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**DISTINGUISHING THE IMPACTS OF REARING VOLUME VERSUS THE STOCKING
DENSITY ON THE SKELETAL QUALITY OF *SPARUS AURATA* DURING THE LARVAL
AND PRE-ONGROWING REARING PHASES**

Ph.D. Thesis

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Table of Contents

SUMMARY IN ENGLISH	5
RIASSUNTO IN ITALIANO / SUMMARY IN ITALIAN	5
RESUMEN EXTENDIDO EN ESPAÑOL / EXTENDED SUMMARY IN SPANISH	6
OBJETIVO DE LA TESIS DOCTORAL	9
¿POR QUÉ PROBAR DIFERENTES DENSIDADES DE CRIANZA?	9
¿POR QUÉ PROBAR DIFERENTES VOLÚMENES DE TANQUE?	10
RESULTADOS Y CONCLUSIONES	11
ANOMALÍAS DEL ESPLACNOCRÁNEO.....	11
DESVIACIONES DEL EJE Y VÉRTEBRAS ANÓMALAS.....	12
COMENTARIOS CONCLUYENTES	14
LIST OF ABBREVIATIONS	15
CHAPTER 1: GENERAL INTRODUCTION.....	16
AIM OF PHD THESIS	18
WHY TEST DIFFERENT REARING DENSITIES?	18
WHY TEST DIFFERENT TANK VOLUMES?	19
CHAPTER 2. CASE STUDY 1: DISTINGUISHING THE IMPACTS OF REARING DENSITY VERSUS TANK VOLUME ON THE SKELETAL QUALITY AND DEVELOPMENT OF GILTHEAD SEABREAM (SPARUS AURATA) DURING THE HATCHERY PHASE	20
1. INTRODUCTION.....	20
2. MATERIALS AND METHODS	22
2.1 <i>Experimental Rearing</i>	22
2.2 <i>Sampling</i>	23
2.3 <i>Post-rearing measurements</i>	24
2.3.1 <i>Anatomical analyses</i>	24
2.4 <i>Ordination models and statistical analyses</i>	24
2.5 <i>MicroCT and Histology</i>	25
3. RESULTS.....	25
3.1 <i>Post-harvest data</i>	25
3.2 <i>Anatomical analyses</i>	26
3.2.1 <i>Meristic counts</i>	26
3.2.2 <i>Skeletal anomalies</i>	26
3.2.3 <i>Head anomalies</i>	30
3.2.4 <i>Anomalies affecting the vertebral column and associated elements</i>	31
3.3 <i>Ordination Models and Statistical Tests</i>	38
3.3.1 <i>Chi-squared and Post hoc Tests</i>	38
3.3.2 <i>Correspondence Analysis</i>	39
3.3.3 <i>Principal Component Analysis</i>	41
4. DISCUSSION.....	42
4.1 <i>Effects on survival rate</i>	42
4.2 <i>Length and weight</i>	43
4.3 <i>Skeletal anomalies</i>	44
4.4 <i>Hypothesis: high density alters gilthead seabream behavior</i>	45
4.5 <i>Economic considerations</i>	47
CHAPTER 3: CASE STUDY 2: EFFECTS OF TANK VOLUMES VERSUS STOCKING DENSITIES ON THE SKELETAL QUALITY DURING THE PREONGROWING PHASE OF GILTHEAD SEABREAM (SPARUS AURATA).....	47
1. INTRODUCTION.....	47
2. MATERIALS AND METHODS	49
2.1 <i>Rearing setup</i>	49

2.2 <i>Sampling and Post-rearing analyses</i>	50
2.3 <i>Skeletal anomalies survey</i>	51
2.4 <i>Geometric morphometrics</i>	52
3. RESULTS	54
3.1 <i>Performance indicators</i>	54
3.2 <i>Anatomical analyses</i>	55
3.2 <i>Cephalic skeleton</i>	57
3.3 <i>Axial skeleton</i>	59
3.4 <i>Correspondence Analysis</i>	68
3.5 <i>Principal components analysis</i>	68
3.6 <i>Geometric morphometrics</i>	70
3.7 <i>Calcium-phosphorous mineral content</i>	73
4. DISCUSSION	74
CHAPTER 4. CONNECTING THE PIECES OF THE PUZZLE	78
1. ANOMALIES OF THE SPLANCHNOCRANIUM	81
2. AXIS DEVIATIONS AND ANOMALOUS VERTEBRAE	82
3. CONCLUSIVE REMARKS	84
ACKNOWLEDGMENTS	84
REFERENCES	86
SUPPLEMENTARY MATERIALS	102

Summary in English

Recent profitability issues within the European aquaculture industry coupled with a pervasive push towards a blue economy focusing on long-term environmental and economic sustainability has resulted in the necessity for the aquaculture industry to revamp their practices and focus on cutting internal costs while simultaneously improving product quality. Due to the undeniably negative impacts of skeletal deformities in farmed fish (welfare issues, economic losses, and consumer perception), ameliorating the morphological quality by reducing the development of skeletal deformities is of paramount importance. Feasibility and straightforward solutions which can be quickly adapted and applied in commercial farms offers an ideal opportunity for European farmers to improve the quality of their fish. Until now, previous studies have primarily focused on comparing morphological quality based on different rearing strategies such as low stocking densities in large volumes *versus* high stocking densities in smaller volumes. The work done in this thesis attempts to reconcile the effects of the different rearing strategies in order to individuate which between available swimming space (volume) and crowding effects (stocking density) is the main driver in eliciting skeletal deformities in gilthead seabream.

This thesis consists of work investigating the stocking density and rearing volumes and their respective impacts on skeletal elements and deformities in the commercial species (Gilthead seabream, *Sparus aurata*). In case study 1 (CS1) seabream were reared from eggs up to early juveniles (60 dph) (hatchery phase) and in case study 2 (CS2) from ~8.7g up to the sub-adult (~55g) stage (pre-ongrowing phase) in commercial-scale aquaculture conditions. The design envisaged to test the effects at an industrial scale of larger (1000 L) and smaller (500 L) tank volumes on seabream, stocked at 3 different densities (1. low density; 2. intermediate density; and 3. high density). Performative data was taken from fish from both of the case studies prior to the fish being anatomically assessed for skeletal quality by means of visual inspection on whole mount stained (CS1) and radiographed specimens (CS2). Recorded data was analyzed by various multivariate statistical analyses to investigate differences among the conditions.

The results from the thesis indicated that high stocking density is the primary driver inciting stunted growth and a greater presence of skeletal anomalies in the vertebral column and head in both the hatchery and pre-ongrowing phases. Furthermore, increased tank volume/swimming space did reduce the presence of anomalies affecting the jaws in both studies. Several potential hypotheses to explain the results found are presented in the discussion sections.

Riassunto in italiano / Summary in Italian

I recenti problemi di redditività all'interno dell'industria dell'acquacoltura europea, insieme a una spinta pervasiva verso un'economia blu incentrata sulla sostenibilità ambientale ed economica a lungo termine, hanno portato l'industria dell'acquacoltura alla necessità di rinnovare le proprie pratiche e concentrarsi sulla riduzione dei costi interni migliorando allo stesso tempo la qualità del prodotto. A causa degli impatti innegabilmente negativi delle deformità scheletriche nei pesci d'allevamento (problemi di benessere, perdite economiche e percezione dei consumatori), è di fondamentale importanza migliorare la qualità morfologica riducendo lo sviluppo di deformità scheletriche. La fattibilità e le soluzioni semplici che possono essere rapidamente adattate e

applicate negli allevamenti commerciali offrono un'opportunità ideale per gli agricoltori europei di migliorare la qualità del loro pesce. Finora, studi precedenti si sono concentrati principalmente sul confronto della qualità morfologica sulla base di diverse strategie di allevamento come densità di allevamento basse in grandi volumi rispetto a densità di allevamento elevate in volumi più piccoli. Il lavoro svolto in questa tesi tenta di conciliare gli effetti delle diverse strategie di allevamento al fine di individuare quale tra lo spazio disponibile per il nuoto (volume) e gli effetti di affollamento (densità di allevamento) è il principale fattore determinante nell'indurre deformità scheletriche nell'orata.

Questa tesi consiste in un lavoro di studio della densità di allevamento e dei volumi di allevamento e dei rispettivi impatti sugli elementi scheletrici e sulle deformità nelle specie commerciali (Orata, *Sparus aurata*). Nel caso di studio 1 (CS1) le orate sono state allevate da uova fino a giovani precoci (60 dph) (fase di incubazione) e nel caso di studio 2 (CS2) da ~ 8,7 g fino allo stadio subadulto (~ 55 g) (pre-fase di crescita) in condizioni di acquacoltura su scala commerciale. Il progetto prevedeva di testare gli effetti su scala industriale di volumi del serbatoio maggiori (1000 L) e più piccoli (500 L) sull'orata, stoccati a 3 diverse densità (1. bassa densità; 2. densità intermedia; e 3. alta densità). I dati performativi sono stati prelevati dal pesce da entrambi i casi di studio prima che il pesce fosse valutato anatomicamente per la qualità scheletrica mediante ispezione visiva su campioni colorati (CS1) e radiografati (CS2) a montatura intera. I dati registrati sono stati analizzati da varie analisi statistiche multivariate per indagare le differenze tra le condizioni.

I risultati della tesi hanno indicato che l'elevata densità di allevamento è il fattore principale che incita alla crescita stentata e una maggiore presenza di anomalie scheletriche nella colonna vertebrale e nella testa sia nella fase di incubazione che in quella pre-crescita. Inoltre, l'aumento del volume della vasca/dello spazio per nuotare ha ridotto la presenza di anomalie che interessano le mascelle in entrambi gli studi. Diverse potenziali ipotesi per spiegare i risultati trovati sono presentate nelle sezioni di discussione.

Resumen extendido en español / Extended Summary in Spanish

La demanda mundial de productos del mar ha ido en aumento y se ha más que duplicado desde la década de 1960 hasta 2017, pasando de 5,2 kg/cápita a más de 19,4 kg/cápita (FAO, 2020). El aumento del consumo per cápita de pescados y mariscos ha estado en consonancia con el aumento de la población mundial, con un aumento global del consumo de estos productos del 2,4 % anual durante los últimos 20 años (FAO, 2020). Esta tendencia de crecimiento es especialmente frecuente en los países en desarrollo (FAO, 2020). Además, la industria de la acuicultura debe crecer sustancialmente en términos de producción para satisfacer la demanda del mercado y contribuir a aliviar la presión de la pesca sobre las poblaciones silvestres. La dorada (*Sparus aurata*) es una especie muy apreciada y comúnmente cultivada en el Mediterráneo, debido a su apreciado sabor (Pavlidis y Mylonas, 2011).

La producción de dorada, así como de otras especies marinas criadas en acuicultura (de cultivo/ de producción acuícola), generalmente se segmenta en tres fases principales que pueden ser

completadas por dos entidades comerciales separadas o un negocio integrado: la fase de criadero, el preengorde y el engorde. En cada una de estas fases, los acuicultores pueden aplicar diferentes estrategias de crianza para aumentar las ganancias al mejorar la eficiencia de la producción. Estas estrategias incluyen dos tipos de objetivos: por una parte maximizar la utilización de recursos y espacio para obtener una mayor cantidad de pescado, con el objetivo de aumentar la relación entre ingresos y costos, y por otra, mejorar la calidad del producto, aumentando el valor de mercado de los productos finales. Estos dos objetivos dan lugar respectivamente a las estrategias que se conocen generalmente como acuicultura intensiva y semi-intensiva.

La acuicultura intensiva puede utilizar alimentación constante/automatizada, 24 horas de luz, altas densidades de población, mayor tasa de reemplazo de agua y temperaturas de agua más cálidas. Además, en la cría intensiva, los reproductores a menudo se controlan de cerca y se seleccionan por sus rasgos y genes ideales. Durante la temporada de desove, los huevos se recolectan diariamente y los lotes de alta calidad se crían siguiendo protocolos establecidos que están estrictamente vigilados por los criaderos.

La fase de incubación en particular representa un período de tiempo muy sensible en el que los peces experimentan ontogénesis y organogénesis. Por lo tanto, los parámetros ambientales durante esta fase son potentes impulsores para provocar diferentes trayectorias ontogenéticas y deben ajustarse con precisión y monitorearse cuidadosamente. En general, las larvas se alimentan primero con presas monoespecíficas cultivadas que consisten en rotíferos (*Brachionus plicatilis*), seguidas de *Artemia sp.*, y luego se destetan con una microdieta. Una estrategia de cría más frecuente es la tecnología de 'agua verde' en la que se cría fitoplancton dentro del tanque, lo que permite la propagación del zooplancton y, por lo tanto, el mantenimiento de una fuente constante de alimento para las larvas (Papandroulakis et al., 2001). Otros parámetros de crianza varían mucho entre los criaderos y se mantienen en secreto (comunicación personal). Por ejemplo, mientras algunos criaderos aplican el destete temprano (para reducir los altos costos de las presas vivas), otros aumentan el período de destete para evitar grandes mortalidades al cambiar de presas vivas a una microdieta inerte.

Por otro lado, el enfoque semi-intensivo prevé la adopción de la producción extensiva tradicional a la acuicultura moderna mediante la implementación de tanques muy grandes (es decir, 'Grandes Volúmenes' sensu Cataudella et al. (2002) o 'Mesocosmos', sensu Divanach y Kentouri (2000)), ambos junto con bajas densidades, agua verde y, a veces, zooplancton salvaje. El dualismo entre cultivo semi-intensivo e intensivo, basado en la diferencia entre densidades bajas en tanques grandes y densidades altas en tanques más pequeños, se debe a que para producir un número suficiente de alevines, los criaderos que emplean la estrategia semi-intensiva requieren tanques muy grandes para obtener suficientes alevines (criados a bajas densidades) al final de la crianza. Mientras que la acuicultura intensiva requiere un control constante de la calidad del agua y el estado de salud de las larvas y postlarvas, lo que es más factible en tanques más pequeños.

En general, la industria de la acuicultura es altamente competitiva, lucrativa y está impulsada a aplicar estrategias que mejoren su comerciabilidad. Tales estrategias dependen de una multitud de factores, como el tamaño de la granja, su ubicación, los consumidores objetivo y los requisitos

biológicos específicos del ciclo de producción (es decir, el engorde de la dorada durante la temporada de invierno, la eliminación de los piojos de mar parásitos del salmón criado en el mar, etc.).

Un problema biológico particular que presenta una pérdida significativa en el valor de mercado y las ganancias generales es la presencia de deformidades esqueléticas en los peces criados. De hecho, en la Unión Europea las pérdidas económicas por anomalías esqueléticas en la piscicultura se estimaron en unos 50 millones de euros anuales (Hough, 2009); este valor es probablemente el mismo o mayor en la actualidad, ya que el problema ha persistido (Boglione et al., 2013b) y el exceso de sobre-oferta/baja demanda en los últimos años está obligando a los acuicultores a optimizar la eficiencia productiva interna para mejorar sus márgenes de beneficio (Llorente et al., 2020).

Las razones principales de los impactos negativos de las anomalías esqueléticas en los peces son: 1) perjudican el bienestar animal al aumentar el estrés, la susceptibilidad a las enfermedades e incluso ocasionan mortalidad; 2) generan pescado no comercializable, lo que les cuesta dinero a los acuicultores para producir, pero no les da ningún beneficio. Además, en algunos casos, se deben considerar costos adicionales de filetear pescado deformado; 3) influyen en cómo los consumidores y la sociedad ven la industria de la 'acuicultura' en su conjunto (Boglione et al., 2013b), lo que limita la capacidad de los productos producidos por la acuicultura para reemplazar los productos del mar capturados en la naturaleza en los mercados de comercialización estándar. El desarrollo de anomalías esqueléticas es innegablemente complejo y sus causas suelen ser multifactoriales, sinérgicas y específicas de cada etapa de la vida (Russo et al., 2010). Entre la multitud de factores que provocan anomalías esqueléticas se encuentran factores dietéticos/nutricionales como la inanición (Faria et al., 2011), niveles inadecuados de ácidos grasos polinsaturados (EPA y DHA) en las dietas artificiales (Izquierdo, 1996; Roo et al., 2009) u otros desequilibrios dietéticos como los de vitamina A (Fernández et al., 2012; Georga et al. 2011), vitamina D (Haga et al., 2004 (*Paralichthys olivaceus*); Darias et al., 2010) o la proporción adecuada de taurina, vitamina E y vitamina C (Izquierdo et al., 2019, (*Ictalurus punctatus*) Murai et al., 1978; (*Notemigonus crysoleucas*) Chen et al., 2003). Otros factores causales son biológicos, como la insuflación inadecuada de la vejiga natatoria, (Kitajima et al., 1981; Chatain y Dewavrin, 1989; Daoulas et al., 1991; Chatain, 1994) o bioquímicos, como el bajo nivel de oxígeno disuelto (Araújo- Luna et al., 2018) o la presencia de metales tóxicos (Muramoto, 1981; Jezierska et al., 2009; Sfakianakis et al., 2015). Se ha descrito que los factores genéticos están relacionados con anomalías del maxilar superior (Fragkoulis et al., 2018) o con el síndrome LSK (lordosis-escoliosis-cifosis) (Afonso et al., 2000). Además, los factores físicos, como los regímenes de luz (Fjellidal et al., 2004; Blanco-Vives et al., 2010), el color del tanque (Cobcroft et al., 2012), la temperatura del agua (Sfakianakis et al., 2004; Sfakianakis et al. al., 2006; Georgakopoulou et al., 2010; Kourkouta et al., 2021) o las corrientes de agua (Divanach et al., 1997), pueden inducir anomalías esqueléticas. También se ha demostrado que una alta densidad de población provoca una mayor incidencia de deformidades en *Epinephelus marginatus* (Boglione et al., 2009),

Oncorhynchus mykiss (Boglione et al, 2014), *Pagrus pagrus* (Roo et al., 2010); y dorada (Prestinicola et al., 2013).

Evidencias recientes en la literatura han expuesto la alteración del comportamiento de los peces que ocurre en peces criados cuando se modifican factores ambientales particulares (Martini et al., 2021). Diferentes modalidades de natación pueden inducir una actividad diferenciada en las distintas partes de la columna vertebral, provocando la aparición de anomalías esqueléticas. Por ejemplo, la remodelación de los tejidos esqueléticos puede ocurrir en respuesta al aumento de la carga mecánica ejercida por la musculatura axial hipertrófica (Witten y Huysseune, 2009).

Objetivo de la tesis doctoral

Esta tesis doctoral tuvo como objetivo dilucidar los efectos impulsores de la densidad de cría frente al volumen del tanque durante las fases de incubación (Estudio de caso 1) y pre-engorde (Estudio de caso 2) en doradas, con el fin de individualizar cuál de estos dos factores es más efectivo. en la mejora de la calidad del esqueleto.

Los estudios científicos que comparan el efecto de las condiciones de crianza en criaderos sobre las anomalías esqueléticas a menudo se enfocan en estrategias de crianza muy diferentes, como semi-intensiva (mesocosmos sensu Divanach y Kentouri (2000) o Gran Volumen sensu Cataudella et al. (2002): volúmenes de 40- 60 m³ junto con densidades 3-16 larvas/L; Prestinicola et al., 2013) versus crianza intensiva (volúmenes de 0.5-9 m³ junto con densidades ~ 100 larvas/L), con conclusiones que demuestran que las estrategias semi-intensivas rinden más desarrollo osteológico (Roo et al., 2010) con menos anomalías esqueléticas (Boglione et al., 2009) en peces marinos, como los juveniles de dorada (Boglione et al., 2001; Prestinicola et al., 2013). Aunque estos estudios son fundamentales para establecer un estándar para una mejor calidad de producción de pescado, hacen poco para ofrecer a los piscicultores intensivos opciones con las que podrían mejorar su producción y reducir la incidencia de anomalías esqueléticas sin tener que reestructurar su negocio para incorporar los peces más grandes y tanques costosos en espacio. Sin embargo, el efecto del espacio disponible para nadar (a saber, el volumen del tanque) disociado del efecto de la densidad de población queda por dilucidar. Cada uno de estos factores puede actuar como un impulsor individual, como se ilustra en las siguientes secciones a continuación.

¿Por qué probar diferentes densidades de crianza?

Se ha demostrado que la densidad de población modula muchas respuestas en los peces de cultivo. El hacinamiento puede conducir a cambios ambientales multifacéticos que afectan la calidad del agua (Pickering y Stewart, 1984), el oxígeno disponible, la respuesta del sistema inmunitario (Montero et al., 1999b), los patrones de alimentación como el consumo de alimentos (Jorgensen et al., 1993; Wedemeyer, 1997).), el crecimiento de los peces (Holm et al., 1990; Björnsson, 1994; Vijayan y Leatherland, 1988), la tasa de supervivencia (Soderberg y Meade, 1987) y las respuestas conductuales, como el aumento de la agresión (Ellis et al., 2002). Las respuestas de estrés a la alta densidad de población pueden conducir a una multitud de consecuencias fisiológicas posteriores para el organismo. Por ejemplo, los niveles elevados de cortisol en plasma causados por el hacinamiento pueden suprimir la respuesta inmunitaria innata de los organismos (Maule et al.,

1989; Pickering y Pottinger, 1989; Angelidis et al., 1987; Yin et al., 1995; Tort et al., 1996). Se ha demostrado que se genera estrés crónico en la dorada cuando se mantiene en altas densidades de población, lo que provoca niveles más altos de cortisol en plasma, así como una composición alterada de ácidos grasos en el hígado. El hacinamiento parece alterar la respuesta metabólica de los lípidos para satisfacer una mayor demanda energética (Montero et al., 1998). A veces, una mayor densidad de peces puede ser beneficiosa, mejorando las interacciones sociales entre individuos: en condiciones naturales, algunas especies (o etapas de vida, es decir, juveniles) tienden a formar cardúmenes para evadir a los depredadores y beneficiarse de las proezas de la caza.

En lo que respecta a las anomalías esqueléticas, el aumento de la densidad de cría, por ejemplo, puede desencadenar efectos de hacinamiento con la posterior instalación de estrés agudo/crónico y cambios de comportamiento en los peces (Herrera et al., 2014). Se ha informado que mayores pesos y longitudes se correlacionan con densidades más bajas en lugar de más altas en ciertos modelos de peces, como el pez cebra (*Danio rerio*) (Martini et al., 2021) y el medaka (*Oryzias latipes*) (Di Biagio et al., 2022). En lo que respecta a las especies comerciales criadas, varias especies muestran la misma relación inversa entre el aumento de la densidad y estas cualidades negativas de rendimiento durante las etapas postlarvales, como durante el engorde de ~ 271 g de dorada (Araújo-Luna et al., 2018), de ~82g de trucha arcoíris (*Oncorhynchus mykiss*) (Zoccarato et al., 1994) y de preengorde de <2,4g de salmón coho (*O. kisutch*) (Fagerlund et al., 1981). Además, el preengorde de ~ 6,6 g de lubina europea (Papoutsoglou et al., 1998) y el engorde de ~ 53,8 g de trucha alpina (*Salvelinus alpinus*) (Jørgensen et al., 1993) mostraron mejores tasas de crecimiento a densidades de población más altas en lugar de densidades de población más bajas. Estos estudios mejoran la posible existencia de diferencias específicas de especie al responder a densidades variables.

¿Por qué probar diferentes volúmenes de tanque?

Cuando los peces se ven obligados a entrar en un espacio artificial limitado, como un tanque o una jaula, pueden surgir algunas respuestas de estrés fisiológico y efectos conductuales bastante desconocidas también en especies gregarias o en etapas del ciclo vital (Arechavala-Lopez et al., 2020). La diferente autoecología (particularmente con respecto a la explotación del espacio o las interacciones intraespecíficas) de las especies y etapas de vida es desconocida para muchas especies de peces y puede ser ignorada por los piscicultores.

En lo que respecta al volumen del tanque, según nuestro conocimiento, la información disponible sobre los efectos básicos es limitada.

Esta tesis consiste en un trabajo de investigación sobre el impacto de la densidad de población y los volúmenes de cría en los distintos elementos esqueléticos y las deformidades en las especies comerciales (dorada, *Sparus aurata*). En el estudio de caso 1 (CS1), se criaron doradas desde huevos hasta juveniles tempranos (60 dph) (fase de incubación) y en el estudio de caso 2 (CS2) desde ~ 8,7 g hasta la etapa subadulta (~ 55 g) (pre- fase de engorde) en condiciones de acuicultura a escala comercial. El diseño preveía probar los efectos a escala industrial de volúmenes de tanque más grandes (1000 L) y más pequeños (500 L) en dorada, sembrada en 3 densidades diferentes (1.

baja densidad; 2. densidad intermedia; y 3. alta densidad). Los datos de desempeño se tomaron de los peces de ambos estudios de casos antes de evaluar anatómicamente la calidad del esqueleto de los peces mediante una inspección visual en especímenes teñidos (CS1) y radiografiados (CS2) de montaje completo. Los datos registrados se analizaron mediante varios análisis estadísticos multivariados para investigar las diferencias entre las condiciones.

Resultados y Conclusiones

Los resultados de la tesis indicaron que la alta densidad de población es el principal impulsor del retraso en el crecimiento y una mayor presencia de anomalías esqueléticas en la columna vertebral y la cabeza tanto en la fase de incubación como en la fase previa al crecimiento. Además, el aumento del volumen del tanque/espacio para nadar redujo la presencia de anomalías que afectaban a las mandíbulas en ambos estudios. Varias hipótesis potenciales para explicar los resultados encontrados se presentan en las secciones de discusión. El alcance de este trabajo de tesis fue encontrar de manera concluyente una manera de reducir la incidencia de anomalías esqueléticas durante las fases más críticas del ciclo de producción de dorada, no explicar la multitud de procesos por los cuales podrían surgir las anomalías. Sin embargo, se realizaron algunas investigaciones histológicas y la evaluación de la relación Ca:P presente en vértebras lordóticas y no lordóticas con el fin de contribuir al conocimiento básico sobre anomalías esqueléticas en peces marinos criados en cautividad. En los siguientes párrafos, resumiré las principales hipótesis sobre los procesos que conducen a una mayor incidencia de anomalías esplanocraneales y vertebrales especialmente frecuentes y marcadas en condiciones de HD.

Anomalías del esplanocráneo

Con respecto a las anomalías del esplanocráneo encontradas tanto en el criadero como en las fases previas al crecimiento, la crianza de baja densidad resultó ser el factor impulsor para reducir la presencia de anomalías mandibulares y operculares, mientras que los volúmenes más grandes solo mejoraron la reducción de las mordidas inferiores y las anomalías operculares. Además, los resultados obtenidos en CS2 en dorada de pre-engorde, revelaron que estas anomalías aumentaron en frecuencia en individuos en alta densidad con respecto a lo detectado al inicio del experimento (lote T0), así como en comparación con las frecuencias encontradas en los individuos en Tf criados en densidades más bajas. Curiosamente, los resultados obtenidos en CS1 confirman parcialmente lo descrito por Prestinicola et al. (2013) que indicaron que en ambas condiciones semi-intensivas (Grandes Volúmenes y Mesocosmos) solo los huesos con osificación intramembranosa mostraron una incidencia de anomalías consistentemente menor; mientras que se observaron efectos no lineales en los elementos esqueléticos cuyo proceso de osificación requiere un precursor cartilaginoso.

La discrepancia en los efectos observados en huesos (dentario, maxilar, premaxilar, interopércular, opércular, preopércular, subopércular) ubicados en la misma región corporal (cabeza) y que comparten el mismo origen embriológico (cresta neural) y modalidad de osificación (osificación intramembranosa) (Hall, 2005), confirma observaciones previas de que el mismo factor ambiental (es decir, el volumen del tanque) puede inducir diferentes incidencias de anomalías que afectan

elementos esqueléticos del mismo tipo óseo y modo de osificación (Fernández y Gisbert, 2011), en la misma especie y etapa de vida (Boglione et al., 2013b). Esto podría deberse al hecho de que desempeñan diferentes funciones que no están igualmente influenciadas por el volumen variable del tanque o las densidades de población (es decir, morder versus ventilación). En la discusión de CS2 se informa la posibilidad de que las diferencias encontradas entre los tanques de 500L y 1000L podrían deberse a algunas diferencias en la difusión del oxígeno que pueden haber mejorado la eficiencia de la bomba bucal, mitigando así el desarrollo de anomalías operculares en 1000L. Además, la discusión de los datos obtenidos en el CS1 destacó cómo el mayor número de larvas efectivamente sembradas en condiciones de alta densidad podría haber inducido una escolarización más estricta y una mayor competencia por la alimentación, produciendo respuestas de estrés como se describió previamente en doradas criadas de forma intensiva por Montero et al. (1999b), lo que también desencadena una mayor producción de ROS relacionadas con el estrés (Seo et al., 2020). También se ha encontrado que los altos niveles de ROS están correlacionados con un aumento de las deformidades esqueléticas en la dorada (Izquierdo et al., 2019). Posteriormente, teniendo en cuenta las diferencias notificadas anteriormente entre la dorada de los dos ensayos experimentales, solo las causas biomecánicas pueden considerarse como sólidas para explicar el aumento de los maxilares y las anomalías operculares en condiciones de alta densidad y el efecto de mejora en las incidencias de mordida inferior y anomalías operculares detectadas en volúmenes más grandes.

Desviaciones del eje y vértebras anómalas

Con respecto a la columna vertebral, nuevamente hubo similitudes en las respuestas a la densidad basadas en los resultados de los CS 1 y 2. Sin embargo, los resultados de la fase de incubación revelaron que las anomalías de la columna vertebral se debieron principalmente a fusiones en el centro caudal, mientras que las anomalías que afectaban a todo el eje eran relativamente raras. Por el contrario, en la fase de precrecimiento, los peces resultaron ser afectados principalmente por lordosis hemal que involucra varios centros. Además, durante el ciclo de incubación (CS1), a diferencia de las anomalías mandibulares, las condiciones de mayor volumen de 1000 L redujeron la frecuencia de fusiones del centro caudal, fusiones parciales y hemivértebras. Este resultado confirma las menores ocurrencias de anomalías de los cuerpos vertebrales caudales observadas por Prestinicola et al. (2013) en condiciones semi-intensivas, con respecto a los lotes hermanos criados intensivamente. Sin embargo, este efecto no fue confirmado en el CS2, representando nuevamente una respuesta diferencial al mismo estímulo ambiental en elementos esqueléticos ubicados en la misma región esquelética (vértebras caudales), compartiendo el mismo origen embriológico (mesodermo paraxial embrionario), así como modalidad de osificación (osificación pericondral). Una vez más, estos resultados discrepantes entre CS1 y CS2 parecen estar relacionados con las diferentes etapas de la vida y con el diferente papel que desempeñan las vértebras caudales y la aleta en las diferentes etapas de la vida (larvas frente a juveniles posteriores / subadultos). En particular, la aleta caudal es la primera aleta en diferenciarse durante la ontogénesis larval. Desde el punto de vista morfofuncional, la aleta caudal es extremadamente importante para las larvas cuyo pequeño tamaño y baja velocidad de natación les permite operar en los regímenes

hidrodinámicos que son intermedios entre los regímenes de flujo viscoso e inercial. Mientras que en juveniles y subadultos, el cambio hacia el régimen de flujo inercial completo se establece de forma permanente. Gracias a su baja inercia corporal y su rápido sistema neuromuscular, la explotación de altas frecuencias de batido de cola a las velocidades más rápidas permite a las larvas compensar las limitaciones relacionadas con el tamaño de la velocidad de natación (Voosenek et al., 2018). En consecuencia, el pedúnculo caudal y la aleta están mucho más tensos durante la fase larvaria en comparación con las fases posteriores de la vida. Además, de acuerdo con el microambiente al que se enfrentan las larvas, pueden surgir y predominar diferentes comportamientos de natación. Las larvas tienen dos tipos principales de respuestas de inicio rápido, el inicio C y el inicio S, que difieren en sus movimientos iniciales, involucrando la actividad de los músculos de diferentes regiones del cuerpo y probablemente diferentes circuitos neuronales motores (Voosenek et al., 2018). En el inicio en C, el pez escapa de un estímulo transgresor doblando el cuerpo para formar una forma de C antes de la propulsión hacia adelante. Esto se caracteriza por actividad muscular unilateral simultánea a lo largo de un lado del cuerpo con poca o ninguna actividad en el otro lado del cuerpo. Por el contrario, el patrón motor de inicio en S tiene actividad muscular simultánea en la región rostral del cuerpo y una flexión activa posterior en la región caudal en la dirección opuesta. Esto permite que la larva curve el cuerpo en forma de S, seguido de una curva en forma de L, restringida en gran medida al pedúnculo caudal y la aleta. El comportamiento de inicio en S se utiliza como respuesta de escape y en ataques de depredadores, caracterizando los movimientos provocados por la cola hacia la presa o alejándose de un depredador (Hale, 2002). En densidades de población más altas, el comportamiento agresivo y de escape podría estimularse más que en condiciones de baja densidad. Por lo tanto, se podría esperar un mayor requerimiento de los músculos caudales con el consiguiente aumento de la tensión en los cuerpos vertebrales caudales en condiciones de alta densidad.

Al considerar las desviaciones del eje, la lordosis afectó a los peces en frecuencias relativamente altas según los resultados de CS2 y se encontraron diferencias estadísticamente significativas entre las condiciones de densidad, pero no entre los diferentes volúmenes. Por otro lado, la lordosis y la cifosis estuvieron ausentes en la fase de incubación de CS1, como era de esperar teniendo en cuenta que las desviaciones del eje vertebral surgen solo en los juveniles mayores (Loizides et al., 2014). Como se discutió en el Capítulo 3 (CS2), las doradas criadas en alta densidad exhibieron comportamientos de escolarización más fuertes (Arechavala-Lopez et al., 2020) con una sobrecarga de estímulos mecanosensoriales (Anneser et al., 2020) y mayor actividad física general (Bégout y Lagardère, 1995). Este estado, junto con el mayor caudal de agua, indujo un comportamiento de nado alterado en la dorada criada en densidades más altas, caracterizado por una actividad más frenética y una diversidad reducida en los tipos de movimiento (Sánchez-Muros et al., 2017), con el consiguiente mayor y/o diferente requerimiento de las diferentes regiones del sistema musculoesquelético. Se reconoce que este ejercicio muscular adicional induce efectos positivos sobre la osteogénesis, la mineralización y la recuperación de la degeneración esquelética; sin embargo, la actividad frenética y los tipos de movimiento reducidos parecen haber provocado concomitantemente una respuesta de modelado anormal de los elementos esqueléticos (Suniaga et

al., 2018). El control mecano-sensorial del modelado y remodelado esquelético ejercido por la musculatura esquelética parece ser ostensiblemente el principal proceso que produce la reducción de la presencia de peces lordóticos detectados en doradas LD1000 y LD500 respecto a T0 y la mayor aparición de individuos lordóticos en condiciones de HD.

Esta hipótesis se valida aún más por el hallazgo de que no hubo diferencias significativas en la relación calcio-fósforo (Ca: P) entre el centro lordótico y el centro normal. Sin embargo, observamos una mayor variabilidad (aunque no estadísticamente significativa) en las proporciones Ca:P en las vértebras lordóticas. Esto podría sugerir que las diferencias en la carga mecánica ejercida por la masa muscular sobre el hueso subyacente pueden iniciar respuestas no lineales que estimulan los canales de compuerta de voltaje, que posteriormente modulan la relación entre las concentraciones de iones internos y externos que pueden afectar el Ca: P en los huesos (Yavropoulou y Yovos, 2016). De todos modos, la estructura interna de los centros vertebrales deformados parece estar sana aparte de la evidente forma de flexión, como lo confirman los análisis histológicos.

Comentarios concluyentes

Futuras investigaciones en las que los análisis anatómicos/histológicos/estadísticos (como los realizados en este trabajo) deberían ir acompañados de datos bioquímicos, conductuales y epigenéticos en un número de muestras consistentemente alto. Los estudios adicionales son de suma importancia para dilucidar una imagen completa de los procesos que modulan los tipos y frecuencias de anomalías esqueléticas cuando solo se altera un parámetro físico (es decir, la densidad).

List of abbreviations

CS1: Case study 1
CS2: Case study 2
HD: High density
MD: Medium density
LD: Low density
SL: Standard length
TL: Total length
DW: Dry weight
WW: Wet weight
Dph: Days post hatching
RM: Raw Matrix
BM: Binary Matrix
T₀: Initial batch of gilthead seabream before being placed in experimental conditions
T_F: Final samples of gilthead seabream after being reared in experimental conditions
K: Fulton's condition factor
CA: Correspondence analysis
PCA: Principal components analysis
TPS: Thin plate spline
LDA: Linear discriminate analysis
FCR: Food conversion ratio
SGR: Standard growth rate
KS: Kolmogrov-smirnov test for equal distribution

Chapter 1: General Introduction

Global seafood demand has been on the rise and has more than doubled from the 1960s to 2017, from 5.2 kg/capita to over 19.4 kg/capita (FAO, 2020). Augmentation in seafood per capita consumption has been in line with an increasing global population, with a global increase in seafood consumption of 2.4%/year over the last 20 years, this growth trend is especially prevalent in developing countries (FAO, 2020). Additionally, the aquaculture industry must grow substantially in terms of production to meet the market demand as well as contribute to alleviating the pressure of fisheries on wild fish stocks. Gilthead seabream (*Sparus aurata*) is a highly valued and commonly farmed species in the Mediterranean, due to its appreciated flavor (Pavlidis and Mylonas, 2011).

The production of gilthead seabream, as well as other marine reared species, is usually segmented in three main principal phases which can be completed by two separate business entities or an integrated business: the hatchery phase, the pre-ongrowing, and the grow-out. In each of these phases, farmers may apply different rearing strategies in order to increase profits by improving production efficiency. These strategies are often employed to maximize resource and space utilization to obtain a greater quantity of fish (with the aims of increasing the ratio between revenue and costs) or to improve product quality (increasing the market value of the final products). These two strategies are generally referred to as intensive and semi-intensive aquaculture, respectively. Intensive aquaculture may apply constant/automated feeding, 24 hours of light, high stocking densities, increased water replacement rate, and warmer water temperatures. Additionally, in intensive farming, broodstock are often closely monitored and selected for ideal traits and genes. During the spawning season, eggs are collected daily, and the high-quality batches are reared following set-protocols which are strictly guarded by the hatcheries.

The hatchery phase in particular, represents a very sensitive time period in which fish undergo ontogenesis and organogenesis. Therefore, environmental rearing parameters during this phase are potent drivers for eliciting different ontogenetical trajectories and must be finely-tuned and carefully monitored. In general, the larvae are at first fed cultured monospecific prey consisting of rotifers (*Brachionus plicatilis*), followed by brineshrimp (*Artemia sp.*), they are then weaned to a microdiet. One rearing strategy recently developed is ‘green water’ technology in which phytoplankton is reared inside the tank allowing for the propagation of zooplankton and thus the maintenance of a constant source of nourishment for the larvae (Papandroulakis et al., 2001). Other rearing parameters vary greatly between hatcheries and are kept secret (*personal comm.*). For example, while some hatcheries apply early weaning (to reduce the high costs of live preys), others increase the weaning period to avoid large mortalities when switching from live prey to a pelleted microdiet.

On the other hand, the semi-intensive approach foresees the adoption of traditional extensive production to modern aquaculture by implementing very large tanks (i.e., ‘Large Volumes’ *sensu* Cataudella et al. (2002) or ‘mesocosms’, *sensu* Divanach and Kentouri (2000)) both coupled with low densities, green water, and sometimes wild zooplankton. The dualism between semi-intensive and intensive farming based on the difference between low densities in large tanks and high

densities in smaller tanks is due to the fact that in order to produce a sufficient number of fry, hatcheries employing the semi-intensive strategy require very large tanks in order to obtain enough fry (reared at low densities) by the end of rearing. While, intensive farming requires constant monitoring of water quality and the health status of larvae and post larvae, which is effectively more feasible in smaller tanks.

Overall, the aquaculture industry is highly competitive, often lucrative, and driven to apply strategies which improve their marketability. Such strategies depend on a multitude of factors, such as the farm's size, location, targeted consumers, and the specific biological requirements of the production cycle (*i.e.*, gilthead seabream ongrowing during the winter season, removing parasitic sea lice from salmon reared in sea-cages, or genetic selection for broodstock management that produce an elevated number of fast-growth fish).

One particular biological issue that presents a significant loss in market value and overall profits is the presence of skeletal deformities in reared fish. In fact, in the European Union economic losses due to skeletal anomalies in fish farming were estimated to be around 50 million euros annually (Hough, 2009); this value is likely the same or higher nowadays, as the problem has persisted (Boglione et al., 2013b) and oversupply/low demand in recent years is forcing farmers to optimize internal production efficiency to improve their profit margins (Llorente et al., 2020). The principal reasons for the negative impacts of skeletal anomalies on fish are: 1) they impair animal welfare by increasing stress, susceptibility to disease, and even causing mortality; 2) they generate unmarketable fish, thus costing farmers money to produce but not yielding them any returns. Additionally, in some cases, extra costs must be considered for filleting deformed fish; 3) they influence how consumers and society view the industry of 'aquaculture' as a whole (Boglione et al., 2013b), thereby, limiting the ability of aquaculture-produced products to replace wild-caught seafood in standard consumer markets.

The development of skeletal anomalies is undeniably complex, and their causes are often multifactorial, synergistic and life stage-specific (Russo et al., 2010). Among the multitude of factors that cause skeletal anomalies there are dietary/nutritional factors such as starvation (Faria et al., 2011), inadequate levels of unsaturated fatty acids (EPA and DHA) in the artificial diets (Izquierdo, 1996; Roo et al., 2009) or other dietary imbalances of vitamin A (Fernández et al., 2012; Georga et al. 2011), vitamin D (Haga et al., 2004 (*Paralichthys olivaceus*); Darias et al., 2010), proper ratio of taurine, vitamin E, and vitamin C, impacting oxidative processes, growth, survival, and skeletal anomalies (Izquierdo et al., 2019, (*Ictalurus punctatus*) Murai et al., 1978; (*Notemigonus crysoleucas*) Chen et al., 2003). Other causative factors are biological (improper inflation of the swim bladder), (Kitajima et al., 1981; Chatain and Dewavrin, 1989; Daoulas et al., 1991; Chatain, 1994) or biochemical factors such as low dissolved oxygen (Araújo-Luna et al., 2018) and toxic metals (Muramoto, 1981; Jezierska et al., 2009; Sfakianakis et al., 2015). Genetic factors have been described to be connected to anomalies of the upper jaw (Fragkoulis et al., 2018) or to LSK syndrome (lordosis-scoliosis-kyphosis) (Afonso et al., 2000). Additionally, physical factors, such as light regimes (Fjellidal et al., 2004; Blanco-Vives et al., 2010), tank color (Cobcroft et al., 2012), water temperature (Sfakianakis et al., 2004; Sfakianakis et al., 2006; Georgakopoulou

et al., 2010; Kourkouta et al., 2021), and water currents (Divanach et al., 1997) have been described to be able to induce skeletal anomalies. High stocking density has also been demonstrated to provoke greater incidences of deformities in *Epinephelus marginatus* (Boglione et al., 2009), *Oncorhynchus mykiss* (Boglione et al., 2014), *Pagrus pagrus* (Roo et al., 2010); and gilthead seabream (Prestinicola et al., 2013).

Recent evidences in literature have expounded on the modification of fish behavior occurring in reared fish when particular environmental factors are modified (Martini et al., 2021). Different swimming modalities may induce differentiated activity in the different parts of the vertebral column, provoking the onset of skeletal anomalies. For example, remodeling of skeletal tissues can occur in response to increased mechanical loading exerted by hypertrophic axial musculature (Witten and Huysseune, 2009).

Aim of PhD thesis

This PhD thesis was aimed at elucidating the driving effects of rearing density *versus* tank volume during the hatchery (Case Study 1) and pre-ongrowing (Case Study 2) phases on gilthead seabream, in order to individuate which of these two factors is more effective in ameliorating the skeletal quality.

Scientific studies comparing the effect of hatchery rearing conditions on skeletal anomalies often focus on vastly different rearing strategies, such as semi-intensive (mesocosms *sensu* Divanach and Kentouri (2000) or Large Volume *sensu* Cataudella et al. (2002): volumes of 40-60 m³ coupled with densities 3-16 larvae/L; Prestinicola et al., 2013) *versus* intensive rearing (volumes of 0.5-9 m³ coupled with densities ~100 larvae/L), with conclusions demonstrating that semi-intensive strategies yield more advanced osteological development (Roo et al., 2010) with fewer skeletal anomalies (Boglione et al., 2009) in marine fish, such as gilthead seabream juveniles (Boglione et al., 2001; Prestinicola et al., 2013). Although these studies are paramount to setting a standard for a better fish production quality, they do little to offer intensive farmers any options with which they could ameliorate their production and reduce the incidence of skeletal anomalies without having to restructure their business to incorporate the larger and space-costly tanks. Nevertheless, the effect of the available swimming space (*viz.* tank volume) disassociated from the effect of stocking density remains to be elucidated. Each of these factors can act as an individual driver as illustrated in the following sections below.

Why test different rearing densities?

Stocking density has been shown to modulate many responses in cultured fish. Crowding can lead to multifaceted environmental changes impacting water quality (Pickering and Stewart, 1984), available oxygen, immune system response (Montero et al., 1999b), feeding patterns such as feed intake (Jorgensen et al., 1993; Wedemeyer, 1997), fish growth (Holm et al., 1990; Björnsson, 1994; Vijayan and Leatherland, 1988), survival rate (Soderberg and Meade, 1987), and behavioral responses such as increased aggression (Ellis et al., 2002). The stress responses to high stocking density can lead to a multitude of down-the-line physiological consequences for the organism. For instance, heightened plasma cortisol levels caused by crowding can suppress the organisms' innate

immune response (Maule et al., 1989; Pickering and Pottinger, 1989, Angelidis et al., 1987; Yin et al., 1995; Tort et al., 1996). Gilthead seabream have been shown to generate chronic stress when held at high stocking densities, eliciting higher plasma cortisol levels as well as altered liver fatty acid composition. Overcrowding seems to alter the lipid metabolic response in order to meet a higher energetic demand (Montero et al., 1998). Sometimes, higher density of fish may be beneficial, improving the social interactions between individuals: in natural conditions, some species (or life stages, i.e. juveniles) tend to form schools or shoals in order to evade predators and benefit from hunting exploits.

As far as skeletal anomalies are concerned, the augmentation of the rearing density, for instance, may trigger crowding effects with subsequent instauration of acute/chronical stress and behavioral changes in fish (Herrera et al., 2014). Greater weights and lengths have been reported to be correlated with lower rather than higher densities in certain fish models, such as zebrafish (*Danio rerio*) (Martini et al., 2021) and medaka (*Oryzias latipes*) (Di Biagio et al., 2022). As far as reared commercial species are concerned, several species exhibit the same inverse relationship between increasing density and these negative performative qualities during the post-larval stages, such as during the on-growing of ~271g gilthead seabream (Araújo-Luna et al., 2018), of ~82g rainbow trout (*Oncorhynchus mykiss*) (Zoccarato et al., 1994) and of pre-on-growing of < 2.4g coho salmon (*O. kisutch*) (Fagerlund et al., 1981). Additionally, pre-on-growing of ~6.6g European seabass (Papoutsoglou et al., 1998), and on-growing of ~53.8g arctic char (*Salvelinus alpinus*) (Jørgensen et al., 1993) showed improved growth rates at higher stocking densities rather than at lower ones. These studies enhance the possible existence of species-specific differences in responding to varying densities.

Why test different tank volumes?

When fish are forced into a limited artificial space such as a tank or a cage, some physiologic stress responses and quite unknown behavioral effects may arise also in gregarious species or life-stages (Arechavala-Lopez et al., 2020). The different autoecology (particularly regarding space exploitation or intraspecific interactions) of species and life stages is unknown for many fish species and may be disregarded by the farmers.

As far as tank volume is concerned, to our knowledge limited information is available on the effects in reared fish. In Large Volume tanks, many natural larval behaviors (i.e., schooling, predatory activity, escape reactions, hierarchy instauration, etc.) have been observed in gilthead seabream larvae, post-larvae, and juveniles (Boglione pers. com.), facilitated by larger space availability. Additionally, in large tanks, it is expected that cannibalism and feeding competition are reduced when compared smaller tanks. When Large Volumes are equipped with special radial air-lifters that allowed hydrodynamic laminar flow to be generated preventing the generation of vortices (Prestinicola et al., 2013), fish have the availability to choose among different hydrodynamic areas of the tank (i.e., slower along the walls, faster towards the center of the tank) where to rest, feed, or swim, according to their preference. Some of the behavior defined as “anomalous” (excessive stillness or frenetic activity; Etscheidt, 1992) or unnatural body positions (Furevik et al., 1993) have not been observed in aquaculture settings. It should be taken into

consideration that reared fish species, particularly marine species, are not yet ‘domesticated’, contrary to terrestrial farmed vertebrates. In aquaculture, environmental conditions tend to be pragmatically simplified and standardized, without considering many species-specificities generated throughout a long evolutionary process. Consequently, the chance to exhibit natural species- or life stage-specific behavior is expected to reduce the stress of farming conditions, with respect to more trivialized (i.e., small tank) regimes. Additionally, in large tanks, it is expected that cannibalism and competition for feed are lower than in smaller tanks.

In order to obtain sound results that could be directly applicable to the farmers, the experimental rearing was carried out at a pilot scale as proof of concept for future studies/operations facilitating upscaling production. Consequently, this strategy offers the advantage of rearing a very large number of fish in tanks and not in aquaria (such as those used for experimental studies), following the standard procedures routinely applied in commercial farms. This study tested the effect of rearing on skeletal anomalies’ onset in a larger volume of 1000L (1m³) and in a smaller volume of 500L (0.5m³), that are tanks size widely spread in aquaculture farming. The experimental densities were chosen on the basis of the farmers’ requests.

Chapter 2. Case study 1: Distinguishing the impacts of rearing density versus tank volume on the skeletal quality and development of gilthead seabream (*Sparus aurata*) during the hatchery phase

1. Introduction

The production of gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) together make up the second most important aquaculture industry in the European Union (STECF, 2018). However, data from 2017 and 2018 have revealed increased production while the profitability from seabream and seabass production has experienced some decline due to high production costs and oversupply (Llorente et al., 2020). Therefore, a focus on increasing the production value rather than increasing production quantity could offer a sustainable solution to improve profitability and adjust for long-term environmental and economic goals in the EU (STECF, 2018; Llorente et al., 2020).

Due to high levels of mortality and undesirable fry quality, the hatchery phase (eggs to juveniles, 5-10 mg) remains to be the predominant bottleneck in marine fish culture (Hamre et al., 2013), including gilthead seabream fry production. Although hatchery managers have been improving production for many years, several challenges remain open. One of the primary factors leading to undesirable fry quality is the prevalence of skeletal anomalies which impact the morphological quality and the wellbeing of the fish, while also damaging the consumers’ perception of aquaculture products (Boglione et al., 2013b). Elucidating some of the causative factors has led to ameliorated skeletal quality in larviculture, i.e., genetic factors (Afonso et al., 2000) and proper nutritional requirements (Izquierdo et al., 2010). However, production facilities still produce fish with a significant presence of skeletal anomalies (Boglione et al., 2013b). When considering the overall economic impact of skeletal anomalies on the aquaculture industry, the Federation of

European Aquaculture Producers (FEAP) estimated in 2009 an annual cost of 50 million euros for EU farmers (Hough, 2009).

Scientific studies comparing the effect of hatchery rearing conditions on skeletal anomalies often focus on vastly different rearing strategies, such as semi-intensive (mesocosms *sensu* Divanach and Kentouri (2000) or Large Volume *sensu* Cataudella et al. (2002): volumes of 40-60 m³ coupled with densities of 3-16 larvae/L; Prestinicola et al., 2013) *versus* intensive rearing (volumes of 0.5-9 m³ coupled with densities of ~100 larvae/L), with conclusions demonstrating that semi-intensive strategies yield more advanced osteological development (Roo et al., 2010) with fewer skeletal anomalies (Boglione et al., 2009) in marine fish, such as gilthead seabream juveniles (Boglione et al., 2001; Prestinicola et al., 2013). Although these studies are paramount to setting a standard for better fish production quality, they do little to offer intensive farmers any options with which they could ameliorate their production and reduce the incidence of skeletal anomalies without having to restructure their business to incorporate the larger and space-costly tanks. Nevertheless, the effect of the available swimming space (*viz.* tank volume) disassociated from the effect of stocking density remains to be elucidated. Each of these factors can act as an individual driver: the augmentation of the rearing density, for instance, may trigger crowding effects with subsequent instauration of acute/chronical stress and behavioral changes in fish. Greater weights and lengths have been reported to be correlated with lower rather than higher densities in certain fish models, such as zebrafish (*Danio rerio*) (Martini et al., 2021) and medaka (*Oryzias latipes*) (Di Biagio et al., 2022). As far as reared commercial species are concerned, some species exhibit the same inverse relationship between increasing density and these performative features during the post-larval stages. A few examples from the literature found that ~271g gilthead seabream (Araújo-Luna et al., 2018), ~82g rainbow trout (*Oncorhynchus mykiss*) (Zoccarato et al., 1994) and of pre-ongrowing < 2.4g coho salmon (*O. kisutch*) (Fagerlund et al, 1981), reported negative effects on growth when the rearing densities were too high. While on the other hand, pre-ongrowing of ~6.6g European seabass (Papoutsoglou et al., 1998), and on-growing of ~53.8g arctic char (*Salvelinus alpinus*) (Jørgensen et al., 1993) showed improved growth rates at higher stocking densities rather than at lower ones. These studies enhance the possible existence of species-specific differences in responding to varying densities.

As far as tank volume is concerned, to our knowledge limited information is available on the effects in reared fish. In Large Volume tanks, many of the natural larval behaviorism (i.e., schooling, predatory activity, escape reactions, hierarchy instauration, etc.) have been observed in gilthead seabream larvae, post-larval, and juveniles (Boglione *pers. com.*), facilitated by the larger space availability. Furthermore, in large tanks, fish have the availability to choose among different hydrodynamic areas of the tank (i.e., slower along the walls, faster towards the center of the tank) where to rest, feed, or swim, accordingly to their preference. Some of the behavior defined as “anomalous” (excessive stillness or frenetic activity; Etscheidt, 1992) or unnatural body positions (Furevik et al., 1993) have never been observed. It should be taken into consideration that reared fish species, particularly marine species, are not yet ‘domesticated’, contrary to terrestrial farmed vertebrates. In aquaculture, environmental conditions tend to be pragmatically simplified and

standardized, without considering many species-specificities generated throughout a long evolutionary process. Consequently, the chance to exhibit natural species- or life stage-specific behavior is expected to reduce the stress of farming conditions, with respect to more trivialized (i.e., small tank) regimes. Additionally, in large tanks, it is expected that cannibalism and competition for feed are lower than in smaller tanks.

Behavior, and in particular swimming modality, exerts well known effects on muscle growth. According to the swimming modalities, some axial muscles may result to be more stimulated than others, determining a differential mechanical loading on underlying vertebrae. The mechanical loading exerted by muscles induces and controls bone formation and mineralization in fish as well in terrestrial vertebrates (Hall and Witten, 2018; Suniaga et al., 2018; Witten et al. 2006).

In this scenario, this study aims at elucidating the driving effects of rearing density *versus* tank volume during the hatchery phase on gilthead seabream in order to individuate which of the two plays a greater impact in ameliorating the skeletal quality. The goal of this study was to discriminate the effects on skeletal anomalies onset, occurrence, and types on gilthead seabream from hatching up to the juvenile stage between two different tank volumes (1000L vs 500L), and among three different densities, chosen on the basis of the farmers' requests. In order to obtain sound results that could be directly applicable to the farmers, the experimental rearing was carried out at a pilot scale as proof of concept for future studies/operations facilitating the upscaling of production. Consequently, this strategy offers the advantage of rearing a very large number of fish (initial number of larvae = 625,000) in tanks and not in aquaria (such as those used for experimental studies), following the standard procedures routinely applied in commercial farms.

2. Materials and Methods

2.1 Experimental Rearing

Animal experiments were performed according to the European Union Directive (2010/63/EU) and Spanish legislation (Royal Decree 53/2013) on the protection of animals for scientific purposes at the ECOAQUA Institute, University of Las Palmas of Gran Canaria (Canary Islands, Spain).

The experiment was carried out with gilthead seabream reared from eggs up to an early juvenile stage (60 days-post-hatching, hereafter denoted: dph). Eggs were obtained from natural spawns from the gilthead seabream broodstock at Grupo de Investigación en Acuicultura (GIA) (Supplementary Tab. 2.1) from the ECOAQUA Institute. Eggs were collected and incubated overnight, held in a mesh net in an incubating tank with a renovation of 25% seawater/h. Eggs were then transferred into the experimental tanks (April 10, 2019) and overstocked with respect to the average hatching rate from that broodstock group (~97.5%), in order to have the following initial densities: Low Density (LD, 25 larvae/L); Medium Density (MD, 125 larvae/L); High Density (HD, 250 larvae/L). Each density condition was tested in one cylindrical 1000L (1m³) and one cylindroconical 500L (0.5m³) fiberglass tanks. The resulting six experimental conditions are hereafter referred to as LD1000, MD1000, HD1000, LD500, MD500, and HD500. The tanks were supplied with continuous running filtered seawater via a flow-through system in which water entered from the center of the basin of the tanks and exited through a mesh-covered outlet filter.

Siphoning the tank bottom and cleaning of the mesh outlet covers was done daily. A subsample of same-batch eggs was maintained separately in order to check the effective hatching rate and the survival rate at 3 dph (calculated according to the method described by Ferosekhan et al., (2020)). Photoperiod was based on natural light during the springtime months of late April to late June in the Canary Islands. Natural seawater was filtered and pumped into the system at a steady rate of 15% per hour until the eggs had hatched; it was then reduced to 5% per hour up to the first feeding upon depletion of the yolk sac, and slowly increased up to 25% replacement per hour (which equated to 4.16 L/min in the 1000L conditions and 2.08 L/min in the 500L conditions). Every morning at 8 am, 1L of phytoplankton (*Nannochloropsis* sp., containing $3-4 \times 10^7$ cells/mL) was poured directly into the inlet of each tank for every 100L of tank volume. The concentrations in the tanks of enriched (Origreen and Orione, Skretting) rotifers (*Brachionus plicatilis*) were checked several times throughout the day by taking water samples to determine the present rotifer density and maintained at 10 individuals/mL by adding rotifers when necessary. Upon the seabream larvae reaching an average size of 4.5mm (14 dph), enriched (Origreen and Orione, Skretting) *Artemia salina* nauplii and metanauplii were also added to the tank twice per day, at an initial density of 0.5 individuals/mL of seawater, up to 31 dph, at which point the concentration was augmented to 1 individual/mL of seawater. Starting at 25 dph, fish began to be weaned with a Gemma microdiet dry feed (Skretting) and fed *ad libitum* hourly during the daylight hours. Throughout experimental rearing, tanks had an average water temperature of $22.4 \pm 1.0^\circ\text{C}$ (Supplementary Fig. 1a); and the average dissolved oxygen was 5.4 ± 0.6 mg/L, regularly monitored in order to maintain a saturation level of $70.0 \pm 7.3\%$ (Supplementary Fig. 2.1b). Seabream were reared up to ~60 dph (SL>16mm) to be sure that the complete skeletal mineralization had occurred (Faustino and Power, 1998).

Due to an overnight power outage and subsequent limited inlet flow in some of the tanks, reduced oxygen concentration led to the total tank failure of HD1000 and LD500 on 22/04/2019 and necessitated the restocking of both of those tanks as well as adding two comparable tanks with the same densities and differing volumes: so, a second trial of HD1000, LD500, HD500, and LD1000 was started on 26/04/2019 following the exact same protocol described for Trial 1. The HD500 condition from Trial 2 later crashed on 05/05/2019 due to another power outage incident, however the original conditions from Trial 1 (T1) along with the conditions from Trial 2 (T2) (except for HD500 T2) all succeeded. Due to the absence of significant differences in the survival rate, final size, and weight between LD1000 T1 and LD1000 T2, the collected data were merged and reported as LD1000 (Supplementary Tab. 2.2).

2.2 Sampling

At the end of the experimental rearing, the juveniles were euthanized with an overdose of clove oil (1mL/L of seawater): 100 individuals/condition were photographed and measured for length and weight; another 300 individuals/condition were fixed (1.5% paraformaldehyde PFA, 1.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4), then dehydrated after 24 hours following a series of graded ethanol and stored in 70% ethanol for subsequent anatomical analyses.

2.3 Post-rearing measurements

Total length (TL) and standard length (SL) measurements were obtained using a ruler and a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan). Weight measurements were taken by pre-weighing blank slides, then placing 4 seabream individuals per slide which were weighed immediately for the wet weights (WW). The slides (with the seabream) were then dried in the oven and weighed again to calculate the dry weight (DW) (COBOS precision scale, Barcelona, Spain).

2.3.1 Anatomical analyses

Fixed samples (total n. = 949) were whole-mount stained with Alizarin red S (protocol modified from Taylor and Van Dyke, 1985). Mass monitoring of meristic counts and skeletal anomalies was conducted using an AxioZoom V.16 (Zeiss) stereomicroscope.

Data on skeletal anomalies were recorded following an alphanumeric code adapted from Prestinicola et al. (2013) (Supplementary Tab. 2.3).

The analyses were carried out based on the following assumptions: 1) incomplete fused bone elements were counted separately as discrete elements (*i.e.* a fusion of 2 rays that were still discernably separate at some location along the ray were considered for meristic counts as 2 rays); 2) supernumerary bones (additional bones) with normal morphology were not considered to be anomalies and included in the meristic count; conversely, anomalous supernumerary elements were included among anomalies; 3) only clearly and unquestionably identifiable variations in shape were considered as skeletal anomalies.

Skeletal anomaly data were expressed in a raw matrix (RM) and used to calculate the frequencies (%) of each type of anomaly over the total number of anomalies in each group. The RM was subsequently transformed into a binary matrix (BM) which was used to calculate the prevalence (%) of individuals affected by each anomaly type.

The following metrics were calculated for each experimental lot: 1) relative frequency (%) of individuals with at least one anomaly; 2) number of anomaly types observed; 3) malformation index (number of total anomalies/number of malformed individuals); 4) relative frequency (%) of individuals with at least one severe anomaly (anomalies affecting the head, the vertebral axis, or the vertebral centra); 5) relative frequency (%) of observed severe anomalies over the total number of observed anomalies; 6) severe anomalies' load (number of severe anomalies/number of individuals with severe anomalies); 7) frequency (%) of each anomaly type over the total number of anomalies.

2.4 Ordination models and statistical analyses

A Correspondence Analysis (CA; Benzecri, 1973) was performed on a RM constituted of grouped anomalies (grouped RM). The observed anomalies were grouped based on the body region and skeletal type (listed in Supplementary Tab. 2.4). A Principal Component Analysis (PCA) was applied to performance data and some grouped skeletal anomalies, selected on the base of the presence of significant differences in occurrence among the six conditions. The difference in the occurrence of grouped anomalies among the rearing conditions was statistically tested using a Chi-squared test (Pearson, 1900). Statistical analyses and graphs were done using Python 3.8.0.

2.5 MicroCT and Histology

Seventy fixed samples were scanned using a Micro CT Skyscan 1172 (Bruker) at the Indiana University School of Medicine (scan parameters included a 60 kVp X-ray source with a 0.5 mm Al filter, and a final image voxel size of 6-10 μm) and reconstructed using AMIRA (Thermo Fisher Scientific). Four of the scanned samples which were selected for subsequent histological analyses are reported in this study. After rehydration from ethanol, they were decalcified for 4 weeks by submersion in 10% EDTA, 0.5% PFA at 4 °C and embedded in a GMA (glycol methacrylate) resin according to Witten et al. (2001). Sections (5 μm) were cut on a standard rotary microtome (Microm HM360, Marshall Scientific) and stained with toluidine blue.

3. Results

After the effective survival rate at 3 dph was calculated on a subset of larvae from the same spawn, the estimated densities in the experimental tanks at the beginning of the experiment resulted slightly lower than the desired ones. Nevertheless, this affected the fish in all the conditions proportionately, thus maintaining the differences between the LD, MD, and HD lots (the specific numbers are reported in Supplementary Tab. 2.2).

3.1 Post-harvest data

Estimated final survival was greatest in the LD500 condition, with a 29.7% survival of early juveniles at 60dph, followed by the LD1000 condition (10.0%). The other conditions showed lower survival, with the lowest found to be 3.3% in the HD1000 (Supplementary Tab. 2.2).

Final TL, SL, WW, and DW were significantly different among the conditions ($p < 0.05$, Kruskal-Wallis). In particular, the LD1000 rearing condition produced both the longest and the heaviest seabream juveniles, followed by the LD500 condition, while the HD500 condition produced the smallest and most lightweight seabream. The other 3 conditions (MD1000, HD1000, MD500), however, were not significantly different from each other (Fig. 2.1; $p < 0.05$, Kruskal-Wallis, Dunn's *post hoc* with Bonferroni correction).

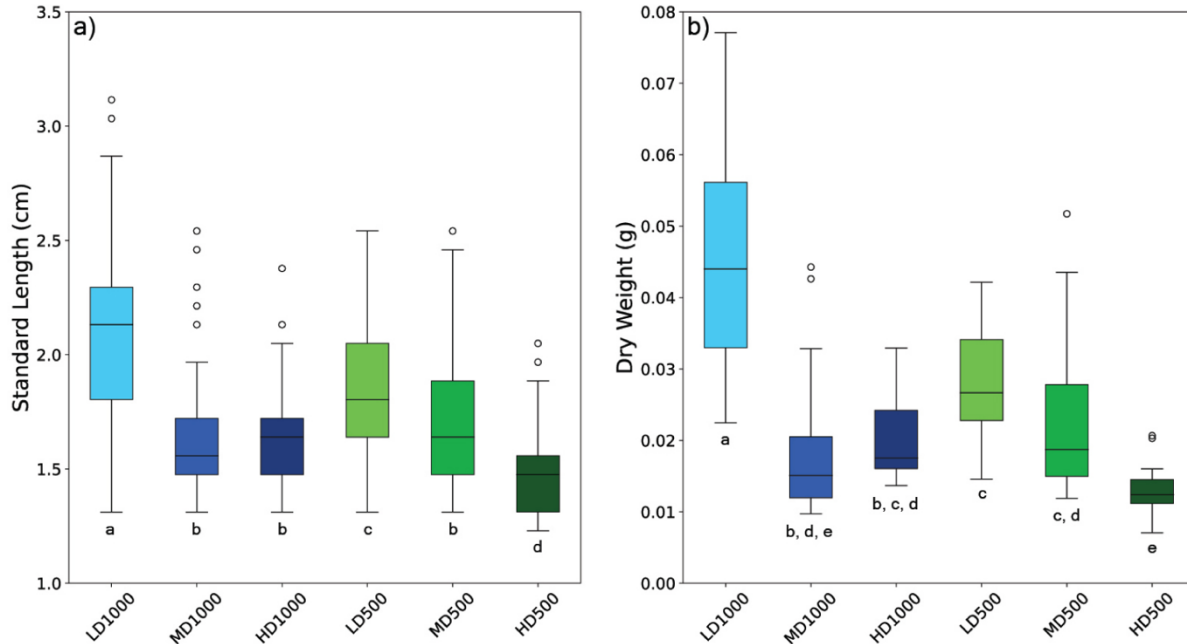


Figure 2.1. (a) Box and whisker plots of SL (cm) and (b) DW (g) of gilthead seabream individuals sampled after 60 dph. Box indicates the central percentile, the line inside the box is the median while the whiskers represent the minimum and maximum values, and the isolated circles represent outliers. Different letters below each box and whisker indicate statistically significant differences between conditions: therefore, conditions which are significantly different do not share any of the same letters ($p < 0.05$, Kruskal-Wallis, Dunn's post hoc with Bonferroni correction).

3.2 Anatomical analyses

3.2.1 Meristic counts

The meristic counts from the experimental lots did not differ greatly from the variations witnessed in wild seabream. Regarding the vertebral body count, in the MD500 lot, the number of 26 vertebrae was found to be the maximum instead of 25 (found in the wild lots), and 23 vertebrae instead of 24, was found to be the minimum count in LD500, LD1000, and HD1000. Interestingly, the presence of extra or fewer vertebral bodies slightly increases in frequency when densities are augmented. Moreover, a recovered vertebral fusion, identifiable by a longer vertebral body, was present in 3 out of 7 juveniles which had a vertebral body count of 23 vertebrae.

3.2.2 Skeletal anomalies

The frequencies (%) of each anomaly type over the total number of the anomalies detected within the group (Supplementary Tab. 2.5) and the frequencies (%) of individuals affected by each anomaly type and are given for each experimental lot (Supplementary Tab. 2.6). This data was used to construct a descriptive metrics table of observed skeletal anomalies (Supplementary Tab. 2.1). Nearly all of the examined seabream had at least one anomaly: the occurrence of individuals with no skeletal anomalies was between 1-2% in all of the lots, except for the HD500 condition in which all of the seabream had at least one anomaly. As many as 64 different anomaly types

were observed. Both LD1000 and LD500 had the lowest malformation index (7) and frequencies of individuals with severe anomalies (28% and 40% respectively, (Tab. 2.1), while the MD1000 and HD500 lots showed the highest malformation index (18). The highest frequencies of individuals with severe anomalies were found in HD1000 (56%), followed by HD500 (52%). The HD500 lot also had the greatest number of anomaly types which were observed (64).

Table 2.1: Descriptive skeletal metrics of the experimental lots

	LD1000	MD1000	HD1000	LD500	MD500	HD500
Number of individuals	197	149	152	149	152	150
Frequency (%) of malformed individuals	98	99	98	96	98	100
Malformation index	7	18	12	7	14	18
Observed anomaly types	53	55	49	52	56	64
Frequency (%) of individuals with severe anomalies	28	42	56	40	49	52

The observed severe anomalies affecting the vertebral column and centra were fusions (Fig. 2.2a), partial fusions (Fig. 2.2b), hemivertebrae (Fig. 2.2c), and localized scoliosis (Fig. 2.2d). In the head (Fig. 2.3), other severe anomalies were found to affect the maxillary, premaxillary (Fig. 2.3b), dentary (Fig. 2.3c and 2.3e), and the opercular plates (Fig. 2.3d and 2.3f).

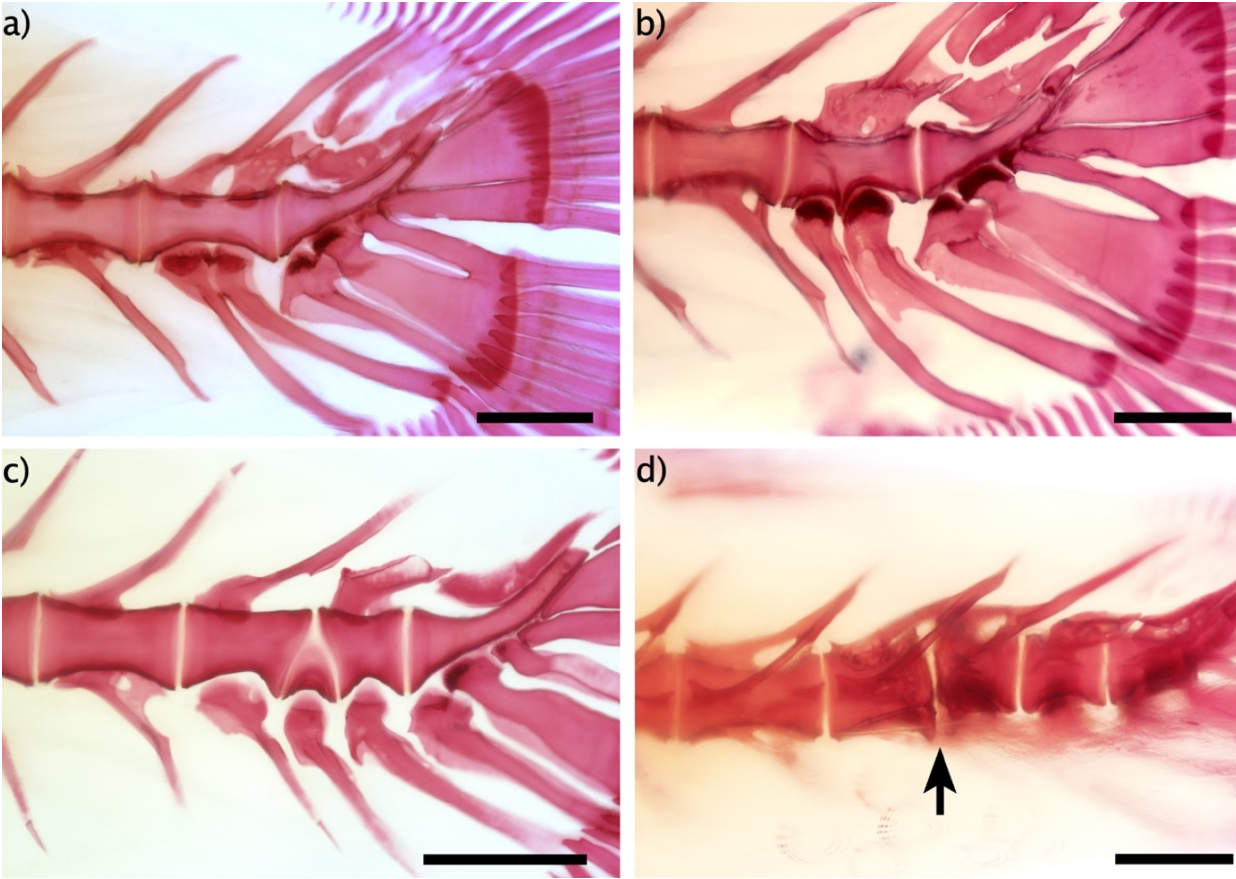


Figure 2.2. (a) Lateral perspective of a caudal vertebra displaying a recovery from a previous fusion of the centra: note the elongated vertebral body, the two neural elements, and the double hemal arches, fusing proximally and diverging distally. (b) Lateral perspective of a caudal vertebra displaying an intermediate step in the recovery process from a fusion: the dorsal region has a remodeled lining, one modified neural arch (created from the fusion between the neural arch of PU3 and the spur of PU2) and a normal centrum lining. In the ventral region, the two fused centra are still detectable in the lining, each with their respective hemal arch. (c) Lateral perspective of a ventrally located hemivertebra, which maintained its hemal arch. (d) Dorsal perspective displaying two vertebrae (arrow) that deviated from the longitudinal orientation of the axis, resulting in a localized scoliosis with deformed centra. Note for reference that a normal vertebral body is displayed in the centra preceding the fused bodies in image a. Scale bar =0.5mm.

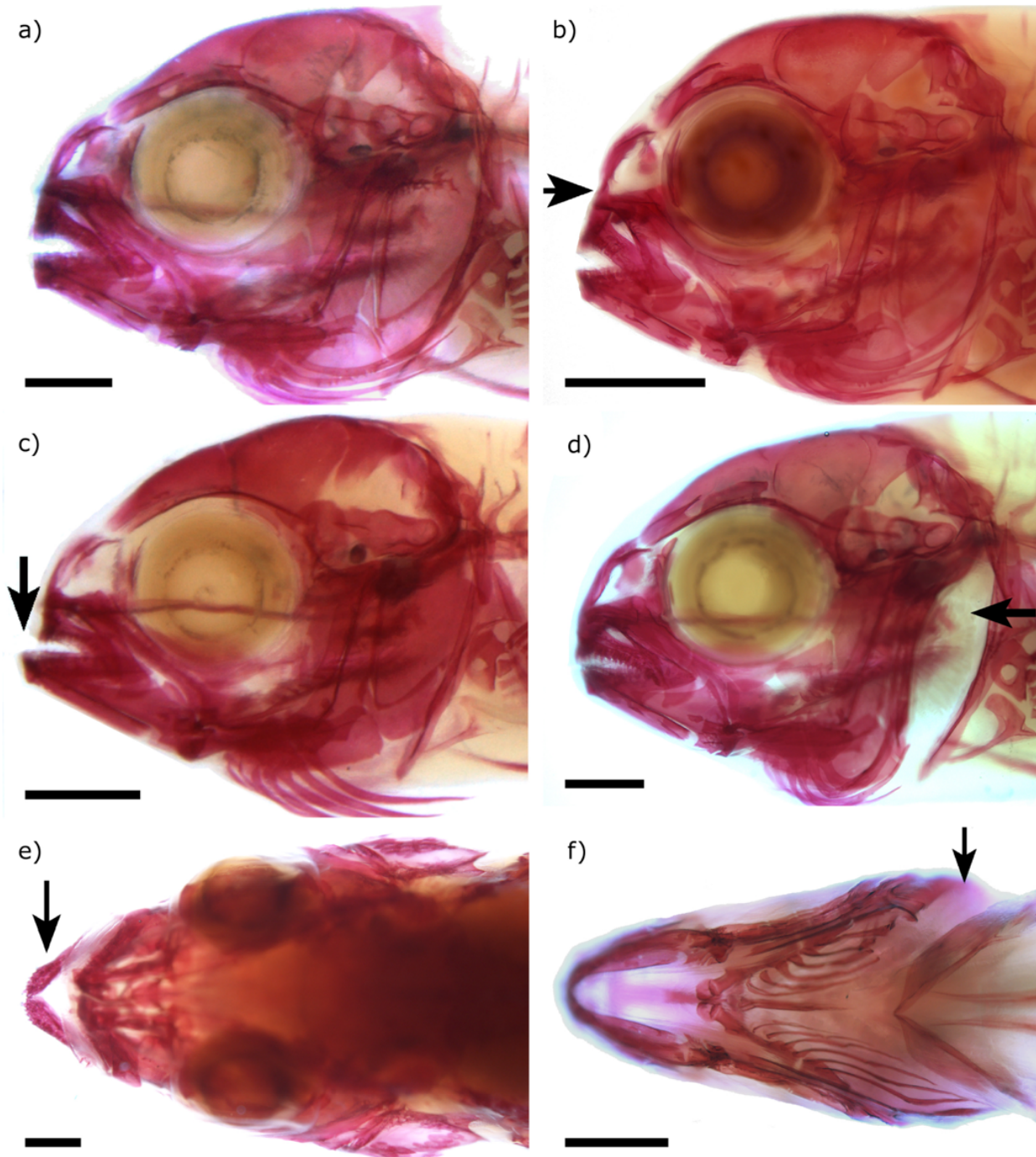


Figure 2.3. (a) Seabream specimen with a normal cranium. (b) Specimen exhibiting a pughead, characterized by a compressed snout (arrow). (c) Lateral (top) and (e) dorsal view (bottom) of a specimen exhibiting an underbite, characterized by a protruding dentary. (d) Lateral (top) and (f) ventral view (bottom) of a specimen with folded left opercular plate (arrow). Scale bar = 1mm.

The following sections report a detailed description of the observed anomalies.

3.2.3 Head anomalies

As many as 57% of the individuals in the HD1000 condition were affected by cranial (neuro- and splanchnocranium) anomalies, while the LD1000 seabream displayed the lowest prevalence (28%) of affected specimen (Fig. 2.4).

In both the 1000L and the 500L conditions, the incidence of seabream exhibiting cranial anomalies increased as the respective stocking density increased (LD<MD<HD) (Fig. 2.4). The prevalence of cranial anomalies among the 500L conditions ranged approximately from 40% to 52% respectively from LD to HD, while the 1000L conditions had a wider range of disparity between the density groups, ranging approximately from 28% to 57% prevalence among individuals from LD to HD (Fig. 2.4).

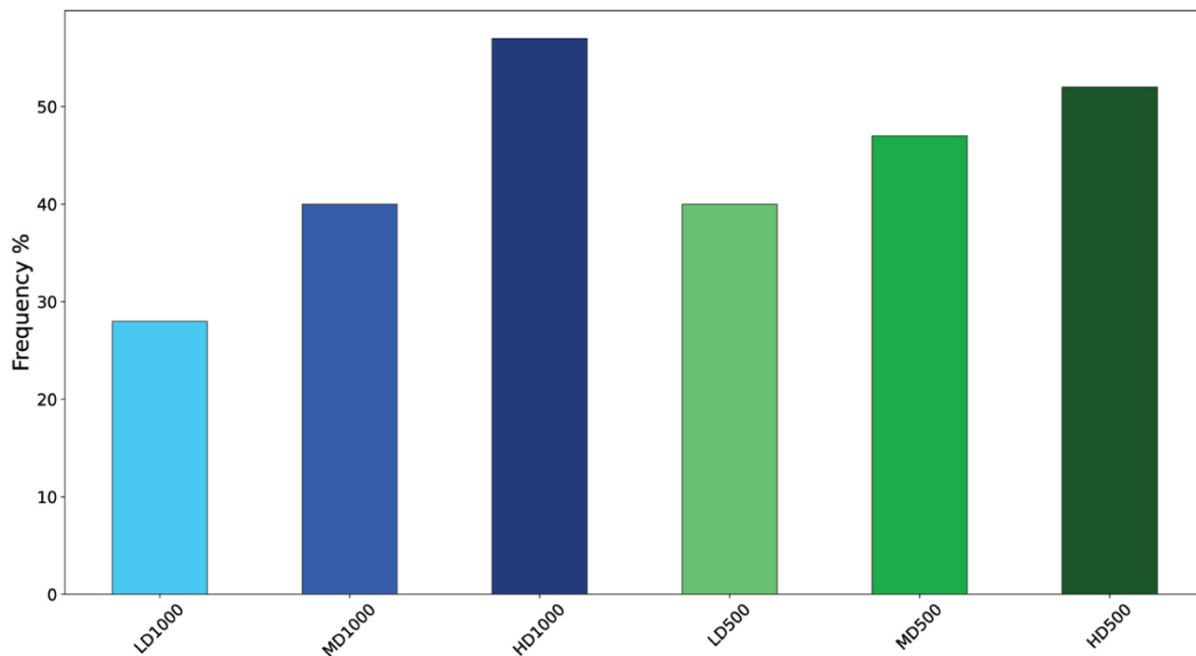


Figure 2.4. Frequencies (%) of individuals affected by anomalies in the neuro- and splanchnocranium, in the experimental lots.

When considering jaw anomalies, a similar trend is exhibited, with an increasing prevalence in affected individuals as the density increases (Fig. 2.5a). Pughead (Fig. 2.3b) and underbite (Fig. 2.3c) were the most frequent of these splanchnocranium anomalies in which the lowest prevalence was found in LD1000 (15%) while the highest was found in HD500 (35%) (Fig. 2.5a). Often, they occurred in association with mesethmoid (neurocranium) anomalies (*i.e.*, in pug-head). A relationship between increased frequency of jaw anomalies and augmentation of density is easily discernable, however there is also a ‘volume effect’ which is evidenced by the higher prevalence of jaw anomalies found in the 500L tanks. The occurrence of individuals with jaw anomalies was lower in the 1000L tank than in the 500L for each tested density: in the LD1000 condition, 15%

of individuals had jaw anomalies vs the 22% found in the LD500, 27% in MD1000 vs 30% in MD500, and 30% in HD1000 vs 35% in HD500.

Opercular plate anomalies were the least prevalent head anomaly in each condition (with frequencies of affected individuals ranging from 2% to 9.2%), although they still exhibited an increasing rate of incidence from LD to HD (Fig. 2.5b). Contrary to jaw anomalies, lower incidences of opercular plate anomalies were found in 500L tanks than in 1000L tanks for both the MD and HD density lots (for MD and HD: 500L<1000L), and no difference in frequency was found between the LD500 and the LD1000 condition (LD500=LD1000, ~2%).

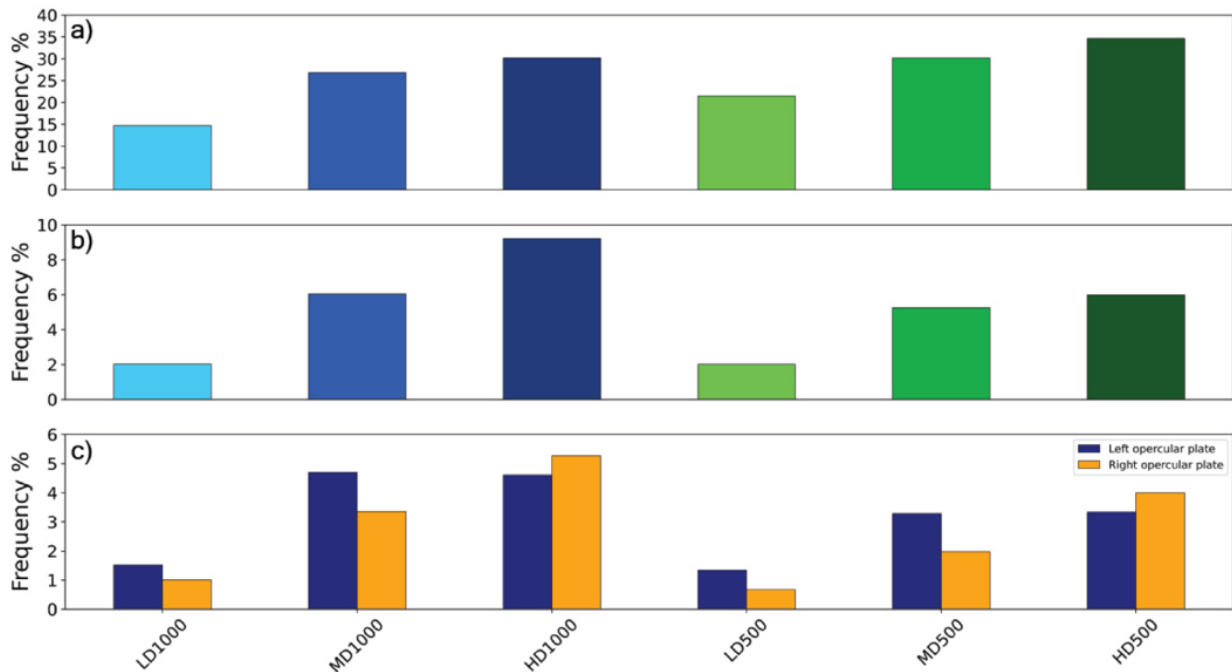


Figure 2.5. Frequency (%) of individuals affected by (a) jaw (pre-maxillary, maxillary, and dentary) anomalies; (b) at least one opercular anomaly; (c) anomalies of the left or right opercular plate, in each experimental lot.

3.2.4 Anomalies affecting the vertebral column and associated elements

The anomalies of the vertebral axis that were considered in this study were kyphosis, lordosis, and scoliosis although neither kyphosis nor lordosis were found and only a few of the specimens exhibited a scoliosis (Fig. 2.6). In general, the most frequently affected regions of the vertebral column were the abdominal and hemal regions (see Prestinicola et al., 2013 for the description of body regions), in which fish with anomalous centra and associated elements were found in all conditions indiscriminately (Fig. 2.7). In particular, the vertebral arches and spines resulted to be more affected by anomalies than the corresponding centra in each region (Fig. 2.7). Individuals affected by anomalous neural arches in the caudal region (anomaly type D5*) were much more common than hemal ones (D6*), although this trend is reversed for the hemal region in which hemal arches (C6*) were more common than neural arches (C5*) (Fig. 2.8).

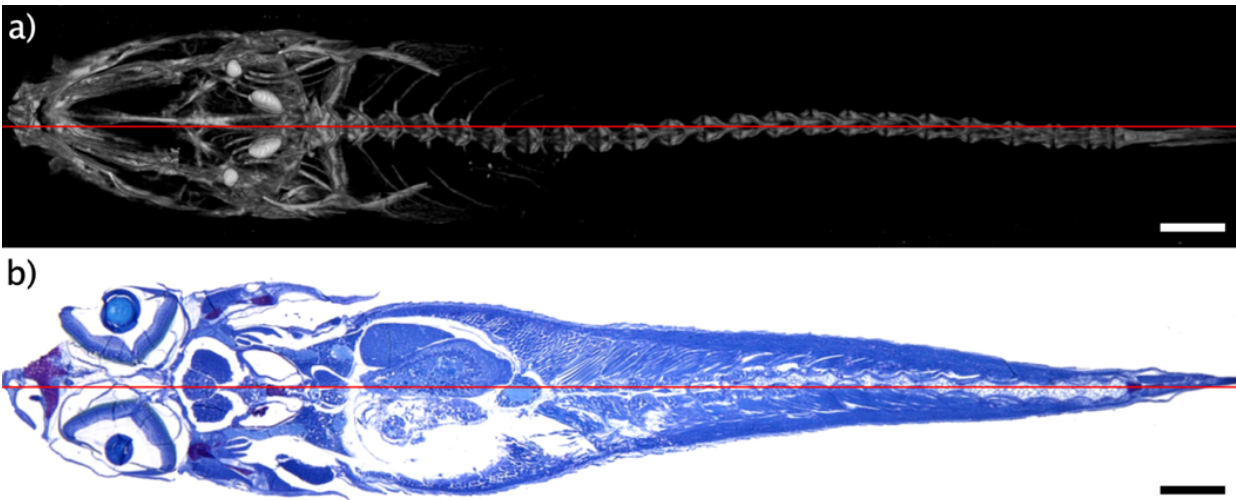


Figure 2.6. (a) Micro-CT reconstruction (dorsal view) of a seabream juvenile (SL = 17.79mm) exhibiting a scoliosis starting in the abdominal region. (b) Tangential section of the same fish, note the difference in the distances between the vertebral bodies and the right and left body edge, which is a distinctive feature of scoliosis. Toluidine blue staining. Scale bar = 1mm.

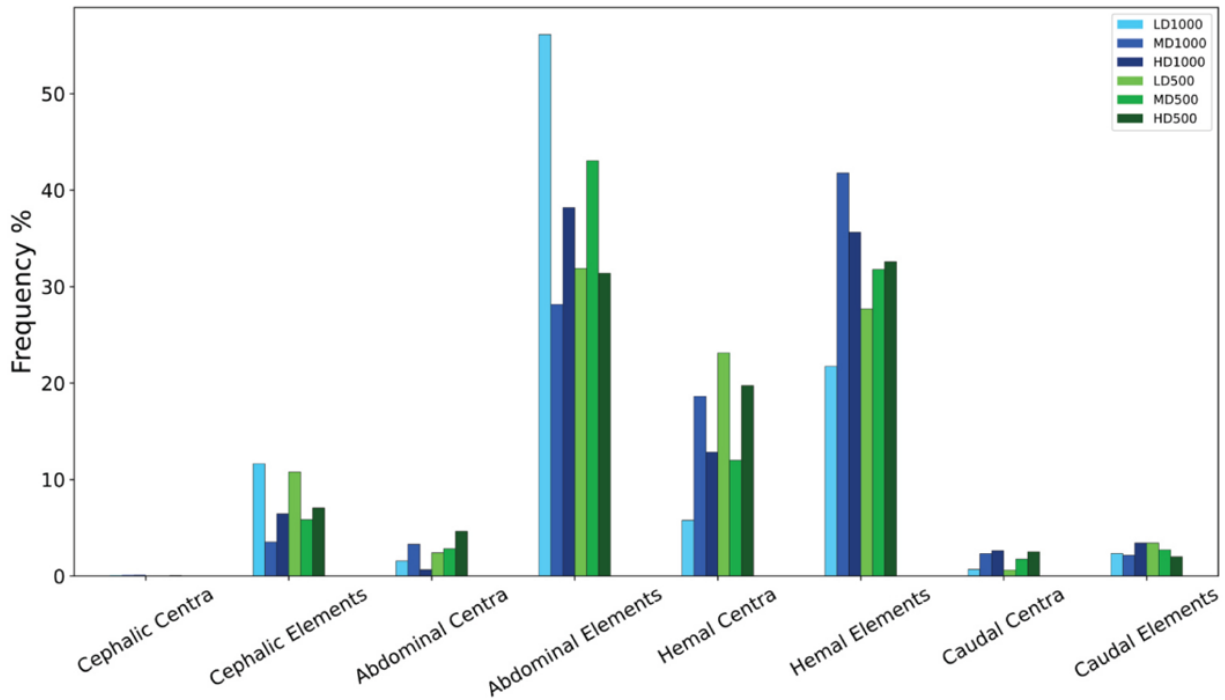


Figure 2.7. Relative frequency (%) of anomalies affecting the vertebrae in different regions of the vertebral column, in seabream reared at each of the experimental conditions. The category 'elements' includes the neural and hemal arches, the ribs, and the spur.

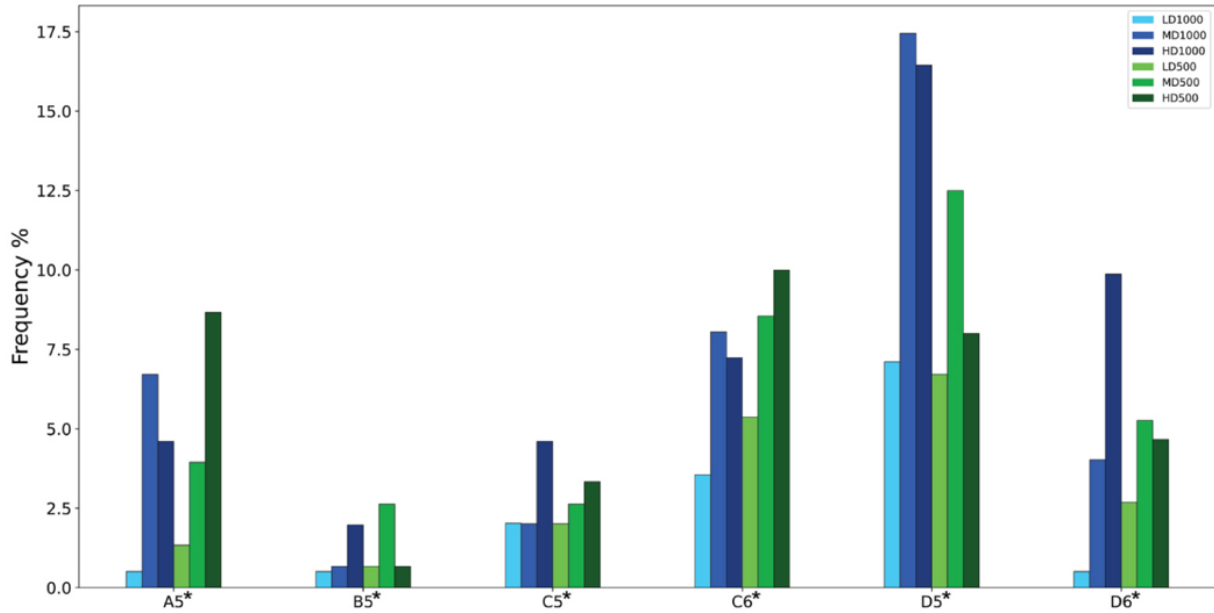


Figure 2.8. Frequency (%) of individuals affected by anomalous arches and spines (5* and 6*), in each experimental condition. A5*=supernumerary or absence of neural element in the cephalic vertebrae region. B5*=supernumerary or absence of neural element in the abdominal vertebrae region. C5*=supernumerary or absence of neural element in the hemal vertebrae region. C6*=supernumerary or absence of hemal element in the hemal vertebrae region. D5*=supernumerary or absence of neural element in the caudal vertebrae region. D6*=supernumerary or absence of hemal element in the caudal vertebrae region.

Some examples of anomalies which were found in the elements attached to the centra are reported in Figs. 2.9 and 2.10. The incidence of arch anomalies was often higher in the MD and HD conditions than in the LD conditions (Fig. 2.8), although no clear ‘volume’ effect could be detected.

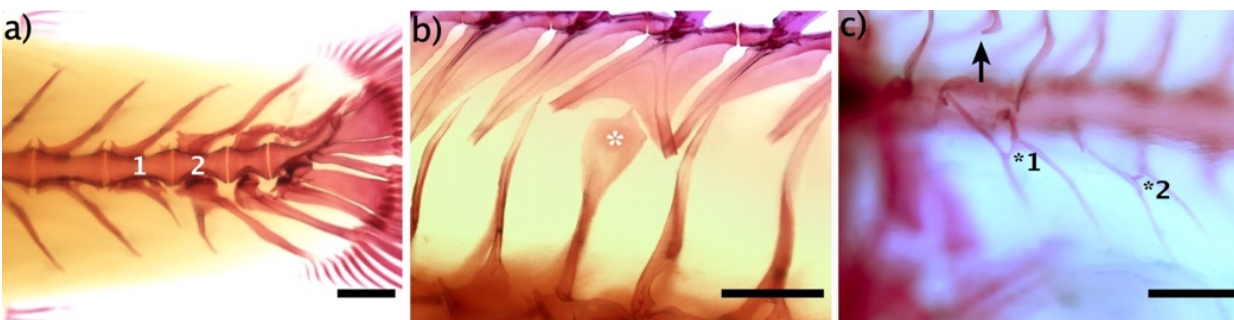


Figure 2.9. Examples of deformed vertebra associated elements. (a) the terminal hemal vertebra (1) shows a hemal arch that lost the spine, which appears to be fused to the hemal arch of the following posterior vertebra (2, first caudal vertebra). (b) Flag-shaped neural spine (asterisk). Note that the above dorsal bifurcated pterygophore seemingly adapted to host the space occupied by the anomalous flag-shaped spine. (c) An abdominal vertebra (arrow) showing a missing segment of the left side of the arch (arrow), and two sets of ribs fused with each other (black asterisks 1 and 2). Note that normal arches are displayed in the preceding centra for images (a) and (b). Scale bar =0.5mm.

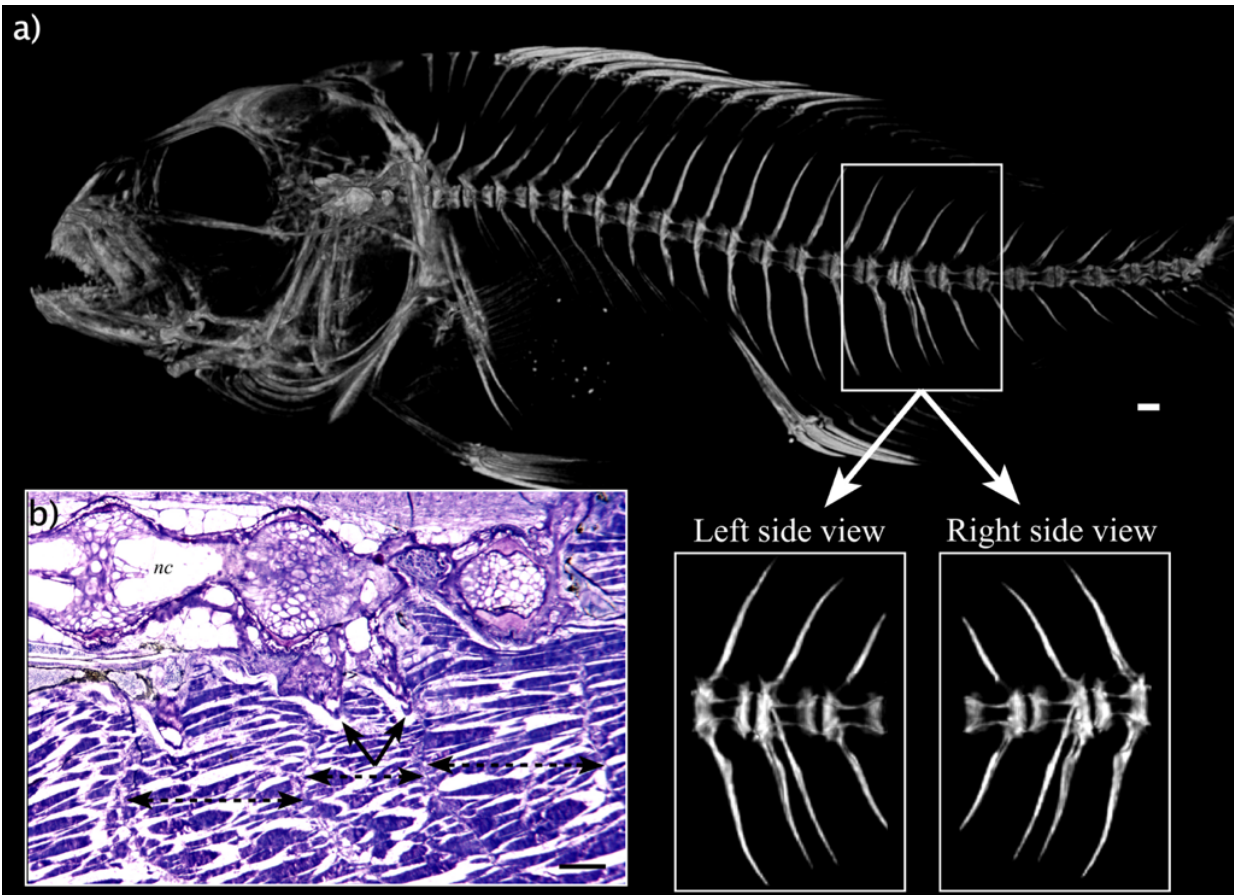


Figure 2.10. (a) Micro-CT reconstruction of seabream specimen (lateral view, SL = 28.0mm) with a duplicated hemal arch element (6*): note that the duplication is present only on the right lateral plane of the hemal vertebral body. (b) Parasagittal section of the vertebrae highlighted in a). Note that this and the preceding centrum are both deformed. Solid black arrows indicate the normal (on the left) and the extranumerary hemal arch element (on the right). Dashed two-way arrows indicate the myosepta delimiting myomeres, whose width changes according to the position of the arches. nc indicates the notochord. Scale bar = 0.5mm.

The frequencies of individuals with severe vertebral anomaly types (*viz.* scoliosis, centra fusion, and hemivertebrae) demonstrate a clear effect of density, with more affected fish in the higher density conditions (Fig. 2.11), following the same trend described for severe anomalies of the splanchnocranium. The HD lots showed the greatest occurrences (~7.2% for both HD1000 and HD500) while the lowest occurrences were found in the LD conditions (~0.7% for LD500 and ~1.0% for LD1000; Fig. 2.11). There was no discernible effect of tank volume on any of the conditions.

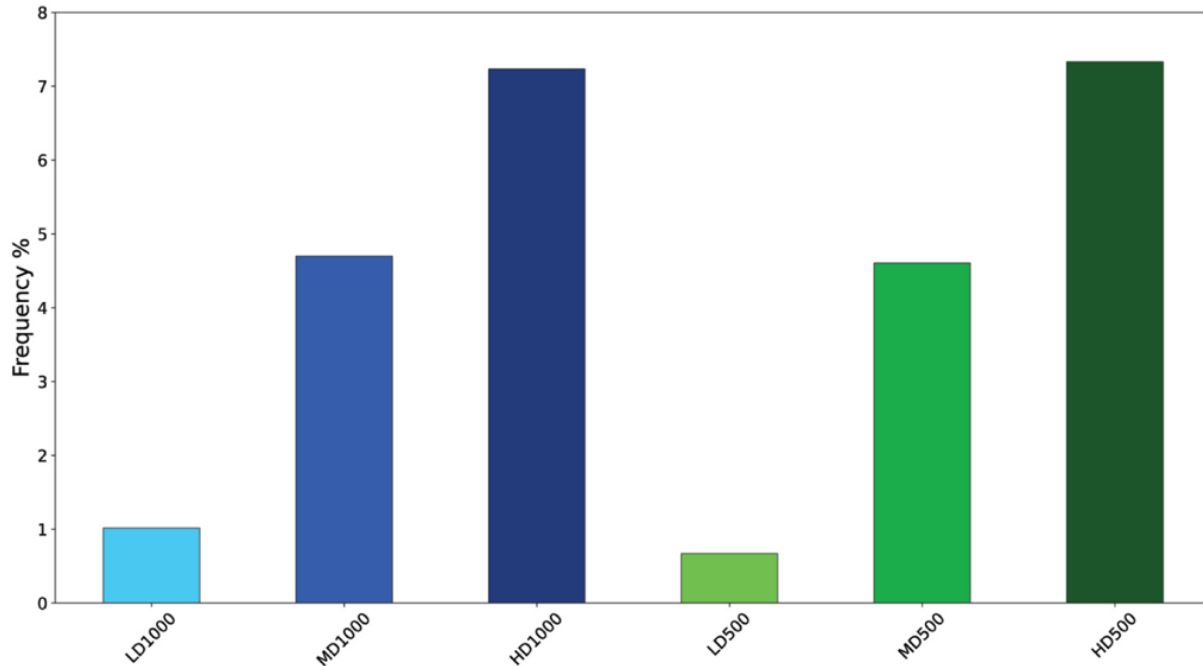


Figure 2.11. Frequency (%) of individuals affected by at least one severe anomaly (scoliosis, centra fusion, and hemivertebrae) in the vertebral column, in each experimental lot. Anomalies defined as ‘severe’ include centra fusions, partial fusions, hemivertebrae, and axis deviation.

The incidence of severe vertebrae anomalies was primarily concentrated in the caudal region in which completely or partially fused centra and hemivertebrae anomalies were witnessed (Fig. 2.12), particularly in the HD500 and HD1000 seabream (Fig. 2.13). In this case, alongside a primary density effect, a secondary volume effect is discernable, with a higher prevalence in the larger volume conditions (Fig. 2.13; LD1000>LD500, MD1000>MD500, HD1000>HD500).

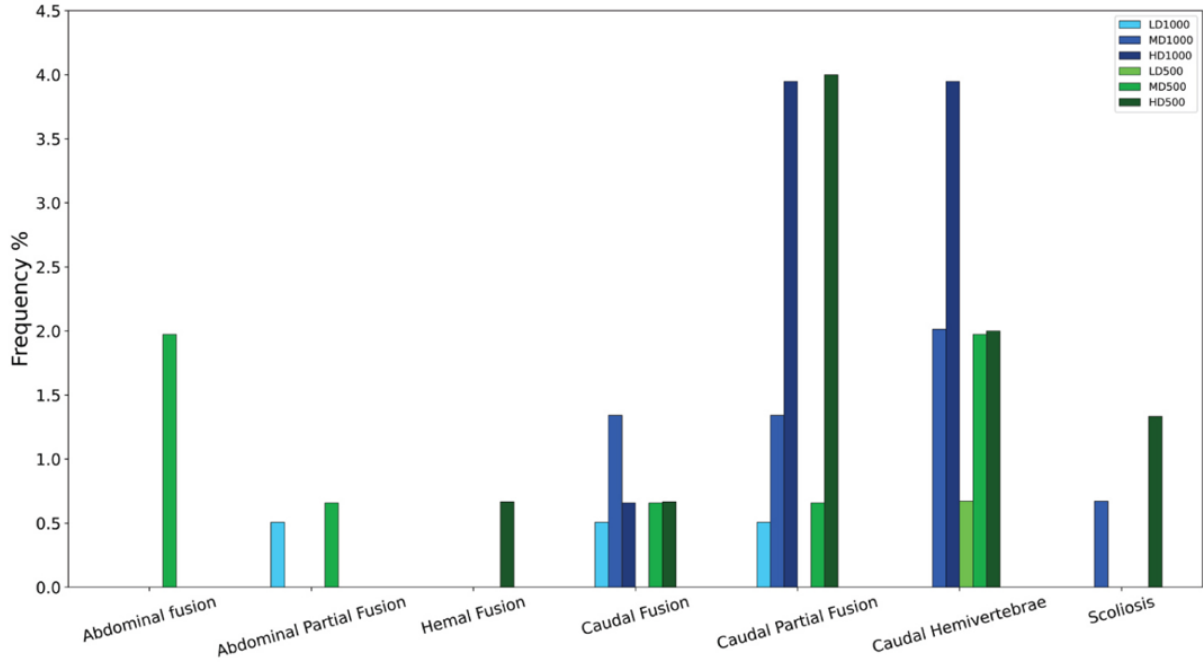


Figure 2.12. Frequency (%) of gilthead seabream affected by each type of severe vertebral anomaly, in each experimental lot.

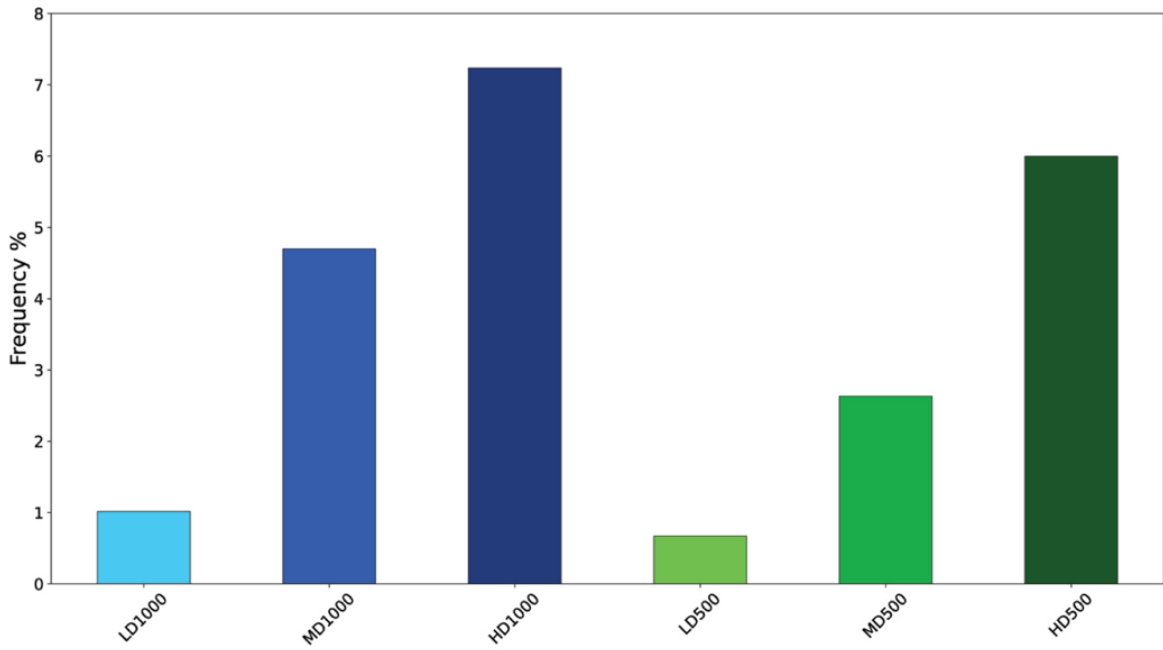


Figure 2.13. Frequency (%) of individuals exhibiting severe anomalies of the caudal vertebrae, in each lot.

The histological section and wholemount stained seabream showed a common occurrence in the fused vertebrae of the caudal region in which the fusion is completed dorsally, however the two fusing centra are still distinguishable on the ventral side of the vertebra (Fig 2.14c). However, the presence of hyaline-rich cartilage of the neural and hemal arches is present both dorsally and ventrally to the fused vertebra, indicating the persistence of the 2 neural and 2 hemal arches not yet completely fused (Fig. 2.14c).

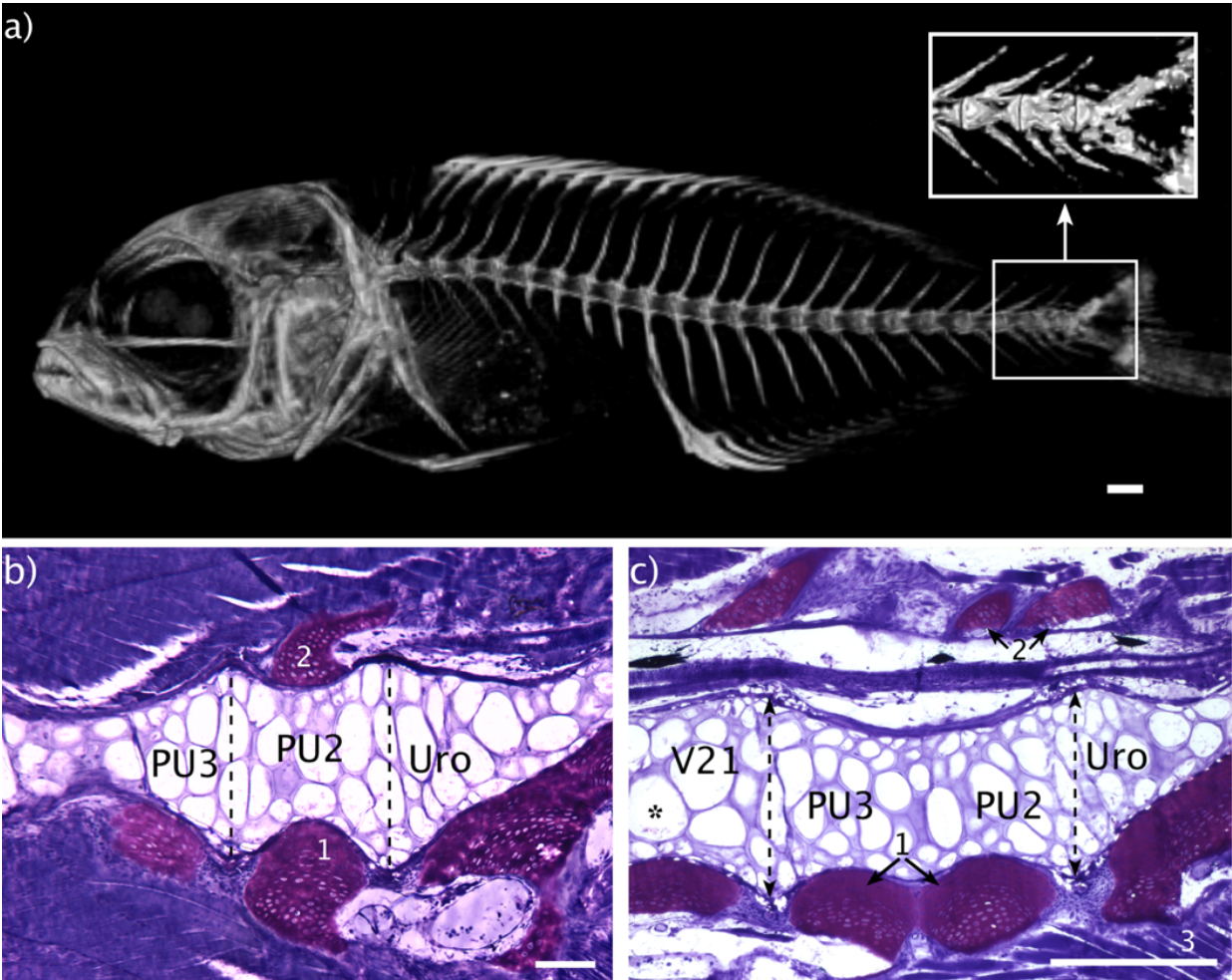


Figure 2.14. (a) Micro-CT reconstruction of seabream (lateral view, SL = 15.43mm) showing a putative fusion of caudal vertebral bodies; (b) normally shaped caudal vertebral bodies of another specimen (SL = 12.98mm); (c) sagittal sections of the vertebrae highlighted in the rectangular selection of the Micro-CT reconstruction. The histological evaluation confirms the presence of a fusion between the two preural vertebrae (PU3 and PU2; compare with (b)). The modeling of the intervertebral ligament between the fusing centra is advanced dorsally. Ventrally, the two vertebral centra are still discernible. V21: last (terminal) hemal vertebra; PU3 and PU2 = preural vertebrae 3 and 2; Uro = urostyle; asterisk = notochord vacuole; 1 = Hyaline cartilage at the base of hemal arches; 2 = Hyaline cartilage of the neural arches; 3 = myomere. (b) and (c): Toluidine blue staining. Scale bar for MicroCT image = 0.5mm. Scale bar for histological sections = 0.2mm.

3.3 Ordination Models and Statistical Tests

3.3.1 Chi-squared and Post hoc Tests

In order to test the independent distribution of statistical differences in the frequencies of grouped skeletal anomalies between the rearing conditions, a Chi-squared test was performed, using 5 categories of anomalies. The selected subset of anomalies was comprised of: *complete and partial vertebrae fusions*, *opercular anomalies*, *jaw anomalies*, *arch anomalies**, *vertebral body shape anomalies*, and the sum of *severe anomalies* (Supplementary Tab. 2.4). The *p*-values obtained with the Chi-squared test are listed in Table 2.2, displaying that 5 out of these 6 categories resulted in significant differences ($p < 0.05$) among the experimental lots, particularly *severe anomalies*, *arch anomalies*, and *vertebral body shape anomalies*. Only the *vertebrae fusions* did not show a significant difference between the conditions, probably due to their low occurrences.

Table 2.2. Results of *Chi-squared* test for testing the differences among all experimental lots for grouped anomalies. Significant ($p < 0.05$) differences are highlighted in bold.

<i>Grouped anomalies</i>	<i>p-values</i>
Vertebral body shape	6.583×10^{-37}
Arches (5* and 6*)	1.207×10^{-6}
Severe Anomalies	1.450×10^{-6}
Jaws Anomalies	0.0002
Opercular Anomalies	0.0241
Vertebrae fusion	0.0893

Consequently, pairwise *post hoc* paired tests were carried out for the 5 categories which had resulted in significant differences (Tab. 2.3), using the False Discovery Rate (FDR) correction for multiple comparisons (Benjamini and Hochberg, 1995). The *opercular anomalies* were the only ones that resulted to be not significantly different from the other conditions, despite showing a significant difference in the general Chi-squared (Tab. 2.2). The LD1000 group was significantly different with respect to nearly all other conditions for the considered anomalies. The only exception was represented by the LD500 condition, which showed a significant difference between LD1000 only for the *vertebral body shape* anomalies. Another noteworthy characteristic was the difference between LD500 and HD500. The last general feature that is important to highlight is regarding the *vertebral body shape* category. Accordingly, the latter anomaly category indicated not only a discriminating pattern between conditions of equivalent densities but also of different volumes (LD1000 vs LD500, MD1000 vs MD500, HD1000 vs HD500).

Table 2.3. Results of *Post hoc* pairwise tests for testing differences of grouped anomalies between pairs of experimental lots.

Pairwise Comparisons	VB shape	Arches	Severe	Jaws	Operculum
LD1000 and MD1000	****	***	*	*	NS
LD1000 and HD1000	****	****	****	**	NS
LD1000 and LD500	****	NS	NS	NS	NS
LD1000 and MD500	****	**	**	**	NS
LD1000 and HD500	****	**	***	***	NS
MD1000 and HD1000	****	NS	*	NS	NS
MD1000 and LD500	****	**	NS	NS	NS
MD1000 and MD500	****	NS	NS	NS	NS
MD1000 and HD500	NS	NS	NS	NS	NS
HD1000 and LD500	NS	**	*	NS	NS
HD1000 and MD500	NS	NS	NS	NS	NS
HD1000 and HD500	****	NS	NS	NS	NS
LD500 and MD500	NS	NS	NS	NS	NS
LD500 and HD500	****	*	NS	*	NS
MD500 and HD500	****	NS	NS	NS	NS

Significant ($p < 0.05$) differences are highlighted by asterisks: *: $0.05 > p\text{-value} > 0.01$; **: $0.01 > p\text{-value} > 0.001$; ***: $0.001 > p\text{-value} > 0.0001$; ****: $p\text{-value} < 0.0001$; NS: not statistically significant. VB = vertebral body

3.3.2 Correspondence Analysis

A preliminary CA was carried out on the raw matrix consisting of 949 individuals and 70 anomalies (grouping shown in Supplementary Table 2.4). However the ordination models displayed an extremely low variance for the respective axes; therefore diverse CAs were performed on different subsets of anomalies and the highest variance expressed by the first two correspondence axes (CA1: 35.85%; CA2: 33.54%) was obtained by applying the CA to a matrix consisting of 860 individuals and 4 categories of anomalies, in which 37 (instead of 70) anomalies were grouped (Supplementary Tab. 2.4): *severe vertebrae and column* anomalies (total and partial vertebral fusions, hemivertebrae, and scoliosis), *head* anomalies, anomalies of associated *arches* (5* and 6*) (supernumerary/absence of neural and/or hemal arches and spines), and anomalies of *unpaired fins* (affecting rays, pterygophores, hypurals, and epurals). As a consequence, a total of 89 individuals without these anomalies were removed from the analysis due to a null data vector (a specimen without any of the considered anomalies) cannot be analyzed with vector normalization methods such as those required for a CA. The ordination model is shown in Fig. 2.15 and the ordination of lot centroids is shown in Fig. 2.15b. Even though individuals are mostly concentrated (often overlapping) in the 3rd quadrant (Fig. 2.15a), the lot centroids are positioned

in the 1st, 3rd, and 4th quadrants (Fig. 215b). Both the LD1000 and LD500 centroids were plotted on the 3rd quadrant relatively near to each other. MD500 is also located in quadrant 3 but nearer to the axes' origin. In this quadrant, the *unpaired fins* are also positioned (quadrant 3, Fig. 2.15a). In the opposite plane and quadrant, MD1000 and HD1000 centroids are located (quadrant 1, Fig 2.15b), distally from the 'unpaired fin anomalies', and sharing quadrant 1 (although they are not proximal) with *arches (5* and 6*)*. The HD500 centroid is plotted in the 4th quadrant, sharing the quadrant proximally with *head anomalies* and distally with the *severe vertebrae and column anomalies*.

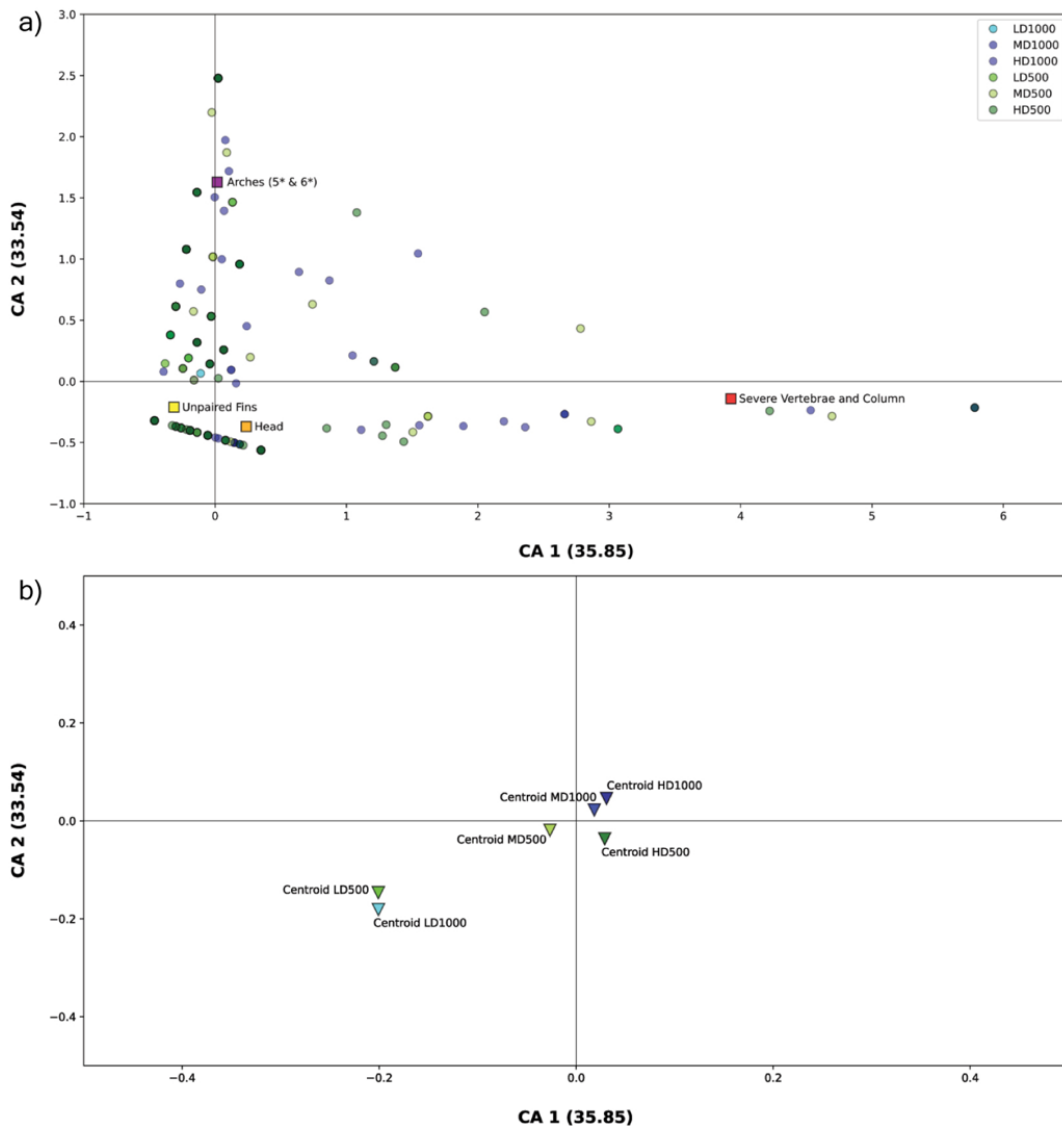


Figure 2.15. Results of Correspondence Analysis (CA) applied to the matrix of 850 individuals x 4 anomaly categories: **(a)** ordination model of all individuals and descriptors (anomalies grouped as shown in Supplementary Tab. 2.4); **(b)** ordination model of lot centroids. Note the increased magnification with respect to **(a)**.

3.3.3 Principal Component Analysis

A PCA was performed to recognize general patterns associated with the rearing conditions. The following 6 variables were selected to perform the ordination: *standard length*, *survivorship*, *dry weight*, *vertebral body shape anomalies*, *malformation index*, and *opercular anomalies* (Fig. 2.16). Type one scaling was used, since the principal focus was on the relative position of the conditions. By using this type of scaling, the distance between the objects in the plot approximates their Euclidean distances in full-dimensional space. The variance explained by the principal component 1 and 2 was 54.7% and 30.57%, respectively. The large share of variance explained by the first two axes highlights that the structure behind the data is relatively simple and justifies an unconventional use of the PCA, which usually requires a larger number of observations with respect to the considered variables. The ordination enables the observation of several key features based on the different rearing conditions. From a general perspective, a greater survival rate is closely associated with greater DW (g). On the other hand, the malformation index, and the percentage of opercular anomalies describe the opposite direction of the first component. Moreover, the ordination suggests the existence of a positive relationship between the length of the individuals and the low densities conditions while the malformation index and the opercular anomaly vectors represent a dominant influence in the high and medium density conditions (Fig. 2.16).

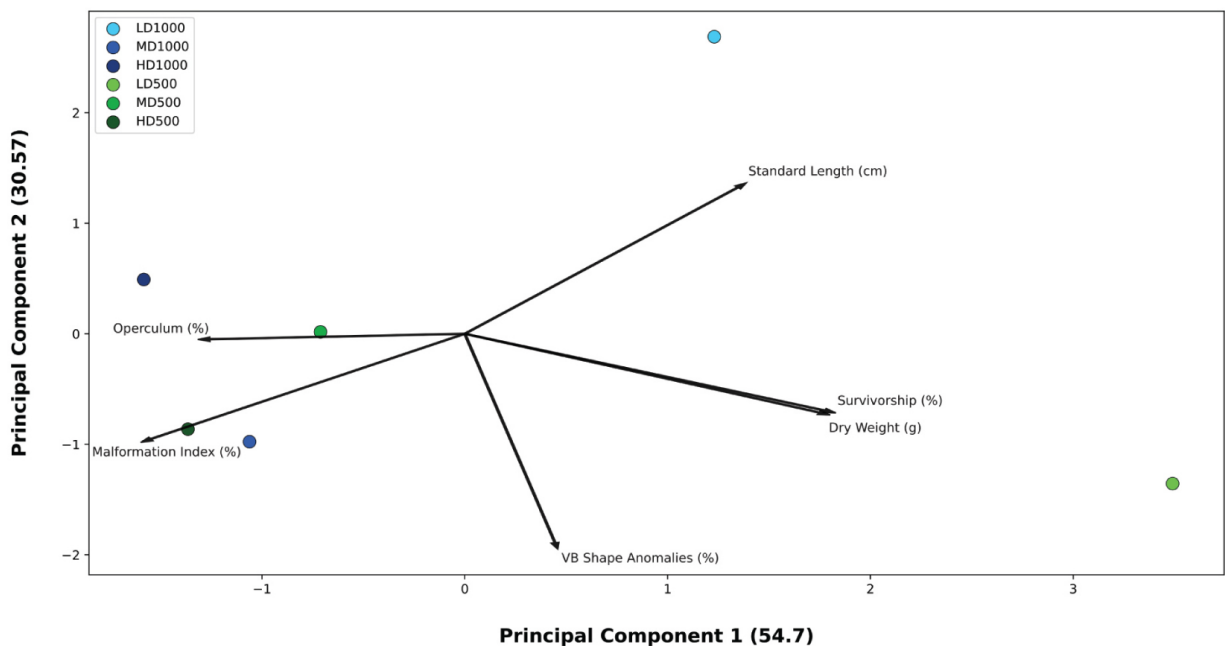


Figure 2.16. PCA performed to recognize the association among experimental rearing conditions and some descriptors. The resulting ordination suggests that the low-density conditions are associated with favorable output features such as survival, weight, and length of the individuals while negatively related to skeletal anomalies.

4. Discussion

While many studies have enabled fish farmers to close the main knowledge gaps (e.g., generally resolving the issue of swim bladder anomalies, by the use of skimmers removing surface oil), many challenges still remain (Boglione et al., 2013a). In particular, skeletal anomalies represent a major setback in fish production and serious deformations (those which can be easily noticed by consumers) typically arise during larval and postlarval lifestages (Daoulas et al., 1991). Different causative factors can induce the same skeletal anomaly, in different species; furthermore, environmental factors often act synergistically and can either have a negative impact (worsening the deformation) or a positive impact (recovery of a deformation) (Fragkoulis et al., 2019; Witten et al., 2006), depending on the species and/or the life stage. Even though skeletal anomalies can arise up until harvest, the hatchery phase is paramount for supply and final production. Furthermore, this phase represents a critical bottleneck in the production cycle of marine finfish. This study is the first attempt to describe the effects of the tank volume *versus* stocking density on the incidence of skeletal anomalies in gilthead seabream during the hatchery phase. In order to meet the farmers' needs, the tested densities were selected based on what was suggested by the producers (API, Italian Fish Farmers Association). These density conditions were tested as a pilot study for future industrial/commercial applications, while the volumes were chosen to be relatively small, maximizing the exploitation of the spaces generally available in the large part of commercial farms. Furthermore, the use of the tested densities in tanks with volumes greater than 1000L would require an excessively high number of eggs, only possible to get from multiple spawning events. Consequently, the densities tested in this trial (25 vs 125 vs 250 larvae/L) were much higher than those reported in previous density-related comparisons (3-16 larvae/L *versus* 100 larvae/L), while the compared volumes were smaller (500 and 1000L) than those previously investigated (small volumes: 500 or 900L *versus* mesocosms or Large Volumes: 40,000 or 60,000L) (Prestinicola et al., 2013). Bearing in mind the objective to maintain only the rearing density or the tank volume as the driving factors eliciting skeletal deformities, the water open-flow circulation system, natural seawater, natural photoperiod, tank color, feeding regime, technical maintenance, oxygen saturation, and temperature were consistent among all the tanks during the experimental trial. Necessarily, the velocity rate of water flow differed between the 1000L tanks and the 500L tanks, due to the requirement of maintaining the same water replacement rate: water had to be pumped in faster to replace 1000L in the equivalent time as the 500L tanks; this issue will be discussed later in further detail.

4.1 Effects on survival rate

The final survival rates obtained starting from the exogenous feeding of the larvae (3dph) ranged from 3.3% to 29.7%. In the literature, typical survival rates for pre-weaned larvae typically range from 3% to 50% (Pousão-Ferreira et al., 2003), although ranges with upper limits of 23% are considerably decent (Izquierdo et al., 2017). The stocking densities evidently influenced the survival rate at 60 dph calculated from the 3 dph survival at the start of endogenous feeding up to early juvenile seabream. Both LD tanks had higher survival rates, and the overall highest obtained

was found in the LD500 (29.7% vs 10% in LD1000). This trend seems to indicate that the density of 25 larvae/L in 500L tank represents a limit beyond which some technical (higher flux speed of water from the inlet) or behavioral (tighter interactions, frequent changes in the swimming direction, cannibalism, hierarchy instauration with consequential increased competition for feeding, etc.) stresses may induce a lower survival rate, as crowding has already been shown to impact immune responses and survival (Montero et al., 1999b). It should be taken into consideration that even when the densities were the same in the 500L and 1000L tanks, the larger volume tanks contained double the number of individuals present in the equivalent-density small volume tanks. The tendency for lower survival in the larger volumes detected in LD was also upheld when comparing the large and small volumes in HD condition, but this trend was reversed in the MD conditions. Only a precise regular monitoring of dead larvae could have allowed a better understanding of the reasons for this irregularity.

4.2 Length and weight

Early juveniles from the LD conditions were the longest (TL and SL) overall, while the fish from the MD and HD conditions were the shortest, indicating a sound density effect. A secondary volume effect can be witnessed in both LD and HD conditions where the tanks of 1000L produced fish longer than the 500L ones. Again, the MD conditions showed a peculiarity, in which there was an absence of significant differences between the two tested volumes. Notably, the larger TL of LD juveniles was not correlated to the number of vertebrae, whose range on the contrary included the lowest count: both LD lots ranged from 23 to 25 counts with respect to 24-25 or 24-26 detected in the other lots. The exact same trend was also found in the WW and DW.

The fact that for both the LD and HD conditions, the tested larger volumes produced longer and heavier fish than the smaller volumes (LD500 and HD500) and not in the MD conditions could be due to the fact that although the initial densities were the same, a greater survival in the large volume (MD1000) was recorded with respect to MD500. This consequentially entails that the final densities in MD500 and MD1000 were not the same (final densities: MD1000 = 9 juveniles/L; MD500 = 4 juveniles/L). The lower density established in MD500 could explain the fact that the specimens' lengths and weights were slightly greater in MD500 than in the MD1000, although not significantly different.

As reported in the Introduction, on-growing gilthead seabream grown from adults (~271g) for 9 weeks at the densities of 5, 10, and 20kg/m³ (Araújo-Luna et al., 2018) showed the same inverse relationship between increasing density and fish weights and lengths. However, the present study is the first to report the effects of rearing density from eggs up to post-weaning in a marine species. Additionally, it could be interesting to thoroughly investigate if food conversion efficiency, schooling, or cannibalistic behaviors are species- or life stage-specific responses to stocking density. In order to assess individual spatial responses to density, video-recordings and spatial density maps of the model fish medaka reared at different densities in the same water volume are currently in progress at our lab.

4.3 Skeletal anomalies

The results from this study indicate that higher densities yield an increase of individuals with skeletal anomalies, witnessed when comparing between LD and MD (a factor of 5), and even more pronounced effects (by a factor of 10) when comparing LD with HD. This study has evidenced, in fact that, while a diversity of anomaly types may be found with occurrences uninfluenced by the rearing density (considered as ‘background’ anomalies), the incidences of some less frequent but more severe anomalies are indeed reduced in lower densities: the lowest malformation index and frequency of individuals with at least one severe anomaly (*i.e.*, jaws, opercular plate, and caudal vertebral fusion) were reported for the LD juveniles. The hypothesis of ‘density’ as the primary driving factor for severe skeletal anomalies onset is soundly corroborated by multivariate analyses, namely the CA, PCA, and Chi-squared *Post hoc* pairwise tests.

However, even the tank volumes resulted to play a secondary effect in the hatchery phase of gilthead seabream, as confirmed by PCA and by the pairwise *Post hoc* tests, particularly for certain vertebral body and jaw (premaxillary and maxillary) anomalies. Opercular anomalies are quite important for farmers because they facilitate the infestation of parasites in the gills, and such anomalies are externally detectable on marketed fish. Although in the tested conditions from this study opercular anomalies affected relatively few individuals, their frequencies displayed a direct relationship with both density and tank volume. The discrepancy in the effects of tank volume observed in bones (opercular plate and jaws) which are located in the same body region and share the same embryological origin as well as ossification modality (*i.e.*, splanchnocranium, which is composed by dermal bone sharing the same neural crest origin; Hall, 2005), confirms previous observations that the same environmental factor (*i.e.*, tank volume) may induce different incidences of anomalies affecting skeletal elements of the same bone type and ossification mode (Fernández and Gisbert, 2011), in the same species and life stage. This could be due to the fact that they play different functions (*i.e.*, maxillary/premaxillary and opercle) which are not equally influenced by varying stock densities (*i.e.*, biting *vs* ventilation).

Interestingly, even the fusions and hemivertebrae in the caudal centra showed an augmented prevalence in increased density and volume conditions. The caudal fin enables controlled quick movements in the water column and is a key component in predatory escape responses (Gibb et al., 2006). Furthermore, caudal fin development represents a critical stage rousing key behavioral changes in the early ontogeny of fishes (Somarakis and Nikolioudakis, 2010). Due to the particular nature of this region, it is likely that environmental factors such as water flow rate, space availability and utilization, stocking density, and type/quantity of individual interactions may yield differential phenotypes among the various components of the caudal fin in the same species, at the same early developmental stage. Caudal region sensitivity has been previously proposed to be a species-specific characteristic of gilthead seabream as even some wild seabreams have been found to show anomalies in this region (Boglione et al., 2001). However, the fusing of the two centra preceding the urostyle has only been observed in a single wild seabream individual (data taken on a total of 742 wild juvenile and adult gilthead seabream analysed by Boglione and coll., unpublished data). Although the hypothesis of strong selection pressure against deformed fish in

the wild has been presented (Vladimirov, 1975; Shelbourne, 1964), it is not yet known if such fusions are lethally selected in the wild or recovered in later stages in wild fish. Lethal selection has also been proposed as a hypothesis for the lower prevalence of caudal fin deformities in mesocosms which may be more selective in certain ways (swimming and feeding) than smaller funnel-shaped tanks (Koumoundouros et al., 1997).

Our study was not conducted to determine all of the specific effects of space availability or rearing density on the physiological state or behavioral status, but rather a focused investigation on the skeletal response and quality of reared gilthead seabream from spawning up to reaching the early juvenile stage. Nevertheless, some speculative hypotheses explaining the obtained results based on the available literature are formulated here below.

4.4 Hypothesis: high density alters gilthead seabream behavior

Some authors propose that the different species and life stage specificity in response to stocking density is due to aggregation and schooling behavior (Papoutsoglou et al., 1998). Furthermore, differences can also exist at the intraspecific level in which different personality traits may play a role in individual behavioral responses. Presently, two main personality types have been reported in fish: proactive (anticipatory preparation to exert control) and reactive (momentary cognitive engagement as the need arises) (Martins et al., 2012). Particular personality types can manifest themselves in a wide array of ways depending on the environmental conditions and group interactions, yielding different feeding behavior, swimming habits, and stress responses. In fact, recent evidences obtained from zebrafish have confirmed that certain social cues are detected by the neuromasts of the lateral line which detect movements from neighboring fish and enable fish to monitor the density of other individuals within the space (Anneser et al., 2020). Furthermore, previous studies in rainbow trout found that fish reared in high densities had a reduced number of superficial neuromasts on their lateral-line compared to wild-caught trout (Brown et al., 2013), signaling a deterioration of an organ critical to the detection of low frequency motions, controlling orientation and rheotaxis (Montgomery et al., 1997). Although the synergistic impacts of high-density rearing, overload of mechanosensory stimuli, and social responses have not been fully investigated, it could be hypothesized that the resulting stress induces profound impacts on swimming behavior and subsequently on the musculoskeletal system. Therefore, when considering the stress impact of rearing an elevated number of fish in a confined area, the high density (greater number of individuals) rather than available swimming space remains to be the primary factor in producing unfavorable physiological responses, eliciting increased mortality, stunted growth, and augmentation in frequencies of some skeletal anomalies.

Another important factor causing this response could be due to the autecological behaviors based on species-specific or life-cycle dependent interactions, when held at high density, regardless of the dimensional volume available. In fact, gilthead seabream reared in high density conditions have been observed to exhibit stronger schooling behaviors (Arechavala-Lopez et al., 2020), forming tighter schools with closer interactions between individuals. This could consequentially lead to a behavioral response in which fish do not fully exploit the available swimming space. Interestingly, gilthead seabream juveniles reared in high density conditions have been found to

exhibit more overall movements although contemporaneously displaying a reduced diversity in movement types, when compared to seabream held at low density; on the contrary, those held at low densities exhibited less movement due to decreased swimming speeds coupled with a greater diversity in movement types (Sánchez-Muros et al., 2017). Such an intense increase in swimming triggered by high densities, or by a high number of individuals, may cause different usage of certain trunk muscles than required in their natural environment or in low-density conditions. Thus, an accumulation of muscular strains on the same vertebral region (i.e., hemal) could subsequently provoke an abnormal modeling response of the underlying skeletal elements resulting in anomalies.

Moreover, technical differences may have also been involved, *i.e.* interaction between water flow and tank shape coupled with the higher demand for the water inlet velocity necessary to maintain the water replacement rate in the 1000L cylindrical tank may have yielded swimming behaviors which were different than those found in the 500L cylindroconical tanks. Recent studies on gilthead seabream juveniles have found that increased water flow can induce an ‘exercise behavior’ resulting in an improved growth rate (Moya et al., 2019), muscle building, energy mobilization, robustness, and reduced stress, although lordotic juvenile seabream were found to be more frequent when reared in tanks with higher flow rates (Palstra et al., 2020). In the present study, lordotic fish were not found, possibly due to the early juvenile stage of the final sampling (average SL=1.79 cm). Additionally, the flow rates in this study were generally lower than flow rates used to assess exercise response, even during the end of rearing in the 1000L tanks, in which water flow was increased up to 100%/h, and flow velocities were ~ 0.73 and ~ 0.3 m/s from the inlet for 1000L and 500L tanks, respectively. Taking into consideration that this velocity is not uniform throughout the tank and peak velocity was limited to the water inlet and differentially distributed between the borders and the center of the tank (Oca and Masalo, 2013), fish were able to swim in the zone of their preferred water flux. Although the behavioral swimming responses to higher water flow are multifaceted, the increased flow rate could have enabled better growth (Davison and Herbert, 2013), resulting in higher final length and weights in the LD and HD 1000L conditions compared to their 500L density equivalents. In the MD1000 lot, higher survival, and thus effective density, may have inhibited flow-related growth stimulation. An increased muscle mass and robustness due to increased swimming could also lead to higher mechanical loading that potentially counteracts skeletal degeneration (Suniaga et al., 2018).

Another factor which could have played a role on skeletal quality is the stress response associated with crowding. Stress induced by high stocking in gilthead seabream has already been reported to be associated with an increase of macrophages in the spleen (Montero et al., 1999a), of plasma cortisol, and changes in fatty acid composition in the liver (Montero et al., 2001). Acknowledging the highly stressful environment for gilthead seabream stocked at high density, an increase in ROS (reactive oxygen species) is expected (Braun et al., 2010). This elevation in ROS, in turn, could play a role affecting the survival and ontogenetic processes (*i.e.*, skeleton differentiation, modeling, and remodeling) of the fish reared at high densities. In fact, dietary components, that are known to have either oxidative or antioxidative properties (dependent on the dosage) fed to

gilthead seabream larvae, have been found to influence the incidence of severe skeletal deformities (Izquierdo et al., 2019). Therefore, a deleterious effect of ROS induced by the increased stress brought on by crowding should be considered as a plausible metabolic factor linking the higher incidences of skeletal anomalies found in high density rearing conditions. As physiological analyses were not carried out in this study, it is not possible to ascertain if the causative factor of greater incidences of anomalies is a direct consequence of an elevation in metabolic factors related to stress or behavioral alteration due to overcrowding. Regardless, even elevated metabolic factors driven by the consequences of a stressing condition can induce behavioral alteration.

4.5 Economic considerations

Based on this current study, the economic advantages of rearing gilthead seabream larvae at a density of 25 individuals/L are extensively evident due to the significantly larger juveniles, higher survival, and a sound reduction in the incidence of skeletal anomalies, which are all considered paramount to the assurance of profitability. Larger volumes on the other hand enhanced a positive effect of lower density on weight and length as well as the reduction of some skeletal anomalies in both LD1000 and HD1000 conditions, although there was no clear effect of the tested volumes on survival.

In general, larger volumes require more energy, space, live preys, water exchange, and technical labor which all lead to increased production costs for the farmer. Although large volumes do enable more larvae to be maintained in a set condition, the risk of mass mortality (*i.e.*, provoked by lethal sulfo-rodo-bacteria; Papandroulakis et al., 2001) can be mitigated by using more numerous smaller tanks. Bearing all of these additional costs in mind, hatcheries wishing to improve the quality of the fry and increase their profit margins should consider reducing their stocking density, together with the consolidated practice of preemptively culling out the malformed seabream.

Chapter 3: Case study 2: Effects of tank volumes versus stocking densities on the skeletal quality during the preongrowing phase of Gilthead seabream (*Sparus aurata*)

1. Introduction

Gilthead seabream production is one of the main aquaculture industries in the Mediterranean, producing 258,754 tons of seafood in 2019 (FAO, 2021). Modern ongrowing/grow-out farms (farms in which reared fish are fattened up to reach the commercial size of 200-350g) often employ intensive strategies with high energy demands, tailored feeds, added oxygen, and high stocking densities (Pavlidis and Mylonas, 2011). Generally, juveniles weaned to artificial feed (5 g) are pre-ongrown in land-based facilities (tanks) for 8-10 months up to reach 50-60 g at first, then definitively ongrown in sea cages. Differently from the hatcheries (where each production cycle lasts 2-3 months), the duration of the ongrowing phases is much longer (up to 16 months). In recent years, there has been a great deal of uncertainty with regards to the economic profitability for many seabream farmers, since production costs often outweigh the profits due to rapid market expansion in the 1980s, followed by an oversupply establishing a lower market value in the last two decades

(Llorente et al., 2020). Taking into consideration that gilthead seabream hatcheries produce varying amounts of juveniles of suboptimal quality (i.e., with externally detectable skeletal anomalies), ongrowing farms are usually willing to pay premium prices for high quality fry to be ongrown. This extra cost, unfortunately, is not always effective: as skeletal anomalies can in fact arise throughout the lifecycle (Daoulas et al., 1991), thereby adding even more serious threats to the economic security of ongrowing farmers. This economic drain caused by deformed fish is due to the expensive feeding regimen (wasting feed on deformed fish that are destined to die or be thrown out prior to being marketed), the undetectability of certain deformities in early life stages, which become more evident only later on (Bardon et al., 2009), and the subsequent reduced marketability of deformed ongrown seabream (Le Vay et al., 2007; Lijalad and Powell, 2009; Boglione et al., 2013a). In ongrowing farms the primary production costs come from the feed (46% of production cost) and fry (14% of production cost) (Nielsen et al., 2021). The costs for feeding deformed fish which are destined to be unmarketable can be effectively lowered by identifying technical procedures capable of reducing the presence of skeletal anomalies that arise during ongrowing. While fish with severe cranial anomalies could be culled out during larval rearing because they are easily detectable by external examination, vertebral axis deformities are rare and often undetectable in the early juvenile stage (Dellacqua et al., submitted). In ongrowing fish however, vertebral axis deformities are quite common along with severe deformities of the head. Due to the difficulty in recognizing axis deformities preemptively and culling out juveniles (by means of manual sorting), other solutions must be recognized and applied during ongrowing. Currently, ongrowing production facilities foresee regular culling to both reduce the stocking density (as the fish grow the relative biomass per tank volume increases), and to eliminate deformed fish from the production cycle (Koumondorous et al., 1997a). This strategy, although necessary to reduce the stocking density, can inflict additional stress on the animals (Costa et al., 2013), and requires a great amount of technical labor on the part of the technicians. Furthermore, pre-ongrowing in tanks requires frequent tank cleaning as well as high water exchange rates and oxygen saturation. While the pros and cons of choosing intensive *versus* semi-intensive rearing are weighed out by the farmers based primarily on the space availability, farm dimensions, and willingness to wait for economic returns (*i.e.*, the quantity and size of fish that can be produced in the shortest time while minimizing the production costs), other incentives are gaining in popularity and economic competitiveness in European aquaculture. In particular, improved animal welfare, reduction of labor and production costs, organic labeling schemes, and added-value marketing (gained by a higher monetary evaluation of higher quality products), all represent modernized approaches to animal production that can offer farmers a competitive edge and niche-market opportunities. Obviously, the principal goal of producing both a high quantity and quality of fish remains paramount for the farmers. In order to efficiently optimize the desired quantity with the ideal quality, the proper rearing parameters must be identified.

Presently, many studies have been conducted to isolate species and life-stage specific requirements, enabling improved rearing strategies (Boglione et al., 2013b). However, the main objective of novel discoveries (*viz.* their direct application to the production cycle on the farms) is

not always realized or economically viable. This is often due to various different reasons such as expensive feed additives, complex genetic enhancement strategies, and/or environmental or technical incompatibility (*i.e.* ability to increase water flow in RAS systems, augment sea temperatures, or eliminate pathogens in cages). Differences in environmental parameters, in particular, have offered more competitive production of gilthead seabream in the past based on their geographic location in which there are warmer and less variable water temperatures (Llorente and Luna, 2014), although this is likely to impact the skeleton in response to climate change in the future (Boglione, 2020).

Nevertheless, two environmental parameters that can be easily adapted and optimized by farmers regardless of their geographical limitations, feed costs, genetic selection strategies, or threat of climate change, are the stocking density and available swimming space. Previous studies have looked at the effects of different rearing densities (Arechavala-Lopez et al., 2020; Araújo-Luna et al., 2018; Prestinicola et al., 2013; Montero et al., 2001; Montero et al., 1999; Canario et al., 1998) and some have compared the effects of semi-intensive ('Large Volumes' and 'Mesocosms') rearing strategies to intensive rearing strategies (Prestinicola et al., 2013; Boglione et al., 2001; Divanach and Kentouri, 2000; Loy et al., 1999) in gilthead seabream. However, to our knowledge no published paper has focused on discerning which among tank volume and stocking density plays a greater role in the development of skeletal anomalies in pre-ongrown gilthead seabream. This study aims at delineating which among tank volume and stocking density is the main factor driving the formation and proliferation of skeletal anomalies in the pre-ongrowing phase of gilthead seabream. In order to facilitate the implementation of improved rearing strategies presented in this work, this study was conducted at a pilot scale following the standardized industrial management of the pre-ongrowing phase by using relatively high stocking densities (5kg/m³, 10kg/m³, 20kg/m³), which were suggested to be worthwhile to study based on the interest from the Italian Fish Farmers Association (API). Additionally, in order facilitate the implementation of the methodology applied in this study to gilthead seabream farmers, the cylindrical tanks used for the two different experimental volumes were all of the same diameter (1m), however they had different heights and contained different volumes of water (1000L vs 500L).

2. Materials and Methods

2.1 Rearing setup

Experimental rearing was conducted at the Portuguese Institute of the Sea and Atmosphere (IPMA) facilities in Olhão (Portugal) during the late summertime from 03/08/2019 to 05/10/2019. Gilthead seabream juveniles (average weight of 6.7 ± 2.5 g and length of 7.8 ± 1.1 cm) obtained from different spawns and parents, were previously selected based on external inspection carried out by experienced technicians for skeletal anomalies, and the 'non-deformed' fish (hereafter named as T₀) were stocked at 3 different densities LD: 3.5kg/L; MD: 7kg/L; HD: 14kg/L and maintained at LD: 5kg/L; MD: 10kg/L; HD: 20kg/L once those densities were reached in both 500L and 1000L water volumes, with a replacement rate of 100% tank volume/hour of sand filtered natural seawater

with flow velocities of ~0.73 and ~0.3 m/s for 1000L and 500L tanks respectively. The experimental tanks for the 6 different experimental conditions were labeled LD1000, MD1000, HD1000, LD500, MD500, HD500. They were identical with regards to the cylindrical shape and diameter, while the greater water volume corresponded with an increased tank height. The stocking densities were maintained at a ratio of x - 2x - 4x for LD, MD, and HD, respectively, by sampling at 3 different time points and removing excess fish to maintain the original biomass/L for each of the respective densities in the lots (Supplementary Tab. 3.1). The light regime was based on natural light and photoperiod; tanks were also shaded with a mesh tarp to reduce overheating. The water temperature in the tanks ranged 19-29° C throughout the ~63 days of rearing (Supplementary Fig. 3.1a). Oxygen diffusers were placed at the bottom of tanks in order to maintain an O₂ saturation level above 70% and above 5 mg/L (Supplementary Fig. 3.1b). Final sampling was done upon the seabream reaching an average final weight of ~55g/individual (Supplementary Tab. 3.1). Fish were fed 3% of their body weight/day with AquaGold 5 pellets (AquaSoja, Ovar, Portugal).

2.2 Sampling and Post-rearing analyses

A sample of 161 seabream from T₀ were lethally anesthetized with 300mg/L of MS-222 and fixed in 4% PFA and X-rayed (Gilarioni CPX 160/4 System, Unleaded film Kodak Mx 125, 55 Kv, 4 mA) for anatomical evaluation of skeletal elements.

Seabream from the experimental conditions were sampled at 4 different time points (including the final sampling) in which individual wet weight (WW) was used to calculate the growth in biomass and subsequently the density. Excessive fish were then randomly removed in order to reduce the increasing biomass and maintain the original desired density. After ~63 days of experimental rearing (T_F), samples of fish were measured for fork length (FL), total length (TL), and wet weight (WW) (Supplementary Tab. 3.1). Additionally, the Fulton's condition factor (K) was determined based on the formula: $K = W_t/L^3$, in which W_t is individual's WW and L the TL. The K factor is a useful computation as it describes the relationship between length and weight as they pertain to body-shape indicating the slenderness *versus* the stoutness of individual fish (Ricker, 1975). The Food Conversion Rate (FCR) and the Standard Growth Rate (SGR) were calculated using the T₀ and T_F weights. Differences among experimental groups were tested with Kruskal-Wallis test for equal medians and Dunn's *post hoc* with Bonferroni correction.

A total of 853 seabream (T_F; Tab. 8) from the different conditions were euthanized with a lethal dose of 300mg/L of MS-222, X-rayed with a digital DXS Pro X-ray (Bruker) (0.7mm double top filter, 17mm focal plane, with a single acquisition 120s exposure time), and digitally visualized with the Carestream package (Bruker) at the University of Algarve. Dissections of the vertebral column were made on 12 lordotic and 12 normal fish individuated from the radiograph images, to assess potential differences in the calcium-phosphorous ratio between lordotic and non-lordotic vertebral centra (n = 3 vertebral centra/fish). After removing attached tissues, the vertebrae were digested in nitric acid and then the calcium and phosphorous mineral content was quantified using microwave plasma-atomic emission spectrometry (MP-AES) at the University of Algarve.

Other samples which were affected by different anomaly types were also dissected and both deformed and non-deformed bones were fixed in 1.5% PFA and 1.5% glutaraldehyde in 0.1M

sodium cacodylate buffer (pH 7.4) then transferred to 70% ethanol. These samples were then decalcified for 4 weeks by submersion in 10% EDTA and 0.5% PFA at 4 °C before being embedded in a GMA (glycol methacrylate) resin, according to the protocols by Witten et al. (2001). Sections (5 µm) were cut on a standard rotary microtome (Microm HM360, Marshall Scientific) and stained with toluidine blue and Verhoeff's elastin stain.

2.3 Skeletal anomalies survey

Meristic counts and skeletal anomalies types and occurrences were inspected on the digital (T_f) and film (T_0) radiograph images using FIJI (Schindelin et al., 2012), with an alphanumeric code expressing the body region and the anomaly type (Supplementary Tab. 3.2). The examination was carried out based on the following assumptions: 1) incomplete fused bone elements were counted as discreet elements; 2) supernumerary bones with normal morphology were not considered to be anomalies but were included in meristic count variations; conversely, anomalous supernumerary elements were included among anomalies; 3) only clearly and unquestionably identifiable variations in shape were considered as skeletal anomalies: i.e., only the axis deviation associated to deformation of the vertebrae involved. Skeletal anomaly data were then expressed in a raw matrix (RM) and used to calculate the frequencies (%) of each type of anomaly on the total number of anomalies, in each group (Supplementary Tab. 3.3). The RM was subsequently transformed into a binary matrix (BM) which was used to calculate the prevalence of individuals affected by each anomaly type (Supplementary Tab. 3.4). The following metrics were calculated, for each group: 1) relative frequency (%) of individuals with at least one anomaly; 2) number of anomaly typologies observed; 3) average anomalies charge (total amount of anomalies/number of malformed individuals); 4) relative frequency (%) of individuals with at least one anomaly; 5) frequency (%) of each anomaly type with respect to the total number of anomalies. The representative data from the RM and BM are expressed in tables and histograms. Correspondence Analysis (CA) (Benzecri, 1973) was performed on a BM matrix of grouped anomalies and a Principal Component Analysis (PCA) on selected grouped anomaly types (Supplementary Tab. 3.5). Differences in the frequencies of individuals affected by certain anomalies among the rearing conditions were tested using a χ^2 test and *pairwise post hoc* (Pearson, 1900).

Axial deviations were also described by measuring the angle of bending from the apex, located at the point of highest angular flexion with vectors drawn to either the dorsal or ventral (dorsal in the case of lordosis and ventral in the case of kyphosis) tip of the two vertebral centra preceding the apex angle and the two successive centra following the apex angle (Fig. 3.1). A modified categorization of angular class grouping made by Sfakianakis et al. (2006) was applied to characterize 7 classes of axis deviations based on severity, according to the ranking shown in Table 3.1. A Kolmogorov-Smirnov test was applied to test for differences between the angular class frequency distributions among the lots.

Statistical analyses and graphs were done using Past 4.02 (Hammer et al., 2001) and Python 3.8.

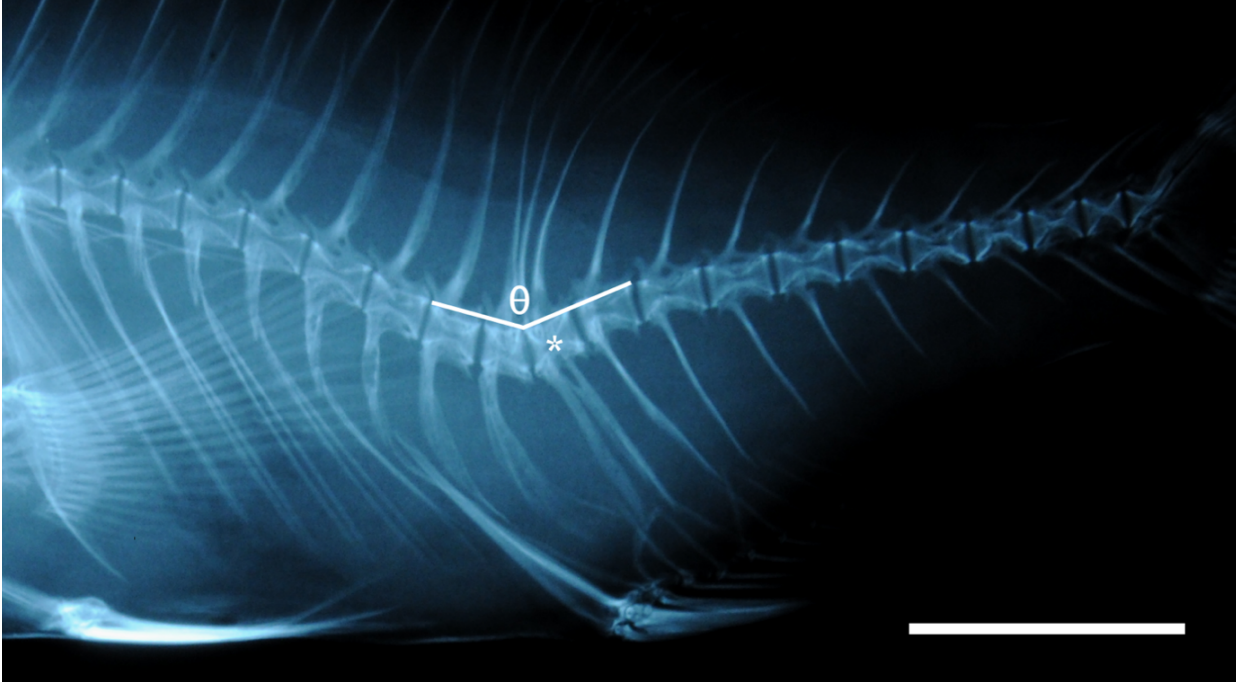


Figure 3.1 Seabream individual exhibiting a hemal lordosis (classified as quite severe with an angle of 142.5°) and a hemal vertebral body fusion marked by the asterisk. Bar=1cm

Table 3.1. Ranking of different angular classes of axis deviation.

Most severe	<137.9°
Quite severe	138.0°-144.9°
Severe	145.0°-151.9°
Moderate	152.0°-158.9°
Slight	159.0°-165.9°
Relatively Mild	166.0°-172.9°
Normal	173.0°-180°

2.4 Geometric morphometrics

A total of 853 digital X-rays were analyzed with geometric morphometrics (Loy et al., 2001) used to investigate differences in body-shape between the T_f individuals from different rearing conditions. The following landmarks were manually selected on each digital radiograph as displayed in Figure 3.2.

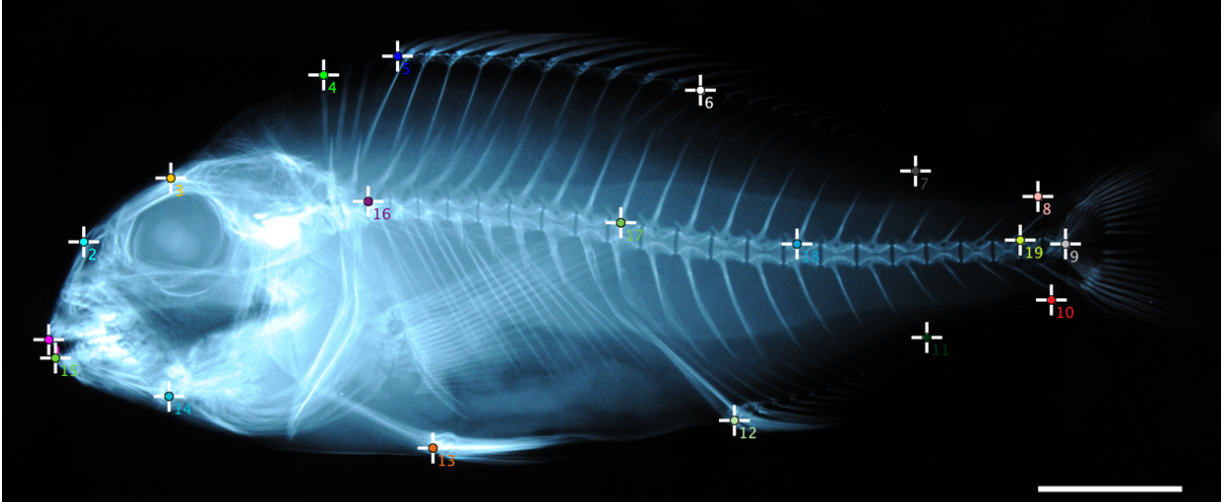


Figure 3.2. Radiograph of seabream individual with selected landmarks superimposed. The following text describes the morphological feature associated with each number. 1: frontal tip of premaxillary; 2: rostral head point in line with the center of the eye; 3: dorsal head point in line with the center of the eye; 4: distal edge of the 1st (anteriormost) predorsal bone; 5: insertion point of the 1st dorsal hard ray; 6: edge of the 1st dorsal soft ray pterygophore; 7: edge of the last dorsal soft ray pterygophore; 8: dorsal concave inflection-point of caudal peduncle; 9: middle point between the bases of hypurals 2 and 3 (fork); 10: ventral concave inflection-point of caudal peduncle; 11: edge of the last anal pterygophore; 12: edge of the 1st anal ray pterygophore; 13: insertion of the pelvic fin on the body profile; 14: preopercle ventral insertion on body profile; 15: frontal tip of dentary; 16: neural arch insertion on the 1st abdominal vertebral body; 17: neural arch insertion on the 1st hemal vertebral body; 18: neural arch insertion on the 6th hemal vertebral body; 19: middle point between the pre- and post-zygapophyses of the 1st and 2nd caudal vertebral bodies. Scale bar =10mm.

A Procrustes transformation (Rohlf and Slice, 1990) was applied to adjust landmark configurations for centroid size, eliminating potential effects that are irrelevant to shape. A 2-dimensional between-group PCA (bgPCA, which is analogous to a Relative Warp Analysis (Costa et al., 2006)), and a LDA (sometimes referred to as a Canonical Variates Analysis (CVA) were applied to the transformed x and y coordinates calculating between-group variance to visualize the effects of the rearing condition on body shape. An Eigenvalue scale was applied to the results of the PCA in order to enhance visualization (Legendre and Legendre, 1998). Thin Plate Spline (TPS) interpolation diagrams were obtained from the residuals of the Procrustes transformation (Bookstein, 1991) and visualized as spline deviations from the consensus along the weight matrix (the components of the bgPCA). The LDA differences between experimental groups were tested with a MANOVA (Wilks' λ); the Mahalanobis squared distances were calculated for each pairwise group (*i.e.*, LD1000L vs LD500L) and the differences between lots tested for significance with a *post hoc* test with Bonferroni correction.

3. Results

3.1 Performance indicators

The need to continuously maintain the experimental densities in each experimental tank made it impossible to calculate the survival rate at the end of the trial. However, only a few ($n=5$) individuals in total (1 mortality in LD1000, 3 in HD1000, 1 in MD500, and 1 in HD500). Another mortality event occurred on 16/08/19 in HD1000 in which 70 individuals from HD1000 died due to the oxygen stone becoming loose and detaching from the supply tube which had shot out of the tank. Although this event reduced the density in the tank, the reduction was very slight (roughly a 1kg/m^3 loss), furthermore a biomass sampling and density adjustment was carried out the week following the incident to reestablish exact density ratios between the tanks (x:2x:4x, for LD:MD:HD, respectively).

There were no differences found in the FCR or SGR (1.43 ± 0.20 and 2.82 ± 0.07 , respectively) among the tanks. HD lots resulted in fish which were significantly smaller (TL) than the LD ones, regardless of the tank volumes (Fig. 3.3a). Although there were no significant differences in fish weight, significant differences were found in the Fulton's condition factor (K) between the 3 densities tested in 1000L, and between HD and the other tested densities in 500L (Fig. 3.3b).

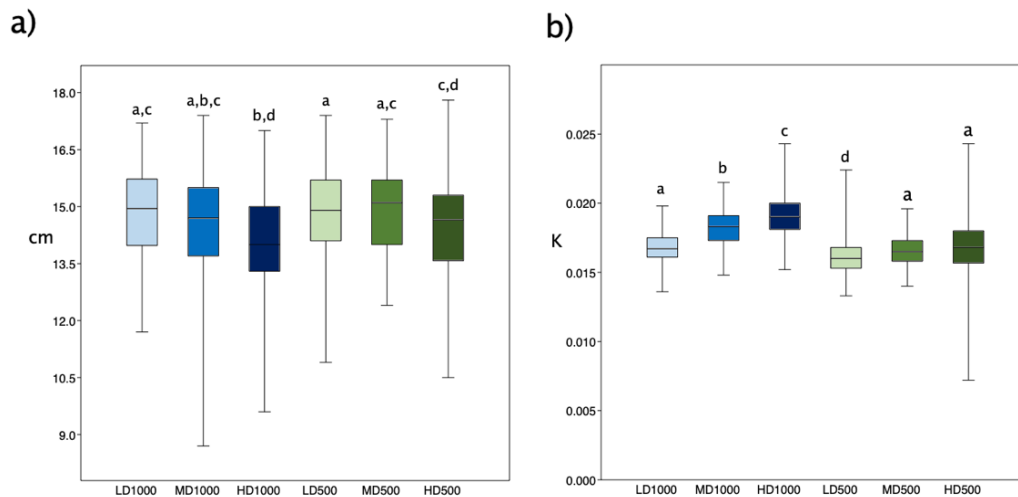


Figure 3.2. a) Box and whisker plots of TL of T_f samples of the different experimental lots. **b)** differences in Fulton's K factors between conditions. Box indicates the central percentile, the line inside the box is the median while the whiskers represent the minimum and maximum value. Different letters indicate significant differences ($p < 0.05$, Kruskal-Wallis, Dunn's post hoc with Bonferroni correction) among experimental lots.

3.2 Anatomical analyses

Table 3.2. Metrics calculated at T₀ and in the experimental lots. The highest value for each metric is in bold

Lots	T ₀	LD1000	MD1000	HD1000	LD500	MD500	HD500
Number of specimens	161	140	115	197	124	96	181
% of individuals with at least one anomaly	79	63	69	80	69	75	88
Anomalies charge	3	2	2	2	2	2	2
Observed types of anomalies	21	18	23	23	17	21	23

Skeletal anomaly data were inspected on 853 individuals from T_f and 161 individuals from T₀. Due to the disparity in the sharpness of the images between the film (T₀) and digital (T_f) radiographs, the anomalies considered in this study were only those that affected the vertebral column and centra, and splanchnocranial bones in a clearly identifiable way (i.e., anomalous vertebral arches or pterygophores were not considered). The observed anomalies affecting the vertebral region were deformities of vertebral centra (fusions, partial fusions, and hemivertebrae) and axis deviations. The anomalies observed in the head were opercular plate anomalies (Fig. 3.4b) and anomalies affecting the maxillary, premaxillary, and dentary, defined as underbite (Fig. 3.4c) or overbite (Fig. 3.4d). The frequencies of individuals resulted affected by each type of anomalies are shown in fig. 3.5.

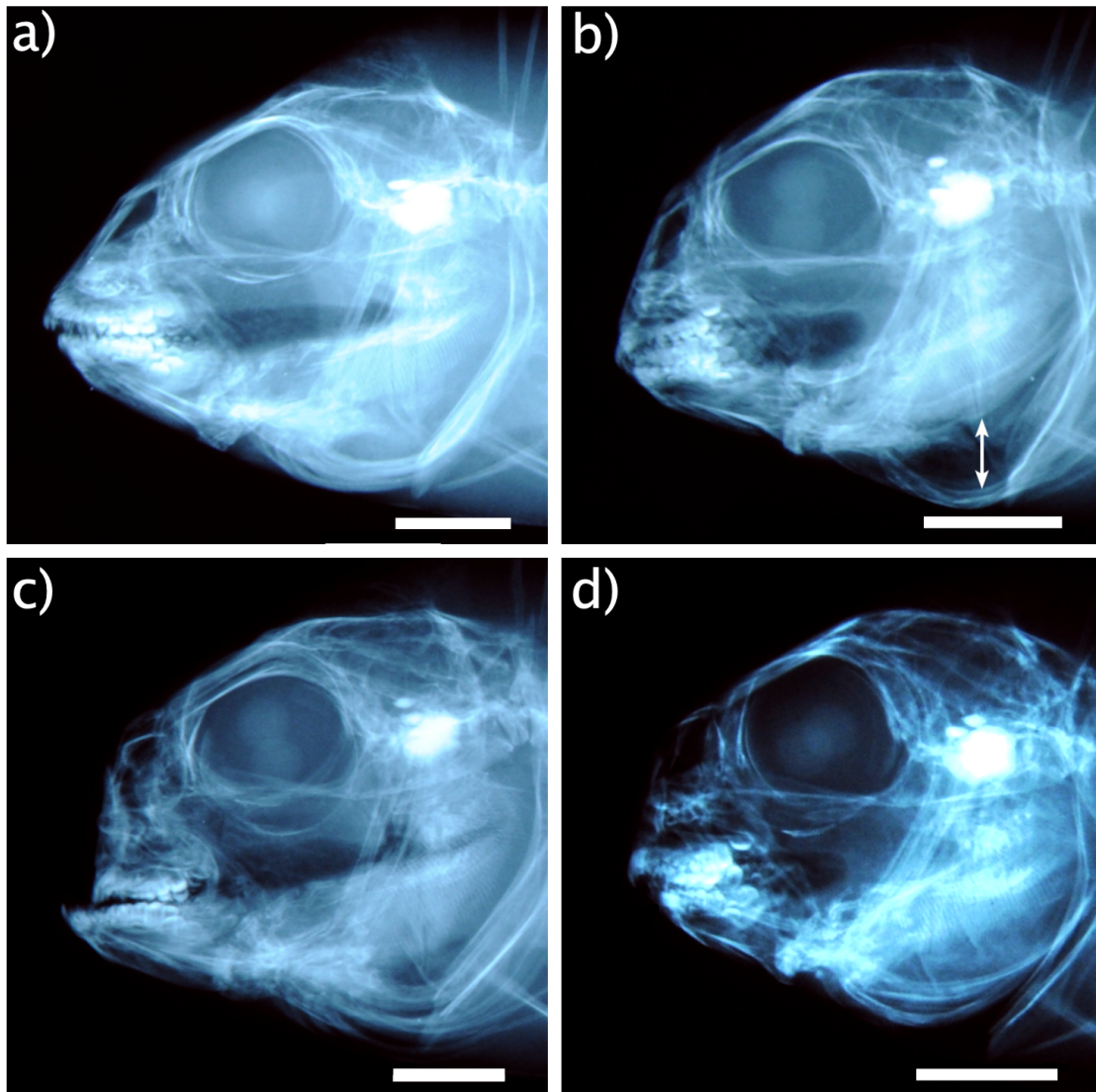


Figure 3.4. Splanchnocranium anomalies affecting gilthead seabream: **a)** normal jaws and opercular plate; **b)** reduced opercular plate; **c)** underbite: contemporary deformation of premaxillary and maxillary and protrusion of dentary (arrow); **d)** overbite: contemporary deformation (twisting, in this case) of the premaxillary, maxillary and a reduction in length of dentary (arrow) resulting in an upper-jaw overlap. Bar=5mm

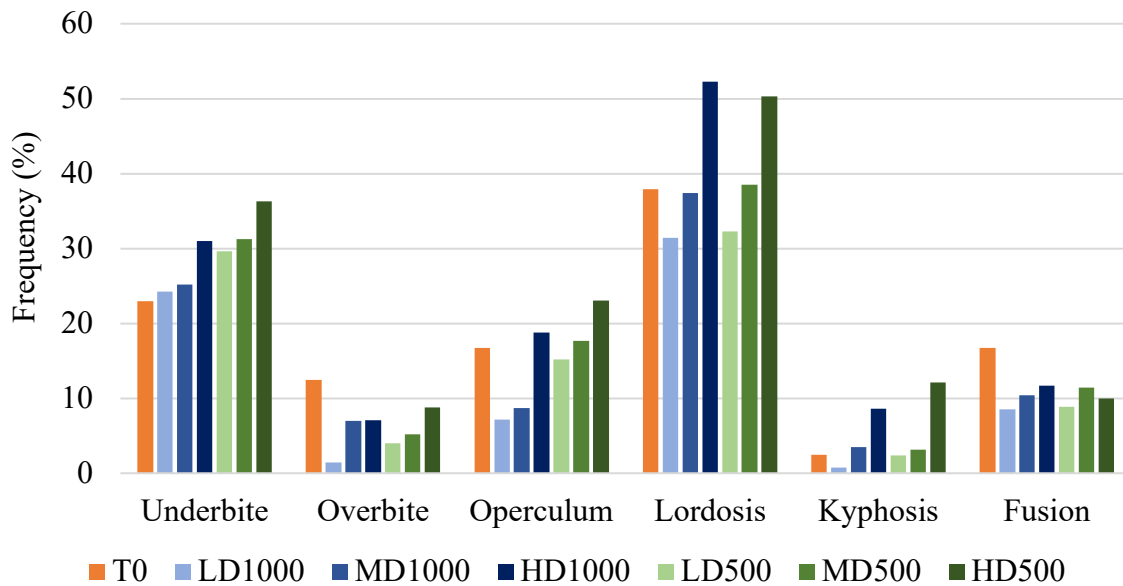


Figure 3.5. Frequency of individuals affected by skeletal anomalies in T_0 and into each experimental lot.

The T_0 lot (constituted of fish selected as non-deformed on the basis of external examination) resulted to be the one with the highest *anomalies charge* (an average of 3 anomalies for each deformed fish) (Tab. 3.2) with remarkable occurrences of individuals with anomalies, particularly lordosis (38% of individuals), mainly in the hemal region (32% of individuals; Fig. 3.5). Furthermore, vertebral centra fusions affected a larger number (17%) of T_0 than the T_f individuals. Concerning the T_f lots, the majority had at least one anomaly, with occurrences ranging from 63-88% (Tab. 3.2). A clear density effect is discernable by the fact that both of the HD conditions displayed the highest percentages of malformed individuals. The same density effect is present in the number of observed anomaly types. However, a secondary volume effect is detectable: the 500L lots displayed greater percentages of deformed individuals than the 1000L lots of equivalent densities (HD500>HD1000, MD500>MD1000, and LD500>LD1000).

The following sections report detailed descriptions of the observed anomalies in the different body regions.

3.2 Cephalic skeleton

The T_f samples revealed a fairly high frequency of individuals exhibiting an *underbite*, particularly in the HD500 lot (36.5%). The incidence of *underbite* among the conditions demonstrated a clear density effect as the frequency of affected individuals linearly increased with increasing density for both volume treatments (Fig. 3.6a; LD<MD<HD). Additionally, a volume-effect revealed by an augmented occurrence of fish in the 500L volume conditions compared with their 1000L equivalent density counterparts (Fig. 3.6a; LD1000<LD500, MD1000<MD500, HD1000<HD500). Although rarer, even *overbites* tended to be more frequent when the density

was greater in the 500L conditions (Fig. 3.6b LD<MD<HD), whilst the frequency in the 1000L lots was greater than those observed in the other two lots, quite similar (Fig. 3.6b; LD<MD≅HD).

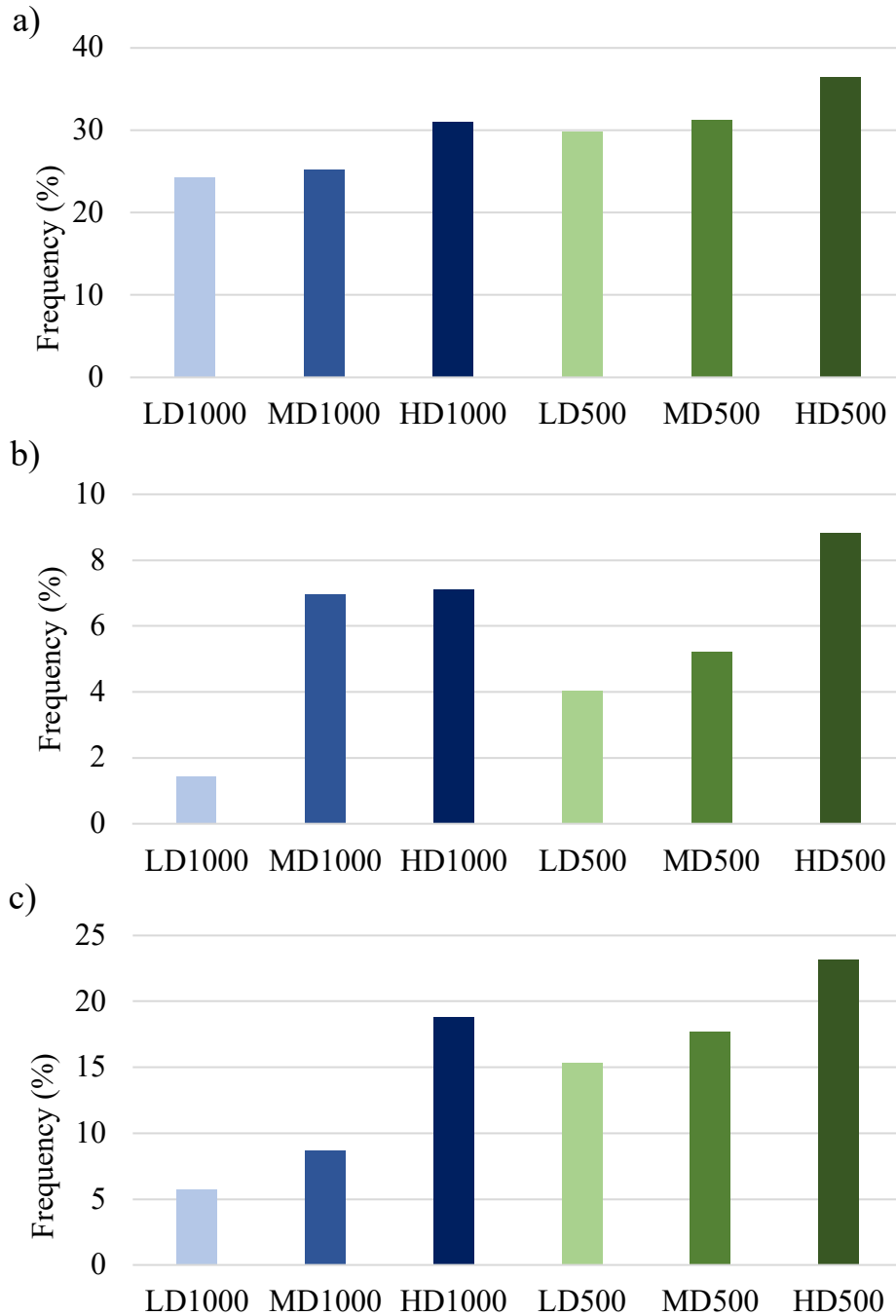


Figure 3.6. a) Frequency of individuals exhibiting an underbite. **b)** Frequency of individuals exhibiting an overbite. **c)** Frequency of individuals displaying a left-opercular plate anomaly.

Conversely to *underbite*, the incidences of seabream with *overbites* did not display the same volume effect, as the frequencies varied haphazardly and the differences were relatively small (Fig. 3.6b; LD1000<LD500, MD1000>MD500, and HD1000<HD500). However, the differences in the

frequencies of individuals affected by *underbites* and *overbites* resulted to be not significant among the experimental lots (Pearson's χ^2 test: p values = 0.2 and 0.09, respectively).

In the case of *opercular anomalies*, which were inspected only from the left side of the fish, a more frequent anomaly prevalence was associated with greater densities was clearly discernable in both volume groups (Fig. 3.6c; LD<MD<HD). Analogous to *underbite*, a secondary volume effect was also detectable as the smaller volume lots displayed a higher incidence than the large volume density equivalents (Fig. 3.6c; LD1000<LD500, MD1000<MD500, and HD1000<HD500). Conversely to jaw anomalies, these differences in fish affected by opercular anomalies resulted to be significant among the conditions (χ^2 ; p value < 0.001). The Dunn' *post hoc* with Bonferroni correction confirmed that significant differences exist between LD1000 vs HD1000, LD1000 vs MD500, LD1000 vs HD500, and MD1000 vs HD500 (Tab. 3.3).

Table 3.3. p values from the χ^2 test *post hoc* with Bonferroni correction for frequencies of individuals with opercular anomalies in the different experimental lots

	LD1000	MD1000	HD1000	LD500	MD500	HD500
LD1000		NS	0.0030	NS	0.0196	0.0001
MD1000			NS	NS	NS	0.0083
HD1000				NS	NS	NS
LD500					NS	NS
MD500						NS

3.3 Axial skeleton

Vertebral body counts ranged from 22 vertebral bodies to 25 in the LD1000 and MD1000 lots, while in the other lots the counts ranged from 22 vertebral bodies to 24 (Fig. 3.7). In the LD1000, MD1000 and LD500 lots, about the 85% of the specimens had a vertebral count of 24 (as in wild gilthead seabream (Prestinicola et al., 2013; Boglione et al., 2001)). When analyzing increasing densities, a tendency towards a lower vertebra counts appears (Fig. 3.7) mainly due to higher occurrences of vertebral centra fusions. As stated in the Material and Method session, incomplete fused bone elements (i.e., vertebral centra) were counted as discreet elements. This means that lower vertebrae counts associated with centra fusions refer exclusively to fusions which have already recovered, in which the centrum remodeled and assumed the normal amphicoelous shape but the presence of more than one neural or hemal arch shows that a fusion occurred. Specimens with both fusions and lower vertebral counts in the LD and MD lots exhibited a range of frequency from 0 –16.7% while in the HD conditions the frequencies were much higher (28% and 52.6% for HD1000 and HD500, respectively). However, the overall frequency of individuals exhibiting fused vertebrae did not vary greatly between the lots (8.6 –11.7%), with lower percentages in the LD lots with respect to MD and HD ones (Fig. 3.8). There were no significant differences between the lots (χ^2 test: p = 0.95).

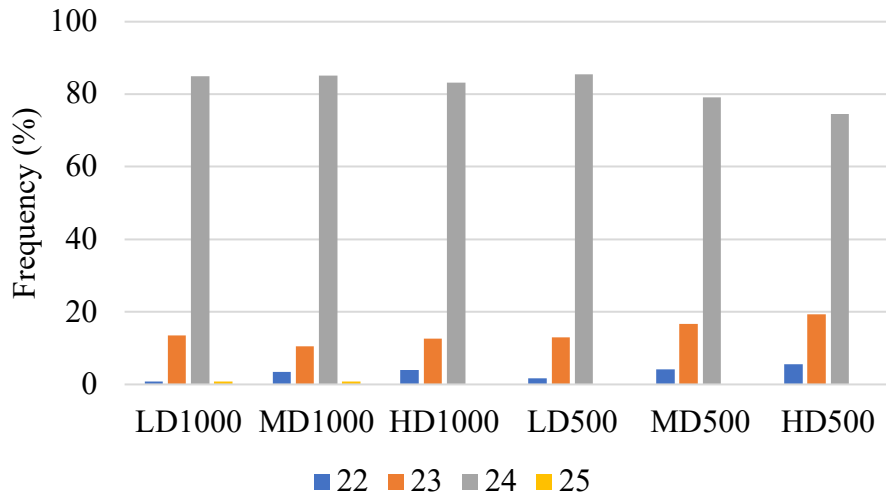


Figure 3.7. Frequency classes of vertebrae counts in the experimental lots.

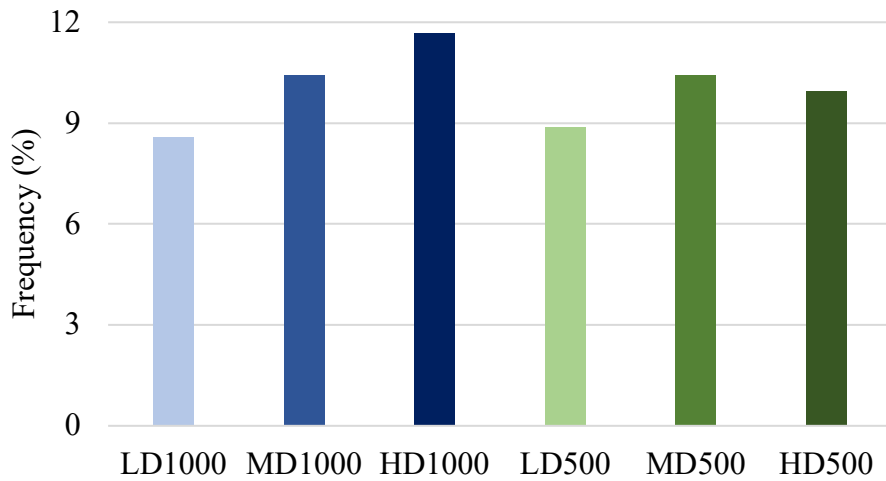


Figure 3.8. Frequencies of individuals with fusions of vertebral body centra in the experimental lots.

Concerning axial deformations, 42% of all individuals presented a lordosis and 6% a kyphosis. The histograms of the frequency classes in Figs. 3.9 and 3.10 show a progressive increment of individuals with lordosis and kyphosis with increasing density. The highest frequency of lordotic individuals was found in HD1000 (52.3%) followed by HD500 (50.3%), while the other conditions displayed lower frequencies which were roughly the same for each density group, indicating a clear density effect, but no secondary volume effect (Fig. 3.9; HD1000 (52.3%) > HD500 (50.3%) > MD1000 (37.4%) \cong MD500 (37.4%) > LD1000 (31.4%) \cong LD500 (32.3%)).

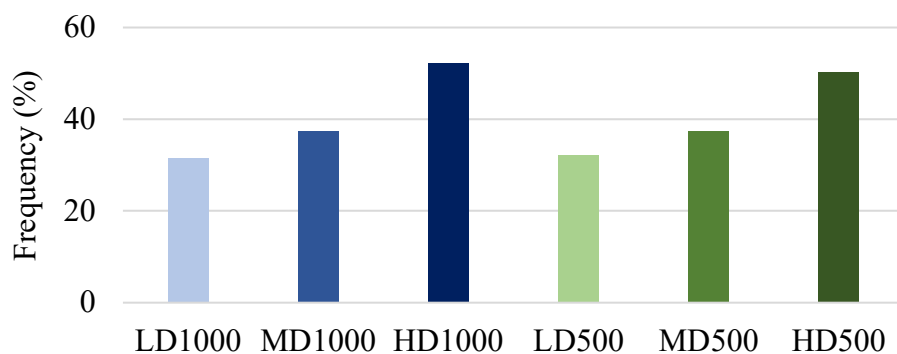


Figure 3.9. Frequency of individuals exhibiting lordosis in each lot.

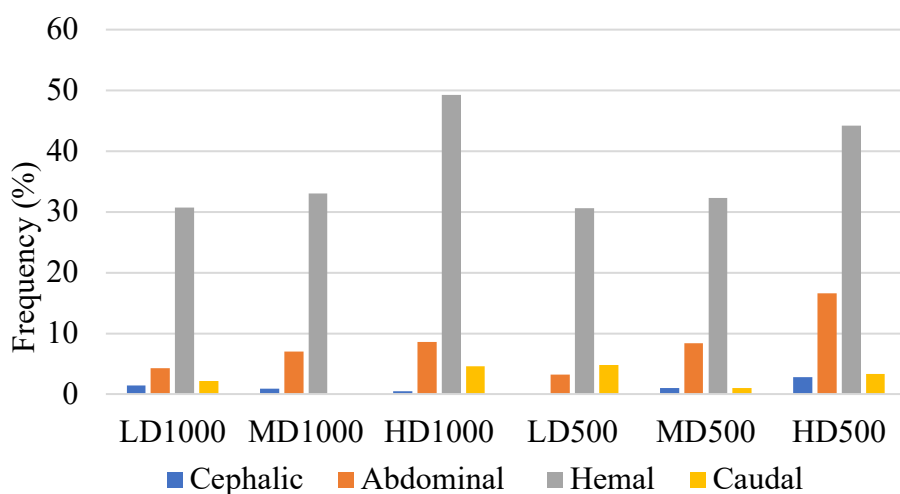


Figure 3.10. Frequency of individuals exhibiting lordosis in the different vertebral regions.

The region most commonly affected by lordosis was the hemal region in which the HD1000 lot followed by the H500 lot had the greatest frequency of individuals exhibiting a hemal lordosis (Fig. 3.10; 49.2% and 44.2%, respectively). Differences between the frequencies of LD and HD individuals affected by lordosis were statistically confirmed with a χ^2 test ($p < 0.00001$) and the subsequent pairwise *post-hoc* (Tab. 3.4).

Table 3.4. χ^2 test *post hoc* *p*-values with Bonferroni corrections for frequencies of lordotic individuals. NS = not significant.

	LD1000	MD1000	HD1000	LD500	MD500	HD500
LD1000		NS	0.0008	NS	NS	0.0042
MD1000			NS	NS	NS	NS
HD1000				0.0026	NS	NS
LD500					NS	0.0108
MD500						NS

Furthermore, the varying levels of severity applied to the angular classes (Tab. 3.1) of lordotic specimens among the conditions highlighted significant differences existing between the distribution of severity among the lots (Fig. 3.11; Tab. 3.5) but with a less clear trend.

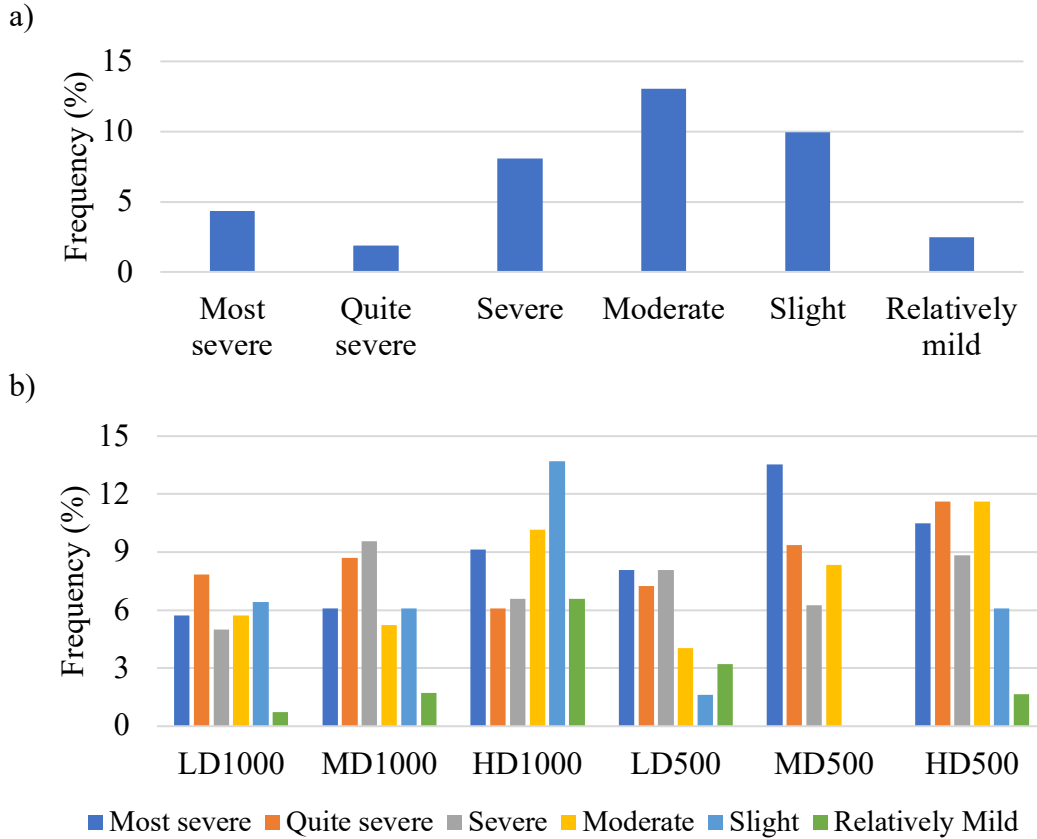


Figure 3.11. Distribution of different angular classes of lordotic curvatures: **a)** in the lot T_0 at the beginning and **b)** of the different lots at the end of the experimental rearing.

Table 3.5. KS tests with distribution of severity of lordosis (excluding the normal class). Only the significant ($p < 0.05$) comparisons are reported.

Pairwise <i>Post hoc</i>	<i>P</i> values
LD1000 vs HD1000	0.002
MD1000 vs HD1000	0.002
HD1000 vs LD500	0.002
HD1000 vs MD500	0.02
LD500 vs HD500	0.02

Kyphotic seabream were less frequent than the lordotic ones; however, an effect of density can be detected between all of the 1000L lots (LD<MD<HD), while in the 500L conditions the trend is

upheld only between HD and the other two conditions (Fig. 3.12; LD500: 2.4% \cong MD500: 2.1% < HD500: 12.2%). Overall, the highest prevalence of individuals displaying kyphosis was found in the HD500 (12.2%) followed by the HD1000 (8.6%), the χ^2 test confirmed significant differences between the frequencies of kyphotic individuals among all the conditions ($p < 0.0001$). The pairwise *post hoc* with Bonferroni corrections highlighted that significant differences were found between LD1000 vs HD1000, LD1000 vs HD500, HD500 vs MD1000, HD500 vs LD500, and HD500 vs MD500 (Tab. 3.6).

Table 3.6. χ^2 test *post hoc* p -values with Bonferroni correction for frequencies of kyphotic individuals

	LD1000	MD1000	HD1000	LD500	MD500	HD500
LD1000		NS	0.0087	NS	NS	0.0008
MD1000			NS	NS	NS	0.0344
HD1000				NS	NS	NS
LD500					NS	0.0206
MD500						0.0377

As far as the effect of tank size on occurrences of kyphotic individuals is concerned, none of the differences observed resulted to be statistically significant (Tab. 3.6). Furthermore, there were no significant differences in the distributions of severity classes (Fig. 3.12).

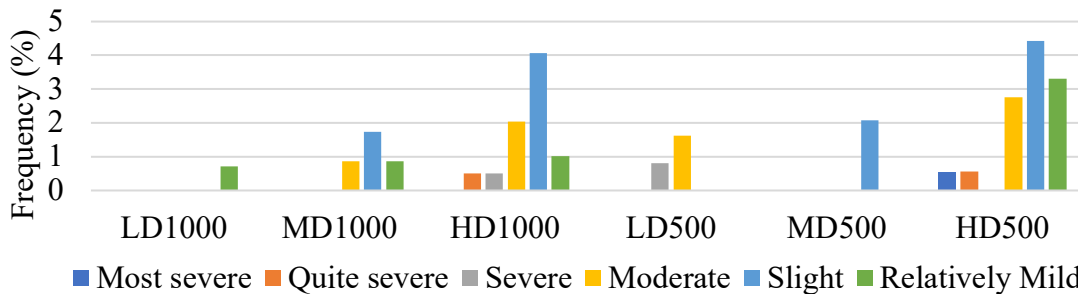


Figure 3.12. Distribution of different angular classes of kyphotic curvatures among the conditions.

Histological analyses highlighted interesting features of some vertebrae anomalies. In the compressed vertebral bodies shown in Fig. 3.13, a dorsal-ventral asymmetry is present. Presumably this is a step of the fusion process in which the hemal arches begin to first fuse, while the intravertebral space still persists. In a complete fusion of centra (Fig. 3.14), abnormal structural features of the vertebral centra associated to the point of fusion, such as reactive scar tissue of the notochord strand, lateral bending in the orientation of the collagen fibers constituting the trabeculae, elongation of vacuolated cells surrounding the notochord, and the persistence of an outer elastin layer (Fig. 3.15). On the other hand, histological sections of lordotic vertebral centra show the presence of only ventral stretching and dorsal compression without any presence of the anomalous features above described in fusing and fused vertebral centra (Fig. 3.16).

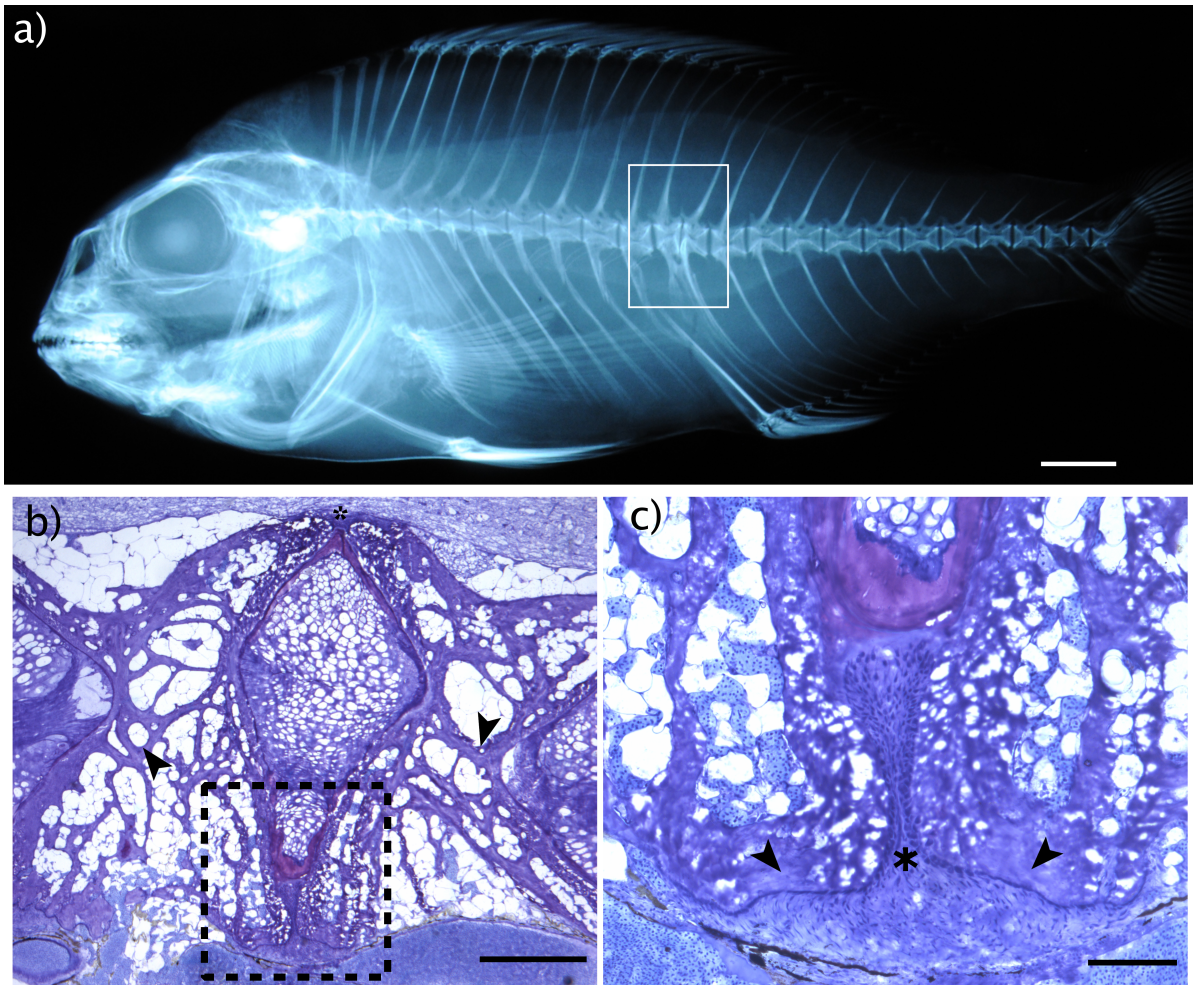


Figure 3.13. Compressed vertebral centra. **a)** Radiographic image of a T_0 seabream exhibiting two compressed vertebral centra (rectangle). Bar =5mm; **b)** and **c)** Toluidine blue stained sections of the deformed vertebral bodies shown in **a)**. The black arrowheads in **b)** point to bending trabeculae associated with remodeling of the misshapen centra. Note the dorsal-ventral asymmetry, where the ventral regions of the two centra appear enlarged. Bar =1mm; **c)** higher magnification of the region indicated with the black dashed rectangle in **b)**: the asterisk indicates at the insertion point of the hemal arches in the intravertebral space between the two centra. The black arrowheads point to the bone of the hemal arches. Bar =0.1mm

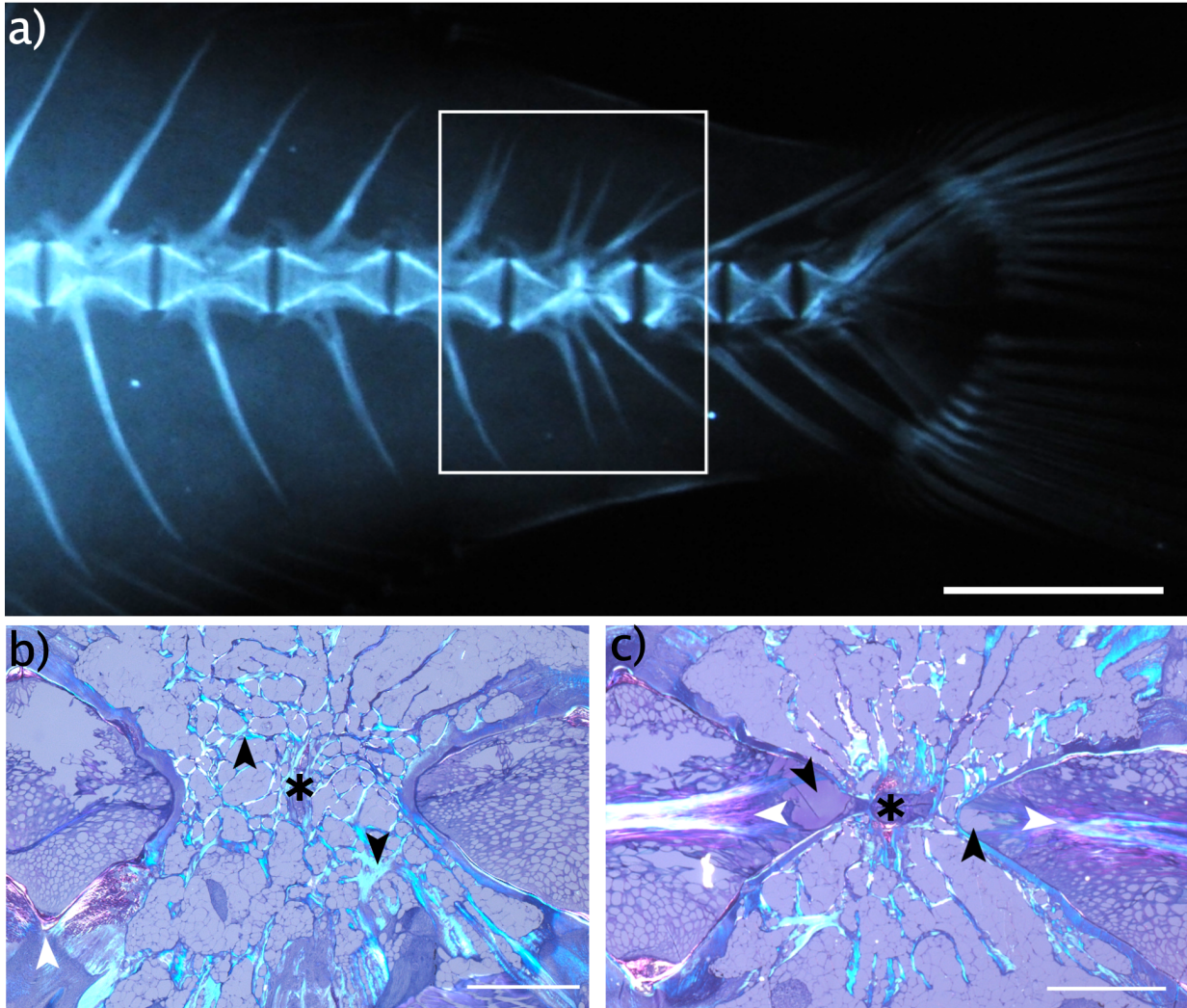


Figure 3.14. Complete fusion of vertebral bodies. **a)** Radiograph image of a T_0 seabream exhibiting a multiple fusion among vertebral centra (rectangle). Bar =5mm; **b)** and **c)** Toluidine blue stained parasagittal section of the multiple fusion shown in **a)**, photographed with polarized light. The black arrowheads in **b)** point to laterally bending trabeculae that surround the fusing vertebral centra (asterisk). The white arrowhead points to a normal vertebral body endplate. Bar =1mm; **c)** the black arrowheads point to putative reactive scar tissue of the notochord strand (white arrowheads), occurring proximal to the point of the fusing centra (asterisk). Note the hyaline rich cartilage (in pink) of the hemal and neural arches, located both dorsally and ventrally to the fusing centra. Bar =1mm.

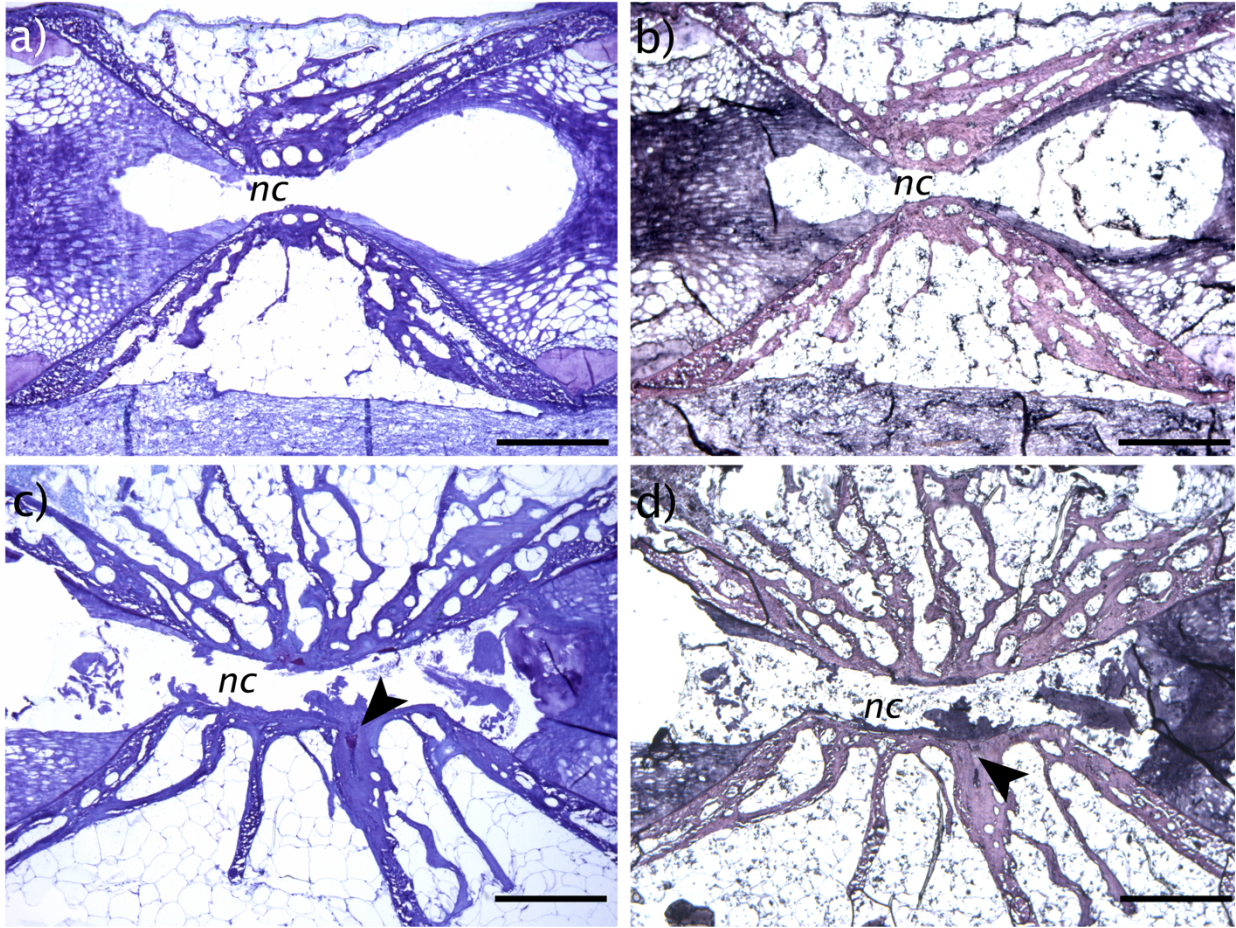


Figure 3.15. *a) Toluidine blue stained section and b) Verhoeff's elastin-stained section of a normal vertebral centra. c) Toluidine blue stained section and d) Verhoeff's elastin-stained section of a vertebral centra fusion. The black arrowhead in c) points to the persisting hyaline rich cartilage of the hemal arch; and in d) the arrowhead indicates an elastin layer. Bar =0.5mm*

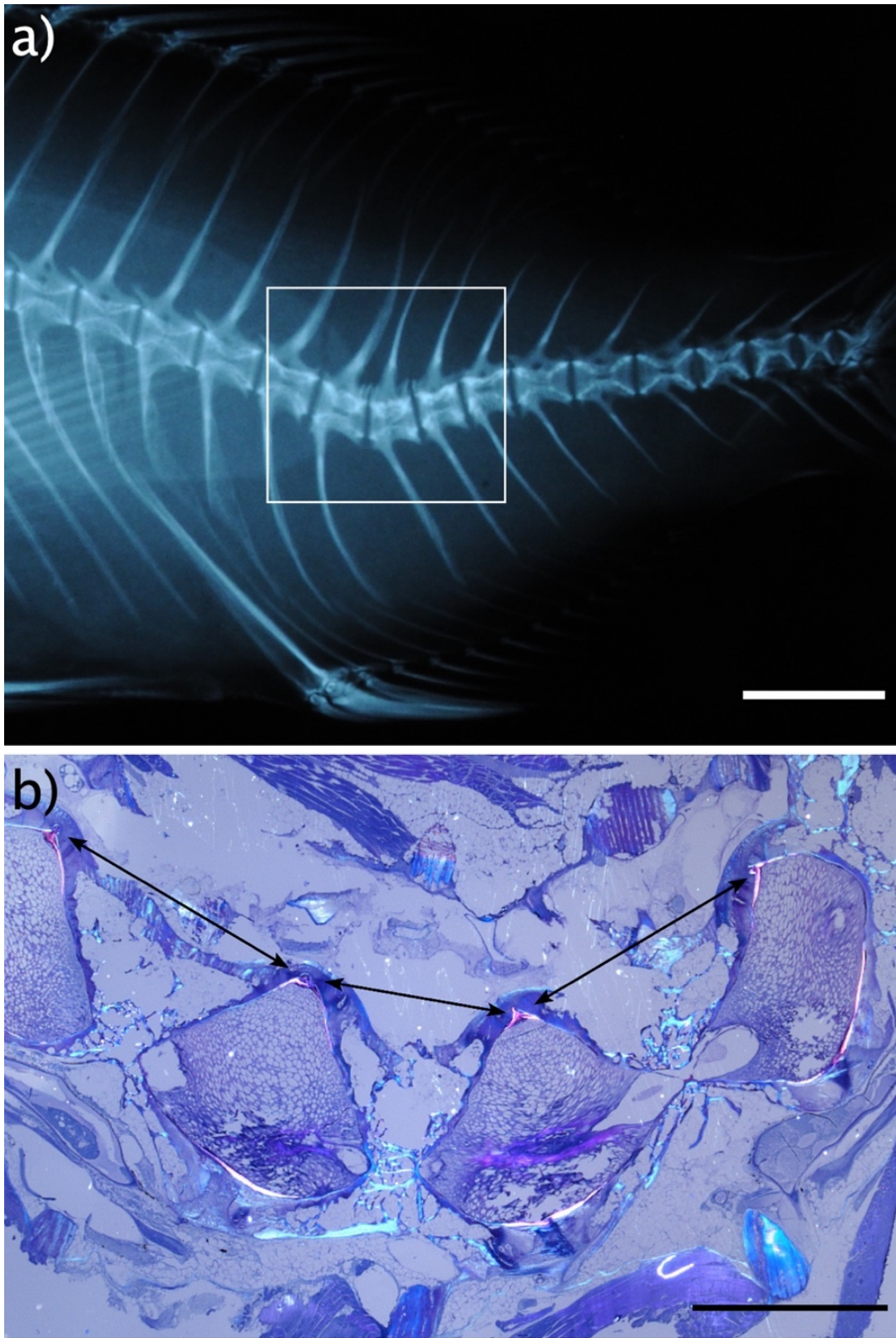


Figure 3.16. *a)* Radiograph of a lordotic seabream. Bar = 5mm; *b)* Toluidine blue parasagittal section photographed with polarized light of the lordotic vertebral body centra shown in *a)*. The double arrows point to the dorsal compression of the malformed centra. Bar = 2mm.

3.4 Correspondence analysis

An initial CA was attempted on the binary matrix consisting of 853 individuals and 26 anomalies but, due to the singularity of certain anomaly types (*i.e.*, hemal lordosis) and concomitant multiplicity of others (*i.e.*, deformed hemal vertebral bodies), the exhibited variance resulted particularly low. Then the CA was applied to a frequency matrix (641 x 6) built by grouping the anomalies in the following 6 categories: *jaw anomalies*, *opercular plate anomalies*, *lordosis*, *kyphosis*, *fusions of vertebral centra*, and *vertebral body shape deformities*. A total of 212 individuals, which did not exhibit any of the considered anomalies, were removed from the matrix due to a null data vector as they cannot be analyzed with vector normalization methods required for the CA. The ordination model obtained by the CA (Fig. 3.16) exhibited a total of variance on the first two axes (CA1: 23.62%; CA2: 21.72%) equal to 45,32 %. The position of the skeletal descriptors (Fig. 3.16b) and of the lot centroids (Fig. 3.16c) on the first two correspondence axes, enhance the close proximity in the 1st quadrant of the two HD lots (HD500 and HD1000) with the categories *lordosis*, *kyphosis*, and *jaw anomalies*, suggesting a strong effect of density on these anomalies. The centroids from the LD conditions, on the other hand, were positioned in the opposite half plane of CA1 (2nd and 3rd quadrant) together with MD500 and the category *vertebral body shape anomalies*.

3.5 Principal components analysis

A PCA was applied to 6 selected important classes of anomaly frequencies in order to corroborate and to augment the resolution of the ordination model for some more specific anomalies obtained by CA. The categories of the anomalies considered were *fusions*, *lordoses*, *overbites*, *kyphoses*, *opercular anomalies*, and *underbite anomalies* (Fig. 3.17). This PCA explained a total of 91.31% of variance in the first two axes (76.62% and 14.69%, respectively for the 1st and 2nd axes). In this ordination a clear effect of density is again distinguishable through the HD conditions position towards the negative direction of component 1, in the same direction of the skeletal categories' vectors, while MD conditions are located more towards the center of the plane and LD groups plotted in the positive direction of component 1. Although component 2 explains much less of the variance (14.69% vs 76.62%), it hints to a secondary volume effect, detectable by the positioning of 500L lots (green circles) in the positive half plane, in opposition to the 1000L lots.

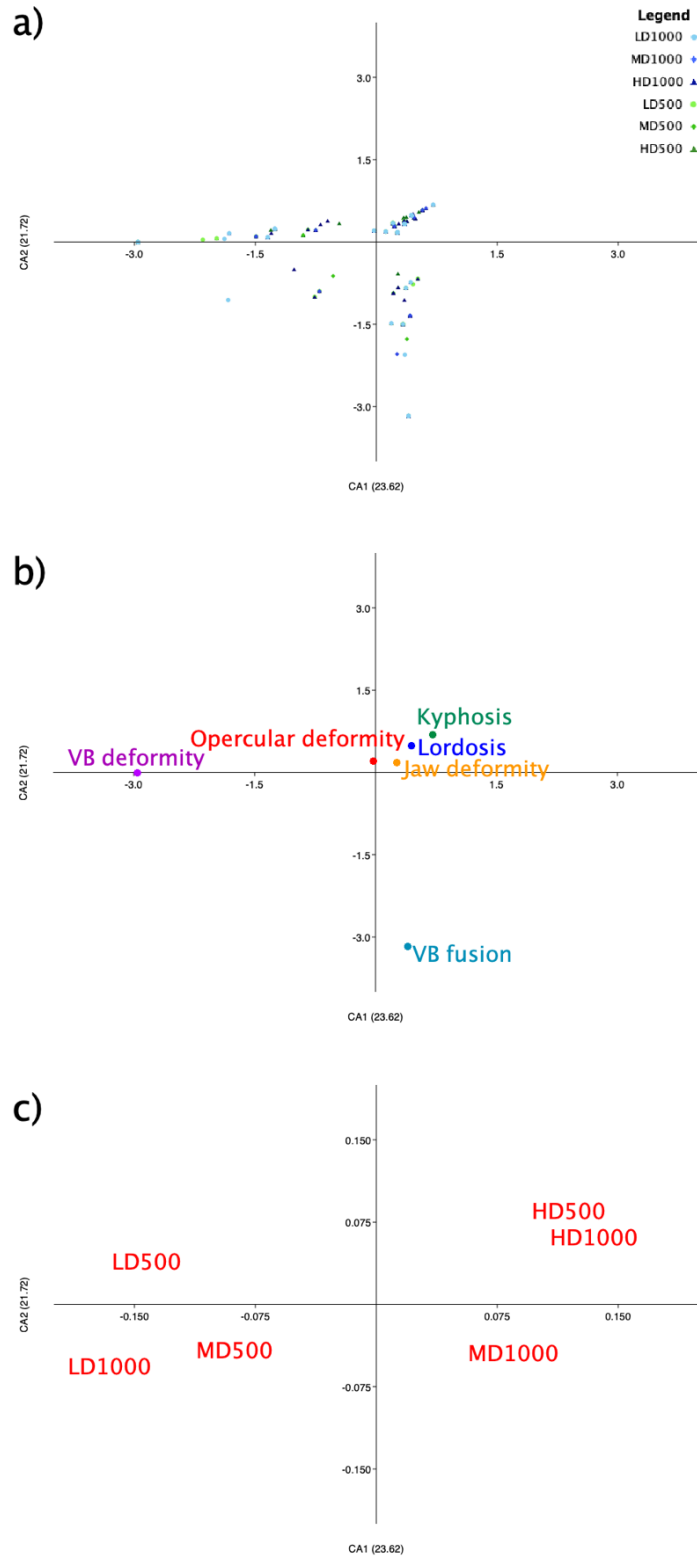


Figure 3.16. *a) CA ordination model relative to the first two CA axes showing the locations of all individuals. b) Ordination model of the descriptors (categories of skeletal anomalies. c) CA ordination model of the group centroids; note the increased magnification.*

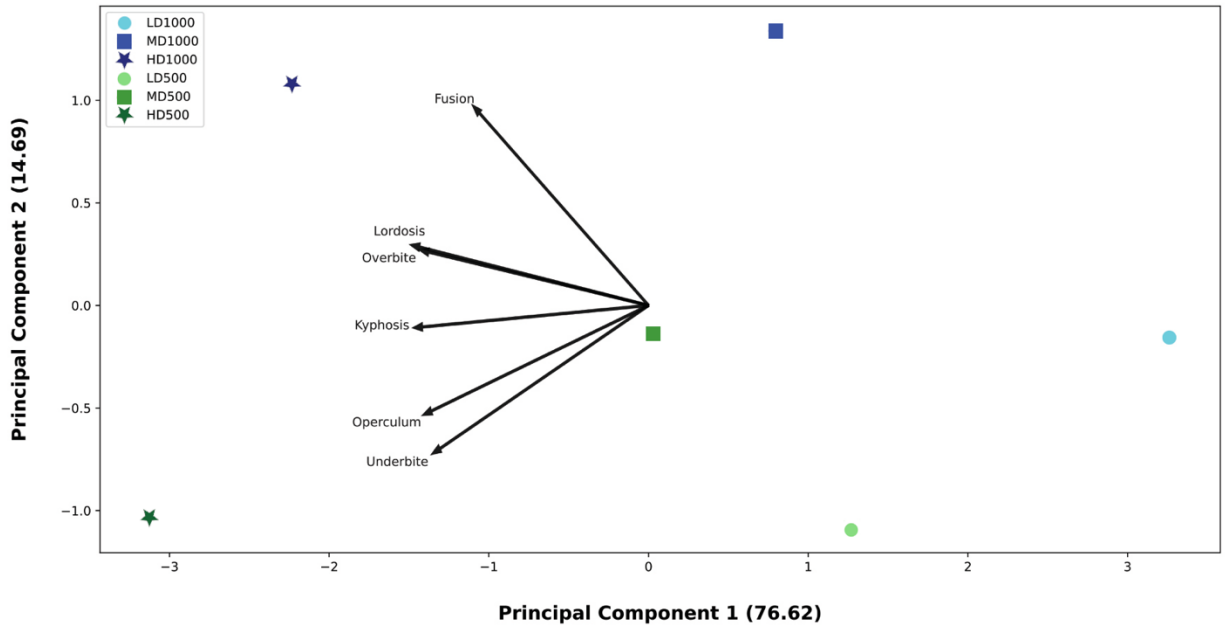


Figure 3.17 PCA ordination with selective skeletal anomaly descriptors and rearing conditions plotted. The resulting ordination suggests that the high-density conditions were associated with unfavorable output features such as fusions, lordoses, kyphoses, overbites, underbites, and opercular anomalies.

3.6 Geometric morphometrics

The *bg*PCA was applied to the residuals from the Procrustes transformation derived from landmarks plotted on the digital radiograph images. In this PCA the ordination revealed that inter-groups differences can be explained by 62.98% of the variance in the 1st axis and 24.01% of the variance in the 2nd axis (Fig. 3.18a). The LD lot centroids were both located on the positive plane of component 1, while the other lots are positioned on the opposite side towards the negative space of Component 1. While LD500 and LD1000 are located in opposite materials s of Component 2 (Quadrants 1 and 4, respectively), they are both distally positioned from the other conditions. Furthermore, lots reared with the same density and different volumes are positioned proximally to each other, thus suggesting a sound effect of rearing density on fish shape, conversely to the rearing volume that does not produce any detectable effect. Thin plate spline (TPS) deformations splines, which were superimposed to the PCA, assisted in further discerning shape differences among the conditions. The TPS deformation grid displayed in the positive hemi-plane of component 1 (proximal to the LD lot centroids) shows that LD individuals exhibited a slenderer shape than the roundish shape of the MD and HD individuals located on the negative hemi-plane of component 1 (Fig. 18a). Further, the TPS deformation splines corresponding to component 2 displayed both convergent and divergent expansion factors within the caudal peduncle region and the front rostral region (grids not shown).

The LDA (Linear Discriminant Analysis) ordination explained 54.51% and 27.05% of total variance in the first two axes, respectively (Fig. 3.18b). Additionally, the LDA further enhanced the detection of shape differences between the groups displaying a strong effect of density as the

LD condition centroids are positioned in the 2nd and the 3rd quadrants, in the negative space of LD1, while the MD and HD centroids are located on the positive hemi-plane of LD1, in the 1st and the 4th quadrants. LDA confirm that centroids of the lots reared with the same density are located proximally to each other, independently from the tank volumes.

Lastly, a MANOVA (Wilks' λ) applied to the uniform and non-uniform scores derived from the *weight matrix* revealed significant differences inter-groups, as well as pairwise test between the Mahalanobis squared distances (Tab. 3.7, Bonferroni corrected p -values, $p < 0.00001$). In particular, the greatest Mahalanobis squared distance was 14.9 which was found between LD500 and HD500.

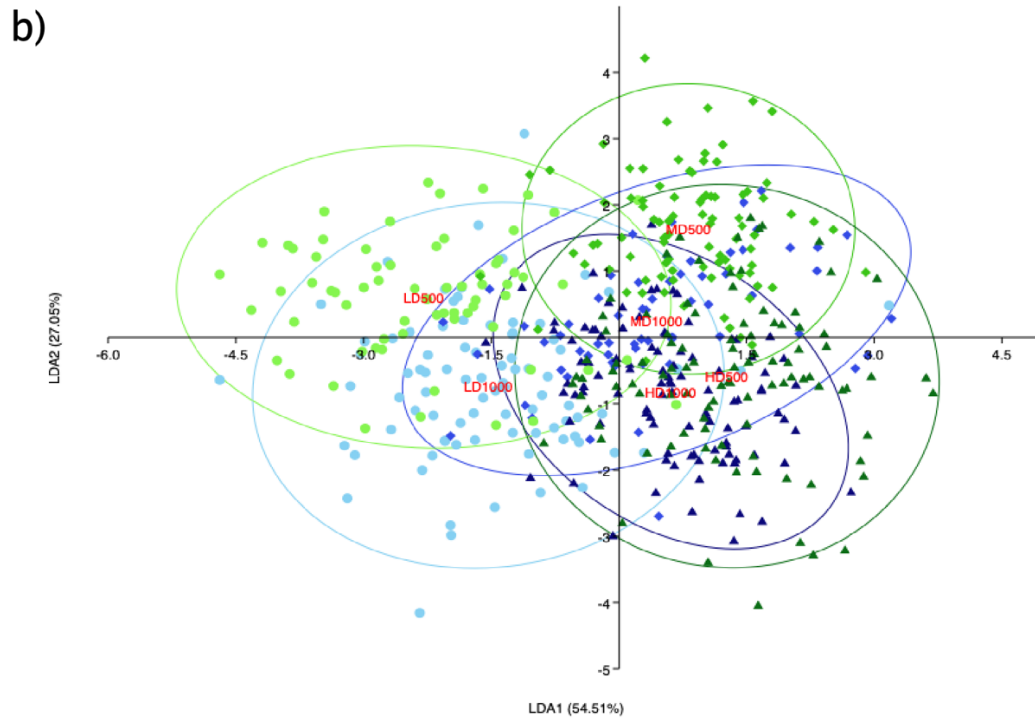
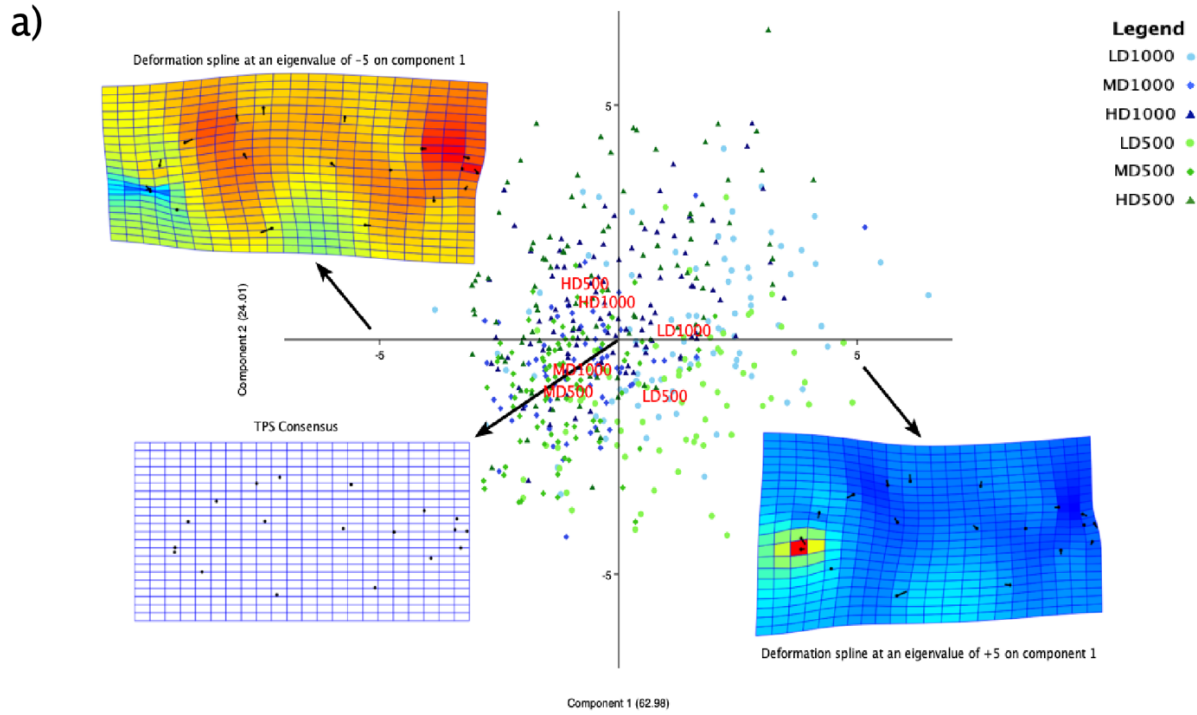


Figure 3.18. a) bgPCA scatter with deformation grids plotted on the extremities of the first component. TPS deformations are color coded in which cool colors represent convergence with respect to the consensus and warm colors represent divergence. **b)** Linear Discriminant Analysis (LDA) scatter results with 95% ellipses and lot centroids, highlighting group shape differences.

Table 3.7. Mahalanobis squared distances intra-groups

	MD1000	HD1000	LD500	MD500	HD500
LD1000	6.7184	6.5169	4.0499	11.34	8.7823
MD1000		2.5247	9.1422	3.6365	3.9081
HD1000			11.321	6.9757	2.9881
LD500				11.575	14.898
MD500					5.905

Significantly different distances were found between all groups (Bonferroni correction, $p < 0.0001$)

3.7 Calcium-phosphorous mineral content

Results from the MP-AES mineral quantification revealed the absence of significant differences in the calcium / phosphorous ratios between malformed lordotic vertebral bodies and normal vertebral bodies, although there does appear to be a greater variability of ratios in lordotic vertebral centra than in normal ones (Fig. 3.19).

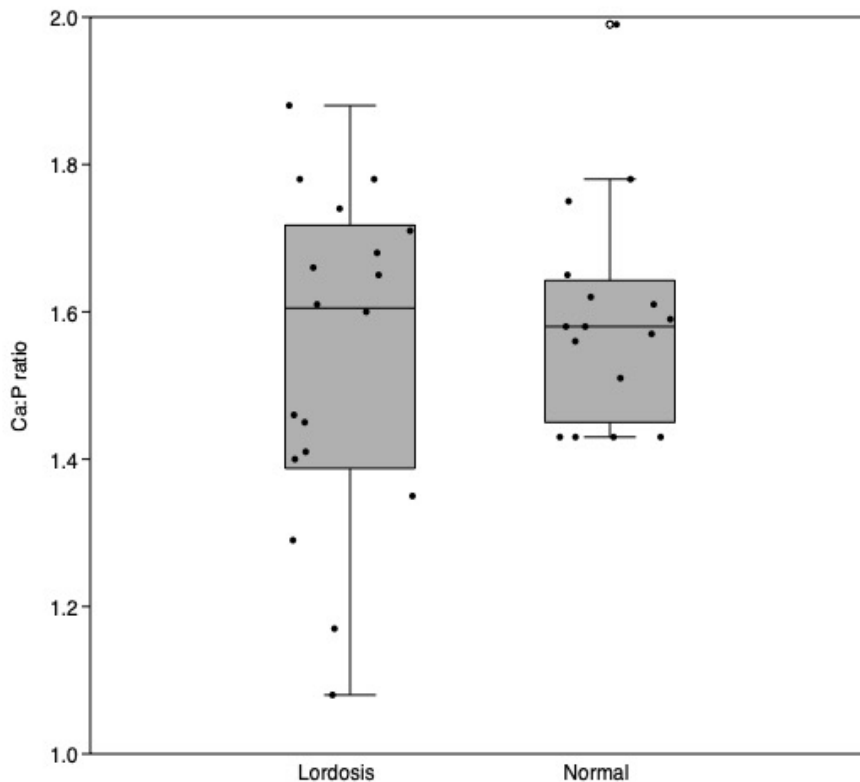


Figure 3.19 Calcium - phosphorous ratio between lordotic and normal centra.

4. Discussion

This study has demonstrated that 8.7g (average) gilthead seabream stocked in low densities for two months were significantly longer than those reared in higher densities. The fact that they were not heavier but presented significant differences in the condition factor K, indicated that LD seabream acquired a slenderer profile than the fish reared in MD and HD which appeared more roundish, as confirmed by the geometric morphometrics analyses. Both of these features could be due to the higher prevalence of lordotic fish found in high density conditions, due to the presence of axis deviations affecting the length and the body height, giving the deformed fish a stumpy body aspect. A previous study on adult gilthead seabream ($217 \pm 1.9\text{g}$) reared for 6 weeks at 5, 10, and 20kg/m^3 also found fish with greater weights in the LD conditions than in the HD conditions (Araújo-Luna et al., 2018). Even though the densities tested in the experiment by Araújo-Luna et al. (2018) were comparable to the densities we tested in our study, they did not remove fish to maintain the experimental densities as done in the present study, thus seemingly increasing interactions (food competition, individual dominance, etc.) in the tanks with greater fish biomass. Based on the results from geometric morphometrics, the PCA and LDA demonstrated that after only 63 days of rearing seabream sub-adults in the 6 different experimental conditions, significant differences in shape between the lots were already established primarily as a response to the stocking density, even though the fish belonged to the same initial starting group of fish from different origins. This is quite striking, since typically morphometric analyses enhance shape differences between different populations and/or species from different ecological (*i.e.*, feeding habits; Loy et al., 1998), evolutive, or phylogenetic contexts (Colangelo et al., 2019; Kerschbaumer and Sturmbauer, 2011), or in the same species, between differences in size classes (Costa et al., 2004), origins (Loy et al., 2000), life-stages, and/or long-term environmentally distinct factors (*i.e.*, between wild and reared seabream; Yiannis et al., 2011). It is likely that the differences we found after only 63 days of experimental rearing, in a quite advanced life stage dominated by isometric growth, are (at least to some extent) due to the greater frequency of deformed individuals determined by HD conditions. The Fig. 3.20, in which the spline of the centroid of a non-lordotic LD1000 individual is compared to the TPS of a lordotic HD500 individual, epitomizes this postulation.

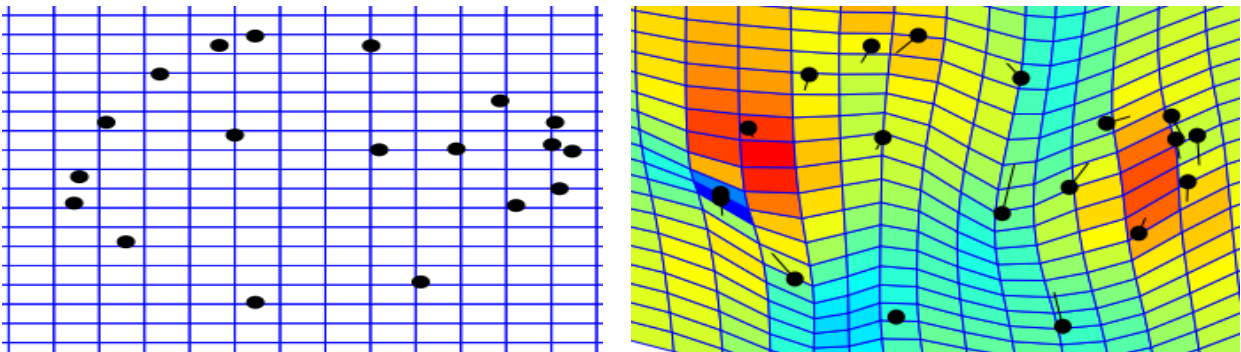


Figure 3.20. Normal LD1000 seabream TPS on left and lordotic HD500 seabream TPS on right.

Considering the frequencies and typologies of skeletal anomalies found in reared gilthead seabream, previous studies have primarily focused on intensive *versus* semi-intensive rearing (Prestinicola et al., 2013). The present study highlighted that when the effects of the rearing density are applied and analyzed separately from those of the tank volume, even when all other environmental factors are held constant, both exert some effects on the skeletal phenotype in gilthead seabream. However, these effects vary according to the skeletal region, the bones involved, and the type of ossification. For example, while an effect of stocking density is evident in the frequency of head anomalies and axial deviations, no clear effects are identifiable in the incidence of vertebral fusions, lordotic centra included. Probably, the fusions occurring between lordotic vertebral centra are the consequence, and not the cause, of the axis deviation, resulting in their appearance only later on. Furthermore, ‘volume effects’, even if secondary to the density, also evidently elicit differential responses according to different anomaly types. For example, head anomalies in this study were revealed to respond primarily to the effects of density in the incidences of underbite, overbite, and opercular anomalies, but only underbites and opercular anomalies resulted to be subjected to the effects of tank volume.

Overall, the most frequently observed anomaly found in all of the lots was lordosis. This anomaly is particularly problematic for farms as its presence greatly reduces the products’ value potential. In this study we found that the most common region of the body affected by lordosis was the hemal region. This finding is in accordance with other studies on the presence of hemal lordoses in seabream and has already been recognized to be frequently present in reared fishes (Koumoundouros, 2010). Furthermore, the severity of lordoses can range from light deformations, that do not present any clear effects on the external morphology, to very severe, with strikingly apparent impacts on external shape (Fragkoulis and Koumoundouros, 2021). The increased severity of lordosis based on the angle of curvature was found to be significantly related with the number of affected vertebral centra in European seabass (Sfakianakis et al., 2006), therefore angular measurements represent a reliable proxy for overall shape changes in the vertebral column. The classification of lordosis severity could also have useful applications for studies investigating recovery, due to recent findings that in certain cases lordoses can be recovered (Printzi et al., 2021; Fragkoulis et al., 2019; 2022). The potential for recovery is also supported by the results we found regarding the comparison between the incidence of lordotic individuals in T_0 and the final frequencies of lordotic individuals found in the LD conditions, suggesting the possibility of a recovery from lordosis after being reared in LD for 63 days. In this current study, the classification of lordosis severity demonstrated that at this life stage gilthead seabream show a wide array of severity levels and that differences between the distributions of severity classes significantly differed in response to the density. This is the first study that has statistically evidenced differences between severity classes, laying the foundations for future applications and studies of axial deviation severity, eventually to be associated with potential recovery. For example, farmers could screen for slight axis deviations and apply different rearing strategies (such as reducing stocking density) to effectively recover those fish before the severity increases beyond a threshold of no return, saving both time and money. Lordoses had been previously proposed to be present only in

association with improper inflation of the swimbladder (Chatain and Dewavrin, 1989; Chatain, 1994; Kitajima et al., 1994). However, Boglione et al., (1995) found lordotic seabass juveniles with functional swimbladder, and Andrades et al., (1996) described seabream larvae displaying a lordotic notochord even prior to the normal inflation of the swimbladder, suggesting that other etiologies may be responsible for eliciting this malformation. Recent studies, in fact, have suggested that the primary causes for lordoses in reared fish are connected to the swimming activity (Divanach et al., 1997; Kihara et al., 2002; Sfakianakis et al., 2006; Palstra et al., 2020; Printzi et al., 2021) and that lordosis is an adapted response to increased mechanical loading exerted by trunk muscles (Kranenbarg et al., 2005). This study found that there were no significant differences in the calcium-phosphorous ratio (Ca:P) between lordotic centra and normal centra, suggesting that lordosis could be more likely due to a biomechanical response rather than to metabolic alterations. This hypothesis seems to be corroborated by the healthy appearance of lordotic vertebrae from our histological analyses. Similarly, to our findings, Boursiaki et al. (2019) found no differences in the Ca:P ratio between scoliotic and non-scoliotic centra in seabream individuals. In our study, we observed a greater variability (even if not statistically relevant) in the values of Ca:P ratio in the lordotic vertebrae: this may have been a result of nonlinear responses to some differences in the mechanical loading which may stimulate voltage gate channels controlling the ratio between internal and external ion concentrations that potentially affects Ca:P ratio (Yavropoulou and Yovos, 2016).

The different water exchange rate could also have impacted the development of some anomalies like lordosis. An increased inlet flow velocity in the larger volume tanks was necessary to maintain the same water exchange as the small volumes. Previous studies have found that increased flow actually improved the growth rates in seabream (Blasco et al., 2015; Palstra et al., 2020). Enhanced growth due to exercise can occur when fish are subjected to higher water replacement regimes (with the exercised fish experiencing a replacement rate of 700L/h) which establishes hyperplasia and hypertrophy in the white muscles (Moya et al., 2019; Ibarz et al., 2011). Nevertheless, Palstra et al., (2020) found an association with high flow regimes (~0.78 m/s) and increased frequency of lordotic gilthead seabream. The water replacement rate and flow velocities tested in those studies were however higher than the maximum replacement and inlet velocities in our experimental rearing (maximum water exchange rate: 100%/h; flow velocities of ~0.73 and ~0.3 m/s for 1000L and 500L tanks at the inlets, respectively). Again, this potential effect of flow did not reveal itself in our experimental rearing as lordosis, dissimilar to cranial anomalies, no differences were present based on the different volumes (nor, consequently, flow velocity).

Considering the importance of the axial skeleton in response to swimming modalities coupled with the lack of a secondary volume effect, the presence of water flow and tank shape differences, it can be presumed that increased swimming space does not effectively influence the dorsal-ventral bending of the vertebral column, at least in the volumes we tested. On the contrary, the stocking density did play a driving role in the prevalence of lordosis. This factor undoubtedly influences the fish behavior by impacting the intensiveness of interactions (avoidance, escape, competition, aggression, etc.) among individuals, facilitating the introduction of hierarchies, which in turn can

elicit stress responses, and change the predominant swimming modality (pinning towards bursting). While LD fish have been noted to present less overall movement within the tanks but a greater diversity in their responses to behavioral and social tests (Sánchez-Muros et al., 2017), tighter interactions with consequent rapid changes of directions and burst swimming are more frequent in HD conditions, even when (as in our experimental rearing and in aquaculture farms) the number of fish is reduced regularly to mitigate fish biomass augmentation. Burst swimming is characterized by high speeds maintained for a few seconds (<20 seconds) (Beamish, 1978), rapid changes of direction by angular bending in the hemal region, and energy primarily supplied to myotomal white muscle through anaerobic processes (Gui et al., 2014). All of these features yield potentially dire consequences for the (hemal) musculoskeletal system (Arechavala-Lopez et al., 2020), with an onset of axis deviations in the hemal region induced by higher biomechanical pressure of muscle on underlying vertebrae.

Conversely, underbites and opercular anomalies resulted to be, to some extent, influenced by water volumes: smaller tanks aggravated the augmentation in occurrences of individuals affected by underbites and opercular plate anomalies. The presence of particular differences in tank height and water flow among the two tested volumes may offer some insights to putatively explain the positive effects of larger volumes on these anomalies. Oxygen diffusers were placed at the bottom of the tanks that were 1.2m and 0.6m in height in 1000L and 500L conditions, respectively. According to Soderberg (2014), oxygen bubble residence time is longer in taller vessels than in shorter ones, with consequent higher oxygen transfer efficiency. Furthermore, in order to maintain the oxygen saturation above 5mg/L and have equivalent water renewal rate in all of the experimental tanks, higher oxygen supply and water flow velocity were necessary in the 1000L tanks. These factors could have created a higher uniformity in oxygen dispersion in 1000L than in 500L. Therefore, in small volumes and HD conditions (where rapid changes of directions and burst swimming are more frequent), hyperventilation through increased opercular plate movements could have been required for the fish, provoking greater fatigue and exhaustion with subsequent increased feeding competitiveness, and stress. Conversely, the alleviation of these anomalies observed in 1000L tanks could be due to steady oxygen consumption, a reduced variability in head orientation that has been found to be associated with more steady swimming in response to increased water flow in gilthead seabream (Arechavala-Lopez et al., 2021), and increased swimming in response to water flow (Papoutsoglou et al., 1998).

Lastly, another potential factor that could have provoked the increase of frequency of anomalies found in HD is the internal response to stress. It has been demonstrated that gilthead seabream exhibit markers of stress in response to high rearing density (Mancera et al., 2008) which is also known to impact the immune response (Montero et al., 1999). Stress is also known to be associated with elevated production of reactive oxygen species (ROS) in sparids (Seo et al., 2020) and in turn, ROS is known to be triggered from nutritional imbalances, among other things, and linked to an increased presence of skeletal deformities in seabream (Izquierdo et al., 2019). Therefore, it is plausible that factors such as ROS production may have played a role in the increased presence of severe anomalies in the HD (more stressful) conditions.

Nevertheless, what has been discussed above should be considered in the context that the initial lot, T_0 , displayed a high presence of anomalies, whose occurrence in some cases were reduced, and in others were augmented in the final T_F seabream. The consequence is that the quality of the fish used to start the ongrowing phase define the quality threshold from which amelioration or worsening can be obtained by the predominant modulating factor: rearing density.

Chapter 4. Connecting the pieces of the puzzle

Sustainable aquaculture is a key driver for the blue economy, however, pertinent occurrences of deformed fish cause significant economic losses for farmers and hesitancy for aquaculture products by consumers, due to animal welfare issues and market-based aesthetic considerations. Given this scenario, it is critical that research assists the farmers offering the knowhow to reduce the presence of anomalies in their reared fish.

Though much work has been done in the last 30 years in attempt to elucidate the underlying factors eliciting the development of skeletal deformities in farmed fish, the persistence of varying incidences of severe skeletal anomalies signifies that there is still a lot of research yet to be done. Many different reasons justify this ostensible failure:

1) The farming of a fish species does not necessarily imply that the species has been domesticated (Teletchea, 2021). Animal domestication is a complex and multistage process at the end of which, animals result to be altered behaviorally, morphologically, and physiologically with respect to their wild ancestors (Ahmad et al., 2020). The debate on what is a domesticated fish is still ongoing (Balon, 2004; Bilio, 2008; Duarte et al., 2007; Gjedrem et al., 2012; Teletchea, 2017). Furthermore, in fish, domestication is not considered a definitive status since these animals continue evolving all the time and are even able to return and readapt to the wild (feralization is a major issue in the aquaculture industry globally) (Lorenzen et al., 2012; Glover et al., 2017). Accordingly, to different authors, 2 (Balon, 2004), 42 (Bilio, 2008), one-third (Teletchea, 2021), over 250 (Duarte et al., 2007) of the reared fish species could be considered domesticated.

2) More so than terrestrial vertebrates, fish skeletal tissues include several types of bone, many different types of cartilage, and many tissue types that are intermediate between connective tissue and bone as well as between bone and cartilage. Furthermore, other differences are present among teleost fish: i.e., cyprinids and salmonids (Basal Teleosts, 'primary freshwater fish') have bone that contains osteocytes, as well as mononucleated and numerous multinucleated osteoclasts. In almost all advanced marine Teleosts groups (such as gilthead seabream, European seabass, flatfish, tunas, etc.) as well as Teleosts which had reinvaded freshwater habitats ('secondary freshwater fish': e.g. cichlids, like tilapias, and Adrianichthyidae, such as medaka), osteocytes are no longer present (acellular bone) and the predominant osteoclast type is mononucleated (Bogliione et al., 2013a). In Basal Teleosts (as in mammals), bone resorption and remodeling are carried out by the multinucleated osteoclasts, whilst in Teleosts with acellular bone resorption and remodeling occur by osteoblasts and osteoclasts (Ofer et al., 2019; Witten and Huysseune, 2009). Not only do Teleost display a greater number of tissues and intermediate tissues than mammals (Witten and Huysseune, 2009), but Teleost that live in freshwater versus in the marine environment can have different bone

types with different predominant types of bone-resorbing cells. Consequently, insights gained from studies on the skeletal system of freshwater fishes or basal teleosts may not necessarily apply to marine species.

3) Many teleost species never stop growing, entailing that skeletal modelling is metabolic- and growth-related, and constant throughout life (Witten and Huysseune, 2009). The skeletal growth occurs through bone modeling, when the shape of a bone simply needs to be altered, or bone remodeling (bone turnover), when resorption is followed by new bone, without any change in shape. Nevertheless, acellular teleost bone must be remodeled in the frame of allometric growth, adaptation to mechanical loading, and continuous tooth replacement (Gunter and Meyer, 2014; Huysseune and Witten, 2006; Tu et al., 2012; Witten and Villwock, 1997). Lordosis, scoliosis, kyphosis, and fusion of vertebral bodies must involve bone resorption and bone remodeling, as a primary pathology or in response to altered mechanical loading (Kranenbarg et al., 2005; Witten et al., 2006).

4) Teleosts can repair their skeleton (e.g. fracture repair) (Moss, 1962; Dean and Shahar 2012) and dermal skeletal elements (teeth, scales, fin rays), but not the endoskeleton, which can be largely regenerated (Akimenko et al. 2003; Huysseune et al. 2007).

5) As reported in the Introduction, a multitude of factors can cause skeletal anomalies in reared fish (dietary/nutritional, biological, biochemical, genetic, physical, environmental, behavioral factors) that may act singularly or synergistically, and differently in specific species or life stages. The results obtained during the experimental work for this thesis succeeded in ameliorating the skeletal quality of reared juveniles of gilthead seabream by modulating two physical parameters, the tank volume and the rearing density, chosen among other possible parameters, due to their low-tech, low-cost, quicker, and easy duplicability. The obtained results enhanced that it is possible to significantly improve the survival, growth, and incidences of skeletal anomalies by rearing seabream from spawning up to 55g at lower densities, and that larger tank volumes may enhance some of these positive effects. In Fig. 4.1 the similarities and differences between the two experimental rearing case studies carried out during this PhD thesis are summarized.

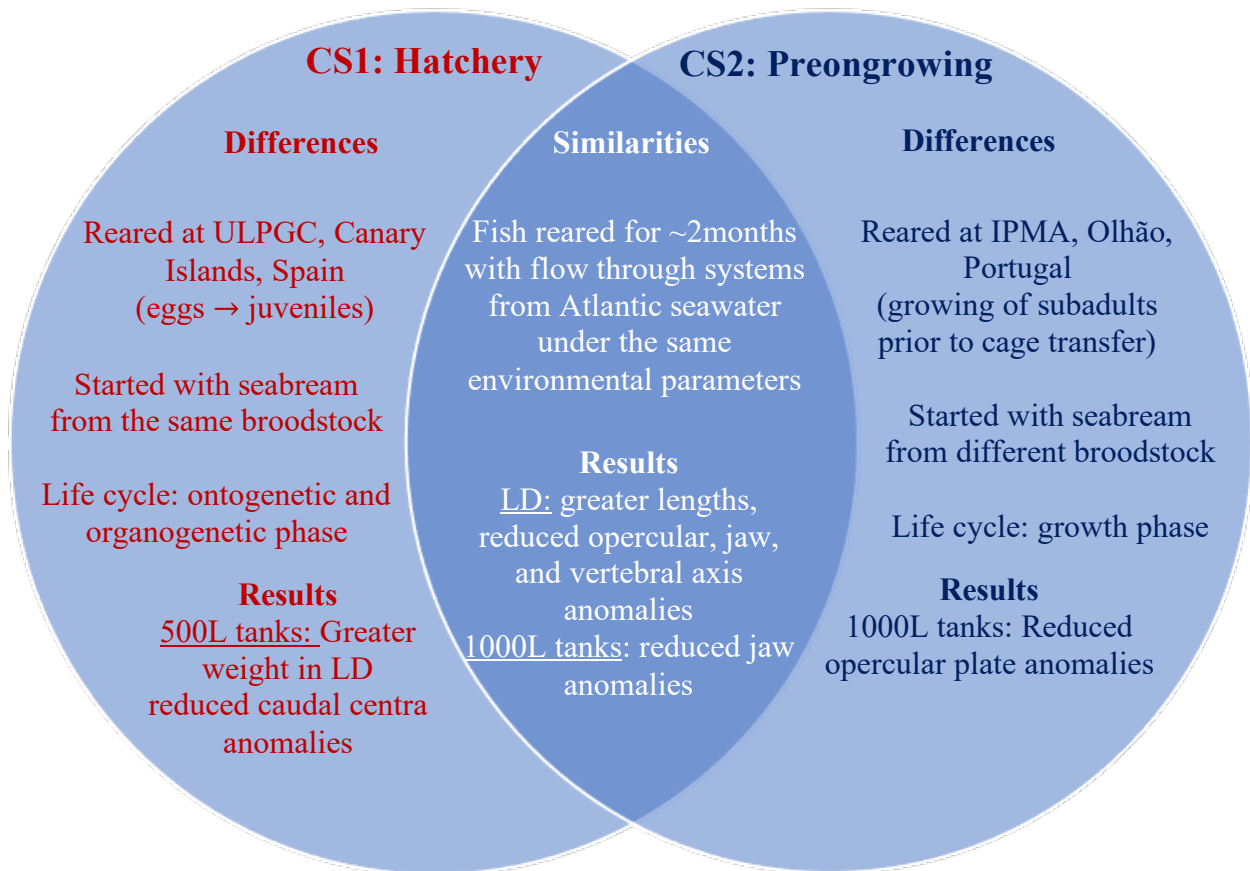


Figure 4.1. Venn-diagram displaying differences and similarities between the two case studies.

The most thought-provoking result is that, despite the differences noted above, gilthead seabream at different life stages (larval ontogenesis until the juvenile stage in CS1; and fry to a subadult stage in CS2), with different genetic origins (from the same broodstock in case 1; from different broodstock in case 2), reared in different facilities/countries (CS1: Canary Islands, Spain; CS2: Olhão, Portugal) responded with some of the same phenotypes to the same physical drivers (density or volume). The larval stage of CS1 represents a critical and unique life phase, characterized by the persistence of certain embryonic organs and subsequent replacement by different definitive organs of the same function, with consequence effecting mortality, allometric growth, changes in habitat, trophic ecology, feeding modality, type of swimming, social responses, and behavior. The subadult phase (CS2), conversely, can be characterized by a more canalized (Kozhara, 1994) and exclusive isometric growth. In this species, skeletal plasticity seems to react to the same challenging environmental condition by developing some homeorhetic trajectories that result in being preserved even if the environmental cue is confronted in very different life stages, independently from the genetic background. Regarding the anomalies that we found, the results from both CS1 and CS2 suggest that both cranial and axis anomalies were able to persist or worsen within the conditions and did not greatly impede feeding or swimming ability (ultimately, survival), at least up to the life stages/size classes we tested.

The possible explanations for the limited effects of water volumes on the incidence of skeletal anomalies in gilthead seabream are manifold. The necessity to carry out the experimental trial with a greater number of fish (initial number of larvae in CS1: 625,000; initial number of subadults in CS2: 4080) necessary to mimic as much as possible rearing at a commercial scale and regular farm management, permitted carrying a pilot scale experimental design as a proof of concept. Even though a ‘larger’ volume (1000L), which was twice the amount of the smaller volume (500L) was used, both of these tank volumes would be considered ‘small volumes’ in commercial farms (500-900L, Prestinicola et al., 2013). Furthermore, the large volumes tested in this thesis were not comparable with the ample swimming space as those which would be found in ‘true’ Large Volumes *sensu* Cataudella et al. (2002) or ‘mesocosms’ *sensu* Divanach and Kentouri (2000) systems (60,000 or 40,000L, respectively). The choice of the densities to be tested for this work was based on the precise requirements requested by farmers. This approach determined that even the tested LD, MD, and HD were proportionally greater than the densities applied in studies where intensive (100 larvae/L) conditions were compared to Large Volume and Mesocosm rearing systems (3-16 larvae/L).

The scope of this thesis work was to conclusively find a way to reduce the incidence of skeletal anomalies during the most critical phases of the production cycle of gilthead seabream, not to explicate the multitude of processes by which the anomalies could arise. However, some histological investigations and the evaluation of the Ca:P ratio present in lordotic and non-lordotic vertebrae were carried out in order to contribute to the basic knowledge on skeletal anomalies in reared marine fish. In the following paragraphs, I will summarize the main hypotheses regarding the processes leading to greater incidences of particularly frequent and stark splanchnocranial and vertebral anomalies in HD conditions.

1. Anomalies of the splanchnocranium

Regarding the anomalies of the splanchnocranium found in both the hatchery and the preongrowing phases, the low-density rearing resulted to be the driving factor in reducing the presence of jaw and opercular anomalies, while larger volumes only ameliorated the reduction of underbites and opercular anomalies. Furthermore, the results obtained in CS2 on preongrowing seabream, revealed that these anomalies augmented in frequency in HD individuals with respect to what was detected in the beginning of the experiment (lot T₀) as well as compared to the frequencies found in the individuals at T_F reared in lower densities. Interestingly, the results obtained in CS1 partially confirm what was described by Prestinicola et al. (2013), which indicated that in both semi-intensive conditions (Large Volumes and Mesocosms) only the bones having intramembranous ossification showed a consistently lower incidence of anomalies; while non-linear effects were observed in the skeletal elements whose ossification process requires a cartilaginous precursor.

The discrepancy in the effects observed in bones (dentary, maxillary, premaxillary, interopercle, opercle, preopercle, subopercle) located in the same body region (head) and sharing the same embryological origin (neural crest), and ossification modality (intramembranous ossification) (Hall, 2005), confirms previous observations that the same environmental factor (*i.e.*, tank volume)

may induce different incidences of anomalies affecting skeletal elements of the same bone type and ossification mode (Fernández and Gisbert, 2011), in the same species and life stage (Boglione et al., 2013b). This could be due to the fact that they play different functions which are not equally influenced by varying tank volume or stocking densities (*i.e.*, biting *vs* ventilation). In the discussion of CS2 it is reported the possibility that the differences found between 500L and 1000L tanks could have been due to some differences in the oxygen diffusion and water flow that may have yielded improved buccal pump efficiency, thus mitigating the development of opercular anomalies in 1000L. Furthermore, the discussion of data obtained in the CS1 highlighted how the greater number of larvae effectively stocked in HD conditions could have induced tighter schooling and increased competition for feeding, yielding stress responses as previously described in intensively reared gilthead seabream by Montero et al. (1999b), also triggering higher production of stress-related ROS (Seo et al., 2020). High levels of ROS have also been found to be correlated with an augmentation of skeletal deformities in seabream (Izquierdo et al., 2019). Subsequently, taking into consideration the above reported differences between the seabream of the two experimental trials, only the biomechanical causes can be soundly considered to explain the augmentation of jaws and opercular anomalies in HD conditions and the ameliorating effect on incidences of underbite and opercular anomalies detected in larger volumes.

2. Axis deviations and anomalous vertebrae

Regarding the vertebral column, again there were similarities in the responses to density based on the results from both of the CSs 1 and 2. However, the results from the hatchery phase revealed that vertebral column anomalies were primarily fusions in the caudal centra, while anomalies affecting the entire axis were relatively rare. Contrarily, in the preongrowing phase, fish resulted to be mainly affected by hemal lordoses involving several centra. Furthermore, during the hatchery cycle (CS1), unlike jaw anomalies, the larger volume 1000L conditions elicited reduced frequencies of caudal centra fusions, partial fusions, and hemivertebrae. This result confirms the lower occurrences of anomalies of the caudal vertebral bodies observed by Prestinicola et al. (2013) in semi-intensive conditions, with respect to the intensively reared sister lots. However, this effect was not confirmed in the CS2, thus representing again a differential response to the same environmental stimulus in skeletal elements located in the same skeleton region (caudal vertebrae), sharing the same embryological origin (embryonic paraxial mesoderm), as well as ossification modality (perichondral ossification). Again, these discrepant results between CS1 and CS2 seem to be linked to the different life-stages and to the different role played by the caudal vertebrae and fin in the different life stages (larvae *vs* subadult). In particular, the caudal fin is the first fin to differentiate during larval ontogenesis. Morpho-functionally the caudal fin is extremely important for larvae whose small size and slow swimming speed allows them to operate in the hydrodynamic regimes which are intermediate between viscous and inertial flow regimes. While in juveniles and subadults, the shift towards the full inertial flow regime is permanently set. Thanks to their low body inertia and fast neuromuscular system, the exploitation of high tail beat frequencies at the fastest speeds allows larvae to compensate for size-related limitations in swimming speed (Voesenek et al., 2018). Consequently, the caudal peduncle and fin are much

more strained during the larval phase compared to later life phases. Furthermore, according to the micro-environment the larvae face, different swimming behaviors can arise and become predominate. Larvae have two main types of fast-start responses, the C-start and the S-start, which differ in their initial movements, involving activity of muscles from different body regions and likely different motor neural circuits (Voosenek et al., 2018). In the C-start, the fish escapes from a transgressing stimulus by bending the body to form a C shape prior to forward propulsion. This is characterized by simultaneous unilateral muscle activity along one side of the body with little to no activity on the other side of the body. In contrast, the S-start motor pattern has simultaneous muscle activity in the rostral region of the body and posteriorly active bending in the caudal region in the opposite direction. This allows the larva to curve the body into an S shape, followed by an L-shaped bend, largely restricted to the caudal peduncle and fin. S-start behavior is used as an escape response and in predatory strikes, characterizing tail-elicited motions either towards prey or away from a predator (Hale, 2002). In higher stocking densities aggressive and escaping behavior could be more stimulated than in low density conditions. Therefore, a greater requirement of caudal muscles with consequential increased strain on the caudal vertebral bodies could be expected in high density conditions.

When considering axis deviations, lordosis affected fish in relatively high frequencies based on the results from CS2 and statistically significant differences were found between the density conditions, but not between the different volumes. On the other hand, lordosis and kyphosis were absent in the CS1 hatchery phase, as expected considering that vertebral axis deviations arise in older juveniles (Loizides et al., 2014).

As discussed in Chapter 3 (CS2), gilthead seabream reared in high density exhibited stronger schooling behaviors (Arechavala-Lopez et al., 2020) with an overload of mechanosensory stimuli (Anneser et al., 2020) and greater overall physical activity (Bégout and Lagardère, 1995). This status, together with the greater water flow, induced altered swimming behavior in seabream reared in higher densities, characterized by more frenetic activity and a reduced diversity in movement types (Sánchez-Muros et al., 2017), with consequential greater and/or different requirements of the different regions of the musculoskeletal system. This additional muscular exercise is recognized to induce positive effects on osteogenesis, mineralization, and recovery from skeletal degeneration. However frenetic activity and reduced movement types seem to have concomitantly provoked an abnormal modeling response of the skeletal elements (Suniaga et al., 2018). The mechano-sensory control of skeletal modeling and remodeling exerted by skeletal musculature seems to be ostensibly the main process yielding the reduction in the presence of lordotic fish detected in LD1000 and LD500 seabream with respect to T_0 and the greater occurrence of lordotic individuals in HD conditions.

This hypothesis is further validated by the finding that there were no significant differences in the calcium-phosphorous ratio (Ca:P) found between lordotic centra and normal centra. However, we observed a greater variability (even if not statistically significant) in the Ca:P ratios in the lordotic vertebrae. This could suggest that differences in the mechanical loading exerted by muscle mass on underlining bone may initiate nonlinear responses stimulating voltage gate channels, which

then modulate the ratio between internal and external ion concentrations potentially affecting Ca:P in bones (Yavropoulou and Yovos, 2016). Regardless, the inner structure of deformed vertebral centra appear to be healthy other than the evident bending shape, as confirmed from the histological analyses.

3. Conclusive Remarks

Future research in which anatomical/histological/statistical analyses (such as those carried out in this work) should be accompanied by biochemical, behavioral, and epigenetic data on a consistently high number of samples. Further studies are of paramount importance towards elucidating a complete picture of the processes which modulate the types and frequencies of skeletal anomalies when only one physical parameter (i.e., density) is altered.

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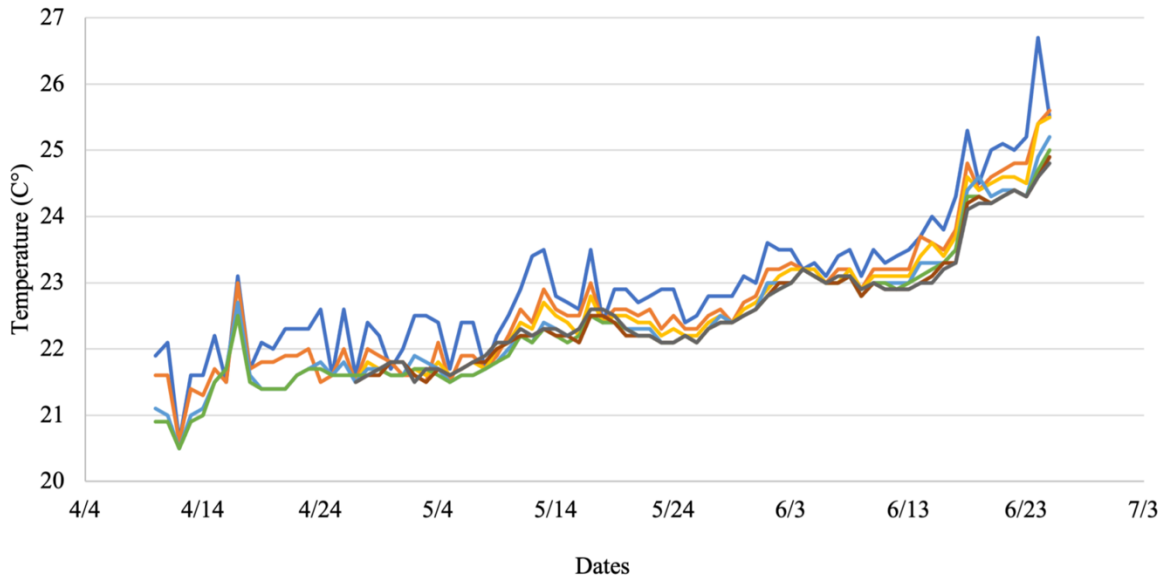
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Supplementary materials

a)



— LD1000 — M1000 — HD1000 T2 — HD500 — MD500 — LD500 T2 — LD1000 T2

b)



— LD1000 — M1000 — HD1000 T2 — HD500 — MD500 — LD500 T2 — LD1000 T2

Supplementary Figure 2.1. a) Temperature changes inside the tanks of the experimental conditions throughout rearing. b) Oxygen saturation (%) in all of the tanks of the experimental conditions throughout rearing.

Supplementary Table 2.2. Experimental data at the start and the end of the experimental rearing. T1= Trial 1, T2= Trial 2

Experimental conditions	Estimated initial number of larvae based on hatching rate (T1:97.5%; T2:98 %)	Estimated number of larvae based on survival at 3 dph (T1:92.5%; T2:83.5%)	Estimated initial densities (larvae/L) on the base of the hatching rate	Estimated initial densities (larvae/L) after 3dph	Estimated survival (%) after 60dph based on survival at 3 dph	Final number of individuals	Final densities (juveniles/L)	Average final TL (cm)	Final total biomass (g)
<i>LD 1000L T1</i>	25,000	23,128	25	23	8.9	2,053	2	2.50	384.5
<i>LD 1000L T2</i>	25,000	20,878	25	21	11.1	2,322	2	2.60	492.6
LD 1000L average	25,000	22,003	25	22	10.0	2,188	2	2.55	438.6
MD 1000L T1	125,000	115,638	125	116	7.9	9,110	9	2.03	770.7
HD 1000L T2	250,000	208,775	250	209	3.3	6,957	7	2.00	660.2
LD 500L T2	12,500	10,439	25	21	29.7	3,102	6	2.26	385.3
MD 500L T1	62,500	57,819	125	116	3.7	2,158	4	2.06	231.3
HD 500L T1	125,000	115,638	250	231	7.1	8,249	17	1.82	527.1

Supplementary Table 2.3. List of considered anomaly types

<i>Region</i>	
A	Cephalic vertebrae
B	Abdominal vertebrae
C	Hemal vertebrae
D	Caudal vertebrae
E	Pectoral fin
F	Anal fin
G	Caudal fin
H	Dorsal spines
I	Dorsal soft rays
L	Pelvic fin
<i>Type</i>	
1	Kyphosis
2	Lordosis
3	Partial vertebral fusion
3*	Total vertebral fusion
4	Vertebral shape anomaly
5	Anomalous neural arch/spine
5*	Supernumerary or absence of neural element
6	Anomalous hemal arch/spine
6*	Supernumerary or absence of hemal element
7	Anomalous rib
8	Anomalous pterygophores
9	Anomalous hypural
9*	Anomalous parahypural
10	Anomalous epural
11	Anomalous ray
13	Presence of calculi in urinary duct
14	Anomalous maxillary/premaxillary
15	Anomalous dentary
16	Other cephalic deformities
17L/R	Anomalous opercular plate
17*L/R	Anomalous branchiostegal rays
18	Predorsal bone anomalies
19	Decalcified hypurals
20	Decalcified pterygophore
21	Anomalous epipleural ribs
22	Anomalous dorsal ribs
23	Anomalous pleural rib
24 L/R	Decalcified opercular plate
25	Epural with decalcifications
26	Supernumerary element
27	Decalcified urostyle
28	Decalcified vertebrae
29	Anomalous postcleithrum
30	Box-shaped vertebral bodies
31	Decalcified elements of arches
32	Ectopic element
33	Hyperostosis
34	Flagged arch
EO	Extra hemal ossicle in the caudal region
S	Scoliosis
HMV	Hemivertebrae
CIL/R	Anomalous cleithrum
Cor L/R	Anomalous coracoid

Supplementary Table 2.4. Descriptors used to perform the different Multivariate Analyses.
See Supplementary Tab. 3 for the codes of the anomalies. VB: vertebral body anomalies.

<i>CA</i>				
Head anomalies	Severe vertebrae and column anomalies	Arches (5* and 6*) anomalies	Anomalies of unpaired fins	
14	B3	A5*	F8	H20
15	B3*	B5*	F11	H26
16	C3	C5*	F32	H32
17L/R	D3	C6*	G9	H11
17*L/R	D3*	D5*	G10	I32
	DHMV	D6*	G11	H8
	S		G19	18
			G32	I11
			H18	I20
				I32

<i>PCA</i>					
Standard Length	Survival	Dry Weight	VB shape	Malformation index	Operculum
Length in cm of 100 specimens per condition	Percentage of surviving individuals per condition	Weight in g of 100 specimens per condition	A4 B4 C4 D4	Number of observed malformations/ numbers of malformed individuals	17L/R 17*L/R

<i>Chi-squared</i>					
VB shape	Associated arches*	Severe	Jaws	Operculum	Vertebrae fusion
A4	A5*	14	14	17L/R	B3
B4	B5*	15	15	17*L/R	B3*
C4	C5*	16			C3
D4	C6*	17L/R			D3
	D5*	B3			D3*
	D6*	B3*			
		C3			
		D3			
		D3*			
		HMV			
		S			

Supplementary Table 2.5. Relative frequency of specific skeletal anomalies among the conditions

%	LD1000	MD1000	HD1000	LD500	MD500	HD500
<i>A4</i>	0.07	0.11	0.11	0.00	0.00	0.08
<i>A5</i>	8.22	2.67	4.80	7.81	4.61	4.40
<i>A5*</i>	0.07	0.38	0.44	0.19	0.29	0.53
<i>A5/33</i>	0.00	0.08	0.00	0.00	0.05	0.00
<i>A5/31</i>	0.00	0.00	0.00	0.00	0.00	0.11
<i>A32</i>	0.00	0.11	0.11	0.00	0.00	0.11
<i>A5/34</i>	0.07	1.20	0.60	0.56	0.88	0.98
<i>A14</i>	1.81	1.20	2.18	2.82	2.16	1.65
<i>A15</i>	0.28	0.34	0.54	0.19	0.20	0.34
<i>A16</i>	3.27	1.02	2.83	2.63	1.77	1.47
<i>17L</i>	0.21	0.26	0.38	0.19	0.25	0.19
<i>17*L</i>	0.14	0.45	0.33	0.19	0.15	0.30
<i>17R</i>	0.14	0.19	0.44	0.09	0.15	0.23
<i>17*R</i>	0.14	0.49	0.38	0.19	0.15	0.38
<i>B3</i>	0.00	0.00	0.00	0.00	0.29	0.00
<i>B3*</i>	0.00	0.00	0.00	0.00	0.05	0.00
<i>B4</i>	1.11	2.33	0.49	1.69	1.91	3.76
<i>B5/34</i>	2.23	5.34	2.40	0.75	2.80	4.21
<i>B5</i>	10.93	12.37	12.37	11.67	16.53	12.78
<i>B5*</i>	0.07	0.19	0.16	0.19	0.29	0.04
<i>B6</i>	0.56	0.04	0.11	0.75	0.05	0.23
<i>B6/34</i>	0.07	0.04	0.00	0.09	0.00	0.04
<i>B7L</i>	10.58	3.16	6.32	3.20	7.80	4.89
<i>B7R</i>	15.39	3.87	9.59	6.87	8.88	5.04
<i>B33</i>	0.00	0.60	0.05	0.09	0.15	0.26
<i>B32</i>	0.07	0.00	0.00	0.09	0.05	0.04
<i>C3</i>	0.00	0.00	0.00	0.00	0.00	0.04
<i>C4</i>	3.76	15.30	10.35	16.18	9.96	16.42
<i>C5</i>	3.97	15.08	10.74	8.00	8.68	9.06
<i>C5*</i>	0.28	0.11	0.60	0.28	0.29	0.19
<i>C6</i>	4.60	12.89	10.19	7.81	8.58	9.70
<i>C5/34</i>	1.11	1.65	1.20	0.47	1.81	2.14
<i>C6/34</i>	5.01	6.88	5.50	3.01	6.47	6.43
<i>C6*</i>	0.49	0.49	0.60	0.94	0.83	0.60
<i>C33</i>	0.35	1.24	0.05	0.94	0.20	0.68
<i>C5/32</i>	0.00	0.00	0.05	0.00	0.20	0.15
<i>D3</i>	0.07	0.15	0.11	0.00	0.05	0.04
<i>D3*</i>	0.07	0.08	0.33	0.00	0.05	0.23

<i>D4</i>	0.35	1.73	1.36	0.38	1.23	1.80
<i>D5</i>	0.49	0.26	0.11	0.28	0.34	0.45
<i>D5*</i>	0.97	1.02	1.47	0.94	1.03	0.49
<i>D6</i>	0.07	0.23	0.22	0.94	0.39	0.45
<i>D6*</i>	0.07	0.38	0.98	0.38	0.49	0.30
<i>D6/32</i>	0.07	0.04	0.00	0.00	0.05	0.04
<i>DHMV</i>	0.00	0.11	0.33	0.09	0.15	0.11
<i>E11 Lx</i>	0.21	0.08	0.00	0.00	0.00	0.04
<i>E11 Rx</i>	0.21	0.04	0.00	0.09	0.00	0.04
<i>F8</i>	1.74	0.19	0.33	1.69	0.34	0.41
<i>F11</i>	0.00	0.08	0.00	0.09	0.00	0.11
<i>F32</i>	0.00	0.04	0.00	0.28	0.00	0.00
<i>G9</i>	6.41	1.88	2.72	4.52	2.16	2.29
<i>G10</i>	6.55	1.54	2.62	4.99	2.80	2.03
<i>G11</i>	0.14	0.00	0.00	0.28	0.05	0.11
<i>G19</i>	3.76	0.19	1.96	1.51	0.49	0.26
<i>G32</i>	0.21	0.38	0.82	0.47	0.88	0.41
<i>H18</i>	0.42	0.68	0.71	1.03	1.62	1.24
<i>H20</i>	0.07	0.00	0.00	0.00	0.00	0.00
<i>H26</i>	0.07	0.00	0.00	0.00	0.00	0.08
<i>H32</i>	0.21	0.08	0.16	0.56	0.25	0.15
<i>H11</i>	0.07	0.00	0.00	0.09	0.05	0.11
<i>I32</i>	0.00	0.00	0.00	0.09	0.05	0.04
<i>H8</i>	1.46	0.26	0.65	1.41	0.39	0.38
<i>I8</i>	0.84	0.15	0.22	0.94	0.20	0.15
<i>I11</i>	0.21	0.00	0.00	0.00	0.00	0.04
<i>I20</i>	0.00	0.00	0.05	0.00	0.00	0.00
<i>I32</i>	0.00	0.00	0.05	0.09	0.05	0.15
<i>EO</i>	0.28	0.30	0.87	0.66	0.25	0.30
<i>S</i>	0.00	0.04	0.00	0.00	0.00	0.08
<i>Cor L/R</i>	0.00	0.04	0.00	0.28	0.05	0.19
<i>CI L/R</i>	0.00	0.00	0.00	0.00	0.15	0.08

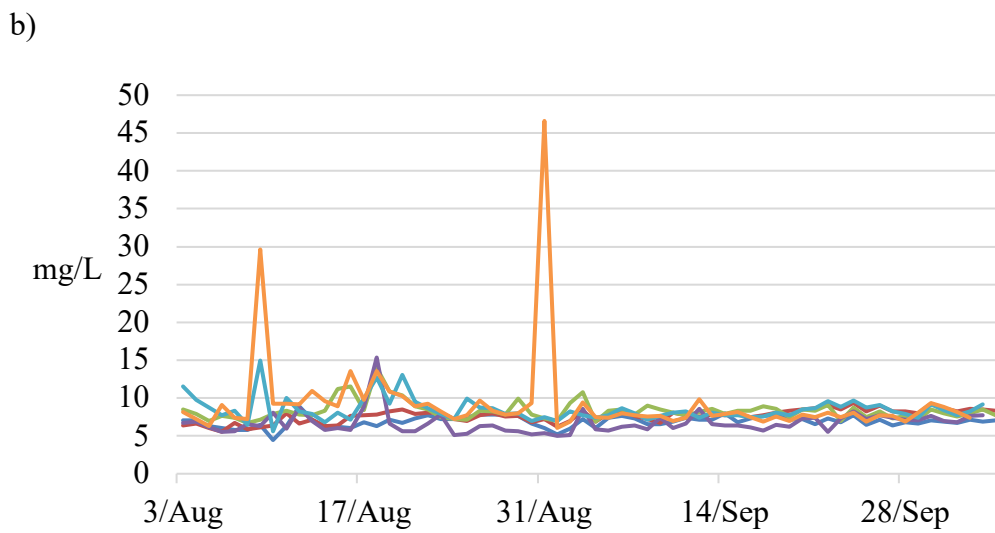
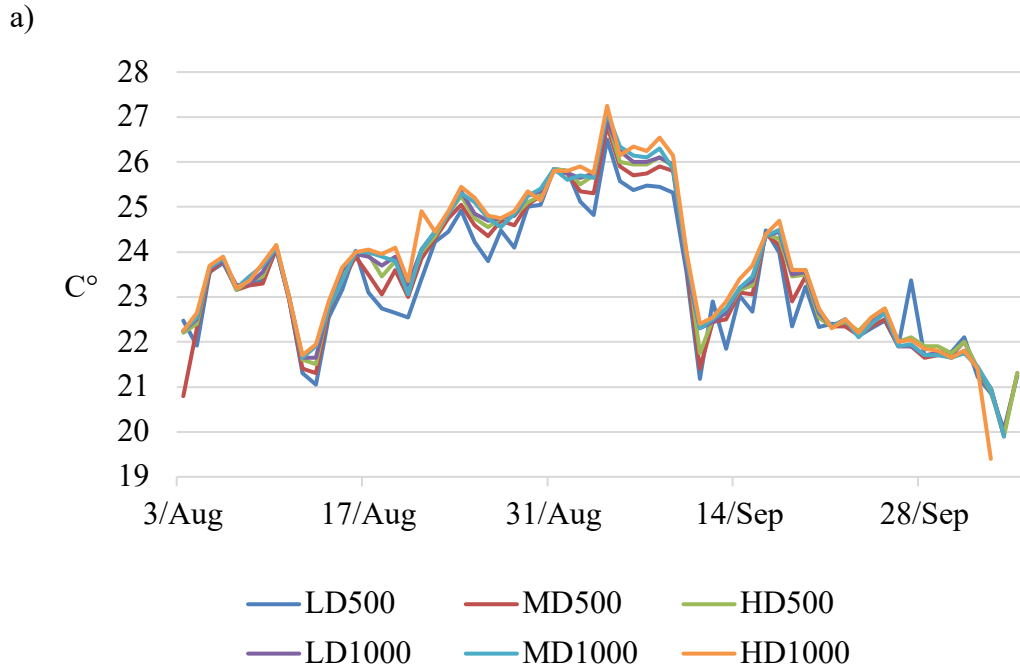
Supplementary Table 2.6. Frequency of individuals exhibiting specific skeletal anomalies among the conditions

%	LD1000	MD1000	HD1000	LD500	MD500	HD500
<i>A4</i>	0.51	2.01	1.32	0.00	0.00	1.33
<i>A5</i>	38.58	34.90	43.42	36.24	37.50	52.00
<i>A5*</i>	0.51	6.71	4.61	1.34	3.95	8.67
<i>A5/33</i>	0.00	1.34	0.00	0.00	0.66	0.00
<i>A5/31</i>	0.00	0.00	0.00	0.00	0.00	1.33
<i>A32</i>	0.00	2.01	1.32	0.00	0.00	2.00
<i>A5/34</i>	0.51	18.12	6.58	4.03	11.84	12.00
<i>A14</i>	13.20	21.48	26.32	20.13	28.95	29.33
<i>A15</i>	2.03	6.04	5.92	1.34	2.63	6.00
<i>A16</i>	23.86	18.12	34.21	18.79	23.68	26.00
<i>17L</i>	1.52	4.70	4.61	1.34	3.29	3.33
<i>17*L</i>	1.02	8.05	3.95	1.34	1.97	5.33
<i>17R</i>	1.02	3.36	5.26	0.67	1.97	4.00
<i>17*R</i>	1.02	8.72	4.61	1.34	1.97	6.67
<i>B3</i>	0.00	0.00	0.00	0.00	1.97	0.00
<i>B3*</i>	0.00	0.00	0.00	0.00	0.66	0.00
<i>B4</i>	3.55	19.46	3.29	4.03	11.18	28.67
<i>B5/34</i>	8.12	40.94	16.45	4.03	19.08	31.33
<i>B5</i>	27.41	57.72	46.05	32.21	53.29	58.00
<i>B5*</i>	0.51	0.67	1.97	0.67	2.63	0.67
<i>B6</i>	1.02	0.67	1.32	0.67	0.66	0.67
<i>B6/34</i>	0.51	0.67	0.00	0.67	0.00	0.67
<i>B7L</i>	32.49	22.82	27.63	11.41	42.11	34.67
<i>B7R</i>	46.70	27.52	43.42	20.81	46.71	40.00
<i>B33</i>	0.00	7.38	0.66	0.67	0.66	4.00
<i>B32</i>	0.51	0.00	0.00	0.67	0.66	0.67
<i>C3</i>	0.00	0.00	0.00	0.00	0.00	0.67
<i>C4</i>	11.17	68.46	42.11	36.91	39.47	66.00
<i>C5</i>	11.17	58.39	40.79	24.16	46.71	44.00
<i>C5*</i>	2.03	2.01	4.61	2.01	2.63	3.33
<i>C6</i>	18.27	61.74	47.37	31.54	50.00	50.67
<i>C5/34</i>	7.61	18.79	11.18	3.36	14.47	22.67
<i>C6/34</i>	23.86	63.09	38.16	15.44	41.45	52.67
<i>C6*</i>	3.55	8.05	7.24	5.37	8.55	10.00
<i>C33</i>	1.52	12.75	0.66	4.03	2.63	8.00

<i>C5/32</i>	0.00	0.00	0.66	0.00	1.97	0.67
<i>D3</i>	0.51	1.34	0.66	0.00	0.66	0.67
<i>D3*</i>	0.51	1.34	3.95	0.00	0.66	4.00
<i>D4</i>	2.03	23.49	9.87	1.34	9.21	19.33
<i>D5</i>	3.55	4.70	1.32	2.01	4.61	8.00
<i>D5*</i>	7.11	17.45	16.45	6.71	12.50	8.00
<i>D6</i>	0.51	4.03	1.97	5.37	5.26	7.33
<i>D6*</i>	0.51	4.03	9.87	2.68	5.26	4.67
<i>D6/32</i>	0.51	0.67	0.00	0.00	0.66	0.67
<i>DHMV</i>	0.00	2.01	3.95	0.67	1.97	2.00
<i>E11 Lx</i>	1.52	1.34	0.00	0.00	0.00	0.67
<i>E11 Rx</i>	1.52	0.67	0.00	0.67	0.00	0.67
<i>F8</i>	11.17	3.36	3.95	10.74	3.95	4.67
<i>F11</i>	0.00	0.67	0.00	0.67	0.00	2.00
<i>F32</i>	0.00	0.67	0.00	2.01	0.00	0.00
<i>G9</i>	38.07	31.54	32.24	28.86	27.63	37.33
<i>G10</i>	47.21	27.52	27.63	34.90	36.18	33.33
<i>G11</i>	1.02	0.00	0.00	2.01	0.66	2.00
<i>G19</i>	26.90	3.36	23.68	10.74	7.24	4.67
<i>G32</i>	1.52	6.71	9.87	3.36	11.84	7.33
<i>H18</i>	3.05	11.41	8.55	6.71	19.74	18.67
<i>H20</i>	0.51	0.00	0.00	0.00	0.00	0.00
<i>H26</i>	0.51	0.00	0.00	0.00	0.00	1.33
<i>H32</i>	1.02	1.34	1.97	3.36	3.29	2.67
<i>H11</i>	0.51	0.00	0.00	0.67	0.66	2.00
<i>I32</i>	0.00	0.00	0.00	0.67	0.66	0.67
<i>H8</i>	8.12	4.03	7.24	6.71	3.95	6.67
<i>I8</i>	5.08	2.68	2.63	6.71	2.63	2.67
<i>I11</i>	0.51	0.00	0.00	0.00	0.00	0.67
<i>I20</i>	0.00	0.00	0.66	0.00	0.00	0.00
<i>I32</i>	0.00	0.00	0.66	0.67	0.66	1.33
<i>EO</i>	2.03	5.37	10.53	4.70	3.29	5.33
<i>S</i>	0.00	0.67	0.00	0.00	0.00	1.33
<i>Cor L/R</i>	0.00	0.67	0.66	2.01	0.66	3.33
<i>Cl L/R</i>	0.00	0.00	0.00	0.00	1.32	1.33

Supplementary Table 3.1. Pre-ongrowing rearing established tank densities and performative parameters

Conditions	Initial setup			1 st sampling				2 nd sampling				Final sampling		
	Initial number of fish	Initial weight/ fish (g)	Initial density (kg/m ³)	Weight/ fish (g)	New tank density (kg/m ³)	Number of fish after removal	Density established after removal of fish (kg/m ³)	Weight/ fish (g)	Current tank density	Number of individuals after removal	Density established after removal of fish	Measured seabream individuals	Final average weight	Final average TL (cm)
LD 1000L	370	9.0	3.3	19.6	7.2	255	5	47.3	12.0	148	7	66	56.0 ± 14.8	14.8 ± 1.2
MD 1000L	760	8.7	6.6	19.1	14.5	525	10	41.4	21.7	338	14	89	57.6 ± 16.2	14.5 ± 1.5
HD 1000L	1460	9.0	13.2	19.7	27.1	1017	20	40.6	41.1	690	28	179	54.3 ± 15.2	14.1 ± 1.3
LD 500L Tank 1	183	8.7	3.2	20.1	7.3	125	5	43.8	10.9	80	7	71	54.8 ± 13.9	15.1 ± 1.2
LD 500L Tank 2	187	8.6	3.2	21.1	7.9	118	5	42.0	9.9	83	7	74	53.3 ± 11.5	14.7 ± 1.1
MD 500L	380	8.6	6.6	20.6	15.7	343	10	42.8	20.8	164	14	115	55.4 ± 12.8	14.9 ± 1.1
HD 500L	740	8.9	13.2	20.1	29.5	497	20	39.6	39.3	353	28	214	52.0 ± 15.3	14.4 ± 1.3



Supplementary Figure 3.1. a) Average daily temperature throughout rearing. **b)** Average daily dissolved oxygen throughout rearing.

Supplementary Table 3.2. List of considered anomaly types

<i>Region</i>	
A	Cephalic vertebrae
B	Abdominal vertebrae
C	Hemal vertebrae
D	Caudal vertebrae
<i>Type</i>	
1	Kyphosis
2	Lordosis
3	Vertebral centra partial or complete fusion
4	Vertebral shape anomaly
5	Underbite
6	Overbite
7	Opercular plate anomaly

Supplementary Table 3.3. Relative frequency of specific skeletal anomalies among the conditions

%	LD1000	MD1000	HD1000	LD500	MD500	HD500	T0
<i>A1</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>A2</i>	0.00	0.87	0.51	0.00	1.04	2.21	0.62
<i>A3</i>	0.00	0.00	0.00	0.00	1.04	0.00	1.24
<i>A4</i>	0.71	0.00	1.02	2.42	2.08	0.00	10.56
<i>A/B1</i>	0.00	0.00	1.02	0.00	0.00	1.10	1.24
<i>A/B2</i>	1.43	0.00	0.00	0.00	0.00	0.55	0.62
<i>A/B3</i>	0.00	0.87	0.00	0.00	0.00	0.00	1.24
<i>B1</i>	0.00	1.74	4.57	2.42	1.04	3.31	0.62
<i>B2</i>	0.71	3.48	2.54	0.81	4.17	3.31	6.21
<i>B3</i>	4.29	5.22	2.54	1.61	4.17	2.21	11.80
<i>B4</i>	7.86	3.48	3.05	4.84	3.13	1.10	37.27
<i>B/C1</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.62
<i>B/C2</i>	2.86	3.48	6.09	2.42	4.17	12.71	1.86
<i>B/C3</i>	0.71	0.00	2.54	0.81	2.08	1.66	0.00
<i>C1</i>	0.00	0.00	1.02	0.00	0.00	3.31	0.00
<i>C2</i>	26.43	29.57	40.61	25.00	27.08	29.83	29.81
<i>C3</i>	1.43	5.22	3.55	5.65	4.17	2.76	4.35
<i>C4</i>	14.29	11.30	10.15	18.55	12.50	11.60	83.85
<i>C/D1</i>	0.71	1.74	1.52	0.00	1.04	2.21	0.00
<i>C/D2</i>	2.14	0.00	3.55	3.23	1.04	2.21	0.62
<i>C/D3</i>	1.43	0.87	1.02	0.00	1.04	2.21	0.00
<i>D1</i>	0.00	0.00	1.02	1.61	0.00	1.10	0.00
<i>D2</i>	0.00	0.87	1.52	0.00	0.00	1.66	0.00
<i>D3</i>	1.43	0.87	4.06	1.61	3.13	2.21	1.24
<i>D4</i>	3.57	1.74	3.55	2.42	4.17	3.31	17.39
<i>5</i>	24.29	25.22	30.96	29.84	31.25	36.46	22.98
<i>6</i>	1.43	6.96	7.11	4.03	5.21	8.84	12.42
<i>7</i>	5.71	8.70	18.78	15.32	17.71	23.20	16.77

Supplementary Table 3.4. Frequency of individuals exhibiting specific skeletal anomalies among the conditions

%	LD1000	MD1000	HD1000	LD500	MD500	HD500	T0
<i>A1</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>A2</i>	0.00	0.87	0.51	0.00	1.04	2.21	0.62
<i>A3</i>	0.00	0.00	0.00	0.00	1.04	0.00	1.24
<i>A4</i>	0.71	0.00	0.51	1.61	1.04	0.00	8.70
<i>A/B1</i>	0.00	0.00	1.02	0.00	0.00	1.10	1.24
<i>A/B2</i>	1.43	0.00	0.00	0.00	0.00	0.55	0.62
<i>A/B3</i>	0.00	0.87	0.00	0.00	0.00	0.00	1.24
<i>B1</i>	0.00	1.74	4.57	2.42	1.04	3.31	0.62
<i>B2</i>	0.71	3.48	2.54	0.81	4.17	3.31	6.21
<i>B3</i>	4.29	5.22	2.54	1.61	3.13	2.21	10.56
<i>B4</i>	3.57	2.61	1.52	2.42	1.04	0.55	19.25
<i>B/C1</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.62
<i>B/C2</i>	2.86	3.48	6.09	2.42	4.17	12.71	1.86
<i>B/C3</i>	0.71	0.00	1.52	0.81	1.04	1.10	0.00
<i>C1</i>	0.00	0.00	1.02	0.00	0.00	3.31	0.00
<i>C2</i>	26.43	29.57	40.61	25.00	27.08	29.83	29.81
<i>C3</i>	1.43	3.48	3.55	4.84	3.13	2.76	4.35
<i>C4</i>	7.14	6.09	6.60	8.06	7.29	7.73	34.78
<i>C/D1</i>	0.71	1.74	1.52	0.00	1.04	2.21	0.00
<i>C/D2</i>	2.14	0.00	3.55	3.23	1.04	2.21	0.62
<i>C/D3</i>	1.43	0.87	1.02	0.00	1.04	2.21	0.00
<i>D1</i>	0.00	0.87	1.52	0.00	0.00	1.66	0.00
<i>D2</i>	0.00	0.00	1.02	1.61	0.00	1.10	0.00
<i>D3</i>	1.43	0.87	4.06	1.61	3.13	2.21	1.24
<i>D4</i>	3.57	1.74	3.05	2.42	3.13	2.76	13.66
<i>5</i>	24.29	25.22	30.96	29.84	31.25	36.46	22.98
<i>6</i>	1.43	6.96	7.11	4.03	5.21	8.84	12.42
<i>7</i>	5.71	8.70	18.78	15.32	17.71	23.20	16.77

Supplementary Table 3.5. Descriptors used to perform ordination models (see Sup. Tab. 3.2 for the codes of the anomalies).

CA					
Kyphosis	Lordosis	Fusion	VB deformity	Jaws	Operculum
A1	A2	A3	A4	5	
B1	B2	B3	B4	6	7
C1	C2	C3	C4		
D1	D2	D3	D4		
PCA					
Kyphosis	Lordosis	Fusion	Underbite	Overbite	Operculum
A1	A2	A3			
B1	B2	B3	5	6	7
C1	C2	C3			
D1	D2	D3			