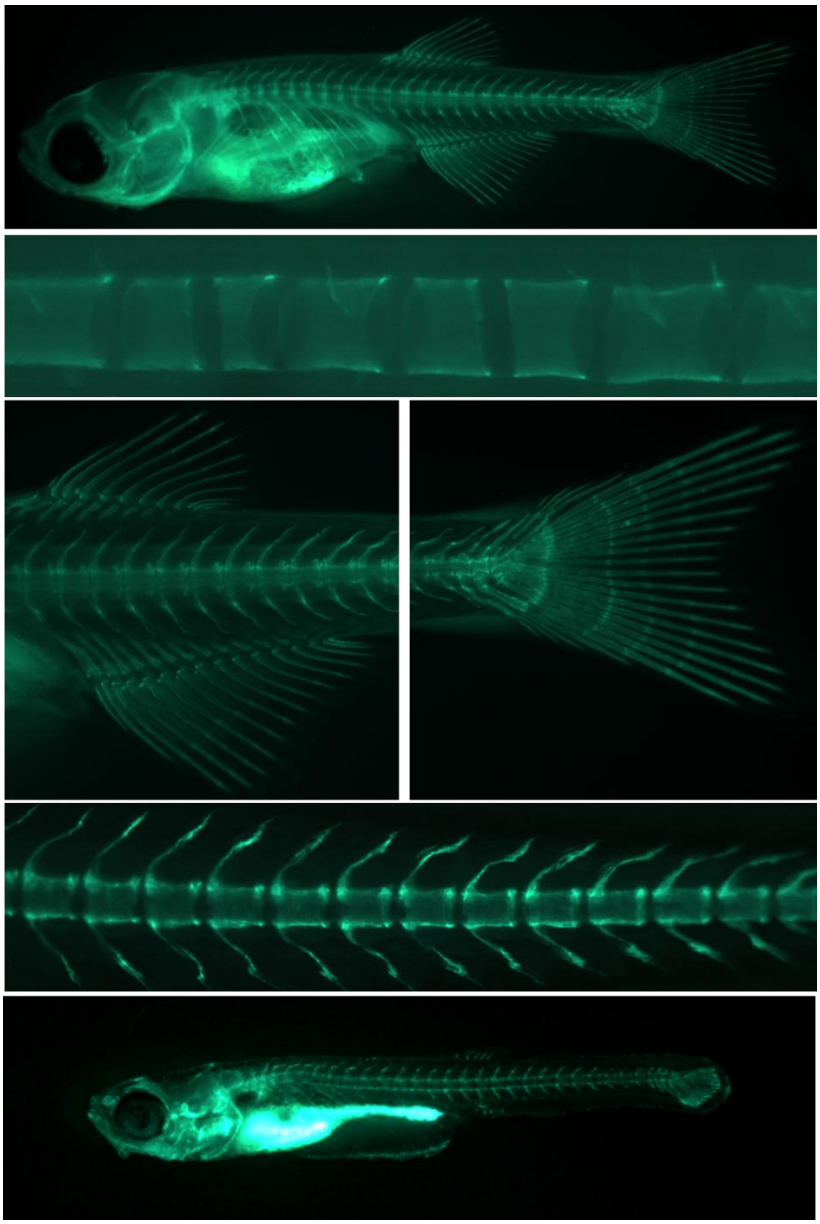
Diagnostic markers of skeleton ontogenetic deviations in zebrafish *Danio rerio* (Hamilton 1822)



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Περιβαλλοντική Βιολογία ENVIRONMENTAL BIOLOGY

"Κοίτα καλά την σπονδυλική, για να βρεις την αιτία της ασθένειας"
(Ιπποκράτης, 460 π.Χ.)

"Look well to the spine for the cause of the disease"
(Hippocrates, 460 BC)

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Summary

Skeletal deformities are a significant issue for aquaculture, with negative impacts on fish biological performance (swimming, respiration etc.), phenotypic quality and welfare. Their appearance is attributed to both environmental (temperature, pH etc) and genetic factors. Identifying accurate diagnostic markers linked to deviations of skeletogenesis and development of skeletal deformities, could offer powerful tools to the research on the causative factors of abnormalities as well to the commercial hatcheries. In the present study we used zebrafish as a model species to test whether particular skeletal defects can be associated with the expression of genes involved in bone formation and resorption. Primarily we focused on kyphosis, since it can be easily distinguished macroscopically, it is frequent among rearing species and known to induce high mortality rates. Zebrafish was chosen because of its similar ontogenetic pattern to that of the most fish species, its well-characterized skeletal development, easy maintenance, short life cycle and well-studied genome. Also, it is known how to at-will cause skeletal deformities, allowing the examination of their development.

Two groups of fish were reared in triplicate, under two different feeding regimes; one standard (control, with combined use of Artemia nauplii and dry feed), and another one (D, with dry feed only) which has been associated with elevated rates of skeletal defects. Skeletal analysis verified the initial hypothesis on the effect of the tested regimes on the development of skeletal abnormalities, with D group presenting significantly elevated abnormality rates ($p<0.05$, G-test). The significance effect of feeding regimes was observed in the case of the abnormal branchiostegal rays (10±9% in the control group vs 42 ±12% in the D group, mean±SD), bended neural processes (10±8% at the Control group vs 47±8% at the D group), abnormal gill cover (4±3 % in the control vs 25±4 % in the D group) and caudal-peduncle scoliosis (6±4 % in the control vs 23±3 % in the D group). In addition to the bibliographically expected abnormalities, D group presented high rates of abnormal vertebral ossification pattern, in the form of resorption lacunae and irregular bone formation (4±2 % in the control vs 34±32 % in the D group). Calcein staining of the specimens that were collected during an additional fourth experimental replicate showed that vertebral abnormalities can be easily detected, on alive larvae, at the beginning of their formation; well before they are

expressed on the external phenotype. In the case of resorption lacunae, they were first detected on specimens of ca 6.0 mm SL.

QPCR analysis did not reveal significant difference in transcript levels between the two experimental groups for the most of the studied genes (*bglap*, *col1a1a*, *acp5a*, *runx2b*, *mmp13a*). In the case of *Sp7* gene a significantly different expression level ($p<0.05$, Kruskal-Wallis) was found between the control (0.45±0.09SE) and D group (0.84±0.11SE).

Our results on gene expression differentiation between the two experimental groups are discussed in association to the potential causative factors and critical ontogenetic windows of the observed skeletal abnormalities.

Περίληψη

Οι σκελετικές παραμορφώσεις αποτελούν σημαντικό πρόβλημα στις υδατοκαλλιέργειες, καθότι έχουν αρνητικό αντίκτυπο στις επιδόσεις των ψαριών (κολύμβηση, αναπνοή κ.λπ.), τη φαινοτυπική ποιότητα, αλλά και την ευζωία τους. Η μορφολογία τους αποδίδεται σε περιβαλλοντικούς (θερμοκρασία, pH κ.λπ.) και γενετικούς παράγοντες. Ο προσδιορισμός διαγνωστικών δεικτών που συνδέονται με αποκλίσεις της σκελετογένεσης από το στερεότυπο τρόπο, θα μπορούσε να προσφέρει ισχυρά εργαλεία στην έρευνα σχετικά με τα αίτια πρόκλησης των παραμορφώσεων, καθώς και προς χρήση από τους ιχθυογεννητικούς σταθμούς. Στην παρούσα εργασία χρησιμοποιήσαμε το zebrafish ως οργανισμό μοντέλο για να ελέγξουμε εάν συγκεκριμένες παραμορφώσεις μπορούν να συσχετιστούν με την έκφραση γονιδίων που εμπλέκονται στον σχηματισμό και την ανάπτυξη των οστών. Πρωταρχικά εστιάσαμε την κύφωση, καθότι αποτελεί μια παραμόρφωση η οποία είναι ευδάκριτη μακροσκοπικά, εμφανίζεται συχνά στα εκτρεφόμενα είδη, και ευθύνεται για αυξημένη θνησιμότητα. Το zebrafish επιλέχτηκε καθότι εμφανίζει παρόμοιο οντογενετικό πρότυπο με αυτό των περισσότερων ειδών ψαριών, έχει καλά καθορισμένη σκελετογένεση, είναι εύκολα διαχειρίσιμο κατά την διεξαγωγή πειραμάτων, έχει στο σύντομο κύκλο ζωής καθώς και καλά μελετημένο γονιδίωμά.

Πραγματοποιήθηκαν εκτροφές ψαριών εις τριπλούν, υπό δύο διαφορετικές μεθοδολογίες διατροφής των νυμφικών σταδίων, (α) μία πρότυπη (control, με συνδυασμό παροχής ναύπλιων *Artemia* και ξηρής τροφής), και (β) μία (D, αποκλειστική χορήγηση ξηρής τροφής) που έχει συσχετιστεί με αυξημένα ποσοστά εμφάνισης σκελετικών παραμορφώσεων. Η μελέτη της εξέλιξης του σκελετού επιβεβαίωσε την αρχική υπόθεση σχετικά με την επίδραση των δοκιμασμένων διατροφικών συνθηκών στην ανάπτυξη σκελετικών ανωμαλιών, με την ομάδα D να παρουσιάζει σημαντικά αυξημένα ποσοστά εμφάνισης παραμορφώσεων ($p<0,05$, G-test). Οι κυριότερες παραμορφώσεις που παρατηρήθηκαν ήταν οι παραμορφώσεις των βραγχιοστεγών ακτινών (10±9% στην Control συνθήκη, έναντι 42±12% στην D συνθήκη, mean±SD), οι κεκαμμένες νευρικές αποφύσεις (10%±5 στην Control συνθήκη, έναντι 47±8% στην D συνθήκη), τα κεκαμμένα βραγχιακά επικαλύμματα (4±3% στην Control συνθήκη, έναντι 25±4% στην D συνθήκη), καθώς και οι σκολίωση του ουραίου μίσχου (6±4% στην Control συνθήκη, έναντι 23±3% στην D συνθήκη).

Πέραν τον αναμενόμενων παραμορφώσεων, στην συνθήκη D εμφανίστηκαν σε υψηλά ποσοστά περιπτώσεις μη φυσιολογικού προτύπου οστεοποίησης της σπονδυλική στήλης, με την μορφή κενών απορρόφησης και ακανόνιστου σχηματισμού του οστού (4±2% στην Control συνθήκη, έναντι 34±32% στην D συνθήκη). Σε μια τέταρτη πειραματική επανάληψη που πραγματοποιήθηκε, συλλέχθηκαν δείγματα έπειτα από χρώση καλσεΐνης· όπου και διαπιστώθηκε ότι οι παραμορφώσεις της σπονδυλικής στήλης μπορούν εύκολα να εντοπιστούν σε ζωντανές νύμφες κατά την αρχή του σχηματισμού τους πολύ πριν εκφραστούν στον εξωτερικό φαινότυπο. Σε ότι αφορά τις παραμορφώσεις των κενών απορρόφησης, φάνηκε ότι για πρώτη φορά ανιχνέυτηκαν σε δείγματα 6.0 mm SL.

Η ανάλυση του μεταγραφώματος δεν έδειξε σημαντικές διαφοροποιήσεις των επιπέδων γονιδιακής έκφρασης μεταξύ των δύο πειραματικών ομάδων, για τα περισσότερα από τα γονίδια που μελετήθηκαν (*bglap, col1a1a, acp5a, runx2b, mmp13a*). Στην περίπτωση του γονιδίου *sp7* βρέθηκε μια σημαντικά διαφορετική έκφραση ($p<0,05$, Mann-Whitney) μεταξύ της control συνθήκης (0,45±0,09SE) και της D (0,84±0,11SE).

Τα αποτελέσματα συζητούνται σε σχέση με τους υποκείμενους μηχανισμούς που συνδέονται με την ενεργοποίηση συγκεκριμένων τύπων κυττάρων άμεσα συσχετιζόμενα με τον σχηματισμό και την απορρόφηση των οστών.

Contents

Chapter 1: Introduction

I. Skeletal deformities in aquaculture and laboratory fish

Skeletal deformities

The term "disease" has prevailed upon years to describe conditions that deviate from the natural function or structure of an animal, even though there is no case of an actual infection. Thus in a broad sense morpho-anatomical abnormalities are considered as a disease, regardless the fact that they may not induced by contaminants or pathogens, and instead they are mostly linked to acute developmental defects (Koumoundouros 2010). Apart from the deformities related to pigmentation and the lateral line, there could be a separation between two major categories; the deformities that are linked to the presence and function of the swimbladder along with those that affect the skeleton form and structure. The latter case is the most abundant.

Skeletal abnormalities can develop either as post-metamorphic deformations in bones that were initially normal (e.g bone resorption syndrome, (Kourkouta et al. 2022, Printzi et al. 2021, 2022) or as deviations from a normal developmental pattern during the skeleton ontogeny (Fragkoulis et al. 2018; Fragkoulis et al. 2016; Loizides et al. 2014). They are of a great interest especially among farmed fish, since they can develop due to simple factors such as unbalanced levels of nutrients during early development (Cahu, Infante, and Takeuchi 2003; Lall and Lewis-McCrea 2007). Dietary deficiencies linked to phosphorus or vitamins A and C could cause deformities of the opercula, jaw and vertebrae (Fjelldal et al. 2012; Gapasin and Duray 2001; Geurden et al. 1998; Smedley et al. 2018). Deformities observed in farmed fish are rarely seen in the wild though. That is because in nature, deformed fish tend to be removed by the forces of natural selection, so they have a short life-span often resulting in the absence of deformed adult fish because of predation and poor robustness (Branson and Turnbull 2008)

Deformities of the vertebral column are another form of skeletal abnormalities and they are distinguished in lordosis, scoliosis, kyphosis (Figure 1.1) and compression. Those deformities can be observed individually or combined, and can exhibit differences in severity (Divanach et al. 1997; Koumoundouros et al. 2002).

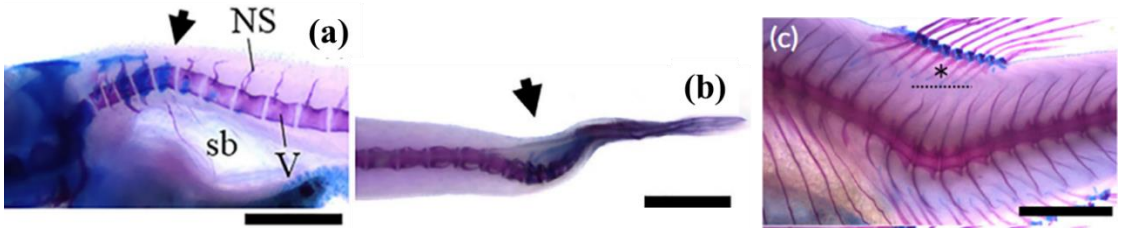

Figure 1.1: Typical cases of kyphosis (a), scoliosis (b) and lordosis (c). NS refers to neural process, sb to swim bladder and V to vertebral centrum. Scale bars = 0.5mm (a,b) and 1mm (c). Source: (Printzi, Fragkoulis, et al. 2021; Printzi, Kourkouta, et al. 2021a)

Lordosis can appear in both haemal and pre-haemal region of the vertebral column, and can be induced by a variety of factors (Divanach et al. 1997; Sfakianakis et al. 2006). Lordotic individuals appear to have a dorsoventral flexion of the spine in V shape (Andrades, Becerra, and Fernández-Llebrez 1996; Barahona-Fernandes 1982). In pre-haemal lordosis normally two or three vertebrae are involved (usually the last pre-haemal vertebrae), where the flexion of the vertebral column occurs.

Pre-haemal kyphosis is the opposite case of lordosis, where a dorsoventral flexion in Λ shape is observed. This type of kyphosis develops dorsally to swimbladder,, often on vertebrae 5 and 6 (Koumoundouros et al. 2002).. Finally, scoliosis is referred as the lateral bending of the vertebral column.

The ontogenetic range at which skeletal deformities have been identified is rather broad, since they have been observed during the embryonic and larval stage as well (Georga and Koumoundouros 2010; Koumoundouros et al. 1997; Loizides et al. 2014), or even during the metamorphosis and juvenile stages (Mazurais et al. 2009; Sfakianakis et al. 2006). Collectively, the main factors identified behind the appearance of skeletal deformities are adverse abiotic conditions, improper diet or genetic factors. From there on the matter tends to become species-specific (Koumoundouros 2010).

Inducing skeletal deformities in lab

Many efforts have been made on determining a fast, simple and with respect to animals' welfare, vertebrate model for the *in vivo* induction of skeletal deformities. The first successful experiments were made on poultry, by inducing vertebral deformities through surgical removal of the pineal gland (Machida et al. 1993). Pineal gland

contributes to the development of the bones by secreting melatonin. Similar experiments were conducted on the Atlantic salmon, yet the mortality rates were high (Fjelldal et al. 2004) and six months after the excision problematic connections of the vertebrae of the fish were observed. Other experiments on rabbits included surgeries on the chest and corresponding areas (Hakkarainen 1981). All those surgical interventions resulted in most of the cases to the induction of scoliosis (Bobyn et al. 2014; Janssen et al. 2011). In addition, in *Poecilia reticulata*, spine wedges similar to humans have been observed, which are associated to a form of scoliosis (Gorman and Breden 2009). Thus, this could be the link between the skeletal disorders of humans and fish, paving the way for the future use of teleosts as models for the study of main skeletal deformities.

Some recent studies though, have proved that the induction of skeletal abnormalities could also occur without surgical interventions. Specifically factors like the feeding regime followed could cause a variety of deformities (Printzi, Kourkouta et al. 2021; Mazurais et al. 2009) or even specific anomalies such as lordosis (Printzi et al. 2021)

Descriptor and predictor markers of altered skeletal development

In commercial hatcheries, skeletal abnormalities could be the outcome of fluctuations in the complex rearing conditions (Kourkouta et al. 2022; Fragkoulis et al. 2016; Loizides et al. 2014) . Appropriate predictor markers could allow aquaculture to monitor those variables and make predictions upon the quality of fish at the end of the hatchery phase, thus assisting in appropriate management decisions. In spite of the deep knowledge on the molecular mechanisms of fish skeletogenesis (Bensimon-Brito et al. 2012; Carvalho et al. 2017; Tarasco et al. 2021), we currently miss accurate molecular diagnostics of altered fish skeletogenesis (Koumoundouros et al. 2017). Research on bone development is mainly focused on markers of proliferation and differentiation of basic cell types linked to bone formation (osteoblasts and osteoclasts) (Hall 2015), and not specifically about the normal or abnormal morphogenesis of the skeleton. Thus, there is a lack of knowledge regarding the variability that predictors and biomarkers could show upon different incidences of skeletal deformities or the severity they appear to have, and more studies are required for this correlation to be examined.

Causative factors of skeletal deformities

Many studies refer to biotic and abiotic parameters as main causative factors of skeletal deformities. Some of them are known to be performed during species-specific developmental stages, at different frequencies and severity. Developmental temperature is among the most significant factors linked to abnormalities such as swimbladder deformities during embryonic and larval phase, haemal lordosis, caudal fin abnormalities and deformities of the branchiostegal rays among many others (Koumoundouros 2010). The water parameters during rearing of fish could also affect the frequency of deformities. Specifically, velocity of water current in the tanks affects the prevalence level of haemal lordosis (Koumoundouros 2010). Also, water surface free of lipid films is crucial for the normal swimbladder inflation, since a non-inflation induces pre-haemal lordosis (Koumoundouros 2010).

Inappropriate microdiet formulation and nutritional deficiency play an important role at the appearance of deformities, and lack of some nutritional elements is linked to specific abnormalities. Fatty acids, such as PUFA, in either lack or excess cases could cause gill cover abnormalities and scoliosis (Koumoundouros 2010). HUFA dietary levels are associated to teratogenesis in many fish species (Koumoundouros 2010). High levels of Vitamin A could cause lordosis and scoliosis (Lewis-McCrea and Lall 2010), while a prolonged exposure to low levels of Vitamin C could be responsible for abnormal skeletal development in many teleost fish. Additionally different levels of phosphorus could cause craniofacial anomalies (Costa et al. 2018). More specifically low levels of phosphorus are linked to low mineralization levels of the endplates of vertebrae while on the contrary higher levels could induce increased bone mineralization in the form of increased bone stiffness and fusion of vertebral centra (Cotti et al. 2020) (Figure 1.2)

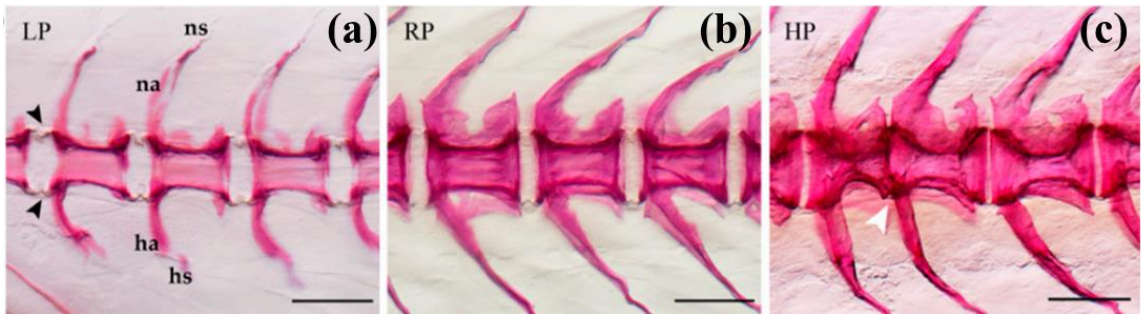

Figure 1.2: Vertebral body endplates (black arrows) with low mineralization in low phosphorus treatment (LP) (a), intermediate mineralization in treatment with regular phosphorus (RP) (b), high mineralization in high phosphorus treatment (HP) (c).

Vertebral body fusion (white arrow). Neural arches (na), heamal arches (ha), have low mineralization and their spines are deformed in the LP treatment compared to RP and HP. Scale bar = 200 μm. Image source: (Cotti et al. 2020)

Genetic factors have also been studied the last years and how they are linked to skeletal abnormalities. For instance, the genetic basis of jaw deformities has been studied in different fish species (e.g., García-Celdrán et al., 2016; Nguyen et al., 2015; Sawayama and Takagi, 2016)

II. Main aspects of bone development in Teleosts

Osteoblasts and osteoclasts in bone formation

In teleosts with acellular bone, bone lining cells and osteoblasts that reside on the bone surface are the most obvious candidate cells for sensing mechanical load. This idea is based on the findings in some teleost species with acellular bone, where the cells on the bone surface extend cytoplasmic cell process into the matrix, similar to mechano-sensing osteocytes (in mammals, osteocytes are known to detect mechanical load and strain) (Witten and Hall 2015).

Additionally, as it is well aware from mammals and can be verified in teleosts too, osteoblasts participate in bone formation, while osteoclasts contribute to cartilage degradation and bone resorption. Thus, the bone homeostasis is maintained by a balance between those two major processes of bone remodelling, driven by various factors and hormones such as mechanical stress, thyroid hormones, calcium-mobilization and so on (Tanaka, Nakayamada, and Okada 2005).

Osteoblasts

Osteoblasts participate in bone formation by synthesizing multiple bone matrix proteins. They also play a central role in the differentiation of osteocytes (Witten and Huysseune 2009). They are surrounded by extracellular matrix (ECM) proteins, including collagen type I and fibronectin (Tanaka, Nakayamada, and Okada 2005). The latter is highly expressed by osteoblasts. They can also engage β1 integrin, with the participation of a specific ligand or antibody, inducing in this way processes like differentiation, proliferation and bone matrix-synthesis of osteoblasts (Tanaka, Nakayamada, and Okada 2005).

Osteoblasts come from multipotent mesenchymal cells, and after that about half of the amount produced differentiate into osteocytes and are mobilized, while those that remain undergo apoptosis (Tanaka, Nakayamada, and Okada 2005).

Osteoclasts

Before the action of osteoblasts, osteoclasts are the cells responsible for removing bone (bone resorption). In mammals the differentiation of osteoclasts is linked to either osteoblasts or molecules expressed by stroma cells, which regulate the maturation of osteoclasts after direct contact with them (Tanaka, Nakayamada, and Okada 2005).

Chondroclasts and odontoclasts, related to osteoclasts, are responsible for cartilage and dentine resorption respectively. Although these cells appear to have different regulatory mechanisms, names and environments, there is strong evidence that they belong to the same cell linage (Nordahl et al. 2000; Shibata and Yamashita 2001). Typical mammalian osteoclast cells could be also seen as specialized multinucleated macrophages. As so they derive from hematopoietic bone marrow cells, which also give rise to phagocytosis cells such as macrophages and monocytes (Holtrop, Cox, and Glowacki 1982).

Resembling the mechanisms of macrophages, osteoclasts migrate from site to site depending on where the bone resorption is going to take place. When the osteoclasts make contact with the surface of the bone, they create a peripheral sealing zone through their apical cell membrane (concealing the subcellular space), along with a ruffled border (successive membrane infoldings). The areas where the ruffled borders are formed, are indicative of active centers for the bone resorption to take place (Witten and Huysseune 2009).

In teleosts can also be found typical mammalian-like multinucleated osteoclasts especially in cyprininds, although the number of nuclei in the resorbing cells might differ (more than mammals). The variation of the number of nuclei in teleost osteoclasts could be related to differences among species, skeletal elements, ontogenetic stages and physiological conditions (Witten and Hall 2002; Hall and Witten 2007). Moreover, in teleosts can be found and mononucleated osteoclasts on which bone resorption could rely solely, and even sometimes shifts can be noticed from resorbing mononucleated cell to multinucleated during development (Witten, Hansen, and Hall 2001).

Apart from bone resorption another role of osteoclasts widely noticed in teleosts is demineralization of the bone without affecting the bone matrix, a process that is

known as halastatic demineralization (Kacem and Meunier 2003). Since skeletal remodelling describes the replacement of one skeletal tissue by another, remodelling in teleosts could not only occur by resorption and *de novo* bone formation, but trans-differentiation (metaplasia) of skeletal tissues as well (Witten and Hall, 2002; Gillis et al., 2006; Hall and Witten, 2007).

Processes like resorption, halastatic demineralization and trans-differantiation could occur in mammals either during normal or pathological development. Though some mechanisms of bone remodelling are more common in teleosts and prominent, and so they are recognized as regular processes during bone development (Van der heyden and Huysseune, 2000; Witten, Hansen and Hall, 2001, Witten et al., 2003; Hall and Witten, 2007; Witten and Huysseune, 2007).

Phenotypic plasticity of skeletal structures in Teleosts

As this term suggests, phenotypic plasticity refers to the fact that organisms responding to a changing environment can maintain a consistent performance across a wide range of environmental factors (Morgan et al. 2022). Fish belong to a group called ectotherms, and thus exhibit a great ability to alter their phenotype depending on a challenging environment. For instance, different water temperatures could influence the swimming performance of fish (Dimitriadi et al. 2018; Kourkouta et al. 2021) along with their cardiac anatomy (Dimitriadi et al. 2018) and shape and morphometry of the otoliths (Geladakis et al. 2022). Other factors that have been proven to affect different features of the teleostei are the conciseness of water in oxygen at early developmental stages (Vulesevic and Perry 2006), photoperiod and salinity, food abundance (Meyer 1987), the breeding method of fish (Koumoundouros, Divanach, and Kentouri 1999), the environment of development (Marks et al. 2005), or even the absence of "competitors" (Paszkowski et al. 1990). Those in fact have been observed to have a linkage with many meristic features, swimming performance, body shape, developmental rate, in myogenesis and sex ratio.

Plasticity of skeletal structure in Teleost fish is rampant in nature (Johanson et al. 2009) and it is known that epigenetic factors during development could induce skeletal plasticity. This could extend from plasticity in serial elements such as vertebrae, scales or fin rays to skeletal tissues that can range from acellular bone to cartilage. Since the ability of skeletal tissue remodelling is that vast, a new term has also emerged so for

the general process of functional adaptation is determined, and it is referred to as Wolff's law (Ruff, Holt, and Trinkaus 2006). "Wolff's law of bone transformation" (Wolff 1982) or otherwise known as "bone functional adaptation" (Ruff, Holt, and Trinkaus 2006), describes the ability of tissues to modulate their phenotype in response to the mechanical load.

Various experiments also provide evidence that "bone functional adaptation" can be well documented in teleosts, and actually it can be induced by simple external factors such as feeding conditions. Moreover, it has been shown that mechanical loading could also affect early teleost bone development (Fiaz et al. 2012) apart from bone growth and shape in adults and juveniles.

Phenotypic plasticity could also appear in the form of recovery of skeletal deformities. In Atlantic salmon, Witten et al. (2006) reported for the first time the remodelling of two fused vertebral centra into one non-deformed. However multiple haemal and neural processes were presented. In this case, this phenomenon was restricted to a fusion of two or three vertebrae, although there are signs of multiple vertebrae fusion as well. Later it was also shown that recovery of haemal lordosis could be achieved during the on-growing period in Gilthead seabream and European seabass (Fragkoulis et al. 2018; Fragkoulis et al. 2022). From these studies was proved that the recovery rate could range from 43.6% to 73.5% depending on the initial severity of the abnormality. Recently there was another study which presented the same results in zebrafish too, while it additionally revealed that that approximately 973 genes exhibited altered RNA expression levels due to the recovery process (Printzi et al. 2022). Those genes seem to participate in processes such as the initiation of DNA replication, chromosome organization and DNA metabolic processes (Printzi et al. 2022).

III. Zebrafish

Taxonomy, ecology and distribution

Zebrafish is a freshwater fish of South Asia, that belongs to the Cyprinidae family (Subfamily Rasbirinae, Howes 1991). It is considered as a vertebrate model-organism widely used in science because of its unique features that enable biological research procedures in a wide range of areas (developmental and molecular biology, genetics, ecotoxicology, environmental biology of fish, etc). Its name in based on the five even,

pigmented, horizontal, blue stripes at both sides of body, that highly resemble those of zebras and which extent until the end of the caudal fin.

The name of the Genus (*Danio*) originates from the word “dhani”, which in Bengali language means “from the puddy” (Talwar & Jhingram 1991). The species of this genus are known for their small size, the presence of a typical notch on the margin of the denture known as “danion notch", as well as for their pattern of pigmentation, that is based on the alternation of dark and light horizontal stripes (Spence et al. 2008).

Zebrafish, *Danio rerio* is probably the most common representative of this genus. It inhabits in the areas of Northeast India, Bangladesh, Pakistan and Bhutan (Figure 1.3). It is believed though, that the expansion of zebrafish is highly affected and perhaps limited by anthropogenic action. Zebrafish is a tropical demersal fish. The ideal conditions for its survival and reproduction refer to pH from 6 to 8, and water hardness from 5 to 19 (dH). Reproduction of the species can be easily accomplished in laboratory conditions. In its natural habitat, it reproduces in open waters where the eggs scatter and fall to the substrate (Riehl and Baensch 1991). The eggs are benthic with a 600-700 μm diameter, and hatch within 48-72 hours after fertilization, although this can be determined by various factors such as temperature (Kimmel et al. 1995). It is not observed the slightest parental care (Riehl and Baensch 1991), instead it’s really common to develop cannibalistic behaviors where the parents devour their eggs.

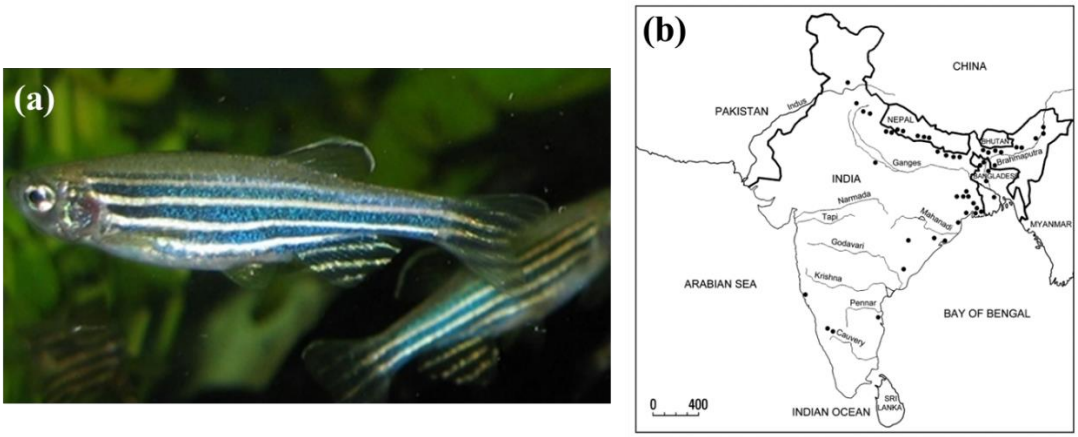

Fig 1.3: (a) The typical alternating stripes of zebrafish. Image source: Wikipedia (https://en.wikipedia.org/wiki/Main_Page), (b) Zebrafish distribution in its natural habitat. Image source (Spence et al. 2008)

In the regions of zebrafish distribution, a monsoon climate prevails with a significant seasonal differentiation in freshwater ecosystems. Especially a fluctuation in temperature is noted in zebrafish habitats, which could reach from 6 ^{o}C in winter to 38 ^{o}C in summer (Spence et al. 2008). Zebrafish can be found in canals, lakes, streams and in general where there is static or low-flow water (Talwar and Jhingram 1991).

Ontogeny and skeletogenesis

Zebrafish provides the opportunity of easy genetic handlings, while the nature of its embryology and development makes it ideal as a model organism (Nusslein-Volhard and Dahm 2002). The most important feature though, that facilitates the purposes of the present study, is that it allows the live imaging even in cellular level of many complex processes, such as the formation of the vertebral column and spinal cord (Du et al. 2001). Regarding the ontogeny of the species, hatching of the eggs is noted approximately at 72 hpf (hours post fertilization, in 28°C water temperature), where the embryonic stage is being completed (Kimmel et al. 1995).

Macroscopically, a major event in axis development is the bending of the notochord posterior tip, a process called flexion. It begins at ~4.6 mm SL and is completed at 6.3 mm SL (Parichy et al. 2009).

At the microscopical level, it is known that at 7 dpf (days post fertilization) the axial skeleton makes its

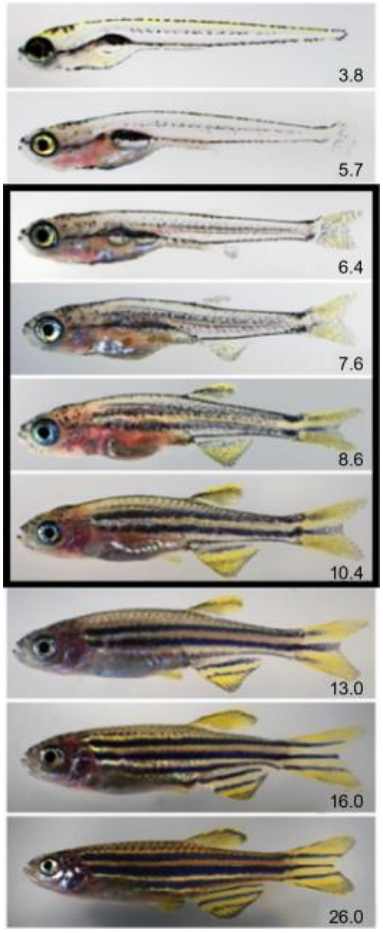

Figure 1.4 Postembryonic developmental stages in zebrafish (*D. rerio*). Numbers indicate SL in mm. The black box indicates the period of peak metamorphic remodeling during the transition from larva to juvenile. This transition begins at 10-14 days and is completed approximately in 2 weeks. At postembryonic stages, fish can develop in different rates so days of development are not an adequate staging criterion. Instead, their morphology and size are more preferable criteria for developmental studies (Parichy et al., 2009). Image source: McMenamin and Parichy (2013)

appearance. At 16 dpf (approximately 6.2 mm SL) the bones of the tail are distinct along with the processes, while around at 10 mm SL cartilage and bones are visible *in vivo* (Du et al. 2001; Parichy et al. 2009). Following the metamorphosis stage (Figure 1.4), in which the transformation of larvae into juvenile or adult is noted. At 11 mm SL the fish goes through the juvenile stage, where the metamorphosis is completed and the differentiation of the gonads occurs (Hsiao and Tsai 2003). The transition from the juvenile to the adult stage does not present any specific feature, but during that phase individuals have completed processes regarding with mineralization, pigmentation and scale formation. Eventually adulthood is signaled by the production of viable gametes, and the appearance of secondary sexual characteristics (rounder belly at females, differences in coloration). Those secondary characteristics are more apparent at 17.5 ± 0,6 mm SL for males and at 18.3± 0,7 mm SL for females (Parichy et al. 2009). Moreover, the total number of the vertebrae of the species is debatable, since it is estimated that they fluctuate from 29 to 34, although these numbers can be affected by different factors, such as the temperature (Sfakianakis et al. 2011).

Both humans and zebrafish belong to the subphylum Vertebrata and appear to have many resemblances upon the morphology and the structure of the vertebral column. Zebrafish is suggested by literature as a model organism for the study of bone structure related to age (Chang et al. 2013). Actually, this can be possible, since the vertebrae of some of the main quadrupedal resemble those of their remote ancestors that used to live in aquatic environments (Liem 2001). In both organisms the notochord forms the initial shape of the vertebrae and they even show a natural anterior kyphosis (Boswell and Ciruna 2017). Along the evolutionary line of the species, the vertebrae become more durable and harden as their individual elements lengthen.

Due to the use of calcein staining the pattern of mineralization of the vertebrae is known to follow a course from the anterior to posterior (Parichy et al. 2009). A similar pattern is also noted at the mineralization of the vertebral processes with a time delay. First indications of vertebral column are observed at 4 mm NL (Notochord Length) and refer to the appearance of the first mineralized vertebra centers (Bird and Mabee 2003).

IV. Aim of the present study

The aim of the present study was to determine whether the development of specific skeletal abnormalities can be linked to alterations in the expression of genes involved

in bone formation and skeletogenesis. To examine this, two different feeding regimes were applied for the at-will induction of skeletal deformities following (Printzi, Kourkouta, et al. 2021), by manipulating the Artemia to dry feed transition rate.

Among the skeletal deformities, kyphosis was mainly targeted because it is a frequent vertebral abnormality in different species under rearing conditions (Koumoundouros et al. 2002; Printzi, Kourkouta, et al. 2021) inducing both phenotypic alterations and high mortality. Its development starts during the early larval period, and specifically during the ossification of the vertebrae, dorsally to the swimbladder (Koumoundouros et al. 2002). Moreover, it is linked to abnormalities of other skeletal elements that follow the same developmental pattern (i.e., membranous or fibrous ossification). Kyphosis can be easily detected on alive and anesthetized larvae, thus allowing sample selection and proper fixation for transcript analysis.

Following the results on the frequency and the typology of skeletal abnormalities in the first task, a series of samples was also taken during a fourth experimental replicate. The aim of this objective was to examine the ontogeny and anatomy of a new abnormality type, linked with poor ossification of membranous bones along with the possible underlying mechanisms and causative factors of this abnormal condition. The feeding regimes followed for the induction of abnormalities were the same as the first task. Calcein staining was carried out, for the *in vivo* sample selection and proper fixation.

Identifying specific molecular markers from the transcript analysis, could be a strong tool for predicting a phenotype, assist in making management decisions in aquaculture and in improving our understanding on the causative factors of skeletal abnormalities.

Chapter 2: Materials & Methods

Experimental design

Larval rearing was performed under two different feeding regimes, according to (Printzi, Kourkouta, et al. 2021a). Feeding regimes involved cofeeding on both dry microdiet and *Artemia* nauplii (Control) and feeding exclusively with dry microdiet (D) (Figure 2.1). The second regime was chosen because it results to comparatively elevated rates of kyphosis and other skeletal abnormalities (Printzi et al. 2021). When the individuals reached 7.5-8.5 mm standard length (SL, tip of snout to the base of the caudal lepidotrichia), at ca 24 days post fertilization (dpf), random samples from each population were anesthetized stained *in vivo* with calcein in order to be examined for the presence of vertebral abnormalities. At first kyphosis was targeted, since it is frequent and can be easily distinguished and observed in a stereoscope. In cases where the fish had the desirable standard length and had been recorded as deformed, they were fixed in RNA later for gene expression analysis. Additionally, a random sample of fish from each population was formalin fixed, stained for bone and cartilage and examined for the presence of skeletal abnormalities. All the treatments were applied in triplicate.

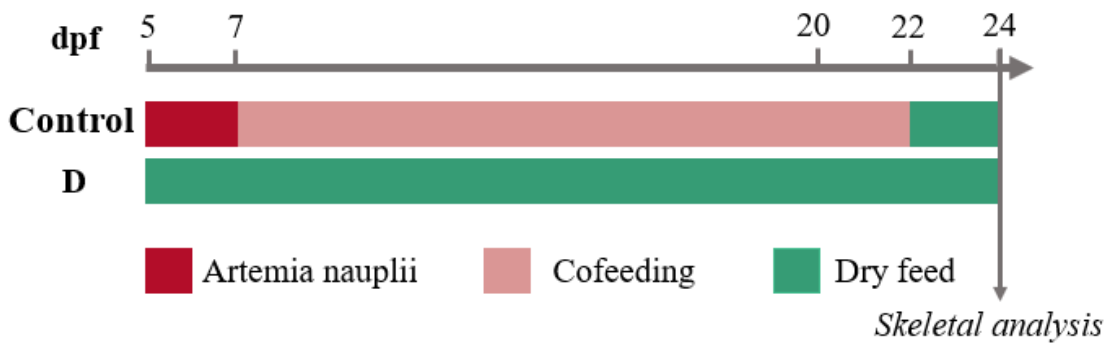

Figure 2.1. Experimental design of the feeding regimes followed including co-feeding of dry feed and *Artemia* nauplii (Control) and exclusively dry feed (D). dpf, days post fertilization.

Maintenance of the experimental populations

For each replicate, approximately 500 embryos (±20) were collected and divided into two different net pens of 4.5 L volume each. The walls of the pens were made of net (100μm mesh size), which allowed the water circulation while at the same time prevented the escape of *Artemia* nauplii. Feed provision to the larvae started when most individuals presented an inflated swimbladder (5 dpf). Larvae were fed 5 times per day with either *Artemia* nauplii, dry feed or their combination (Figure 2.1). The net pens of each replicate were positioned into a common 40-L aquarium, connected to a closed recirculation system. Oxygen concentration, temperature, pH and conductivity were monitored on a daily basis. Ammonia, nitrate and nitrite measurements were taken once a week (Table 2.1).

All embryos were obtained from a common broodstock (AB strain). Breeders were kept in a closed recirculation system (ZebTEC, Techniplast, Italy), under standard for the species conditions of temperature (28.0 ± 0.5 ^{o}C), conductivity (450–600 μS/cm), pH (7.0–7.5) and oxygen concentration (6.5–7.0 mg/L). The breeders were kept under a constant photoperiod of 14 L :10 D (hours light: dark). Breeders were fed with commercial dry feed (Zebrafeed 400–600; Sparos Lda) twice a day.

Table 2.1: Mean values (±SD) of the abiotic conditions monitored throughout the experimental processes. All fish were kept under a 14 hours light–: 10 hours dark photoperiod.

Parameters	Nutritional treatment	Replicate 1	Replicate 2	Replicate 3
Temperature (^{o}C)	Control - D	28.0 (±0.3)	28.0 (±0.1)	28.0 (±0.1)
pH	Control - D	8.40 (±0.0)	8.30 (±0.1)	8.30 (±0.0)
Conductivity (μS/cm)	Control - D	448 (±24)	440 (±15)	468 (±15)
O_2 saturation (%)	Control - D	89 (±6)	98 (±2)	95 (±4)
Ammonia (mg/L)	Control - D	<0.02	<0.05	<0.10
Nitrate(mg/L)	Control - D	<0.20	<0.05	<5
Nitrite (mg/L)	Control - D	<0.05	<7.5	<2.5

In vivo staining for skeleton examination – Calcein staining

Random samples were taken during the experiment in order to examine the length of the individuals and whether they appear to have kyphosis or not. First, fish were anesthetized and measured for the SL. In cases where the standard length was in the

range of 7.5-8.5 mm, fish were kept to be examined for deformities using the method of *in vivo* calcein staining. All the vertebral deformities were targeted, along with deformities of the fins and the cranial area.

For the verification of the selection of the samples before their fixation in RNA later, calcein staining was applied. The use of calcein pigment ($C_{30}H_{26}N_2O_{13}$) for in vivo staining, allows the observation of fish skeletogenesis without the sacrifice of the individuals being needed. This type of pigment binds to tissues that contain calcium (Ca^{+2}). The protocol that followed was according to Du et al. (2001), where at first a solution of 0.1 g calcein pigment per 50 ml of water was made. Since the calcein solution is acidic, the pH was adjusted to ca 7.0 by adding sodium hydroxide solution (NaOH 0.5M).

For calcein staining, fish larvae at 7.5-8.5 mm (20-22 dpf) were immersed in the calcein solution from 3 up to 10 minutes, according to their size (bigger fish needed more time in order for the calcein to diffuse through the tissues and bind on the calcium). Hereupon, individuals were submitted to three successive washes in tap water, and after that remained for 10 minutes in tap water so for the pigment to incubate in the target-tissue before the stereoscope observation. For the observation and photography (OLYMPUS SZX16 fluorescence stereo microscope), the individuals were anesthetized (2-phenoxyethanol, C ≈ 0.15-0.25 ml/L).

Fish that had the desirable length (7.5-8.5 mm SL), were anesthetized and fixated in RNA later (1:10 fish volume to RNA later volume) according to the recommended use instructions. Samples were fixated individually and remained in room temperature for approximately 24 hours to give time for the RNA later to enter inside all tissues and then stored in -80ºC All procedures were carried out at 28 ºC.

Calcein staining was performed to check and enable the criteria applied for the isolation of kyphotic fish, and then fixate them in RNA later. All the fish that were observed in the stereoscope and thought to be kyphotic, were later proceeded for staining.

Gene expression analysis

As kyphosis appeared only in light severity, in order to avoid a non-objective categorization, we proceeded to use random samples for gene expression analysis.

The target genes chosen for the analysis (Table 2.2**)**, were closely related to osteoblast and osteoclast cells (*sp7, runx2b and mmp13a, acp5a* respectively), osteocalcin (*bglap*) and Type I collagen (*col1a1a*). More specifically sp7 is a transcription factor expressed by early osteoblasts, *runx2b* induces bone formation and is closely related to *sp7* by regulating its expression, osteocalcin (*bglap*) is a calcium binding protein produced by late osteoblasts, col1a1a is an ECM structural constituent abundant in bone and tissues, while mmp13a and acp5a are involved in bone resorption (Bensimon-Brito et al. 2012; Carvalho et al. 2017; Tarasco et al. 2021). As reference genes were used *gapdh* and *eef1a1l1* (Table 2.2).

Table 2.2: Target and reference genes and some of their basic functions in zebrafish

Target gene	Function	Ref
bglap	Osteoblast-related gene (osteocalcin / calcium binding proteins produced by late osteoblasts, involved in bone mineralization)	(Chen et al. 2019)
col1a1a	Expressed in olsteoblasts (Type collagen I, produced by bone cells, found in bone matrix, connective tissues and skin)	(Gistelinck et al. 2016)
runx2b	Osteoblast-related gene (transcription factor, induce bone formation, upstream of sp7/osterix, regulates expression of osteocalcin)	(Li et al. 2022; Pinto et al. 2005)
sp7	Osteoblast-related gene	(Chen et al. 2019)
mmp13a	ECM-related gene, e matrix metalloproteinases and mainly degrade the ECM	(He et al. 2018)
acp5a	Osteoclast-related gene	(He et al. 2018)
gapdh	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an enzyme involved in breaking down glucose to obtain energy	(He et al. 2018; Tamai et al. 2022)
eef1a1l1	An evolutionarily conserved GTPase that catalyzes the efficient delivery of charged tRNA to the ribosome during protein elongation and is critically involved in translation fidelity.	(He et al. 2018; Talapatra, Wagner, and Thompson 2002; Zhu et al. 2022)

A series of procedures was performed for the analysis of the expression of the target genes and RT-qPCR analysis. For the gene expression study, 3-4 RNA-later preserved samples (§ *Calcein staining*) per nutritional treatment and replicate (9-12 samples in total per nutritional treatment). were used. The primers of the gene targets were designed with the help of the Primer3 software. Primers are oligonucleotides of 18-30 bases which demarcate the area of the DNA to be replicated. More specific details on the sequences of the primers (forward and reverse) along with the amplicon size, are shown in Table 2.3.

Table 2.3: The sequences of the primers used. Their size and NCBI code are also given

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	NCBI	Amplicon Size (bp)
gapdh	CGTCTTGAGAAACCTGCCAAG	AACCTGGTGCTCCGTGTATC	NM_001115114.1	102
eef1a1l1	CTACCCTCCTCTTGGTCGCT	GGAACGGTGTGATTGAGGGAA	NM_131263.1	162
bglap	CAGCTGACACAGAAGCGAAC	AGGAGTCAGGAAGACCTGCG	NM_001083857.3	93
col1a1a	GAGGATGGTTGTACGTCGCA	AGGTGCACCAACGTCCATAG	NM_199214.1	117
sp7	CGTCGCTCCTGTTTTTGACG	CCATAACGTGTTTCCTCCTCCA	NM_212863.2	138
mmp13a	AGCCTTCCAGTACAGAGGTTTA	CTTCAGGCGGTAAGTATTAAAGAAA	NM_001290479.1	154
acp5a	GAGCAGAAGCTGCTGGTATGG	TAGGTAGTCCACCCCAGTCC	NM_214773.1	164
runx2b	GACGTCTTCCAGGTTCGACA	GACTGGAAAAGCGGCTCTCG	NM_212862.2	99

Total RNA was isolated by using TRI Reagent buffer for the homogenization and lysis of the cells, while in a next step an RNA extraction kit (NucleoSpin RNA, Macherey Nagel/740955.50) was used for the purification of the RNA and the destruction of any DNA residues. RNA purification was later tested by using NanoDrop Microvolume Spectrophotometer (Table 2.4).

Table 2.4: Nanodrop values (ng/μl) per sample. 260/230: the ratio used to assess the purity of RNA, 260/280: the ratio used for the assessment of the quality of the RNA sample

Nutritional treatment	Sample name	RNA concentration (ng/μl)	260/280	260/230	Replicate
Control	C1	246.6	2.10	2.14	1
Control	C2	278.7	2.12	2.19	1
Control	C3	300.9	2.05	1.94	1
Control	C4	209.7	2.09	2.07	2
Control	C5	266.8	2.00	1.59	2
Control	C6	213.8	2.07	1.99	2
Control	C7	271.7	2.05	1.85	3
Control	C8	207.8	2.06	1.86	3
Control	C9	274.3	2.08	1.99	3
D	D1	247.4	2.10	2.03	1
D	D2	228.1	1.98	1.57	1
D	D3	211.2	2.02	1.78	1
D	D4	346.4	2.04	1.83	2
D	D5	196.8	2.02	1.69	2
D	D6	229.1	2.09	1.95	2

D	D7	147.2	2.10	1.87	3
D	D8	222.2	2.07	2.18	3
D	D9	353.6	2.10	2.25	3

The results of Real Time PCR were analyzed by using the Relative Quantification method. This method is based in the comparison of the Ct values of the gene target to the Ct values of the reference gene (method $2^{-\Delta'CT}$) (Livak & Schmittgen 2001). Initially the difference between the Ct value of the gene target compared to the Ct of the reference gene was estimated:

$$\Delta'CT = CT_{\text{gene target}} - CT_{\text{reference gene}}$$

where, as Ct of the reference gene was used the mean value of the geometric mean value of the Cts of the genes *gapdh* and *eef1a1l1*. Gene expression results were expressed as the mean value of $2^{-\Delta'CT}$ + 1 SEM. All those evaluations were made automatically using the CFX Maestro programme.

The statistical significance of the differences in gene expression between the two different groups (Ctrl, D) was tested by the non-parametric Mann Whitney test (on the values of $2^{-\Delta'CT}$). Parametric tests were not used because the appropriate assumptions (normal distribution and homogeneity of variances) were not met.

Study of skeletal abnormalities - Alizarin Red and Alcian Blue staining

At the end of the trials, a random sample of 50 individuals was taken from each feeding regime and replicate. Fish were anesthetized till death (phenoxyethanol–), and then preserved in phosphate buffered 5% formalin(pH=6.8) . Then the samples were stained by following a double staining protocol, using Alizarin Red S and Alcian Blue dyes. Alizarin Red is a specific dye for all the ossifying or ossified structures, while Alcian blue specifically stains all the cartilage elements. Fish staining followed the methodology of Walker and Kimmel (2007).

The examination of the skeleton was performed with the help of a stereoscopic microscope. Additionally, axial-skeleton abnormalities were re-examined, on individual photographs (Olympus SZX16) of the specimens of each sample . The significance of the difference in the abnormality frequencies between the experimental conditions was tested by means of G-test (Sokal and Rohlf 1981).

Micro-CT imaging

To better visualize the anatomy of skeletal abnormalities of particular interest, micro-CT imaging was used. Scanning of the samples were carried out at the Hellenic Centre of Marine Research, using a computed microtomography (Skyscan 1172, 1–1.3 μm resolution, 180° total rotation, 970 ms exposure time, 40 kV voltage, 250 μA) (Printzi et al., 2021). Using X-rays, it is possible to get a 3D imaging of the desirable structure. During the scanning, the sample is located and rotated between an array of detectors and a high-energy X-ray source. From each angle it is obtained a projection image. Those images are greyscale images on which the absorption of X-rays of the object are depicted. Dense areas (small passage of X-rays) correspond to the white color, while less dense regions (high X-ray penetration) correspond to black. The 3D reconstruction of the projection images was obtained with the help of a specific software (NRecon, SKYSCAN)

Mortality and growth rates

After the end of the samplings for RNA fixation, a random sample of ca 50 fish was taken. Fish were anaesthetized and measured for standard length (SL) under a stereoscopic microscope (Olympus SZX16) equipped with a digital camera). Individual specific growth rate (SGR) was then estimated by using the formula $SGR = \frac{lnSL_2 - lnSL_1}{t_2 - t_1}$, where SL_2 is the SL of each individual at the end of the trials, SL_1 is the SL of zebrafish larvae at first feeding (3.5 mm, Parichy et al. 2009), t_2 is the fish age at end of the trials (days post fertilization, dpf) and t_1 is the fish age at feeding onset (4 dpf). The significance of the differences between in SGR between the two treatments of each replicate was tested by means of Students' t-test. Dead fish were recorded every day, so that mortality rate could be estimated for every replicate. The significance of the differences between the mortality rates of the two treatments was tested by means of Mann-Whitney U-test.

Experimental design of an additional task

Experimental design

Following the results on the frequency and the typology of skeletal abnormalities in the first task, to examine the ontogeny and anatomy of a new abnormality type, linked with poor ossification of membranous bones (and lacunae formations), a series of samples was also taken. In this case the same feeding regimes were followed, and fish were either fed normally with *Artemia* nauplii (Control) or exlusively with dry microdiet (Figure 2.2). During the experiment abiotic conditions were monitored constantly. All fish were kept under a 14 hours light: 10 hours dark photoperiod (Table 2.5).

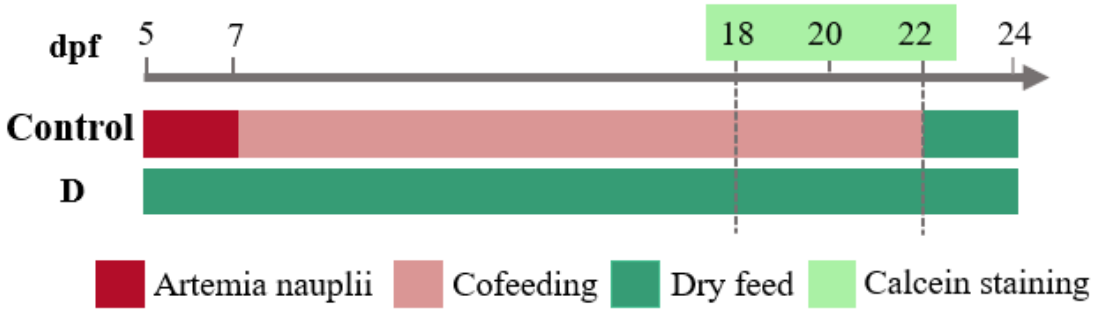

Figure 2.2: Experimental design of the feeding regimes followed including co-feeding of dry feed and *Artemia* nauplii (Control) and exclusively dry feed (D). dpf, days post fertilization. Light green indicates the time period when calcein staining took place, to take the necessary samples of the developmental stages targeted.

Table 2.5: Mean values (±SD) of the abiotic conditions monitored throughout the experimental processes. All fish were kept under a 14 hours light: 10 hours dark photoperiod.

Parameters	Nutritional treatment	Replicate 1
Temperature (ºC)	Control - D	27.9 (±0.2)
pH	Control - D	8.38 (±0.04)
Conductivity (µS/cm)	Control - D	454 (±22)
O_2 saturation (%)	Control - D	85 (±5)
Ammonia (mg/L)	Control - D	<0.02
Nitrate (mg/L)	Control - D	<0.20
Nitrite (mg/L)	Control - D	<0.05

Sampling

This task was only performed in one replicate and two more target groups were added. In order to test if the lacunae formations appear at an earlier stage samples were also taken at two developmental stages, the one as the first task (7.5-8.5 mm) and the other one at an earlier stage of 5.5-6.5 (ca 17-18 dpf), which is also a developmental milestone (Parichy et al. 2009). Moreover, one another objective was added, this of the transcriptomic analysis inside the same group. Thus, in each sampling at the two different developmental stages an effort was made to subdivide the samples to normally developed and deformed (presenting lacunae). As it appeared, the lacunae formations were not that obvious at this developmental stage. Approximately 10 samples of normally developed fish were taken from each developmental stage and feeding regime (10 normal samples for control at 5.5-6.5mm, 10 for control at 7.5-8.5mm, 10 for D at 5.5-6.5mm, 10 for 7.5-8.5mm). Likewise, less than 5 samples were taken of deformed fish per condition, feeding regime and developmental stage (at 5.5-6.5mm stage almost none deformed fish were found at any treatment). Aim of this separation was to test possible differences in gene expression between the experimental groups and within each group separately at both developmental stages. The samples remain to be analyzed for gene expression though. This experiment was executed in one replicate only. Mortality rate was 5%.

Calcein staining and RNA fixation

Calcein staining was performed following the protocol used for the first task, in order to monitor the development and ontogeny of the lacunae formations without killing the fish. After the examination for deformities at each developmental stage, fish were either kept as samples or returned to the rearing regime (in the case of the first samplings only, when fish standard length ranges to 5.5-6.5 m) to test whether they would develop this deformity later on development. Following, the samples that were kept, were fixated in RNA later.

Chapter 3: Results

Effect of feeding conditions on the frequency of skeletal abnormalities

Skeletal analysis showed that abnormalities developed on all the anatomical parts of the fish (cranium, vertebral column, fins). Cranial abnormalities consisted of gill-cover abnormalities (Figure 3.1a), where a curving of the operculum was observed along with twisted branchiostegal rays (Figure 3.1b). No abnormalities were detected on the jaws.

Fin abnormalities mainly affected the anal, caudal and, to a less extent, the dorsal fin. Anal and dorsal fin abnormalities developed in the form of missing rays (Fig. 3.2a, 2b) and/or pterygiophores (proximal and/or distal, 3.2b). Anal and dorsal fin abnormalities were often associated with underdeveloped primordial marginal finfold (Fig. 3.2c, 2c'). Caudal fin abnormalities consisted of shortened lepidotrichia on an abnormal finfold (Figure 3.2d, 2e), missing lepidotrichia (Figure 3.2f), as well as internal defects of individual supporting elements (Figure 3.2g, 2g'), that did not affect the external fin morphology.

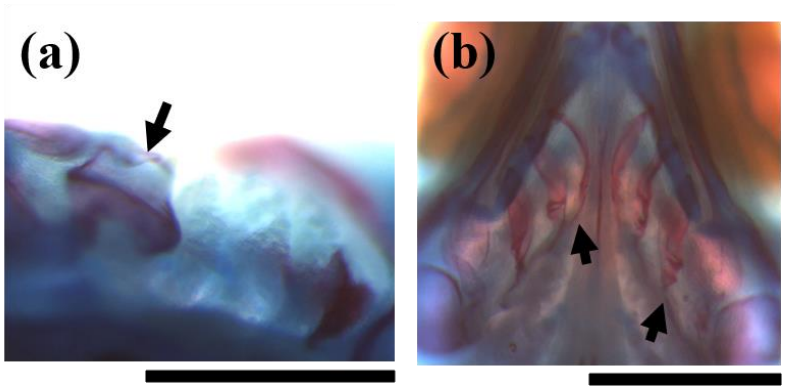

Figure 3.1:. Abnormal gill-cover (a, arrow) and branchiostegal rays (b, BRr). Scale bars equal to 0.5 mm.

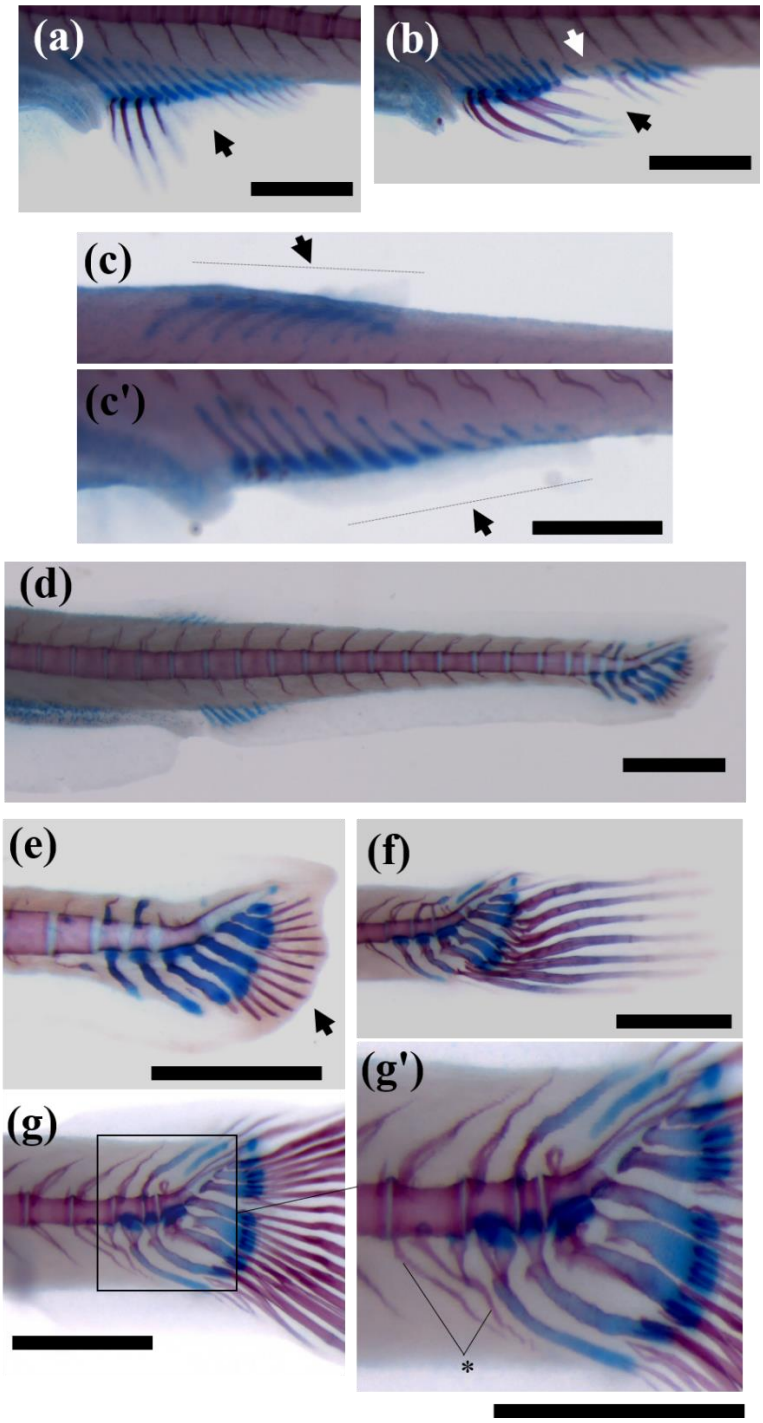

Figure 3.2: Representative cases of abnormalities of the unpaired fins. (a-b), Missing anal fin rays (a) and pterygiophores (b). Black arrows indicate the missing rays and the white the missing pterygiophores. (c-c') Dorsal and anal fin abnormalities (missing rays) with underdeveloped primordial marginal finfold. (e-g) Caudal fin abnormalities with shortened (d, e) and missing rays (f), or abnormalities of single supporting elements (*, g, g'). Scale bars equal to 0.5 mm.

Vertebral deformities consisted of caudal-peduncle scoliosis, pre-heamal kyphosis of light severity and bended neural processes on the centra dorsally to the swimbladder. Additionally, a variety of abnormalities of vertebral centra appeared in the form of lacunae (Figure 3.3a, 3e. 3e') and ossification irregularities (Figure 3.3b, 3c), deficient ossification of the endplates (Figure 3.3d), compressed and swollen centra (Figure 3.3f) and deformed vertebral processes (Figure 3.3g). Ossification abnormalities of the vertebral centra and lacunae forms were also presented through micro-computed tomography (CT) analysis (Figure 3.4).

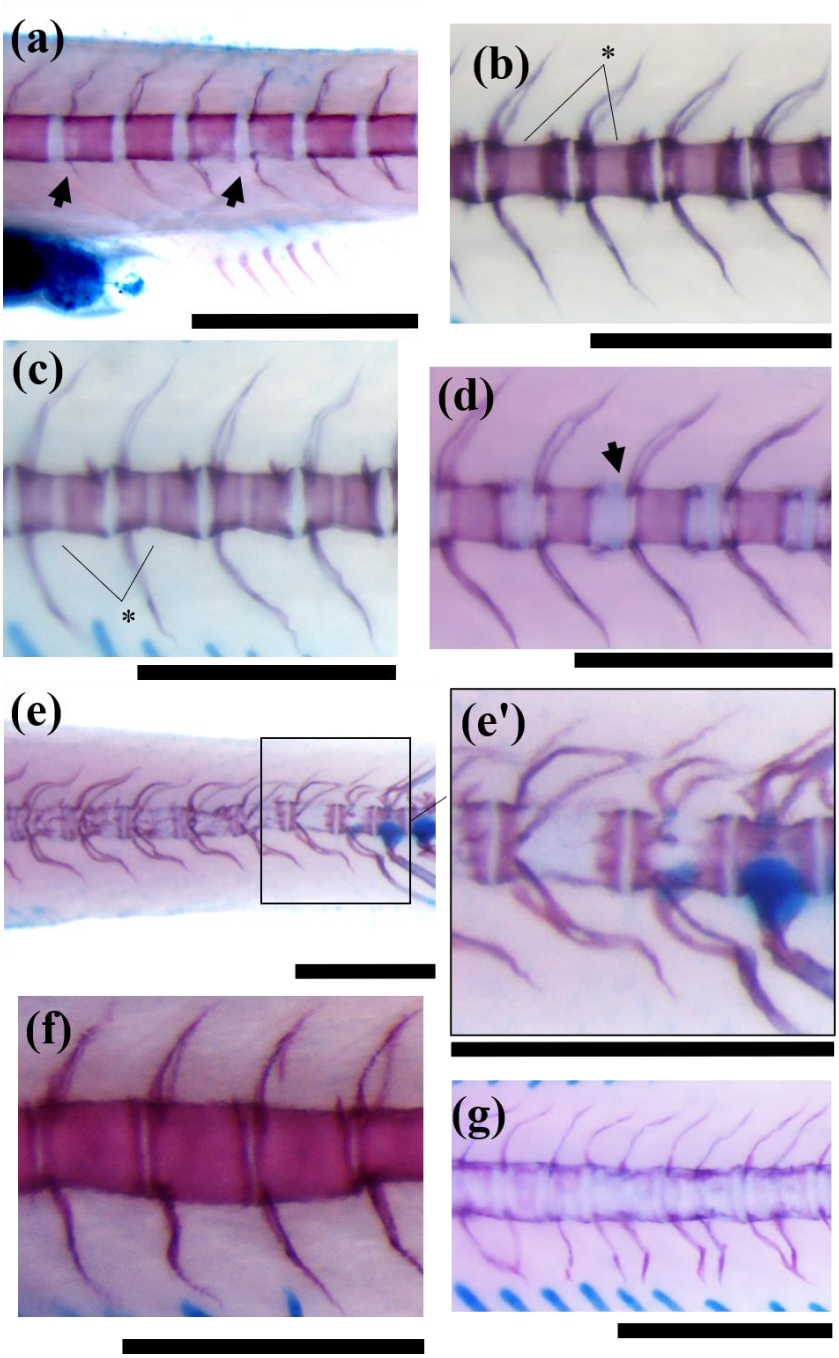

Figure 3.3: Vertebral deformities. Abnormalities of vertebral center in the form of lacunae (arrow, a, e, e'), ossification irregularities along the vertebral column (*, b,c), poor ossification of the endplates (arrow, d), compresses and swollen vertebral centra (f) and deformed vertebral processes (g). Scale bars equal to 0.5 mm

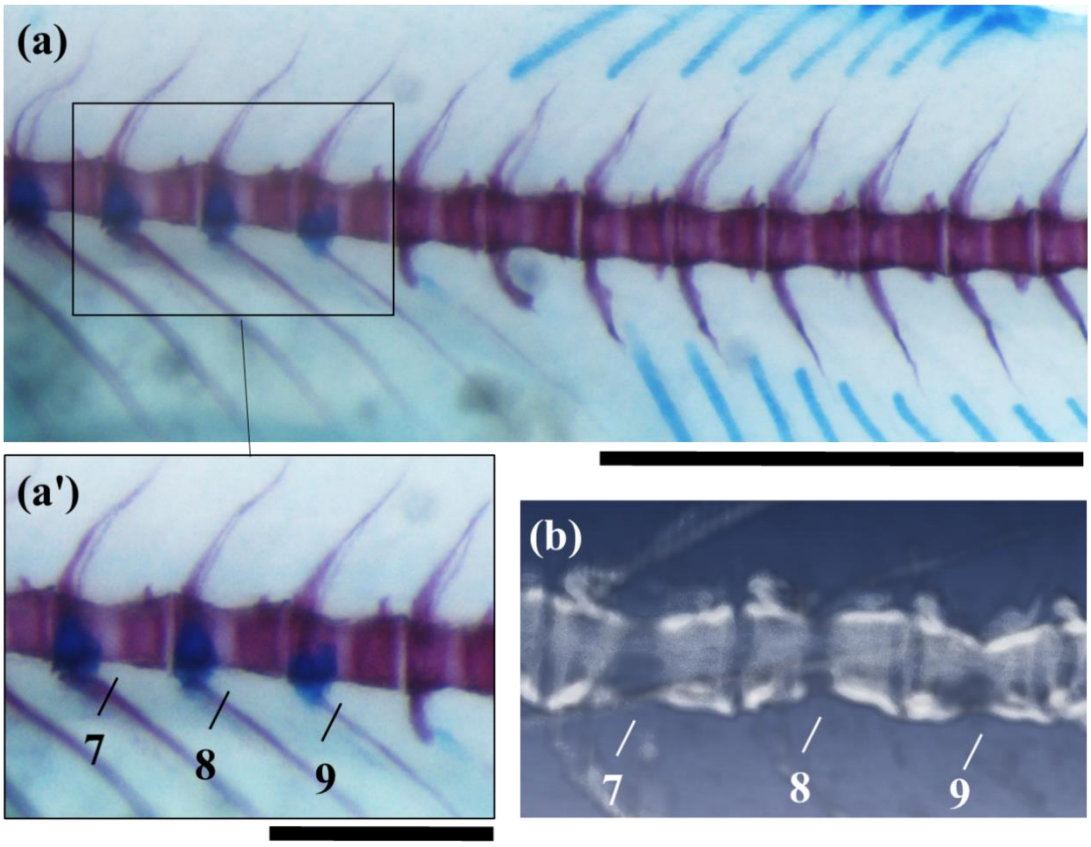

Figure 3.4: Irregular ossification and lacunae. A sample stained for bone and cartilage analysis (a, a') and micro computed tomography (CT) scanning (b). Numbers refer to each vertebra. Scale bars equal 1 mm.

Following the results of the fourth trial, calcein staining revealed that lacunae first appeared at ca 5.5-6.5 mm SL (11-12 dpf) with a frequency of 3% (6 out of 200 fish in D group) (Figure 5a, a'). In the next sampling period (7.5-8.5 mm SL, 20-22 dpf) this frequency increased to 10% (20 out of 200 fish in D regime). At the Control condition only one fish (out of 200) was found with lacunae formations and all the remaining fish had a normally developed vertebral column (Figure 3.5b). Samples were fixated in RNA later for future gene expression analysis. Additionally, calcein staining allowed the monitoring of other deformities such as bended neural processes dorsally to the swimbladder (Figure 3.5c)

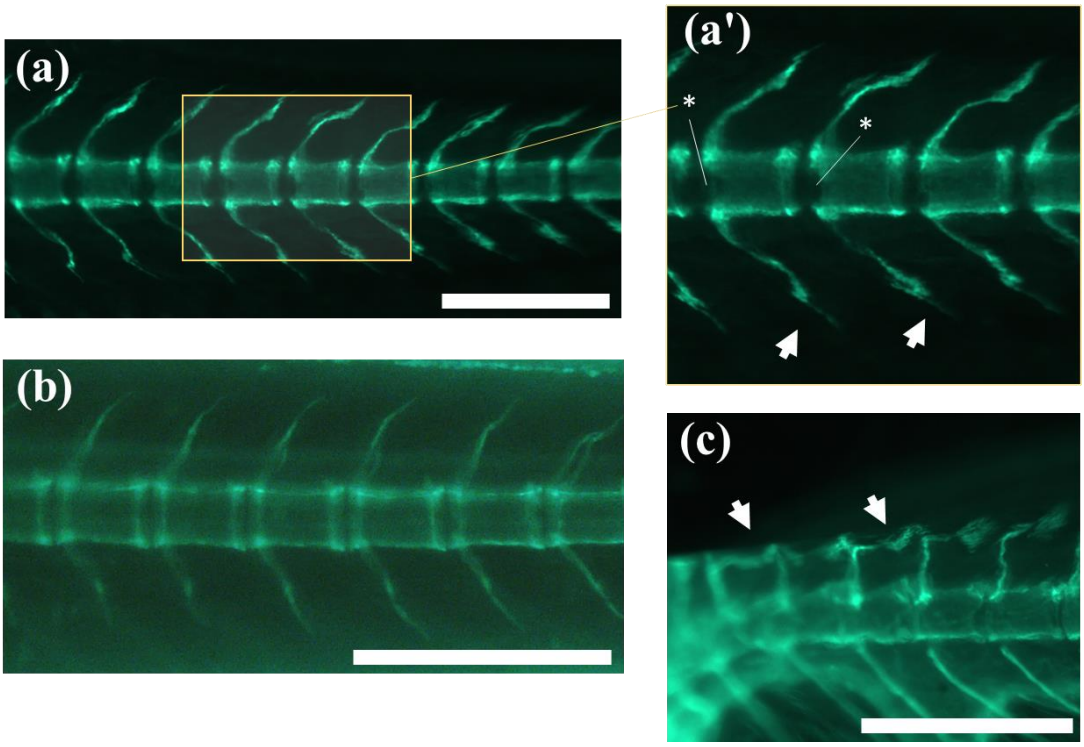

Figure 3.5: Deformities recorded by using *in vivo* calcein staining. Lacunae forms at an eraly stage ca 5.5 – 6.5 mm SL (* a, a'), normally developed vertebral column (b), bended neural processes (arrow, c). Scale bars equal to 0.5 mm.

Larval feeding protocols significantly affected the development of skeletal abnormalities in zebrafish larvae, with D group presenting comparatively elevated abnormality frequencies (Fig. 3.6). The most frequent deformities referred to the branchiostegal rays (Figure 3.6a) (10±9% in the control group vs 42 ±12% in the D group, mean±SD), the neural processes which appear to be bended (Figure 3.6b) (10±8% at the Control group vs 47±8% at the D group) and finally to cases of irregular development of vertebrae (swollen or compressed) and their processes (Figure 3.6c) (22±20% at the Control group vs 38±1% at the D group). Some deformities with lower frequency of appearance were those of the gill cover (Figure 3.d) (25±4% at the Control group vs 4±3%at the D group), the caudal fin (Figure 3.6e) (6±0.2% at the Control group vs 16±6% at the D group) and cases of caudal peduncle scoliosis (Figure 1f) (6±4% at the Control group vs 23±3% at the D group). The frequency of kyphotic fish was the lowest at 9±4% (at the D group vs 2±1% at the control group) (Figure 3.6g), yet significant between the groups. Interestingly deformities of dorsal and anal pterygiophores had a frequency of 32 ±7% (at the D group vs 22±5% at the Control

group) (Figure 3.6h), while the case of ossification abnormalities of the vertebral centra and lacunae forms were recorded at a level of 34 ±32% (at the D group vs 4%±2% at the Control group) (Figure 3.6i).

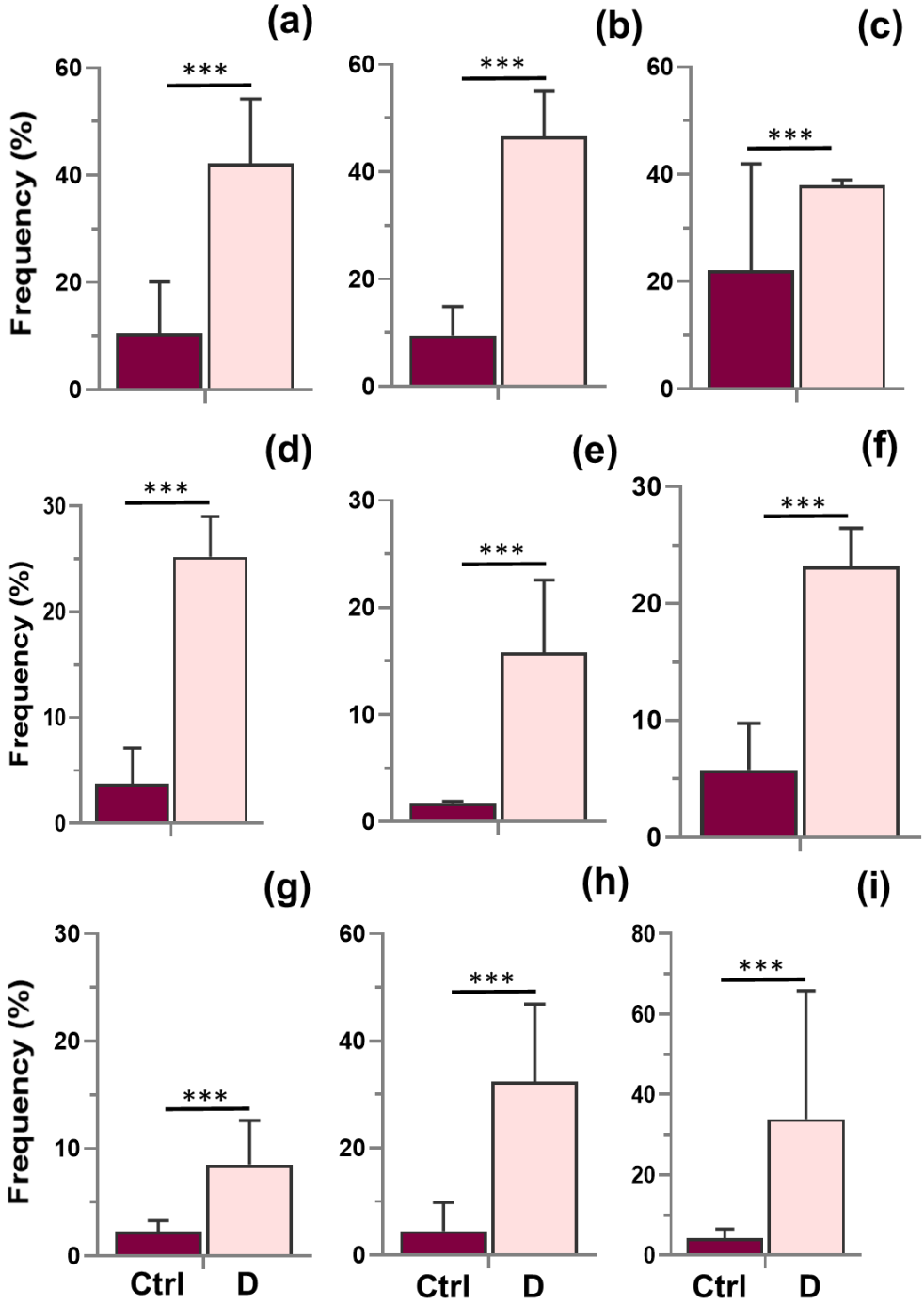

Figure 3.6: Effect of larval feeding protocol on the frequency of skeletal abnormalities in zebrafish larvae. Branchiostegal rays abnormalities (a), bended neural processes (b), pattern formation (compressed or swollen vertebrae and/or their deformed processes) (c), gill cover abnormalities (d), caudal fin abnormalities (e), caudal peduncles scoliosis (f), kyphosis (g), dorsal/anal pterygiophores abnormalities (h), poor ossification

(lacunae forms and abnormalities of vertebral centra) (i). (***, statistical significance) ($p<0.05$, G-test). Error bars = 1 SD.

Expression analysis of specific target genes related to zebrafish skeletogenesis

Only light cases of kyphosis were observed in a few samples, and in order to avoid non-objective categorization of the deformity, it was decided that for the gene expression analysis random samples would be chosen between the experimental groups. The expression of the genes of interest is depicted in Figure 3.7, normalized according to the geometric mean values of the reference genes (*gapdh, eef1a1l1*). Outliers were examined individually and excluded from the analysis in the cases of technical errors (failure of one of the technical replicates, human handling errors).

The qPCR results of the target genes *bglap, col1a1a, acp5a* and *runx2b*, showed no significant differences in expression levels between the two experimental groups ($p>0.05$, Mann-Whitney U test). *Sp7* gene showed significantly different levels of expression between the two experimental groups (p-value<0.05, Mann-Whitney test). The target gene *mmp13a* was excluded from the analysis since it appeared to have Cq values higher than 30 (Cq value refers to the PCR cycle number at which the sample's reaction curve intersects the threshold line). Those values could be attributed to either a defective primer, and/or not inadequate amount of mRNA for the specific gene.

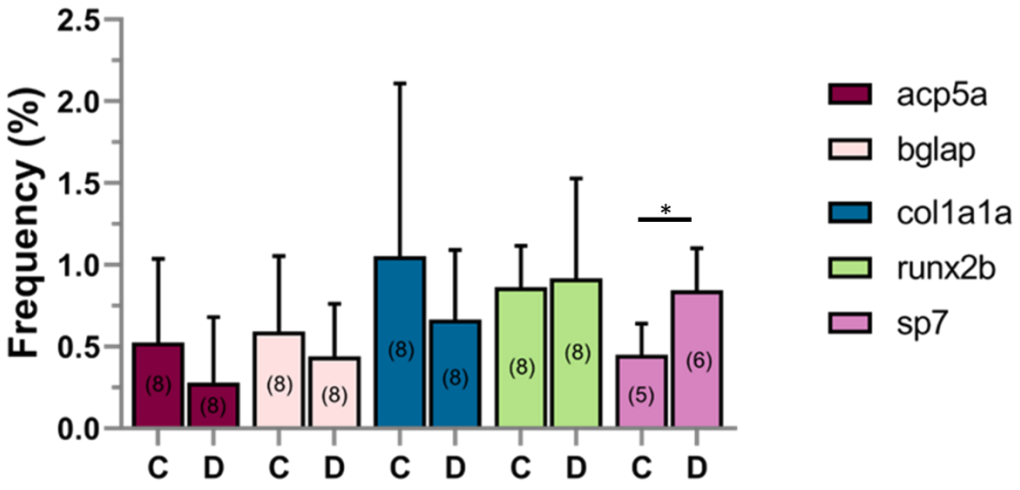

Figure 3.7: Effect of feeding conditions (C, control and D, Dry feed) on the relative gene expression levels (mRNA). Expression levels were measured by qPCR and the values taken were normalized to the reference genes (*gapdh*, *eef1a1l1*). Values with

asterisk are statistically significant between the experimental groups (p-value<0.05, Mann-Whitney test). Brackets indicate the number of samples used for the gene expression analysis per condition and target gene. Error bars = 1 SE.

Mortality and growth rates

Larval feeding protocol did not significantly influenced fish mortality between the experimental groups (Figure 3.8a,b).

As it was indicated by the larval size (SL) measurements, feeding regimes significantly affected zebrafish growth rate. Control groups in all replicates presented significantly larger larvae than group D (p<0.001, ANOVA) (Figure 3.8c). Specifically, the results showed that in replicate 1 SGR was 0.14 days^{-1} ±0.01 SD at the control group while at the D group it was 0.11 days^{-1} ±0.01 SD. Likewise the control group at the replicates 2 and 3 showed an SGR of 0.11 days^{-1} ±0.01 for both and 0.10 days^{-1} ±0.01 SD for both at the D group as well.

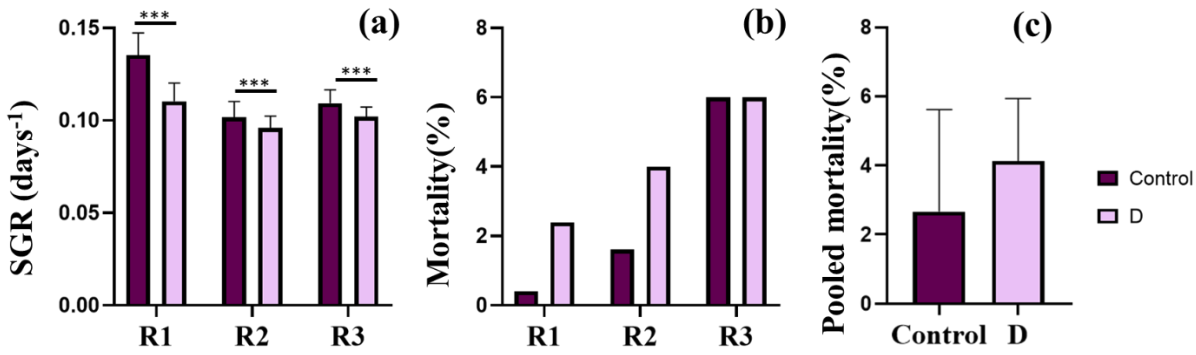

Figure 3.8: Effect of larval feeding protocols on the mortality and growth of zebrafish larvae. Mean standard growth rate (SGR) of zebrafish larvae in the different protocols and replicates (Rep1-Rep3) (a), Mortality rate of the different protocols and replicates (Rep1-Rep3) (b) , mean mortality rate in the different protocols. (c). (***, p<0.001, ANOVA). Error bars = 1 SD

Discussion

Various indices have been used in commercial hatcheries as predictors of the quality of fish, in terms of body shape, since it is affected by the developmental of skeletal deformities. Potential molecular predictors though have not been established yet, although they could be a valuable tool in deciding whether a batch deserves to be reared to commercial size or not. In this study we examined whether specific conditions that induce skeletal abnormalities may be associated with differentiated gene expression. For this purpose, we used two different feeding regimes, one of them known to be associated with elevated rates of skeletal deformities according to Printzi et al. 2021, and fixated samples for gene expression analysis. Since the development of skeletal deformities was our main interest, we focused on the expression levels of genes which participate in bone mineralization (*bglap*, *col1a1a*), ossification (*sp7*, *runx2b*) and resorption (*mmp13a*, *acp5a*) processes (Bensimon-Brito et al. 2012; Carvalho et al. 2017; Tarasco et al. 2021). Some really important cell types that participate in bone formation are osteoblasts and osteoclasts. The former participates in bone formation, while the latter in bone resorption. Our results confirmed in terms of all the deformities tested, that the one feeding regime had indeed high rates of deformities appearance. Kyphosis of light severity was recorded. Among the target genes chosen for the analysis, only one exhibited differentiated gene expression between the experimental groups.

Among the genes tested, our results showed an upregulation of the *sp7* gene at the microdiet treatment, where also skeletal deformities were frequent. *Sp7* is an osteoblast-related gene, a fact that could justify its upregulation where bone mineralization anomalies appear. *Sp7* also has a role in osteoblast differentiation (Li et al. 2009) especially during the first days of development (5 dpf). Thus, an upregulation of *sp7* could be a reaction to any delay of bone formation (Gebuijs et al. 2022). In our study, at the group treated only with dry diet, there were frequent cases of ossification abnormalities and lacunae forms along the vertebral column. This fact could also justify the *sp7* upregulation, since it has been linked to bone regeneration as well (Hosoya et al. 2013). *Sp7* has an important role at the developmental stage approximately at 5 dpf,

when the larvae are fed for the first time. The samples for gene analysis were taken during 20-22 dpf, where fish were fed with dry feed at both experimental groups though. However, the upregulation of *sp7* recorded, could be a response to an abnormal skeletogenesis that occurred while fish were fed at an earlier ontogenetic stage during first feeding. This could be attributed to either a delayed rate of osteological development, or at the fact that lacunae formations appear and thus, this upregulation is associated with an altered gene expression of an abnormal phenotype.

At the present study, four more genes were analyzed. Physiologically, two of these genes are involved and linked in a common pathway with *sp7*. *Bglap*, *runx2b* and *col1a1a* are bone mineralization-related genes. *Bglap* especially, encodes osteocalcin secreted by osteoblasts and therefore is implicated in calcium ions homeostasis and bone mineralization (Chen et al. 2019). Interestingly, *sp7* is known to be directly linked to the function of *bglap* since it regulates its expression by binding to the gene's promoter. It is also supported that the interaction of these two genes enhances the mineralization of bones. The gene *runx2b* is also osteoblast-related and linked to *sp7* by regulating its expression (Dong et al. 2023). According to that we could expect *bglap* and *runx2b* to exhibit differences in the expression levels between the experimental groups, yet none of this happened. However, it has also been shown that in cases of *sp7* mutants bone mineralization still occurs, suggesting the existence of other additional pathways apart from the regulation system of these three genes (Chen et al. 2019). *Col1a1a* is one of the three collagen type I genes that are expressed in zebrafish, a gene also closely related with osteoblasts especially in larvae. Moreover, mutants of this genes have been associated with Osteogenesis imperfecta (OI) in zebrafish, a rare genetic disease linked to bone fragility (Huang, Liu, and Zhang 2021). *Col1a1a* is osteoblast-related and expected to be upregulated in cases of deficient ossification, such as the ossification irregularities presented in this study. Since some genes appear to be linked with *sp7*, we expected more than one gene to exhibit differentiated gene expression between the experimental groups. Yet, the fact that in cases of mutant *sp7* mineralization still occurs, indicates the existence of more pathways that could regulate such conditions. Thus, in the future it would be interesting to test more genes, involved in different pathways to examine their response.

Two of the genes were osteoclast-related as well. We examined those based on the lacunae formations that appeared at the experimental groups. *Acp5a* gene in zebrafish, encodes tartrate-resistant acid phosphatase (TRAP) which is secreted by

dendritic cells, macrophages and most importantly be bone-resorbing osteoclasts (He et al. 2018). Our results did not show any significant differences between the experimental groups. Of course, *acp5a* is one of the many genes related to osteoclasts function that could be examined, and perhaps this one is not associated with deformities observed in this study. Thus, the theory of an osteoclast-upregulation responsible for the poor ossification phenotype needs more backing, and maybe more genes to be tested. For all the above results though, we should also consider that maybe the gene expression does not alter.

Another osteoclast-related gene used for our study was *mmp13a*. This one was excluded from all the analysis though, and we did not take the pPCR results into consideration since the Cq values that occurred were higher than acceptable. The higher the Cq values (>29-30) the more uncertainty appears about the quantity of the target (Ruiz-Villalba, Ruijter, and van den Hoff 2021), which could be in lower amounts. Conversely there could be a problematic primer which either amplifies itself due to higher affinity between its tails, or higher primer concentrations than needed (Bustin and Huggett 2017). Additionally, following a different approach in the future to examine the response of this gene as well, we could either attempt to make the analysis having more samples or samples of an earlier ontogenetic stage, where the feeding regimes followed were different. Finally, a more appropriate approach would be to perform RNA sequencing so to make a targeted selection of genes in general.

In general, though, we ought to be cautious about the results we got, since we cannot be sure about the tissue specificity. It was difficult to isolate the bone structures of interest in larvae of this size, and thus we performed RNA extraction on the whole fish. This implies that the samples used for the gene expression analysis included apart from bone tissues, muscles and other types of tissues. This fact could have affected the expression levels we got for the reference genes, and thus the overall analysis for the rest of the genes.

Printzi et al. (2021) recorded a variety of skeletal deformities, induced by specific nutritional treatments, two of which were also used in the present study. Most of our results confirm those of literature except for two cases. First, we noticed a lower rate of kyphosis appearance at the microdiet treatment than expected, and almost all of them were of light severity. What we observed though, was a high rate of bended neural processes. The fact that we did not record as many kyphotic fish as expected, and instead we got more deformed neural processes, could be attributed to the earlier

sampling we did, compared to literature. In our study we targeted fish with standard length (SL) at a range of 7.5-8.5mm, in which kyphosis may have not been formed yet. Moreover the elevated rate of bended neural processes could justify that, since this phenotype is considered to be an early ontogenetic sign of kyphosis (Koumoundouros et al. 2002).

Another deformity we observed was this of the poor ossification, mainly in the form of ossification abnormalities of the vertebral centra and lacunae forms. To our knowledge this is among a few studies where this phenotype has been recorded in zebrafish. This deformity appeared in a variety of phenotypes, and one of them included the non-ossificated vertebrae endplates. Cotti et al. (2020), recorded the same phenotype in cases where there was a low phosphorus (P) diet. In another study with a similar experimental design, low phosphorus content was linked with upregulation of osteocalcin (*bglap*) (Costa et al. 2018), a fact that does not keep up with our results. Yet, we could not exclude P as a causative factor, since in both of these studies the low-P effect was performed at post-metamorphic normally developed animals (~20-24 dpf), in contrast with our study where the effect of diet occurs at the incident of first feeding. Non-supplemented diet of copper (17 mg Cu/kg) was also found to be linked with a delay of mineralization (Tseng et al. 2023), a fact that could also justify the ossification irregularities we recorded in this study. Yet, in the case of Tsang et al. (2023) fish were treated with different Cu levels at a post-metamorphic developmental stage (47 dpf). Additionally, there are a few studies that attempted to link abnormal vertebral development with the expression of some genes related to osteoblasts' maturation and matrix mineralization (e.g Riera-Heredia et al. 2019), yet most of them were focused in deformities such as lordosis, kyphosis etc, and not in cases of ossification irregularities and bone resorption.

Fin abnormalities were also recorded. Mostly shortened or missing rays were noticed, which often were linked to deformities of the pterygiophores at fish of greater length. At fish of smaller length along with these deformities, also a deformed marginal finfold was recorded. Saddleback (SBS) syndrome is a deformity that in *Dicentrarchus labrax* has been directly associated with missing rays and pterygiophores because of an abnormally developed finfold (Fragkoulis et al. 2016). In our study we obtained similar results. Thus, since the causative factors of this deformity remain unknown in most of the rearing species, zebrafish could become a model organism for the study of this skeletal deformity.

For the identification of causative factors for the new abnormalities recorded in this study, more research is required, yet a few assumptions could be made. At first, a low P diet probably could not be excluded since it is still unknown which effect it could have at earlier ontogenetic stages. Also, abiotic parameters could not be recognized as causative factors, since they were monitored on a daily basis, and were kept identical throughout the experiment. Both experimental groups were reared under the same abiotic conditions. The differences at the skeletal deformities expected between the present study and this of Printzi et al. (2021) could be attributed to the fact that in our study we used the AB strain for the experiments, unlike literature where the strain Wagenigen (WAG) was used.

In conclusion, zebrafish could become a model organism for the study of significant skeletal abnormalities recorded in aquaculture species such as lordosis (Printzi et al. 2019; Printzi, Kourkouta, et al. 2021b; Printzi et al. 2022). Although in this study we noticed some deformities that appeared in a differential way (e.g., kyphosis, fin abnormalities) yet, they still develop in zebrafish the same way following similar ontogenetic pathways. Additionally, another deformity was recorded, this of lacunae formation, which has been recorded to appear in a differential way in other species too (Kourkouta et al. 2022). Also, according to the results of A. Printzi (personal communication), corresponding deformities appear in the vertebrae of other species such as *D. labrax* (Figure 6.1). In this case it is suggested that zebrafish could become a valuable model for the understanding of the underlying mechanisms of skeletal deformities in other species too, even the commercial ones.

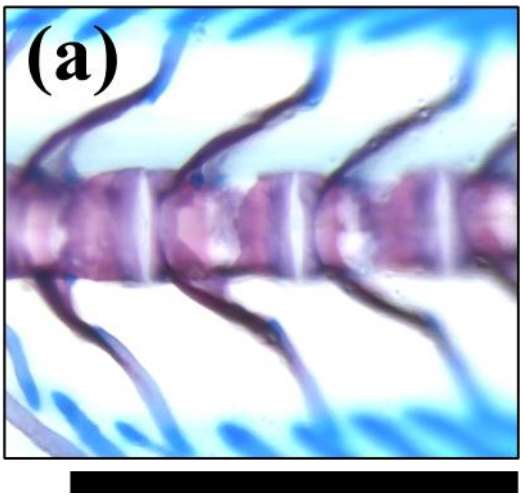

Figure 6.1: Representative case of ossification irregularities in the form of lacunae in *Dicentrarchus labrax* (a). Scale bars equal to 1 mm.

Chapter 5: Conclusions

As expected applied nutritional regimes affected skeletal development. Despite this repeatability of the experiments, it seems that abnormal patterns of skeletogenesis can always develop, since in this study an additional deformity of bone tissue resorption appeared. Also, a case of fin deformities possible linked to abnormal development of marginal finfold was recorded in zebrafish. Deformities of the finfols are linked to saddleback syndrome, making zebrafish it a valuable model for the study of this deformity as well. From the target genes tested for differentiated gene expression, only one (*sp7*) exhibited statistically significant differences. Finally, it was observed that the exclusive intake of dry microdiet was responsible for the increased rate of deformities, compared to the control group. The different feeding regimes did not influence the mortality rate, yet the standard growth rate was significantly higher in the control group than the D group. Calcein staining could be a valuable tool for the monitoring of development of deformities such as lacunae upon the vertebral column. Because of that, it is known that ossification irregulations appear at a standard length of 5.5 – 6.5 mm (11-12 dpf) on zebrafish.

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Annotation/Dictionary

- Dpf = Days post fertilization
- Hpf = Hours post fertilization
- ECM = Extracellular matrix
- Bone remodelling = continuous bone replacement
- SL = Standard length
- SE = Standard error
- 260/230 = the ratio used to assess the purity of RNA
- 260/280 = the ratio used for the assessment of the quality of the RNA sample