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Baby fish working out: an epigenetic source of adaptive variation in the cichlid jaw

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Understanding the developmental processes that underlie the production of adaptive variation (i.e. the 'arrival of the fittest') is a major goal of evolutionary biology. While most evo-devo studies focus on the genetic underpinnings of adaptive phenotypic variation, factors beyond changes in nucleotide sequence can also play a major role in shaping developmental outcomes. Here, we document a vigorous but enigmatic gaping behaviour during the early development of Lake Malawi cichlid larvae. The onset of the behaviour precedes the formation of bone, and we predicted that it might influence craniofacial shape by affecting the mechanical environment in which bone develops. Consistent with this, we found that both natural variation and experimental manipulation of this behaviour induced differential skeletal development that foreshadows adaptive variation in adult trophic morphology. In fact, the magnitude of difference in skeletal morphology induced by these simple shifts in behaviour was similar to those predicted to be caused by genetic factors. Finally, we demonstrate that this mechanical-load-induced shift in skeletal development is associated with differences in *ptch1* expression, a gene previously implicated in mediating between-species differences in skeletal shape. Our results underscore the complexity of development, and the importance of epigenetic (*sensu* Waddington) mechanisms in determining adaptive phenotypic variation.

1. Introduction

Over 150 years after the publication of *On the origin of species* [1], adaptive phenotypic variation remains the primary focus of evolutionary investigations. However, a comprehensive and mechanistic understanding of how phenotypic variation arises over development remains elusive. During the early to mid-1900s, the modern synthesis provided key insights into this question by implicating genetic variation as an important source [2–4], and by the end of the twentieth century, the field of evo-devo had begun to identify the specific genes and allelic variants that contribute to adaptive phenotypic variation in the wild [5–8]. Indeed, conceptual and technological innovations have led to a recent explosion in the number of studies seeking to link genotype to phenotype, raising the question of whether we are entering (or in) a 'golden age' of evolutionary genetics [9]. An underappreciated consensus from these myriad genetic analyses is that for the majority of traits, genotype alone can only explain a fraction of the total amount of phenotypic variation in natural populations. Thus, our understanding of the 'arrival of the fittest' (e.g. [10]) remains deceptively incomplete.

In the 1940s and 1950s, Waddington [11,12] introduced the term epigenetics to describe the full range of factors, above the level of genotype, that contribute to developmental and thus phenotypic variation. The epigenetic landscape metaphor was used to conceptualize this process, whereby development was represented by a branching landscape of hills and valleys, cells were represented by rolling marbles and developmental fate was depicted by the path that each cell took as it rolled down the bifurcating landscape. In this metaphor, the overall shape of the landscape (e.g. the number and sequence of branches) is determined

by genotype, whereas the height of the hills and width of the valleys represents environmental influences on development. Wide valleys and short hills characterize a developmental system in which the environment can have a large influence on developmental outcome. Alternatively, narrow valleys with high ridges represent canalized developmental events that are more robust to environmental input. As described by Jamniczky *et al*. [13], 'rediscovering' Waddington in the context of modern evolutionary genetics holds great promise to more completely understand how both genetic and epigenetic factors combine to affect phenotypic variability and influence organismic evolvability.

In this study, we report an epigenetic (*sensu* Waddington) factor for adaptive variation in the cichlid mandible that is produced by differences in early larval behaviour, which alter the mechanical environment in which the jaw develops. Cichlid fishes are well known for their rapid evolution among vertebrates, which has produced an outstanding spectrum of variation in virtually all aspects of their biology. Many cichlid species can be easily hybridized in the laboratory, making them an excellent model to understand the genetic and developmental basis of phenotypic variation. Adaptations associated with the craniofacial skeleton have been of particular interest because they are directly related to feeding specializations to different food sources, which in turn facilitate niche partitioning and speciation [14,15]. Many efforts have been devoted to uncovering the genetic basis for craniofacial variation, yet in all of these studies, genetic factors have only been found to contribute to a relatively small percentage of the observed phenotypic variation [16–21]. These data are consistent with genetic analyses of complex traits in general, which often reveal many interacting genetic factors with small effect sizes on such phenotypes. They are also consistent with the prediction that the environment is an important source of phenotypic variation in complex traits like the craniofacial skeleton [22].

Here, we complement these studies by showing that (i) the context for craniofacial development is dynamic in cichlid larvae, and occurs within the context of a vigorous gaping behaviour whereby fish start gaping their mouths immediately after the cartilaginous lower jaw forms and just before the beginning of bone deposition; (ii) gaping frequency varies between species in a way that foreshadows differences in bone deposition, particularly around the retroarticular process (RA), a critical bone for the action of jaw opening (reviewed in [19]); (iii) surgical disruption of gaping in the fast-gaping species results in shorter RAs, similar to the slow-gaping species; (iv) when forced to gape at a higher frequency, the slow-gaping species develop longer RAs, similar to the fast-gaping species; and (v) this epigenetic shift in mandible shape involves changes in expression of *ptch1*, a regulator of RA length [19] that is sensitive to foraging environment [23]. Notably, the magnitude of phenotypic change induced via behavioural manipulations is comparable to those induced by molecular manipulations from previous studies. Further, the fold change in *ptch1* expression between experimental (i.e. fast gaping) and control (i.e. slow gaping) larvae is greater than that observed between cichlid species [19]. Our results underscore the complexity of how craniofacial shape arises over development, and offer a novel experimental framework for examining sources of phenotypic variation beyond those determined by changes in nucleotide sequence.

2. Material and methods

(a) Monitoring gaping frequency

We monitored larvae gaping behaviour in three Lake Malawi cichlid species, *Labeotropheus fuelleborni* (LF, $n = 29$), *Maylandia zebra* (MZ, $n = 32$) and *Tropheops tropheops* (TT, $n = 30$). All three species are mouth brooders, which means females incubate their eggs in their mouths. Once brooding females were identified (via enlarged buccal cavities), the embryos were extracted and staged according to Fujimura & Okada [24], and then maintained in a standard 1 l culturing flask with system water and steady air supply. To measure the gaping frequency, the larvae were transferred into a small Petri dish with approximately 10 ml system water, and then allowed a 10 min acclimation period before being placed under a Leica M165 FC microscope. Gaping frequency was measured by counting gapes of individual larva under the scope in real time with a stopwatch. Disrupted observations (e.g. larva escaped field of view) were not recorded, such that only continuous observations of more than 60 gapes were included in subsequent analyses. Most observations consisted of 80 gapes and were finished within 1 min. The exact time it took to reach 80 gapes was then used to calculate gapes per minute for each animal. There was no significant size difference of the larvae among the three species across the developmental stages examined here (from stage 17, approx. 6 days post-fertilization (dpf) to stage 23, approx. 10 dpf). We chose these stages following [19,20], in order to compare the effects of genetic and epigenetic factors (see Results and discussion).

(b) Surgery

Stage 17 (6 dpf) LF larvae from the same brood were anaesthetized with tricaine at 0.2 mg ml^{-1} according to [25], and the interopercle-mandibular ligament (IOPL) on the right side was cut with extra-fine forceps. Larvae were transferred into fresh larval fish water immediately after surgery to recover before returning to standard culturing flasks. For sham surgeries, an incision of similar size was made to the tissue just anterior to the RA, where no ligaments or skeletal elements were present. Control larvae were exposed to tricaine for approximately the same period of time, but no surgery was performed. All larvae were allowed to develop for an additional 4 days to stage 23 (10 dpf), then euthanized and stained with alizarin red and alcian blue for skeletal measurements.

(c) Manipulation of gaping frequency

MZ larvae (6 dpf) from a single brood were divided into two groups: (i) control group where individual larvae were kept in large containers with approximately 150 ml of system water; (ii) experiment group where individual larvae were kept in small containers with approximately 15 ml of system water. Water was replaced twice a day. The restriction of water/space increased the frequency of gaping, although the specific cause is unclear. The higher gaping frequency in the experiment group does not appear to be induced by hypoxia or a decline in water quality because all standard environmental variables, such as pH, temperature, conductivity, O_2, ammonia, nitrate and nitrite levels, remain in the normal range of our standard system water after 12 h incubation, and gaping frequency did not change before and after water change. In addition, aerated system water with significantly higher O_2 level (8.5–9 ppm, when compared with 6.5–7.5 ppm in standard system water) did not affect gaping frequency.

(d) Quantification of *ptch1* expression

At 10 dpf, lower jaw tissue back to and including the first two pharyngeal arches (i.e. the mandibular, hyoid arches) of MZ larvae from the gaping manipulation experiment were microdissected and preserved in TRIzol (Ambion). This represents the general domain in which *ptch1* is normally expressed in the

craniofacial region of cichlid embryos [19,20]. RNA was extracted using a standard chloroform–isopropanol protocol according to the TRIzol user guide, and then quantified using the Qubit RNA assay kit (Thermo Fisher Scientific). Normalized RNA was used as a template for reverse transcription reactions using SuperScript III (Invitrogen). Real-time PCR reactions were performed using the Taqman Universal PCR Master Mix (Applied Biosystems) and were run on a Roche Lightcycler 480. Relative expression of *ptch1* was calculated using the 2-ΔΔCT method, normalized with β-actin expression as an endogenous control. Primer-probe sets follow Roberts *et al*. [19] and Carleton *et al*. [26].

3. Results and discussion

(a) Gaping behaviour in cichlid larvae

We noted a striking, but confounding, behaviour in cichlid larvae. Specifically, they start to repeatedly open and close their mouths soon after the lower jaw forms (approx. stage 17, 6 dpf; electronic supplementary material, movie S1). This behaviour appears to be common across cichlid species at this developmental stage, but is not observed in the laboratory model zebrafish. At this stage, Meckel's cartilage is well formed, but bone deposition has only just begun around the dentary and angloarticular bones [19,27,28]. The gaping frequency is surprisingly high, often exceeding 200 times min^{-1}. We reasoned that this behaviour is unlikely to be solely due to respiration or ion-regulation needs of the larvae, because (i) the gill filaments are not yet fully developed at this early stage, which has been noted in several fish species including cichlids [29–31], (ii) the primary means of gas and ion exchange for young fish larvae is through the skin [30,32], and (iii) cichlid larvae develop an elaborate vascular system around their large yolk sac that greatly increases surface area for material exchange, and is thought to act as the primary respiratory organ through much of larval developmental [24,29]. While the evolutionary origins (and adaptive value) of this behaviour remain to be explored, we hypothesized that it may contribute to variation in early bone development, as mechanical-stress-induced bone deposition is well known in various vertebrates [33,34].

(b) Species-specific variation in gaping frequency foreshadows differences in jaw development

To test this hypothesis, we first monitored gaping behaviour in three Lake Malawi cichlid species, LF ($n = 29$), MZ ($n = 32$) and TT ($n = 30$). LF is an obligate algae scraper with a short jaw and long RA, whereas MZ is a more generalized suction feeder with a relatively long jaw and short RA (figure 1). *Tropheops* species in general, and TT in particular, exhibit lower jaw shapes that are intermediate to LF and MZ [15,19,28]. These shape differences are evident as early as 10 dpf (electronic supplementary material, figure S3, also see [19,28]), and are generally considered adaptive as they reflect different biomechanical configurations well suited for their specific feeding strategy (reviewed in [17,19]). We found that larval gaping frequency differs significantly (electronic supplementary material, table S1) among these species in a way that coincides with interspecific jaw shape variation, particularly the length of the RA (figure 1; electronic supplementary material, figure S3). Between 6 and 10 dpf, LF larvae gaped approximately 260 times min^{-1} on average, which is much higher than the frequency of MZ (approx. 180 times min^{-1}). TT gaped at intermediate frequencies over this developmental window (figure 1*d*). During each gape, the IOPL pulls directly at the ventral tip of the RA, which functions as the opening in-lever to generate jaw rotation (figure 1*c*). Higher gaping frequency should therefore produce more mechanical stimuli on the RA, which in turn could lead to greater levels of mechanical-load-induced bone formation around this element. Our observations are consistent with this rationale, as they show that the species with longer RAs gapes at a consistently higher rate than species with shorter RAs.

(c) Surgical disruption of the four-bar linkage mechanism changes jaw shape

To corroborate these developmental observations, we performed two experimental manipulations of larval gaping behaviour and assessed their effects on bone deposition. The first was a loss-of-function experiment wherein we asked whether physically breaking the mechanical linkage between the IOP and RA in the fast-gaping species (i.e. LF) was sufficient to reduce bone deposition around the RA and phenocopy RA length of the slow-gaping species (i.e. MZ). For this experiment, we surgically cut the IOPL on the right side of LF larvae at stage 17 (6 dpf), while the left side was left intact. In this way, the intact side was used to control for genotype, behaviour and general health of each fish. It also reduced the sample size required for such an invasive procedure. Fish were allowed to recover for 4 days, at which point they were assessed for differences in RA length. Our prediction was that the RA on the surgical side would receive less mechanical stress from the IOPL and thereby develop at a slower rate than the RA on the intact side. To account for general surgery effects (e.g. inflammation), we performed sham surgeries where incisions of a similar size were made to tissue immediately anterior to the RA (electronic supplementary material, figure S1). Notably, our surgical manipulation of the IOPL resulted in a breakage at the tip of the interopercle bone (IOP) (figure 2*a*, arrowhead), which is a sesamoid bone that ossifies within the IOPL. This suggests that our surgery was successful in that the incision was made at the correct location and the damage likely attenuated the amount of force transmitted from the IOPL to the RA. Moreover, and consistent with our prediction, surgical disruption of the IOPL resulted in changes in RA length (figure 2*a*,*b*), such that it was significantly shorter on the surgical side compared with the intact side (figure 2*c*, $p < 0.001$, Tukey's HSD). It is also worth noting that fish resumed normal gaping behaviour within 12 h of surgery, probably due to the action of the IOPL on the contralateral side, as well as other jaw opening mechanisms such as hyoid depression. Thus, surgery led to a measurable decrease in RA length in spite of the fact that gaping behaviour resumed. This suggests that it is the physical linkage between the IOPL and the RA that influences bone deposition, not the behaviour *per se*.

(d) Behavioural manipulation of gaping frequency changes jaw shape

Next, we performed a complementary gain-of-function experiment, where we asked whether increasing gaping frequency in the slow-gaping species (i.e. MZ) was sufficient to increase length of the RA and phenocopy the morphology of the fast-gaping species (i.e. LF). To accomplish this, we used a behavioural approach. Specifically, we manipulated the

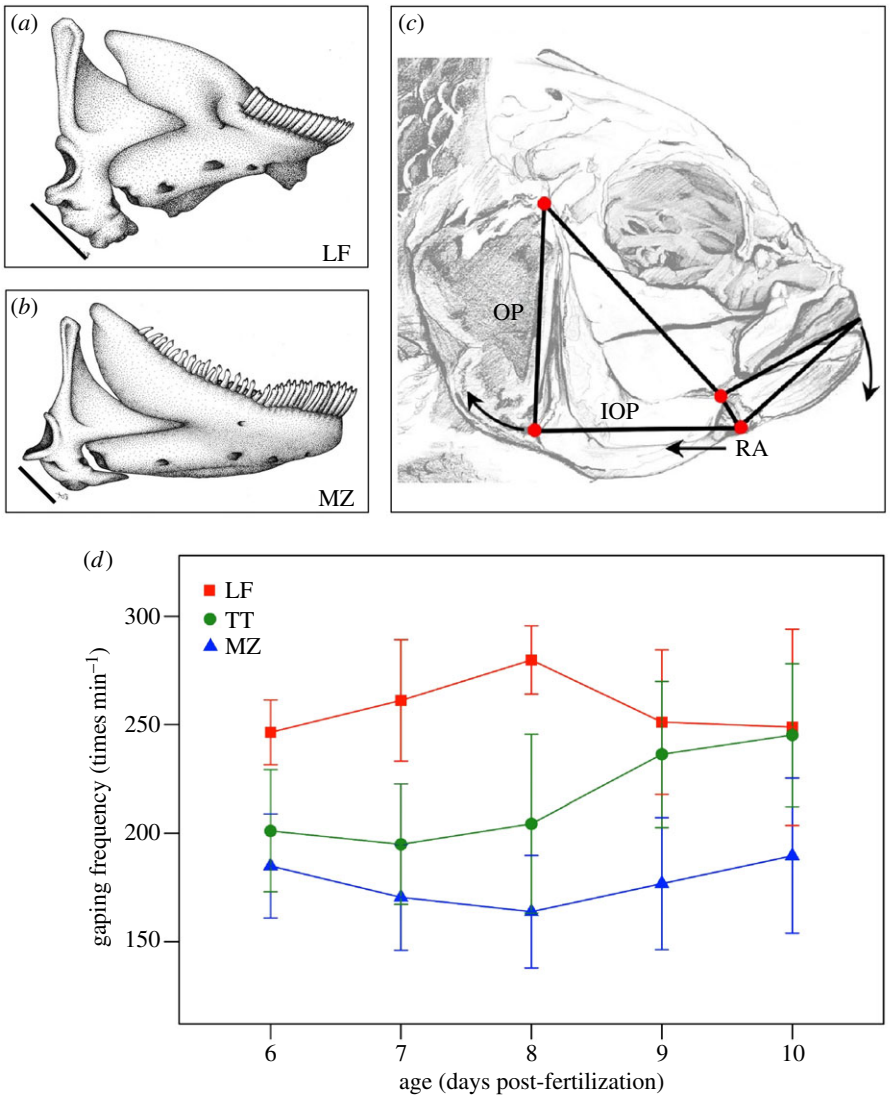

Figure 1. Variation in gaping frequency of cichlid larvae coincides with variation in adult jaw shape. (*a,b*) Illustration of the mandible in LF and MZ. Black bar indicates different RA length. No scale bar is included as these are pen and ink renderings from digital images; however, jaws were taken from equivalently sized adult fish approximately 8 cm in standard length. For quantitative comparisons of jaw morphology, see [17,19,28,35,36]. (*c*) Four-bar linkage system for lower jaw depression: the opercle (OP) represents the input link, the interopercle (IOP, including the IOPL) represents the coupler link and the RA represents the output link. The important attribute of this system to note for this paper is the functional linkage between the IOP and RA via the insertion of the IOPL (after [37]). Thus, mechanical forces generated, in part, by the dimensionality of the IOP (e.g. a deeper bone will allow for the origins of a thicker ligament) will be propagated to the RA during lower jaw depression throughout the life of the animal. (*d*) Gaping frequency of cichlid larvae during early ontogeny. Whiskers show standard deviation. See electronic supplementary material, table S1 for an additional statistical analysis of interspecific variation in gaping frequency. (Online version in colour.)

gaping frequency in MZ larvae by restricting them to a smaller container with less water (approx. 15 ml versus approx. 150 ml of embryo water). This simple change in rearing environment led to consistent and predictable shifts in gaping frequency (figure 3*a*). Specifically, animals reared in smaller volumes of water exhibited higher gaping frequency compared with larvae raised in standard conditions. This shift in behaviour was significant by 8 dpf ($p < 0.05$, two-tailed *t*-test), and when assayed for phenotype at 10 dpf, the fast-gaping animals were found to possess longer RAs compared with the control group (figure 3*b*; $p < 0.0001$, two-tailed *t*-test). In contrast to the RA, overall developmental rate remained the same between the two groups (based on standard length and the number of caudal fin ray elements, following Fujimura & Okada [24]). As more frequent gaping likely produces a higher amount of mechanical stimulus, these data are consistent with our hypothesis that variation in RA development can be induced epigenetically.

Both of our experimental manipulations of gaping behaviour resulted in measurable effects on RA development in less than 4 days. This suggests that changes in the local mechanical environment can have an immediate effect on bone development during early larval stages. Although it is unclear how much this specific behaviour contributes to variation in adult RA length, our data illustrate how developmental context (i.e. mechanical environment in which the RA develops) can be an important source of phenotypic variation, which has been noted in other vertebrates as well [38]. It is also worth noting that when extrapolating to adult stages, our data are highly predictive. For instance, our experimental manipulations produced changes in average RA length of approximately 20 µm over 4 days, or about 5 µm d^{-1}. In the laboratory, fish reach adult sizes in about 1 year. At 5 µm d^{-1}, this would predict a difference in adult RA lengths of the order of approximately 2 mm, which is similar in magnitude to differences observed among adults (i.e. a few millimetres; R.C.A. 2001–2011, unpublished data, based on samples used in [19,35,36]).

The gaping behaviour observed during larval development does not persist into juvenile stages; however, other

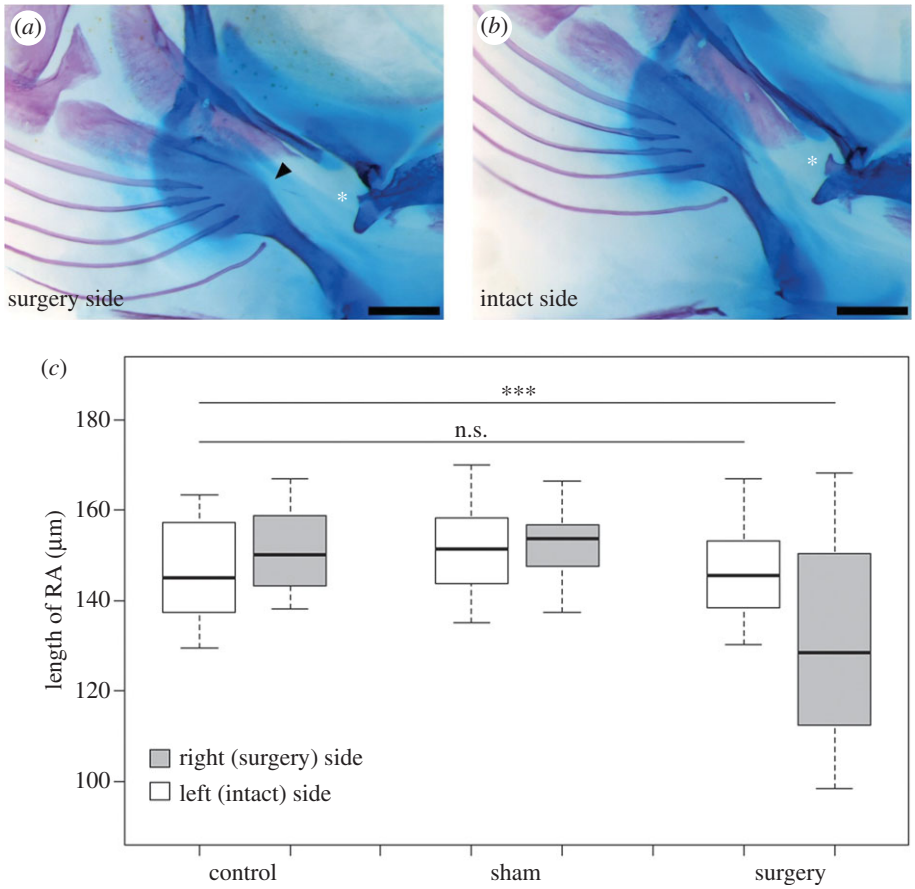

Figure 2. Results of the IOPL surgery in LF. (Top panel) Flat mount of cleared and stained pharyngeal skeletons of one representative individual (10 dpf LF) from the experiment group showing the surgery side (*a*) and intact side (*b*), note that the image on the intact side (*b*) is digitally reversed for better comparison. The black arrowhead points to a breakage in the IOP after surgery. Asterisks: RA. Scale bar, 200 µm. (*c*) Boxplot summarizing the result of the IOP surgery experiment. In the experimental group ($n = 26$), the IOPL on the right side was cut; in the sham group ($n = 23$), a cut with similar size was made in tissue just anterior to the RA on the right side; in the control group ($n = 14$), fish larvae were exposed to the same dose of anaesthetic for the same time period, but no surgery was performed. Experiments were performed in stage 17 (early 6 dpf) LF larvae. Lengths of the RA were measured at stage 23 (10 dpf). ***Statistical significance ($p < 0.05$), Tukey's HSD. For more statistical details, see electronic supplementary material, table S2 and figure S2.

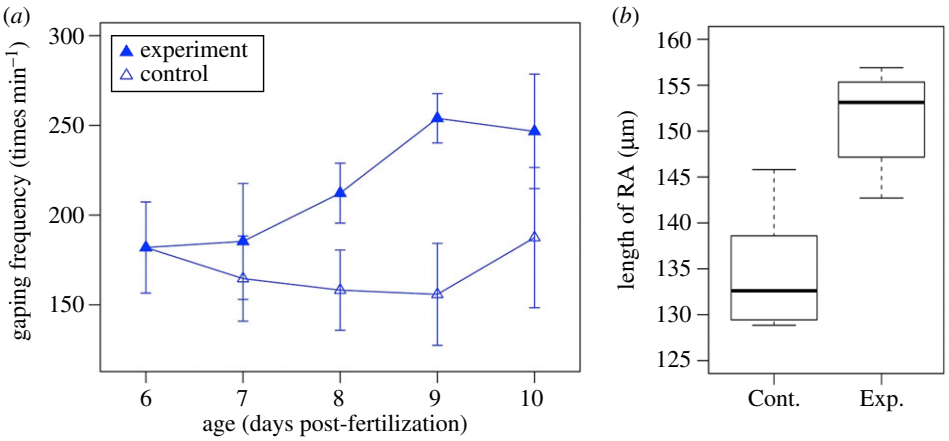

Figure 3. Manipulation of gaping frequency in MZ. (*a*) Larvae in the experimental group showed higher gaping frequency. (*b*) Larvae in the experimental group developed a longer RA (measured at 10 dpf, $p < 0.0001$, two-tailed *t*-test). Control (Cont.): MZ larvae reared individually in larger flask/beakers with approximately 150 ml of fish water ($n = 8$). Experiment (Exp.): MZ larvae kept in smaller beakers with approximately 15 ml of fish water ($n = 10$). (Online version in colour.)

behaviours that can affect the mechanical environment of the mandible begin with the onset of the juvenile period. Specifically, juvenile development is marked by the onset of exogenous feeding [24], and differences in feeding behaviour and diet can cause differential mechanical load to be propagated to the jaws throughout the life of the animal. Such behavioural polymorphisms can also lead to differential bone development (e.g. [39,40]). In support of this, we recently reared juvenile cichlids under alternate feeding strategies (i.e. biting versus suction feeding) for five months, and of seven foraging-related traits examined, the RA was by far the most plastic [23]. Taken together, these data are highly complementary and suggest that changing the mechanical environment of jaw development during either larval (this paper) or juvenile stages [23] can have a pronounced effect on bone development.

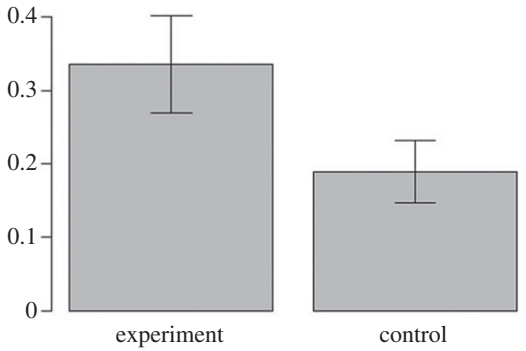

Figure 4. Quantitative PCR analysis of *ptch1* expression in fast- and slow-gaping MZ. Relative expression levels of *ptch1* in the experiment group (fast gaping) = 0.3358 ± 0.0666; control group (slow gaping) = 0.1890 ± 0.0425. ($p < 0.05$, two-way *t*-test).

(e) Difference in gaping frequency coincides with differential *ptch1* expression

Our previous collaborative work suggests that different RA lengths in LF and MZ arise over development due to genetic variation at the *ptch1* locus [19], which is a receptor in the Hedgehog (Hh) signalling pathway. Specifically, different *ptch1* alleles were associated with different expression levels around the RA, which in turn foreshadowed differences in the amount of bone deposition around this skeletal process. Besides its well-known role in skeletal development [41–43], the Hh pathway has also been shown to participate in mechanosensing via its physical association with the primary cilium [44,45]. Consistent with this, Parsons *et al*. [23] demonstrated that the cichlid *ptch1* locus is sensitive to the foraging environment. Specifically, while genetic variation at *ptch1* was shown to influence RA length in both biting (i.e. hard food) and suction (i.e. soft food) feeding regimes, the genotypic effects were far more pronounced when animals were reared on the mechanically more demanding biting diet [23]. Based on these results, we predicted that changes in *ptch1* expression might be associated with gape-induced changes in RA length. To test this prediction, we performed a qPCR experiment on dissected lower jaws of animals experiencing different gaping frequencies. We found that when forced to gape at higher frequencies, MZ larvae showed elevated levels of *ptch1* expression (approx. 1.78-fold increase, figure 4). Thus, differences in *ptch1* expression both between [19] and within species are associated with the development of different RA lengths. Notably, the relative expression difference of *ptch1* induced by gaping behaviour, epigenetically, is greater than the interspecific difference between LF and MZ, presumably caused by genetic variation in the regulatory region of *ptch1* (approx. 1.22-fold difference).

Taken together, we propose a combined genetic and epigenetic origin for adaptive variation in the cichlid RA, whereby the Hh signalling pathway may be used by both mechanisms (figure 5). *Ptch1* underlies variation in RA length [19], as well as differences in the shape of the IOP [20], which is functionally integrated with the RA via the IOPL ([37]; figure 1*c*). During lower jaw depression, the opercle pulls the IOP posteriorly, which in turn pulls the ventral tip of the RA via the IOPL. As the in-lever of the system, input on the RA causes the mandible to rotate around the jaw joint (i.e. fulcrum), thereby opening the mouth. The length of the RA has implications for the speed and power of jaw depression, and our combined investigations into this system suggest that variation in RA length can arise due to genetic and behavioural variation. To explain these results, we offer a model (figure 5) in which *ptch1* expression due to genetic differences has an influence on both the RA and IOP early in development, but behavioural variation in gaping behaviour also leads to differences in *ptch1* expression and concomitant shifts in bone deposition around the RA. Both genetic and epigenetic mechanisms combine to produce variation in adult RA length. Whether mechanical-load-induced bone development is sufficient to explain variation in cichlid RA length by itself is an interesting but as yet inconclusive question. In addition, whether *ptch1* expression is driving or merely a transcriptional read-out of skeletal development remains unclear. This gene encodes a receptor protein of the Hh pathway that physically interacts with primary cilia [44,45]. Therefore, differences in transcript abundance of this gene could facilitate mechanosensing of the cell. However, *ptch1* is also a transcriptional target of the Hh pathway. Given the important role for Hh signalling in skeletal cell differentiation [42], it is also expected that greater bone deposition will involve greater *ptch1* expression. Nevertheless, involvement of the same locus in both the plastic response of RA length within species, and the evolutionary divergence of RA length between species, offers a potential mechanism for the genesis of adaptive variation in this functional system (e.g. flexible stem theory, [46]).

One caveat of our study is that larval gaping behaviour is observed in an artificial culturing environment. Unfortunately, with currently available technology, it is impossible to know what the larval gaping behaviour is like inside the mother's mouth. Although we devoted much effort to recreate the natural rearing condition (e.g. temperature, water quality, etc.), we cannot rule out that the micro-environment within the brooding female's mouth is different in some way (e.g. mucus or other secretions cannot be replicated). Nevertheless, we feel confident that the observed interspecific behavioural differences in gaping frequency are real, because (i) such differences are observed from the first moment the larvae were removed from the buccal cavity, and (ii) gaping frequency within species remained relatively constant (at least for LF and MZ) during the 4-day period examined in this study. We therefore are confident in the main conclusion of this paper, that subtle differences in mechanical environment (within a genotype) can induce differential bone development on the order of what has previously been observed between genotypes.

4. Conclusion

Although the field of evolutionary biology is pivoting away from a mostly gene-centric view of adaptive variation [47,48], the prevailing paradigm remains focused on identifying genetic sources of such variation. Data presented here show that a seemingly trivial gaping behaviour of cichlid larvae can also introduce a large degree of variation in the RA. In fact, the change in the mean RA length induced by experimental manipulation of gaping frequency (approx. 14% increase, figure 3*b*) is very similar to the allele effects of *ptch1* on effective RA length (11.3%, [19]), and is comparable to the effects of cyclopamine manipulation on RA length (approx. 24%, [19]). Understanding how adaptive variation originates is an

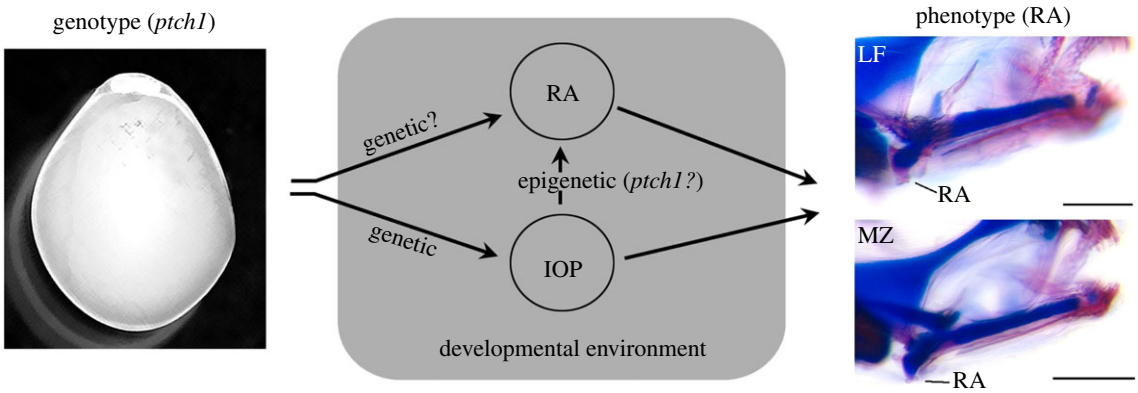

Figure 5. Model for *ptch1*-mediated variation in cichlid jaw opening mechanisms. An early blastula stage cichlid embryo is depicted to the left. This represents the baseline genotypic state of the embryo. To the right are images of cleared and stained cichlid larvae (13 dpf) showing different RA lengths. Scale bar, 200 µm. The centre panel depicts the internal environment of the embryo and illustrates the genetic paths of phenotypic variation in larval structures, as well as interactions between structures that could lead to epigenetic sources of variation, possibly mediated by Hh/*ptch1*. Ultimately, both sources combine to produce variation in the juvenile/adult structure (after [13]).

ongoing challenge in evolutionary research. Our findings highlight the importance of mechanical context in the development of complex traits, and we expect future research to continue discovering novel mechanisms hidden in the valleys of Waddington's epigenetic landscape.

Ethics. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Amherst.

Data accessibility. The dataset supporting this article is available in the Dryad Digital Repository at: http://dx.doi.org/10.5061/dryad.s8fn4 [49].

Authors' contributions. Y.H. and R.C.A. conceived of the project, performed the research, analysed the data and wrote the paper.

Competing interests. We declare we have no competing interests.

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