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

Eric Schuppe

CONSERVED ANDROGENIC DIFFERENTIATION PATHWAYS ARE REPURPOSED DURING THE EVOLUTION OF ADULT SEXUAL PLASTICITY

by

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Under the Direction of Matthew S. Grober, PhD

ABSTRACT

Early exposure to androgens is necessary to organize male phenotype, and inhibition of androgen signaling adversely affects external genitalia development. Nonetheless, vertebrates that remain sexually plastic as adults require mechanisms that induce changes and prevent fixation of genital morphology. In *Lythrypnus dalli*, a highly social hermaphroditic fish, sex differences in genitalia morphology are maintained in the absence of dimorphic levels of potent fish androgen 11-ketotestosterone. Since a functional androgen receptor (AR) is critical to the masculinization of phenotype across all vertebrates, local differences in the AR may be one mechanism to maintain dimorphic genitalia in this species. We demonstrate that AR expression is high throughout the male genitalia, but low or absent in female genitalia. Animals with ambiguous genitalia exhibited higher AR expression than females within the body of the papilla.

In addition, both male and female developing *L. dalli* exhibited high mesenchymal AR expression suggesting that elevated that levels of AR in this region of ambiguous animals may be characteristic of multipotentiality, allowing for genitalia morphogenesis in either direction. To examine the necessity of AR in maintaining male-typical genitalia, nesting males were intraperitoneally injected with 50µg/g/day of flutamide (AR antagonist) or a vehicle for five days. Compared to control animals, flutamide treated males had a significant percent decrease in papilla length to width ratio. In a species that must remain neutral to sexual canalization, regional differences in AR expression appear to mediate this phenotypic difference.

INDEX WORDS: Androgen receptor, Sexual plasticity, Sexual differentiation

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1 INTRODUCTION

Androgens are critical regulators of sexual differentiation. In most vertebrates, exposure to androgens during a sensitive period in development is necessary to permanently masculinize aspects of reproductive phenotype (reviewed in Murashima, Kishigami, Thomson, & Yamada, 2014). While these animals are investing heavily in one sexually differentiated state, aspects of reproductive phenotype remain malleable only during a brief window in development, after/during which any alternative sexual states become mechanistically prohibited for life. For instance, if androgens are exogenously administered to females during this period it can lead to the development of male-typical external genitalia (Phoenix, Goy, Gerall, & Young, 1959; Roberts, Padmanabhan, & Lee, 2008; Young, Goy, & Phoenix, 1964). Androgens regulate masculinization of phenotype by signaling through the androgen receptor (AR), and mutations leading to disruption of normal androgen signaling can adversely affect male sexual differentiation (De Bellis et al., 1994; Murakami, 1987). During male sexual differentiation, AR expression within the genitalia is high expressed and necessary for the induction of growth factors (e.g. Sonic hedgehog and Wnt) that regulate morphogenesis (Gonzalez-Cadavid, Swerdloff, Lemmi, & Rajfer, 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 reproductive phenotype (Gonzalez-Cadavid et al., 1991; Miyagawa et al., 2009).

Unlike mammals which canalize genital morphology early in development, reproductive phenotype remains plastic throughout adulthood in numerous teleost species (Ghiselin, 1969; Warner, Robertson, & Egbert, 1975). Since early fixation limits sexual fate, the release of canalization is necessary to induce phenotypic transitions in adult hermaphroditic fishes. This might occur through inhibition mechanisms that de-feminize/masculinize aspects of reproductive

phenotype during sexual differentiation. In addition to preventing fixation of phenotype, hermaphroditic fishes need the ability to perceive salient information later in life that can induce sex change. In hermaphroditic fishes that form complex social groups, adult sex change is regulated by the transduction of information regarding changes in the social hierarchy. The process of adult sex change leads changes in neural circuits underlying reproductive behavior (Black, Reavis, & Grober, 2004; Godwin, Sawby, Warner, Crews, & Grober, 2000; Grober & Sunobe, 1996; Marsh, Creutz, Hawkins, & Godwin, 2006) as well as a complete reorganization of gonadal and genitalia morphology (St. Mary, 1994, 1998).

Lythrypnus dalli, a highly social bi-directional hermaphroditic teleost fish, provides a unique opportunity to examine whether initial differentiation and adult sex change utilizes highly a conserved androgenic differentiation pathway to regulate genital masculinization. In this species, initial sexual differentiation and adult sex change are regulated by social factors (Rodgers, Drane, & Grober, 2005; Solomon-Lane et al., in prep). Loss of a male from a stable social group creates a context that permits the dominant female to undergo natural sex change within 5 days, but administration of 17β-estradiol (E2) inhibits masculinization of the genitalia during this process (Fig. 1, Reavis & Grober, 1999; E. Rodgers and M.S. Grober, unpublished data). In an inhibitory social context, administration of the potent fish androgen, 11-ketotestosterone (KT), to a subordinate female induces rapid genital masculinization (Carisle et al., 2000; Pradhan, Connor, Pritchett, & Grober, 2014). Paradoxically, KT concentrations are not sexually dimorphic in this species, even though there is a transient increase in KT levels during female to male sex change (Lorenzi, Earley, & Grober, 2012; Lorenzi, Earley, Rodgers, Pepper, & Grober, 2008). Since *L. dalli* possesses many of the factors involved in mammalian sexual

differentiation, conserved endocrine mechanisms may induce changes and prevent fixation of phenotype in adults.

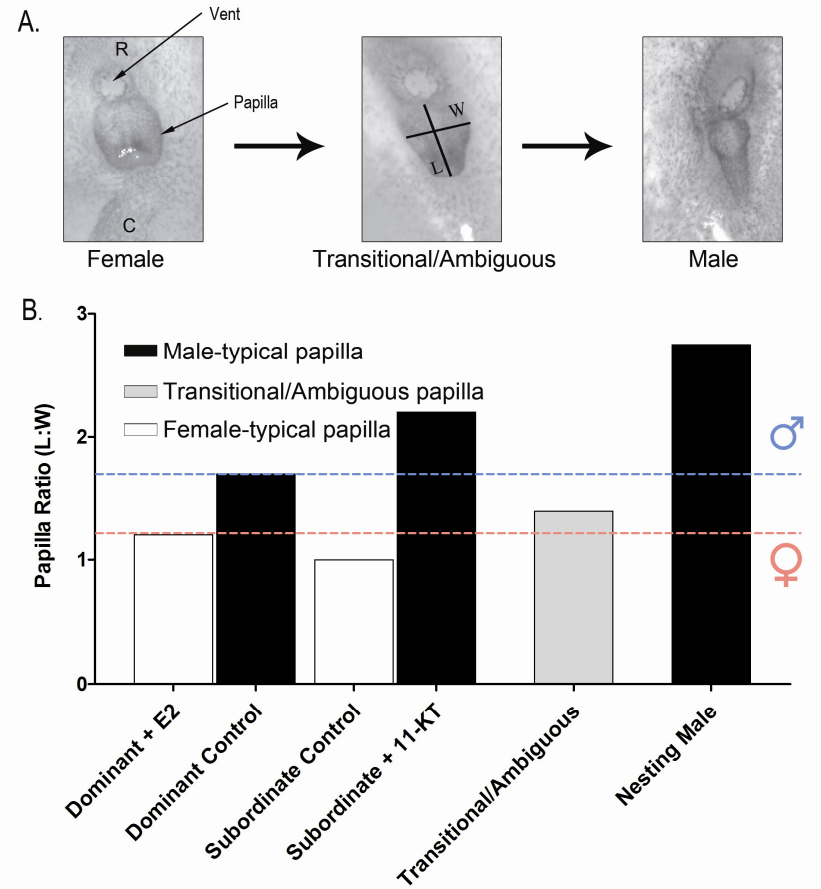

Figure 1. Sexual dimorphism in the genital papilla, the external genitalia in L. dalli Papilla ratio is calculated by measuring the length and width of the papilla, located on the ventral surface. **(B)** A fish that has a L:W greater than 1.6 (--) would have a male typical papilla. Those with a L:W less than 1.4 (--) would have a female typical papilla. Transitional or ambiguous fish have a L:W of approximately 1.4.

One proposed route to masculinization of phenotype in hermaphroditic fishes is through an estrogen sex determination pathway, wherein, androgens are thought to regulate phenotypic transitions through inhibition of *cyp19a1a* gene expression, thereby reducing E2 levels (Guiguen, Fostier, Piferrer, & Chang, 2010; Kroon, Munday, Westcott, Hobbs, & Liley, 2005). While experimental evidence has provided support for this model (Kobayashi, Nozu, & Nakamura, 2011; Kroon et al., 2005; Nozu, Kojima, & Nakamura, 2009), this is likely not the main pathway for regulating masculinization of reproductive phenotype in *L. dalli.* During natural sex change in *L. dalli*, phenotypic and behavioral changes occur within days, but reduction in systemic and gonadal E2 levels are much slower (Lorenzi et al., 2012; Lorenzi et al., 2008). Furthermore, changes in brain aromatase occur very rapidly, but newly sex reversed individuals often have high levels of gonadal aromatase (Black, Balthazart, Baillien, & Grober, 2005). Therefore, genital phenotype may be masculinized during natural sex change by recapturing an ancestral androgen mediated sex determination pathway. In addition, once terminal phenotype is reached, sex differences in the distribution and expression of AR in genitalia of *L. dalli* might be one mechanism by which anatomical dimorphism.

The current study had two aims, first to determine whether natural sex change is utilizing aspects of a conserved androgenic signaling cascade to rapidly masculine the external genitalia. To do this, we examined the whether epithelial and mesenchymal AR protein expression in transitional *L. dalli* mirrors AR expression in developing vertebrates (Cooke, Young, & Cunha, 1991; Kim et al., 2002). Additionally, we wanted to determine whether sexually dimorphic expression of AR could be one mechanism by which male-typical external genitalia morphology can be maintained in the absence of dimorphic concentrations of KT. Second, since developing *L. dalli* begin in an undifferentiated state in which all animals initially lack gonadal tissues, we

wanted to determine how regional expression of AR changes throughout development. Based on research that demonstrates AR is low when the gonad of European sea bass are undifferentiated, we though levels in the genitalia of *L. dalli* will be low initially and continue to increase throughout development (Blazquez & Piferrer, 2005; Piferrer, Blazquez, Navarro, & Gonzalez, 2005). Similar to developing mammals (Cooke, Young, & Cunha, 1991; Kim et al., 2002), we predicted expression in developing animals would be highest in mesenchymal cells in the papilla. To further examine this we wanted to determine whether disrupting normal androgen signaling by systemically administering an androgen receptor antagonist would be sufficient to cause demasculinization in nesting males.

2 METHOD

2.1 General methods

Adult and juvenile *L. dalli* used in the current experiments were collected off the coast of Catalina Island, California between April-July of 2013 and 2014 using hand nets (permit numbers # SC-11879). Since all juveniles were collected in the field we were unable to ascertain there exact age; however, Solomon-Lane and colleagues (in prep) demonstrated both weight and size of an animal are associated with timing of gonadal development. Based on these finding, we initially choose animals based on size and papilla morphology. Field collected transitional animals were characterized by papilla morphology and presence of both sperm and eggs in the gonads. For each fish, we measured standard length, body weight(g), and papilla length to width ratio. Fish were immediately euthanized by exposure to a lethal dose of tricaine methanesulfonate (MS-222). All fish were fixed in 4% paraformaldehyde, then cryopreserved in 30% sucrose in phosphate buffer (PB), and stored briefly at 4°C before immunohistochemistry.

2.2 Experiment 1: Immunohistological localization of AR in developing and adult papilla

Field collected adult male (N = 6), female (n = 6), and ambiguous (n = 4) fish as well as juveniles (N = 21) were sectioned in two series on a cryostat, and slides were stored at -20℃ until further processing. On the day of processing, slides were allowed to equilibrate to room temperature and sections were washed in 0.1M phosphate buffer (PB) twice for 7 minutes each. Sections were then incubated in a blocking solution (normal goat serum and 0.2% Triton-X in 0.1M PB) for 20 minutes. Next, the sections were incubated in primary antibody, AR (PG-21, Millipore) diluted (AR 1/250) in normal goat serum and 0.2% Triton-X in 0.1M PB overnight at 4℃. The next day slides were rinsed twice in 0.1M PB for 7 minutes. Next, biotinylated secondary antibody (Kirkegaard & Perry Laboratories) was added to the slides for 30 minutes. The sections were then twice rinsed in 0.1M PB for 7 minutes each and then incubated in streptavidin-peroxidase (Kirkegaard & Perry Laboratories) for 30 minutes. AR positive cells visualized using 3,3'-diaminobenzidine (Sigma Chemical). The sections were then dehydrated in an ethanol series, cleared in citrasolv, and mounted with paramount. As a control, sections (n = 2 per sex) were processed by omitting the primary antibody. Expression of gonadal AR on each section was used as a positive control. Images were acquired using an Axioplan microscope and Axiovision software. Androgen receptor expression was evaluated in three regions of interest, including expression in the dorsal and ventral epithelium as well as mesenchymal layer of the papilla. The level of AR staining for each region was rated on semi-quantitative scale 0-3 scale (0: no staining present, 1: mild staining, 2: moderate staining, and 3: intense staining).

2.3 Experiment 2: Effect of systemic flutimide on papilla morphology

Social groups consisting of an adult male and three subordinate females (< 2mm smaller than the male) were created in our laboratory at the USC Wrigley Institute for Environmental

Science on Catalina Island, California. On the first day of the study, males were anaesthetized with MS-222 (0.5mg/100 ml water) to take papilla pictures (Motic image 2.0) as well as determine weight and SL. While still anaesthetized, males were intraperitoneally injected with either 50ug/g/day flutamide (N = 5) or vehicle (0.1M PB, N = 6) using a 28.5G needle (Becton Dickinson). Following the injection, males were placed in a 200mL cup that was filled halfway with sea water and allowed at least 15 minutes to recover. Prior to returning males to their social groups, their water was changed to prevent potential flutamide contamination when being placed back in their tank. Each animal was injected every day for four days, and papilla pictures were taken every other day. On the afternoon of the fifth day, final measurements were taken and animals were sacrificed as described above. At the start of the study, all males had with a papilla L:W ratio greater than 2.0 and the two treatment groups did not significantly differ in standard length ($t(10) = 0.94$, $p = .37$). The dose of flutamide was determined from previous research (Dang, Traas, & Vermeire, 2011; O'Connor, Frame, & Ladics, 2002) as well as a pilot study conducted to determine an optimal dose for *L. dalli.* ImageJ (NIH) was used to calculate changes in papilla morphology.

2.4 Data analysis

An analysis of variance (ANOVA) was used to compare regional differences in papilla AR expression between males, ambiguous, and female animals. Juveniles were split into three groups for analysis based on results for timing of gonadal differentiation in Solomon-lane and colleagues (in prep). Animals that did not have gonadal tissue were categorized as undifferentiated and those with adult typical external genitalia phenotype, but smaller then nesting adults were characterized as differentiating animals An ANOVA was run to examine differences in AR expression between these different developmental time periods. A linear

regression was used to examine the relationship between both papilla L:W and standard length and AR expression in the papilla in adults. Since our data did not meet the statistical assumptions to run a repeated measures ANOVA, two paired sample t-tests to examine the effect of each treatment on papilla morphology. Independent samples t-tests were used to determine whether there were differences in percent change in standard length or papilla L:W. When applicable scheffe *post hoc* analyses were used to further examine differences. All analyses were carried out using SPSS 21.0.

3 RESULTS

3.1 Experiment 1: AR expression in the genital papilla of adult and developing *L. dalli.*

Expression of AR significantly differed between the three phenotypes in the dorsal epithelium ($F(2,15) = 6.439$, $p = 0.01$), ventral epithelium ($F(2,15) = 5.32$, $p = 0.042$), and mesenchyme ($F(2,15) = 8.78$, $p = 0.02$) of the adult papilla (Fig. 2d). Scheffe *post hoc* analyses revealed that males exhibited higher AR expression in the dorsal epithelium (Fig 2a/c, $p = 0.02$), ventral epithelium (Fig. 2a/c, $p = 0.02$), and in mesenchymal layer of the papilla compared to females (Fig. 2a/c, $p = 0.03$). Furthermore, *post hoc* analyses revealed ambiguous animals had significantly higher expression in the dorsal epithelium (Fig. 2a/b, $p = 0.03$) and mesenchyme (Fig. 2 a/b, $p = 0.02$), but did not differ between males in all regions of the internal papilla. There was a positive relationship between papilla L:W and AR expression in the dorsal epithelium (Fig. 3A, $r^2 = 0.46$, $p = 0.003$) as well as ventral epithelium (Fig. 3B, $r^2 = 0.47$, $p = 0.002$), but not mesenchymal expression (Fig. 3C, $r^2 = 0.13$, $p = 0.13$). In addition, the size of a fish (standard length) was positively related to AR expression in the dorsal epithelium ($r^2 = 0.58$, $p < 0.0001$), ventral epithelium ($r^2 = 0.344$, $p = 0.01$), and mesenchymal expression ($r^2 = 0.27$, $p = 0.045$).

Papilla L:W ratio of undifferentiated juveniles ($M = 0.55$, S.E.$\pm$ 0.10) was significantly

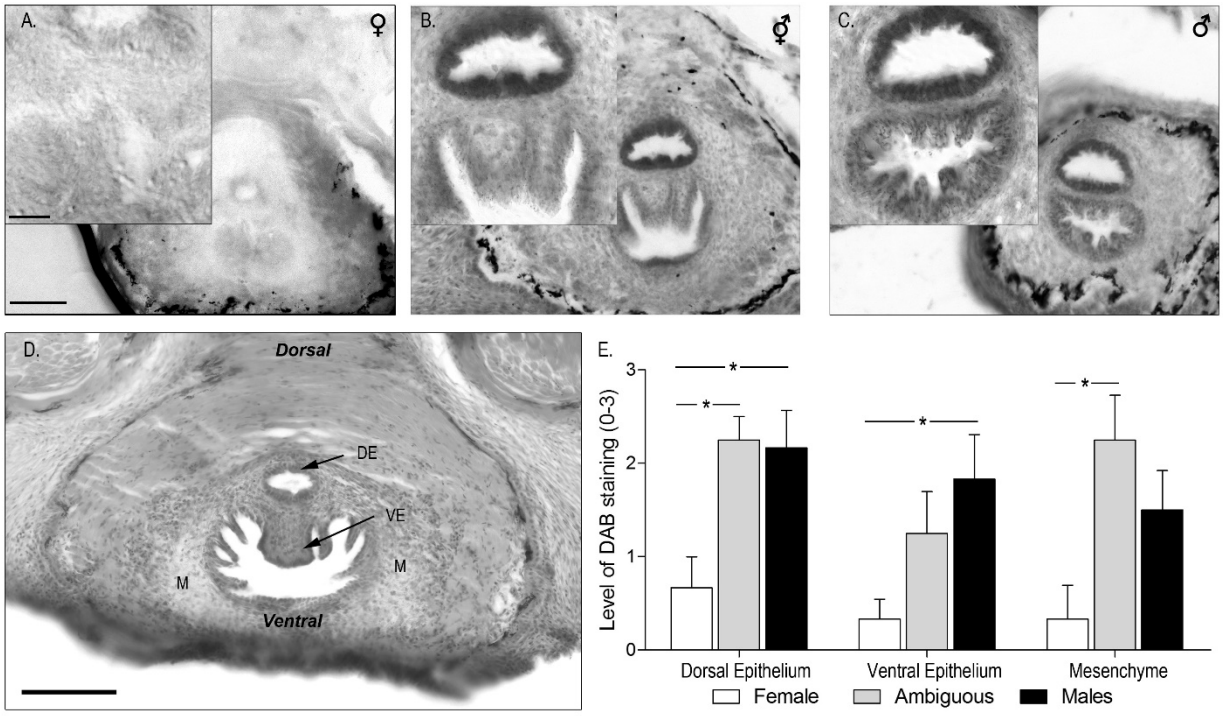

Figure 2. Immunohistological localization of the androgen receptor in adult female (A), ambiguous (B), and male papillae (C).
(D) Female papilla stained with hematoxylin and eosin. **(E)** Average (±SEM) level of diaminobenzidine (DAB) staining within the dorsal epithelium, ventral epithelium, and mesenchyme between males (n = 6), females (n = 6), and transitional (n = 4) animals. Asterisks indicate significant differences ($p < 0.05$). DE = dorsal epithelium, VE = ventral epithelium, M = mesenchyme. Scale bar on A-C = 200 µm, insert for A-C = 20 µm, D = 100 µm

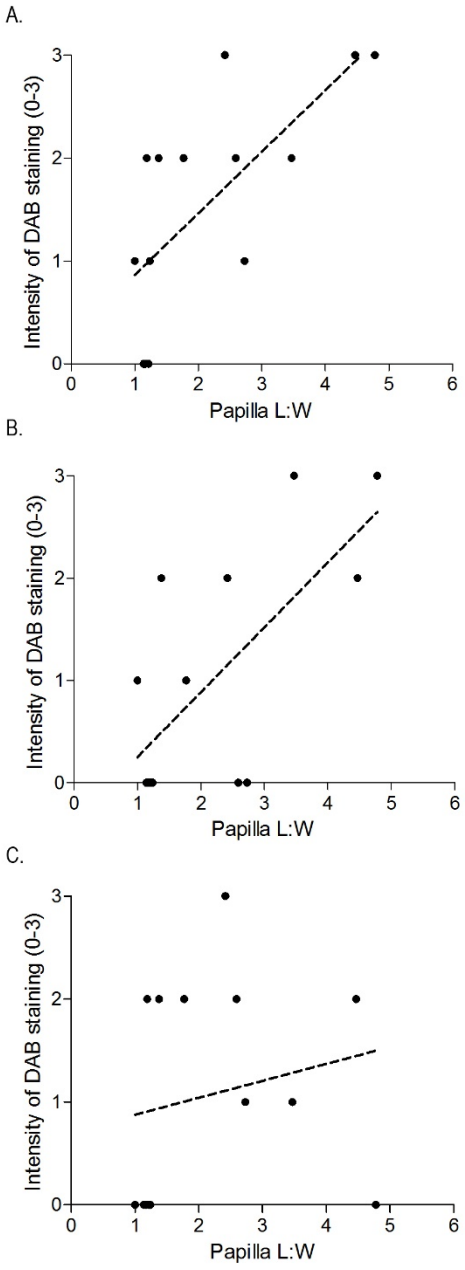

Figure 3. Association between papilla length t o with ratio (L:W) and intensity of diaminobenzidine (DAB) staining within the dorsal epithelium (A), ventral epithelium (B), and mesenchymal (C) layers of the papilla.

smaller than juveniles differentiating as females (M = 1.17, S.E.± 0.07) and males (M = 1.86, S.E.± 0.16), $F(2,13)$ 27.92 , $p < 0.0001$. There were no differences AR expression within the dorsal epithelium ($F(2,20) = 0.38$, $p = 0.68$), ventral epithelium ($F(2,20) = 1.16$, $p = 0.33$), or mesenchyme ($F(2,20) = 2.18$, $p = 0.14$) between undifferentiated juveniles and differentiating males or females (Fig 4a-c). In juveniles there was no relationship between papilla L:W and AR expression in the dorsal epithelium ($r^2 = 0.001$, $p = 0.44$); however, there was a positive relationship in ventral epithelium ($r^2 = 0.17$, $p = 0.03$) and mesenchymal AR expression ($r^2 = 0.23$, $p = 0.01$). There was no relationship between standard length and level of AR expression in any region of the papilla.

While there was no difference in mesenchymal AR expression between developing males and adult males ($t(11) = 0.84$, $p = 0.42$), adult females had significantly less AR expression within the mesenchyme compared to developing females ($t(13) = 2.27$, $p = 0.02$). Adult males and females also had higher AR expression then developing males in the dorsal (males, $t(11) = 2.93$, $p = 0.007$; females, $t(13) = 1.89$, $p = 0.044$), but not ventral epithelium (males, $t(11) = 1.29$, $p = 0.11$; females, $t(13) = 0.86$, $p = 0.20$).

3.2 Experiment 2: Effect of systemic flutimide on papilla morphology.

Flutamide treatment significantly decreased papilla L:W from baseline to day five (Fig 4b, $t(4) = 3.62$, $p = 0.014$), and treatment with vehicle had no effect (Fig 4b, $t(4) = -0.22$, $p = 0.83$). Animals treated with flutamide had a significant percent decrease in papilla L:W compared to controls (Fig 4c, $t(10) = 2.91$, $p = 0.01$).

4 CONCLUSIONS

Our results demonstrate that AR expression is sexually dimorphic in all regions of the adult papilla. In addition, field collected transitional fish had a notable increase in dorsal

epithelial and mesenchymal AR expression compared to females. In undifferentiated fish as well as juveniles undergoing male or female sexual differentiation, expression was low or absent in both epithelial layers, but high in the papilla mesenchyme. By IP injecting nesting males with an AR antagonist we demonstrated that disruption of normal androgen receptor signaling is sufficient to partially demasculinize males.

Androgen receptor expression in the genitalia of adult L. dalli

In the current study, we wanted to determine whether a conserved androgenic pathway serves to masculinize and maintain male-typical reproductive phenotype in adult *L. dalli*. Previous research has proposed that reproductive phenotype in hermaphroditic fishes can be maintained and masculinized by androgens directly regulating aromatase expression (Guiguen et al., 2010; Kroon et al., 2005). While 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., 2012; Lorenzi et al., 2008). Our findings show that in the absence of dimorphic levels of androgens, a mesenchymal-epithelial androgen signaling pathway could maintain anatomical dimorphism and regulate phenotypic transitions in adult *L. dalli*. In females, E2 may directly regulate transcription of AR, thereby maintaining female-typical genitalia morphology. This hypothesis is supported by *in vitro* and *in vivo* studies that E2 can directly inhibit AR protein and mRNA expression (Lin, Rajfer, Swerdloff, & Gonzalez-Cadavid, 1993; Prins, 1992; Taylor et al., 2012).

Since natural sex change is associated with rapid morphological changes in reproductive phenotype, we first wanted to assess whether region specific AR expression during phenotypic transitions was similar to differentiating vertebrates. Our findings show expression of AR within

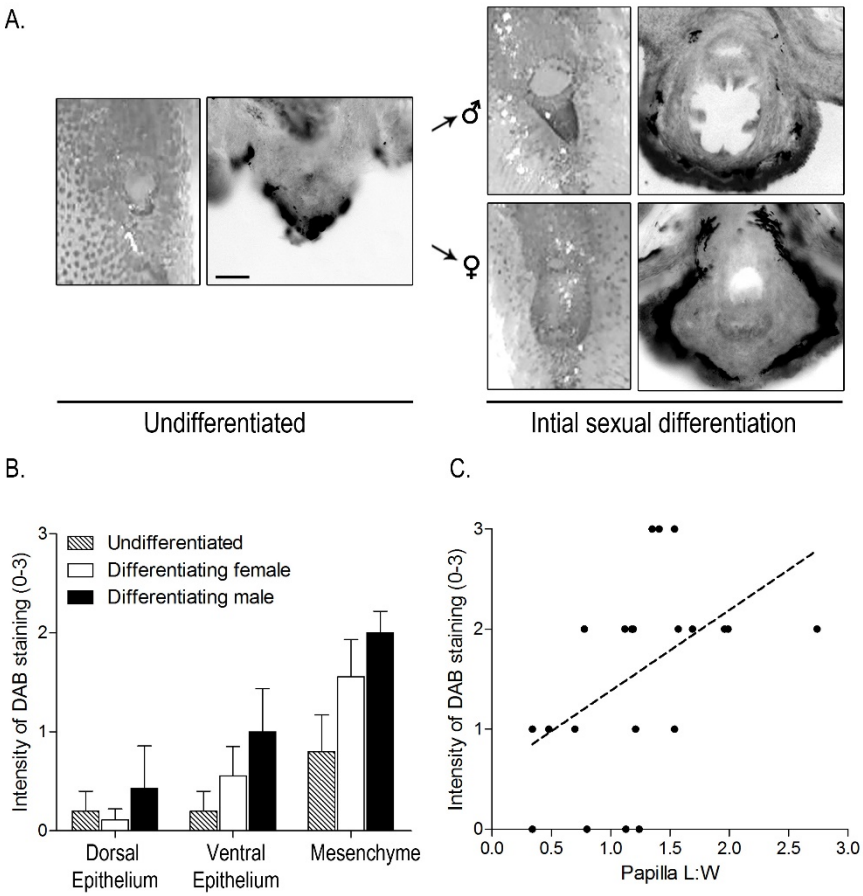

Figure 4. Androgen receptor expression in developing *L. dalli*

(A) External genitalia morphology and immunohistological localization of the androgen receptor in undifferentiated animals, differentiating males (top), and differentiating female papillae (bottom). **(B)** Average (±SEM) level of diaminobenzidine (DAB) staining within the dorsal epithelium, ventral epithelium, and mesenchyme between undifferentiated fish (n = 5), differentiating females (n = 9), and differentiating males (n = 7). **(B)** Association between papilla length to with ratio (L:W) and intensity of DAB staining within the mesenchymal **(C)** layers of the papilla in juveniles. Scale bar for cross sections = 50 µm

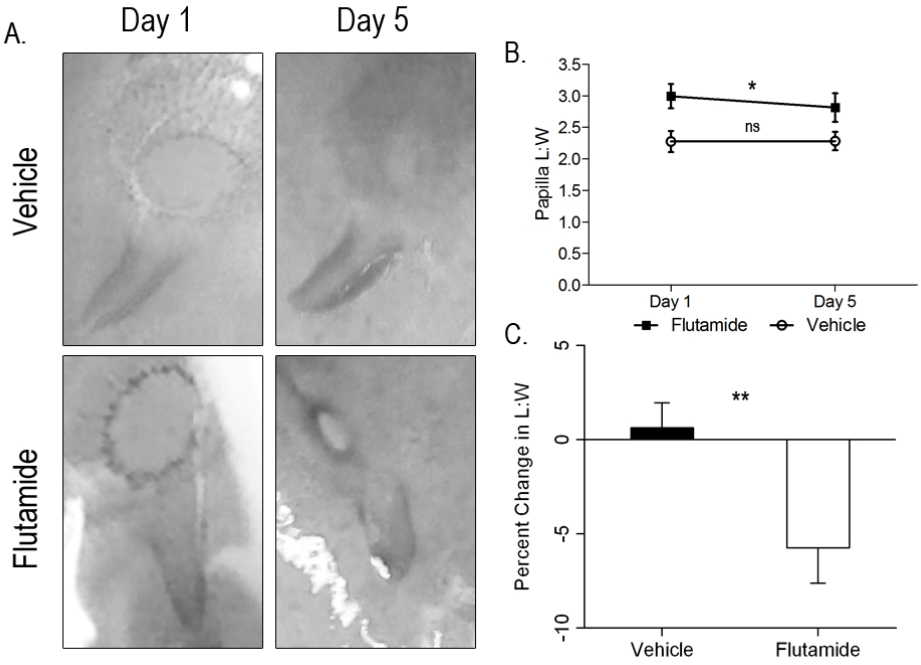

Figure 5. The effect of flutamide treatment on external papilla morphology in nesting males. Average (±SEM) papilla length to width ratio (L:W) before and after treatment with flutamide (n = 5) or vehicle (n =6). **(C)** Average (±SEM) percent change in papilla L:W between vehicle or flutamide treated animals. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$).

the dorsal epithelium and mesenchyme was elevated in transitional fish compared to both sexes (Fig. 2a/b). Research across vertebrates has established that androgen signaling during development regulates expression of numerous growth factors within the mesenchymal layer of the external genitalia (Haraguchi et al., 2000; Miyagawa et al., 2009; Murashima et al., 2011; Ogino, Katoh, & Yamada, 2004). While 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 females may be necessary to facilitate genital morphogenesis and confer aspects of male-typical reproductive physiology. In females, low constitutive AR expression within the papilla may allow this tissue to remain sensitive to the morphological 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, and administration of androgens increases genitalia size and AR expression in copulatory muscles (Holmes & Wade, 2005). Unlike most vertebrates which exhibit declines in AR expression once they become reproductive at sexual maturity (Gonzalez-Cadavid et al., 1991), high constitutive levels of mesenchymal AR might be necessary to further lengthen the papilla and elaborate internal anatomy/physiology that is necessary for male reproductive function. Although we transitioning females can attain male-typical phenotype (papilla L:W $\geq$ 2.0) relatively quickly, we have demonstrated male papillae continue to lengthen throughout adulthood (Grober, M.S., unpublished data). Thus, remaining sensitive to androgens throughout life might be on mechanism by which both sexes avoid canalization. Taken together, our results are consistent with the idea that the process of adult sex change does not utilize a novel mechanism to regulate phenotypic transitions, but rather is recapturing ancestral mechanisms used for male sexual differentiation (Rodgers, Earley, & Grober, 2007; Wilkins, 2002).

In addition to maintaining male-typical anatomy, sex differences in epithelial AR expression may also be necessary to regulate evolutionary conserved aspects of male-typical reproductive physiology. Although the exact function of the two chambers remains unknown in both sexes, we believe the dorsal chamber releases gametes and the ventral chamber secretes sex specific molecules. For instance, the ventral chamber in males may secrete proteins or mucus synthesized in the male-specific accessory gonadal structure. Based on our findings, the release of sperm and mucus in males may be under regulation of AR. This is supported by evidence from other vertebrates that suggests AR expression within the epithelial layer of the genitalia remains high throughout adulthood and regulates the secretion of seminal fluid (Cunha & Young, 1991; Dietrich, Haitel, Huber, & Reiter, 2004; Holmes & Wade, 2005; Murashima et al., 2011). Eggs in female *L. dalli* ovaries lack adhesive threads (Pradhan D.S. and Grober M.S., personal observation) that are used for attachment to the substrate in other goby species (Tavolga, 1950). Therefore, these filaments must be added during transport of the egg from the ovary to the substrate. Given its anatomy and location, the ventral chamber in female genitalia is most likely the source of the adhesive threads in this species. While epithelial AR expression is high within the oviduct of female leopard geckos (*Eupblepharis macularius*), expression of AR in female *L. dalli* is often low or absent (Rhen & Crews, 2001). 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).

Androgen receptor expression in developing L. Dalli

In vertebrates that exhibit environmental sex determination, integration of external stimuli (e.g. social cues or temperature) is critical in regulating (Piferrer et al., 2005). Our findings are consistent with previous work in developing *L. dalli* that demonstrates animals

initially lack gonadal tissue (Solomon-Lane et al., in prep). During this period when juveniles are in an undifferentiated state, genitalia outgrowth may proceed in a default mode and arrest until sexual differentiation begins. Research in sex changing fishes demonstrates transduction of salient social information may induce gonadal and genitalia differentiation (Hobbs, Munday, & Jones, 2004; Iwata, Nagai, Hyoudou, & Sasaki, 2008; Rodgers et al., 2007; Solomon-Lane et al., in press). While early social cues may cause the external genitalia to develop in one direction, both the internal genitalia (Fig. 4A) and gonad appear to remain in a bi-potential state (Solomon-Lane et al., in prep). This ambiguity in developing animals might be due to differences in the rate at which the external and internal genitalia differentiate. Another possibility is fish may remain in an ambiguous state until they fully become reproductive or receive specific social cues that induce complete differentiation. Solomon-Lane and colleagues (in prep) demonstrated that placing juveniles in a highly structured social context can accelerate differentiation. Specifically, among groups composed of only juveniles, the most dominant individual quickly exhibited male-typical behavior and morphology (Solomon-Lane et al., in prep).

The findings in the current study are consistent with research in mammals, which mesenchymal AR expression appears first in the genitalia, and through 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). Since initial sexual differentiation coincides with gonadal development, aspects of reproductive phenotype may be regulated by steroid hormones. Therefore, the role of AR in regulating genitalia morphogenesis in *L. dalli* is likely utilizing mechanisms that are highly conserved among vertebrates. For instance, androgen signaling in mesenchymal cells may be more important for regulating initial reproductive phenotype in *L. dalli* (Cooke et al., 1991; Miyagawa et al., 2009). Research examining

epithelium or mesenchyme-specific ablation of AR illustrates that only the loss of mesenchymal AR will disrupt male-typical morphology (Miyagawa et al., 2009). Furthermore, elevated levels of AR in the mesenchymal region of developing animals may also be characteristic of multipotentiality, allowing for genitalia morphogenesis in either direction.

Effect of systemic flutimide on papilla morphology.

The effects of flutamide on adult genitalia morphology in *L. dalli* further demonstrate that normal androgen signaling may be necessary to maintain male-typical genitalia in bi-directional hermaphroditic fishes. Since AR expression within the genitalia of adult male *L. dalli* remains high and sensitive to the effects of androgens, administering an AR antagonist likely inhibits masculinization of phenotype in a similar manner to disruption of normal signaling in developing vertebrates. For instance, perinatal administration of flutamide when AR expression is high within the genitalia can inhibit normal masculinization in mammals that canalize phenotype early in development (Simon et al., 2012). Our findings are also consistent with research that shows functional AR is necessary for development of male-typical genitalia morphology in other teleost fishes (Bayley, Junge, & Baatrup, 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 become the male-typical genital (Ogino et al., 2004). Since inhibition of AR decreased growth factor expression in developing fish (Ogino et al., 2004), high expression of AR may still be necessary for papilla morphogenesis during male-to-female sex change.

Conclusions

Functional AR expression is critical to normal male-typical sexual differentiation in vertebrates. In the current, study we demonstrate 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 dimorphic concentrations of KT, sexually dimorphic AR expression within the mesenchymal and epithelial layers of the papilla 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 genitalia morphogenesis. The finding in the current study suggest 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 a conversed androgenic pathway that is recapturing mechanisms used for initial differentiation in all vertebrates. Finally, we demonstrate that short-term administration of an androgen receptor antagonist is sufficient to partially de masculinize males. Since hermaphroditism has evolved independently several times in teleost fishes (Francis, 1992), it remains possible that different endocrine mechanisms may regulate sex change and maintience of terminal phenotype differently among species

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