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The oestrogen pathway underlies the evolution of exaggerated male cranial shapes in *Anolis* lizards

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Sexual dimorphisms vary widely among species. This variation must arise through sex-specific evolutionary modifications to developmental processes. *Anolis* lizards vary extensively in their expression of cranial dimorphism. Compared with other *Anolis* species, members of the *carolinensis* clade have evolved relatively high levels of cranial dimorphism; males of this clade have exceptionally long faces relative to conspecific females. Developmentally, this facial length dimorphism arises through an evolutionarily novel, clade-specific strategy. Our analyses herein reveal that sex-specific regulation of the oestrogen pathway underlies evolution of this exaggerated male phenotype, rather than the androgen or insulin growth factor pathways that have long been considered the primary regulators of male-biased dimorphism among vertebrates. Our results suggest greater intricacy in the genetic mechanisms that underlie sexual dimorphisms than previously appreciated.

1. Introduction

Sexual dimorphisms among animals come in many different forms [1]. Differences in the primary reproductive organs are necessary for sexual reproduction. Secondary sexual variation in weapons (e.g. horns, antlers and tusks) and differences in body size are often exaggerated in species that exhibit substantial male–male competition [2,3]. Many species also exhibit colour or acoustic dimorphisms, whereby males show off brightly coloured ornaments or elaborate songs to attract females [4,5]. Furthermore, males and females can differ significantly in their body proportions, which can, in turn, have important ecological implications for the sexes [6,7]. The distinct forms of sexual selection—whether intrasexual combat, intersexual resource competition or intersexual choice—that shape evolutionary patterns of sexual diversity have been widely studied [8–12]. However, the developmental mechanisms by which these diverse patterns arise are less well understood, particularly among vertebrates [13].

Most secondary sexual characteristics in vertebrates develop after hatching/birth through tissue-specific changes in trait growth. While body size dimorphism is regulated by differences in circulating hormone levels [11,14,15], localized differences in growth are regulated by sex- and tissue-specific levels of hormonal receptors that translate into downstream changes in signalling and patterning molecules. Therefore, to understand anatomically mosaic patterns of sexual diversity, we must more thoroughly examine evolutionary changes in hormonal *receptor* levels in distinct target tissues, as it is the receptors that moderate the tissue-specific response to globally circulating signals.

Two distinct molecular pathways have been consistently found to regulate the development of male-biased dimorphism. Differential expression of the androgen pathway (i.e. testosterone and its derivatives) underlies the development of exaggerated male traits in many vertebrate lineages, including the lion's mane [16], swordtail swords [17], and dimorphism in body size and secondary sexual traits
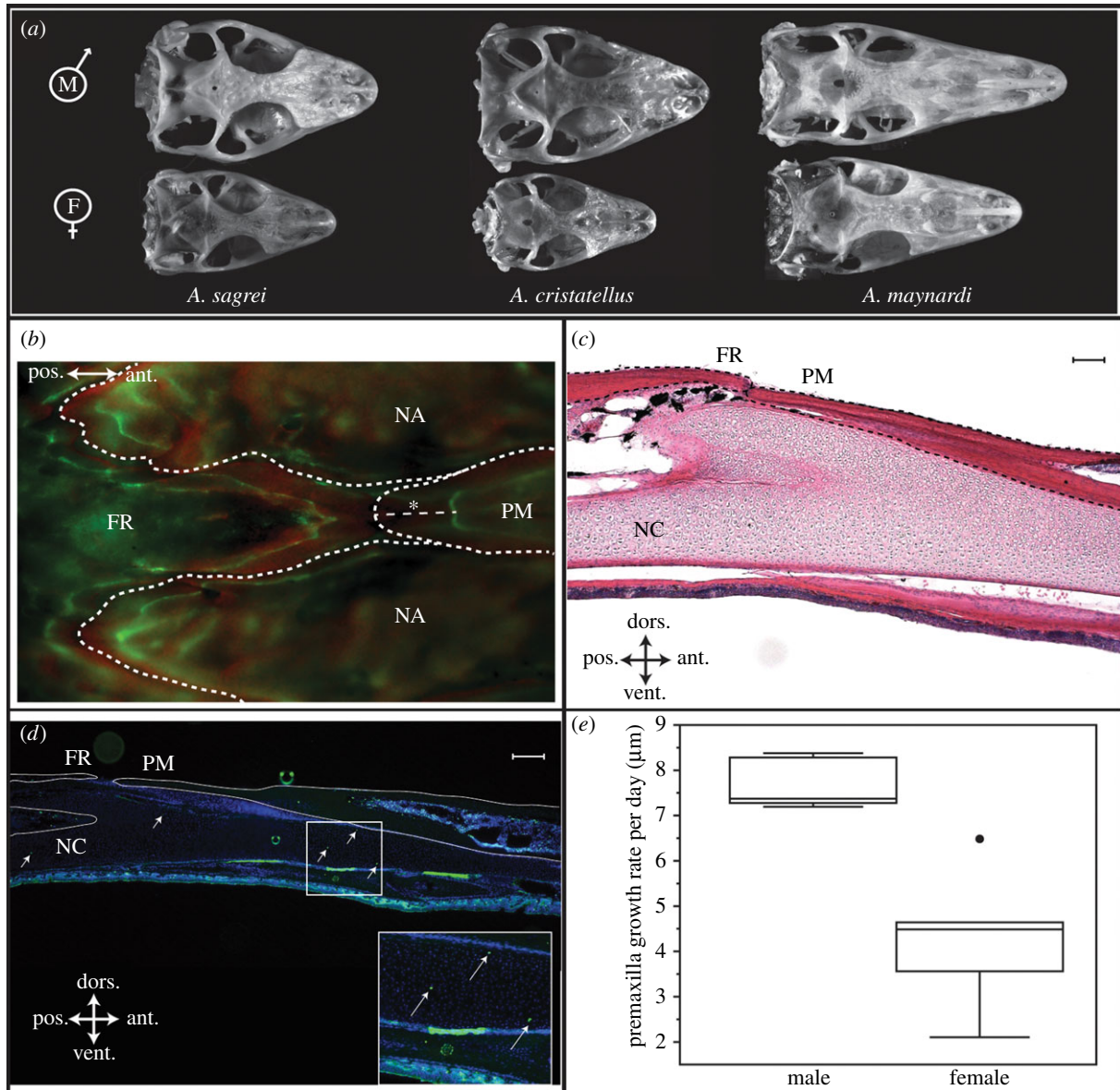
Figure 1. The evolution and development of craniofacial dimorphism in *Anolis* lizards. (*a*) Many *Anolis* species, such as *Anolis cristatellus* and *Anolis sagrei*, exhibit considerable size dimorphism, but low to moderate levels of shape dimorphism. *Anolis cristatellus* and *A. sagrei* have average facial length to skull width ratios of 1.02 between the sexes (male to female). The *carolinensis* clade of anoles evolved extreme shape dimorphism as males evolved extraordinarily long faces relative to the conspecific females. The *carolinensis* clade species *Anolis maynardi* represents one of the most dimorphic anole species in regard to facial length, obtaining average facial length to skull width ratios of 1.25. (*b*) Calcein – alizarin (green fluorescence, red fluorescence, respectively) pulse labelling reveals that facial elongation occurs throughout the adult face but is greatest at the nasal (NA) – frontal (FR) – premaxilla (PM) suture. Staining of a sagittal cross section of the elongating anole face (*c*) also reveals that anoles lack a discrete, morphologically defined growth region in their nasal cartilage (NC) as there is in postnatal mammalian nasal cartilage. (*d*) Proliferation in the nasal cartilage is not localized but is instead found throughout the nasal cartilage as indicated by EdU positive cell nuclei (green). (*e*) Comparison of the growth rates of the premaxilla (* in (*b*)) between males and females reveals that males elongate their faces at nearly twice the rate as females, but still only reach maximum elongation rates of approximately 9 μm d^{-1}.

in lizards [11,15]. Alternatively, the insulin-like growth factor (IGF) pathway is thought to produce an honest signal of male condition owing to its sensitivity to nutritional condition, such as in the development of exaggerated rhinoceros beetle horns [18], deer antlers [19] and avian size dimorphism [20].

Caribbean *Anolis* lizards, or anoles, represent a textbook example of adaptive phenotypic diversification [21]. Anoles also vary extensively in the pattern and magnitude of sexual dimorphism [7,10,22] particularly in their head dimensions (figure 1*a* [23]). Ancestrally, anoles possessed skulls of moderate proportion and low levels of sexual dimorphism, later diverging along multiple cranial dimensions during their adaptive diversification [23,24]. Several lineages of

anoles have evolved pronounced levels of cranial dimorphism through male-specific changes in facial length. Facial length dimorphism arises through two distinct developmental strategies: a mode of development whereby males and females diverge shortly after hatching, which is ancestral to *Anolis*, or a differing strategy in which males and females diverge late, following sexual maturity (electronic supplementary material, figure S1; [23]). A single clade, the *carolinensis* clade, uses this pattern of late divergence with males and females not reaching their highest levels of divergence until 1–2 years after sexual maturity. We wanted to investigate whether this novel and dramatic phenotype— the elongated face in *carolinensis* clade males following

sexual maturity–could also be explained in terms of a novel molecular mechanism, powerful enough to modify the constraints of a long-established ancestral pattern. More specifically, we tested the hypothesis that modifications to the androgen or IGF pathways underlie the emergence of exaggerated dimorphism in the *carolinensis* clade of anoles.

2. Material and methods

Details of our experimental methods are described in the electronic supplementary materials. An overview of our methodology is described below.

(a) Species selection and morphological measurements

We compared cranial and post-cranial levels of sexual dimorphism for 30 *Anolis* species using a combination of linear and geometric morphometrics [25]. Sexual dimorphism in head shape was calculated using geometric morphometric analysis following Sanger *et al.* [23]. We calculated post-cranial measurements of dimorphism from: (i) a set of five linear measurements (snout to vent length, hindlimb length, forelimb length, pectoral width, and pelvic width), and (ii) by counting the number of foot and hand lamellae. All variables were log transformed and size corrected prior to subsequent analysis. We used principal component (PC) analysis to extract the primary axes of shape variation from the morphometric data. Following Sanger *et al.* [23], we then calculated sexual dimorphism for both cranial and post-cranial datasets as the Euclidean distance between males and females of each species taking into account all significant PC axes.

(b) Proliferation assay, histology and pulse labelling

Among amniotes, developmental mechanisms of facial elongation at the cellular and molecular levels have only been previously examined in the laboratory mouse, *Mus musculus* [26]. To obtain a better understanding of the mechanisms of facial elongation in *Anolis*, we examined patterns of facial growth, histology of the nasal cartilage and patterns of proliferation and hypertrophy in the emerging model species, *Anolis carolinensis*. To assess ossification patterns associated within the elongating anole face, we administered calcein (green fluorescence) 30 days prior to sacrifice followed by alizarin red complexone (red fluorescence) 24 h prior to sacrifice. The distance between green and red labels, therefore, represents the amount of growth that occurred for each skeletal element between pulses. We compared facial elongation rates between adult male and adult female green anoles using a two-tailed *t*-test on growth of the premaxilla (figure 1*b*).

(c) Cloning and *in situ* hybridization

To prepare riboprobes for *in situ* hybridization (ISH), we cloned 500–1000 base pair fragments of the receptors for the five major hormonal pathways from embryonic *A. carolinensis* cDNA: androgen (*ar*), oestrogen (*erα* and *erβ*), IGF (*igfr1*), growth hormone (*ghr*) and parathyroid hormone (*pth1r*). We performed ISH on 12 μm cryo-sections using digoxigenin-labelled riboprobes following Abzhanov [27].

(d) Tissue collection and quantitative real-time PCR

To understand the relative significance of the different regulatory pathways in facial elongation, we compared expression levels of hormonal receptors between males and females for representative species possessing the ancestral or derived developmental strategies: two non-*carolinensis* species, *Anolis cristatellus* and *Anolis sagrei*, and a species from the *carolinensis* clade, the green anole, *A. carolinensis*. Of the species within the *carolinensis* clade,

A. carolinensis was chosen, in part, because of its growing genomic resources [28]. *Anolis sagrei* and *A. cristatellus* have independently converged on the short-faced morphology [24] and relatively low levels of facial length dimorphism [23]. We prepared cDNA libraries from the growing facial skeleton of the three anole species at the juvenile, subadult and adult stages to fully capture time periods when sexual differentiation is occurring in each developmental strategy (electronic supplementary material, figure S2). We compared relative gene expression between the sexes and between stages using real-time PCR. Gene expression levels were assayed using an Eppendorf Mastercycler using SYBR green with 40 cycles of amplification. Gene expression was assayed in triplicate for each sample and normalized for *gapdh* and β-actin. Finally, we analysed the expression data using the comparative CT method [29].

3. Results and discussion

(a) Mosaic patterns of dimorphism

To understand whether the levels of dimorphism are body-wide, suggesting a globally acting regulatory mechanism, or cranial-specific, suggesting a locally acting mechanism, we compared cranial and post-cranial levels of dimorphism. Our analyses reveal that levels of cranial and post-cranial dimorphism are only weakly correlated among anoles illustrating a mosaic pattern of secondary sexual trait evolution during anole diversification (electronic supplementary material, figure S2; $r^2 = 0.23$, phylogenetic regression $p = 0.008$). Further details of the morphometric analyses are presented in the electronic supplementary material, tables S1–S3.

(b) Mechanisms of facial elongation

The developmental mechanisms of facial elongation in *Anolis* are distinct from those previously described in the laboratory mouse model species, *M. musculus*. In the laboratory mouse, postnatal facial elongation is localized to the region of the nasal–frontal suture [26]. In this region, facial outgrowth is driven by an underlying growth plate in the nasal septum comprised organized proliferative and hypertrophic chondrocytes [26]. By contrast, facial elongation in *Anolis* is not localized and the underlying nasal cartilage lacks an organized growth plate (figure 1*b,c*; electronic supplementary material, figure S3). There is also no evidence of hypertrophic chondrocytes, as identified using ISH for Collagen X (electronic supplementary material, figure S4), and proliferative cells are distributed throughout the nasal cartilage (figure 1*d*). Within sexually mature *A. carolinensis*, the sum of chondrocyte proliferation in the nasal cartilage yields an elongation rate in males that is approximately twice as fast as that observed in females (figure 1*e*; two-tailed *t*-test $p < 0.001$). The developmental differences observed between *A. carolinensis* and *Mus* may reflect differences in the organization of skull between anoles and mice (e.g. skeletal arrangement, growth rates, etc.) or fundamental evolutionary differences in the mechanisms of skeletal development between the two clades. Further research into the mechanisms of amniote skeletal growth is needed to more thoroughly resolve these alternatives.

Despite lack of an organized cellular growth zone, receptors for the five major hormonal signalling pathways are expressed in relatively restricted, yet overlapping domains of the nasal cartilage anterior to the nasal–frontal–premaxilla suture region (figure 2).
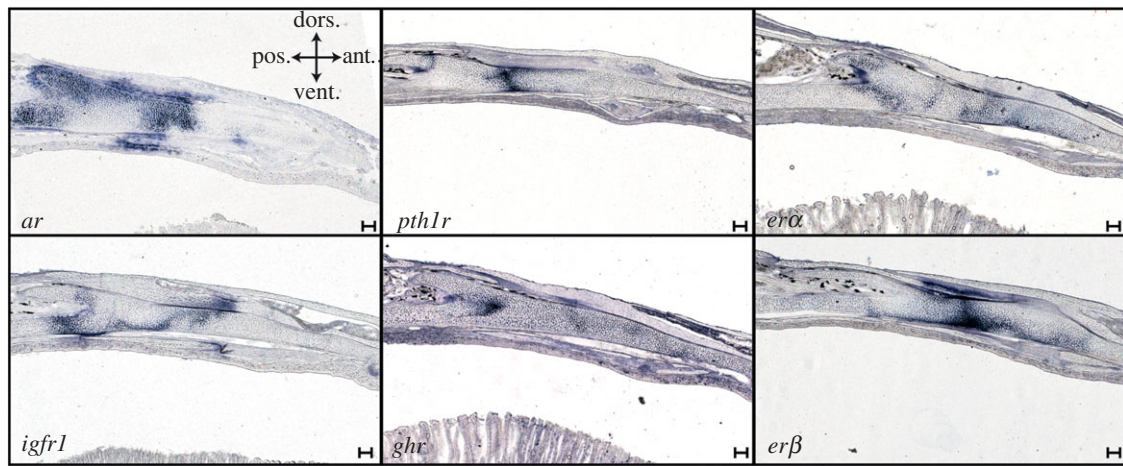
Figure 2. The expression patterns of hormonal receptors in adult male *A. carolinensis* reveal that all six pathways coordinate to regulate growth of the anole face. All six hormonal receptors are expressed in distinct, but overlapping, domains of the nasal septum and perichondrium below and anterior to the nasal–frontal–premaxilla suture.

(c) Sex-specific gene expression of hormonal receptors

Our analysis revealed that neither *ar* nor *igfr1* could be directly responsible for the regulation of facial length dimorphism observed in *Anolis* lizards, as they are not differentially expressed between males and females at any stage (figure 3*a*; electronic supplementary material, table S4). Instead, our analysis found large differences in expression of an oestrogen receptor, *erβ*, which was specific to the subadult and adult stages of *A. carolinensis* when facial length dimorphism is developing. Such differential expression of *erβ*, or any other hormonal receptor studied, was not found at any stage in *A. sagrei* or *A. cristatellus* (figure 3*b,c*). Furthermore, adult long bone epiphyses do not exhibit sex-specific differences in expression of these hormonal receptors, indicating the observed difference in *erβ* is not a systemic difference found throughout the developing skeletons of male and female green anoles (electronic supplementary material, table S5). The time-, tissue- and species-specific nature of *erβ* expression suggests that the oestrogen pathway may be the hormonal pathway underlying the evolution of male-biased dimorphism in the *carolinensis* anoles.

The skeleton is an endocrine organ that locally regulates the effects of the sex steroid and IGF pathways [30,31]. Differences in regulation of hormonal pathways in skeletal tissues can, therefore, also result from sex-specific expression of accessory molecules that are required for signalling downstream of the hormonal receptors. To further test whether such changes related to the androgen or IGF pathways could be responsible for the evolution of pronounced dimorphism in the *carolinensis* anoles, we screened the relative expression levels of metabolic enzymes responsible for metabolizing steroid hormones in the target tissues and of nuclear co-activators needed to produce active transcriptional complexes in the nucleus. We found that neither the metabolic enzymes responsible for locally processing androgens and oestrogens, 5α reductase and aromatase, respectively, nor the steroid nuclear co-activators, *src1* and *cbp*, show differential expression throughout adulthood despite minor variation in expression during the period of sexual maturation (electronic supplementary material, table S7). Likewise, the IGF accessory molecules, *igfbp5* and *foxo1*, which help regulate insulin sensitivity in developing skeletal tissues [32,33], also

show no consistent signature of differential expression in the elongating facial tissues (electronic supplementary material, table S6). These observations further demonstrate that the androgen and IGF pathways are probably not involved in regulation of the differential facial elongation through molecules downstream of the hormonal receptors.

The effects of oestrogen on lizard skeletal growth have not previously been examined, but in mammals precise temporal regulation of the oestrogen pathway is critical for proper longitudinal and cortical bone growth in both males and females [34,35]. *erβ*, in particular, mediates growth plate fusion in young adult female mice, effectively eliminating long bone elongation [36]. To better understand the temporal dynamics of hormone receptor regulation in annoles, we compared expression levels for hormone receptors between juvenile and adult stages. In all three species examined, most hormone receptors show a temporal decrease in expression or similar expression levels over ontogeny, consistent with decreasing rates of growth following sexual maturity (electronic supplementary material, table S7). But only in *A. carolinensis* does *erβ* exhibit different temporal patterns between the sexes, being temporally upregulated in females and temporally downregulated in males. Therefore, we hypothesize that increased *erβ* in females at the time of sexual maturity reduces their rate of facial elongation relative to males, resulting in the male-biased dimorphism present in *carolinensis* species.

(d) Sex-specific gene expression of signalling and skeletogenic molecules

To regulate the growth of a skeletal trait, hormonal receptors must activate a downstream cascade of signalling molecules and skeletogenic transcriptional factors. To elucidate some of the potential downstream targets of oestrogen signalling in the face, we surveyed relative expression levels in a panel of genes known to be involved with facial morphogenesis and skeletal growth in the three *Anolis* species. Four of six signalling and patterning molecules measured—*bmp4*, *bmp2*, *msx2* and *ihh*—exhibited differences in expression levels between male and female *A. carolinensis* (table 1). While these differences are relatively subtle, ranging from only
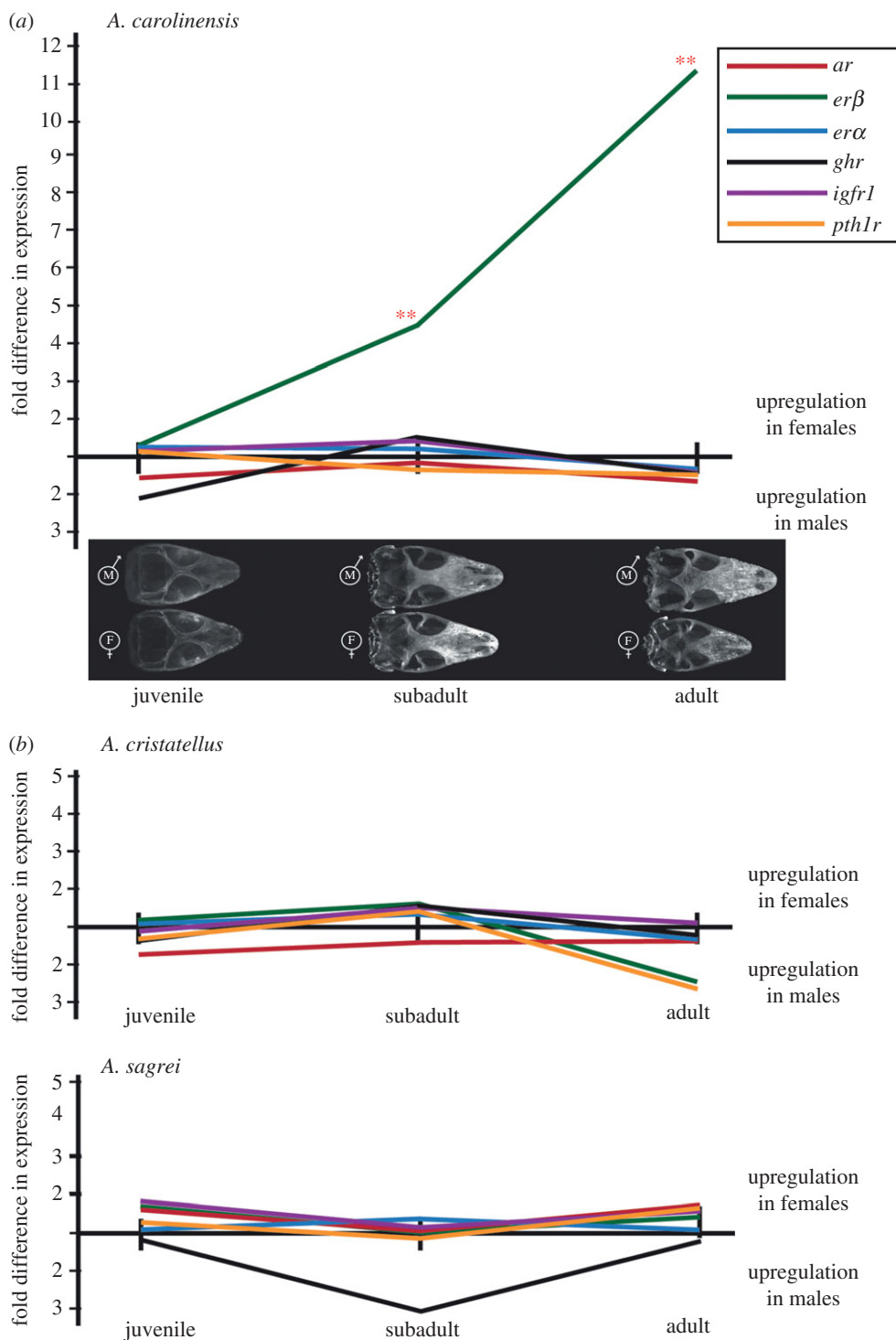
Figure 3. Comparison of male and female hormone receptor levels over ontogeny. Skulls of male (M) and female (F) *A. carolinensis* at each ontogenetic stage are depicted. There is no evidence that the androgen or IGF pathways are responsible for the development of facial length dimorphism in *A. carolinensis* at any stage. The only significant difference between male and female green anoles at the time of sexual differentiation is in the differential expression in *erβ* (*a*). This difference is species-specific, not being present in either *A. cristatellus* or *A. sagrei* (*b*). The lack of significant expression differences in juveniles of the non-*carolinensis* species suggests that subtle differences in facial length dimorphism arise through the combination of many relatively small differences in receptor levels, through differential expression of the circulating molecules or through downstream skeletogenic factors. Statistical details are summarized in the electronic supplementary material (* denotes $p < 0.001$, table S4).

1.4–1.7-fold, they are not found in either *A. sagrei* or *A. cristatellus*. The differential expression of these signalling molecules correlates with downstream effects on genes associated with skeletogenesis; both *spp1* and *col II* exhibit an approximately twofold relative increase in expression in male *A. carolinensis*, consistent with their elevated rate of male facial elongation (table 2). These expression differences are also specific to *A. carolinensis* and are not found in the other species examined.

(e) Experimental summary

Our analyses illustrate that extreme male-biased facial length dimorphism in the *carolinensis* clade of anoles has evolved through regulatory changes that resulted in novel sex- and clade-specific expression of the oestrogen pathway gene network (figure 4). More specifically, within developing *A. carolinensis* males, relatively low levels of *erβ* responding to relatively low levels of circulating oestradiol lead to a cascade of molecular signalling events, which in turn lead to

Table 1. Differential expression of signalling and patterning molecules between adult males and females of three anole species. (Though the precise function of these molecules is not yet known in anoles, note that only in *A. carolinensis* are these molecules differentially expressed, consistent with the observed differential rates of facial elongation. Significant results are highlighted in italics.)

signalling and patterning molecules						
	A. carolinensis (5m/6f)		*A. sagrei* (4m/4f)		*A. cristatellus* (4m/4f)	
gene	fold change	*p*-value	fold change	*p*-value	fold change	*p*-value
bmp4	*1.6F*	*0.010*	1.0M	0.838	1.2M	0.643
mbp2	*1.7M*	*0.001*	1.2F	0.315	1.2M	0.420
msx2	*1.6M*	*0.037*	1.1F	0.498	1.3M	0.600
ihh	*1.4F*	*0.003*	1.2F	0.617	1.3F	0.399
tgfβr2	1.08F	0.324				
dkk3	1.35F	0.812				

Table 2. Differential expression of skeletogenic molecules between adult males and females of three anole species. (Note that in only *A. carolinensis* are these molecules differentially expressed, being consistently unregulated in males. Significant results are highlighted in italics.)

skeletogenic molecules						
	A. carolinensis (5m/6f)		*A. sagrei* (4m/4f)		*A. cristatellus* (4m/4f)	
gene	fold change	*p*-value	fold change	*p*-value	fold change	*p*-value
spp1	*2.1M*	*0.020*	1.6M	0.672	1.1M	0.770
col I	2.1M	0.101	1.0M	0.904	1.2F	0.349
col II	*2.3M*	*0.002*				

increased rates of facial elongation relative to females. Such shift in expression of *erβ* following sexual maturity does not appear to represent a temporal transposition of earlier differentiation mechanisms and represents a novel mechanism of sexual differentiation among anoles, specifically, and vertebrates more broadly. Our analyses of *Anolis* skull shape dimorphism illustrate that although clades may possess 'homologous series in variation' [23,24,37] over macroevolutionary time scales, approximately 40 Myr in this case, novel developmental strategies will occasionally be recruited to overcome otherwise conserved evolutionary constraints.

4. Emerging perspectives on the evolution and development of sexual dimorphism

Studies investigating the developmental bases of morphological evolution have significantly expanded in recent decades [38–40], yet the mechanisms controlling sexually dimorphic characters and their evolution have not received the same detailed attention. Extensive research has focused on how the evolution of body size dimorphism depends on circulating hormone levels [11,14,41], but this type of analysis is insufficient to explain anatomically mosaic patterns of sexual trait evolution. Localized changes in body proportion must arise through evolutionary modifications to sex- and tissue-specific regulation of hormone receptors, nuclear regulatory molecules and downstream molecules. As many secondary sexual traits represent some of the most exaggerated and rapidly evolving traits found in nature, understanding the details of their development and evolution may provide novel insights into the mechanisms that regulate the production of phenotypic diversity.

Recent investigations, including this study, have focused on developmental networks to uncover a diversity of previously unappreciated cellular and molecular mechanisms that regulate secondary sexual variation in animals. In many vertebrates, differences in circulating testosterone levels regulate size dimorphism [11,14,42]. However, sex-specific oestrogen signalling is also needed to explain variation in the second-to-fourth digit ratio in mammals (2D : 4D; [43]) and facial length dimorphism in *Anolis* lizards (this study). Among many insects, sex-specific levels of juvenile hormone regulate body size dimorphism [44], but cannot alone explain sexually dimorphic traits such as mandible size in stag beetles [45]. In this latter case, sexual dimorphism in mandible length results from sex- and tissue-specific expression of *Doublesex* isoforms conveying differential sensitivity to juvenile hormone signalling [46]. Likewise, sexual dimorphism in horned beetles is also regulated by the *Doublesex* locus [47], although differences in horn allometry among males are regulated by a distinct upstream factor, the nutrition-sensitive insulin pathway [18,48].

Each of these examples highlights that independent examination of circulating hormones and signalling molecules provides only a partial picture of the mechanisms regulating secondary trait development. Considering that secondary sexual characters result from the interaction of globally and locally acting signalling factors, determining how these developmental networks—from hormone to hormonal
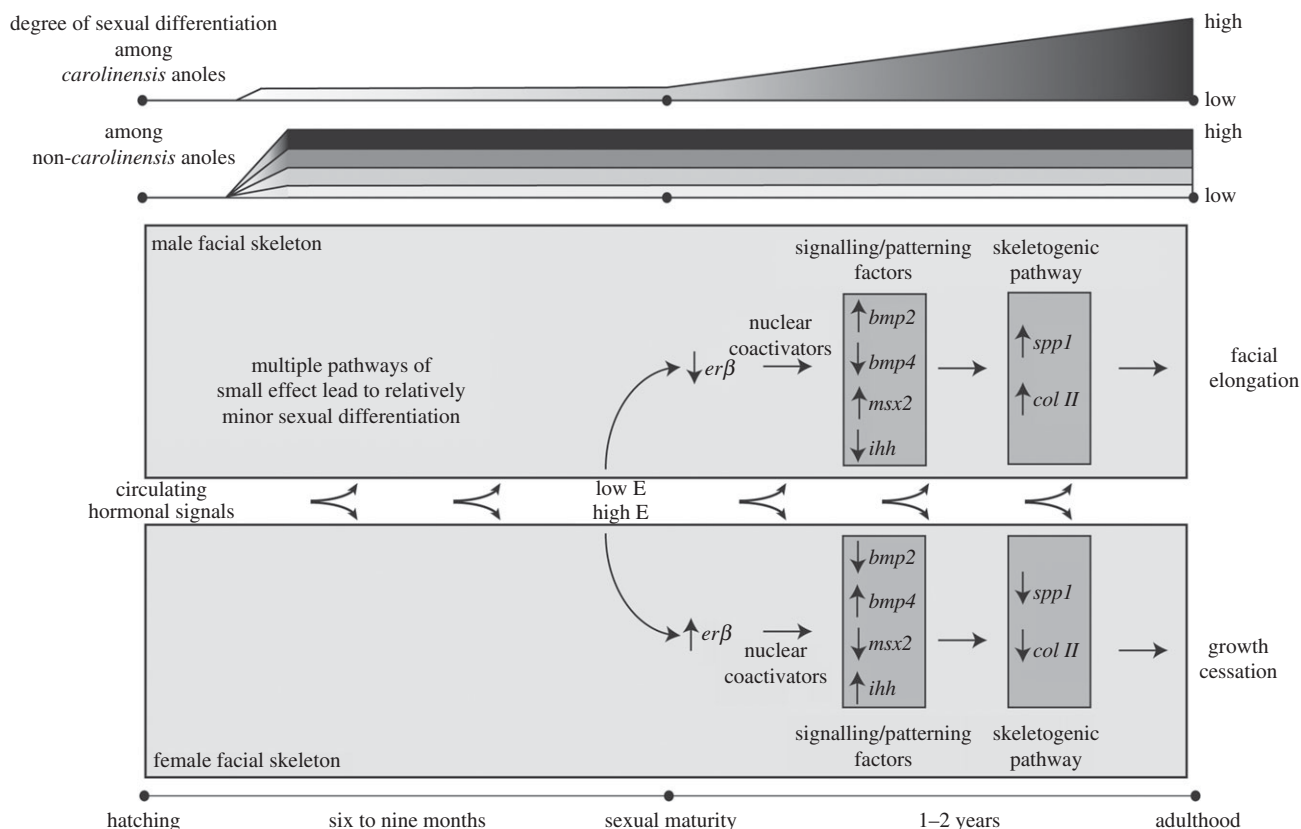
Figure 4. The molecular pathway underlying the development of facial length dimorphism in *A. carolinensis*. Regardless of the magnitude of facial length dimorphism (grey to black gradient), facial length dimorphism in most anole species develops during a discrete period early in post-hatching ontogeny. By contrast, dimorphism in the *carolinensis* anoles develops slowly, over 1–2 years, following sexual maturation. Our analyses reveal that facial length dimorphism in the green anole develops using evolutionarily novel, sex-specific expression of the oestrogen pathway. More specifically, the differential expression of *erβ* leads to downstream expression differences in patterning and skeletogenic markers producing elongation of the male face and cessation of growth in the females. These differences are adult stage-specific and are not the result of temporal shifts in gene expression from earlier ontogenetic stages.

receptors to signalling and patterning molecules—evolve in response to sexual selection pressures is one of the most pressing challenges ahead.

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