

## RESEARCH ARTICLE

# Terrestrial acclimation and exercise lead to bone functional response in *Polypterus senegalus* pectoral fins

Trina Y. Du\* and Emily M. Standen

## ABSTRACT

The ability of bones to sense and respond to mechanical loading is a central feature of vertebrate skeletons. However, the functional demands imposed on terrestrial and aquatic animals differ vastly. The pectoral girdle of the basal actinopterygian fish *Polypterus senegalus* was previously shown to exhibit plasticity following terrestrial acclimation, but the pectoral fin itself has yet to be examined. We investigated skeletal plasticity in the pectoral fins of *P. senegalus* after exposure to terrestrial loading. Juvenile fish were divided into three groups: a control group was kept under aquatic conditions without intervention, an exercised group was also kept in water but received daily exercise on land, and a terrestrial group was kept in a chronic semi-terrestrial condition. After 5 weeks, the pectoral fins were cleared and stained with Alcian Blue and Alizarin Red to visualize cartilage and bone, allowing measurements of bone length, bone width, ossification and curvature to be taken for the endochondral radial bones. *Polypterus senegalus* fin bones responded most strongly to chronic loading in the terrestrial condition. Fish that were reared in a terrestrial environment had significantly longer bones compared with those of aquatic controls, wider propterygia and metapterygia, and more ossified metapterygia and medial radials, and they showed changes in propterygia curvature. Exercised fish also had longer and more ossified medial radials compared with those of controls. *Polypterus senegalus* fin bones exhibit plasticity in response to novel terrestrial loading. Such plasticity could be relevant for transitions between water and land on evolutionary scales, but key differences between fish and tetrapod bone make direct comparisons challenging.

**KEY WORDS:** Amphibious fishes, Mechanical loading, Pectoral radial bones, Skeletal plasticity

## INTRODUCTION

The vertebrate skeleton provides structural support for organs and other soft tissues, and much of its form and structure reflect its mechanical environment. In most vertebrates, the skeleton is composed of bone, a highly dynamic tissue that undergoes growth, resorption and replacement during an individual's lifetime. Although genetics determine a bone's basic shape and properties, much of skeletal development is influenced by epigenetic factors, including mechanical loading, nutrition, temperature and pathology (Hall, 2005).

Mechanical loading is one of the most important determinants of bone phenotype (Biewener and Bertram, 1994; Carter et al., 1996). For example, observations in paralysed organisms (Hall and Herring, 1990) and mutants with disrupted muscle development (Nowlan et al., 2010) have demonstrated that bone development is significantly impaired by the absence of loading from muscle activity. Gravitational loading is also important for normal bone maintenance and growth (Carmeliet and Bouillon, 2001), with astronauts losing as much as 1–2% of bone mass per month in space (Lang et al., 2004). Mechanical loading that leads to increased or novel deformation of bone tissue can also cause dynamic bone modelling and remodelling through a process called bone functional adaptation (Chamay and Tschantz, 1972; Lanyon and Rubin, 1985; Burr et al., 2002). This ability of bones to sense and respond to a mechanical stimulus allows the skeleton to accommodate changes in functional demands throughout the lifetime of an organism.

The specific impacts of mechanical loading on bone development are complex, depending on factors such as species, sex, disease, nutrition, which bone and which region of bone is being loaded, and properties of the loading regimen (Iwamoto et al., 1999; Hsieh et al., 2001; Hamrick et al., 2006; Ruff et al., 2006; Skerry, 2006; Plochocki et al., 2008). However, some general rules of bone functional adaptation have become apparent after decades of experimental study in mammals and birds (Turner, 1998; Burr et al., 2002). Increasing strain beyond a habitual level typically leads to increased bone formation, whereas decreasing strain leads to bone resorption. Dynamic and cyclically applied loads, in particular, are the primary drivers of functional adaptation, whereas static loading typically has no or even negative effects (Lanyon and Rubin, 1984; Robling et al., 2001). Additionally, the number and frequency of loading events, as well as the duration, pattern and gradient of strains also affect bone adaptation (Gross et al., 1997; Judex et al., 1997; Hsieh and Turner, 2001; Warden and Turner, 2004).

The vast majority of studies examining the effects of mechanical loading on bone development have been undertaken in tetrapod models. However, interest in fish bone is increasing, both because of the emerging applications of fish models for human health and for studying the evolution and diversity of bone mechanosensory systems in general (Apschner et al., 2011; Witten and Hall, 2015; Hall and Witten, 2018). Like those of tetrapods, fish skeletons have a high capacity to respond to changes in mechanical loading. Exercise generally promotes bone formation in fish, with swim-training leading to greater osteoblastic activity, earlier ossification and higher mineral content than in non-exercised fish (Deschamps et al., 2009; Totland et al., 2011; Fiaz et al., 2012; Grünbaum et al., 2012). Other examples of loading-induced skeletal plasticity in fish include diet-induced plasticity in teeth and jaw bones (Meyer, 1987; Huysseune et al., 1994; Albertson et al., 2003; Gunter et al., 2013; Gunter and Meyer, 2014), and plasticity in vertebral columns of fish afflicted with spinal deformities, such as scoliosis and lordosis (Kranenburg et al., 2005; Witten et al., 2005; Cardeira et al., 2015).

Department of Biology, University of Ottawa, Gendron Hall, 30 Marie Curie, Ottawa, ON, Canada K1N 6N5.

\*Author for correspondence (trina.du@mail.mcgill.ca)

 T.Y.D., 0000-0003-2193-0795; E.M.S., 0000-0002-3025-1957

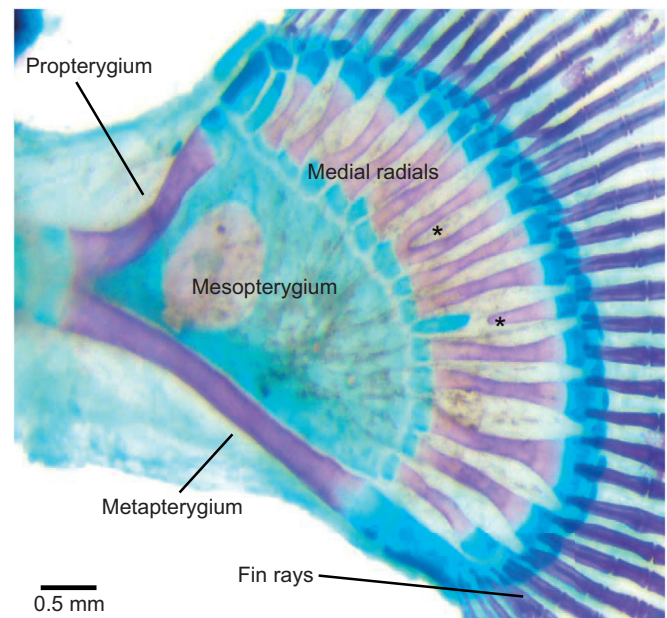
Received 1 November 2019; Accepted 25 April 2020

Vertebrates that live on land must have skeletons that resist the force of gravity as well as stresses induced by weight-bearing activities such as running and jumping. In contrast, most aquatic organisms experience relative weightlessness in the water and the majority of forces experienced by their skeletons are generated by muscle contractions. In this context, the skeletons of amphibious vertebrates may provide new opportunities to understand the relationship between skeletal plasticity and loading, because moving between water and land imposes a wide range of functional demands. Amphibious fish, in particular, play a key part in understanding the role of bone functional adaptation across the fin-to-limb transition and the evolution of weight-bearing appendages. The mechanical responsiveness of the tetrapod skeleton is often thought to have evolved in response to the increased gravitational loading experienced by the first tetrapods (Clack, 2012). However, recent experiments rearing amphibious fishes on land have begun to demonstrate that the fish skeleton is capable of responding to weight bearing in an adaptive manner, similar to tetrapods (Standen et al., 2014; Turko et al., 2017).

In this study, we examined skeletal plasticity in the pectoral fins of *Polypterus senegalus*, an African freshwater fish from the family Polypteridae, notable for their phylogenetic placement as the most basal extant actinopterygians. The capacity of *P. senegalus* to tolerate terrestrial conditions for prolonged periods of time (Standen et al., 2014; Du and Standen, 2017) makes it a useful system to study the effects of terrestrial weight bearing on fish skeletons and their evolutionary implications. Notably, previous work in *P. senegalus* demonstrated that terrestrial acclimation leads to plastic modification of the pectoral girdle bones, resembling the morphology of fossil stem tetrapods, suggesting that phenotypic plasticity could have played a facilitating role during the fin-to-limb transition (Standen et al., 2014). Girdle bones, such as the cleithrum, were the focus of this prior study because of their shared presence in fossil proto-tetrapods. However, their dermal origin and indirect involvement in aquatic or terrestrial locomotion limit their ability to inform us about the evolutionary origins of bone functional adaptation in weight-bearing limbs.

The pectoral fins of *Polypterus* are particularly apt for comparison with tetrapods because of their relevant morphology and function. Like that of other actinopterygians, their fin skeleton is composed of two main components, an initially cartilaginous endoskeleton that connects the fin to the pectoral girdle, and an exoskeleton composed of dermally derived fin rays. *Polypterus* pectoral fins retain the plesiomorphic tribasal condition for fishes with a propterygium, mesopterygium and metapterygium making up the ‘basal’ radials of the fin endoskeleton (Cuervo et al., 2012). *Polypterus* pectoral fins are distinct in that both the propterygium and metapterygium have elongated, rod-like shapes, with a broad triangular mesopterygium between them (Davis et al., 2004). *Polypterus* also possess a fan of smaller rod-like ‘medial’ radial elements distal to the three basal radials (Fig. 1). These elongated basal and medial radials are, in our opinion, more comparable in shape to tetrapod long bones than those of other fishes (for example, see Fig. 1 of Metscher et al., 2005). Additionally, the pectoral fins are heavily muscled (Wilhelm et al., 2015) and actively loaded during both swimming and terrestrial locomotion. On land, *P. senegalus* exhibit a contralateral gait, planting their pectoral fins on the ground in combination with body undulations to produce forward thrust (Standen et al., 2016).

Here, we quantified the effect of novel mechanical loading on the size, shape and ossification of the radial bones in the pectoral fins of *P. senegalus* across the water–land boundary under two different



**Fig. 1. Example of a cleared and stained *Polypterus senegalus* left pectoral fin.** Major skeletal elements are labelled, including the three basal radials (propterygium, mesopterygium and metapterygium), the fan of medial radials and the fin rays. The fin is shown in lateral view, with the head to the left and tail to the right. Cartilaginous tissue is stained blue and ossified tissue is stained red. Note the fused and damaged medial radials indicated with asterisks. These types of abnormal bones were excluded from the analysis.

loading regimes. In the first condition, fins experienced primarily static loading from terrestrially induced hypergravity; and in the second, fins experienced primarily dynamic loading from terrestrial exercise.

## MATERIALS AND METHODS

### Data collection

Juvenile *Polypterus senegalus* Cuvier 1829 (all under 12 cm total body length) were acquired from the pet trade (Mirido Importations Canada, Inc.) and quarantined for 2 weeks prior to the beginning of experimental treatment. All fish were individually housed and randomly assigned into a control group ( $n=13$ ) or one of two treatment groups (exercise and terrestrial,  $n=6$  each). Animals in the control and exercise groups were reared in standard aquatic conditions, with exercise group fish experiencing a slightly smaller aquatic area through the placement of a terrestrial exercise surface within the tank, which reduced swimming activity. Exercise group fish experienced a single daily exercise period when they were removed from the water and induced to travel approximately 30 cm over a smooth substrate to elicit dynamic loading of the fins. The terrestrial group was reared in a chronically semi-terrestrial condition similar to previous experiments (Standen et al., 2014; Du and Standen, 2017) with gravel substrates and a water depth of ~3 mm. Terrestrial fish experienced raised water levels for a few minutes each day to facilitate feeding and cleaning.

Although the terrestrially acclimated fish could move freely inside their tanks, and thus may have also experienced some dynamic loading, we assume that the fins of terrestrially reared fish experienced primarily static loading through passive ground contact. This assumption is supported for two reasons: first, terrestrial fish remained relatively stationary inside their hiding structures (T.Y.D. and E.M.S., personal observation), and second, their fins were probably unloaded when they did move, as previous

work has demonstrated that *P. senegalus* predominantly use axial-driven locomotion on gravel substrates, with relatively little engagement of the pectoral fins (Standen et al., 2016).

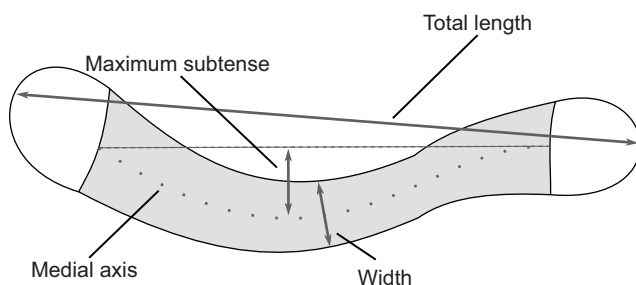
Water temperature was maintained at 26°C for the duration of the experiment. Fish were fed a mix of frozen beef heart and sinking pellets 5 times per week, and food consumption was monitored such that all groups received the same amount of food. We did not distinguish between male and female individuals, as *P. senegalus* do not exhibit secondary sexual characteristics as juveniles (Bartsch et al., 1997). There were no significant differences in body length between groups at the end of the experiment ( $F_{2,22}=1.121$ ,  $P=0.344$ ) and body size was not considered further in the analysis.

Following 5 weeks under experimental conditions, fish were euthanized via immersion in a lethal concentration (420 mg l<sup>-1</sup>) of MS-222 solution. Pectoral fins were then removed, preserved in formalin, and cleared and stained with Alcian Blue and Alizarin Red to visualize cartilage and bone according to a conventional protocol (Fig. 1). Cleared and stained fins were photographed under a Leica M60 dissection microscope using a MC170 HD camera. The best-quality fin from either the left or right side was photographed, but all right fins were left-reflected for the analysis. All experiments were performed under University of Ottawa animal care protocol BL-1934. The investigators were not blind to the group allocation during the experiment or when assessing the outcome.

### Measured variables

Total bone length, bone width, proportion ossified and curvature were measured for the propterygium, metapterygium and medial radials. Total length was measured as the linear distance between the ends of the cartilaginous epiphyses of each bone. Proportion of bone ossified was calculated as the ratio between the length of the ossified section of bone (stained red) and total bone length (Fig. 2). Bone width was measured at the midpoint of the ossified section of bone.

For the medial radials, curvature was calculated as the ratio between the maximum subtense and the length of the chord connecting the proximal and distal end of the ossified section of bone. Maximum subtense was considered to be the distance from the point of maximal curvature along the medial axis to the chord (Fig. 2). We used geometric morphometrics to compare the multivariate shape of the propterygium and metapterygium because they have more complex shapes compared with the



**Fig. 2. Diagram of a typical fin bone from *P. senegalus* and its measured variables.** The ossified region is shaded grey. The thin dashed line denotes the distance between the proximal and distal extents of ossified bone used to calculate the proportion of bone ossified and curvature of the medial radials. Radial width was measured at the midpoint of the ossified section of bone. Maximum subtense was measured as the distance between the point of greatest curvature along the medial axis (dotted line) of the bone and the thin dashed line.

medial radials. Twenty-five equally spaced landmarks were digitized along the medial axis of the propterygium and metapterygium (Fig. 2). A generalized Procrustes analysis was then performed to remove scale, position and orientation using the *gpa* function in the *geomorph* package for R (<https://cran.r-project.org/package=geomorph>). The first and last points were used as fixed landmarks and the others were used as semi-landmarks. Semi-landmarks were aligned by sliding them along their tangent directions so that the bending energy between the points of the curve was minimized (Bookstein, 1997). All bone size and shape measurements were collected using Fiji (Schindelin et al., 2012) and are available from the Dryad digital repository (<https://doi.org/10.5061/dryad.0vt4b8gwc>).

### Statistical analysis

For all univariate variables measured on the propterygium and metapterygium, the effect of treatment was tested with ANOVA. ANOVA contrasts were used to make pairwise comparisons between control–exercise and control–terrestrial groups. Linear mixed effects models were used to test the effect of treatment on medial radials, as each fin contains multiple medial radials. This was implemented using the *lme* function in the *nlme* package for R (<https://CRAN.R-project.org/package=nlme>). Treatment was considered a fixed main effect and individual was included as a random effect to account for non-independence between multiple radials measured from fins of the same individual.

For multivariate bone shape, we performed Procrustes ANOVA with the aligned shape vertices as the response variable and treatment as a categorical variable. Procrustes ANOVA differs from a standard ANOVA because the sum-of-squared Procrustes distances is used as the measure of sums of squares (Goodall, 1991). This analysis was performed using the *procr.lm* function in *geomorph*. A residual randomization permutation method (Collyer and Adams, 2018; <https://cran.r-project.org/web/packages/RRPP>) was used with 999 iterations to evaluate the significance of all tests described above. The *pairwise* function in the *RRPP* package v0.5.2 for R (Collyer and Adams, 2018; <https://cran.r-project.org/web/packages/RRPP>) was used to make pairwise comparisons between control–exercise and control–terrestrial groups. All statistical analyses were performed in R v3.5.3 (<http://www.R-project.org/>).

### RESULTS

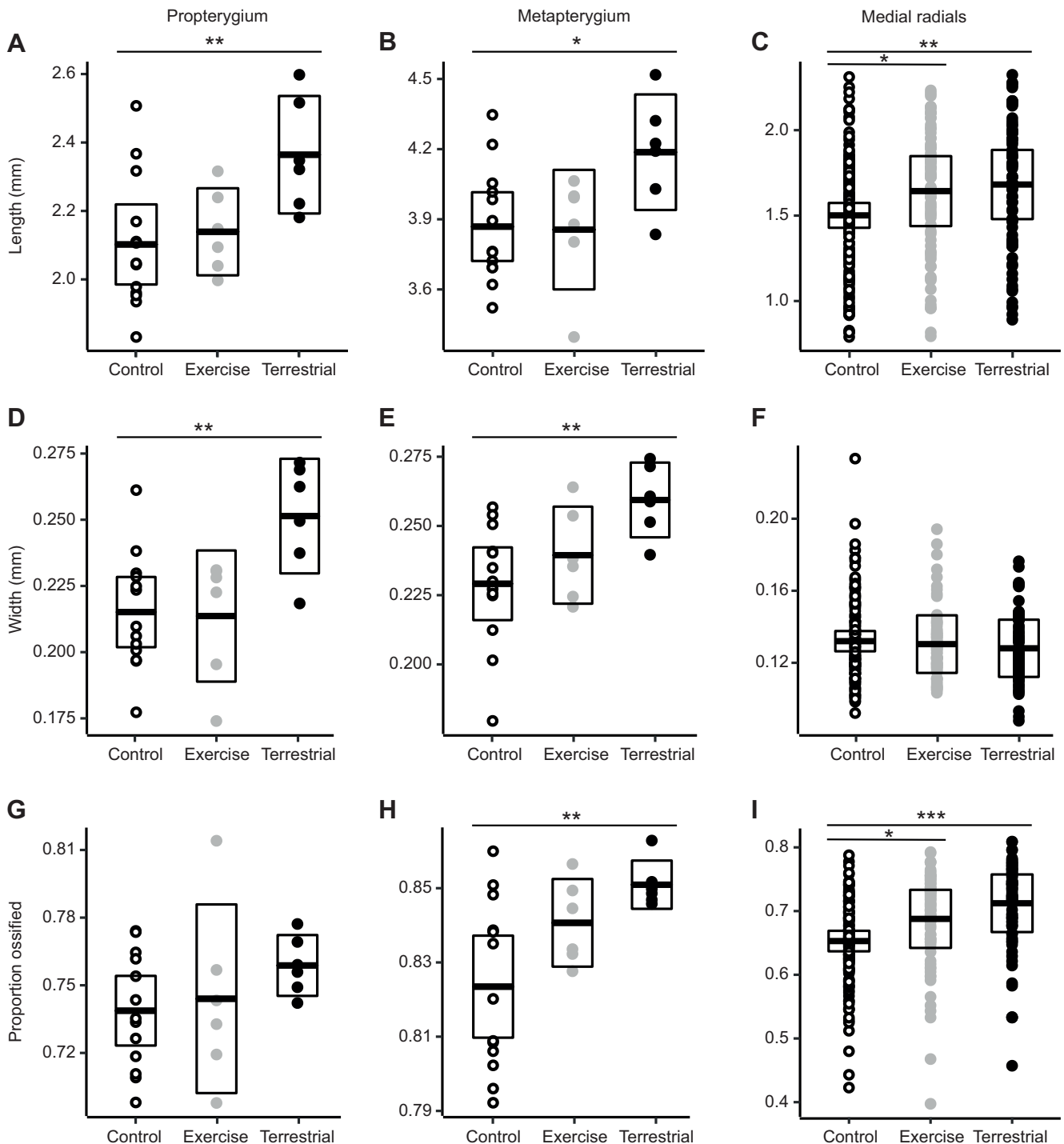
Compared with control fish, terrestrial fish had significantly longer and wider propterygia ( $P<0.007$ ; Fig. 3A,D, Table 1) as well as metapterygia ( $P<0.014$ ; Fig. 3B,E, Table 1). Exercised fish did not differ from controls in length or width of either the propterygium or metapterygium ( $P>0.281$ ; Fig. 3A–D, Table 1). Both exercise and terrestrial groups had significantly longer medial radials than control fish ( $P<0.036$ ; Fig. 3C, Table 1), but showed no difference in width ( $P>0.432$ ; Fig. 3F, Table 1).

There was no difference in propterygium ossification for either exercise or terrestrial groups compared with controls ( $P>0.153$ ; Fig. 3G, Table 1). For the metapterygium, terrestrial fish had a greater proportion of ossification compared with the control group ( $P=0.005$ ; Fig. 3H, Table 1), but there was no difference between exercised and control fish ( $P=0.064$ ). For the medial radials, both exercise and terrestrial groups were more ossified than the control group ( $P<0.023$ ; Fig. 3I, Table 1).

### Curvature

There were no differences in curvature ratio of the medial radials (control:  $0.0456\pm 0.0287$ , exercise:  $0.0438\pm 0.0296$ , terrestrial:





**Fig. 3. Univariate measurements of *P. senegalus* bone size and ossification.** (A–C) Bone length, (D–F) bone width and (G–I) proportion of bone ossified for propterygium (left), metapterygium (middle) and medial radials (right). Boxes indicate means and 95% confidence intervals. Approximate confidence intervals for fixed effects were calculated for the medial radials using the *intervals* function in the *nlme* package for R (<https://CRAN.R-project.org/package=nlme>). *P*-values are indicated by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). All data points for each group are plotted (control  $n = 13$ , exercise  $n = 6$ , terrestrial  $n = 6$ ).

$0.0455 \pm 0.053$ ; means  $\pm$  s.d.) nor in the multivariate shape of the metapterygium (Table 2). There was a significant difference in multivariate shape of the propterygium between terrestrial fish and controls (Table 2). In terrestrial fish, the typical ‘S’ shape of the propterygium was more pronounced compared with that of control fish, and the point of maximum curvature shifted distally compared with that of the average control propterygium (Fig. 4).

## DISCUSSION

### Fin bones respond to static loading

Plastic responses were apparent for all bones and measured variables under the chronic terrestrial condition, in contrast with a limited effect of terrestrial exercise, which only elicited a response in the medial radials. This suggests that static and dynamic loading had different effects on the fin skeleton, with static loading due to chronic gravitational loading causing a greater response than

**Table 1. Total bone length, bone width and ossification for pectoral fin bones of *Polypterus senegalus* following a 5 week treatment in control, exercise or terrestrial conditions**

	Length (mm)			Width (mm)			Ossification (proportion)		
	Control	Exercise	Terrestrial	Control	Exercise	Terrestrial	Control	Exercise	Terrestrial
Propterygium	2.10±0.194	2.13±0.121	2.36±0.164	0.215±0.022	0.214±0.024	0.251±0.021	0.739±0.026	0.744±0.040	0.759±0.013
Metapterygium	3.87±0.243	3.86±0.244	4.19±0.235	0.229±0.022	0.239±0.017	0.259±0.013	0.823±0.023	0.841±0.011	0.851±0.006
Medial radials	1.51±0.337	1.64±0.366	1.68±0.380	0.132±0.023	0.130±0.019	0.128±0.018	0.655±0.066	0.689±0.075	0.712±0.068

Data are means±s.d.

dynamic loading due to terrestrial locomotion. Intriguingly, this contradicts classic experiments in avian and mammalian long bones, which found that bones only respond to dynamic loading (Lanyon and Rubin, 1984; Robling et al., 2001).

One possible explanation for this discrepancy is the structural distinctiveness of fish bone relative to typical tetrapod bone. The cellular mechanism of mechanosensation in tetrapod bone relies on the generation of fluid flow through a system of canals, called the canicular network, in response to bone deformation (Burger et al., 1995; Bonewald and Johnson, 2008; Bonucci, 2009). The pressure from this fluid flow on osteocytes leads to a downstream signalling cascade resulting in changes in the number and activity of osteoblasts and osteoclasts, the cell types responsible for depositing and resorbing bone, respectively (Hall, 2005). The magnitude of bone functional response is determined by the rate of fluid flow, which in turn is proportional to the strain rate and magnitude (Turner et al., 1994, 1995; Mosley and Lanyon, 1998; Aiello et al., 2015). In this way, it has been proposed that only dynamic loading can generate the cyclic loading and relaxation required for fluid flow within bones, and thus alter the rate of bone deposition (Burr et al., 2002).

However, this may not be the case in fish. Although we know that fish bone is capable of responding to mechanical loads to a similar degree to bones of mammals and other tetrapods, it remains unclear whether this mechanism involves osteocytes. In contrast to tetrapods, the bones of many teleost species lack osteocytes altogether (Shahar and Dean, 2013), and even the cellular bones of non-teleost fish, such as *Polypterus*, have reduced numbers of osteocytes with few canaliculi of limited connectivity (Totland et al., 2011; Dean and Shahar, 2012). In the absence of the typical tetrapod mechanotransduction system, the unique capacity of dynamic loads to induce bone plasticity in tetrapods may not apply to fish. Rather, alternative mechanosensors such as chondrocytes, osteoblasts or bone lining cells could contribute to unique responses to loading in fish bones (Hall and Witten, 2018).

**Table 2. Pairwise comparisons of multivariate propterygium and metapterygium shape in *P. senegalus* following a 5 week treatment in control, exercise or terrestrial conditions**

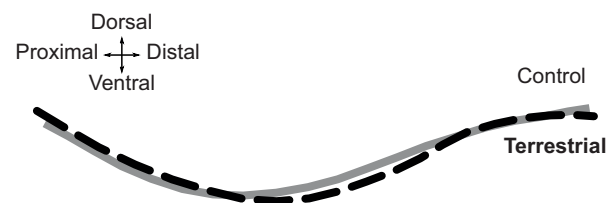
	Distance between means	Effect size	<i>P</i>
Propterygium			
Control versus exercise	0.022	-0.298	0.522
Control versus terrestrial	0.060	2.618	0.013
Metapterygium			
Control versus exercise	0.016	-0.952	0.821
Control versus terrestrial	0.048	0.905	0.180

Distance between least squares means for each group across permutations is shown.

Effect size was calculated as standard deviates of the sums of squares.

Given the substantial differences in experimental design between the work presented here and the classic experiments of Lanyon and Rubin (1984) and Robling et al. (2001), which directly loaded isolated bones, we should also compare our findings with studies on chronic hypergravity. In these experiments, study organisms are typically placed inside a centrifuge to expose them to various levels of *g*-force, comparable to our terrestrial group of *P. senegalus*. In general, the results of hypergravity experiments have been mixed, showing support for both a suppressing effect of constant centrifugation on bone growth and potential osteogenic effects, depending on the magnitude of centrifugation and type of bone in question (Martinez et al., 1998; Vico et al., 1999; Aceto et al., 2015; Canciani et al., 2015; Gnyubkin et al., 2015). Direct comparisons of static versus dynamic hypergravity are rare, presumably because of difficulties in subjecting animals to dynamic centrifugation for extended periods of time. However, a few experiments in isolated fish tissue cultures have shown positive osteoblastic responses to both types of loading but variable effects on osteoclastic function, including an enhanced ability of dynamic hypergravity to suppress osteoclast activity (Suzuki et al., 2008; Kitamura et al., 2013; Yano et al., 2013).

Alternatively, one possible explanation for our relative lack of response to exercise is that the regimen we used was not sufficiently intense to stimulate bone growth. In this experiment, fish were motivated to walk 30 cm per day, which is equivalent to 5–10 contralateral fin stroke cycles. To place this in context, several mammalian studies have found observable changes in bone structural parameters after only a few (1–10) repetitions of high-impact activities (Forwood et al., 1996; Umemura et al., 1997; Honda et al., 2001). In planning this experiment, we assumed that, given the rapid onset of fatigue in these fish (T.Y.D. and E.M.S., personal observation), that any form of terrestrial locomotion could be considered high impact. However, comparisons of walking in salamanders and mudskippers show similar ground reaction forces (Kawano and Blob, 2013), suggesting that terrestrial locomotion in amphibious fishes may be more like walking than jumping in relative intensity. Repeating the experiment with longer or more frequent bouts of exercise could elicit a greater response.

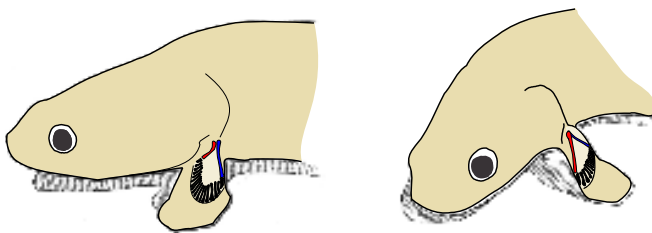
**Fig. 4. Average difference in propterygium shape between control and terrestrial *P. senegalus*. Control, grey solid line; terrestrial, black dashed line. The difference is magnified 2× to facilitate visualization.**

### Increased loading results in longitudinal growth of fin bones

Another noticeable difference between our findings and most mammalian studies is that terrestrially acclimated *P. senegalus* exhibited increased longitudinal bone growth. Most mammalian studies have recorded non-significant (Canciani et al., 2015; Gnyubkin et al., 2015) or negative (Amtmann and Oyama, 1973; Smith, 1975; Wunder et al., 1979; Martinez et al., 1998; Vico et al., 1999) effects of artificial hypergravity on bone growth, as well as negative effects of direct static compression (Robling et al., 2001; Stokes et al., 2007; Ohashi et al., 2002). This difference could be related to how fish fin bones are loaded in nature compared with tetrapod long bones. Axially applied compressive loading of mammalian long bones has been shown to inhibit elongation, potentially due to alterations of the growth plate (Robling et al., 2001; Stokes et al., 2007; Ohashi et al., 2009). However, this scenario is not directly applicable to *Polypterus* or other fishes as fin bones are virtually never loaded in pure axial compression either in the water or on land, which likely reduces the effects on the growth plate. Other potential differences between fish and mammalian bones that could influence the impact of loading on longitudinal growth are that fish bones have indeterminate growth, a more variable arrangement of chondrocytes in the growth plate (Farnum, 2007) and a greater reliance on periosteal ossification over endochondral ossification compared with mammals (Witten and Huysseune, 2007).

### Medial radials exhibit unique responses to increased loading

The only bones that responded to the exercise treatment were the medial radials, which increased in length and ossification. This localized response may be due to the position of the pectoral fins while contacting the ground during terrestrial locomotion. Previous examinations of high-speed video (Standen et al., 2016) have shown that, on smooth surfaces, *P. senegalus* uses both its body and pectoral fins to lift its head off the ground and move forward. This involves abduction of the pectoral fin until it is perpendicular to the body wall. The fin is then planted on the ground and used as a pivot point to rotate and support the body. During this fin-plant phase, the fin rays and the distal region of the fin lobe containing the medial radials are in direct contact with the ground (Fig. 5). As seen in mudskippers (Kawano and Blob, 2013), this likely induces strong medial ground reaction forces on the fin, resulting in high bending, shear and torsional stresses. The stronger response of the medial radials to exercise could be due to their more direct exposure to ground reaction forces compared with the propterygium and



**Fig. 5.** Illustration of the position of the pectoral fin bones of *P. senegalus* during the fin-plant phase of terrestrial locomotion. The left image shows the initiation of the fin-plant followed by the forward pivoting movement of the head and body over the fin in the right image. Images are adapted from Standen et al. (2016) and based on high-speed videos. The depicted images take place approximately halfway through one fin stroke cycle, where a cycle is defined as starting when the nose is at its maximum amplitude towards the right. However, the timing and duration vary between fish (see Standen et al., 2016). The propterygium is red, the metapterygium is blue, and the medial radials are black.

metapterygium, which may be buffered from stress by the medial radials and adjacent connective tissues.

Despite strong responses in terms of length and proportion of ossification, medial radial width did not respond to any treatment of increased loading. We hypothesize that this may be related to variability in the number of medial radials across individuals. As for many other actinopterygians, radial bones in *Polypterus* fins originate as subdivisions of a single cartilaginous disc during development (Bartsch et al., 1997; Dewit et al., 2011). The number of medial radials that are formed can vary between individuals, and in our study ranged from 13 to 16. We found that medial radial width was significantly correlated with the number of radials per fin, where fins with fewer radials also had wider radials (Pearson's  $r = -0.17$ ,  $P = 0.003$ ). To examine the relative importance of the total number of medial radials in determining their mean width, we compared several models of medial radial width using the second-order Akaike information criterion (AICc, AIC corrected for small sample sizes), computed using the *AICcmodavg* package for R (<https://cran.r-project.org/package=AICcmodavg>). We found that a model with no fixed treatment effect but with individual and radial number as random effects has the lowest AICc value, indicating that it is the best-supported model (Table 3). This suggests that medial radial width is predominantly determined by the number of radials present in the fin, which may make it difficult to detect any effects of loading on width.

### Mechanical consequences

Based on our findings, different hypotheses emerge regarding the mechanical and functional consequences of the observed changes in the fin. The increased ossification of the metapterygium and medial radials, as well as the increased bone width of the propterygium and metapterygium, may contribute to increased stiffness. This would be consistent with a hypothesis that fin bones exhibit adaptive plasticity in response to gravitational loading. A stiffening effect of terrestrial acclimation has also been demonstrated in the gill arches of the amphibious mangrove rivulus (although, interestingly, not in *Polypterus*) (Turko et al., 2017, 2019). Conversely, the increased length of bones potentially reduces their strength in bending. Such a reduction in bending strength would appear to be maladaptive for resisting the increased ground reaction forces on land.

Changes in the observed shape of the propterygium, captured through the use of geometric morphometrics and sliding semi-landmarks, are also intriguing. In general, long bone curvature is considered a functional paradox (Bertram and Biewener, 1988) because curvature reduces strength in bending (Biewener, 1983). However, curvature could lead to potential tradeoffs with more predictable patterns of stress (Bertram and Biewener, 1988; Jade et al., 2014) or increased mechanical dampening which dissipates energy from loading (Dodge et al., 2012). The particularities of the shape change in the terrestrial group of *P. senegalus*, namely the distal shift of the point of greatest curvature and the accentuation of its 'S' shape, may also have consequences for the lines of action of

**Table 3.** AICc results for models of medial radial width in *P. senegalus*

Candidate model	<i>k</i>	AICc	ΔAICc	<i>w</i>	log(L)
Width ~ 1+ (FishID/#Radials)	4	-1454.44	0	0.56	731.29
Width ~ Treatment + (1 FishID)	5	-1453.10	1.34	0.30	731.65
Width ~ Treatment + (1 ID/#Radials)	6	-1451.01	3.43	0.11	731.65

AICc, Akaike information criterion (corrected); ΔAICc, difference between the AICc of a given model and the AICc of the model with the lowest AICc; *w*, Akaike weight; log(L), log-likelihood; *k*, estimated parameters.

the abduction and rotational muscles that insert on its dorsal and lateral surfaces (Wilhelm et al., 2015).

### Future directions

Whether the changes observed in the experimentally loaded groups of *P. senegalus* actually have any mechanical or functional consequences remains to be seen, and will need to be the subject of further investigation. This includes testing of material properties of the bones themselves, as well as analyses of the forces experienced by the fins during aquatic and terrestrial locomotion. Future studies would also benefit from the collection of longitudinal data in these fish. Because of the constraints of our chosen method for visualizing the fin skeleton, we only compared differences between groups at the end of the 5 week treatment period, with the assumption that there was no significant variation between groups present prior to the beginning of the experiment. Although the random assignment of fish to experimental groups mitigates this possibility, it is likely that individual fish will exhibit different growth responses to loading. Thus, there would still be considerable value in visualizing bone morphology pre- and post-treatment to gain more direct measurements of how increased loading affects fin bone growth. Radiographic imaging through traditional x-ray or *in vivo* CT scanning (Koba et al., 2013) as well as the use of markers for bone formation such as fluorochromes (Erben, 2003) are some potential approaches to visualizing bone shape and condition prior to treatment.

Future work should also consider how bone plasticity in fish works in the context of other tissues present in the fin. In general, fish exhibit a greater variety of skeletal and connective tissue types compared with mammals, including an increased role of cartilage in load-bearing elements (Witten et al., 2010). Compared with mammalian long bones, ossified tissue comprises relatively little of the fin radials. The majority of the radials are composed of an inner rod of cartilage that is only thinly sheathed by periosteal bone around the bone shaft (Witten and Huysseune, 2007). The contribution of this internal cartilage to the mechanical properties of the structure as a whole is unknown. How a diverse complement of tissues interacts in response to load, especially in a structure with as complex a function and arrangement of skeletal elements as the pectoral fin leaves many questions for the future and highlights the need for more studies of skeletal responses to mechanical loading in fish.

### Conclusions

The ability of skeletal systems to respond to changing mechanical loads throughout life is clearly advantageous for individual organisms, but there is also increasing evidence that such phenotypic plasticity may play an important evolutionary role as well (West-Eberhard, 2003; Wund et al., 2008; Moczek et al., 2011). One of the reasons why we are interested in studying skeletal plasticity in *Polypterus* is to gain insight into how the ancestral fish body plan responded to the challenges experienced by the first vertebrates to venture onto land. In this study, we sought to determine whether the pectoral fin bones of *P. senegalus* are capable of exhibiting a plastic response to weight bearing that may facilitate a terrestrial lifestyle. Acclimation to chronic terrestrial loading results in increases in the length, width and ossification of fin radial bones. This also occurs, to a lesser extent, following short bouts of daily exercise on land. Whether this type of plasticity is relevant on a functional or evolutionary scale remains unanswered. However, it is clear from this and other studies that fish skeletons have an advanced capacity to sense and respond to novel mechanical loading as drastic as the transition from a buoyant aquatic environment to a weight-bearing terrestrial one.

### Acknowledgements

We would like to thank Kimberly Reid for her contributions to the experimental design, animal care and data collection. We would also like to thank Keegan Lutek, Christine Archer and the staff at the University of Ottawa Aquatic Core Facility for help with animal care, as well as Odette Laneuville for lab space and materials.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: T.Y.D., E.M.S.; Methodology: T.Y.D., E.M.S.; Software: T.Y.D.; Validation: T.Y.D.; Formal analysis: T.Y.D.; Investigation: T.Y.D.; Resources: E.M.S.; Data curation: T.Y.D.; Writing - original draft: T.Y.D., E.M.S.; Writing - review & editing: T.Y.D., E.M.S.; Visualization: T.Y.D.; Supervision: E.M.S.; Project administration: E.M.S.; Funding acquisition: T.Y.D., E.M.S.

### Funding

This work was funded by the Natural Sciences and Engineering Research Council of Canada [RGPIN 04324 to E.M.S.] and the Fonds de Recherche du Québec-Nature et Technologies [Bourses de recherche postdoctorale to T.Y.D.].

### Data availability

Bone size and shape measurements are available from the Dryad digital repository (Du and Standen, 2020): [dryad.0vt4b8gwc](https://doi.org/10.5061/dryad.0vt4b8gwc)

### References

- Aceto, J., Nourizadeh-Lillabadi, R., Marée, R., Dardenne, N., Jeanray, N., Wehenkel, L., Aleström, P., van Loon, J. J. W. A. and Muller, M. (2015). Zebrafish bone and general physiology are differently affected by hormones or changes in gravity. *PLoS ONE* **10**, e0126928. doi:10.1371/journal.pone.0126928
- Aiello, B. R., Iriarte-Diaz, J., Blob, R. W., Butcher, M. T., Carrano, M. T., Espinoza, N. R., Main, R. P. and Ross, C. F. (2015). Bone strain magnitude is correlated with bone strain rate in tetrapods: implications for models of mechanotransduction. *Proc. R. Soc. B* **282**, 20150321. doi:10.1098/rspb.2015.0321
- Albertson, R. C., Strelman, J. T. and Kocher, T. D. (2003). Genetic basis of adaptive shape differences in the cichlid head. *J. Hered.* **94**, 291-301. doi:10.1093/jhered/esg071
- Antmann, E. and Oyama, J. (1973). Changes in functional construction of bone in rats under conditions of simulated increased gravity. *Z. Anat. Entwicklungs.* **139**, 307-318. doi:10.1007/BF00519970
- Apschner, A., Schulte-Merker, S. and Witten, P. E. (2011). Not all bones are created equal—using zebrafish and other teleost species in osteogenesis research. In *Methods in Cell Biology* (ed. H. W. Detrich, III, M. Westerfield and L. I. Zon), pp. 239-255. Elsevier.
- Bartsch, P., Gemballa, S. and Piotrowski, T. (1997). The embryonic and larval development of *Polypterus senegalus* Cuvier, 1829: its staging with reference to external and skeletal features, behaviour and locomotory habits. *Acta Zool. Stockholm.* **78**, 309-328. doi:10.1111/j.1463-6395.1997.tb01014.x
- Bertram, J. E. A. and Biewener, A. A. (1988). Bone curvature: sacrificing strength for load predictability? *J. Theor. Biol.* **131**, 75-92. doi:10.1016/S0022-5193(88)80122-X
- Biewener, A. A. (1983). Allometry of quadrupedal locomotion: the scaling of duty factor, bone curvature and limb orientation to body size. *J. Exp. Biol.* **105**, 147-171.
- Biewener, A. A. and Bertram, J. E. A. (1994). Structural response of growing bone to exercise and disuse. *J. Appl. Physiol.* **76**, 946-955. doi:10.1152/jappl.1994.76.2.946
- Bonewald, L. F. and Johnson, M. L. (2008). Osteocytes, mechanosensing and Wnt signaling. *Bone* **42**, 606-615. doi:10.1016/j.bone.2007.12.224
- Bonucci, E. (2009). The osteocyte: the underestimated conductor of the bone orchestra. *Rend. Lincei* **20**, 237-254. doi:10.1007/s12210-009-0051-y
- Bookstein, F. L. (1997). Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. *Med. Image Anal.* **1**, 225-243. doi:10.1016/S1361-8415(97)85012-8
- Burger, E. H., Klein-Nulend, J., Van Der Plas, A. and Nijweide, P. J. (1995). Function of osteocytes in bone—their role in mechanotransduction. *J. Nutr.* **125**, 2020S. doi:10.1093/jn/125.suppl\_7.2020S
- Burr, D. B., Robling, A. G. and Turner, C. H. (2002). Effects of biomechanical stress on bones in animals. *Bone* **30**, 781-786. doi:10.1016/S8756-3282(02)00707-X
- Canciani, B., Ruggieri, A., Giuliani, A., Panetta, D., Marozzi, K., Tripodi, M., Salvadori, P. A., Cilli, M., Ohira, Y., Cancedda, R. et al. (2015). Effects of long time exposure to simulated micro- and hypergravity on skeletal architecture. *J. Mech. Behav. Biomed.* **51**, 1-12. doi:10.1016/j.jmbbm.2015.06.014
- Cardeira, J., Mendes, A. C., Pousão-Ferreira, P., Cancela, M. L., Gavaia, P. J. (2015). Micro-anatomical characterization of vertebral curvatures in Senegalese sole *Solea senegalensis*: vertebral curvatures in *solea senegalensis*. *J. Fish Biol.* **86**, 1796-1810. doi:10.1111/jfb.12686



- Carmeliet, G. and Bouillon, R.** (2001). Space flight: a challenge for normal bone homeostasis. *Crit. Rev. Eukar. Gene* **11**, 1-3. doi:10.1615/CritRevEukarGeneExpr.v11.i1-3.70
- Carter, D. R., Van der Meulen, M. C. H. and Beaupré, G. S.** (1996). Mechanical factors in bone growth and development. *Bone* **18**, 5S-10S. doi:10.1016/8756-3282(95)00373-8
- Chamay, A. and Tschantz, P.** (1972). Mechanical influences in bone remodeling. Experimental research on Wolff's law. *J. Biomech.* **5**, 173-180. doi:10.1016/0021-9290(72)90053-X
- Clack, J. A.** (2012). *Gaining Ground: The Origin and Evolution of Tetrapods*. Bloomington, US: Indiana University Press.
- Collyer, M. L. and Adams, D. C.** (2018). RRPP: an R package for fitting linear models to high-dimensional data using residual randomization. *Methods Ecol. Evol.* **9**, 1772-1779. doi:10.1111/2041-210X.13029
- Cuervo, R., Hernandez-Martinez, R., Chimal-Monroy, J., Merchant-Larios, H. and Covarrubias, L.** (2012). Full regeneration of the tribasal *Polypterus* fin. *Proc. Natl. Acad. Sci. USA* **109**, 3838-3843. doi:10.1073/pnas.1006619109
- Davis, M. C., Shubin, N. H. and Force, A.** (2004). Pectoral fin and girdle development in the basal actinopterygians *Polyodon spathula* and *Acipenser transmontanus*. *J. Morphol.* **262**, 608-628. doi:10.1002/jmor.10264
- Dean, M. N. and Shahar, R.** (2012). The structure-mechanics relationship and the response to load of the acellular bone of neoteleost fish: a review. *J. Appl. Ichthyol.* **28**, 320-329. doi:10.1111/j.1439-0426.2012.01991.x
- Deschamps, M.-H., Labbé, L., Baloché, S., Fouchereau-Péron, M., Dufour, S. and Sire, J.-Y.** (2009). Sustained exercise improves vertebral histomorphometry and modulates hormonal levels in rainbow trout. *Aquaculture* **296**, 337-346. doi:10.1016/j.aquaculture.2009.07.016
- Dewit, J., Witten, P. E. and Huysseune, A.** (2011). The mechanism of cartilage subdivision in the reorganization of the zebrafish pectoral fin endoskeleton. *J. Exp. Zool. Part B* **316**, 584-597. doi:10.1002/jez.b.21433
- Dodge, T., Wanis, M., Ayoub, R., Zhao, L., Watts, N. B., Bhattacharya, A., Akkus, O., Robling, A. and And Yokota, H.** (2012). Mechanical loading, damping, and load-driven bone formation in mouse tibiae. *Bone* **51**, 810-818. doi:10.1016/j.bone.2012.07.021
- Du, T. Y. and Standen, E. M.** (2017). Phenotypic plasticity of muscle fiber type in the pectoral fins of *Polypterus senegalus* reared in a terrestrial environment. *J. Exp. Biol.* **220**, 3406-3410. doi:10.1242/jeb.162909
- Du, T. Y. and Standen, E. M.** (2020). Terrestrial acclimation and exercise lead to bone functional response in *Polypterus* pectoral fins. Dryad, Dataset. doi:10.5061/dryad.0vt4b8gwc
- Erben, R. G.** (2003). Bone-labeling techniques. In *Handbook of Histology Methods for Bone and Cartilage* (ed. Y. H. An and K. L. Martin), pp. 99-117. Totowa, NJ: Humana Press.
- Farnum, C. E.** (2007). Postnatal growth of fins and limbs through endochondral ossification. In *Fins into Limbs: Evolution, Development, and Transformation* (ed. B. K. Hall), pp. 118-151. Chicago, US: University of Chicago Press.
- Fiaz, A. W., Léon-Kloosterziel, K. M., Gort, G., Schulte-Merker, S., van Leeuwen, J. L. and Kranenborg, S.** (2012). Swim-training changes the spatio-temporal dynamics of skeletogenesis in zebrafish larvae (*Danio rerio*). *PLoS ONE* **7**, e34072. doi:10.1371/journal.pone.0034072
- Forwood, M. R., Owan, I., Takano, Y. and Turner, C. H.** (1996). Increased bone formation in rat tibiae after a single short period of dynamic loading in vivo. *Am. J. Physiol. Endoc. M.* **270**, E419-E423. doi:10.1152/ajpendo.1996.270.3.E419
- Gnyubkin, V., Guignandon, A., Laroche, N., Vanden-Bossche, A., Normand, M., Lafage-Proust, M.-H. and Vico, L.** (2015). Effects of chronic hypergravity: from adaptive to deleterious responses in growing mouse skeleton. *J. Appl. Physiol.* **119**, 908-917. doi:10.1152/jappphysiol.00364.2015
- Goodall, C.** (1991). Procrustes methods in the statistical analysis of shape. *J. R. Stat. Soc. B. Met.* **53**, 285-339. doi:10.1111/j.2517-6161.1991.tb01825.x
- Gross, T. S., Edwards, J. L., McLeod, K. J. and And Rubin, C. T.** (1997). Strain gradients correlate with sites of periosteal bone formation. *J. Bone Miner. Res.* **12**, 982-988. doi:10.1359/jbmr.1997.12.6.982
- Grünbaum, T., Cloutier, R. and And Vincent, B.** (2012). Dynamic skeletogenesis in fishes: insight of exercise training on developmental plasticity. *Dev. Dyn.* **241**, 1507-1524. doi:10.1002/dvdy.23837
- Gunter, H. M. and Meyer, A.** (2014). Molecular investigation of mechanical strain-induced phenotypic plasticity in the ecologically important pharyngeal jaws of cichlid fish. *J. Appl. Ichthyol.* **30**, 630-635. doi:10.1111/jai.12521
- Gunter, H. M., Fan, S., Xiong, F., Franchini, P., Fruciano, C. and Meyer, A.** (2013). Shaping development through mechanical strain: the transcriptional basis of diet-induced phenotypic plasticity in a cichlid fish. *Mol. Ecol.* **22**, 4516-4531. doi:10.1111/mec.12417
- Hall, B. K.** (2005). *Bones and Cartilage: Developmental and Evolutionary Skeletal Biology*. Amsterdam: Academic Press.
- Hall, B. K. and Herring, S. W.** (1990). Paralysis and growth of the musculoskeletal system in the embryonic chick. *J. Morphol.* **206**, 45-56. doi:10.1002/jmor.1052060105
- Hall, B. K. and Witten, P. E.** (2018). Plasticity and variation of skeletal cells and tissues and the evolutionary development of actinopterygian fishes. In *Evolution and Development of Fishes* (ed. Z. Johanson, C. Underwood and M. Richter), pp. 126-143. Cambridge, UK: Cambridge University Press.
- Hamrick, M. W., Skedros, J. G., Pennington, C. and McNeil, P. L.** (2006). Increased osteogenic response to exercise in metaphyseal versus diaphyseal cortical bone. *J. Musculoskel. Neuron.* **6**, 258-263.
- Honda, A., Umemura, Y. and Nagasawa, S.** (2001). Effect of high-impact and low-repetition training on bones in ovariectomized rats. *J. Bone Miner. Res.* **16**, 1688-1693. doi:10.1359/jbmr.2001.16.9.1688
- Hsieh, Y.-F. and Turner, C. H.** (2001). Effects of loading frequency on mechanically induced bone formation. *J. Bone Miner. Res.* **16**, 918-924. doi:10.1359/jbmr.2001.16.5.918
- Hsieh, Y.-F., Robling, A. G., Ambrosius, W. T., Burr, D. B. and Turner, C. H.** (2001). Mechanical loading of diaphyseal bone in vivo: the strain threshold for an osteogenic response varies with location. *J. Bone Miner. Res.* **16**, 2291-2297. doi:10.1359/jbmr.2001.16.12.2291
- Huysseune, A., Sire, J.-Y. and And Meunier, F. J.** (1994). Comparative study of lower pharyngeal jaw structure in two phenotypes of *Astatoreochromis alluaudi* (teleostei: Cichlidae). *J. Morphol.* **221**, 25-43. doi:10.1002/jmor.1052210103
- Iwamoto, J., Yeh, J. K. and Aloia, J. F.** (1999). Differential effect of treadmill exercise on three cancellous bone sites in the young growing rat. *Bone* **24**, 163-169. doi:10.1016/S8756-3282(98)00189-6
- Jade, S., Tamvada, K. H., Strait, D. S. and Grosse, I. R.** (2014). Finite element analysis of a femur to deconstruct the paradox of bone curvature. *J. Theor. Biol.* **341**, 53-63. doi:10.1016/j.jtbi.2013.09.012
- Judex, S., Gross, T. S. and Zernicke, R. F.** (1997). Strain gradients correlate with sites of exercise-induced bone-forming surfaces in the adult skeleton. *J. Bone Miner. Res.* **12**, 1737-1745. doi:10.1359/jbmr.1997.12.10.1737
- Kawano, S. M. and Blob, R. W.** (2013). Propulsive forces of mudskipper fins and salamander limbs during terrestrial locomotion: implications for the invasion of land. *Integr. Comp. Biol.* **53**, 283-294. doi:10.1093/icb/ict051
- Kitamura, K., Takahira, K., Inari, M., Satoh, Y., Hayakawa, K., Tabuchi, Y., Ogai, K., Nishiuchi, T., Kondo, T., Mikuni-Takagaki, Y. et al.** (2013). Zebrafish scales respond differently to in vitro dynamic and static acceleration: analysis of interaction between osteoblasts and osteoclasts. *Comp. Biochem. Phys. A* **166**, 74-80. doi:10.1016/j.cbpa.2013.04.023
- Koba, W., Jelicks, L. A. and Fine, E. J.** (2013). MicroPET/SPECT/CT imaging of small animal models of disease. *Am. J. Pathol.* **182**, 319-324. doi:10.1016/j.ajpath.2012.09.025
- Kranenborg, S., Waarsing, J. H., Muller, M., Weinans, H. and van Leeuwen, J. L.** (2005). Lordotic vertebrae in sea bass (*Dicentrarchus labrax* L.) are adapted to increased loads. *J. Biomech.* **38**, 1239-1246. doi:10.1016/j.jbiomech.2004.06.011
- Lang, T., LeBlanc, A., Evans, H., Lu, Y., Genant, H. and Yu, A.** (2004). Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. *J. Bone Miner. Res.* **19**, 1006-1012. doi:10.1359/JBMR.040307
- Lanyon, L. E. and Rubin, C. T.** (1984). Static vs dynamic loads as an influence on bone remodeling. *J. Biomech.* **17**, 897-905. doi:10.1016/0021-9290(84)90003-4
- Lanyon, L. E. and Rubin, C. T.** (1985). Functional adaptation in skeletal structures. In *Functional Vertebrate Morphology* (ed. M. Hildebrand, D. M. Bramble, K. F. Liem and D. B. Wake), pp. 1-25. Cambridge, US: Belknap Press.
- Martinez, D. A., Orth, M. W., Carr, K. E., Vanderby, R., Jr, Vasques, M., Grindeland, R. E. and Vailas, A. C.** (1998). Cortical bone responses to 2g hypergravity in growing rats. *Aviat. Space Envir. Med.* **69**, A17-A22.
- Metscher, B. D., Takahashi, K., Crow, K., Amemiya, C., Nonaka, D. F. and Wagner, G. P.** (2005). Expression of Hoxa-11 and Hoxa-13 in the pectoral fin of a basal ray-finned fish, *Polyodon spathula*: implications for the origin of tetrapod limbs. *Evol. Dev.* **7**, 186-195. doi:10.1111/j.1525-142X.2005.05021.x
- Meyer, A.** (1987). Phenotypic plasticity and heterochrony in *Cichlasoma managuense* (Pisces, Cichlidae) and their implications for speciation in cichlid fishes. *Evolution* **41**, 1357-1369. doi:10.1111/j.1558-5646.1987.tb02473.x
- Moczek, A. P., Sultan, S., Foster, S., Ledon-Rettig, C., Dworkin, I., Nijhout, H. F., Abouheif, E. and Pfennig, D. W.** (2011). The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B Biol. Sci.* **278**, 2705-2713. doi:10.1098/rspb.2011.0971
- Mosley, J. R. and Lanyon, L. E.** (1998). Strain rate as a controlling influence on adaptive modeling in response to dynamic loading of the ulna in growing male rats. *Bone* **23**, 313-318. doi:10.1016/S8756-3282(98)00113-6
- Nowlan, N. C., Bourdon, C., Dumas, G., Tajbakhsh, S., Prendergast, P. J. and Murphy, P.** (2010). Developing bones are differentially affected by compromised skeletal muscle formation. *Bone* **46**, 1275-1285. doi:10.1016/j.bone.2009.11.026
- Ohashi, N., Robling, A. G., Burr, D. B. and Turner, C. H.** (2002). The Effects of dynamic axial loading on the rat growth plate. *J. Bone Miner. Res.* **17**, 284-292. doi:10.1359/jbmr.2002.17.2.284
- Plochocki, J. H., Rivera, J. P., Zhang, C. and Ebba, S. A.** (2008). Bone modeling response to voluntary exercise in the hindlimb of mice. *J. Morphol.* **269**, 313-318. doi:10.1002/jmor.10587
- R Core Team** (2019). *R: a Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Robling, A. G., Duijvelaar, K. M., Geevers, J. V., Ohashi, N. and Turner, C. H.** (2001). Modulation of appositional and longitudinal bone growth in the rat ulna by



- applied static and dynamic force. *Bone* **29**, 105-113. doi:10.1016/S8756-3282(01)00488-4
- Ruff, C., Holt, B. and Trinkaus, E.** (2006). Who's afraid of the big bad Wolff?: "Wolff's law" and bone functional adaptation. *Am. J. Phys. Anthropol.* **129**, 484-498. doi:10.1002/ajpa.20371
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al.** (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676-682. doi:10.1038/nmeth.2019
- Shahar, R. and Dean, M. N.** (2013). The enigmas of bone without osteocytes. *Bonekey Rep.* **2**, 343. doi:10.1038/bonekey.2013.77
- Skerry, T. M.** (2006). One mechanostat or many? Modifications of the site-specific response of bone to mechanical loading by nature and nurture. *J. Musculoskel. Neuron.* **6**, 122-127.
- Smith, S. D.** (1975). Effects of long-term rotation and hypergravity on developing rat femurs. *Aviat. Space Envir. Md.* **46**, 248-253.
- Standen, E. M., Du, T. Y. and Larsson, H. C. E.** (2014). Developmental plasticity and the origin of tetrapods. *Nature* **513**, 54-58. doi:10.1038/nature13708
- Standen, E. M., Du, T. Y., Laroche, P. and Larsson, H. C. E.** (2016). Locomotor flexibility of *Polypterus senegalus* across various aquatic and terrestrial substrates. *Zoology* **119**, 447-454. doi:10.1016/j.zool.2016.05.001
- Stokes, I. A. F., Clark, K. C., Farnum, C. E. and Aronsson, D. D.** (2007). Alterations in the growth plate associated with growth modulation by sustained compression or distraction. *Bone* **41**, 197-205. doi:10.1016/j.bone.2007.04.180
- Suzuki, N., Omori, K., Nakamura, M., Tabata, M. J., Ikegame, M., Ijiri, K., Kitamura, K.-I., Nemoto, T., Shimizu, N., Kondo, T. et al.** (2008). Scale osteoblasts and osteoclasts sensitively respond to low-gravity loading by centrifuge. *Biological Sciences in Space* **22**, 3-7. doi:10.2187/bss.22.3
- Totland, G. K., Fjellidal, P. G., Kryvi, H., Løkka, G., Wargelius, A., Sagstad, A., Hansen, T. and Grotmol, S.** (2011). Sustained swimming increases the mineral content and osteocyte density of salmon vertebral bone. *J. Anat.* **219**, 490-501. doi:10.1111/j.1469-7580.2011.01399.x
- Turko, A. J., Kültz, D., Fudge, D., Croll, R. P., Smith, F. M., Stoyek, M. R. and Wright, P. A.** (2017). Skeletal stiffening in an amphibious fish out of water is a response to increased body weight. *J. Exp. Biol.* **220**, 3621-3631. doi:10.1242/jeb.161638
- Turko, A. J., Maini, P., Wright, P. A. and Standen, E. M.** (2019). Gill remodeling during terrestrial acclimation in the amphibious fish *Polypterus senegalus*. *J. Morphol.* **280**, 329-338. doi:10.1002/jmor.20946
- Turner, C. H.** (1998). Three rules for bone adaptation to mechanical stimuli. *Bone* **23**, 399-407. doi:10.1016/S8756-3282(98)00118-5
- Turner, C. H., Forwood, M. R. and Otter, M. W.** (1994). Mechanotransduction in bone: do bone cells act as sensors of fluid flow? *FASEB J.* **8**, 875-878. doi:10.1096/fasebj.8.11.8070637
- Turner, C. H., Owan, I. and Takano, Y.** (1995). Mechanotransduction in bone: role of strain rate. *Am. J. Physiol. Endoc. M.* **269**, E438-E442. doi:10.1152/ajpendo.1995.269.3.E438
- Umemura, Y., Ishiko, T., Yamauchi, T., Kurono, M. and Mashiko, S.** (1997). Five jumps per day increase bone mass and breaking force in rats. *J. Bone Miner. Res.* **12**, 1480-1485. doi:10.1359/jbmr.1997.12.9.1480
- Vico, L., Barou, O., Laroche, N., Alexandre, C. and Lafage-Proust, M.-H.** (1999). Effects of centrifuging at 2g on rat long bone metaphyses. *Eur. J. Appl. Physiol. O.* **80**, 360-366. doi:10.1007/s004210050604
- Warden, S. J. and Turner, C. H.** (2004). Mechanotransduction in the cortical bone is most efficient at loading frequencies of 5-10 Hz. *Bone* **34**, 261-270. doi:10.1016/j.bone.2003.11.011
- West-Eberhard, M. J.** (2003). *Developmental Plasticity and Evolution*. Oxford: Oxford University Press.
- Wilhelm, B. C., Du, T. Y., Standen, E. M. and Larsson, H. C. E.** (2015). *Polypterus* and the evolution of fish pectoral musculature. *J. Anat.* **226**, 511-522. doi:10.1111/joa.12302
- Witten, P. E. and Hall, B. K.** (2015). Teleost skeletal plasticity: modulation, adaptation, and remodeling. *Copeia* **103**, 727-739. doi:10.1643/CG-14-140
- Witten, P. E. and Huysseune, A.** (2007). Mechanisms of chondrogenesis and osteogenesis in fins. In *Fins into Limbs: Evolution, Development, and Transformation* (ed. B. K. Hall), pp. 79-92. Chicago: University of Chicago Press.
- Witten, P. E., Gil-Martens, L., Hall, B., Huysseune, A. and Obach, A.** (2005). Compressed vertebrae in Atlantic salmon *Salmo salar*: evidence for metaplastic chondrogenesis as a skeletogenic response late in ontogeny. *Dis. Aquat. Organ.* **64**, 237-246. doi:10.3354/dao064237
- Witten, P. E., Huysseune, A. and Hall, B. K.** (2010). A practical approach for the identification of the many cartilaginous tissues in teleost fish. *J. Appl. Ichthyol.* **26**, 257-262. doi:10.1111/j.1439-0426.2010.01416.x
- Wund, M. A., Baker, J. A., Clancy, B., Golub, J. L. and And Foster, S. A.** (2008). A test of the "Flexible Stem" model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the Threespine stickleback radiation. *Am. Nat.* **172**, 449-462. doi:10.1086/590966
- Wunder, C. C., Welch, R. C. and Cook, K. M.** (1979). Femur strength as influenced by growth, bone-length and gravity with the male rat. *J. Biomech.* **12**, 501-507. doi:10.1016/0021-9290(79)90038-1
- Yano, S., Kitamura, K., Satoh, Y., Nakano, M., Hattori, A., Sekiguchi, T., Ikegame, M., Nakashima, H., Omori, K., Hayakawa, K. et al.** (2013). Static and dynamic hypergravity responses of osteoblasts and osteoclasts in medaka scales. *Zool. Sci.* **30**, 217-223. doi:10.2108/zsj.30.217