Ancestral androgenic differentiation pathways are repurposed during the evolution of adult sexual plasticity

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SUMMARY

Although early exposure to androgens is necessary to permanently organize male phenotype in many vertebrates, animals that exhibit adult sexual plasticity require mechanisms that prevent early fixation of genital morphology and allow for genital morphogenesis during adult transformation. In Lythrypnus dalli, a teleost fish that exhibits bi-directional sex change, adults display dimorphic genitalia morphology despite the absence of sex differences in the potent fish androgen 11-ketotestosterone. Based on conserved patterns of vertebrate development, two steroid-based mechanisms may regulate the early development and adult maintenance of dimorphic genitalia; local androgen receptor (AR) and steroidogenic enzyme expression. Consistent with the ancestral pattern of AR expression during the multipotential phase of differentiation, juvenile differentiation into either sex involved high mesenchymal AR expression. In adults, AR expression was high throughout the male genitalia, but low or absent in females. Consistent with the hypothesis that adult sexual plasticity repurposes pathways from primary differentiation, we show that adults with transitioning genitalia also exhibited higher AR expression relative to females. Local androgen biosynthesis may also participate in genitalia transformation, as transitioning adults had greater 11β-HSD-like immunoreactivity in the epithelial layer of the dorsal lumen compared to both sexes. By administering an AR antagonist to adult males, we show AR is necessary to maintain male-typical morphology. In a species that is resistant to early sexual canalization, early androgenic differentiation mechanisms are consistent with other vertebrates and the tissue-specific regulation of AR expression appears to be repurposed in adulthood to allow for transitions between sexual phenotypes.

INTRODUCTION

In the majority of vertebrates, exposure to androgens during a pre/perinatal sensitive period masculinizes critical components of reproductive phenotype (Murashima et al. 2014). One result of this organizational process is the fixation or canalization of sexual phenotype, such that transitions between sexual states are mechanistically blocked for life. For instance, exogenous administration of androgens to female mammals during the sensitive period leads to the development of male-typical external genitalia; however, after this time point, their morphology is unaffected by androgens (Phoenix et al. 1959; Young et al. 1964; Roberts et al. 2008). These masculinizing effects occur through a highly conserved androgenic pathway wherein androgen binding to androgen receptors (AR) in bi-potential tissues initiates the genital outgrowth that is characteristic of male development. Mutations that lead to disruption of normal androgen signaling adversely affect male sexual differentiation (Murakami 1987; De Bellis et al. 1994). Specifically, elevated local synthesis of both potent androgens (Levine et al. 1996) and AR within the genitalia is necessary for the induction of growth factors (e.g., Sonic hedgehog and Wnt) that regulate genital morphogenesis during male sexual differentiation (Gonzalez-Cadavid et al. 1991; Miyagawa et al. 2009). As growth arrests at sexual maturity in mammals, AR declines to a low constitutive level, and disruption of androgen signaling cannot reverse genital phenotype (Gonzalez-Cadavid et al. 1991; Miyagawa et al. 2009). Activation of this androgenic pathway is necessary for the development of both external genitalia morphology and the internal structures that facilitate sperm transfer in almost all vertebrates, thereby ensuring that genital structure and function can be coordinated with reproductive behavior in adult males.

Unlike mammals that canalize genital morphology early in development, reproductive phenotype remains plastic throughout adulthood in numerous teleost fishes (Ghiselin 1969; Warner et al. 1975). As organizational fixation limits sexual fate, the release from this early canalization is necessary to allow for phenotypic transitions during adulthood in sequential hermaphrodites. One mechanism that could allow for life-long sexual
plasticity is the novel utilization of ancient pathways that regulate key aspects of reproductive phenotype during early sexual differentiation. Specifically, androgens and AR may still regulate male genital morphology in sexually plastic species, but the lifelong regulation of this signaling pathway is now controlled via novel regulatory mechanisms (e.g., the Variable Signal Model, Rodgers et al. 2007).

*Lythrypnus dalli*, a highly social, bi-directionally hermaphroditic fish, provides several advantages for examining whether both initial sexual differentiation and adult sex change utilize the ancestral vertebrate androgenic differentiation pathway to generate male-typical genital morphology. First, similar to most vertebrates, the external genitalia (genital papillae, GP) of *L. dalli* is sexually dimorphic (Fig. 1). Relative to females, the male GP is longer, and tapered at the end (Fig. 1). Second, *L. dalli* genitalia have two internal chambers composed of mesenchymal and epithelial tissue that are arranged in a manner consistent with the external genitalia of most vertebrates. Third, although primary sexual differentiation and adult sex change are regulated by social factors in *L. dalli* (Rodgers et al. 2007; Solomon-Lane et al. 2016), administration of the potent fish androgen, 11-ketotestosterone (KT) induces rapid reorganization and masculinization of GP morphology in adult females (Carisle et al. 2000; Pradhan et al. 2014). Paradoxically, KT concentrations are not sexually dimorphic in this species, so the maintenance of male genitalia is not dependent on “male typical” androgen levels. There is a transient increase in KT levels during female to male sex change (Lorenzi et al. 2008, 2012), so the induction of male genitalia may still require increases in androgens. As *L. dalli* possess many of the androgenic signaling mechanisms that characterize the ancestral pattern of vertebrate sexual differentiation, the repurposing of these conserved endocrine mechanisms may circumvent early fixation of sexual phenotype and facilitate genital transformation in adults.

One proposed mechanism of masculinization in hermaphroditic fishes is via an estrogen-mediated sex determination pathway. In this estrogen-based model, androgens are suggested to regulate adult masculinization during phenotypic transitions by directly inhibiting gonadal aromatase (cyp19a1a) gene expression, and thereby reducing the conversion of testosterone to 17β-estradiol (Kroon et al. 2005; Guiguen et al. 2010). Data from several previous studies on *L. dalli* are not consistent with the estrogen-based model (Black et al. 2005; Lorenzi et al. 2008, 2012). An alternative mechanism for the generation of male typical genital morphology during adult phenotypic transitions is the repurposing of the ancestral androgen-mediated differentiation pathway via capture by a novel regulatory mechanism (Rodgers et al. 2007). By recruiting this early ontogenetic pathway for genital differentiation, sex differences in the local expression of AR and KT synthesis (via 11β-hydroxysteroid dehydrogenase [11β-HSD2] regulation) in genitalia of *L. dalli* might be a key mechanism by which anatomical dimorphism is maintained or reversed.

In this study, we examined whether *L. dalli* uses the conserved vertebrate androgenic signaling pathway to initiate and/or maintain male-typical genital morphology. We first determined whether initial sexual differentiation in this sexually plastic species utilizes the conserved pathway to masculinize the external genitalia. In other vertebrates, mesenchymal androgen signaling is necessary for the induction of growth factors that are critical for the initiation of genitalia outgrowth (Cooke et al. 1991; Miyagawa et al. 2009). If genital masculinization follows the conserved pattern, then AR expression should be elevated in the mesenchyme and low or absent in GP epithelium early in male differentiation (Crocoll et al. 1998; Murashima et al. 2014; Zheng et al. 2015). Furthermore, if this pathway is being repurposed during sexual differentiation in *L. dalli*, then

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**Fig. 1.** Location and structural differences in the external genitalia, the genital papilla, of *L. dalli*. (A) Location of the genital papillae on a male *L. dalli*. (B) Female-typical genital papilla can transform, via a transitional state, to a male-typical morphology. Genital papilla morphology is quantified by calculating the ratio of length (L) to width (W) of the ventral surface of the genital papilla. R, rostral and C, caudal, (C) Conceptual figure exhibiting approximate population averages. Fish with a L:W >1.6 (solid line) have male typical genital papilla, whereas those with L:W <1.4 (dashed line) have a female typical genital papilla. Transitional fish have L:W ratios between 1.4 and 1.6.
mesenchyme–epithelial interactions should be necessary for normal male differentiation to occur (Cooke et al. 1991; Miyagawa et al. 2009). Thus, we expect to see a shift from mesenchymal expression to elevated epithelial expression during lumen differentiation (Crocoll et al. 1998).

Second, we sought to determine whether the ancestral androgenic pathway is being repurposed in a novel manner in adult *L. dalli*. This mechanism would permit the initiation of genital morphogenesis during sex change and maintain male-typical physiology and anatomy in the absence of sex differences in systemic KT levels. Specifically, high expression throughout the GP in males may maintain structure and function in the absence of dimorphic KT. Along the same lines, low or absent AR within the mesenchyme of females may prevent the masculinizing effects of androgens in a fish that does not canalize phenotype. As there is a transient increase in KT during natural sex change (Lorenzi et al. 2012), both local KT synthesis via 11ß-HSD2 and elevated AR expression may be necessary to masculinize genital phenotype during this process. If this pathway is repurposed, elevated expression in the mesenchyme at the time of transient increases in KT may promote GP morphogenesis during adult sex change. To further examine the importance of AR in maintaining male genital morphology, we disrupted normal androgen signaling by administering an androgen receptor antagonist and then looked for evidence of genital demasculinization.

**METHODS**

**General methods**

Adult and juvenile *L. dalli* were collected off the coast of Catalina Island, California between April and July of 2013 and 2014 using hand nets (permit number #SC-11879). Juveniles were split into three groups (undifferentiated, differentiating males, and differentiating females) based on gonadal tissue in stained cross sections, GP morphology, and size (Solomon-Lane et al. 2016). To confirm that animals were in an undifferentiated state, we took additional sections that were more anterior and posterior to the location of the GP to assess the presence or absence of gonadal tissue. Differentiating animals were smaller than adults and were characterized on the basis of their sex typical GP morphology (Solomon-Lane et al. 2016). We did not ascertain the exact age of field collected juveniles, but all of the animals had recruited from the plankton earlier that summer.

We also selected adult male, female, and transitional animals based on body size and GP morphology (detailed in Fig. 1). Transitional animals were further characterized by intermediate GP morphology (St. Mary 1993, 1994; GP L:W of approximately 1.4) and presence of both sperm and eggs in the gonad (Fig. 1). As adult fishes of either sex can have gonads containing 1% or greater gametes of the opposite sex (St. Mary 1994, 1998), we only selected animals that had a GP between 1.4 and 1.6 and exhibited >30% of the minority sex gonad. After selection, each fish was immediately euthanized via exposure to a lethal dose of tricaine methanesulfonate (MS-222). At this time, measurements including standard length (SL, mm), body mass (g), and captured digital images of the GP (Motic Image 2.0) were taken for each animal. To calculate GP length (L) to width (W) ratio (L:W ratio), we measured papilla length and the width half-way along this length, as indicated in Figure 1. All fish were immersion fixed in 4% paraformaldehyde overnight, cryopreserved in 30% sucrose in phosphate buffer (PB), and stored briefly at 4°C before cryosectioning. All experiments were conducted in accordance with the Animal Care and Use Committee at Georgia State University.

**Experiments 1 and 2: immunohistological localization of AR and 11ß-HSD2 in juvenile and adult genitalia**

To assess the developmental patterning of androgen signaling in the developing genitalia of *L. dalli*, undifferentiated fish (*n* = 5), differentiating males (*n* = 7), and differentiating females (*n* = 9) were sectioned at 20 µm in two series on a cryostat. Following sectioning, slides were stored at −20°C until further processing. On the day of processing, slides were allowed to equilibrate to room temperature and sections were washed twice in 0.1M PB for 7 min each time. Sections were then incubated in a blocking solution (normal goat serum and 0.2% Triton-X in 0.1M PB) for 20 min. Next, the sections were incubated in primary antibody, AR (PG-21; Millipore, Billerica, MA); based on methods modified from Munchrath and Hoffmann (2010), diluted (1:250) in normal goat serum and 0.2% Triton-X in 0.1M PB and stored overnight at 4°C. This primary antibody has been well characterized in numerous teleost fish species and within a variety of tissues (Gustavson et al. 1994; Munchrath and Hofmann 2010; Pouso et al. 2010; Chakraborty et al. 2011). The following day, slides were rinsed twice in 0.1M PB for 7 min. and then incubated in biotinylated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) for 30 min. The sections were then twice rinsed in 0.1M PB for 7 min each and then incubated in streptavidin–peroxidase (Kirkegaard & Perry Laboratories) for 30 min. Immunolabeled cells were visualized using 3,3′-diaminobenzidine (Sigma Chemical). The sections were then dehydrated in an ethanol series through citrasolv, and cover slipped with permount. A similar procedure was used to label cells for 11ß-HSD2, except for the use of the Aviva, ARP41372_P050 antibody diluted at 1:500. To prevent differences in levels of staining, all juveniles and adults were processed separately, each in a single batch.

After immunostaining, we examined differences in AR and 11ß-HSD2-like labeling between adult male (*AR, n = 5*), female (*n = 6*), and transitional (*n = 4*) fish. For experiments...
1 and 2, control sections \( (n = 2 \text{ per sex}) \) were run by omitting the primary antibody step (Fig. 2C). For all sections, gonadal AR and 11\[\beta\]-HSD2-like expression was used as a positive control because both are highly expressed in reproductive tissues of many teleost fishes, including *L. dalli* (Pelletier et al. 2000; Chakraborty et al. 2009; Arterbery et al. 2010). Images were acquired using an Axioplan microscope and Axiovision software (Zeiss, Thornwood, NY). Androgen receptor expression was evaluated in three regions of interest (Fig. 2): (i) the epithelial lining the dorsal lumen of the GP; (ii) the epithelial lining the ventral lumen of the GP; and (iii) mesenchymal tissue containing the extracellular matrix of the GP. To allow for direct comparisons of our results to previous studies of AR expression in the external genitalia of developing mammals, the level of AR staining for each region was rated based on the following 0–3 scale: 0, no staining present; 1, mild staining (few positively stained cells); 2, moderate staining (approximately 20–50% of all cells in an area); and 3, abundant and intense staining (approximately 50–100% of cells in an area). This scale has been frequently used for evaluating region specific patterning of AR in developing mammalian reproductive tissues (Takane et al. 1991; Sonea et al. 1997; Kim et al. 2002; Sajjad et al. 2007). Evaluations of tissue staining were performed by two observers blind to the sex and age the animals. When assessing the degree of tissue staining observers distinguished between pigmented epithelium (PE) and DAB staining in the GP (Fig. 2, B/C), and observers only scored the degree of cell staining in the mesenchyme.

**Experiment 3: effect of systemic flutamide on genital morphology**

Social groups consisting of an adult male and subordinate females were constructed in the laboratory at the USC Wrigley Institute for Environmental Science on Catalina Island, California. Males were size matched between treatment groups, as previous research documented a strong association between SL and GP ratio in males (Grober, A.G. and Grober M.S., unpublished data). On the first day of the study, males were anaesthetized, and SL, mass, and GP images were acquired as

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**Fig. 2.** External genitalia morphology and immunohistochemical localization of the androgen receptor in undifferentiated *L. dalli*, differentiating males (D-top), and differentiating females (D-bottom). (A) Mean (±SE) differences in genital papilla length to width ratio (L:W) between undifferentiated fish \( (n = 5) \), differentiating females \( (n = 9) \), and differentiating males \( (n = 7) \). (B) Hematoxylin and eosin staining of a differentiating female depicting layers of the genital papilla. (C) Differentiating male genital papilla with primary antibody omitted. (D) Representation AR staining in cross sections of undifferentiated, differentiating male, and differentiating females papilla. (E) Mean (±SEM) level of androgen receptor (AR) staining within the dorsal epithelium, ventral epithelium, and mesenchyme in juvenile *L. dalli*. (F) Association between genital papilla length to width ratio (L:W) and intensity of AR staining within the mesenchyme. DE, dorsal epithelium; VE, ventral epithelium; M, mesenchyme; PE, pigmented epithelium. All images were taken under a 40× objective lens. Scale bar for cross sections = 50 \( \mu \)m. Asterisks indicate significant differences \( ^*P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001 \).
described above. While still anaesthetized, males were intraperitoneally injected with either 50 μg/g flutamide (an AR antagonist, \( n = 5 \)) or vehicle (0.1M PB, \( n = 6 \)) using a 28.5G needle (Becton Dickinson Lo-Dose). The flutamide dose was determined from previous research (O’Connor et al. 2002; Dang et al. 2011), as well as a pilot study conducted to determine an optimal dose for *L. dalli*. Following the injection, males were placed in a cup containing 100 ml sea water and allowed to recover for at least 15 min. Prior to returning males to their social groups, they were placed in a new 100 ml cup containing clean water to prevent potential flutamide contamination when being placed back in their tank. Each male was injected every day for 4 days, and GP pictures were taken every other day. On the afternoon of the 5th day, final measurements were taken, and animals were euthanized. ImageJ (NIH) was used to calculate GP L:W ratio (Fig. 1).

**Data analysis**

Multivariate analysis of variance (MANOVA) was used to examine differences in AR expression between undifferentiated and differentiating males and females. We utilized \( t \) tests to assess our a priori prediction that there should be tissue-specific differences in AR expression if *L. dalli* follows the conserved androgenic differentiation pathway. Additional MANOVAs were used to compare regional differences in AR or 11β-HSD2-like expression between adult male, transitional, and female animals. Linear regression analyses were used to examine the relationships between GP L:W and (i) SL and (ii) AR expression in the GP of juvenile and adult *L. dalli*. As GP ratio differed between groups at the start of the experiment, we used two paired sample \( t \) tests to examine the effect of each treatment on GP morphology. Independent samples \( t \) tests were used to determine whether there were differences in percent change in SL or GP L:W resulting from flutamide administration. When applicable, Scheffe post hoc analyses were used to further examine differences. All analyses were carried out using SPSS 21.0. Data are shown as mean ± SEM throughout.

**RESULTS**

**Experiment 1: AR expression in the genital papilla of juvenile *L. dalli***

Genital papilla ratio in undifferentiated juveniles was significantly smaller than juveniles differentiating as females and males (Fig. 2A; \( F_{(2,18)} = 27.92, \ P < 0.0001 \)). Differentiating males exhibited a greater GP ratio compared to differentiating females (\( P < 0.001 \)) and undifferentiated juveniles (Fig. 2A; \( P < 0.01 \)). In addition, differentiating females had a greater GP ratio compared to undifferentiated juveniles (Fig. 2A; \( P < 0.001 \)).

In both differentiating males and females, AR expression (Fig. 2) was greater within the mesenchyme compared to dorsal (males, \( t_{(6)} = 3.33, \ P = 0.008 \); females, \( t_{(8)} = 3.83, \ P = 0.003 \)) and ventral lumen epithelia (males, \( t_{(6)} = 2.12, \ P = 0.04 \); females, \( t_{(8)} = 2.27, \ P = 0.03 \)). In undifferentiated animals, mesenchymal expression was greater than dorsal (\( t_{(4)} = 2.45, \ P = 0.04 \)), but not ventral epithelium (\( t_{(4)} = 1.50, \ P = 0.10 \)). There were no differences in AR expression within the dorsal epithelium (\( F_{(2,18)} = 0.85, \ P = 0.45 \)), ventral epithelium (\( F_{(2,18)} = 1.33, \ P = 0.29 \)), or mesenchyme (\( F_{(2,18)} = 2.53, \ P = 0.11 \)) between undifferentiated juveniles and differentiating males or females (Fig. 2, C–E). In juveniles, there was no association between GP L:W and AR expression in the dorsal epithelium (\( \beta = 0.14, t_{(20)} = 0.51, \ P = 0.62 \)) or ventral epithelium (\( \beta = 0.53, t_{(20)} = 1.66, \ P = 0.11 \)); however, there was a positive association in mesenchymal AR expression (\( \beta = 0.82, t_{(20)} = 2.41, \ P = 0.02 \), Fig. 2F). There was no association between SL and AR expression in any region of the GP (\( P > 0.05 \)).

**Experiment 2: AR and 11β-HSD2 expression in the genital papilla of adult *L. dalli***

Androgen receptor expression was significantly different among male, female, and transitional adults across the different regions of the GP (dorsal epithelium, \( F_{(2,12)} = 8.28, \ P = 0.006 \); ventral epithelium, \( F_{(2,12)} = 5.85, \ P = 0.02 \); and mesenchyme, \( F_{(2,12)} = 5.89, \ P = 0.02 \); Fig. 3E). Post hoc analyses revealed that, compared to females, males exhibited higher AR expression in the dorsal epithelium (Fig. 3, A and C; \( P = 0.01 \)), ventral epithelium (Fig. 3, A and C; \( P = 0.02 \)), and mesenchymal layer of the GP (Fig. 3, A and C; \( P = 0.03 \)). Transitional animals had significantly higher AR expression in the dorsal epithelium (Fig. 3, A and B; \( P = 0.02 \)) and mesenchyme (Fig. 3, A/B; \( P = 0.02 \)) compared to females but did not differ significantly from males. There was a positive association between GP ratio and AR expression in the dorsal epithelium (Fig. 4A; \( \beta = 0.60, t_{(14)} = 3.43, P < 0.01 \)), as well as the ventral epithelium (Fig. 4C; \( \beta = 0.63, t_{(14)} = 3.30, P < 0.01 \)), but not mesenchymal tissue (Fig. 4E; \( \beta = 0.17, t_{(14)} = 0.70, P = 0.50 \)). In addition, SL was positively associated with AR expression in the dorsal epithelium (Fig. 4B; \( \beta = 0.17, t_{(14)} = 0.59, P < 0.001 \)), ventral epithelium (Fig. 4D; \( \beta = 0.15, t_{(14)} = 0.28, P = 0.02 \)), and mesenchymal tissue (Fig. 4F; \( \beta = 0.13, t_{(14)} = 2.32, P = 0.05 \)).

Male, female, and transitional fishes significantly differed in 11β-HSD2-like expression within the dorsal epithelial (\( F_{(2,9)} = 8.31, \ P = 0.009 \)) and ventral epithelium (\( F_{(2,9)} = 5.76, \ P = 0.025 \)), but not mesenchyme (\( F_{(2,9)} = 1.97, \ P = 0.20 \)). Transitional fish exhibited higher 11β-HSD2-like expression in the dorsal epithelium (Fig. 5, A and C; \( P = 0.01 \)) and ventral epithelium (Fig. 5, A–C; \( P = 0.04 \)) compared to females, and higher expression relative to males (Fig. 5, A–C; \( P = 0.04 \)) within the dorsal epithelium. In addition, males exhibited notably higher levels of 11β-HSD2-like within the ventral epithelium of the GP relative to females (Fig. 5; \( P = 0.058 \)).
Experiment 3: effect of systemic flutamide on genital papilla morphology

Prior to pharmacological manipulation, all males had a GP ratio >2.0, and the two treatment groups did not significantly differ in SL ($t_{(10)} = 0.94, P = 0.37$). Flutamide administration significantly decreased GP ratio from baseline to day 5 (Fig. 6B, $t_{(4)} = 3.95, P = 0.015$), but the vehicle had no effect (Fig. 6B, $t_{(5)} = -0.74; P = 0.94$). Animals treated with flutamide also had a significant percent change in GP ratio compared to controls (Fig. 6C; $t_{(10)} = 3.07, P = 0.013$).

DISCUSSION

This study investigated whether a conserved androgenic pathway serves to establish and/or maintain male-typical genital phenotype in adult *L. dalli*. We provide evidence that the region-specific patterning of AR within the GP of differentiating males and females is similar to that observed during development in other vertebrates. In general, vertebrates maintain low epithelial, but high mesenchymal AR expression within the genitalia during early differentiation. Our findings also illustrate that transitional fish exhibit higher AR and 11β-HSD2-like expression in epithelial and mesenchymal tissue compared to females. By injecting males with an AR antagonist, we demonstrated that disruption of AR signaling is sufficient to initiate genital demasculinization in adult males. Our data are consistent with the hypothesis that adult sexual plasticity results from repurposing an ancestral androgenic pathway to facilitate adult phenotypic transitions and maintain male-typical phenotype even in the absence of sexually dimorphic levels of androgens.

Androgen receptor expression in developing *L. dalli*

Androgen receptor expression within the genitalia of differentiating *L. dalli* follows the conserved vertebrate pattern (Cooke et al. 1991; Kim et al. 2002; Murashima et al. 2011). In developing mammalian genitalia, mesenchymal AR expression precedes epithelial expression and is necessary for inducing growth factors that regulate genital outgrowth early in development. Later, mesenchymal–epithelial paracrine signaling induces cell proliferation and differentiation within the epithelium of the male genitalia (Cooke et al. 1991; Kim et al. 2002; Murashima et al. 2011). Consistent with this, we found that AR expression within the mesenchyme of *L. dalli* is higher than epithelial expression in differentiating males, suggesting that androgen signaling within mesenchymal cells is a highly conserved mechanism to regulate morphogenesis of genital phenotype among vertebrates (Cooke et
al. 1991; Miyagawa et al. 2009). Furthermore, high mesenchymal AR expression in differentiating females suggests that the outgrowth of female genitalia may also be androgen dependent and that elevated levels of AR within the mesenchymal region of developing animals might be characteristic of multipotentiality, allowing for genital morphogenesis during initial sexual differentiation in both sexes. Ultimately, our findings are consistent with the hypothesis that the patterning of AR staining in differentiating male *L. dalli* is reminiscent of the widespread distribution of AR in bi-potential mesenchymal tissues (Cooke et al. 1991; Kim et al. 2002; Murashima et al. 2011). Similar to the sexually canalized vertebrates, early mesenchymal–epithelial androgenic pathways likely regulate initial genital differentiation in a sexually plastic species.

Our findings are consistent with previous work on developing *L. dalli* that demonstrate these animals initially lack gonadal tissue (Lorenzi 2009). During this undifferentiated period, genitalia outgrowth may proceed in a default mode and arrest until sexual differentiation begins. Research on sex changing fishes demonstrates that transduction of salient social information induces gonadal and genitalia differentiation (Hobbs et al. 2004; Rodgers et al. 2007; Iwata et al. 2008). As initial sexual differentiation coincides with gonadal development in *L. dalli*, it seems likely that steroid hormones of gonadal origin may regulate some aspects of genital phenotype.

**Androgen receptor and 11β-HSD2 expression in the genitalia of adult *L. dalli***

In the absence of sex differences in KT (Lorenzi et al. 2008) there are at least two endocrine mechanisms that might be regulating the development and maintenance of sexually dimorphic genitalia. Past work has proposed that reproductive phenotype in other hermaphroditic fishes can be maintained

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**Fig. 4.** Relationship between AR staining intensity and standard length (A, C, and E) or genital papilla length to width ratio (GPL:W; B, D, and F) in the dorsal epithelium (A/B), ventral epithelium (C/D), and mesenchyme (E/F).
and masculinized by androgens directly regulating aromatase expression (Kroon et al. 2005; Guiguen et al. 2010) and thus decreasing estradiol levels. Although aromatase is critical for steroidogenic regulation of gonadal function in vertebrates, the findings from the current and previous studies in L. dalli are not consistent with the hypothesis that aromatase is a central regulator of male-typical phenotype or adult sex change (Black et al. 2005; Lorenzi et al. 2008, 2012). Instead, we provide evidence that in the absence of sex differences in androgen levels, the ancestral localized mesenchymal–epithelial androgen signaling pathway could maintain genital dimorphism and initiate phenotypic transitions in adult L. dalli.

First, we assessed whether region-specific patterning of AR during phenotypic transitions was similar to initial differentiation in other vertebrates. Expression of AR within the dorsal epithelium and mesenchyme was highest in males and transitional fish compared to females (Fig. 3, A–C). In a variety of vertebrates, androgen signaling during development regulates expression of growth factors within the mesenchymal layer of the external genitalia (Haraguchi et al. 2000; Ogino et al. 2004; Miyagawa et al. 2009; Murashima et al. 2011). Although mesenchymal AR precedes epithelial expression during mammalian genitalia differentiation (Cooke et al. 1991; Kim et al. 2002), simultaneous changes in both mesenchymal and epithelial AR expression in adult transitional fishes may be necessary to facilitate genital morphogenesis and promote male-typical reproductive function in L. dalli. Our findings examining 11β-HSD2 are also consistent with previous research that demonstrates local androgen biosynthesis is critical for normal genital masculinization in mammals (Kim et al. 2002; Murashima et al. 2011). Furthermore, in undifferentiated female vertebrates, exogenous administration of androgens can increase AR expression within the developing genitalia (Zheng et al. 2015). Thus, in L. dalli, transient increases in androgens may promote the increased androgenic sensitivity seen in transitional fishes to facilitate aspects genital morphogenesis (Lorenzi et al. 2012). Importantly, our findings do not preclude androgens from participating in genitalia morphogenesis during male to female sex change. Indeed previous findings have demonstrated that androgens modulates female external genitalia outgrowth (Drea et al. 1998). This suggests that there is likely a tight temporal interplay between multiple steroid hormone pathways to generate and maintain sexually dimorphic genitalia.

In females, low constitutive AR expression within the papilla may allow this tissue to remain sensitive to the morphological

Fig. 5. Immunohistological localization of 11β-hydroxysteroid dehydrogenase in adult female (A), ambiguous (B), and male genital papillae (C). Mean (±SEM level of diaminobenzidine (DAB) staining within the dorsal epithelium, ventral epithelium, and mesenchyme between males (n = 5), females (n = 4), and transitional (n = 3) animals. (D) All images were taken under a 20× objective lens. Scale bar for cross sections = 200 µm. Asterisks indicate significant differences (*P < 0.05). T = transitional.
actions of androgens. This is consistent with findings in adult male green anoles (*Anolis carolinensis*) that exhibit AR positive cells in all areas of their external genitalia. In this species, androgen treatment increases genitalia size and AR expression in copulatory muscles (Holmes and Wade 2005). Unlike most vertebrates that exhibit a reduction in AR expression once they become reproductively mature (Gonzalez-Cadavid et al. 1991), in *L. dalli*, high constitutive levels of mesenchymal AR might be necessary to further lengthen the GP and elaborate internal anatomy/physiology that is necessary for male reproductive function. Although transitioning females can attain male-typical phenotype (GP ratio ≥ 2.0) relatively quickly, male GP continue to lengthen throughout adulthood (Grober, A.J. and Grober M. S., unpublished data). Thus, remaining sensitive to androgens throughout life might be one mechanism by which both sexes avoid early phenotypic canalization (Pradhan et al. 2015). Our results illustrate a striking similarity between adult and juvenile AR patterning during genitalia morphogenesis, which is consistent with the idea that adult sex change does not utilize a novel mechanism to regulate phenotypic transitions, but rather is repurposing ancestral mechanisms, at least one of which regulates male sexual differentiation (Wilkins 2002; Rodgers et al. 2007). Data on social regulation of sexual phenotype in *L. dalli* are consistent with the repurposing hypothesis. Both initial sexual development (Solomon-Lane et al. 2016) and adult sexual phenotype (Rodgers et al. 2007) are regulated by the same social cues. This suggests that the androgenic regulation pathway has been released from early fixation via coupling to a novel mechanism of sex determination that is responsive to social behavior and thus flexible throughout life.

In addition to maintaining male-typical anatomy, sex differences in epithelial AR expression may be necessary to regulate evolutionarily conserved male-typical reproductive physiology. This idea is supported by evidence from other vertebrates wherein AR expression within the epithelial layer of the genitalia remains high throughout adulthood and regulates the secretion of seminal fluid (Cunha and Young 1991; Dietrich et al. 2004; Holmes and Wade 2005; Murashima et al. 2011). Although the function of the two chambers (Fig. 3) within the GP of *L. dalli* remains unknown, preliminary evidence suggests that the ventral chamber in males has a blind end and likely is utilized to hydrostatically stiffen the genitalia to facilitate sperm and mucus transfer to the nest surface (Archambeault et al. 2015). The dorsal chamber in males likely releases sperm embedded in proteins as well as mucus synthesized in the male-specific accessory gonadal structure (Archambeault et al. 2015). Accordingly, we show that males exhibit increased expression of AR and 11β-HSD2 in the ventral epithelium relative to females, suggesting that androgenic signaling regulates the release of sperm and mucus in males. Thus, a highly conserved

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**Fig. 6.** The effect of flutamide treatment on genital papilla morphology in nesting males. (A) Representative images of vehicle and flutamide treated genital papillae on days 1 and 5 after the onset of treatment. Dashed lines represent changes in genital papilla length to width ratio following treatment with flutamide. (B) Average (±SEM) genital papilla length to width ratio (L:W) before and after treatment with flutamide (*n* = 5) or vehicle (*n* = 6). (C) Average (±SEM) percent change in genital papilla L:W between vehicle or flutamide treated animals. Asterisks indicate significant differences (*P* < 0.05). All images were taken at the same scale and resolution under a stereomicroscope.
androgenic pathway regulates both the development of male-typical genitalia morphology and the physiological processes that facilitate sperm transfer in *L. dalli*.

Given the difficulties of examining androgenic signaling in female genitalia, few studies have documented the functional role of epithelial AR in females. Unlike other goby species, *L. dalli* eggs within the female ovary lack the adhesive threads that are used for attachment to the substrate (Tavolga 1950). Accordingly, these filaments must be added during transport of the egg from the ovary to the substrate (Archambeault et al. 2015). Given its anatomy and location, the ventral chamber in female genitalia is most likely the source of the adhesive threads in this species (Fig. 3). Similar to males, we suggest that the dorsal chamber in females is the conduit for gamete transport. We showed that epithelial expression of AR and 11β-HSD2 in female *L. dalli* is often low or absent, which contrasts with previous findings in female leopard geckos (*Euplxxelaris macularius*) that show high AR expression within the epithelial layer of the oviduct (Rhen and Crews 2001). As genital phenotype remains plastic and transient androgen levels can facilitate adult phenotypic transitions in *L. dalli*, low AR and 11β-HSD2 is likely necessary to maintain female-typical genitalia in adults. If epithelial signaling is necessary for female-typical reproductive physiology in *L. dalli*, then perhaps aspects of their physiology are regulated by another steroid hormone (e.g., E2 signaling through the estrogen receptor).

Effects of systemic flutamide on genital papilla morphology

The effects of flutamide on adult genital morphology in *L. dalli* provide causal evidence that androgen signaling is necessary to maintain male-typical genitalia. We demonstrate that the genitalia of adult male *L. dalli* remains sensitive to the effects of androgens. Thus these findings show that, administration of an AR antagonist reduces masculinization of phenotype in a manner that is similar to other developing vertebrates. For instance, perinatal administration of flutamide, when AR expression is highest within the genitalia, can inhibit normal masculinization in mammals (Simon et al. 2012). Our data are also consistent with the finding that functional AR is necessary for development of male-typical genital morphology in other teleost fishes (Bayley et al. 2002; Ogino et al. 2004). In developing western mosquito fish (*Gambusia affinis*), flutamide inhibited sonic hedgehog expression and altered the induction of fin rays to acquire male-typical genital morphology (Ogino et al. 2004). These findings not only highlight the importance of high AR expression within the mesenchymal layer to maintain male genital morphology, but also the potential necessity of decreasing androgen signaling pathways to facilitate GP morphogenesis during male-to-female sex change.

Despite finding that increased androgenic sensitivity likely maintains adult male morphology, our study does not directly establish what is generating these changes in genital morphology by blocking AR. One possibility is that changes in genital morphology during adult phenotypic transitions occur by shutting off an androgenic pathway and augmenting local estrogenic signaling. This notion is supported by findings that demonstrate estrogens lead to the induction of many growth factors within bi-potential tissues, including genitalia mesenchyme during development, which may facilitate female-typical morphogenesis (Cooke et al. 1998). However, we suspect that morphogenesis mediated solely through an estrogenic pathway is unlikely, given the slow changes in estrogens in *L. dalli* during phenotypic transitions (Black et al. 2005; Lorenzi et al. 2008, 2012). Although our results demonstrate that blocking androgenic signaling is sufficient to de-masculinize adult males, it does not preclude androgenic action within genitalia mesenchyme for initiating or partially regulating aspects of male to female morphogenesis. In spotted hyenas (*Crocuta crocuta*), androgens have been suggested to participate in clitlotal outgrowth, yet substantial outgrowth still occurs when androgen signaling is pharmacologically blocked (Drea et al. 1998; Glickman et al. 2005). This raises the possibility that both androgenic and estrogenic pathways work in tandem within bi-potential genital tissues to regulate genital outgrowth in female vertebrates. It is clear from these data that future research focusing on multiple steroid hormone pathways is needed to determine the role, and possible interplay, between developmental pathways.

CONCLUSIONS

Functional AR expression is critical for normal male-typical sexual differentiation in vertebrates. We demonstrate that region-specific localization of AR in developing *L. dalli* is similar to non-sex changing fish (Ogino et al. 2004) and other vertebrates (Cooke et al. 1991; Kim et al. 2002). In the absence of sex differences in KT levels (Lorenzi et al. 2008, 2012), sexually dimorphic AR expression within the mesenchymal and epithelial layers of the GP may maintain male-typical physiology and morphology in adults. Findings from both juveniles and transitional adults suggest mesenchymal AR in *L. dalli* is important in regulating genital morphogenesis. This suggests that the role of AR in masculinizing genital phenotype during initial sexual differentiation may be highly conserved among vertebrates. Furthermore, masculinization of phenotype during adult sex change is likely utilizing this conversed androgenic pathway. Finally, we demonstrate that short-term administration of an AR antagonist is sufficient to partially de-masculinize males. Together, these data provide clear evidence that at least one sexually plastic vertebrate has not generated a novel taxon-specific mechanism to regulate masculinization of genital phenotype. Instead, we suggest that *L. dalli* has repurposed a highly conserved androgenic pathway, which is
now regulated by social status, to generate male-typical genital phenotype in adults. Based on our findings, we suggest that future research on the evolution of sexual plasticity should assess whether these animals also repurpose other highly conserved mechanisms that regulate aspects of vertebrate development and physiology.

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