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Development of beak polymorphism in the African seedcracker, *Pyrenestes ostrinus*

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SUMMARY The black-bellied African seedcracker, *Pyrenestes ostrinus*, exhibits a non-sex-related polymorphism in beak size that enables the small-, large-, and mega-billed morphs to utilize different trophic niches. The bill polymorphism between small- and large-billed individuals was previously shown to be under genetic control of a single autosomal locus with the allele for a large bill being dominant. African seedcrackers offer a novel opportunity to study the genetic basis of an adaptive polymorphism driven by disruptive selection and differential niche use in wild populations. In this study, we further explore the morphology and molecular development of the beak skeleton and of the cranial musculature in all morphs, both in adults and juveniles (nestlings). We find a close correlation in growth between the two tissues, even though juvenile birds (nestlings) of all morphs are fed a soft mostly insect diet by their parents until they fledge and become independent. Molecular and histological analyses suggest a heterochronic co-option of the mechanotransduction pathway into beak development program to produce the resource polymorphism. We also find that this plasticity is diminished after the nestling period. We suggest that a mutation affecting cranial muscle mass led to a corresponding change in jawbone morphology, allowing for apparent rapid evolution of novel functional adaptations of multiple tissues, a mechanism previously thought to be hard to achieve.

INTRODUCTION

One of the fundamental challenges in evolutionary biology is to understand the mechanisms that generate adaptive morphological diversity in natural populations. It has been proposed that morphological changes must ultimately originate as alterations to specific developmental programs but there are few examples where developmental pathways responsible for evolutionary changes have been identified and characterized (Stern 2000; Arthur 2002). Traditional evolutionary developmental biology studies of diversity have focused on morphological traits between distantly related taxonomic groups. Although such approaches are of tremendous value, further studies on within-the-species level are required to uncover mechanisms that cause intraspecific variation.

The black-bellied African seedcracker *Pyrenestes ostrinus* (Estrildidae, Passeriformes) inhabits rainforests of Equatorial Africa. This estrildid finch is exhibiting a dramatic polymorphism in bill size that is not determined by sex, age, body size, or geographic origin (Smith 1987). In the equatorial forest, this species is characterized by two distinct morphs, a small- and a large-billed morph, whose ecology and evolution has been extensively examined for over 25 years (Smith 1987, 1990, 1990a, 1990c, 1993; Smith et al. 2001). In addition, a third mega-billed morph was more recently described in transition zones between the tropical forest and the savanna (Smith 1997). Patterns of intraspecific bill variation in seedcrackers are extreme, nonoverlapping, and are as great, or greater than bill size differences in sympatric species of Darwin's Finches (Smith 1990). The extreme variation in beak size is particularly remarkable in lower mandible width, which is the most important character in predicting the time taken to crack the hard sedge seeds on which they feed (Smith and Girman 2000).

Beak morphology and diet

Lower mandible width was found to be a good predictor of the bird's diet with large-billed individuals capable of cracking and handling harder seeds more efficiently than small-billed individuals (Smith 1987, 1993). African seedcrackers feed on the seeds of sedges (*Scleria*). During the reproductive season, when seeds are highly abundant, diet overlap between the small and large morphs is at its greatest. But toward the end

of the major dry season, when food becomes scarce, large-billed morphs specialize on a hard-seeded sedge species *Scleria verrucosa* (mean hardness, 153 Newtons), whereas small-billed morphs feed on the soft-seeded sedge *Scleria goossensii* (mean hardness, 13 Newtons) and broaden their diet to include other soft seeds (Smith 1990c, 1991, 1993). The Mega morph feeds on even harder seeds of *Scleria racemosa* (299 Newtons) (Fig. 1). Because these adaptive features were first described, the African seedcrackers became a textbook example of disruptive selection in the wild and how an extreme polymorphism can be maintained through differences in feeding ecology (Holmes and Harvey 1993; Futuyama 2005).

Regulation of bill morphogenesis

Young birds are fed exclusively by the parents from hatching until shortly after they fledge from the nest and start feeding on their own (Smith 1990a). However, although all morphs of African seedcrackers have a similar bill morphology at hatching, morphological bill differences appear in older nestlings, and are dramatic by the time the birds fledge from the nest and start feeding on their respective adult diets (Smith 1987). Thus, there is no opportunity for food hardness to affect jaw and skull morphology during early growth and before establishment of final morphology as in some other vertebrates, such as mammals and snakes (Katsaros 2001; Katsaros et al. 2002; Aubret et al. 2004; Erickson et al. 2004; Watts et al. 2009).

Observations in the wild have shown that seedcracker morphs breed randomly with regard to bill size (Smith 1993). Colonies of small and large morphs of *P. ostrinus* were established in 1985 and 1989 from individuals captured in south–central Cameroon. All crosses between morphs produced offspring with a lower bill width either of the large- or small-billed class, but no intermediates (Smith 1993). Offspring of crosses of known homotypic small-billed pairs produced only small-billed offspring. The results of breeding experiments of heterotypic *P. ostrinus* pairs (ratio of morphs among offspring of mixed parents) are consistent with a model that the bill polymorphism is determined by a single dominant autosomal diallelic locus. A simple diallelic model of inheritance with the large-bill dominant is also consistent with the phenotypic ratio of morphs on the study area in Cameroon (Smith 1993).

Finally, comparative analysis of genetic correlations among various bill characters in African seedcrackers and Darwin's finches showed a much more significant level of covariation in the Seedcrackers (Smith and Girman 2000), which supports the hypothesis for a single genetic and developmental cause of beak morphological variation for beak morphology in *P. ostrinus*, as opposed to Darwin's finches where different dimensions of beak morphology are regulated by multiple genetic factors (Keller et al. 2001; Abzhanov et al. 2004, 2006).

Genetics and morphology: a different pathway for the mega

Bill morphology can be defined as a ratio of different bill traits relative to the body size (Grant 1986). Birds adapt to consume larger and/or harder seeds either by increasing bill dimensions independently of body size—and in some species also inde-

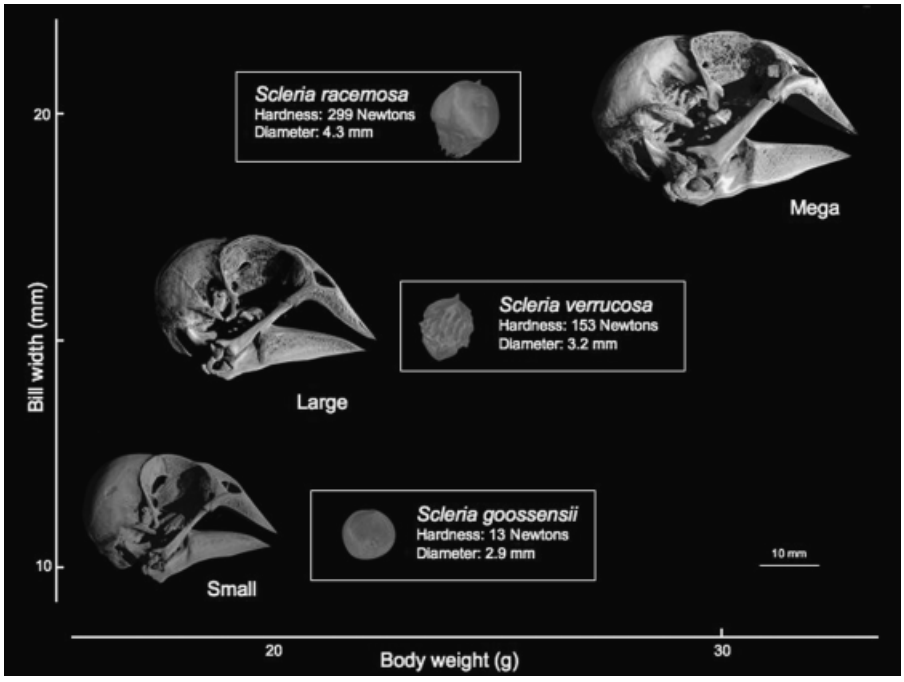


Fig. 1. Representation of average bill and body size (as estimated from body weight) in small, large, and mega morphs of *Pyrenestes ostrinus* (Smith 1997). Seed diets (different species of sedges) and their respective mean diameter in mm and hardness in Newtons are also shown (Smith 1987; Smith and Girman 2000). Each morph and seed is represented by its micro-CT scan data.

pendently changing bill characters relative to each other—or by increasing all the body and bill characters isometrically until a desirable bill size (width and/or depth) is achieved. In the case of the three morphs of the African seedcrackers, both strategies are used: a dramatic bill width increase from the small morph to the large morph of *P. ostrinus* appears to be independent of body size (Fig. 1); whereas all traits measured are increased equally in the mega morph. No data are yet available regarding the genetic nature of the mega morph. However, the isometric increase in size from small to mega suggests a more global developmental effect, such as change in hormonal regulation, as opposed to the locally regulated allometric alterations in bill morphology from small to large morphs.

The three highly distinct morphs of the African Black-bellied seedcracker *P. ostrinus* offer a unique opportunity to understand how adaptive changes in bill morphology arise in evolution. Here, we investigate the cranial anatomy and its development in African seedcrackers to complement existing data on external morphology and to provide baseline data needed to understand the developmental mechanisms driving the observed changes between morphs. In particular, we show that the transition between the small and large morphs is a dramatic example of a local craniofacial (bill)-specific change in postnatal development. Previous research results suggested that a change at a single genetic locus was responsible for the polymorphism observed in small versus large morphs, indicating an early mechanism for morphological transition. Our current data indicate a close and continued relationship between skeletal bill morphology and jaw musculature during evolution of all three morphs of the African seedcrackers, correlation that supports the hypothesis of integration between these tissues. Such a genetically simple and yet immediately adaptively significant change in morphogenesis of two developmentally distinct but functionally interacting tissues makes of the African seedcrackers a unique natural system whose study will allow to address several important and long-standing biological questions relevant to the evolution of all animals.

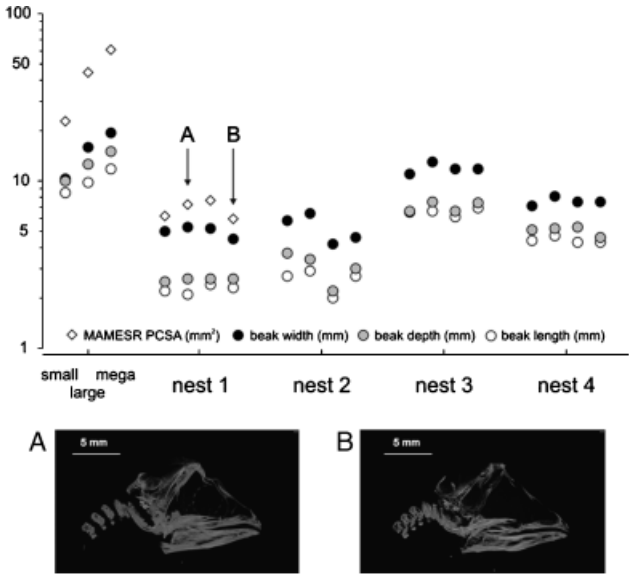


Fig. 2. Beak measurements for adults small-, large- and mega-billed *Pyrenestes ostrinus*, as well as for the juveniles collected in 2008. Measurements for adults are extracted from Smith and Girman (2000) based on more than 620 individuals for the small morph, more than 250 individuals for the large morph, and 35 individuals for the mega morph. Standard errors from the mean are <0.16 for all data depicted on the graph. Data on the physiological cross-section area (PCSA) of the adductors are shown for the individuals of the youngest brood (nest 1). These juveniles are too young to be assigned to a particular morph although their adductor muscles already show size differences. Difference in ossification between two of these juveniles (A and B) can be seen on the micro-CT images. Nest 2 siblings represent two large and two small juveniles. Finally, in nest 3, only one of the siblings appears to be developing as a large morph with bill width already exceeding that of the small morph adults. Nest 4 represents progeny of small $\times$ small parents.

MATERIALS AND METHODS

Sampling

Adult samples for all three morphs were collected by Thomas Smith in Cameroon during several field trips beginning in 1983. Additional samples used in our analyses originated from a breeding colony of African seedcrackers at the Riverbanks Zoological Park (Smith 1993). Birds from the colony were sacrificed following the outbreak of a disease at the zoo. Carcasses were eviscerated, their skull cracked and emptied from soft tissues, and preserved at -20. Consequently, these heads could only be used for superficial analysis, such as micro-CT scanning of the beak. In 2008, nestlings from four nests were sacrificed at the main field site in Ndibi, Cameroon, their skulls collected, fixed in 4% paraformaldehyde, rinsed in saline, and stored in RNAlater (Ambion Inc., Austin, TX, USA). Three of these nests were of mixed parents of large and small morphs, whereas one nest was of small morph parents only. In one of the mixed parent nests, nestlings were too young to display the differences in beak morphology, but for two other nests, bill morphology was more advanced and differences could be more readily observed (Fig. 2). Two pairs of siblings representing large and small morphs from mixed nests were used both for immunohistochemistry and in situ hybridization analyses. In addition, one pair of heterotypic siblings was used for muscle dissection and skeletal preparation (clearing and staining followed by the micro-CT scanning).

Micro-CT scanning

To better understand and visualize the differences in the skeletal morphology between morphs, we generated high-resolution. Three-dimensional (3D) images of the skulls of African seedcrackers of each morph using an XRA-002 micro-CT scan (X-Tek, Tyngsboro, MA, USA) available at the Center for Nanoscale System at Harvard University. Image acquisition was performed at 75 kV and 100 μA. 3D reconstructions were performed with CTPro (Metris,

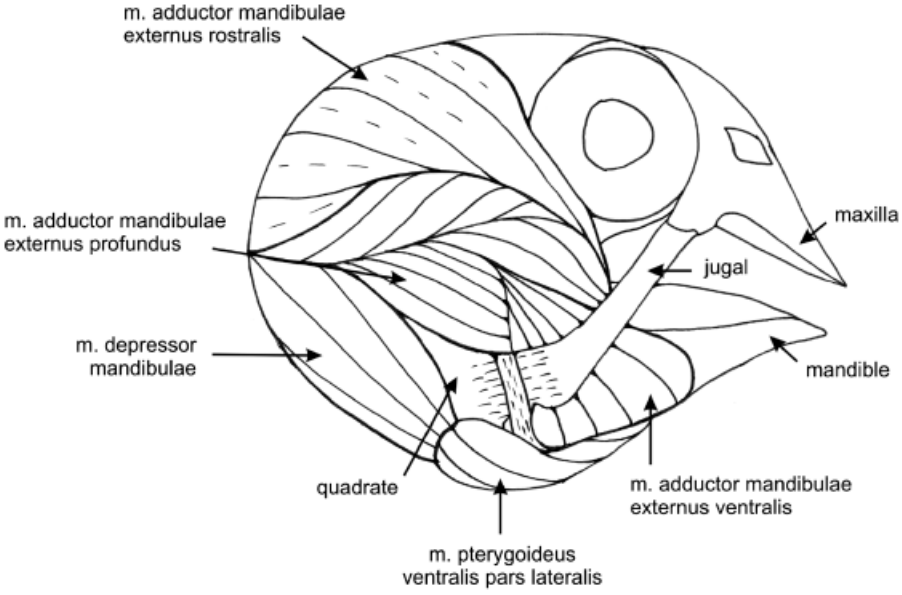


Fig. 3. Schematic drawing of the head of an African seedcracker illustrating the superficial cranial musculature. The different muscles are labeled. m, musculus.

Brighton, MA, USA) and VGStudio Max 2.0 (Volume Graphics, Heidelberg, Germany).

Muscle morphology

Bill and body dimensions were also measured for a single Western bluebill *Spermophaga haematina*, the closest relative of the African seedcrackers (Goodwin 1982). For this Western bluebill, as well as for one individual of each morph of African seedcracker, all cranial muscles (Fig. 3), including subdivisions of the main jaw adductor muscles (*M. pterygoideus, M. adductor mandibulae externus*, and *M. pseudotemporalis*), the jaw opener (*M. depressor mandibulae*), the *M. retractor palatinus*, and the protractors (*M. protractor quadrati* and the *M. protractor pterygoidei*), were dissected and weighed.

Next the connective tissue surrounding the muscles was digested by submerging the muscles in a 30% aqueous nitric acid solution. After 24 h, the nitric acid was replaced by a 50% aqueous glycerol solution and fibers were teased apart using blunt-tipped glass needles. At least 15 fibers selected at random (together with an object of known size for scaling purposes) were drawn for each muscle using a dissecting scope equiped with *camera lucida*. Drawings were scanned and fiber lengths measured using Scion Image for Windows. Physiological cross-sectional areas were calculated based on the mass of the muscles, a density of 1.06 g/cm^3 (Mendez and Keys 1960), and the average fiber length for each muscle bundle. Because complex pennate (penniform) muscles were separated into their component parts no correction for pennation was included.

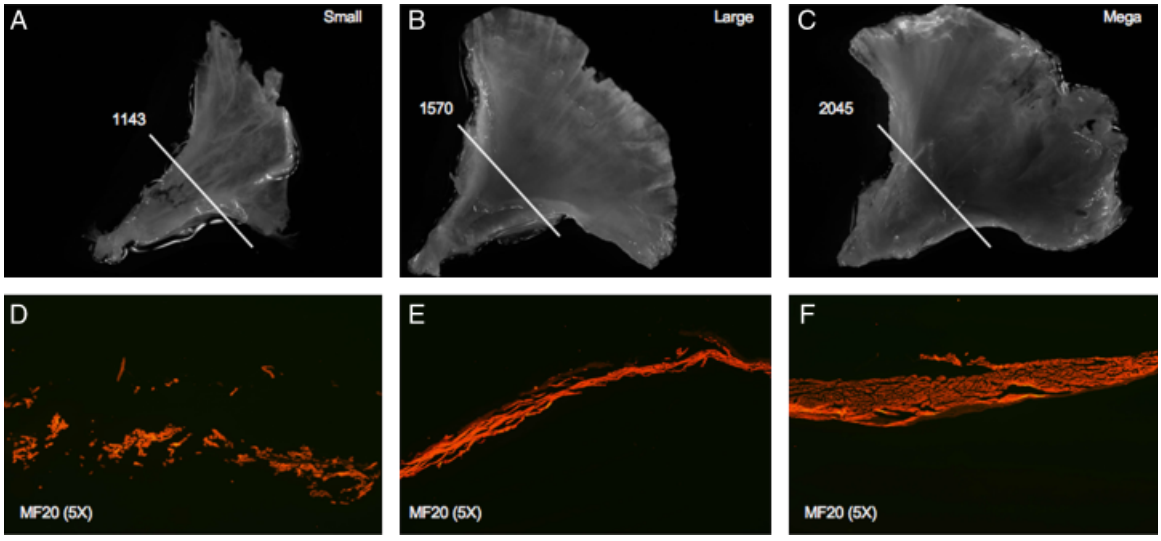


Fig. 4. The size of the adductor is increasing from small to large and even more so from small to mega morphs (A–C). The exact number of myocytes in a cross-section of each muscle is shown. The adductor thickness also increases from small to mega (D–F).

Additionally, we dissected the superficial-most layer of the external adductor as well as the lower jaw depressor in five small (three wild and two zoo-born) and six large morphs (three wild and three zoo-born) of the African seedcracker. Muscles were weighed using a microbalance and for one individual of each wild-caught morph the superficial-most layer of the external adductor was sectioned, and pictures (Fig. 4) were taken with a AxioCam camera (Zeiss, Thornwood, NY, USA) mounted on a SteREO microscope (Zeiss).

Immunocytochemistry

Hematoxylin and Eosin (H&E) staining was performed both on adult and juvenile heads, following standard protocols (Humason 1972). pSmad staining to assess overall BMP activity in the cells of the developing bill skeleton was performed by using an anti-phosphorylated Smad (αSmad1/5/8) antibody (Cell Signaling Technology, Danvers, MA, USA) whose signal was amplified with the Vectastain Elit ABC Kit (Vector Laboratories, Burlingame, CA, USA).

In situ hybridization (ISH)

ISH were performed on sectioned tissues from juveniles heads following the protocol developed for Darwin's finches (Abzhanov 2009). Tissues from heterotypic siblings of the small and large morph morphology were processed in parallel to allow for comparison of gene expression signals. Probes were designed based on the chicken genome, as it has been shown to be conserved enough to allow for reliable hybridization in estrildids (Abzhanov et al. 2004; Abzhanov 2009).

Statistical analyses

All data were log 10 transformed before analysis to meet assumptions of normality and homoscedascity, and all analyses were performed using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA).

We first calculated the residuals of a regression of log 10 transformed beak width and the log10 transformed physiological cross-sectional area of the rostral part of the external jaw adductor versus log10 transformed beak length. This regression included data for both adults and juveniles of all morphs from wild-caught individuals. The residuals were then used as input for a Pearson correlation analysis to test whether birds with greater muscle cross-sectional areas had wider beaks independent of their overall beak size. If so, this would be consist with the hypothesis that a strong integration between muscle and bone development exists.

Analysis of variance were then performed to test for differences in beak shape between individuals collected by Thomas Smith in Cameroon and brought back to the Riverbanks Zoological Park to start a colony and their offspring. At the time when measurements were taken, the founder birds of the captive colony were all adults and older than 1-year-old, and had therefore been subjected to disruptive selection (Smith 1990b, 1993). The other group of birds used for this analysis consists of the offspring of the previous individuals, and were born in the Riverbanks Zoological Park. There they were maintained as hatching on a soft diet (Smith 1993).

To account for potential age and size differences between groups we constructed a new variable (hereafter referred to as SIZE) by combining four different indicators of body size, body mass, wing chord, tail length, and tarsus length, into a single principal component. This principal component was then used as a covariate in our analyses. Beak measurements for which differences between groups were tested included: lower bill width, upper bill width, depth, length from the nostril to the tip for the upper beak, and finally upper and lower beak length (Smith 1987; Grant 1999).

Finally, differences in the mass of the superficial-most layer of the external adductor and the depressor between (1) different morphs of wild-caught individuals and (2) between zoo-raised and wild-caught individuals of each morph were tested using analysis of variance.

RESULTS

Morphology of the skull and beak

The morphology of the skull in all three morphs of the African seedcracker is rather unusual, even for estrildid finches (Nuijens and Zweers 1997), and is characterized by a thick rhamphoteca, a strong downward inclination of the upper beak and a very strongly developed jugal bone. Differences between the three morphs are also striking and are principally reflected in a marked increase in the cross-sectional area of the jugal, a posterior expansion of the skull, a stronger development of the processus zygomaticus and an increase in the size of the temporal fossa and the cristae on the posterior aspect of the skull in the large and mega morphs. These differences reflect the increased demands for space and attachment sites for the external adductor musculature in the two larger morphs. The differences in the skull of the adults might also be reflected in an earlier ossification in the juveniles of the jugal, the squamosal and the quadrate bones in the large morph compared with the small morph (based on data gathered for two individuals from a nest of heterotypic parents) (Fig. 2).

Dramatic differences in morph-specific cranial musculature

The cranial musculature in the African seedcrackers is well developed and increases markedly in size and cross-sectional area from the small to large to the mega morphs (Table 1 and Fig. 2). Our results of the Pearson correlation analysis actually demonstrate a strong and significant correlation between the cross-sectional area of the jaw adductor and mandible width, independent of variation in overall beak size (Pearson's $r = 0.973$, see Fig. 5). The physiological cross-sectional area (PCSA) of the external jaw adductor muscle group, one of the major muscle groups responsible for closing the beak is nearly two times greater in the large, and nearly three times greater in the mega morph compared with the small morph (34.7, 50.2, and 99.3 mm^2 for the small, large, and mega morphs, respectively). As only one individual for each morph was available for dissection, we cannot provide an indication of the within morph variability. However, it must be noted that differences between morphs are much greater than the typical

Table 1. Morphological characterization of the jaw musculature in African seedcrackers and their closest relative, the Western bluebill

Species/morph	Western bluebill	*Pyrenestes ostrinus* small		*P. ostrinus* large		*P. ostrinus* mega
Age	Adult	Adult	Nestling	Adult	Nestling	Adult
M. adductor mandibulae externus						
Mass	67.4	63.5	1.6	111.5	1.8 ± 2.9	176.9
PCSA	50.2	34.73	5.94	68.94	7.02 ± 0.76	99.27
Lower jaw and quadrate protractors						
Mass	4.5	5.5		6.8		7.7
PCSA	2.4	3.41		3.5		3.49
M. pterygoideus						
Mass	54	41.4		82.4		111.4
PCSA	35.64	26.62		61.42		72.43
M. pseudotemporalis						
Mass	30.7	34.2		41.9		57.4
PCSA	17.24	27.62		24.69		31.99
Jaw openers						
Mass	18.2	15.3	1.0	21.6	1.6 ± 0.2	28.9
PCSA	5.2	4.18	4.42	6.12	5.16 ± 0.49	8.19
Total jaw closers						
Mass	160.6	146.7		246.3		363.8
PCSA	106.66	92.25		158.28		207.11

Mass is expressed in mg, physiological cross-sectional area in mm^2.
PCSA, physiological cross-sectional area.

intraspecific differences observed in, for example, Darwin's finches (typically no more than 10% difference, personal observation). Moreover, Fig. 6 clearly illustrates the dramatic nature of the differences in muscle morphology among morphs, spanning nearly half the known diversity in jaw muscle size among all estrildid finches (van der Meij and Bout 2004).

Although the total jaw closer muscle mass in the large morph (275 mg) is larger than average for its body size, the jaw muscle mass of the mega morph (400 mg) is not exceptional for its body size when compared with previously published data for estrildid finches (Fig. 6) (van der Meij and Bout 2004). The small morph had relatively small jaw adductors (168 mg) compared with a closely related species (*Spermaphaga hematina*; 183 mg) and other estrildid finches (van der Meij and Bout 2004). The cross-sectional area of the beak protractors was, however, similar across all morphs (between 3.4 and 3.5 mm^2) suggesting a differential increase in size of the different jaw muscles groups resulting in a hypertrophy of the jaw adductors in the large and mega morphs. A more detailed analysis of the superficial-most layer of the external adductor muscle based on histological cross-sections from all three morphs revealed that the large and mega morphs contained about 40% and 80% more myocytes than the small morph, respectively (Fig. 4 C–E).

Interestingly, although the external jaw adductor in nestlings is also greater in the large morph compared with the small morph (120% of the PCSA of the small morph), the difference is much greater in adults (199%) suggesting strong allometric growth of the jaw adductors later in ontogeny, presumably in fledglings and juveniles.

Induced changes in adult beaks due to feeding on soft versus hard food

ANCOVA's with SIZE as co-variate (Table 2 and Fig. 7) suggests that a difference in beak size (specifically, lower beak width and lower and upper beak length) exists between large billed morphs, which ate hard seeds in the wild, and birds that were raised on soft diet at a breeding colony established at Riverbanks Zoological Park (Smith 1993). The beak of the wild birds was found to be 4% wider than the ones raised in the zoo. Interestingly, no differences in beak dimensions can be found among wild and zoo-born small-billed individuals of *P. ostrinus* (Table 2B). Additionally, whereas small morphs are generally similar in size and independent of whether they were wild-caught or zoo-raised, the wild-caught large morphs were significantly larger than zoo-raised ones in most body dimensions. However, the observed difference in beak dimensions are not merely a consequence of size differences as size was taken into account in our analyses of beak dimensions.

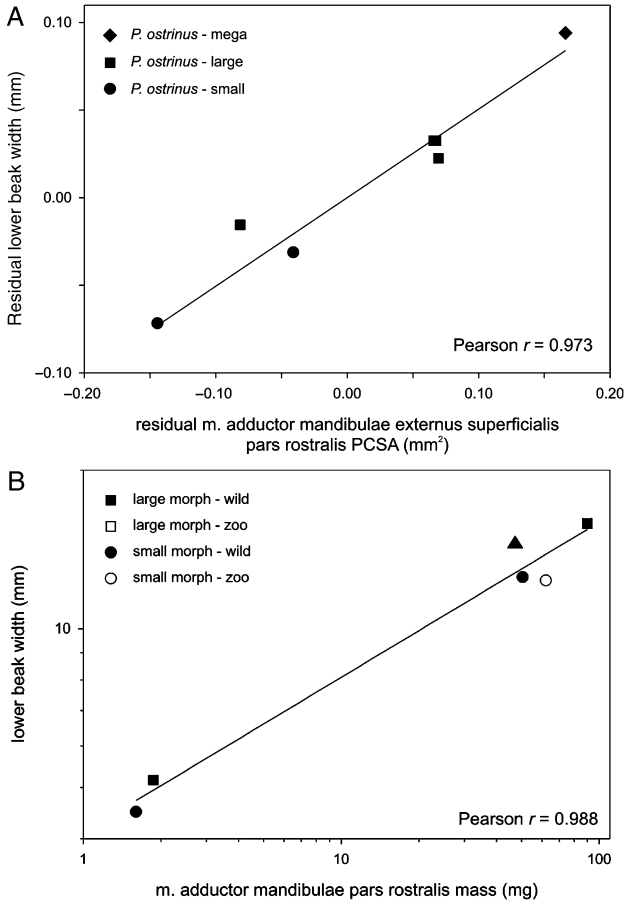


Fig. 5. Residuals of a regression of log10 transformed beak width and the log10 transformed physiological cross-sectional area of the rostral part of the external jaw adductor versus log10 transformed beak length (A). This panel shows that large-billed birds raised in the zoo and fed soft diets deviate from the general pattern (triangle) (B).

Bill morphogenesis correlates with expression of specific skeletogenic molecules

To uncover the developmental bases for the bill size polymorphism, we used systematic histological and in situ hybridizations comparative approaches. We studied expression of several key skeletogenic molecules, including cell type and cell differentiation markers, signaling molecules, and proliferation markers (Fig. 8). Our analysis with H&E stain revealed more deposited mineralized bone material and a higher degree of bone mineralization in the large morph nestlings, especially in their lower jaw bones (Fig. 8, A, B, O, and P). The histological data support the differences in mineralization revealed by the micro-CT scans (Fig. 2, A and B). Correspondingly, we found that both *Bmp4* and *Bmp2* were upregulated in more cells forming the periosteum of the jawbones of the large morph nestlings, which were undergoing rapid bill morphogenesis (Fig. 8, C–F, and Q–R). This upregulation is confirmed by the expression pattern of activated pSMAD as

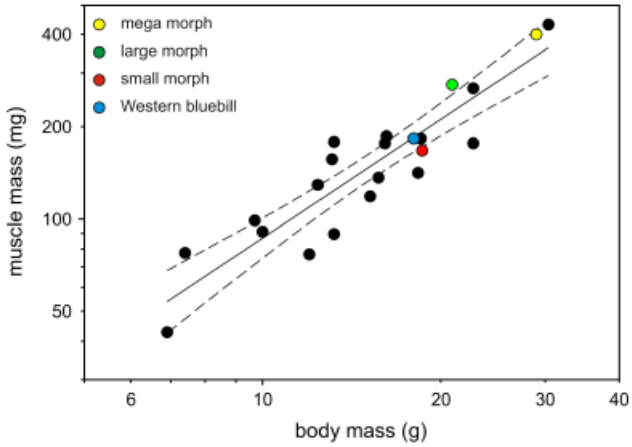


Fig. 6. Jaw muscles size among various estrildids (van der Meij and Bout 2004). The differences in muscle size among the different morphs of African seedcrackers spans over half the range existing between estrildid species. Axes are log axes and dotted lines are the 95% confidence limits.

diagnosed by the anti-phosphorylated Smad (αSmad1/5/8) antibody in the developing jaws of the large morph nestling (Fig. 8, G, H, U, and V). However, we did not detect a marked difference in *Ihh* expression, another key osteogenic signaling molecule (Fig. 8, I, J, W, and X). Osteogenic markers *Runx2* (early osteoblasts— Fig. 8, K, L, Y, and Z) and *Opn* (more mature osteoblasts—M, N, AA, AB) were expressed in more cells of the periosteum in the upper jawbones of large morph as compared with small morph, but showed no difference in their pattern of expression in the lower jaws.

DISCUSSION

We previously performed a comparative analysis of the evolutionary developmental mechanisms controlling changes in bill size and shape in several species of Darwin's Finches, another textbook example of adaptive morphological radiation (Darwin 1859; Bowman 1961; Grant 1999; Abzhanov et al. 2006, 2004). Such an interspecific analysis across distinct species, albeit useful, must necessarily deal with multiple genes contributing to the phenotypic differences due to more complex evolutionary histories. In contrast, studying intraspecific variation in the African seedcrackers, which is based on a single genetic locus, greatly simplifies the search for the genetic and developmental basis of differences in cranial structures.

Our observations reveal several skeletal features in the African seedcrckers that are unusual, even among estrildid finches. One of these features, the downward curvature of the beak, has previously been implicated in increasing bite force (van der Meij and Bout 2008). The morphological observation derived from the micro-CT scans supports the hypothesis of an early adaptation to cracking hard seeds in the common

Table 2. Summary table illustrating differences in body and beak dimensions between wild-caught and zoo-born birds derived from the wild-caught parents

	wild	Zoo	*F*	*P*
Large morph (21, 18)				
Mass (g)	20.91 ± 1.45	19.56 ± 2.96	**4.65**	**0.04**
Wing chord (mm)	62.18 ± 1.56	60.64 ± 1.99	**8.78**	**<0.01**
Tail length (mm)	51.94 ± 1.95	49.00 ± 4.35	**6.72**	**0.01**
Tarsus length (mm)	16.63 ± 0.77	16.59 ± 1.00	0.69	0.41
Lower beak width (mm)	15.86 ± 0.84	15.09 ± 0.63	**4.50**	**0.04**
Upper beak width (mm)	9.58 ± 0.78	9.50 ± 1.17	2.44	0.13
Bill depth (mm)	12.18 ± 1.05	11.48 ± 0.89	0.83	0.37
Culmen length (mm)	11.32 ± 0.72	9.89 ± 1.09	**5.69**	**0.02**
Lower bill length (mm)	9.74 ± 0.33	8.83 ± 0.84	**5.46**	**0.03**
Nostril tip (mm)	4.95 ± 0.52	4.58 ± 0.37	1.36	0.25
Small morph (23, 12)				
Mass (g)	18.20 ± 1.09	18.78 ± 2.21	0.25	0.62
Wing chord (mm)	60.64 ± 1.40	60.68 ± 3.09	0.94	0.34
Tail length (mm)	52.07 ± 1.61	51.55 ± 2.49	0.65	0.43
Tarsus length (mm)	16.48 ± 0.71	16.10 ± 1.48	**3.26**	**0.08**
Lower beak width (mm)	12.69 ± 0.30	12.51 ± 0.71	0.56	0.46
Upper beak width (mm)	7.68 ± 0.56	8.09 ± 1.02	0.11	0.74
Bill depth (mm)	9.89 ± 0.49	9.74 ± 1.40	0.01	0.91
Culmen length (mm)	9.85 ± 0.51	9.13 ± 1.70	0.86	0.36
Lower bill length (mm)	8.55 ± 0.42	7.99 ± 1.56	0.01	0.91
Nostril tip (mm)	3.62 ± 0.36	3.70 ± 0.58	1.96	0.17

Table entries are means ± standard deviations for wild-caught versus zoo-born individuals. Also indicated are *F*- and *P*-values of ANCOVA's with SIZE as a co-variate, testing for differences between wild-caught and zoo-born individuals. Differences in mass, wing chord, tail length, and tarsus length were tested using analysis of variance. Significant differences are indicated in bold. And sample sizes for wild-caught and zoo-born groups are indicated between brackets.

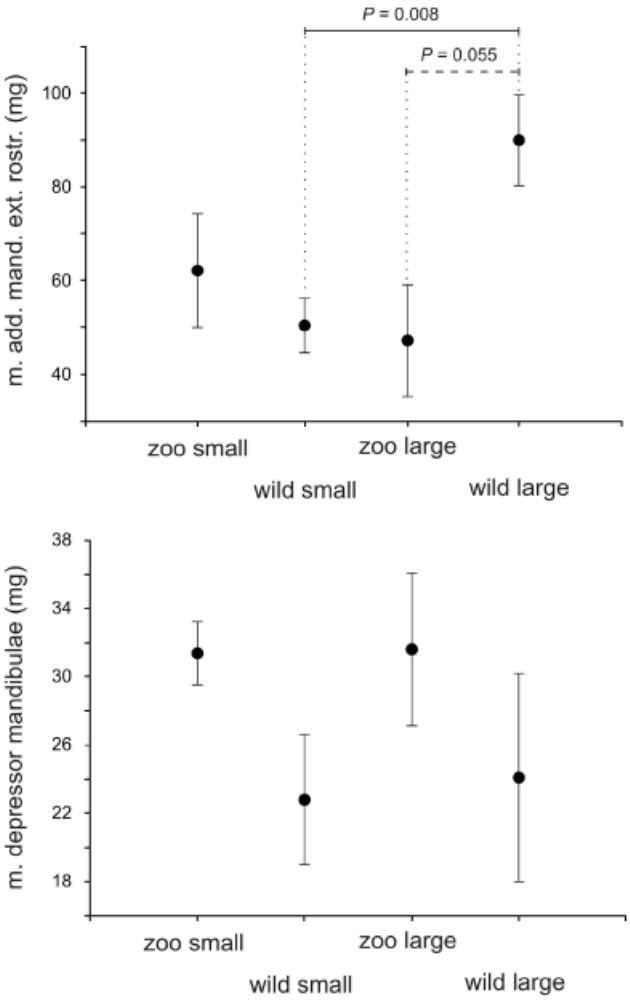


Fig. 7. Adductor and depressor mass for zoo-born and wild African seedcrackers of both small and large morph.

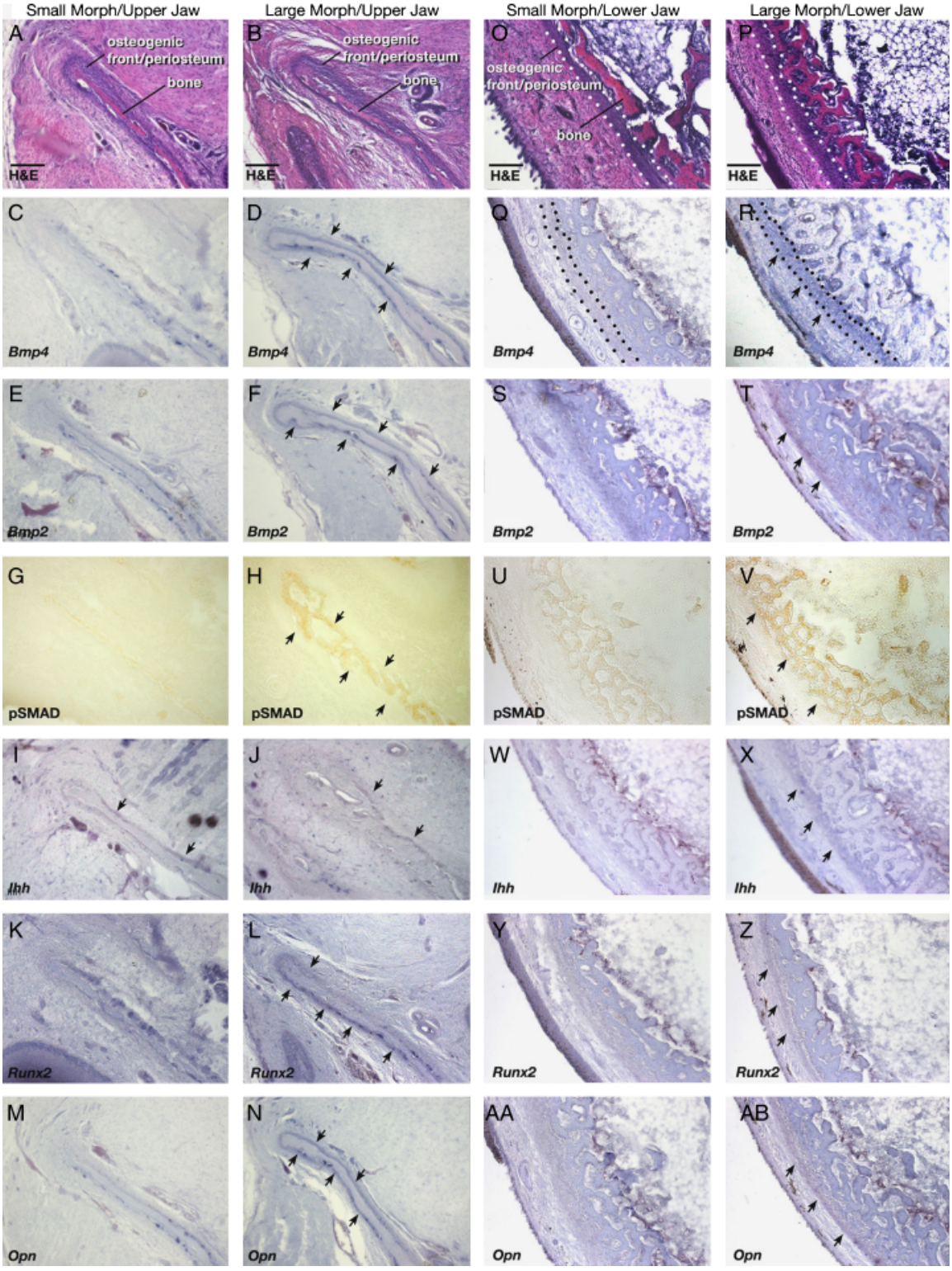
ancestor of all three morphs (Smith 1990c). High bite forces are likely to be advantageous in granivorous birds because they allow for a more efficient seed handling by reducing the time needed to crack and husk seeds (van der Meij and Bout 2006). This correlates with the observed food sources of all three morphs, which are harder than expected for an estrildid finch of such body and adductor muscle masses (van der Meij and Bout 2004). At present, the exact evolutionary history of the different morphs of *P. ostrinus* is still unknown, but based on morphology of their closest known sister group (Goodwin 1982), *S. haematina* or Western bluebill, the basal form is expected to have a small bill morphology (Smith 1990c). Both species are capable of feeding on soft sedge seeds but the skull features specific to *P. ostrinus* might have enabled this bird to fill a more specialized sedge eater niche (unpublished).

Differences observed in cranial structures among morphs are—beside differences in the size of the beak—also provide and increase of the insertion area of jaw muscles in the large and mega morphs. In fact, the jaw closing muscles in the large and mega morphs are two to three times greater in size, respectively, than those observed in the small morph. As muscle size and cross-sectional areas are the traits most directly affecting bite force in finches (van der Meij and Bout 2004, 2008) this provides the large and mega morphs with an increased bite force capacity, thus allowing them to crack harder seeds. The wider beaks observed in large and mega morphs of the African seedcracker likely function to absorb and spread the reaction forces generated during biting (Herrel et al. 2005) and are thus likely adapted to a more efficient cracking and husking of hard seeds (Smith 1987).

The strong correlation between muscle cross sectional area and beak width observed across morphs is consistent with our hypothesis that the development of the beak is integrated with muscle development. Many beak morphological transitions, including those in Darwin's finches and other birds, are also coupled with a corresponding change in jaw musculature (Bowman 1961; van der Meij and Bout 2004). Thus, in addition to being a great model system for the study of evolution of jaw morphology, African seed-

crackers offer an exciting and unique opportunity to understand the mechanisms of an adaptive musculo-skeletal integration in birds.

Several hypotheses can be made regarding the genetic nature of this integrated, multi-tissue polymorphism. For instance, one could hypothesize that several genes or reg-



ulatory elements independently regulating muscles and jaw are situated in close proximity in the genome to be interpreted as a single locus in the pedigree analyses. A more likely explanation involves a mutation at a single gene that controls growth of both muscle and bone tissues. We believe that the most parsimonious explanation for the observed polymorphism is a mutation in a single gene that regulates differential growth of one of these tissues, which in turns influences the growth of another via a strong integration mechanism. Our current knowledge of skeletomuscular biology suggests that mechanotransduction could account for such integration. Mechanotransduction (Jaalouk and Lammerding 2009; Schwartz 2009) allows the translation of a mechanical stress, here caused by increased contractile jaw musculature, as a biochemical signal in a mechanosensory cell, the osteocyte (Allori et al. 2008; Bonewald and Johnson 2008). Mechanical strain can then alter cellular differentiation and skeletal tissue reorganization. A mutation resulting in a differential growth of the jaw adductor muscle size during post hatching development of nestlings (before and without involvement of a difference in diet) could therefore lie at the origin of the beak differences found between beaks of small and large morphs of African seedcrackers. Our measurements of adductor muscles size in juvenile (nestling) offspring of mixed parents developing small and large bills suggest a temporal correlation in development of both tissues (Fig. 2). In addition, our molecular analysis of the developing jawbone tissue provides further evidence for the proposed role of mechanotransduction. Indeed, *Bmp2* and *Bmp4*, whose expression is upregulated in the jawbones of the large morph juveniles, have been found to be upregulated during distraction osteogenesis (Sato et al. 1999; Ikegame et al. 2001; Khanal et al. 2008), a process that involves mechanotransduction. Similarly, analysis of *Runx2* and *Opn* expression and function in mice with compromised stress sensory mechanism that lacked the cilia-like structure acting as a mechano-sensor on osteoblastic and osteocyte-like cells suggested a positive role of this transcription factor in mechanotransduction (Xiao et al. 2006; Malone et al. 2007). The observed upregulation of *Opn* and *Runx2* in the skeleton of the upper jaw of large morph nestlings could therefore be related to activation of these mechanosensory structures.

The close integration that drives the differences in morphology during juvenile development leads to beaks being 20% wider in the large morph than in the small morph. However, once the adult shape is attained, plasticity of the beak in response to change in diet is much reduced, although still present. Indeed, our data show that the mass of the external-most layer of the external adductor in individuals of the large morph, fed a soft diet was not different from that observed for small morph individuals. In turn, measurements of beak morphology showed a statistically significant difference in beak size between wild birds of large morph and those that fledged in captivity and fed on much softer diet of regular bird seed mix (Table 2A). In particular, beaks were on average 4% wider in the wild birds. We hypothesize that, again, a response to stress via mechanotransduction is a likely mechanism responsible for these differences, as opposed to differences in the nutritional value of the respective diets. Indeed, there was no detectable difference in beak sizes among the wild and captive small-billed birds, which all fed on soft diets (Table 2B). This suggests that the muscle- and mechanotransduction-driven plasticity is still present but may be much lower in juveniles after fledging. The exact roles of the stress-induced mechanotransduction in the developing cranial skeleton and musculature in fledglings, juveniles and adults of African Seedcrackers are yet to be fully understood and some pertinent functional tests on related avian species and stages are now under way in our laboratory.

CONCLUSION

Studies on the resource polymorphism in African seedcrackers have the potential for illuminating the selective forces that lead to the evolution of intraspecific diversity and potentially even speciation (Smith and Skulason 1996), as well as the genes and developmental pathways that are responsible for morphogenesis of new and adaptive features. Our findings point to a close functional, and developmental integration between bill size (particularly bill width), skeletal morphology, and jaw adductor musculature during postnatal development. Moreover, the difference between small and large morph is controlled by a single diallelic factor. Therefore we suggest that the cranial skeleton and musculature are closely linked both developmentally and genetically, allowing for efficient functional integration as well as a rapid evolutionary adaptation. Such a regulatory link would negate the need for multiple independent yet simultaneous events to produce a useful morphological transition, a condition long considered

Fig. 8. Molecular analysis of the developing jawbones in small and large morph juveniles (late nestlings in pair-wise comparisons). (A, B, O, P) hematoxylin and eosin staining revealed more heavily mineralized bone in large morph. (C, D, M, Q, R) higher expression levels of *Bmp4* and (E,F,S, T) *Bmp2* in large morph bones both in upper and lower jawbones. (G, H, U, V) Higher BMP activity led to a higher level of pSMAD activation (brown staining indicated with arrows). (I, J, W, X) Expression of *Ihh* is relatively unchanged between the two morphs; (K, L, Y, Z) Expression levels of early osteogenic marker *Runx2* were much higher in the upper jawbone of the large morph but were more comparable in the lower jawbones of the two morphs (arrows). (M, N, AA, AB) expression of the later osteoblastic marker *Opn* was higher in the large morph, especially in the upper jawbone (N). Scale bar is 300 μm.

an impediment to significant and rapid adaptive change that requires complementary and synchronized alterations of multiple tissues (Goldschmidt 1940). Further genetic and functional tests, currently under way, will seek to reveal the exact nature of the beak polymorphism in the African seedcracker *P. ostrinus*.

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REFERENCES

Abzhanov, A. (2009). Darwin's finches. Analysis of beak morphological changes during evolution. In *Emerging Model Organisms*. Vol. 1. Cold Spring Harbor Laboratory Press, Cold Sping Harbor, NY, pp. 481–500.

Abzhanov, A., Kuo, W. P., Hartmann, C., Grant, B. R., Grant, P. R., and Tabin, C. J. 2006. The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches. *Nature* 442: 563–567.

Abzhanov, A., Protas, M., Grant, B. R., Grant, P. R., and Tabin, C. J. 2004. Bmp4 and morphological variation of beaks in Darwin's finches. *Science* 305: 1462–1465.

Allori, A. C., Sailon, A. M., Pan, J. H., and Warren, S. M. 2008. Biological basis of bone formation, remodeling, and repair-part III: biomechanical forces. *Tissue Eng. Part B Rev.* 14: 285–293.

Arthur, W. 2002. The emerging conceptual framework of evolutionary developmental biology. *Nature* 415: 757–764.

Aubret, F., Shine, R., and Bonnet, X. 2004. Evolutionary biology: adaptive developmental plasticity in snakes. *Nature* 431: 261–262.

Bonewald, L. F., and Johnson, M. L. 2008. Osteocytes, mechanosensing and Wnt signaling. *Bone* 42: 606–615.

Bowman, R. I. 1961. Morphological differentiation and adaptation in the Galápagos finches. *Univ. Calif. Publ. Zool.* 58: 1–302.

Darwin, C. 1859. *The Origin of Species*. 1962nd Ed. The Crowell-Collier Publishing Co, New York, NY.

Erickson, G. M., Lappin, A. K., Parker, T., and Vliet, K. A. 2004. Comparison of bite-force performance between long-term captive and wild American alligators (*Alligator mississippiensis*). *J. Zool. Lond.* 262: 21–28.

Futuyama, D. J. 2005. *Evolution*. Sinauer Associates Inc, Sunderland, MA.

Goldschmidt, R. 1940. *The Material Basis of Evolution*. Yale University Press, New Haven, CT.

Goodwin, D. 1982. *Estrilid Finches of the World*. Cornell University Press, Ithaca.

Grant, P. R. 1986. *Ecology and Evolution of Darwin's Finches*. Princeton University Press, Princeton, NJ.

Grant, P. R. 1999. *Ecology and Evolution of Darwin's Finches*. Princeton University Press, Princeton, NJ.

Herrel, A., Podos, J., Huber, S. K., and Hendry, A. P. 2005. Evolution of bite force in Darwin's finches: a key role for head width. *J. Evol. Biol.* 18: 669–675.

Holmes, E., and Harvey, P. 1993. Fitting the bill. *Curr. Biol.* 3: 776–777.

Humason, G. L. 1972. *Animal Tissue Techniques*. 3rd Ed. W.H. Freeman & Co, San Francisco, CA.

Ikegame, M., et al. 2001. Tensile stress induces bone morphogenetic protein 4 in preosteoblastic and fibroblastic cells, which later differentiate into osteoblasts leading to osteogenesis in the mouse calvariae in organ culture. *J. Bone Miner. Res.* 16: 24–32.

Jaalouk, D. E., and Lammerding, J. 2009. Mechanotransduction gone awry. *Nat. Rev. Mol. Cell Biol.* 10: 63–73.

Katsaros, C. 2001. Masticatory muscle function and transverse dentofacial growth. *Swed. Dent. J. Suppl.* 151: 1–47.

Katsaros, C., Berg, R., and Kiliaridis, S. 2002. Influence of masticatory muscle function on transverse skull dimensions in the growing rat. *J. Orofac. Orthop.* 63: 5–13.

Keller, L., Grant, P. R., Grant, B. R., and Pettren, K. 2001. Heritability of morphological traits in Darwin's Finches: misidentified paternity and maternal effects. *Heredity* 87: 325–336.

Khanal, A., Yoshioka, I., Tominaga, K., Furuta, N., Habu, M., and Fukuda, J. 2008. The BMP signaling and its Smads in mandibular distraction osteogenesis. *Oral Dis.* 14: 347–355.

Malone, A. M., et al. 2007. Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism. *Proc. Natl. Acad. Sci. USA* 104: 13325–13330.

Mendez, J., and Keys, A. 1960. Density and composition of mammalian muscle. *Metabolism* 9: 184–188.

Nuijens, F. W., and Zweers, G. A. 1997. Characters discriminating two seed husking mechanisms in finches (Fringillidae: Carduelinae) and Estrildids (Passeridae: Estrildinae). *J. Morphol.* 232: 1–33.

Sato, M., et al. 1999. Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. *J. Bone. Miner. Res.* 14: 1084–1095.

Schwartz, M. A. 2009. Cell biology. The force is with us. *Science* 323: 588–589.

Smith, T. B. 1987. Bill size polymorphism and intraspecific niche utilization in an African finch. *Nature* 329: 717–719.

Smith, T. B. 1990. Patterns of morphological and geographic variation in trophic bill morphs of the African finch Pyrenestes. *Biol. J. Linn. Soc.* 41: 381–414.

Smith, T. B. 1990a. Comparative breeding biology of the two bill morphs of the black-bellied seedcracker. *The Auk* 107: 153–160.

Smith, T. B. 1990b. Natural selection on bill characters in the two bill morphs of the African finch *Pyrenestes*. *Evolution* 44: 832–842.

Smith, T. B. 1990c. Resource use by bill morphs of an African finch: evidence for intraspecific competition. *Ecology* 71: 1246–1257.

Smith, T. B. 1991. Inter- and intra-specific diet overlap during lean times between Quelea erythrops and bill morphs of *Pyrenestes ostrinus*. *Oikos* 60: 76–82.

Smith, T. B. 1993. Disruptive selection and the genetic basis of bill size polymorphism in the African finch, *Pyrenestes*. *Nature* 363: 618–620.

Smith, T. B. 1997. Adaptive significance of the mega-billed form in the polymorphic black-bellied seedcracker *Pyrenestes ostrinus*. *Ibis* 139: 382–387.

Smith, T. B., and Girman, D. J. (2000). Reaching new adaptive peaks. Evolution of alternative bill forms in an African finch. In T. Mousseau, B. Sinervo, and J. Endler (eds.). *Adaptive Genetic Variation in the Wild*. Oxford University Press, Oxford, pp. 139–156.

Smith, T. B., Schneider, C. J., and Holder, K. 2001. Refugial isolation versus ecological gradients. *Genetica* 112–113: 383–398.

Smith, T. B., and Skulason, S. 1996. Evolutionary significance of resource polymorphisms in fish, amphibians and birds. *Annu. Rev. Ecol. Syst.* 27: 111–133.

Stern, D. L. 2000. Evolutionary developmental biology and the problem of variation. *Evolution* 54: 1079–1091.

van der Meij, M. A. A., and Bout, R. G. 2004. Scaling of jaw muscle size and maximal bite force in finches. *J. Exp. Biol.* 207: 2745–2753.

van der Meij, M. A. A., and Bout, R. G. 2006. Seed husking time and maximal bite force in finches. *J. Exp. Biol.* 209: 3329–3335.

van der Meij, M. A. A., and Bout, R. G. 2008. The relationship between shape of the skull and bite force in finches. *J. Exp. Biol.* 211: 1668–1680.

Watts, H. E., Tanner, J. B., Lundrigan, B. L., and Holekamp, K. E. 2009. Post-weaning maternal effects and the evolution of female dominance in the spotted hyena. *Proc. R Soc.* 276: 2291–2298.

Xiao, Z., et al. 2006. Cilia-like structures and polycystin-1 in osteoblasts/osteocytes and associated abnormalities in skeletogenesis and Runx2 expression. *J. Biol. Chem.* 281: 30884–30895.