Bergsson *et al*., 2009; Mahlapuu *et al*., 2016).

Over the last two decades, evidence has been accumulated showing that AMPs perform a variety of functions that go well beyond their direct microbicidal activity, including the modulation of immune responses, which enhances their effectiveness against pathogenic microorganisms. This finding has led the scientific community to adopt the term "host defense peptides" (HDPs) for these compounds, more accurately reflecting the breadth of their biological functions (Scott *et al*., 2002; Hancock *et al*., 2016; Mookherjee *et al*., 2020). Apart from microbicidal functions, such as antibacterial, antiviral, antifungal, and antiparasitic activities, and new immunomodulatory functions, classic AMPs exhibit a wide range of biological functions or activities: anticancer, anti-biofilm, hemolytic, spermicidal, insecticidal, antitoxin, antidiabetic, antioxidant, wound healing promoter, neuroprotective or angiogenesis promoter (Huan *et al*., 2020; Răileanu *et al*., 2023; Wang, 2023). This shift in perspective underlines the importance of HDPs, not only as microbicidal agents but also as critical regulators of innate and adaptive immunity and opens new horizons for the development of innovative therapies against infections. In fact, there is currently a considerable number of these antimicrobial peptides entering clinical trials, reflecting their therapeutic potential (Browne *et al*., 2020; Luo *et al*., 2023).

# 3. Applications of HDPs on fish health

Host defense peptides have shown considerable promise in aquaculture as bioactive molecules against a wide array of pathogens including bacteria, viruses, fungi, and parasites. Studies have documented the efficacy of these peptides both *in vitro* and *in vivo*, emphasizing their potential as therapeutic agents in disease management in aquaculture (Falco *et al*., 2012; Priyam *et al*., 2021).

Notably, certain AMPs possess dual functions: directly combating pathogens and enhancing the immune resilience of fish. This dual functionality enables them to not only neutralize the immediate threat of infection but also reinforce the host's immune response against future infections (Rajanbabu, 2019; Barroso *et al*., 2021). The immunomodulatory capabilities of these peptides are particularly noteworthy, as they can potentially be applied to develop vaccines or incorporated into fish diets as functional additives, thus providing a sustainable approach to disease prevention and health enhancement in aquaculture settings (Noga *et al*., 2011; Valero *et al*., 2018; Álvarez *et al*., 2022; Wang *et al*., 2023). Recent research also highlights the role of supplementation of HDPs in fish diet (Fig. 4) such as hepcidin, piscidin, lactoferrin, and various insect and chimeric

peptides which have been found to improve serum biochemical indices, boost antioxidative enzyme activities, enhance transcriptomic immune responses, and increase resistance to pathogens (Abdel-Wahab *et al*., 2021; Jin *et al*., 2023; Wang *et al*., 2023). These HDPs not only aids in maintaining microbial health in the intestine but also contributes to the physical growth of the fish, marking a significant step forward in the holistic management of fish health (Ting *et al*., 2019; An *et al*., 2023). Fish-derived AMPs have shown promise in aquaculture as alternatives to antibiotics, combating antibiotic-resistant pathogens and enhancing growth performance and immunity in farm animals (Valero *et al*., 2018; Chen *et al*., 2020; Priyam *et al*., 2021). The exploration of HDPs in fish contributes to the development of innovative approaches for the prevention and treatment of diseases in aquaculture, emphasizing the need for sustainable solutions that minimize antibiotic dependency and address antimicrobial resistance. This Doctoral Thesis is oriented towards advancing the knowledge of these peptides in gilthead seabream, not only as antimicrobial agents but also as enhancers of immunity, which could mark a significant advancement in the management of aquaculture health for this species.

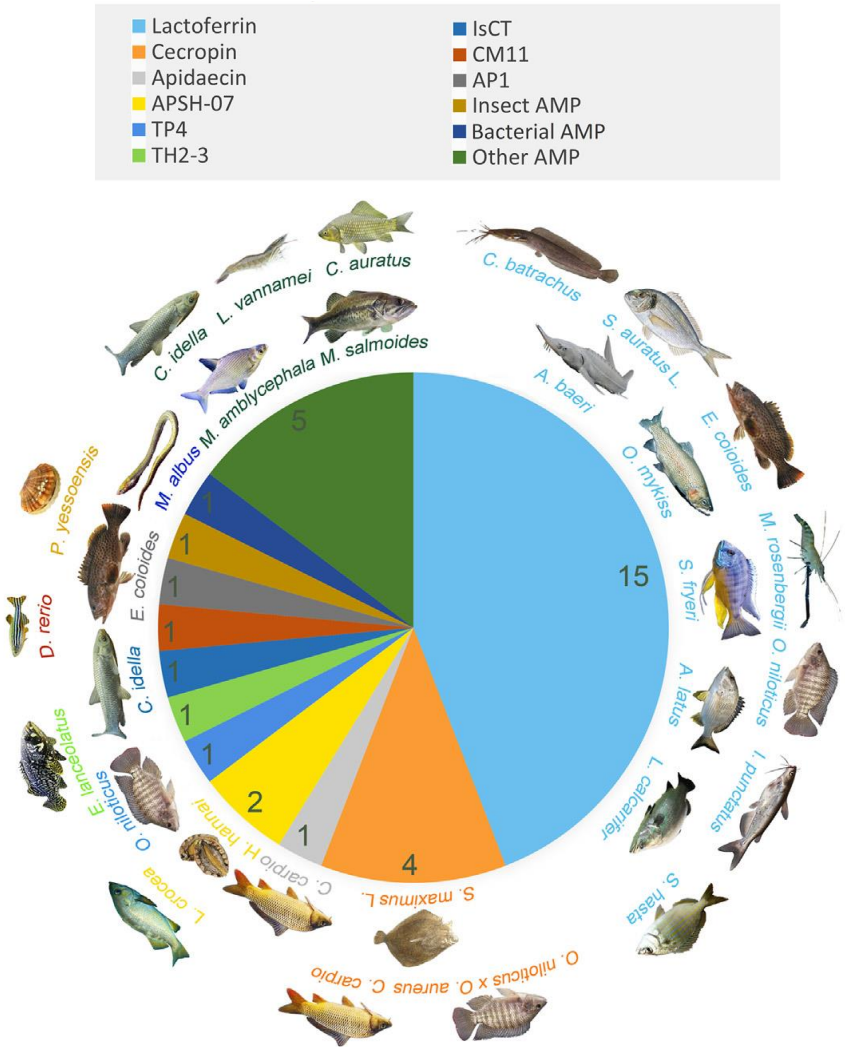


**Figure 4.** A summary of the current application of AMPs as feed additives in aquatic animals. The inserted numbers on the pie chart indicate the number of publications related AMPs used in diets (Wang *et al*., 2023).

# OBJECTIVES

The primary aim of this Doctoral Thesis is to explore host defense peptides in gilthead seabream (*Sparus aurata*) and to enhance our understanding and application in disease management. The specific objectives are:

1. Identification and screening of novel gene sequences within the gilthead seabream genome that encode HDPs, using advanced *in silico* techniques.
2. Conducting evolutionary analyses of the newly discovered genes encoding HDPs and their resultant proteins in gilthead seabream.
3. Examination of the basal transcription of identified HDP genes under standard physiological conditions.
4. Validation of the hypothesis that pathogens such as *Vibrio* and *Betanodavirus* induce changes in the expression of the identified HDP genes.
5. Investigation into the regulation of HDPs in response to non-pathogenic *stimuli*, extending beyond pathogenic interactions.

# SUMMARY OF RESULTS

The importance of host defense peptides such as hepcidin and piscidin in the immune system of the gilthead seabream (*Sparus aurata*) has been extensively investigated in this Doctoral Thesis, driven by the growing concerns over microbial diseases like vibriosis in aquaculture. This compilation of research delves deeply into the genetic and functional intricacies of HDPs in seabream, emphasizing their pivotal role in defending against pathogens, especially the marine bacterium *Vibrio*.

In **Chapter I**, a significant expansion of the hepcidin gene in gilthead seabream was revealed, marking an unprecedented finding among vertebrates. Employing genomic analysis techniques, such as HMMs, we identified fifteen distinct hepcidin genes on chromosome 17 of the seabream genome, comprising one *hamp1* and fourteen *hamp2* genes. Moreover, these novel genes presented a complex evolutionary history and sophisticated transcriptional regulation. Through expression profiling, we determined the tissue-specific roles of these hepcidin variants. Notably, *hamp2.3/4/5* showed stable expression in the head kidney and did not vary significantly under bacterial challenge with *Vibrio anguillarum*, unlike other clade-*hamp* genes which exhibited considerable changes. These findings underscore their potential in modulating host defense mechanisms.

In **Chapter II**, two piscidin genes from gilthead seabream were discovered and explored for their roles in immune response. These genes, *pisc1* and *pisc2*, located on chromosome 22, exhibit different responses to pathogens like *V. anguillarum* and *Betanodavirus*. *Pisc1* maintains a consistent expression pattern, suggestive of a specialized function in adaptive immunity, possibly linked to its significant presence in the thymus. *Pisc2*, predominantly active in frontline immune tissues, shows decreased expression in response to infections, highlighting its role in innate defense. Notably, the putative piscidin proteins (class I) differ markedly in their physicochemical properties; Pisc1 is hydrophilic and Pisc2 is hydrophobic, affecting their antimicrobial actions and interactions with microbial cell membranes. This divergence indicates their specialized and potentially synergistic roles in combating diverse microbial threats.

In **Chapter III**, the roles of hepcidin in regulating iron and defending against pathogens in gilthead seabream were investigated. Iron overload was induced through intraperitoneal injection of iron-dextran, which profoundly influenced immune responses

to pathogens like *Vibrio sp.* Histological assessments of the liver showed substantial alterations, including extensive hemosiderin deposits, and disrupted cellular architecture, indicative of severe impacts on iron metabolism. Concurrently, the bactericidal efficacy of skin mucus decreased significantly in iron-overloaded fish, highlighting impaired antimicrobial defenses. Gene expression analysis revealed an upregulation of the *hamp1* gene, pivotal for iron regulation, while some antimicrobial-associated *hamp2* genes, such *as hamp2.4* and *hamp2.5*, were notably downregulated, suggesting a recalibration of hepcidin functions under conditions of iron excess. Furthermore, a phylogenetic analysis of hepcidin proteins underscored their evolutionary diversity and specialization.

In **Chapter IV**, the acute response of gilthead seabream to *Vibrio harveyi* infection was assessed through the modulation of hepcidin and piscidin genes. The study highlighted significant changes in liver morphology, particularly increased vacuolization indicating cellular stress. The liver's response was further characterized by a notable increase in HDP expression, demonstrating its central role in the immune response. Gene expression across various tissues including liver, spleen, and skin was carefully analyzed, showing tissue-specific responses to the infection. Furthermore, a reduction in the bactericidal activity of skin mucus was determined while maintaining serum activity. This comprehensive study sheds light on the dynamic immune responses in gilthead seabream, illustrating the complex interactions between host defense mechanisms and pathogen challenges.

# EXPERIMENTAL CHAPTERS

# Paper I-Chapter I

## Massive gene expansion of hepcidin, a host defense peptide, in gilthead seabream (*Sparus aurata*).

**Massive gene expansion of hepcidin, a host defense peptide, in gilthead seabream (*Sparus aurata*).** Serna-Duque, J.A., Cuesta, A., Esteban, M.Á., 2022. Fish & Shellfish Immunology 124, 563–571.

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# Massive gene expansion of hepcidin, a host defense peptide, in gilthead seabream (*Sparus aurata)*

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ABSTRACT

Host defense peptides (HDP) are among the most ancient immune molecules in animals and clearly reflect an ancestral evolutionary history involving pathogen-host interactions. Hepcidins are a very widespread family of HDPs among vertebrates and are especially diverse in teleosts. We have investigated the identification of new hepcidins in gilthead seabream (*Sparus aurata*), a fish farmed in the Mediterranean. Targeted gene predictions supported with expressed sequence tags (ESTs) derived from Hidden Markov Models were used to find the *hamp* genes in the seabream genome. The results revealed a massively clustered *hamp* duplication on chromosome 17. In fact, the seabream genome contains the largest number of hepcidin copies described in any vertebrate. The evolutionary history of hepcidins in seabream, and vertebrates generally, clearly indicates high adaptation in teleosts and novel subgroups within hepcidin type II. Furthermore, basal hepcidin gene expression analysis indicates specific-tissue expression profiles, while the presence and distribution of transcription factor binding sites (TFBS) in *hamp* promoters as well as their transcription profile upon bacterial challenge indicates different immune roles depending on the type of hepcidin and tissue. This massive duplication of HDP genes in a bony fish could point to a far more specific and adaptive innate immune system than assumed in the classic concept of immunity in mammals. Hence, a new world of knowledge regarding hepcidins in fish and vertebrates is being initiated.

## 1. Introduction

At present, the development of antimicrobial resistance in aquatic and land livestock has made urgent to research new sanitary control strategies that are not only capable of controlling resistant bacteria but also avoiding their appearance [1,2]. With this in mind, a promising alternative approach is emerging from the study of antimicrobial peptides (AMPs). These AMPs comprise a large number of different host defense gene-encoded short peptides, generally cationic and amphipathic, with a high antimicrobial capacity against some livestock and human pathogens [3]. Among the AMPs, the hepcidin family is one of the most widespread among vertebrates with a highly disulphide-bonded hairpin-shaped β-sheet structure [4]. Hepcidin peptide was identified for the first time in human, its coded-single copy gene is mainly expressed in liver and is formed by three exons and two introns, as conserved in all vertebrates [5–7].

Unlike mammals, fish present multiple duplicated genes similar to discovered human hepcidin due to their polyploidy genomes [8,9]. In fact, *Actinopterygii* class of fish have two types of functional hepcidin genes: *hamp1* and *hamp2*. The first type is present in all fish species and is an ortholog single-copy gene of the human one, mainly involved in iron metabolism though certain indirect role in the immune response seems has been also ascribed. In sharp contrast, *hamp2* is exclusive to actinopterygians, has multiple copies localized in tandem and in a separate region from *hamp1*, and is more closely involved in the innate immune response, including its function as AMP [9–11]. Both hepcidin genes of type I and II have shown different expression patterns in tissues of adults and during larval development. Moreover, stimulation with several bacterial/viral/fungi treatments has differently activated hepcidin expression [12–17].

The aim of the present study is to demonstrate the existence of several copies of the *hamp* gene in a bony fish *(Sparus aurata)* with high interest in marine aquaculture and to understand their evolutionary history. The tissue-specific expression, the transcriptional regulation, and probably also diverse functions, will be analyzed.

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## Paper II- Chapter II

# Two duplicated piscidin genes from gilthead seabream (*Sparus aurata*) with different roles *in vitro* and *in vivo*.

**Two duplicated piscidin genes from gilthead seabream (*Sparus aurata)* with different roles in vitro and in vivo.** Serna-Duque, J.A., Cuesta, A., Sánchez-Ferrer, Á., Esteban, M.Á., 2022. Fish Shellfish Immunol 127, 730–739.

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Full length article

# Two duplicated piscidin genes from gilthead seabream (*Sparus aurata*) with different roles *in vitro* and *in vivo*

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ABSTRACT

From the discovery of pleurocidin in skin mucus of winter flounder, many new related sequences have been found, forming a fish-exclusive family of antimicrobial peptides (AMP) called piscidin. Their mature peptides have a broad-spectrum antimicrobial activity and can be involved in the innate immune response. In the present work, two paralogous tripartite piscidin genes are formally described for the first time in gilthead seabream (*Sparus aurata*), an important marine farmed fish. Gene synteny and protein phylogeny clearly indicated a massive *pisc* gene expansion in a cluster of the chromosome 22 as well as a special evolution of piscidin in gilthead seabream compared to the rest of piscidins studied in other fish species. Despite being highly similar genes, they show totally different expression patterns in tissues and head-kidney leucocytes under both naïve and *Vibrio*/nodavirus-stimulated conditions. Moreover, these paralogous genes coded very different proteins according to their physicochemical properties. In this way, these piscidin genes have distinct roles not only related to their microbicide activity but also to their immune modulation. In addition, the present study improves the knowledge of duplication of AMP genes and adaptative diversification of teleost immune system.

## 1. Introduction

Aquaculture continues to grow steadily and is an essential part of the Blue Growth, but infectious diseases are still a very important limiting factor for its expansion and economic productivity. Increasing public concern about antibiotics, together with the increase in antibiotic-resistant pathogens, have prompted most developed countries to ban their use in animal feed [1]. *A priori*, antimicrobial peptides (AMPs) are an innovative alternative to classical therapies, possessing not only broad antibacterial activity but also activity against viruses, fungi, and parasites. In fact, it is expected that their use could address resistant infections for which there is no effective treatment or for which the causative agent has not yet been identified [2].

In fish, an exclusive family of AMPs, called piscidins, has been identified and it is under evaluation. The first member was characterized in skin mucous secretions from flounder (*Pleuronectes americanus*) and named pleurocidin, which 25-residue peptide resembled other called dermaseptin from amphibians [3]. Piscidins have been only characterized under the superorder Acanthopterygii, particularly in some taxa such as Moronidae, Sciaenidae, Sparidae, Siganidae, Latridae, Belontidae, Cichlidae, and Perichthyidae [4]. Hence, the piscidin family includes many relatively similar peptides to pleurocidin, depending on studied species were named as moronecidin from *Morone saxatilis* × *M. chrysops* [5], dicentracin from *Dicentrarchus labrax* [6], epinecidin from *Epinephelus coiodes* [7], chrysophsin from *Chrysophrys major* [8] and gaduscidin from *Gadus morhua* [9]. These amphipathic α-helical AMPs are generally coded by four-exon and three-intron genes, which are distributed in genomic clusters [10,11]. Indeed, gene duplications of piscidin coded similar structurally isomers in various fish species, thought they seem to show different biologic roles [12,13]. These piscidin genes are mainly expressed in first-line defence tissues as the gill, skin and intestine, but they have also been determined in head-kidney, spleen and some immune cells [11,12,14]. Different classes of piscidin genes were up and down-regulated by various pathogens as it was proved with a pathogen-like stimulus such as LPS, bacterial antigens as well as with parasites, viruses, and poly I:C [4].

The cationic linear α-helical active domain from the gene-coded precursor of piscidin is released after removing conserved N-terminal and anionic variable C-terminal regions by proteolytic post-translational process [15]. From this mature amino acid (aa) sequence, piscidins were

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## Paper III- Chapter III

# Immunometabolic involvement of hepcidin genes in iron homeostasis, storage, and regulation in gilthead seabream (*Sparus aurata*).

**Immunometabolic involvement of hepcidin genes in iron homeostasis, storage, and regulation in gilthead seabream (*Sparus aurata*).** Serna-Duque, J.A., Espinosa Ruiz, C., Martínez Lopez, S., Sánchez-Ferrer, Á., Esteban, M.Á., 2022. Frontiers in Marine Science 9.

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# Immunometabolic involvement of hepcidin genes in iron homeostasis, storage, and regulation in gilthead seabream (*Sparus aurata*)

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Iron is essential for all living things, especially marine organisms, due to its low availability in the marine environment. Iron regulation is key in all vertebrates and is controlled by hepcidin–ferroportin. To improve the knowledge of iron homeostasis in fish, an iron overload was induced in gilthead seabream (*Sparus aurata*), which was chosen as a study species because of its high interest in Mediterranean aquaculture. The amount of iron in different tissues was measured to determine its biodistribution and/or bioaccumulation. Since the liver is directly involved in iron metabolism, the morphological changes induced in this organ as a consequence of the iron increase were studied. The bactericidal activity of fish skin mucus was also determined, observing that it decreased in fish with high iron levels compared to control fish. In addition, to better understand iron regulation, the gene expression of hepcidin, ferroportin, transferrin, and ferritin was evaluated in the head kidney (the main hematopoietic organ in this species) and in the liver. Special interest was taken in the study of the multiple copies of the hamp2 gene present in the gilthead seabream genome. Bioinformatic analysis of the protein sequences derived from these hepcidin genes allowed us to determine the presence of one type I hepcidin and 12 type II hepcidins, all of them with antimicrobial potential. This number of mature hepcidin sequences found in gilthead seabream is the highest within Eupercaria described to date. All the results obtained indicate that the modulation of iron in seabream seems to be much more complicated than in other vertebrates, probably due to the possible involvement of the different hepcidins as mediators between iron metabolism and host immune response.

KEYWORDS

**hepcidin, host-defence peptide, iron, immunometabolism, Gilthead seabream (*Sparus aurata L.*), evolution, teleosts**

## Paper IV- Chapter IV

# Hepcidin and piscidin modulation and antibacterial response in gilthead seabream (*Sparus aurata*) infected with *Vibrio harveyi*.

**Hepcidin and piscidin modulation and antibacterial response in gilthead seabream (*Sparus aurata*) infected with *Vibrio harveyi*.** Serna-Duque, J.A., Espinosa-Ruiz, C., Esteban, M.Á., 2023. Fish Shellfish Immunol 139, 108899.

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Full length article

# Hepcidin and piscidin modulation and antibacterial response in gilthead seabream (*Sparus aurata*) infected with *Vibrio harveyi*

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ABSTRACT

Vibriosis is an infectious disease that generates large economic losses in Mediterranean aquaculture. *Vibrio harveyi* is one of the marine bacteria causing this disease, it is widespread in the Mediterranean Sea and causes ulcers on the skin of the fish it infects. In addition, the skin is a route of entry and colonization of this pathogen. In this study, one group of fish was injected intraperitoneally with phosphate buffered saline (control group) and another with *V. harveyi* (infected group). At 4 h after injection, samples of skin mucus, blood, skin, head kidney, liver, and spleen were collected to study the immune response generated. Liver histology showed notable alterations in hepatocyte morphology, such as increased vacuolization. Bactericidal activity was measured in skin mucus and serum against *V. harveyi* and *V. anguillarum*, different changes in this activity were recorded depending on the bacteria target and sample (skin mucus or serum) used. Gene expression of genes encoding hepcidins and piscidins (antimicrobial peptides) was performed in the mentioned organs. The results indicated a different expression according to the type of AMP and the tissue studied. Hepcidin appeared involved in all tissues studied while piscidins were in the spleen. In this study we have integrated hepcidin-piscidin modulation with the effects of infection on skin mucosa, serum and hepatocyte morphology. Knowing the changes produced in all these parameters improves the understanding of the infection in the first hours in sea bream and could have applications in the diagnosis or treatment of vibriosis in fish farms.

## 1. Introduction

Vibriosis is one of the most widespread zoonotic bacterial diseases with the highest mortality rates in intensive marine fish farming and constitutes a serious risk to human health [1,2]. Members of the genus *Vibrio* (*Vibrio anguillarum*, *Photobacterium damselae*, *V. parahaemolyticus*, *V. vulnificus, V. harveyi*, among others) cause this condition, which affects both marine fish and invertebrates, and are highly endemic in the microbiome of the seas and in fish farms. Naturally, this bacterium does not pose a risk to animal health, however, under conditions of high fish density as is common in fish farms, it becomes a serious opportunistic pathogen [3]. This is because infected farmed fish have many contacts with other fish and thus transmit the disease from one to another. In addition, infected fish eventually develop ulcers and skin necrosis, as this tissue is an important route of entry, colonization and spread of these bacteria [4].

One of the best-known bacteria causing this disease is *Vibrio harveyi*, mainly found in warm waters of Southern of Europe, Asia and America [5–7]. The clinical signs of this infection are unspecific, but included, in addition to skin ulcers as mentioned above, necrosis in muscle, skin and intestine, gastroenteritis, tail rot and vasculitis [8,9]. *V. harveyi* possesses several virulence factors such as extracellular products (e.g., proteases, hemolysins and lipopolysaccharides), iron-binding siderophores, bacteriocin-like substances, biofilm formation and host-microbiome interaction [9]. Moreover, some bacteria of this species isolated from Italian, Indian and Chinese seas were fully resistant to beta-lactam antibiotics, and could be a reservoir of multidrug resistance (MDR) genes [5,6,10] and emerging zoonotic bacteria for humans, as *V. harveyi* has already been found in infected human wounds [11].

To address this emerging challenge, it is necessary to investigate novel and alternative antimicrobial agents such as antimicrobial peptides (AMPs). These AMPs are short genetically encoded peptides (10–50 amino acids) involved in host defense, generally cationic and amphipathic, with high multi-target antimicrobial capacity against both livestock and human pathogens [12,13]. In fish, AMPs have been identified and classified into five main families: hepcidins, β-defensins,

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# MAIN CONCLUSIONS

1. A significant expansion of fifteen hepcidin and two novel piscidin genes has been identified and characterized in the gilthead seabream (*Sparus aurata*) genome. This expansion and diversity of HDPs suggests a complex and versatile humoral innate immunity in this species.
2. Hepcidin and piscidin genes show tissue-specific expression in standard physiological conditions, primarily in head kidney, thymus, liver, and spleen.
3. Iron overload significantly impacts immune responses in gilthead seabream, leading to liver alterations and decreased bactericidal efficacy in skin mucus. Upregulation of *hamp1* and downregulation of *hamp2.4* and *hamp2.5* under iron excess conditions highlight the role of hepcidin genes in maintaining balanced iron levels and optimal immune response.
4. *Vibrio harveyi* infection causes significant liver morphology changes and increased expression of hepcidin and piscidin genes. This response underscores the liver's central role in immune defense. Likewise, *Vibrio* infection reduces the bactericidal activity of skin mucus while maintaining serum activity, indicating different immune responses.
5. Modulation of hepcidin and piscidin genes provides biomarkers for early disease detection. Moreover, genetic insights into these genes could guide selective breeding for enhanced disease resistance and iron homeostasis.
6. Implementing diets, vaccines, and selective breeding programs using identified HDPs from gilthead seabream could ensure healthier and more resilient fish populations. These strategies could be crucial for a sustainable aquaculture and prevention of antimicrobial resistance.

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