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# Plasticity of the skeleton and skeletal deformities in zebrafish (Danio rerio) linked to rearing density

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**Significance Statement:**

The rearing density for zebrafish is often not reported in the literature. Inappropriate rearing density and small tank volumes are known to affect teleost skeletal development. This study shows that rearing density effects the body size and the skeletal phenotype in zebrafish. It is important to distinguish skeletal defects related to rearing condition from defects related to experimental conditions if zebrafish is used as a model to study skeletal development in teleosts or skeletal diseases in humans. This study provides an adaptable methodology for the assessment of skeletal malformations.

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# Plasticity of the skeleton and skeletal deformities in zebrafish (*Danio rerio*) linked to rearing density

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**Abstract**

The teleost zebrafish (*Danio rerio*), an established model for human skeletal diseases, is reared under controlled conditions with defined parameters for temperature and photoperiod. Studies aimed at defining the proper rearing density have been performed with regard to behavioural and physiological stress response, sex ratio and reproduction. Studies concerning the effect of rearing density on the skeletal phenotype are lacking. This study is designed to analyse the response of the skeleton to different rearing densities and provides a description of the skeletal deformities. Wild type zebrafish were reared up to 30 dpf (days post-fertilization) in a common environment. From 30 to 90 dpf, animals were reared at three different densities: high density (HD) 32 fish/L, medium density (MD) 8 fish/L and low density (LD) 2 fish/L. Animals at 30 and 90 dpf were collected and whole-mount stained with Alizarin red S to visualise mineralized tissues. The entire skeleton was analysed for meristic counts and 172 types of deformities. The results showed that rearing density significantly influenced the specimens' average standard length, which decreased with increasing rearing density. Differences concerning meristic counts among the three groups were not observed. Rearing density-independent malformations affected the ribs, neural arches and the spines of the abdominal region as well as vertebrae of the caudal complex. The HD group showed the highest number of deformities per specimens, the highest number of observed types of deformities and, together with the MD group, the

highest frequency of specimens affected by severe deformities. In particular, the HD group showed deformities affecting arches, spines and vertebral centra in the caudal region of the vertebral column. This study provides evidence of an effect of rearing density on the development of different skeletal phenotypes.

Keywords: deformities, plasticity, rearing density, skeleton, zebrafish

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## Introduction

Phenotypic plasticity, a component of phenotypic variation (Klingenberg, 2019), is the ability of living organisms to respond to environmental or internal stimuli through changes in behaviour, morphology or physiology, producing different phenotypes. Phenotypic plasticity can be adaptive or non-adaptive, reversible or irreversible, and its type and degree are specific to the single trait and the environmental conditions involved. In an evolutionary perspective, phenotypic plasticity is a feature of the reaction norm of a trait of single organisms (i.e. the complete set of phenotypic responses of a trait to a specific environmental variable), that can be the target of natural selection, steering towards phenotypic accommodation and genetic assimilation (Pigliucci et al., 2006, Schmalhausen, 1949, Waddington, 1953; West-Eberhard, 2003, 2005).

Phenotypic plasticity is particularly relevant for skeletal tissues. The vertebrate skeleton is composed of five main different skeletal tissue types: notochord, cartilage, bone, dentin and enamel/enameloid. In teleosts, several intermediate tissue types are present and skeletal tissues are considered part of a continuum (Hall and Witten, 2019; Witten et al., 2010). They are able to respond to intrinsic and extrinsic cues (Roux, 1881; Ruff et al., 2006; Weinans and Prendergast, 1996; Wolff, 1892). Skeletal tissues and cells are plastic and dynamic throughout life as they modulate their structure in response to the mechanical load regime. The processes through

which the skeletal cells achieve modifications are modulation, metaplasia, transdifferentiation and remodelling (Hall and Witten, 2007; Witten and Hall, 2015). The ability of tissues to modulate their phenotype in response to mechanical load is known as "Wolff's law of bone transformation" (Wolff, 1892) or as “bone functional adaptation” (Ruff et al., 2006). A famous example is the two-legged goat, whose hind limbs and thoracic skeleton became modified to adapt to the bipedal gait (Slijper, 1942).

Examples of phenotypic plasticity of the teleost skeleton are numerous. In cichlids, differences in the hardness or type of food modify the jaw shape, the number and strength of jaw bone trabeculae and the size of replacement teeth (Huysseune, 1994, 1995; Meyer, 1987). The mechanical load exerted by swimming changes the shape of vertebral ~~bodies~~ centra and can induce lordosis in different teleost species (Kihara et al., 2002; Kranenbarg et al., 2005). Forced swimming accelerates ossification rate of vertebral bodies and cartilage formation in the head and the caudal fin in zebrafish *Danio rerio* (Hamilton 1822) (Fiaz et al., 2012; Suniaga et al., 2018; van der Meulen, 2005).

The rearing of fish implies the modification and control of several environmental factors (e.g., photoperiod, temperature, type of diet, diet composition, hydrodynamics) in order to optimize rearing conditions in aquaculture or laboratory facilities. Aquaculture-related research provides numerous examples of how modifications of environmental conditions change the skeletal phenotype, including the induction of skeletal ~~anomalies~~deformities.

In aquaculture, farming practices can be classified as intensive, semi-intensive and extensive methodologies. They stand out for several parameters, such as rearing density and tank volume, hydrodynamics and diet. In intensive farming practice, rearing density is high and tank volume smaller compared to semi-intensive and extensive rearing conditions. The latter, besides being characterized by decreased number of animal per volume and larger tanks, utilises practises aimed at simulating the natural environment. This includes differentiated hydrodynamics, and large live prey availability and variety (Baluyut and Balnyme, 1995; Cataudella and Bronzi, 2001). The above-mentioned rearing methodologies can affect the morphology of the skeleton. In rainbow trout *Oncorhynchus mykis*s (Walbaum 1792), the occurrence of skeletal ~~anomalies~~ deformities increases significantly in animals reared in intensive conditions compared to animals reared in extensive conditions (Boglione et al., 2014). Similar observations have been reported for advanced marine teleosts: gilthead seabream (*Sparus aurata* L.) and red porgy (*Pagrus pagrus* L.*)* reared in semi-intensive conditions showed a lower number of skeletal deformities per individual and a lower number of deformed individuals (Prestinicola et al*.,* 2013; Roo et al., 2010). Dusky grouper (*Epinephelus marginatus* Lowe 1834) larvae reared in high-density conditions ~~at the highest stocking density~~ showed the highest frequency of deformed individuals, the highest number of deformities per deformed individual, the largest range of types of deformities and the highest incidence of individuals with at least one severe deformity (Boglione et al., 2009).

*Danio rerio* is an established model organism in biological and biomedical research and is now also used as a model for human skeletal diseases. Insights into fundamental pathways of skeletal formation and skeletal diseases can be obtained, provided the differences between the teleost and mammalian skeleton are considered (Witten et al., 2017). Laboratory zebrafish are reared under controlled conditions, with defined parameters for temperature and photoperiod, but recommendations for rearing densities differ (Castranova et al., 2011) and standards based on experimental data are lacking (Lawrence and Mason, 2012). The Zebrafish Book (Westerfield, 2000) recommends a rearing density of 0.55 adult fish/L, whereas the "Guide for the care and use of laboratory animals" (Clark et al., 1997) and Matthews et al. (2002) recommend 5 to 10 individuals/L for adult fish. Another published housing density is 3.5 fish/L (Tsang et al., 2017). Concerning rearing densities for early life stages, published data range from 6.5, up to 94 fish/L (Carvalho et al., 2006; Goolish et al., 1998; Matthews et al., 2002). As Lawrence (2007) emphasized, "the classifications of densities in zebrafish research tend to vary considerably depending on the experimental setting". Remarkably, studies about the effects of the rearing density in *D. rerio* are scarce (Ribas et al., 2017). Published data refer to the animals' sex ratio (Liew et al., 2012; Ribas et al., 2017), growth rate (Hazlerigg et al., 2012; Ribas et al., 2017), stress and behavioural parameters (Ramsay et al., 2006; Shelton et al., 2015) or reproductive rates (Goolish et al., 1998). The effect of rearing densities on the skeleton and the onset of skeletal deformities

in this species has not been reported. The skeletal phenotype of transgenic and mutant zebrafish lines for genes related to human skeletal pathologies has already been extensively described (Fisher et al., 2003; Gray et al., 2014; Gistelinck et al., 2016; Haller et al., 2018; Lleras Forero et al., 2018; Spoorendonk et al., 2008; Wopat et al., 2018). Conversely, to our knowledge, the only works describing the skeletal anomalies in wild type zebrafish are the study of age-related deformities of Hayes et al. (2013) and a comprehensive description of wild adult breeders and F1 juveniles *D. rerio* made by Ferreri et al. (2000). The latter characterized 25 types of anomalies affecting the vertebral column, vertebrae, fins and cranium.

The aim of this study was to analyse the response of the skeleton of juvenile *D. rerio* to a single environmental variable, *i.e.* rearing density. This study provides a description of skeletal deformities developed in *D. rerio* reared at three different ~~stocking~~ densities during the juvenile stage.

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## Materials and methods

### *Ethics statement*

All experiments were carried out at the Experimental Biology and Aquaculture Laboratory, Università degli Studi di Roma Tor Vergata, approved by the Animal-Welfare body and carried out in accordance with Italian and European rules. All the animal experiments were ethically approved and authorised by the General Director of the Ministry of Health, Legislative Decree no.26/2014; European Directive 2010/63/UE.

*Specimens maintenance and collection*

All the specimens used in this study were obtained from the same pool of AB line (commonly referred to as wild type, WT) zebrafish breeders (n= 15), male:female ratio 1:2, housed in a 25 L aquarium equipped with a bio-mechanical filter. Eggs were obtained by natural spawning. Vital eggs were incubated at 28°C until hatching. After hatching, the animals were transferred in one large aquarium at a density of 20 animals/L and maintained there up to 30 days post-fertilization (hereafter, dpf), a time point when a stable number of individuals was achieved (Figure_1_ SuppInfo). At 30 dpf, the specimens were randomly divided into groups and reared at three densities: i) high (32 fish/L), ii) medium (8 fish/L) and iii) low density (2 fish/L) (hereafter referred to as HD, MD and LD, respectively). The choice was based on the need to find a compromise between having a sufficient number of fish for the analyses (especially for the MD and LD group) and the rearing densities adopted usually in the zebrafish facilities (5 fish/L for the adult stage). The remaining fish were euthanized with a lethal dose (500 µl/L) of 2-phenoxyethanol and fixed (1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) representing a "time zero" sampling point (hereafter referred to as T0).

The water used for all the tanks (breeders, eggs, larvae and juveniles) was obtained mixing equal parts of water treated by reverse osmosis water and 50 µm-filtered well water. The photoperiod was 14L:10D and water parameters were maintained as

follows: water temperature 28°C, pH 6.8-8.5, water hardness 60-200 mg/L $CaCO_3$, nitrite and ammonia 0 mg/L, nitrate < 50 mg/L. Fish were fed twice per day *ad libitum* with *Artemia salina* (L.) *nauplii* and dry commercial food of different size according to the developmental stages (Micron, Sera; Tetramin Baby, Junior and Flakes, Tetra®).

*Experimental system and samples collection*

The experimental rearing based on the three different density groups lasted 60 days, from 30 to 90 dpf. The experimental rearing at the three densities was carried out in a recirculating housing system composed of nine interconnected 3.5 L trapezoidal tanks, equipped with a mechanical/biological filter, air and water pumps. Water exchange was 400 ml/min. Temperature, photoperiod and water parameters were the same as reported above.

At the end of the experimental rearing, fish were euthanized and fixed (as above). After 48 hours of fixation at 4°C, all the samples were dehydrated in a graded ethanol series and stored in 70% ethanol at 4°C until the analyses were performed.

The number of specimens used for the analyses was T0, n=32; HD, n=65; MD, n=46 and LD, n=19.

*Staining*

Specimens were whole-mount stained for mineralized tissues with Alizarin red S (modified from Taylor and Van Dyke, 1985). Samples were first rehydrated in a graded ethanol series, washed in distilled water and bleached with a 0.45% $H_2O_2$ and 0.5% KOH solution until

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the depigmentation was achieved, rinsed in distilled water and transferred in saturated borax for 24h. Samples were then stained with 0.01% Alizarin red S in 0.5% KOH overnight or longer, according to the specimen's size, rinsed in distilled water, placed in 1% KOH for 2h, finally cleared and dehydrated in a graded series of KOH-glycerol solutions and stored in 100% glycerol. The standard length ($S_L$, mm) of individuals was then measured on digital images using the software Fiji (Schindelin et al., 2012). Individuals were analysed for meristic counts and skeletal anomalies using a Zeiss Axio Zoom V16 Stereo Zoom Microscope equipped with a 5MP CCD camera.

*Meristic counts and analyses of skeletal anomalies*

Meristic counts were carried out on the number of vertebrae of each region of the vertebral column, fin rays of unpaired and paired (left and right side) fins and their inner supports, and supraneural bones. Nomenclature for skeletal elements follows Arratia et al. (2001) and De Clercq et al. (2017). The vertebral column was subdivided into four different regions, with nomenclature adapted from Bensimon-Brito et al. (2012a). These authors combined the terminologies of Arratia et al. (2001), Bird and Mabee (2003) and Nybelin (1963) as follows: 1) Weberian region (vertebrae bearing the Weberian ossicles), 2) abdominal region (rib-bearing vertebrae with open haemal arches), 3) caudal region (vertebrae with closed haemal arches) and 4) caudal complex (preurals and ural vertebrae with modified haemal and neural arches and spines).

The use of the terms “anomaly”, “malformation” and “deformity” follows Boglione et al. (2013) and Hennekam et al. (2013). Malformations are early developmental defects; deformities are defects that relate to later, epigenetic, factors. We reserve the use of the term anomaly for the description of the methodology adopted in this study and the cases for which nor “malformation” and “deformation” can be used. Skeletal anomalies were classified using an alphanumeric code (modified from Prestinicola et al., 2013), where the capital letter indicates the affected skeletal region, the numbers refer to the skeletal elements and the lowercase letters to the types of anomalies (Table 1).

For each group (T0, HD, MD and LD), the following general metrics were calculated: 1) frequency (%) of individuals with at least one anomaly; 2) number of types of anomaly observed; 3) average anomaly load (total number of anomalies recorded in a group/number of malformed individuals per group); 4) frequency (%) of individuals with at least one severe anomaly; 5) frequency (%) of observed severe anomalies on the total number of observed anomalies; 6) average severe anomaly load (number of severe anomalies/number of individuals with severe anomalies); 7) frequency (%) of each type of anomaly, with respect to the total number of anomalies observed in each group. In this paper, severe anomalies refer to those types of anomalies that affect the vertebral axis (*i.e.*, scoliosis, lordosis, kyphosis) and centra (deformation, elongation and reduction in length, and fusion).

The phenotypic analysis of the skeleton was carried out based on certain assumptions (adapted from Prestinicola et al., 2013): i) non-completely fused vertebral centra were counted as distinct elements in meristic counts while those completely fused as one; ii) supernumerary bones with normal morphology were not considered as anomalies but included as meristic count variations; conversely, anomalous supernumerary elements were included among anomalies; iii) upon simple visual inspection, only the identifiable deformations in shape were considered as skeletal anomalies: if any doubts arose, then the shape variation was not considered anomalous; iv) curvatures of the vertebral column were considered as scoliosis, lordosis and/or kyphosis only if the involved vertebral centra were deformed, in order to exclude from the analyses axis deformations due to neuromuscular anomalies or fixation artefacts.

*Statistical analyses*

Data obtained for the $S_L$ and vertebrae counts were compared with the Kruskal-Wallis test followed by Dunn's *post-hoc* test with the Bonferroni correction.

Data obtained from the analysis of skeletal anomalies were used to build a *Raw Matrix* (hereafter referred to as RM). The RM was transformed into a *Binary Matrix* (hereafter named BM: presence of each type of skeletal malformation = 1; absence = 0). RM was used to calculate the frequencies (%) of each type of anomaly on the total number of anomalies. The BM was used to calculate the frequencies (%) of individuals affected by each type of anomaly in each group.

The frequencies obtained from the RM and the BM are presented with tables or histograms. Statistical differences among groups were tested with one-way PERMANOVA (9999 permutations) using both the RM (Euclidean distance) and the BM (simple-matching) matrices. RM and BM, and other matrices built on a subset of data were subjected to Correspondence Analysis (CA) (Benzécri et al., 1973) in order to visualize the relationships among groups and the role that each anomaly plays in defining the characteristics of the different groups.

Statistics was performed with the software Past 3.20 (Hammer et al., 2001).

## Results

*T0 group*

The average SL of the T0 specimens was 7.6 (±1.7 SD) mm. All caudal fin elements were identifiable in each T0 specimen. The modal value and the range values of the T0 vertebral centra (calculated excluding the specimens with vertebral centra still mineralizing) were 34 and 32-35, respectively (Table 2).

The general metrics for the T0 group are summarised in Table 3. The frequency of specimens affected by at least one anomaly and at least one severe anomaly was 56% and 34%, respectively. The average anomaly load (average number of anomalies per malformed specimen) and the average severe anomaly load (average number of severe anomalies per malformed specimen) was 9 and 2, respectively. The number of observed types of anomalies was 17

(see Figure 1). Severe anomalies represented 12% of all anomalies. Severe anomalies were represented by centra deformation (type 2def and elo/red) and scoliosis (1sco). The frequencies (%) of each type of anomaly on the total number of anomalies counted in the T0 group and the frequency of the specimens affected by each anomaly are reported in Figure 1. The most common (22-41% of T0 specimens) malformations were those affecting the neural arches of the abdominal region (B4def) and the ribs (B7def), scoliosis in vertebrae of the caudal complex (D1sco) and malformations of the epural (G11def). No lordosis, kyphosis, nor fusions of vertebral centra were recorded in the T0 individuals (except for one partial fusion in the caudal complex vertebrae, D2par, in one fish).

*Experimental groups (HD, MD and LD)*

$S_L$ significantly differed among groups (Kruskal-Wallis: H=38.9, p<0.001). Specifically, LD>MD>HD (p<0.01 for each pairwise Dunn's test) (Figure 2).

The data referring to the meristic counts are shown in Table 2. The modal value of the number of vertebral centra (= 33) and the ~~inferior~~ lower limit of its range of variation (=30) were lower in the HD group than in MD and LD group. This is due to the presence of specimens affected by complete fusion of vertebral ~~bodies~~ centra in the HD group, as reported below.

Given that four types of malformation were commonly observed in the T0 group (B4def, B7def, D1sco and G11def), these were considered as "background malformations" for this zebrafish batch

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when the experimental animals were analysed, and removed from the analysis of the experimental groups. ~~Indeed~~Indeed, they occurred at similar percentages in specimens of all experimental groups.

The general metrics referring to the analysis of the skeletal anomalies for each group are presented in Table 4. The frequency (%) of specimens with at least one skeletal anomaly was 100 in the HD and LD groups and 98 the MD group (*i.e.* one specimen in the MD group was only affected by some of the above-mentioned "background malformations"). The highest average anomaly load was found in the HD group (12 anomalies/~~malformed~~ deformed specimen), as well as the widest variety of observed types of anomalies (n=68). The highest frequencies of specimens with at least one severe anomaly (73%) as of severe anomalies relative to the total number of anomalies (21.4%) were observed in the MD group.

Statistically significant differences were found between the HD and the other two experimental groups (MD and LD) (PERMANOVA, $p<0.01$). The frequencies (%) of deformities grouped per skeletal element and per region, and the frequency of affected specimens are represented in Figure 3 (raw data are provided in the Table_1_ SuppInfo), for each experimental group.

None of the following deformities was found in any experimental group: lordosis in the Weberian, abdominal or caudal complex regions (A1lor, B1lor and D1lor), kyphosis (code 1kyp), partial fusion in the Weberian and abdominal region (A2par and B2par), elongated vertebral centrum of the abdominal, caudal and caudal complex regions (B2elo, C2elo and D2elo), demineralization of the urostyle

(D3dec), deformities of fin elements such as coracoid (code 20), post-cleithrum (code 21), pectoral radials (E8sup/abs), pelvic pterygiophores (L8abs and def) and rays (l12abs), anal pterygiophores (F8sup) and rays (F12abs and def), dorsal pterygiophores (H8abs, fus and dec) and rays (H12abs), epural (G11sup) and caudal rays (G12sup), and cranial deformities such as maxilla/premaxilla deformation (code 13), deformations of the opercula (code 16) or branchiostegal rays (17sup, abs and def L), neurocranium deformities (15) and saddle-back syndrome (1sbs).

The Weberian (code A) and abdominal vertebral (code B) regions were the least affected skeletal regions in all the experimental groups (see Table_1_ SuppInfo), with the exception of neural arches in the Weberian vertebrae (malformation A4def) and supraneurals (A18 and A18sup).

The HD and MD groups showed the highest frequency of deformities (Figure 3a) and frequency of individuals with deformities (Figure 3b) affecting centra (Cc) and centra-associated elements of the caudal region (Cae). In particular, the HD group showed the highest percentage of individuals with deformities in the caudal region (Figure 3b), both for centra-associated elements (Cae) (almost all the C4 types and C5def, Figure 4b) and centra (Cc) (C2fus and def, Figure 4b). In the MD group, the highest frequency of deformities (Figure 4a) of the caudal vertebral centra (in particular C2par) was found. Lastly, pectoral and anal fins were more frequently deformed in the HD group.

The LD group showed the highest frequency of neural arch deformities affecting the Weberian vertebrae (Aae in Figure 3, A4def in the Table_1_ SuppInfo) as well as the caudal fin elements (fin rays and inner supports) (Figure 3). The LD group also displayed the highest frequency of deformities affecting the centra of the caudal complex, although the frequency of the specimens affected by these deformities was higher in the MD group (Dc, Figure 3). Different from HD and MD, some deformities were never present in the LD group, *i.e.*, lordosis (C1lor), complete vertebral ~~body~~ centra fusion (C2fus), misplacement of the neural arch insertions (C4ins) and mismatched fusion of neural and haemal spines (C4mis and C5mis), absence of neural or haemal arches or spines (C4abs, C4abs R, C5abs) and scoliosis (C1sco).

In Figure 5, examples of some of the deformities recorded are provided.

*Correspondence Analysis (CA)*

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Different CAs were performed on different matrices in order to visualize the differences or relationships among samples and the role each anomaly played in defining the characteristics of each group. The CA applied to RM or BM, containing all the specimens and the observed types of deformities (matrices 129 specimens x 86 types of deformities). Note that one individual of the MD without any deformities was not included in the matrix, since a null data vector, *i.e.* a record for a specimen without anomalies, cannot be processed by any of the techniques that require vector normalisation, e.g. by

correspondence analysis. The CA applied to RM and BM gave ordination models exhibiting a very low variance for the first three axes (14% and 13%, respectively).Therefore, they are not shown. CA was next applied to a subset of data obtained from the RM containing 19 randomly sampled individuals per group. This number of samples was chosen on the base of the sample size of the LD group, in order to avoid bias due to differences in the sample's dimension. The final matrix was 57 specimens x 12 descriptors. The CA explained an overall variance of 55% for the first three axes of correspondences. In Figure 6, the ordination model obtained on CA1 and CA2 axes (explaining 43% of the variance) is shown for each group on different graphs. The HD centroid plots on the 3rd quadrant (negative semi-plane of CA1), where the deformities of the centra-associated elements (Bae and Cae) and vertebral centra (Bc and Cc) of the abdominal and caudal regions are located. The MD and LD centroids are positioned in the positive half-space of CA 1, with MD in an intermediate position with respect to HD and LD groups. Most individuals of the MD and LD groups are located in the 1st quadrant, overlapping with malformations of the associated elements of the Weberian vertebrae and of the pectoral and caudal fins. In all groups, only a few specimens of the three experimental groups were positioned in the 4th quadrant, where deformities of the anal and dorsal fins and associated elements of the caudal complex vertebrae are situated.

**Discussion**

T~~In t~~his paper~~, we described~~ describes the phenotypic plasticity of the skeleton and the occurrence of skeletal deformities in wild-type *D. rerio* reared under identical conditions, with rearing densities being the only variable. Our results reveal (1) the presence of certain anomalies in zebrafish of different age and experiencing different experimental conditions (T0, HD, MD and LD), (2) a significant difference in size ($S_L$) depending on rearing densities, and (3) a higher incidence of deformities of vertebrae of the caudal region in animals reared at higher densities, in particular deformities of arches and spines and fusion of vertebral ~~bodies~~centra, discussed below.

*Rearing density-independent skeletal malformations: the starting point (T0)*

Animals at the same age (30 dpf), but of different sizes ($S_L$), show that skeletal development is more advanced in larger individuals compared to smaller individuals. This confirms the findings in previous studies that show a better correlation of skeletal development with size than with age, in *D. rerio* (Cubbage and Mabee, 1996; Parichy *et al.*, 2009) and in farmed fish, *i.e.* Atlantic halibut (*Hippoglossus hippoglossus* L.) (Sæle and Pittman, 2010).

The analysis of the skeletal phenotype at the beginning of the experiment allowed identifying malformations of the ribs (B7def), and neural arches and spines in the abdominal region (B4def), scoliosis in the caudal complex (D1sco) and malformations of the epural (G11def) as “background malformations” for the zebrafish used in this study. The presence of malformed ribs and neural arches of the

abdominal region reported in the present work is in agreement with the study of Ferreri et al. (2000). In their work, reared specimens displayed a higher frequency of individuals affected by the aforementioned malformations than wild zebrafish sampled from the river Ganges. Even in wild specimens, about 13% of ribs and 21% of neural arches and spines (although not assigned to distinct regions) were diagnosed as malformed. The high incidence of malformations of neural arches and spines in the abdominal region (close to 100% of the analysed specimens) and the presence of malformed ribs (ranging from 39 to 80%) was also reported for *O. mykiss* reared both at low and high densities (Boglione *et al.*, 2014). Thus, similar to *O. mykiss*, *D. rerio* appears to be susceptible to develop these particular malformations.

Other malformations were found to be present with low frequencies in the T0 specimens, e.g., malformations of the caudal complex, *i.e.* D1sco (22%), D3def (6%), D4def5 (13%), and D2def (13%). Interestingly, no fusions were detected, except for a single occurrence in the caudal complex (D2par). It is recognised that the vertebrae of the caudal complex display a high degree of plasticity and its predisposition to develop vertebral ~~body~~ centra fusions is well documented at least in some species (Bensimon-Brito et al., 2010, 2012b; Gavaia et al., 2002; Koumoundourous et al., 1997, Prestinicola et al., 2013; Witten et al., 2006). As part of normal development, the last vertebral body – the urostyle – in zebrafish forms through five fusion events (Bensimon-Brito et al., 2010, 2012b). The preural vertebral centra, which frequently possess an

accessory arch, show a higher tendency to fuse than the vertebrae of the anteriormost regions (Bensimon-Brito et al., 2012b; Eastman, 1980).

*Effects of the rearing densities*

Specimens reared at high density (HD) showed a significantly reduced growth with respect to the specimens reared at medium and low densities. An inverse relation between growth and rearing density has also been described for zebrafish reared from 6 to 90 dpf at 19, 37 and 74 fish/L (Ribas et al., 2017), as well for other basal teleost species such as *O. mykiss*, and for advanced teleosts such as *H. hippoglossus* and discus (*Symphysodon aequifasciatus* Pellegrin 1904) (Björnsson, 1994; Holm et al., 1990; Tibile et al., 2016). It has been proposed that size differences relate to the reduction in feeding activity or to an increase in energy expenditure associated with enhanced swimming activity due to increased competition or interactions. In our experimental rearing, food was administered *ad libitum*, consequently, insufficient feeding was unlikely a causative factor for the reduced size in the specimens reared at higher densities.

Rearing at different densities after 30 dpf did not influence the modal values of meristic characters. Lower mean values and lower limit of the variation range for the number of vertebral centra observed in the HD reared zebrafish related to the presence of complete vertebral ~~bodies~~ centra fusions (which in the meristic counts were accounted as one element). Ferreri et al. (2000) compared wild and reared

zebrafish and found similar ranges of variation for several meristic elements, with the exception of the anal and pectoral fin rays. Bird and Mabee (2003) confirmed what ~~was previously reported by Ferreri et al. (2000)~~Ferreri et al. (2000) previously reported for vertebral centra counts even in other reared zebrafish. Usually, variation in the number of meristic elements is due to changes in environmental conditions during the early developmental stages. For example, low temperatures lead to an increased number of vertebrae in reared zebrafish (Sfakianakis et al., 2011).

All the specimens analysed (with one exception in the MD group, already discussed) showed at least one anomaly (Table 4). Such a high frequency may be surprising but has been reported before. High frequencies of zebrafish affected by at least one anomaly were already reported for both wild (87%) and reared (93%) specimens by Ferreri et al. (2000).

The HD group displayed the highest average number of deformities per specimen and a larger variety of types of deformities. The latter could be a density effect but it could also relate to the larger number (n = 65) of HD specimens with respect to the MD (n = 46) and LD (n = 19) groups. However, the highest average number of deformities per specimen, as detected in the HD group, parallels what has been already described in aquaculture facilities. Semi-intensive rearing methodologies (characterized also by reduced rearing densities) compared to intensive rearing conditions, decrease the occurrence of skeletal deformities in farmed fish (Boglione et al., 2009; Prestinicola et al., 2013; Zouiten et al., 2011). Similar to what has been described

for an advanced teleost, the *E. marginatus* (Boglione et al., 2009), rearing density alone can affect the skeletal phenotype in zebrafish, and increases the occurrence of particular types of deformities in the caudal region of the vertebral column (partial and complete fusions of vertebral ~~bodies~~centra, deformation of neural and haemal arches). The susceptibility of the caudal region to deformities has been already described in farmed Atlantic salmon (*Salmo salar* L.). Vertebral centra compressions and fusions can relate to high-temperature exposure during the embryonic stages (Grini et al., 2011). The aggravation of such deformities in salmonids reared at high temperature can occur later, for example during the late juvenile seawater phase (Wargelius et al., 2015). The latter may be the result of a synergic effect of the rearing temperature and the high density used during the seawater rearing. Vertebral centra deformities in Atlantic salmon have also been attributed to other not fully elucidated causative factors acting during later ontogenetic stages (Fjelldal, et al., 2007, Fjelldal et al., 2012).

The skeletal elements that displayed the most distinct phenotypic response to increased rearing density were neural and haemal arches and spines (deformations in shape, C4def and C5def), followed by centra of the caudal region (C2par and fus).

Despite the fact that anomalies of arches and spines were also observed in a few specimens of the T0 group, their frequency, and that of specimens affected, are far higher in the HD than in the LD group. Vertebral centra and arches in teleosts are different developmental modules. Vertebral centra originate as chordacentra

by mineralization of the notochord sheath, whilst the associated elements arches and spines are patterned by the somites (Laerm, 1979, Fleming et al., 2015). The duality in vertebral column elements' formation could explain the higher incidence of deformities of arches and spines compared to vertebral centra, in the HD group. Interestingly, malformations, similar to those shown in Fig. 5c, have been described for fused somite mutant zebrafish (tbx6 mutation) (van Eedden et al., 2006, Fleming et al., 2004). In this mutant zebrafish line, the somitogenesis is disturbed and the specimens show malformations of arches and spines, but separated vertebral centra. That shows that centra and associate elements are two distinct developmental modules. However, the mechanisms by which rearing density induces late vertebral column deformities that resemble mutant-related malformations remain to be elucidated. Deformities of arches and spines have also been related to musculature impairments (Favaloro et al., 2006, Backiel et al., 1984). Behavioural studies on *O. mykiss* reared at high stocking densities (Bégout Anras and Lagardère, 2004; Cooke et al., 2000) showed that the complexity of swimming trajectories, space utilization and activity rhythms were altered and that swimming activity, oxygen consumption and muscular activity increased when compared with individuals reared at lower densities. Moreover, the crowded conditions augmented the occurrence of changes in swimming direction with sharper turning angles with respect to individuals kept at lower densities (Bégout Anras and Lagardère, 2004). The swimming patterns suggested recurring avoidance behaviours of

individuals held in the same tank. Avoidance behaviours imply the utilization of fast C-start movements, usually occurring during escape responses, which start with the contraction of the muscles of one side of the body, at the level of the individual's centre of mass ~~(the central region of fish body)~~, in which the propulsive force develops, allowing the fish to change orientation (Eaton and Emberley, 1991). During the fast start movements, the body bends at the level of the central region, below the dorsal fin, at the 50% of the fish $T_L$, as shown for zebrafish by Danos and Lauder (2012). In *Cyprinus carpio* the maximum vertebral column curvature has been calculated to be between 50 and 80% of fish $T_L$ (Shadwick and Lauder, 2006). During fast start movements, the muscles generate a mechanical load on the flexing vertebral column (Shadwick and Lauder, 2006; Wakeling and Johnston, 1999).

Mechanical loading increases bone formation in zebrafish (Fiaz et al., 2010; Suniaga et al., 2018) especially if its frequency is high and the mechanical load is dynamic, rather than static (Lisková and Hert, 1971; Rubin and McLeod, 1994; Turner, 1998; Turner et al., 1994a,b; Turner et al., 1995).

Therefore, if a crowded environment leads to an increased number of interactions between animals and thus changes in swimming trajectories, for example due to food competition, possibly, the centra of the central region (*viz*. caudal) of animals reared at higher densities are more often subjected to the bendings ~~moments~~ generated by the axial musculature. The C-shaped bending of an elongated structure, such as the vertebral column, produces

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compression on the concave side and strain on convex one. Thus, the intervertebral space on the concave side of the bending would be subjected to compression, i.e. mechanical loading. Indeed, the concave and convex sides can reverse from fast movement to another, according to the turning direction. This increased elicitation could explain the occurrence of fusion in the caudal region of the vertebral column.

In this study, the complete fusion of vertebral ~~bodies~~ centra (C2fus) was never observed in specimens reared at low density. Partial fusions (C2par) occurred at a lower frequency in LD compared to the HD and MD groups. Ferreri et al. (2000), using densities far lower than the LD used in this work, did not record vertebral fusion, suggesting that their occurrence and severity could be linked to the increased rearing density. Vertebral ~~body~~ centra fusion can develop at various time points during development. Very early fusions in zebrafish relate to the ectopic mineralisation of the notochord sheath in prospective intervertebral regions (Bensimon-Brito et al., 2012b). It is unlikely that this type of very early fusions accounts for observations made in this experiment: notochord segmentation takes place during early ontogeny and it would not explain differences in the occurrence of vertebral fusions in animals reared at different rearing densities during the juvenile period when the vertebral centra identity is already determined. Further, animals from the T0 group did not show fused vertebrae.

The next (early) process that can cause the fusion of vertebral ~~bodies~~ centra in zebrafish is the bridging of intervertebral spaces by bone

that develops around the mineralised notochord sheath (Bensimon-Brito et al., 2012b; Ytteborg et al., 2010). A third process that may lead to a late fusion (not described in zebrafish but in *S. salar*), is caused by metaplasia, *i.e.* osteoblasts of the vertebral endplate growth zone turn in cells with a chondroblast-like phenotype, producing cartilage in the intervertebral space. This ectopic cartilage later mineralizes and is subsequently remodelled into bone (Fjelldal et al., 2012; Witten et al., 2005; 2006; Ytteborg et al., 2010).

In conclusion, our study shows the effect of rearing density on the growth rate of zebrafish and provides evidence that rearing density affects the skeletal phenotype in this species. High and, to some extent, medium rearing densities slowed down growth and induced deformities, particularly in the caudal region of the vertebral column. Our results suggest that a density of 2 fish/litre, between the age of 30 and 90 dpf can help to reduce the incidence of skeletal malformations in *D. rerio*. This is especially relevant if zebrafish is used for studying skeletal pathologies. Moreover, for this analysis, we propose a methodology that is adaptable and can be used in various contexts to assess skeletal ~~malformations~~ anomalies in zebrafish or other species~~,~~. For example, the alphanumeric code used here can be adapted to different levels of details according to the needs or applications (*i.e.*, by grouping different types of malformations, or by adding subcodes for peculiar or different types of malformations). Such standardization may facilitate comparison among different studies.

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**Author contributions:** CB and PEW conceived the experimental design, AM carried out the experiments, performed the analyses and elaborated the data; AM, AH, PEW and CB discussed the results and wrote the paper. All authors have approved the final version of the manuscript.

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# Plasticity of the skeleton and skeletal deformities in zebrafish (*Danio rerio*) linked to rearing density

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## Abstract

The teleost zebrafish (*Danio rerio*), an established model for human skeletal diseases, is reared under controlled conditions with defined parameters for temperature and photoperiod. Studies aimed at defining the proper rearing density have been performed with regard to behavioural and physiological stress response, sex ratio and reproduction. Studies concerning the effect of rearing density on the skeletal phenotype are lacking. This study is designed to analyse the response of the skeleton to different rearing densities and provides a description of the skeletal deformities. Wild type zebrafish were reared up to 30 dpf (days post-fertilization) in a common environment. From 30 to 90 dpf, animals were reared at three different densities: high density (HD) 32 fish/L, medium density (MD) 8 fish/L and low density (LD) 2 fish/L. Animals at 30 and 90 dpf were collected and whole-mount stained with Alizarin red S to visualise mineralized tissues. The entire skeleton was analysed for meristic counts and 172 types of deformities. The results showed that rearing density significantly influenced the specimens' average standard length, which decreased with increasing rearing density. Differences concerning meristic counts among the three groups were not observed. Rearing density-independent malformations affected the ribs, neural arches and the spines of the abdominal region as well as vertebrae of the caudal complex. The HD group showed the highest number of deformities per specimens, the highest number of observed types of deformities and, together with the MD group, the

highest frequency of specimens affected by severe deformities. In particular, the HD group showed deformities affecting arches, spines and vertebral centra in the caudal region of the vertebral column. This study provides evidence of an effect of rearing density on the development of different skeletal phenotypes.

Keywords: deformities, plasticity, rearing density, skeleton, zebrafish

## Introduction

Phenotypic plasticity, a component of phenotypic variation (Klingenberg, 2019), is the ability of living organisms to respond to environmental or internal stimuli through changes in behaviour, morphology or physiology, producing different phenotypes. Phenotypic plasticity can be adaptive or non-adaptive, reversible or irreversible, and its type and degree are specific to the single trait and the environmental conditions involved. In an evolutionary perspective, phenotypic plasticity is a feature of the reaction norm of a trait of single organisms (i.e. the complete set of phenotypic responses of a trait to a specific environmental variable), that can be the target of natural selection, steering towards phenotypic accommodation and genetic assimilation (Pigliucci et al., 2006, Schmalhausen, 1949, Waddington, 1953; West-Eberhard, 2003, 2005).

Phenotypic plasticity is particularly relevant for skeletal tissues. The vertebrate skeleton is composed of five main different skeletal tissue types: notochord, cartilage, bone, dentin and enamel/enameloid. In teleosts, several intermediate tissue types are present and skeletal tissues are considered part of a continuum (Hall and Witten, 2019; Witten et al., 2010). They are able to respond to intrinsic and extrinsic cues (Roux, 1881; Ruff et al., 2006; Weinans and Prendergast, 1996; Wolff, 1892). Skeletal tissues and cells are plastic and dynamic throughout life as they modulate their structure in response to the mechanical load regime. The processes through

which the skeletal cells achieve modifications are modulation, metaplasia, transdifferentiation and remodelling (Hall and Witten, 2007; Witten and Hall, 2015). The ability of tissues to modulate their phenotype in response to mechanical load is known as "Wolff's law of bone transformation" (Wolff, 1892) or as “bone functional adaptation” (Ruff et al., 2006). A famous example is the two-legged goat, whose hind limbs and thoracic skeleton became modified to adapt to the bipedal gait (Slijper, 1942).

Examples of phenotypic plasticity of the teleost skeleton are numerous. In cichlids, differences in the hardness or type of food modify the jaw shape, the number and strength of jaw bone trabeculae and the size of replacement teeth (Huysseune, 1994, 1995; Meyer, 1987). The mechanical load exerted by swimming changes the shape of vertebral centra and can induce lordosis in different teleost species (Kihara et al., 2002; Kranenbarg et al., 2005). Forced swimming accelerates ossification rate of vertebral bodies and cartilage formation in the head and the caudal fin in zebrafish *Danio rerio* (Hamilton 1822) (Fiaz et al., 2012; Suniaga et al., 2018; van der Meulen, 2005).

The rearing of fish implies the modification and control of several environmental factors (e.g., photoperiod, temperature, type of diet, diet composition, hydrodynamics) in order to optimize rearing conditions in aquaculture or laboratory facilities. Aquaculture-related research provides numerous examples of how modifications of environmental conditions change the skeletal phenotype, including the induction of skeletal deformities.

In aquaculture, farming practices can be classified as intensive, semi-intensive and extensive methodologies. They stand out for several parameters, such as rearing density and tank volume, hydrodynamics and diet. In intensive farming practice, rearing density is high and tank volume smaller compared to semi-intensive and extensive rearing conditions. The latter, besides being characterized by decreased number of animal per volume and larger tanks, utilises practises aimed at simulating the natural environment. This includes differentiated hydrodynamics, and large live prey availability and variety (Baluyut and Balnyme, 1995; Cataudella and Bronzi, 2001). The above-mentioned rearing methodologies can affect the morphology of the skeleton. In rainbow trout *Oncorhynchus mykis*s (Walbaum 1792), the occurrence of skeletal deformities increases significantly in animals reared in intensive conditions compared to animals reared in extensive conditions (Boglione et al., 2014). Similar observations have been reported for advanced marine teleosts: gilthead seabream (*Sparus aurata* L.) and red porgy (*Pagrus pagrus* L.*)* reared in semi-intensive conditions showed a lower number of skeletal deformities per individual and a lower number of deformed individuals (Prestinicola et al*.,* 2013; Roo et al., 2010). Dusky grouper (*Epinephelus marginatus* Lowe 1834) larvae reared in high-density conditions showed the highest frequency of deformed individuals, the highest number of deformities per deformed individual, the largest range of types of deformities and the highest incidence of individuals with at least one severe deformity (Boglione et al., 2009).

126 *Danio rerio* is an established model organism in biological and
127 biomedical research and is now also used as a model for human
128 skeletal diseases. Insights into fundamental pathways of skeletal
129 formation and skeletal diseases can be obtained, provided the
130 differences between the teleost and mammalian skeleton are
131 considered (Witten et al., 2017). Laboratory zebrafish are reared
132 under controlled conditions, with defined parameters for temperature
133 and photoperiod, but recommendations for rearing densities differ
134 (Castranova et al., 2011) and standards based on experimental data
135 are lacking (Lawrence and Mason, 2012). The Zebrafish Book
136 (Westerfield, 2000) recommends a rearing density of 0.55 adult
137 fish/L, whereas the “Guide for the care and use of laboratory
138 animals” (Clark et al., 1997) and Matthews et al. (2002) recommend
139 5 to 10 individuals/L for adult fish. Another published housing density
140 is 3.5 fish/L (Tsang et al., 2017). Concerning rearing densities for
141 early life stages, published data range from 6.5, up to 94 fish/L
142 (Carvalho et al., 2006; Goolish et al., 1998; Matthews et al., 2002).
143 As Lawrence (2007) emphasized, “the classifications of densities in
144 zebrafish research tend to vary considerably depending on the
145 experimental setting”. Remarkably, studies about the effects of the
146 rearing density in *D. rerio* are scarce (Ribas et al., 2017). Published
147 data refer to the animals’ sex ratio (Liew et al., 2012; Ribas et al.,
148 2017), growth rate (Hazlerigg et al., 2012; Ribas et al., 2017), stress
149 and behavioural parameters (Ramsay et al., 2006; Shelton et al.,
150 2015) or reproductive rates (Goolish et al., 1998). The effect of
151 rearing densities on the skeleton and the onset of skeletal deformities

in this species has not been reported. The skeletal phenotype of transgenic and mutant zebrafish lines for genes related to human skeletal pathologies has already been extensively described (Fisher et al., 2003; Gray et al., 2014; Gistelinck et al., 2016; Haller et al., 2018; Lleras Forero et al., 2018; Spoorendonk et al., 2008; Wopat et al., 2018). Conversely, to our knowledge, the only works describing the skeletal anomalies in wild type zebrafish are the study of age-related deformities of Hayes et al. (2013) and a comprehensive description of wild adult breeders and F1 juveniles *D. rerio* made by Ferreri et al. (2000). The latter characterized 25 types of anomalies affecting the vertebral column, vertebrae, fins and cranium.

The aim of this study was to analyse the response of the skeleton of juvenile *D. rerio* to a single environmental variable, *i.e.* rearing density. This study provides a description of skeletal deformities developed in *D. rerio* reared at three different densities during the juvenile stage.

## Materials and methods

### *Ethics statement*

All experiments were carried out at the Experimental Biology and Aquaculture Laboratory, Università degli Studi di Roma Tor Vergata, approved by the Animal-Welfare body and carried out in accordance with Italian and European rules. All the animal experiments were ethically approved and authorised by the General Director of the Ministry of Health, Legislative Decree no.26/2014; European Directive 2010/63/UE.

*Specimens maintenance and collection*

All the specimens used in this study were obtained from the same pool of AB line (commonly referred to as wild type, WT) zebrafish breeders (n= 15), male:female ratio 1:2, housed in a 25 L aquarium equipped with a bio-mechanical filter. Eggs were obtained by natural spawning. Vital eggs were incubated at 28°C until hatching. After hatching, the animals were transferred in one large aquarium at a density of 20 animals/L and maintained there up to 30 days post-fertilization (hereafter, dpf), a time point when a stable number of individuals was achieved (Figure_1_SuppInfo). At 30 dpf, the specimens were randomly divided into groups and reared at three densities: i) high (32 fish/L), ii) medium (8 fish/L) and iii) low density (2 fish/L) (hereafter referred to as HD, MD and LD, respectively). The choice was based on the need to find a compromise between having a sufficient number of fish for the analyses (especially for the MD and LD group) and the rearing densities adopted usually in the zebrafish facilities (5 fish/L for the adult stage). The remaining fish were euthanized with a lethal dose (500 µl/L) of 2-phenoxyethanol and fixed (1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) representing a “time zero” sampling point (hereafter referred to as T0).

The water used for all the tanks (breeders, eggs, larvae and juveniles) was obtained mixing equal parts of water treated by reverse osmosis water and 50 µm-filtered well water. The photoperiod was 14L:10D and water parameters were maintained as

follows: water temperature 28°C, pH 6.8-8.5, water hardness 60-200 mg/L $CaCO_3$, nitrite and ammonia 0 mg/L, nitrate < 50 mg/L. Fish were fed twice per day *ad libitum* with *Artemia salina* (L.) *nauplii* and dry commercial food of different size according to the developmental stages (Micron, Sera; Tetramin Baby, Junior and Flakes, Tetra®).

*Experimental system and samples collection*

The experimental rearing based on the three different density groups lasted 60 days, from 30 to 90 dpf. The experimental rearing at the three densities was carried out in a recirculating housing system composed of nine interconnected 3.5 L trapezoidal tanks, equipped with a mechanical/biological filter, air and water pumps. Water exchange was 400 ml/min. Temperature, photoperiod and water parameters were the same as reported above.

At the end of the experimental rearing, fish were euthanized and fixed (as above). After 48 hours of fixation at 4°C, all the samples were dehydrated in a graded ethanol series and stored in 70% ethanol at 4°C until the analyses were performed.

The number of specimens used for the analyses was T0, n=32; HD, n=65; MD, n=46 and LD, n=19.

*Staining*

Specimens were whole-mount stained for mineralized tissues with Alizarin red S (modified from Taylor and Van Dyke, 1985). Samples were first rehydrated in a graded ethanol series, washed in distilled water and bleached with a 0.45% $H_2O_2$ and 0.5% KOH solution until

the depigmentation was achieved, rinsed in distilled water and transferred in saturated borax for 24h. Samples were then stained with 0.01% Alizarin red S in 0.5% KOH overnight or longer, according to the specimen's size, rinsed in distilled water, placed in 1% KOH for 2h, finally cleared and dehydrated in a graded series of KOH-glycerol solutions and stored in 100% glycerol. The standard length ($S_L$, mm) of individuals was then measured on digital images using the software Fiji (Schindelin et al., 2012). Individuals were analysed for meristic counts and skeletal anomalies using a Zeiss Axio Zoom V16 Stereo Zoom Microscope equipped with a 5MP CCD camera.

*Meristic counts and analyses of skeletal anomalies*

Meristic counts were carried out on the number of vertebrae of each region of the vertebral column, fin rays of unpaired and paired (left and right side) fins and their inner supports, and supraneural bones. Nomenclature for skeletal elements follows Arratia et al. (2001) and De Clercq et al. (2017). The vertebral column was subdivided into four different regions, with nomenclature adapted from Bensimon-Brito et al. (2012a). These authors combined the terminologies of Arratia et al. (2001), Bird and Mabee (2003) and Nybelin (1963) as follows: 1) Weberian region (vertebrae bearing the Weberian ossicles), 2) abdominal region (rib-bearing vertebrae with open haemal arches), 3) caudal region (vertebrae with closed haemal arches) and 4) caudal complex (preurals and ural vertebrae with modified haemal and neural arches and spines).

The use of the terms “anomaly”, “malformation” and “deformity” follows Boglione et al. (2013) and Hennekam et al. (2013). Malformations are early developmental defects; deformities are defects that relate to later, epigenetic, factors. We reserve the use of the term anomaly for the description of the methodology adopted in this study and the cases for which nor “malformation” and “deformation” can be used. Skeletal anomalies were classified using an alphanumeric code (modified from Prestinicola et al., 2013), where the capital letter indicates the affected skeletal region, the numbers refer to the skeletal elements and the lowercase letters to the types of anomalies (Table 1).

For each group (T0, HD, MD and LD), the following general metrics were calculated: 1) frequency (%) of individuals with at least one anomaly; 2) number of types of anomaly observed; 3) average anomaly load (total number of anomalies recorded in a group/number of malformed individuals per group); 4) frequency (%) of individuals with at least one severe anomaly; 5) frequency (%) of observed severe anomalies on the total number of observed anomalies; 6) average severe anomaly load (number of severe anomalies/number of individuals with severe anomalies); 7) frequency (%) of each type of anomaly, with respect to the total number of anomalies observed in each group. In this paper, severe anomalies refer to those types of anomalies that affect the vertebral axis (*i.e.*, scoliosis, lordosis, kyphosis) and centra (deformation, elongation and reduction in length, and fusion).

The phenotypic analysis of the skeleton was carried out based on certain assumptions (adapted from Prestinicola et al., 2013): i) non-completely fused vertebral centra were counted as distinct elements in meristic counts while those completely fused as one; ii) supernumerary bones with normal morphology were not considered as anomalies but included as meristic count variations; conversely, anomalous supernumerary elements were included among anomalies; iii) upon simple visual inspection, only the identifiable deformations in shape were considered as skeletal anomalies: if any doubts arose, then the shape variation was not considered anomalous; iv) curvatures of the vertebral column were considered as scoliosis, lordosis and/or kyphosis only if the involved vertebral centra were deformed, in order to exclude from the analyses axis deformations due to neuromuscular anomalies or fixation artefacts.

*Statistical analyses*

Data obtained for the $S_L$ and vertebrae counts were compared with the Kruskal-Wallis test followed by Dunn's *post-hoc* test with the Bonferroni correction.

Data obtained from the analysis of skeletal anomalies were used to build a *Raw Matrix* (hereafter referred to as RM). The RM was transformed into a *Binary Matrix* (hereafter named BM: presence of each type of skeletal malformation = 1; absence = 0). RM was used to calculate the frequencies (%) of each type of anomaly on the total number of anomalies. The BM was used to calculate the frequencies (%) of individuals affected by each type of anomaly in each group.

The frequencies obtained from the RM and the BM are presented with tables or histograms. Statistical differences among groups were tested with one-way PERMANOVA (9999 permutations) using both the RM (Euclidean distance) and the BM (simple-matching) matrices. RM and BM, and other matrices built on a subset of data were subjected to Correspondence Analysis (CA) (Benzécri et al., 1973) in order to visualize the relationships among groups and the role that each anomaly plays in defining the characteristics of the different groups.

Statistics was performed with the software Past 3.20 (Hammer et al., 2001).

## Results

*T0 group*

The average SL of the T0 specimens was 7.6 (±1.7 SD) mm. All caudal fin elements were identifiable in each T0 specimen. The modal value and the range values of the T0 vertebral centra (calculated excluding the specimens with vertebral centra still mineralizing) were 34 and 32-35, respectively (Table 2).

The general metrics for the T0 group are summarised in Table 3. The frequency of specimens affected by at least one anomaly and at least one severe anomaly was 56% and 34%, respectively. The average anomaly load (average number of anomalies per malformed specimen) and the average severe anomaly load (average number of severe anomalies per malformed specimen) was 9 and 2, respectively. The number of observed types of anomalies was 17

(see Figure 1). Severe anomalies represented 12% of all anomalies. Severe anomalies were represented by centra deformation (type 2def and elo/red) and scoliosis (1sco). The frequencies (%) of each type of anomaly on the total number of anomalies counted in the T0 group and the frequency of the specimens affected by each anomaly are reported in Figure 1. The most common (22-41% of T0 specimens) malformations were those affecting the neural arches of the abdominal region (B4def) and the ribs (B7def), scoliosis in vertebrae of the caudal complex (D1sco) and malformations of the epural (G11def). No lordosis, kyphosis, nor fusions of vertebral centra were recorded in the T0 individuals (except for one partial fusion in the caudal complex vertebrae, D2par, in one fish).

*Experimental groups (HD, MD and LD)*

$S_L$ significantly differed among groups (Kruskal-Wallis: H=38.9, p<0.001). Specifically, LD>MD>HD (p<0.01 for each pairwise Dunn's test) (Figure 2).

The data referring to the meristic counts are shown in Table 2. The modal value of the number of vertebral centra (= 33) and the lower limit of its range of variation (=30) were lower in the HD group than in MD and LD group. This is due to the presence of specimens affected by complete fusion of vertebral centra in the HD group, as reported below.

Given that four types of malformation were commonly observed in the T0 group (B4def, B7def, D1sco and G11def), these were considered as "background malformations" for this zebrafish batch

when the experimental animals were analysed, and removed from the analysis of the experimental groups. Indeed, they occurred at similar percentages in specimens of all experimental groups.

The general metrics referring to the analysis of the skeletal anomalies for each group are presented in Table 4. The frequency (%) of specimens with at least one skeletal anomaly was 100 in the HD and LD groups and 98 the MD group (*i.e.* one specimen in the MD group was only affected by some of the above-mentioned "background malformations"). The highest average anomaly load was found in the HD group (12 anomalies/deformed specimen), as well as the widest variety of observed types of anomalies (n=68). The highest frequencies of specimens with at least one severe anomaly (73%) as of severe anomalies relative to the total number of anomalies (21.4%) were observed in the MD group.

Statistically significant differences were found between the HD and the other two experimental groups (MD and LD) (PERMANOVA, $p<0.01$). The frequencies (%) of deformities grouped per skeletal element and per region, and the frequency of affected specimens are represented in Figure 3 (raw data are provided in the Table_1_ SuppInfo), for each experimental group.

None of the following deformities was found in any experimental group: lordosis in the Weberian, abdominal or caudal complex regions (A1lor, B1lor and D1lor), kyphosis (code 1kyp), partial fusion in the Weberian and abdominal region (A2par and B2par), elongated vertebral centrum of the abdominal, caudal and caudal complex regions (B2elo, C2elo and D2elo), demineralization of the urostyle

(D3dec), deformities of fin elements such as coracoid (code 20), post-cleithrum (code 21), pectoral radials (E8sup/abs), pelvic pterygiophores (L8abs and def) and rays (I12abs), anal pterygiophores (F8sup) and rays (F12abs and def), dorsal pterygiophores (H8abs, fus and dec) and rays (H12abs), epural (G11sup) and caudal rays (G12sup), and cranial deformities such as maxilla/premaxilla deformation (code 13), deformations of the opercula (code 16) or branchiostegal rays (17sup, abs and def L), neurocranium deformities (15) and saddle-back syndrome (1sbs).

The Weberian (code A) and abdominal vertebral (code B) regions were the least affected skeletal regions in all the experimental groups (see Table_1_ SuppInfo), with the exception of neural arches in the Weberian vertebrae (malformation A4def) and supraneurals (A18 and A18sup).

The HD and MD groups showed the highest frequency of deformities (Figure 3a) and frequency of individuals with deformities (Figure 3b) affecting centra (Cc) and centra-associated elements of the caudal region (Cae). In particular, the HD group showed the highest percentage of individuals with deformities in the caudal region (Figure 3b), both for centra-associated elements (Cae) (almost all the C4 types and C5def, Figure 4b) and centra (Cc) (C2fus and def, Figure 4b). In the MD group, the highest frequency of deformities (Figure 4a) of the caudal vertebral centra (in particular C2par) was found. Lastly, pectoral and anal fins were more frequently deformed in the HD group.

The LD group showed the highest frequency of neural arch deformities affecting the Weberian vertebrae (Aae in Figure 3, A4def in the Table_1_ SuppInfo) as well as the caudal fin elements (fin rays and inner supports) (Figure 3). The LD group also displayed the highest frequency of deformities affecting the centra of the caudal complex, although the frequency of the specimens affected by these deformities was higher in the MD group (Dc, Figure 3). Different from HD and MD, some deformities were never present in the LD group, *i.e.*, lordosis (C1lor), complete vertebral centra fusion (C2fus), misplacement of the neural arch insertions (C4ins) and mismatched fusion of neural and haemal spines (C4mis and C5mis), absence of neural or haemal arches or spines (C4abs, C4abs R, C5abs) and scoliosis (C1sco).

In Figure 5, examples of some of the deformities recorded are provided.

*Correspondence Analysis (CA)*

Different CAs were performed on different matrices in order to visualize the differences or relationships among samples and the role each anomaly played in defining the characteristics of each group. The CA applied to RM or BM, containing all the specimens and the observed types of deformities (matrices 129 specimens x 86 types of deformities). Note that one individual of the MD without any deformities was not included in the matrix, since a null data vector, *i.e.* a record for a specimen without anomalies, cannot be processed by any of the techniques that require vector normalisation, e.g. by

correspondence analysis. The CA applied to RM and BM gave ordination models exhibiting a very low variance for the first three axes (14% and 13%, respectively).Therefore, they are not shown. CA was next applied to a subset of data obtained from the RM containing 19 randomly sampled individuals per group. This number of samples was chosen on the base of the sample size of the LD group, in order to avoid bias due to differences in the sample's dimension. The final matrix was 57 specimens x 12 descriptors. The CA explained an overall variance of 55% for the first three axes of correspondences. In Figure 6, the ordination model obtained on CA1 and CA2 axes (explaining 43% of the variance) is shown for each group on different graphs. The HD centroid plots on the 3$^{rd}$ quadrant (negative semi-plane of CA1), where the deformities of the centra-associated elements (Bae and Cae) and vertebral centra (Bc and Cc) of the abdominal and caudal regions are located. The MD and LD centroids are positioned in the positive half-space of CA 1, with MD in an intermediate position with respect to HD and LD groups. Most individuals of the MD and LD groups are located in the 1$^{st}$ quadrant, overlapping with malformations of the associated elements of the Weberian vertebrae and of the pectoral and caudal fins. In all groups, only a few specimens of the three experimental groups were positioned in the 4$^{th}$ quadrant, where deformities of the anal and dorsal fins and associated elements of the caudal complex vertebrae are situated.

## Discussion

This paper describes the phenotypic plasticity of the skeleton and the occurrence of skeletal deformities in wild-type *D. rerio* reared under identical conditions, with rearing densities being the only variable. Our results reveal (1) the presence of certain anomalies in zebrafish of different age and experiencing different experimental conditions (T0, HD, MD and LD), (2) a significant difference in size ($S_L$) depending on rearing densities, and (3) a higher incidence of deformities of vertebrae of the caudal region in animals reared at higher densities, in particular deformities of arches and spines and fusion of vertebral centra, discussed below.

*Rearing density-independent skeletal malformations: the starting point (T0)*

Animals at the same age (30 dpf), but of different sizes ($S_L$), show that skeletal development is more advanced in larger individuals compared to smaller individuals. This confirms the findings in previous studies that show a better correlation of skeletal development with size than with age, in *D. rerio* (Cubbage and Mabee, 1996; Parichy *et al.*, 2009) and in farmed fish, *i.e.* Atlantic halibut (*Hippoglossus hippoglossus* L.) (Sæle and Pittman, 2010).

The analysis of the skeletal phenotype at the beginning of the experiment allowed identifying malformations of the ribs (B7def), and neural arches and spines in the abdominal region (B4def), scoliosis in the caudal complex (D1sco) and malformations of the epural (G11def) as “background malformations” for the zebrafish used in this study. The presence of malformed ribs and neural arches of the

abdominal region reported in the present work is in agreement with the study of Ferreri et al. (2000). In their work, reared specimens displayed a higher frequency of individuals affected by the aforementioned malformations than wild zebrafish sampled from the river Ganges. Even in wild specimens, about 13% of ribs and 21% of neural arches and spines (although not assigned to distinct regions) were diagnosed as malformed. The high incidence of malformations of neural arches and spines in the abdominal region (close to 100% of the analysed specimens) and the presence of malformed ribs (ranging from 39 to 80%) was also reported for *O. mykiss* reared both at low and high densities (Boglione *et al.*, 2014). Thus, similar to *O. mykiss*, *D. rerio* appears to be susceptible to develop these particular malformations.

Other malformations were found to be present with low frequencies in the T0 specimens, e.g., malformations of the caudal complex, *i.e.* D1sco (22%), D3def (6%), D4def5 (13%), and D2def (13%). Interestingly, no fusions were detected, except for a single occurrence in the caudal complex (D2par). It is recognised that the vertebrae of the caudal complex display a high degree of plasticity and its predisposition to develop vertebral centra fusions is well documented at least in some species (Bensimon-Brito et al., 2010, 2012b; Gavaia et al., 2002; Koumoundourous et al., 1997, Prestinicola et al., 2013; Witten et al., 2006). As part of normal development, the last vertebral body – the urostyle – in zebrafish forms through five fusion events (Bensimon-Brito et al., 2010, 2012b). The preural vertebral centra, which frequently possess an

accessory arch, show a higher tendency to fuse than the vertebrae of the anteriormost regions (Bensimon-Brito et al., 2012b; Eastman, 1980).

*Effects of the rearing densities*

Specimens reared at high density (HD) showed a significantly reduced growth with respect to the specimens reared at medium and low densities. An inverse relation between growth and rearing density has also been described for zebrafish reared from 6 to 90 dpf at 19, 37 and 74 fish/L (Ribas et al., 2017), as well for other basal teleost species such as *O. mykiss*, and for advanced teleosts such as *H. hippoglossus* and discus (*Symphysodon aequifasciatus* Pellegrin 1904) (Björnsson, 1994; Holm et al., 1990; Tibile et al., 2016). It has been proposed that size differences relate to the reduction in feeding activity or to an increase in energy expenditure associated with enhanced swimming activity due to increased competition or interactions. In our experimental rearing, food was administered *ad libitum*, consequently, insufficient feeding was unlikely a causative factor for the reduced size in the specimens reared at higher densities.

Rearing at different densities after 30 dpf did not influence the modal values of meristic characters. Lower mean values and lower limit of the variation range for the number of vertebral centra observed in the HD reared zebrafish related to the presence of complete vertebral centra fusions (which in the meristic counts were accounted as one element). Ferreri et al. (2000) compared wild and reared zebrafish

and found similar ranges of variation for several meristic elements, with the exception of the anal and pectoral fin rays. Bird and Mabee (2003) confirmed what Ferreri et al. (2000) previously reported for vertebral centra counts even in other reared zebrafish. Usually, variation in the number of meristic elements is due to changes in environmental conditions during the early developmental stages. For example, low temperatures lead to an increased number of vertebrae in reared zebrafish (Sfakianakis et al., 2011).

All the specimens analysed (with one exception in the MD group, already discussed) showed at least one anomaly (Table 4). Such a high frequency may be surprising but has been reported before. High frequencies of zebrafish affected by at least one anomaly were already reported for both wild (87%) and reared (93%) specimens by Ferreri et al. (2000).

The HD group displayed the highest average number of deformities per specimen and a larger variety of types of deformities. The latter could be a density effect but it could also relate to the larger number (n = 65) of HD specimens with respect to the MD (n = 46) and LD (n = 19) groups. However, the highest average number of deformities per specimen, as detected in the HD group, parallels what has been already described in aquaculture facilities. Semi-intensive rearing methodologies (characterized also by reduced rearing densities) compared to intensive rearing conditions, decrease the occurrence of skeletal deformities in farmed fish (Boglione et al., 2009; Prestinicola et al., 2013; Zouiten et al., 2011). Similar to what has been described for an advanced teleost, the *E. marginatus* (Boglione et al., 2009),

553 rearing density alone can affect the skeletal phenotype in zebrafish,
554 and increases the occurrence of particular types of deformities in the
555 caudal region of the vertebral column (partial and complete fusions of
556 vertebral centra, deformation of neural and haemal arches). The
557 susceptibility of the caudal region to deformities has been already
558 described in farmed Atlantic salmon (*Salmo salar* L.). Vertebral
559 centra compressions and fusions can relate to high-temperature
560 exposure during the embryonic stages (Grini et al., 2011). The
561 aggravation of such deformities in salmonids reared at high
562 temperature can occur later, for example during the late juvenile
563 seawater phase (Wargelius et al., 2015). The latter may be the result
564 of a synergic effect of the rearing temperature and the high density
565 used during the seawater rearing. Vertebral centra deformities in
566 Atlantic salmon have also been attributed to other not fully elucidated
567 causative factors acting during later ontogenetic stages (Fjelldal, et
568 al., 2007, Fjelldal et al., 2012).
569 The skeletal elements that displayed the most distinct phenotypic
570 response to increased rearing density were neural and haemal
571 arches and spines (deformations in shape, C4def and C5def),
572 followed by centra of the caudal region (C2par and fus). Despite the
573 fact that anomalies of arches and spines were also observed in a few
574 specimens of the T0 group, their frequency, and that of specimens
575 affected, are far higher in the HD than in the LD group. Vertebral
576 centra and arches in teleosts are different developmental modules.
577 Vertebral centra originate as chordacentra by mineralization of the
578 notochord sheath, whilst the associated elements arches and spines

579 are patterned by the somites (Laerm, 1979, Fleming et al., 2015).
580 The duality in vertebral column elements' formation could explain the
581 higher incidence of deformities of arches and spines compared to
582 vertebral centra, in the HD group. Interestingly, malformations,
583 similar to those shown in Fig. 5c, have been described for fused
584 somite mutant zebrafish (tbx6 mutation) (van Eedden et al., 2006,
585 Fleming et al., 2004). In this mutant zebrafish line, the somitogenesis
586 is disturbed and the specimens show malformations of arches and
587 spines, but separated vertebral centra. That shows that centra and
588 associate elements are two distinct developmental modules.
589 However, the mechanisms by which rearing density induces late
590 vertebral column deformities that resemble mutant-related
591 malformations remain to be elucidated. Deformities of arches and
592 spines have also been related to musculature impairments (Favaloro
593 et al., 2006, Backiel et al., 1984). Behavioural studies on *O. mykiss*
594 reared at high stocking densities (Bégout Anras and Lagardère,
595 2004; Cooke et al., 2000) showed that the complexity of swimming
596 trajectories, space utilization and activity rhythms were altered and
597 that swimming activity, oxygen consumption and muscular activity
598 increased when compared with individuals reared at lower densities.
599 Moreover, the crowded conditions augmented the occurrence of
600 changes in swimming direction with sharper turning angles with
601 respect to individuals kept at lower densities (Bégout Anras and
602 Lagardère, 2004). The swimming patterns suggested recurring
603 avoidance behaviours of individuals held in the same tank.
604 Avoidance behaviours imply the utilization of fast C-start movements,

usually occurring during escape responses, which start with the contraction of the muscles of one side of the body, at the level of the individual's centre of mass, in which the propulsive force develops, allowing the fish to change orientation (Eaton and Emberley, 1991). During the fast start movements, the body bends at the level of the central region, below the dorsal fin, at the 50% of the fish $T_{L,}$ as shown for zebrafish by Danos and Lauder (2012). In *Cyprinus carpio* the maximum vertebral column curvature has been calculated to be between 50 and 80% of fish $T_L$ (Shadwick and Lauder, 2006). During fast start movements, the muscles generate a mechanical load on the flexing vertebral column (Shadwick and Lauder, 2006; Wakeling and Johnston, 1999).

Mechanical loading increases bone formation in zebrafish (Fiaz et al., 2010; Suniaga et al., 2018) especially if its frequency is high and the mechanical load is dynamic, rather than static (Lisková and Hert, 1971; Rubin and McLeod, 1994; Turner, 1998; Turner et al., 1994a,b; Turner et al., 1995).

Therefore, if a crowded environment leads to an increased number of interactions between animals and thus changes in swimming trajectories, for example due to food competition, possibly, the centra of the central region (*viz*. caudal) of animals reared at higher densities are more often subjected to the bendings generated by the axial musculature. The C-shaped bending of an elongated structure, such as the vertebral column, produces compression on the concave side and strain on convex one. Thus, the intervertebral space on the concave side of the bending would be subjected to compression, i.e.

631 mechanical loading. Indeed, the concave and convex sides can
632 reverse from fast movement to another, according to the turning
633 direction. This increased elicitation could explain the occurrence of
634 fusion in the caudal region of the vertebral column.
635 In this study, the complete fusion of vertebral centra (C2fus) was
636 never observed in specimens reared at low density. Partial fusions
637 (C2par) occurred at a lower frequency in LD compared to the HD and
638 MD groups. Ferreri et al. (2000), using densities far lower than the
639 LD used in this work, did not record vertebral fusion, suggesting that
640 their occurrence and severity could be linked to the increased rearing
641 density. Vertebral centra fusion can develop at various time points
642 during development. Very early fusions in zebrafish relate to the
643 ectopic mineralisation of the notochord sheath in prospective
644 intervertebral regions (Bensimon-Brito et al., 2012b). It is unlikely that
645 this type of very early fusions accounts for observations made in this
646 experiment: notochord segmentation takes place during early
647 ontogeny and it would not explain differences in the occurrence of
648 vertebral fusions in animals reared at different rearing densities
649 during the juvenile period when the vertebral centra identity is
650 already determined. Further, animals from the T0 group did not show
651 fused vertebrae.
652 The next (early) process that can cause the fusion of vertebral centra
653 in zebrafish is the bridging of intervertebral spaces by bone that
654 develops around the mineralised notochord sheath (Bensimon-Brito
655 et al., 2012b; Ytteborg et al., 2010). A third process that may lead to
656 a late fusion (not described in zebrafish but in *S. salar*), is caused by

metaplasia, *i.e.* osteoblasts of the vertebral endplate growth zone turn in cells with a chondroblast-like phenotype, producing cartilage in the intervertebral space. This ectopic cartilage later mineralizes and is subsequently remodelled into bone (Fjelldal et al., 2012; Witten et al., 2005; 2006; Ytteborg et al., 2010).

In conclusion, our study shows the effect of rearing density on the growth rate of zebrafish and provides evidence that rearing density affects the skeletal phenotype in this species. High and, to some extent, medium rearing densities slowed down growth and induced deformities, particularly in the caudal region of the vertebral column. Our results suggest that a density of 2 fish/litre, between the age of 30 and 90 dpf can help to reduce the incidence of skeletal malformations in *D. rerio*. This is especially relevant if zebrafish is used for studying skeletal pathologies. Moreover, for this analysis, we propose a methodology that is adaptable and can be used in various contexts to assess skeletal anomalies in zebrafish or other species. For example, the alphanumeric code used here can be adapted to different levels of details according to the needs or applications (*i.e.*, by grouping different types of malformations, or by adding subcodes for peculiar or different types of malformations). Such standardization may facilitate comparison among different studies.

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Table marked

| Region | Skeletal element | code | Description |
|---|---|---|---|
| **A** | | | Weberian vertebrae (carrying modified arches/spines- Weberian ossicles) |
| **B** | | | Abdominal vertebrae (carrying ribs and open haemal arches, without haemal spines) |
| **C** | | | Caudal vertebrae (with closed haemal and neural arches/spines) |
| **D** | | | Caudal complex (preurals and ural vertebrae) |
| **E** | | | Pectoral fin |
| **F** | | | Anal fin |
| **G** | | | Caudal fin |
| **H** | | | Dorsal fin |
| **I** | | | Pelvic fin |
| | 1 | kyp | Kyphosis |
| | | lor | Lordosis |
| | | sbs | Saddle-back syndrome* |
| | | sco | Scoliosis |
| | 2 | par | Partial vertebral fusion |
| | | fus | Complete vertebral ~~body~~ centra fusion |
| | | def | Vertebral deformation |
| | | elo/red | Vertebral marked elongation/reduction in length |
| | 3 | def | Deformed urostyle |
| | | dec | Unmineralized urostyle |
| | 4 | def | Malformed neural arch and/or spine |
| | | sup/abs | Supernumerary/absent neural elements |
| | | sup/abs (L/R) | Supernumerary/absent left/right neural elements |
| | | bif | Bifid (forked) neural spine, the right and the left spine don't fuse |
| | | mis | Mismatched fusion of two different neural spines |
| | | ins | Misplacement of the neural arch insertion |
| | 5 | def | Malformed haemal arch and/or spine |
| | | sup/abs | Supernumerary/absent haemal elements |
| | | sup/abs (L/R) | Supernumerary/absent left/right haemal elements |
| | | bif | Bifid (forked) haemal spine, the right and the left spine don't fuse |
| | | mis | Mismatched fusion of two different haemal spines |
| | | ins | Misplacement of the haemal arch insertion |
| | 6 | def | Deformed Weberian ossicles |
| | 7 | def | Malformed rib |
| | | sup/abs | Supernumerary/ absent pleural rib |
| | 8 | def | Deformed fin ray inner support |
| | | sup/abs | Supernumerary/absent fin ray inner support |
| | | fus | Fused fin ray inner support |
| | | dec | Unmineralized fin ray inner support |
| | 9 | def | Deformed hypural |
| | | sup/abs | Supernumerary/absent hypural |
| | | fus | Fused hypural |
| | | dec | Unmineralized hypural |
| | 10 | def | Deformed parahypural |
| | | dec | Unmineralized parahypural |
| | | fus | Fused parahypural |
| | 11 | def | Deformed epural |
| | | sup/abs | Supernumerary/absent epural |
| | | fus | Fused epural |
| | | dec | Unmineralized epural |
| | 12 | def | Deformed ray |
| | | sup/abs | Supernumerary/absent ray |
| | | fus | Fused ray |
| | 13 | def | Malformed maxillary and/or pre-maxillary |
| | 14 | def | Malformed dentary |
| | 15 | def | Other cephalic deformities (glossohyal, neurocranium...) |
| | 16 | def L/R | Malformed left/right operculum |
| | 17 | def L/R | Deformed branchiostegal ray - L/R |
| | | sup/abs (L/R) | Supernumerary/absent branchiostegal ray - L/R |
| | | fus L/R | Fused branchiostegal ray - L/R |
| | 18 | def | Supraneurals bones malformations |

| | | |
|---|---|---|
| | sup/abs | Supernumerary/absent supraneurals |
| | fus | Fused supraneurals |
| | dec | Unmineralized supraneurals |
| 19 | def | Malformed cleithrum L/R |
| 20 | def | Malformed left/right coracoids |
| 21 | def | Deformed postcleithrum |

**Codes for grouped anomalies**

| | |
|---|---|
| Ac | Centra of the Weberian region |
| Aae | Centra-associated elements (arches and Weberian ossicles) of the Weberian region |
| Bc | Centra of the abdominal region |
| Bae | Centra-associated elements (arches and spines) of the abdominal region |
| Cc | Centra of the caudal region |
| Cae | Centra-associated elements (arches and spines) of the caudal region |
| Dc | Centra of the caudal complex |
| Dae | Centra-associated elements (arches and spines) of the caudal complex |

*Table 1* ***List of the anomalies considered. In red, severe anomalies****. Skeletal elements codes: 1 = vertebral column; 2 = vertebral centrum; 3 = urostyle; 4 = neural arch and spine; 5 = haemal arch and spine; 6 = Weberian ossicles; 7 = rib; 8 = internal support of fin rays; 9 = hypural; 10 = parahypural; 11 = epural; 12 = ray; 13 = maxillary and/or pre-maxillary; 14 = dentary; 15 = other cephalic anomalies; 16 = operculum; 17 = branchiostegal ray; 18 = supraneural bone; 19 = cleithrum; 20 = coracoid; 21 = postcleithrum. *Saddle-back syndrome refers to the deformation of the dorsal profile (shaped as a "saddle") linked to the lack of dorsal fin pterygiophores and rays. It can be associated to deformed caudal fin, abdominal kyphosis, caudal lordosis and caudal fin anomalies.*

| | | | | | | Pectoral fin | | | | | | Pelvic fin | | Dorsal fin | | Anal fin | | | Caudal fin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Vertebral centra | Supraneurals | Left radials | Right radials | Left rays | Right rays | Left rays | Right rays | Pterygiophores | Rays | Pterygiophores | Rays | Epural | Hypurals and parahypural | Upper rays | Lower rays |
| **T0** | Modal value | 34 | 7 | 4 | 4 | 10 | 10 | 6 | 5 | 8 | 8 | 13 | 14 | 1 | 6 | 9 | 9 |
| | Range | 32-35 | 0-8 | | | 5-10 | 5-11 | 5-8 | 4-8 | 5-8 | 5-10 | 7-14 | 7-16 | | | 8-9 | 8-9 |
| | Specimens with incomplete development of skeletal elements | 2/32 | 26/32 | 15/32 | | 32/32 | | 30/32 | | 5/32 | 20/32 | 15/32 | 25/32 | 0/32 | 0/32 | 0/32 | |
| **HD** | Modal value | 33 | 7 | 4 | 4 | 12 | 12 | 8 | 8 | 8 | 9 | 13 | 15 | 1 | 6 | 9 | 9 |
| | Range | 30-36 | 4-11 | 3-4 | | 10-13 | 10-13 | 6-8 | 6-9 | 7-8 | 9-10 | 12-15 | 13-17 | | | 8-10 | 8-10 |
| **MD** | Modal value | 34 | 7 | 4 | 4 | 12 | 12 | 8 | 8 | 8 | 9 | 13 | 15 | 1 | 6 | 9 | 9 |
| | Range | 33-36 | 4-10 | | 3-4 | 10-14 | 11-14 | 6-8 | 6-8 | 7-8 | 9-10 | 10-15 | 11-17 | 0-1 | 5-6 | 7-9 | 8-10 |
| **LD** | Modal value | 34 | 7 | 4 | 4 | 12 | 12 | 8 | 8 | 8 | 9 | 13 | 15 | 1 | 6 | 9 | 9 |
| | Range | 32-35 | 6-9 | | | 12-13 | 12-13 | 7-8 | 7-8 | 7-9 | 8-9 | 12-15 | 14-17 | | 5-6 | 7-9 | 7-9 |

*Table 2 Modal values and range for the vertebral centra and fins' elements in the T0 and the three experimental groups. Ranges left empty indicate no variation in the number of elements. Note that lower modal values reported for some skeletal element in the T0 group compared to the experimental groups are due to the incomplete development of such skeletal elements at the considered stage (T0, 30 dpf). ~~The third row for the T0 samples~~ "Specimens with incomplete development of skeletal elements" indicates the number of individuals on the total of T0 group (n = 32) having not yet differentiated the final numbers of skeletal elements.*

| *General metrics on skeletal anomalies* | **T0** |
|---|---|
| **N of observed specimens** | 32 |
| **Frequency (%) of specimens with at least one anomaly** | 56 |
| **Average anomaly load** | 9 |
| **N of observed types of anomaly** | 17 |
| **Frequency (%) of specimens with at least one severe anomaly** | 34 |
| **Average severe anomaly load** | 2 |
| **Frequency (%) of observed severe anomalies/total n anomalies** | 12 |

*Table 3 General metrics for the analysis of the skeletal anomalies for the T0 group.*

| *General metrics on skeletal anomalies* | **HD** | **MD** | **LD** |
|---|---|---|---|
| **N observed specimens** | 65 | 46 | 19 |
| **Frequency (%) of specimens with at least one anomaly** | 100 | 98 | 100 |
| **Average anomaly load** | **12** | 9 | 9 |
| **N observed types of anomaly** | **68** | 47 | 44 |
| **Frequency (%) of specimens with at least one severe anomaly** | 72 | **73** | 58 |
| **Average severe anomaly load** | 2 | 3 | 3 |
| **Frequency (%) of severe anomalies/total n anomalies** | 13 | **21** | 19 |

*Table 4 General metrics for the analysis of skeletal anomalies in the experimental groups HD, MD and LD. Highest values are shown in bold.*

Table revised

| Region | Skeletal element | code | Description |
|---|---|---|---|
| **A** | | | Weberian vertebrae (carrying modified arches/spines- Weberian ossicles) |
| **B** | | | Abdominal vertebrae (carrying ribs and open haemal arches, without haemal spines) |
| **C** | | | Caudal vertebrae (with closed haemal and neural arches/spines) |
| **D** | | | Caudal complex (preurals and ural vertebrae) |
| **E** | | | Pectoral fin |
| **F** | | | Anal fin |
| **G** | | | Caudal fin |
| **H** | | | Dorsal fin |
| **I** | | | Pelvic fin |
| | 1 | kyp | Kyphosis |
| | | lor | Lordosis |
| | | sbs | Saddle-back syndrome* |
| | | sco | Scoliosis |
| | 2 | par | Partial vertebral fusion |
| | | fus | Complete vertebral centra fusion |
| | | def | Vertebral deformation |
| | | elo/red | Vertebral marked elongation/reduction in length |
| | 3 | def | Deformed urostyle |
| | | dec | Unmineralized urostyle |
| | 4 | def | Malformed neural arch and/or spine |
| | | sup/abs | Supernumerary/absent neural elements |
| | | sup/abs (L/R) | Supernumerary/absent left/right neural elements |
| | | bif | Bifid (forked) neural spine, the right and the left spine don't fuse |
| | | mis | Mismatched fusion of two different neural spines |
| | | ins | Misplacement of the neural arch insertion |
| | 5 | def | Malformed haemal arch and/or spine |
| | | sup/abs | Supernumerary/absent haemal elements |
| | | sup/abs (L/R) | Supernumerary/absent left/right haemal elements |
| | | bif | Bifid (forked) haemal spine, the right and the left spine don't fuse |
| | | mis | Mismatched fusion of two different haemal spines |
| | | ins | Misplacement of the haemal arch insertion |
| | 6 | def | Deformed Weberian ossicles |
| | 7 | def | Malformed rib |
| | | sup/abs | Supernumerary/ absent pleural rib |
| | 8 | def | Deformed fin ray inner support |
| | | sup/abs | Supernumerary/absent fin ray inner support |
| | | fus | Fused fin ray inner support |
| | | dec | Unmineralized fin ray inner support |
| | 9 | def | Deformed hypural |
| | | sup/abs | Supernumerary/absent hypural |
| | | fus | Fused hypural |
| | | dec | Unmineralized hypural |
| | 10 | def | Deformed parahypural |
| | | dec | Unmineralized parahypural |
| | | fus | Fused parahypural |
| | 11 | def | Deformed epural |
| | | sup/abs | Supernumerary/absent epural |
| | | fus | Fused epural |
| | | dec | Unmineralized epural |
| | 12 | def | Deformed ray |
| | | sup/abs | Supernumerary/absent ray |
| | | fus | Fused ray |
| | 13 | def | Malformed maxillary and/or pre-maxillary |
| | 14 | def | Malformed dentary |
| | 15 | def | Other cephalic deformities (glossohyal, neurocranium...) |
| | 16 | def L/R | Malformed left/right operculum |
| | 17 | def L/R | Deformed branchiostegal ray - L/R |
| | | sup/abs (L/R) | Supernumerary/absent branchiostegal ray - L/R |
| | | fus L/R | Fused branchiostegal ray - L/R |
| | 18 | def | Supraneurals bones malformations |

| | | |
|---|---|---|
| | sup/abs | Supernumerary/absent supraneurals |
| | fus | Fused supraneurals |
| | dec | Unmineralized supraneurals |
| 19 | def | Malformed cleithrum L/R |
| 20 | def | Malformed left/right coracoids |
| 21 | def | Deformed postcleithrum |

**Codes for grouped anomalies**

| | |
|---|---|
| Ac | Centra of the Weberian region |
| Aae | Centra-associated elements (arches and Weberian ossicles) of the Weberian region |
| Bc | Centra of the abdominal region |
| Bae | Centra-associated elements (arches and spines) of the abdominal region |
| Cc | Centra of the caudal region |
| Cae | Centra-associated elements (arches and spines) of the caudal region |
| Dc | Centra of the caudal complex |
| Dae | Centra-associated elements (arches and spines) of the caudal complex |

*Table 1* ***List of the anomalies considered. In red, severe anomalies.*** *Skeletal elements codes: 1 = vertebral column; 2 = vertebral centrum; 3 = urostyle; 4 = neural arch and spine; 5 = haemal arch and spine; 6 = Weberian ossicles; 7 = rib; 8 = internal support of fin rays; 9 = hypural; 10 = parahypural; 11 = epural; 12 = ray; 13 = maxillary and/or pre-maxillary; 14 = dentary; 15 = other cephalic anomalies; 16 = operculum; 17 = branchiostegal ray; 18 = supraneural bone; 19 = cleithrum; 20 = coracoid; 21 = postcleithrum. *Saddle-back syndrome refers to the deformation of the dorsal profile (shaped as a "saddle") linked to the lack of dorsal fin pterygiophores and rays. It can be associated to deformed caudal fin, abdominal kyphosis, caudal lordosis and caudal fin anomalies.*

| | | | | | | Pectoral fin | | | | Pelvic fin | | Dorsal fin | | Anal fin | | | Caudal fin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Vertebral centra | Supraneurals | Left radials | Right radials | Left rays | Right rays | Left rays | Right rays | Pterygiophores | Rays | Pterygiophores | Rays | Epural | Hypurals and parahypural | Upper rays | Lower rays |
| **T0** | Modal value | 34 | 7 | 4 | 4 | 10 | 10 | 6 | 5 | 8 | 8 | 13 | 14 | 1 | 6 | 9 | 9 |
| | Range | 32-35 | 0-8 | | | 5-10 | 5-11 | 5-8 | 4-8 | 5-8 | 5-10 | 7-14 | 7-16 | | | 8-9 | 8-9 |
| | Specimens with incomplete development of skeletal elements | 2/32 | 26/32 | 15/32 | | 32/32 | | 30/32 | | 5/32 | 20/32 | 15/32 | 25/32 | 0/32 | 0/32 | 0/32 | |
| **HD** | Modal value | 33 | 7 | 4 | 4 | 12 | 12 | 8 | 8 | 8 | 9 | 13 | 15 | 1 | 6 | 9 | 9 |
| | Range | 30-36 | 4-11 | 3-4 | | 10-13 | 10-13 | 6-8 | 6-9 | 7-8 | 9-10 | 12-15 | 13-17 | | | 8-10 | 8-10 |
| **MD** | Modal value | 34 | 7 | 4 | 4 | 12 | 12 | 8 | 8 | 8 | 9 | 13 | 15 | 1 | 6 | 9 | 9 |
| | Range | 33-36 | 4-10 | | 3-4 | 10-14 | 11-14 | 6-8 | 6-8 | 7-8 | 9-10 | 10-15 | 11-17 | 0-1 | 5-6 | 7-9 | 8-10 |
| **LD** | Modal value | 34 | 7 | 4 | 4 | 12 | 12 | 8 | 8 | 8 | 9 | 13 | 15 | 1 | 6 | 9 | 9 |
| | Range | 32-35 | 6-9 | | | 12-13 | 12-13 | 7-8 | 7-8 | 7-9 | 8-9 | 12-15 | 14-17 | | 5-6 | 7-9 | 7-9 |

*Table 2 Modal values and range for the vertebral centra and fins' elements in the T0 and the three experimental groups. Ranges left empty indicate no variation in the number of elements. Note that lower modal values reported for some skeletal element in the T0 group compared to the experimental groups are due to the incomplete development of such skeletal elements at the considered stage (T0, 30 dpf). "Specimens with incomplete development of skeletal elements" indicates the number of individuals on the total of T0 group (n = 32) having not yet differentiated the final numbers of skeletal elements.*

| *General metrics on skeletal anomalies* | **T0** |
|---|---|
| **N of observed specimens** | 32 |
| **Frequency (%) of specimens with at least one anomaly** | 56 |
| **Average anomaly load** | 9 |
| **N of observed types of anomaly** | 17 |
| **Frequency (%) of specimens with at least one severe anomaly** | 34 |
| **Average severe anomaly load** | 2 |
| **Frequency (%) of observed severe anomalies/total n anomalies** | 12 |

*Table 3 General metrics for the analysis of the skeletal anomalies for the T0 group.*

| *General metrics on skeletal anomalies* | **HD** | **MD** | **LD** |
|---|---|---|---|
| **N observed specimens** | 65 | 46 | 19 |
| **Frequency (%) of specimens with at least one anomaly** | 100 | 98 | 100 |
| **Average anomaly load** | **12** | 9 | 9 |
| **N observed types of anomaly** | **68** | 47 | 44 |
| **Frequency (%) of specimens with at least one severe anomaly** | 72 | **73** | 58 |
| **Average severe anomaly load** | 2 | 3 | 3 |
| **Frequency (%) of severe anomalies/total n anomalies** | 13 | **21** | 19 |

*Table 4 General metrics for the analysis of skeletal anomalies in the experimental groups HD, MD and LD. Highest values are shown in bold.*

Figure Captions marked

***Figure 1*** *Histogram showing the frequency of each malformation on the total of the observed malformations and the frequency of affected specimens in the T0 group.*

***Figure 2*** *Box plot for the $S_L$ in HD, MD and LD experimental groups. The box represents the 25-75 percent quartiles, the horizontal line inside the box indicates the median value, the cross indicates the mean value and the minimal and maximal values are shown with "whiskers". A dot indicates outlier, defined as data value larger or smaller than 1.5 times the interquartile range. All the differences between groups, are significant according to Kruskall-Wallis test, followed by Dunn's post hoc test with Bonferroni correction ($p< 0.01$), as indicated by different letters.*

***Figure 3*** *Histograms showing the frequency of deformities grouped per typology of skeletal element and region (a) and the frequency of specimens affected (b), for each experimental group. Aae: deformities of the centra-associated elements in the Weberian region; Ac: deformities of the centra in the Weberian region; Bae: deformities of the centra-associated elements in the abdominal region; Bc: deformities of the centra in the abdominal region; Cae: deformities of the centra-associated elements in the caudal region; Cc: deformities of the centra in the caudal region; Dae: deformities of the centra-associated elements in the caudal complex; Dc: deformities of the centra in the caudal complex.*

***Figure 4*** *Histograms showing the frequency of deformities (a) and affected specimens (b) in the caudal region.*

***Figure 5*** *Some of the recorded deformities: a) normal vertebrae; b) B4def, neural arches and spines deformities in the abdominal region and B7def, anomalous ribs; c) C4abs L, missing left neural arch in the caudal region, C4bif, bifid neural spine in the caudal region, C5def, anomalous haemal arches and spines in the caudal region; d) C2fus, complete vertebral body fusion in the caudal region; e) C2par, partial vertebral body's fusion; f) C4ins, misplacement of the neural arch insertion in a caudal vertebra, F8def, deformed anal fin's pterygiophores, C5abs, absence of the haemal arch in a caudal vertebra; g) D2fus, complete fusion in the caudal complex; h) G11def, deformation of the epural. Alizarin red whole-mount staining.*

***Figure 6*** *Ordination model obtained by CA applied to a subset of RM (matrix 57x12). Dots represent individuals, each one of them is connected with a line to the centroid (i.e., the average of x and y-axes coordinates of individuals belonging to each experimental group). Experimental groups and deformities are plotted in separate graphs (a-d) to allow better the visualization.*

***Figure 1*** *Histogram showing the frequency of each malformation on the total of the observed malformations and the frequency of affected specimens in the T0 group.*

***Figure 2*** *Box plot for the $S_L$ in HD, MD and LD experimental groups. The box represents the 25-75 percent quartiles, the horizontal line inside the box indicates the median value, the cross indicates the mean value and the minimal and maximal values are shown with "whiskers". A dot indicates outlier, defined as data value larger or smaller than 1.5 times the interquartile range. All the differences between groups, are significant according to Kruskall-Wallis test, followed by Dunn's post hoc test with Bonferroni correction ($p< 0.01$), as indicated by different letters.*

***Figure 3*** *Histograms showing the frequency of deformities grouped per typology of skeletal element and region (a) and the frequency of specimens affected (b), for each experimental group. Aae: deformities of the centra-associated elements in the Weberian region; Ac: deformities of the centra in the Weberian region; Bae: deformities of the centra-associated elements in the abdominal region; Bc: deformities of the centra in the abdominal region; Cae: deformities of the centra-associated elements in the caudal region; Cc: deformities of the centra in the caudal region; Dae: deformities of the centra-associated elements in the caudal complex; Dc: deformities of the centra in the caudal complex.*

***Figure 4*** *Histograms showing the frequency of deformities (a) and affected specimens (b) in the caudal region.*

***Figure 5*** *Some of the recorded deformities: a) normal vertebrae; b) B4def, neural arches and spines deformities in the abdominal region and B7def, anomalous ribs; c) C4abs L, missing left neural arch in the caudal region, C4bif, bifid neural spine in the caudal region, C5def, anomalous haemal arches and spines in the caudal region; d) C2fus, complete vertebral body fusion in the caudal region; e) C2par, partial vertebral body's fusion; f) C4ins, misplacement of the neural arch insertion in a caudal vertebra, F8def, deformed anal fin's pterygiophores, C5abs, absence of the haemal arch in a caudal vertebra; g) D2fus, complete fusion in the caudal complex; h) G11def, deformation of the epural. Alizarin red whole-mount staining.*

***Figure 6*** *Ordination model obtained by CA applied to a subset of RM (matrix 57x12). Dots represent individuals, each one of them is connected with a line to the centroid (i.e., the average of x and y-axes coordinates of individuals belonging to each experimental group). Experimental groups and deformities are plotted in separate graphs (a-d) to allow better the visualization.*

Figure 1

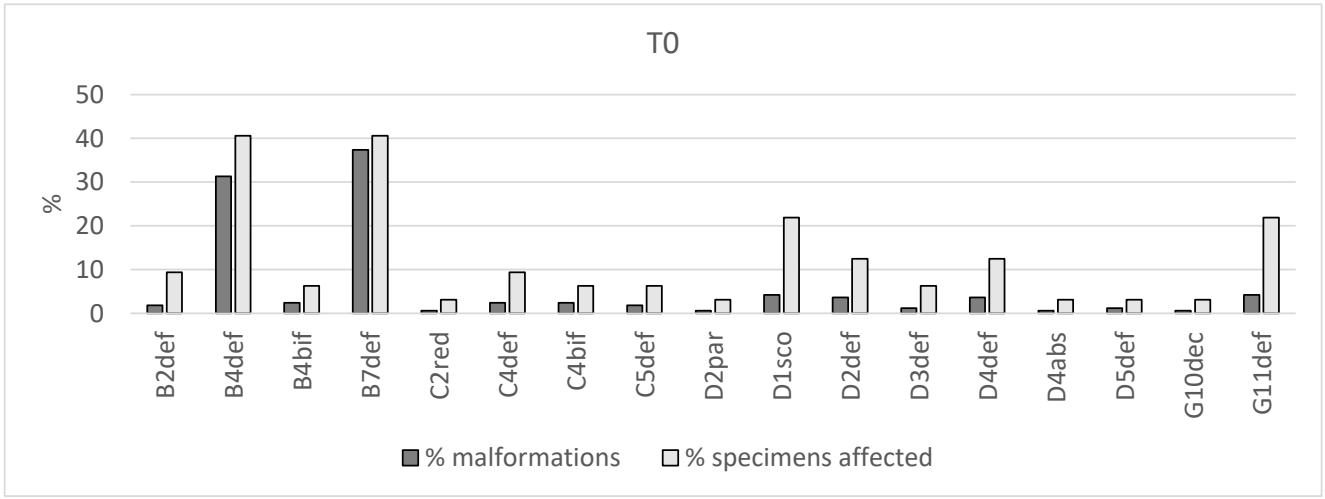

T0
50
40
30
20
10
0
%
B2def
B4def
B4bif
B7def
C2red
C4def
C4bif
C5def
D2par
D1sco
D2def
D3def
D4def
D4abs
D5def
G10dec
G11def
% malformations
% specimens affected

Figure 2

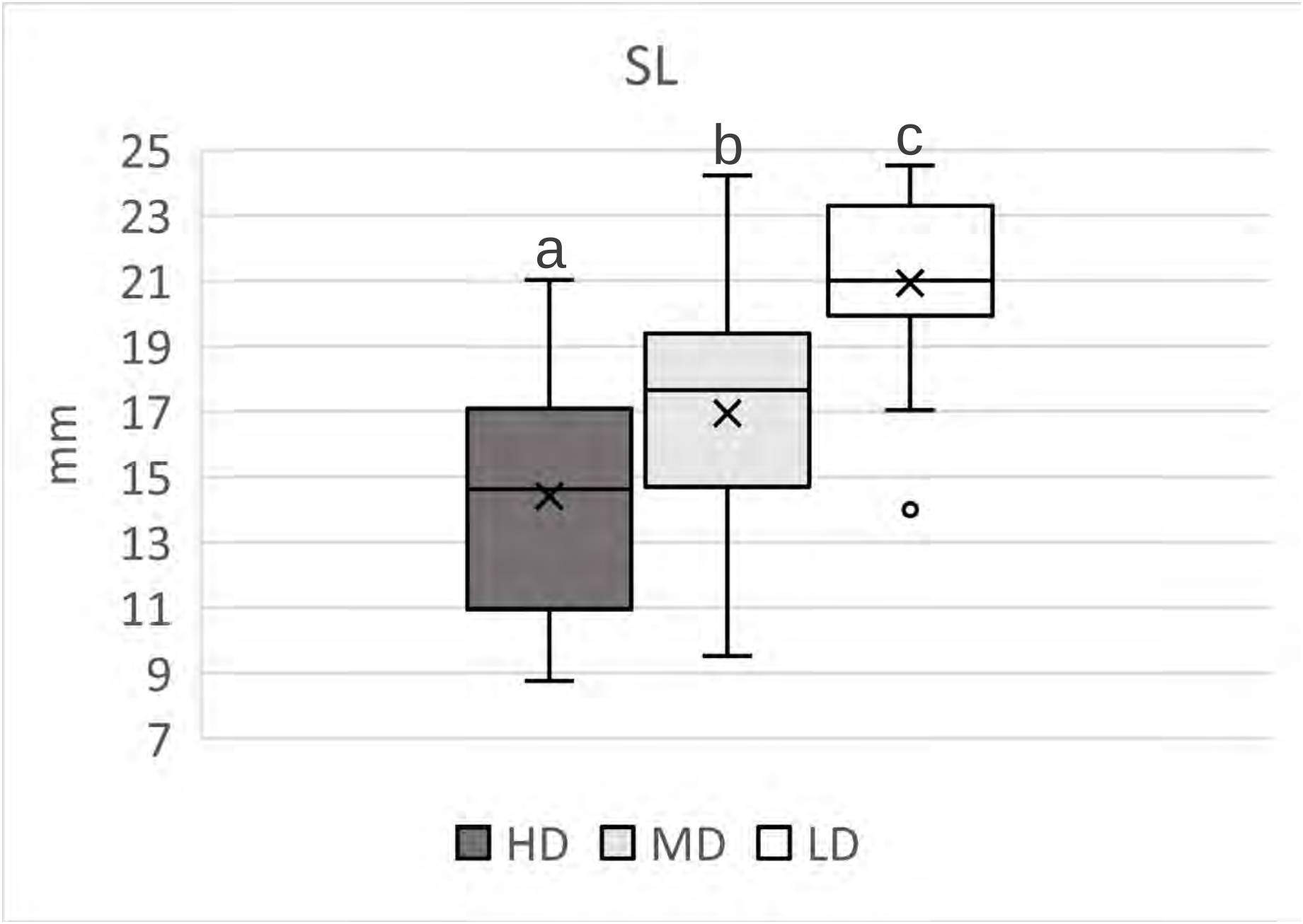


Figure 3

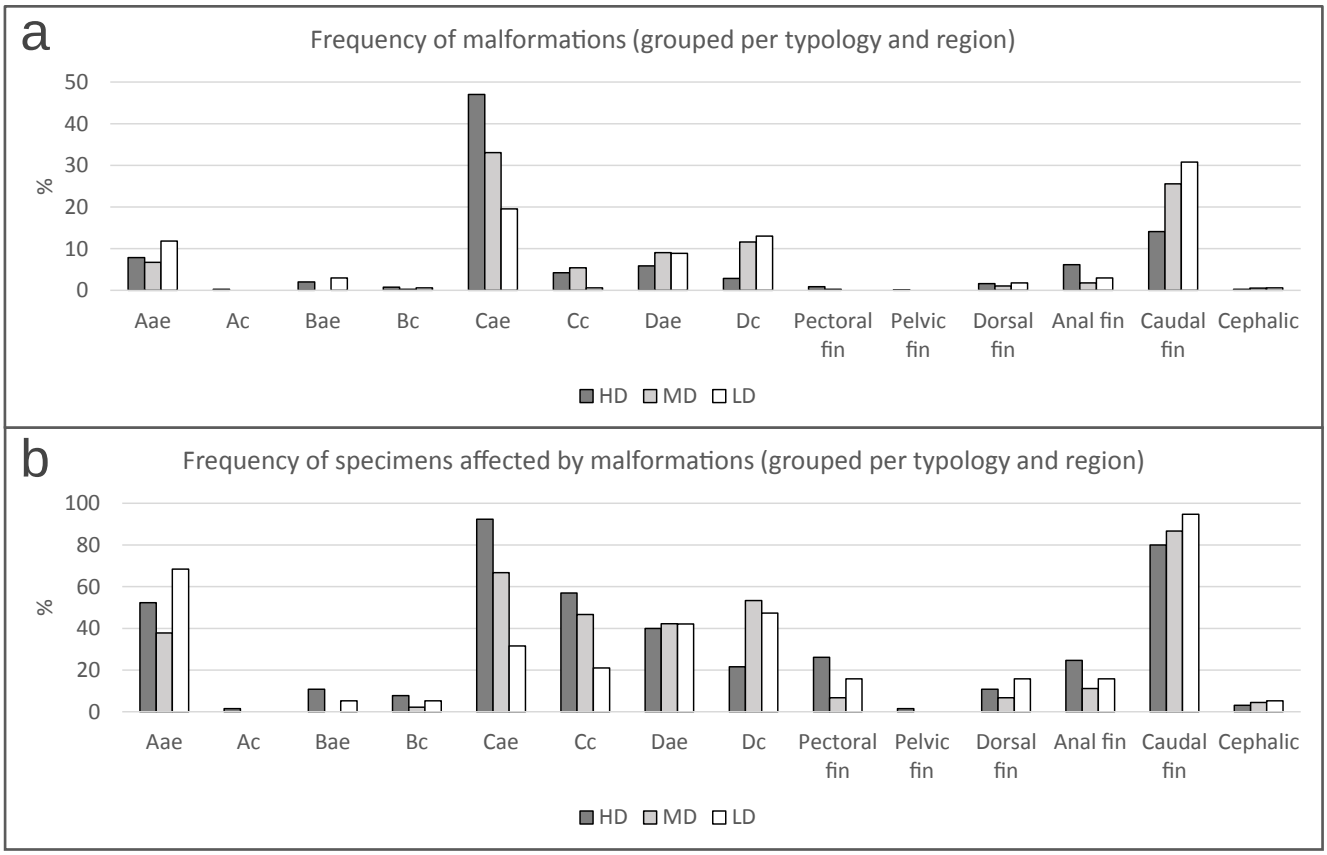

a
Frequency of malformations (grouped per typology and region)
%
50
40
30
20
10
0
Aae
Ac
Bae
Bc
Cae
Cc
Dae
Dc
Pectoral fin
Pelvic fin
Dorsal fin
Anal fin
Caudal fin
Cephalic
HD
MD
LD
b
Frequency of specimens affected by malformations (grouped per typology and region)
%
100
80
60
40
20
0
Aae
Ac
Bae
Bc
Cae
Cc
Dae
Dc
Pectoral fin
Pelvic fin
Dorsal fin
Anal fin
Caudal fin
Cephalic
HD
MD
LD

Figure 4

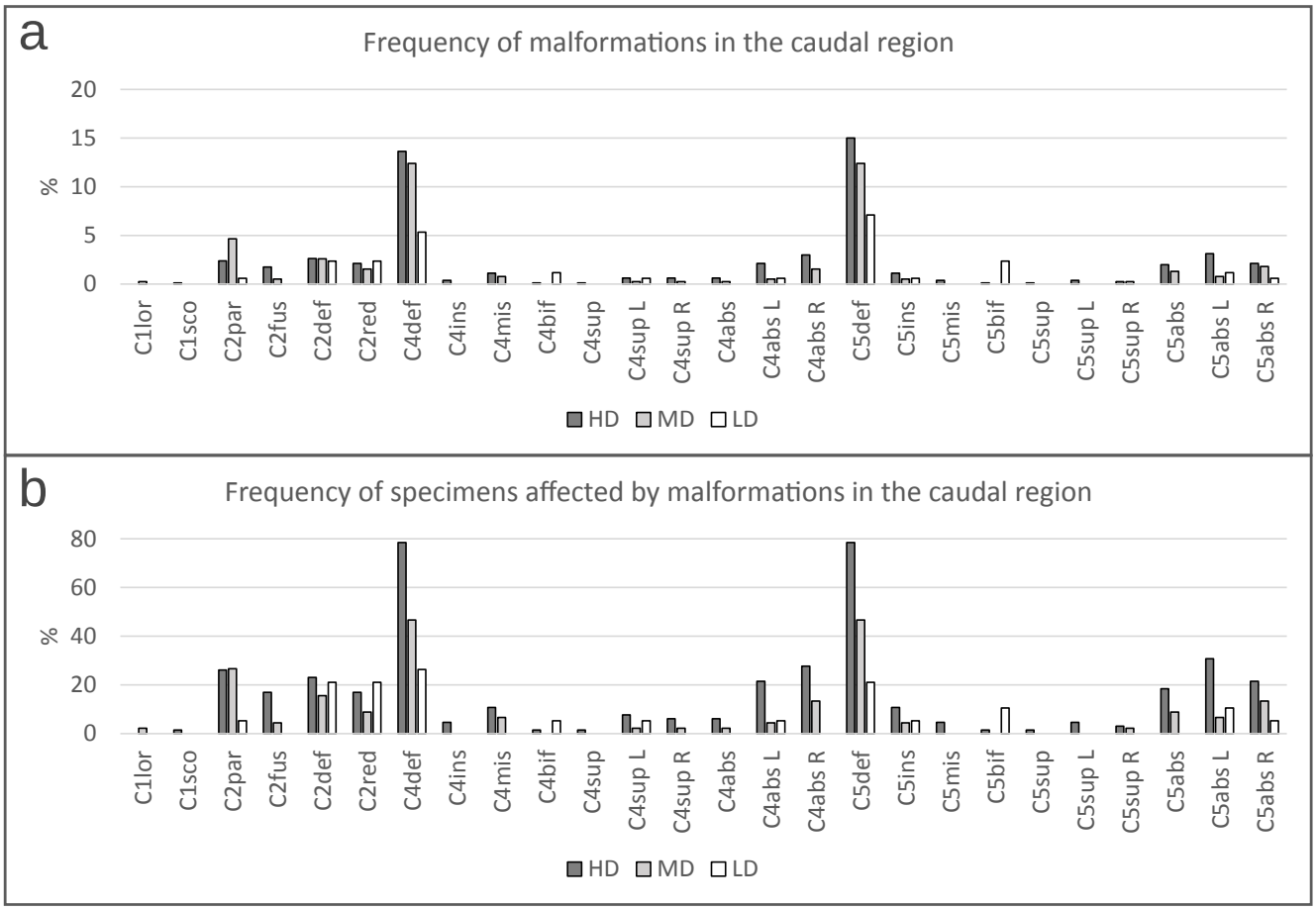

a
Frequency of malformations in the caudal region
%
20
15
10
5
0
C1lor
C1sco
C2par
C2fus
C2def
C2red
C4def
C4ins
C4mis
C4bif
C4sup
C4sup L
C4sup R
C4abs
C4abs L
C4abs R
C5def
C5ins
C5mis
C5bif
C5sup
C5sup L
C5sup R
C5abs
C5abs L
C5abs R
HD MD LD
b
Frequency of specimens affected by malformations in the caudal region
%
80
60
40
20
0
C1lor
C1sco
C2par
C2fus
C2def
C2red
C4def
C4ins
C4mis
C4bif
C4sup
C4sup L
C4sup R
C4abs
C4abs L
C4abs R
C5def
C5ins
C5mis
C5bif
C5sup
C5sup L
C5sup R
C5abs
C5abs L
C5abs R
HD MD LD

Figure 5

Click here to access/download;Figure;Figure_5.tif

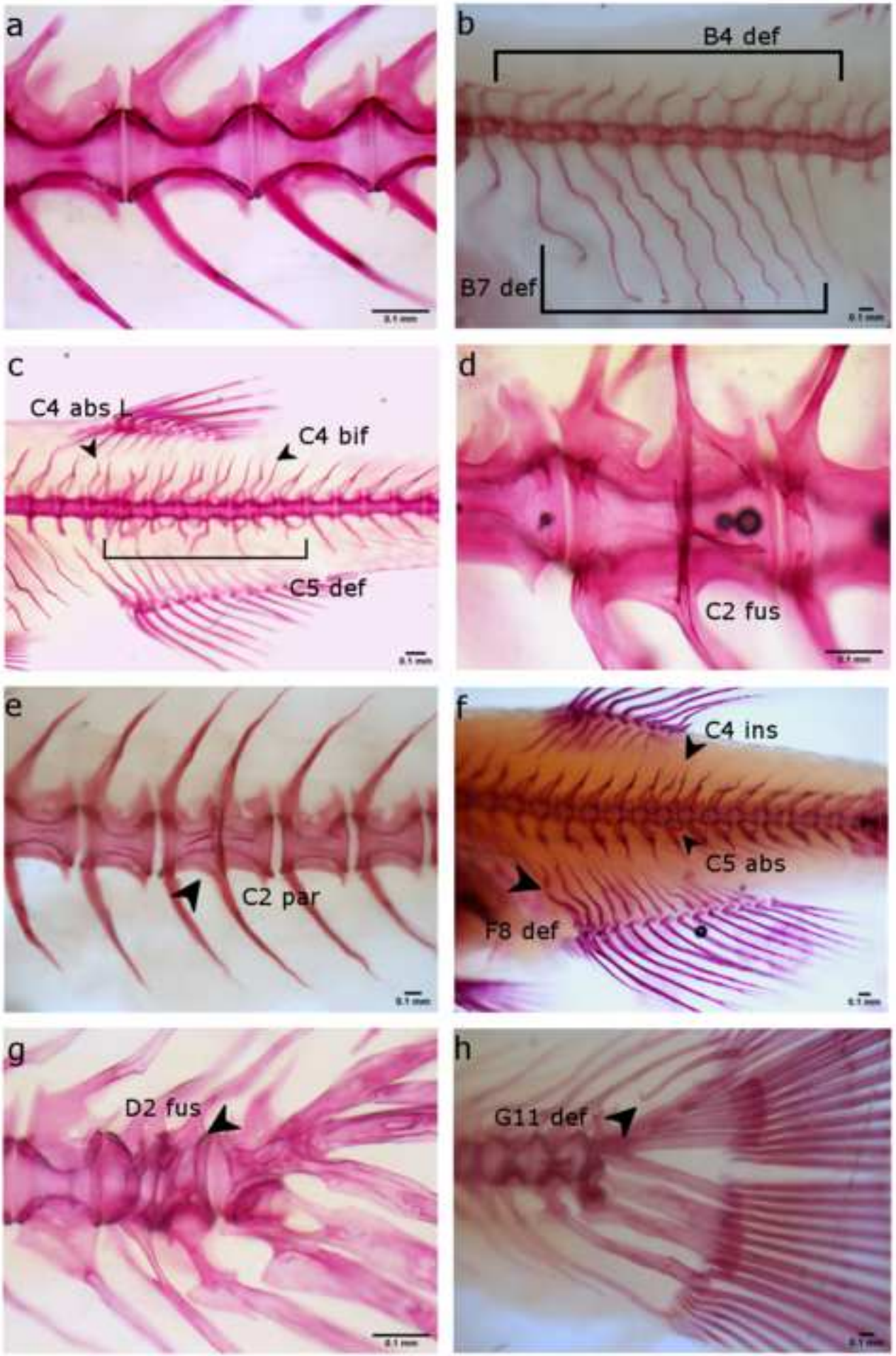
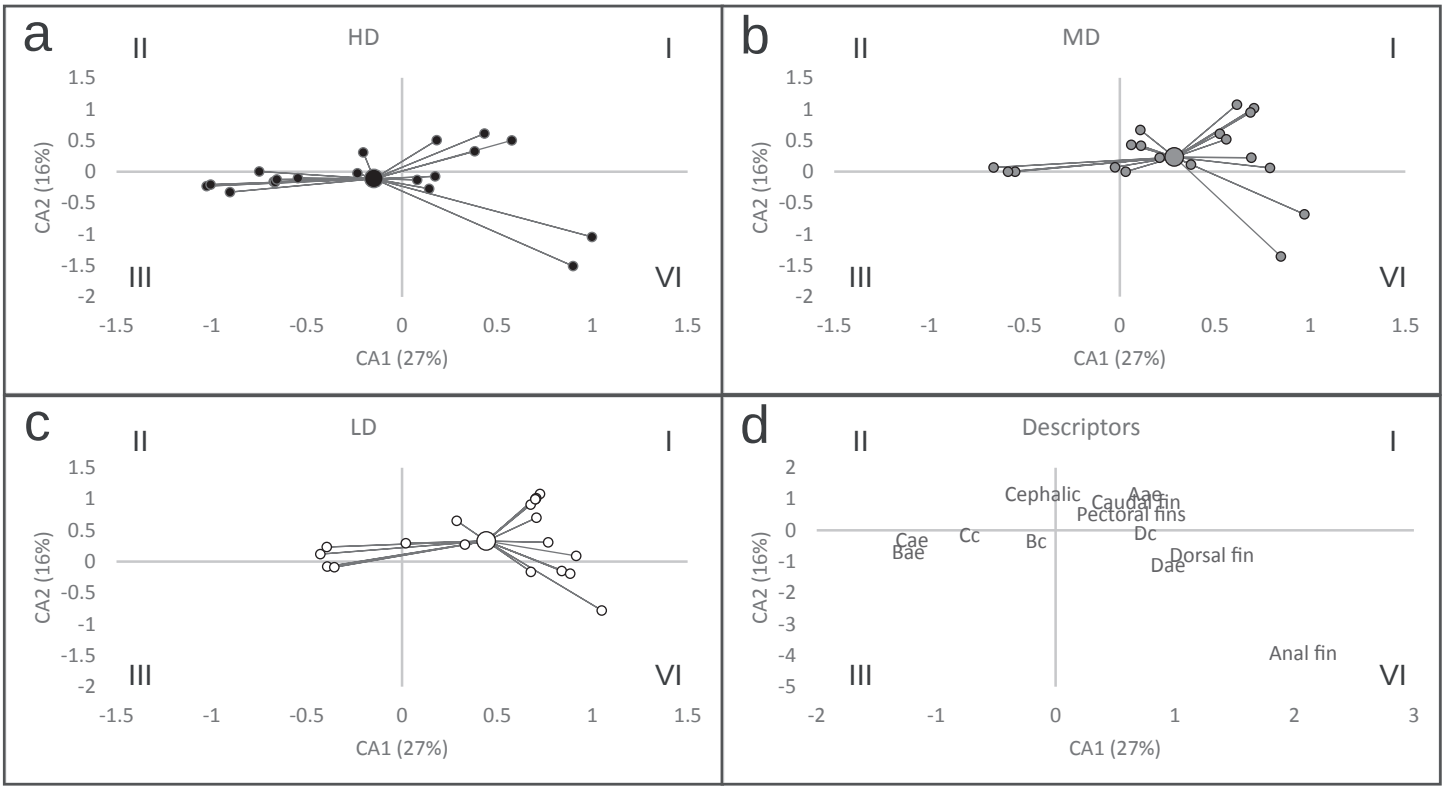


Figure 6


a
HD
II
I
III
VI
CA2 (16%)
CA1 (27%)
b
MD
c
LD
d
Descriptors
Cephalic
Aae
Caudal fin
Pectoral fins
Cae
Cc
Bc
Dc
Bae
Dae
Dorsal fin
Anal fin

| | *HD* | | *MD* | | *LD* | |
|---|---|---|---|---|---|---|
| | % anomalies | % affected specimens | % anomalies | % affected specimens | % anomalies | % affected specimens |
| ***A4 red*** | 0.1 | 2 | | | | |
| ***A4 elo*** | 0.1 | 2 | | | | |
| ***A5*** | 2.4 | 23 | 1.2 | 9 | 3.5 | 26 |
| ***A18*** | 3.8 | 34 | 3.7 | 27 | 7.1 | 53 |
| ***A26*** | 2.1 | 15 | 3.1 | 13 | 2.8 | 16 |
| ***A30*** | | | | | 0.7 | 5 |
| ***B3 tot*** | 0.4 | 3 | | | | |
| ***B4 def*** | 0.4 | 5 | 0.3 | 2 | | |
| ***B4 red*** | | | | | 0.7 | 5 |
| ***B5 mis*** | 0.1 | 2 | | | | |
| ***B5 bif*** | | | | | 1.4 | 5 |
| ***B5 sup*** | 0.1 | 2 | | | | |
| ***B5 sup L*** | 0.1 | 2 | | | | |
| ***B5 abs*** | 0.3 | 3 | | | | |
| ***B5 abs L*** | 0.3 | 3 | | | | |
| ***B5 abs R*** | 0.3 | 3 | | | 0.7 | 5 |
| ***B6*** | | | | | 0.7 | 5 |
| ***B6 abs*** | 0.4 | 5 | | | 0.7 | 5 |
| ***B7 bif*** | 0.1 | 2 | | | | |
| ***B7 abs*** | 0.4 | 5 | | | | |
| ***C2*** | | | 0.3 | 2 | | |
| ***C3*** | 2.5 | 26 | 5.5 | 27 | 0.7 | 5 |
| ***C3 tot*** | 1.9 | 17 | 0.6 | 4 | | |
| ***C4 def*** | 2.8 | 23 | 3.1 | 16 | 2.8 | 21 |
| ***C4 red*** | 2.3 | 17 | 1.8 | 9 | 2.8 | 21 |
| ***C5*** | 14.4 | 78 | 14.7 | 47 | 6.4 | 26 |
| ***C5 ins*** | 0.4 | 5 | | | | |
| ***C5 mis*** | 1.2 | 11 | 0.9 | 7 | | |
| ***C5 bif*** | 0.1 | 2 | | | 1.4 | 5 |
| ***C5 sup*** | 0.1 | 2 | | | | |
| ***C5 sup L*** | 0.7 | 8 | 0.3 | 2 | 0.7 | 5 |
| ***C5 sup R*** | 0.7 | 6 | 0.3 | 2 | | |
| ***C5 abs*** | 0.7 | 6 | 0.3 | 2 | | |
| ***C5 abs L*** | 2.3 | 22 | 0.6 | 4 | 0.7 | 5 |
| ***C5 abs R*** | 3.2 | 28 | 1.8 | 13 | | |
| ***C6*** | 15.9 | 78 | 14.7 | 47 | 8.5 | 21 |
| ***C6 ins*** | 1.2 | 11 | 0.6 | 4 | 0.7 | 5 |
| ***C6 mis*** | 0.4 | 5 | | | | |
| ***C6 bif*** | 0.1 | 2 | | | 2.8 | 11 |
| ***C6 sup*** | 0.1 | 2 | | | | |
| ***C6 sup L*** | 0.4 | 5 | | | | |
| ***C6 sup R*** | 0.3 | 3 | 0.3 | 2 | | |
| ***C6 abs*** | 2.1 | 18 | 1.5 | 9 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***C6 abs L*** | 3.3 | 31 | 0.9 | 7 | 1.4 | 11 |
| ***C6 abs R*** | 2.3 | 22 | 2.1 | 13 | 0.7 | 5 |
| ***CS*** | 0.1 | 2 | | | | |
| ***29*** | 1.7 | 20 | 0.6 | 4 | 2.1 | 16 |
| ***CL L*** | | | 0.3 | 2 | | |
| ***E8 def*** | 0.5 | 3 | | | | |
| ***E8 fus*** | 0.1 | 2 | | | | |
| ***E11 def*** | 0.3 | 2 | | | | |
| ***F8 def*** | 5.7 | 25 | 1.8 | 9 | 2.1 | 11 |
| ***F8 fus*** | 0.1 | 2 | 0.3 | 2 | 0.7 | 5 |
| ***F8 dec*** | 0.3 | 2 | | | | |
| ***F11 sup*** | | | | | 0.7 | 5 |
| ***G9 def*** | 1.9 | 15 | 11.0 | 38 | 8.5 | 32 |
| ***G9 abs*** | | 0 | 0.3 | 2 | 1.4 | 11 |
| ***G9 fus*** | 0.1 | 2 | | | | |
| ***G9* dec*** | 2.0 | 23 | 2.4 | 18 | 2.8 | 21 |
| ***G9* def*** | 0.4 | 5 | 0.3 | 2 | 2.1 | 16 |
| ***G9* fus*** | 0.5 | 6 | 1.8 | 13 | 3.5 | 26 |
| ***G10 dec*** | 3.3 | | 6.1 | 2 | 6.4 | |
| ***G10 def*** | | 38 | 0.3 | 44 | | 47 |
| ***G10 abs*** | 0.1 | | | 2 | | |
| ***G10 fus*** | | 2 | 0.3 | | | |
| ***G11 def*** | 0.4 | 5 | 1.5 | 11 | 3.5 | 26 |
| ***G19*** | 9.5 | 71 | 12.2 | 71 | 14.9 | 74 |
| ***H8 def*** | 0.7 | 5 | 0.6 | 4 | 1.4 | 11 |
| ***H8 sup*** | | | | | 0.7 | 5 |
| ***H11 def*** | 1.1 | 6 | 0.6 | 2 | | |
| ***L11 def*** | 0.1 | 2 | | | | |
| ***15*** | | | | 0 | 0.7 | 5 |
| ***16*** | 0.1 | 2 | 0.6 | 4 | | |
| ***17* def R*** | 0.1 | 2 | | | | |

*Supplementary Table 1 The frequency of each anomalies and the frequency of specimens affected per each experimental group*

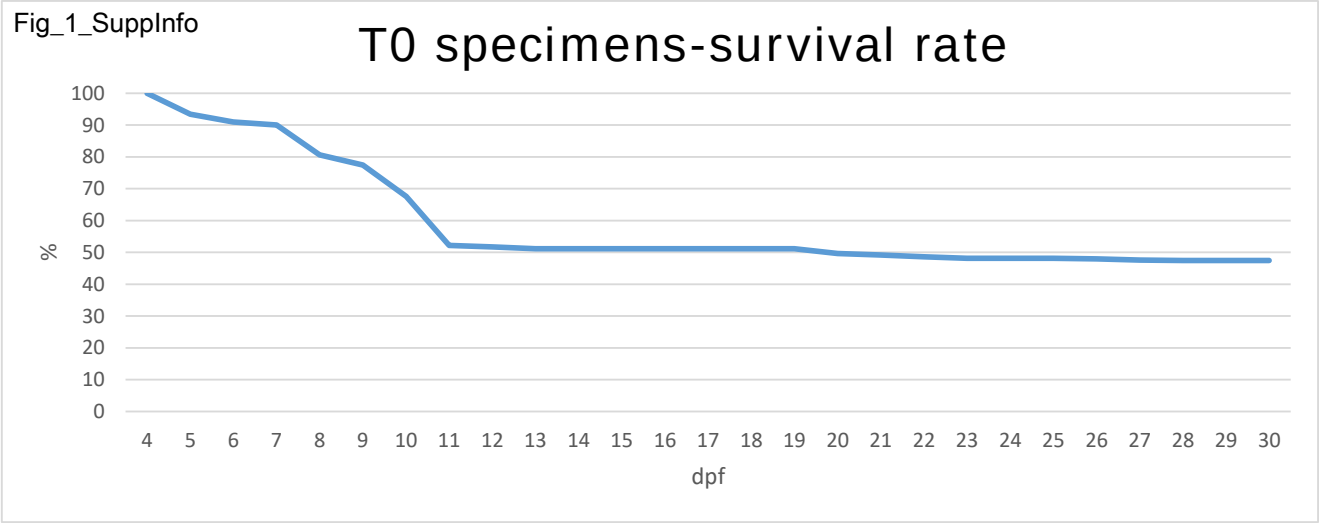



Figure_1_SuppInfo: Survival rate for the T0 samples