



Structural changes in the ovotestis of the bidirectional hermaphrodite, the blue-banded goby (*Lythrypnus dalli*), during transition from ova production to sperm production

Jessica M. Maxfield  · Kathleen S. Cole

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Abstract Hermaphroditism is widespread in fishes and has been extensively studied in many groups. The process whereby the gonad changes morphology in sequential hermaphrodites (i.e., female to male sex change or the reverse) is well documented. However, gonadal changes in bidirectional hermaphroditic fishes, in which gamete production can shift repeatedly, has received little attention. Here we examine the transition process in the bidirectional sex-changer *Lythrypnus dalli*, as it goes from producing eggs to producing sperm. In the ova-producing phase, the majority of the ovotestis consists of vitellogenic oocytes. Gonad transition is initiated with the break-down and reabsorption of mature oocytes. This is then followed by the proliferation of spermatogenic tissue from the dorsal and ventral regions of the ovotestis. The proliferation of spermatogenic tissue continues until it makes up the majority of the ovotestis with the remainder of the gonad comprising previtellogenic oocytes. We were able to define five transitional stages characterized by the relative area of the ovotestis made up of oogenic, spermatogenic, and atretic tissue. In only one other species of bidirectional sex-changing fish, *Eviota epiphanes*, has similar examination of transitional morphology been performed. *Lythrypnus dalli* and *E. epiphanes* are both in the teleost family Gobiidae, and closely related. It has been hypothesized that these two lineages have independently

evolved the ability to change sex. Here we provide a comparison of the transition process between *L. dalli* and *E. epiphanes*, which highlights the conserved and novel elements and provided insights into differences in their life histories.

Keywords Hermaphroditism · Gobiidae · Morphology · Gonad · Ovotestis

Introduction

In most fishes, gonad morphology and function is set by early adulthood and remains constant throughout life. In sex-changing fish species there is a significant reorganization of tissue within the gonad as adults undergo a shift in sexual function. This includes changes to the proportion of tissue types in the gonad and the stage of maturity of gametes. Among many protogynous (female to male) sex changers, the functional shift follows a similar pattern in which the initial degradation of oogenic tissue (i.e., atresia) is followed by the proliferation of spermatogenic tissue (Nakamura et al. 1989; Shapiro et al. 1993; Bhandari et al. 2003; Sundberg et al. 2009). In some species, initial phase females have a small portion of spermatogenic tissue within the gonad or they maintain undifferentiated gonial cells (Bhandari et al. 2003). In others, no spermatogenic tissue is visibly present prior to sex change (Nakamura et al. 1989; Shapiro et al. 1993; Sundberg et al. 2009). In all species examined, only spermatogenic tissue is present after a shift in sexual function.

J. M. Maxfield (✉) · K. S. Cole
University of Hawai'i at Mānoa, 2538 McCarthy Mall,
Edmondson Hall 216, Honolulu, HI 96822, USA
e-mail: je.maxfield@gmail.com

Gonad restructuring in protandrous (male to female) fish species shows a similar pattern in the restructuring of the gonad which begins with the degradation and autophagy of spermatogenic tissue, followed by the proliferation of oogenic tissue (Hattori and Yanagisawa 1991; Law and de Sadovy Mitcheson 2017). These studies of protandrous species found that the ‘male’ gonad is made up primarily of spermatogenic tissue undergoing active spermatogenesis, along with a reserve of immature oocytes. After sex change, the gonad consists of mature oocytes and no spermatogenic tissue is retained. While there is an abundance of information available on changes in gonad morphology in sequential hermaphrodites, there have been very few studies documenting morphological changes in the gonads of bidirectional sex changing fishes (i.e., individuals that can change in both directions) as they transition from producing one gamete type to the other (Liu and Sadovy 2004; Maxfield and Cole 2018).

Among bidirectional sex-changing fishes there is considerable variation in the morphology of the adult gonads. In some species an ovotestis is maintained throughout adulthood with both oogenic and spermatogenic tissue co-occurring at various stages of maturity (Cole 1990; Sunobe and Nakazono 1993). In others, no spermatogenic tissue is present in the gonad while in the oocyte-producing phase (Cole 1990). In the sperm-producing phase, previtellogenic oocytes are present in some species and absent in others (Cole and Hoese 2001; Wittenrich and Munday 2005). A detailed description of transitional gonad morphology has been conducted in only one bidirectional sex-changing species, the divine dwarfgoby, *Eviota epiphanes* (family Gobiidae) (Maxfield and Cole 2018).

The blue-banded goby, *Lythrypnus dalli*, is bidirectional sex changing species that ranges from Morro Bay California down through Ecuador (Van Tassell et al. 2010). Fish live in groups typically comprising one sperm-producing individual (i.e., in s-phase) and several ova-producing individuals (i.e., in o-phase) (St. Mary 1993). It has been the subject of numerous studies with respect to its ability to change its sexual function in both directions (St. Mary 1993; Carisle et al. 2000; Black et al. 2005; Rodgers et al. 2005, 2007; Maxfield et al. 2012; Solomon-Lane et al. 2014; Archambeault et al. 2016 among many others). The external morphology and behavioral changes that take place during sexual transition have been documented in detail for this species (Rodgers et al. 2005). In contrast, changes to gonad

morphology and details of the timing of the transition process have never been documented in *L. dalli*.

The goals of this study are to provide a detailed description of the process of transition from o-phase (oocyte production) to s-phase (sperm production) in *L. dalli*. This includes changes in morphology and to draw comparisons to another species of a goby, *E. epiphanes*. Cole (2010) proposed that based on differing adult hermaphroditic gonad morphology, functional hermaphroditism has evolved as many as five times within the Gobiidae. *Lythrypnus dalli* and *E. epiphanes* are both bidirectional hermaphrodites and occur in two well-separated gobiid clades (Tornabene et al. 2013a; Thacker 2015). The crown age for the clade *L. dalli* belongs to is approximately 35 my and the crown age for the clade *E. epiphanes* belongs to is approximately 39.1 my, supporting a long period of divergence (Thacker 2015). A comparison of *L. dalli* here with *E. epiphanes*, looking at the conserved and novel elements in the transition process, provides insights into how life history characteristics and social structure may have shaped the evolution of the process of sex-change in both species.

Methods

Live fish experiments and tissue collection

All fish collections took place at Isthmus Reef off of Catalina Island, California (33.44782 N 118.48932 W) from May 2014–August 2014. Fish were collected on scuba with hand nets by using clove oil as an anesthetic (California collection permit SC-13011), transported to the University of Southern California’s Wrigley Marine Science Center and held in 10-gal flow-through tanks (experiments were done under University of Southern California IACUC number 20172). After a 24-h recovery period fish were measured (standard length (SL), from the end of the rostrum to the caudle peduncle) with digital calipers and sexed under a dissecting microscope by observing the shape of the genital papilla. The genital papilla is a structure just posterior to the anus that is elongated in s-phase individuals and blunted in o-phase individuals (St. Mary 1993). To verify that the fish we selected were actively producing eggs, and not immature, juvenile, or incorrectly sexed based on genital papilla morphology, adults were initially placed into o-phase/s-phase pairs until spawning occurred. All tanks

were set up in outside facilities under natural summertime photoperiod conditions (roughly 14 h between sunrise and sunset). Water was supplied directly from the surrounding ocean and at relatively ambient conditions with water temperature between 16 and 18 °C. Each 10 gl tank housing a pair of fish had two 1/2" diameter PVC tubes to act as nesting sites and a small rock for shelter. Fish were fed twice daily with live brine shrimp and commercial fish flakes (Ocean Nutrition Prime Reef Flakes, Formula Flake Foods™). Tanks were checked daily for the presence of fertilized eggs, which would verify the presence of one adult in o-phase and one in s-phase. Eggs, when laid, were observed under a dissecting microscope for the presence of a clear chorion and multiple cells on top of the yolk to verify successful fertilization.

From the verified o-phase individuals (based on papilla morphology) from the above set up, new experimental pairs of just o-phase individuals were established. In natural populations of *L. dalli* the s-phase individual in a group is generally larger than o-phase individuals and when changing from o-phase to s-phase an individual rapidly increases in size (St. Mary 1994; Rodgers et al. 2005). For this reason, to better mimic a typical in situ social setting, an effort was made to avoid placing same-sized fish together in experimental pairs. Tank conditions for o-phase/o-phase pairs were identical to those of the s-phase/o-phase set up used for the first part of the experiment described above. Tanks were checked daily for the presence of fertilized eggs, which would indicate a change in functional sex of one individual in the pair of initially two o-phase fish. It takes approximately 14 days for *L. dalli* in captivity to complete sex change (Rodgers et al. 2005). In order to capture a range of transitional states of ovotestis transformation, fish pairs were euthanized over a 14-day time scale on Days 1, 2, 3, 5, 7, 8, 9, 10, 11, 12 and 14 days after set up. These are referred to here after as 'Day euthanized post-set-up'. Each sample period was designed to include three replicates, however, due to variable fish mortality, some days have just two replicates and some four. Number of replicates per day does not impact overall results, as 'day' was used to achieve maximum variation in gonad state not as a method for grouping fish for analysis. There were two replicates for Days 5, 9, 11, and 14, three replicates for days 2, 8, 10, 12 and four replicates from Days 1, 3, and 7 for a total of 32 experimental pairs. Fish were euthanized with tricaine methanesulfate (MS222) mixed at 0.5 g/l L of

seawater, dissected under a stereomicroscope (Olympus SZX16) and the gonads removed. One lobe of the gonad was preserved in Dietrich's fixative (300 ml 95% ethanol, 100 ml formalin, 20 ml glacial acetic acid and 580 ml double-distilled H₂O) for at least 24 h for histological analysis.

Control o-phase and s-phase fish

To obtain a baseline for comparison to transitional fish, three o-phase/s-phase pairs, identified as such based on genital papilla morphology, were established directly from wild caught fish. In all pairs the s-phase individual was between 4 mm and 7 mm larger (SL) than the o-phase individual. These individuals, referred to here after as control pairs, spawned in captivity within 7 d, confirming that they were reproductively active adults. After spawning, these three o-phase/s-phase pairs were euthanized with MS222 (0.5 g/l L of seawater), the gonads were removed under a dissecting stereomicroscope, and preserved in Dietrich's fixative for histological analysis. These non-transitioning control fish were used as a baseline for what constituted an active s-phase and an active o-phase individual, in terms of gonad morphology. Comparisons between transiting fish and control fish provided diagnostic characters for placement of experimental fish into different transitional stages.

Histological analysis

Fixed tissues consisting of one gonadal lobe were rinsed for 24 h in gently running tap water, dehydrated with a series of ethanol baths increasing in concentration from 30 to 100%, passed through two changes of xylene and a final 50:50 xylene-paraffin solution, then embedded in paraffin. The resulting blocks were serially sectioned at 5 µm on a rotary microtome (Leica RM2255). Sections were mounted onto slides, stained with Harris modified hematoxylin and acidified eosin (Sigma-Aldrich) and protected with a cover slip using Cytoseal 60 low viscosity mounting medium (Richard-Allan Scientific). Slides were then observed under a light microscope (Olympus BX61). Digital images were taken with a mounted digital camera (Olympus DP71) and then viewed using MicroSuite™ imaging software, version five (Olympus). All images were cropped and color balanced in Adobe Photoshop (CC 19.1.5). Edits made to images did not alter the data that was taken from them.

For same-sex pairs external morphology characters (i.e., genital papilla morphology, differences in body size and dorsal fin shape) alone were not always enough to determine which fish in a pair were in the process of transition. For this reason both fish in every pair were examined histologically to determine which fish in that pair were in the process of transition.

Transitional stage classification

‘Day’ was initially used to get a variety of transitional states; however, fish were grouped into chronological stages of transition based on changes in ovotestis morphology. A post-hoc cluster analysis was applied to the morphological data to group individuals based on their gonad state.

The total area of the ovotestis and the areas made up of spermatogenic (tissue in the process of capable of producing sperm), oogenic (tissue in the process or capable of producing oocytes), and atretic ovarian tissue (ovarian tissue in the process of braking down), respectively, were quantified using Adobe Photoshop’s magnetic lasso and measurement tools. Because of natural variation in ovotestis size that was not related to transitional state, the percent of the ovotestis made up of each tissue type (area tissue type/total gonad area) was used for analysis rather than the area values. The entire gonadal lobe was initially sectioned, but only an average of five slides were used for measurements and the calculation of the percentage of each tissue type. Selected sections ranged from the posterior to the anterior region of the gonadal lobe to ensure that the entirety of the gonad was considered in analysis. For each fish, the mean percent of each tissue type as averaged from all sections examined, was used for subsequent cluster analysis. We used a *k*-mean cluster analysis (MacQueen 1967) separating 32 samples (*n*) into six clusters (*k*) implemented in JMP® 13. This analysis grouped individuals based on the mean percentage all three tissue types. Individuals grouped into one of six clusters with the nearest mean of ovarian, spermatogenic, and atretic tissue. These clusters were then compared to one another and to control fish to identify diagnostic characters that defined each cluster/transitional stage.

Following the classification of each ovotestis into one of several stages with respect to ovotestis morphology, we did a correlation analysis using Spearman’s correlation (JMP 13), to see how closely ‘Day euthanized’ was linked to transitional stage.

Results

Control pairs

In the three control o-phase/ s-phase pairs, the gonadal tissue of the three s-phase individuals was predominantly spermatogenic, making up between 77% and 99% of the ovotestis. The spermatogenic tissue was arranged into seminiferous lobules consisting of numerous spermatocysts surrounding the sperm-filled lobule lumina. The remaining portion of the ovotestis comprises previtellogenic oocytes (Fig. 1). No atretic tissue was present. In the three o-phase individuals the majority of the ovotestis was made up of vitellogenic oocytes and some previtellogenic oocytes (between 98% and 99%). Spermatogenic tissue was also present but made up <0.3% of the gonad. In one of the three o-phase fish, spermatozoa were present within the spermatogenic region (Fig. 1). Atretic tissue made up between 0% and 1.3% of the ovotestis.

Transitional pairs

There was evidence of transition beginning almost immediately upon set-up into same-sex pairs. The first signs in the ovotestis of transition, atresia of ovarian tissue, was seen as early as one day post-setup. After the initiation of sex change, it took as little as ten days for transition to complete. This was evidenced by the presence of fertilized eggs. A total of 32 o-phase pairs were sampled over a 14-day time period to get a range of transitional states. Gonads for both individuals in a pair were examined histologically to determine which fish was in the process of transition. In all but one transitional pair, the larger individual was the one to initiated transition.

In 28 pairs one individual was in o-phase while the other was clearly in the process of transition (noted as increase in atresia and proliferation of spermatogenic tissue). In one other pair, both individuals had begun to transition. In the remaining three pairs it appeared that neither fish was transitioning, with both fish maintaining high levels of vitellogenic oocytes and no atretic tissue present. These latter three pairs were not used for subsequent analysis, leaving a total of 30 individuals transiting from o-phase to s-phase for analysis (28 from pairs where one fish transitioned and two from a pair where both fish began to transition).

The Spearman’s correlation analysis between day euthanized and transitional stage revealed a positive

correlation between the two variables ($p = 0.0001$, Fig. 2). Explained variance however was poor (Spearman's $\rho = 0.677$), supporting the use of morphological data and not 'Day' as a predictor of transitional stage.

The k-means cluster plot grouped individuals into four large clusters ($n =$ between three and 15 individuals) and two smaller clusters ($n = 1$), (Fig. 3). Upon comparison of each cluster to control fish we determined it was necessary to separate Cluster 5 into two clusters (denoted by a red line, Fig. 3) due to differing amounts of spermatogenic tissue present. In Cluster 5, 11 of the fish had proportions of spermatogenic tissue similar to control o-phase control fish. The remaining four fish had proportions of spermatogenic tissue higher than control o-phase fish. A Kruskal-Wallis rank sum test revealed the percentage of oogenic and atretic tissue was not statistically different between these two groups (oogenic X stage $p = 0.7439$, atretic tissue X stage $p = 0.3275$). The amount of spermatogenic tissue however was statistically different between these groups (spermatogenic X stage $p = 0.0043$). Based on a similarity in the amount of vitellogenic oocytes present, Cluster 2 was grouped with Cluster 1 for further analysis. Based on a similarity in the amount of oogenic and atretic tissue present, Cluster 4 was grouped with Cluster 3.

Though we are treating gonad transition as a discreet process by placing individuals into stages, it should be noted that in reality, morphological transition occurs along a continuum. While the stages described below are not always clearly distinguishable in terms of the proportional presence of any single tissue type, the dividing line between groups reflects the predominant co-occurrence pattern of all three tissue types. And it is the co-occurrence pattern that serves as a basis for comparisons with control fish. This is reflected in the k-means cluster analysis, which grouped fish based on the mean percentage of the three tissue types.

Stage O_1: Initiation of sexual transition

Fish placed in this stage are from Clusters 1 and 2 on the k-means clustering plot (Fig. 3) and were at the very start of transition. The gonad was still primarily made up of oogenic tissue, but to be placed in this stage there needed to be more atretic tissue present than found in control o-phase fish. Stage O-1 fish ($n = 7$) had an ovotestis primarily consisting of vitellogenic oocytes.

Oogenic tissue consisting of vitellogenic and previtellogenic oocytes made up 79% -92% of the gonad and atretic oocytes made up 2.9% - 13.4% of the gonad. Spermatogenic tissue was present in all individuals in the dorsal and ventral regions of the gonad (Fig. 4). Spermatogenic tissue ranged from 0.37% - 4.4% (average: 1.8%) of the gonad. In four individuals, tailed sperm (spermatozoa) were present within the lumina of the seminiferous lobules. Fish determined post-histology to be in Stage O_1 were euthanized on Days 1, 2, 3, and 12.

Stage O_2: Complete atresia

Stage O_2 individuals were in the lower half of Cluster 5 in the k-means cluster plot (below the red line Fig. 3). Fish in this stage were characterized as having more atresia than Stage O_1 individuals, and having no vitellogenic oocytes in contrast to both O_1 and control o-phase fish. In stage O_2 individuals ($n = 11$) the majority of the gonad was made up of atretic follicles, between 87% and 99%, accompanied by some previtellogenic oocytes. In only six individuals was spermatogenic tissue also present, between 0.07% - 1.7% (average: 0.5%) (Fig. 4b) which didn't differ from the amount of spermatogenic tissue present in control o-phase fish. Of those with spermatogenic tissue, four of the six had tailed sperm present. The gonad of the remaining five individuals had no spermatogenic tissue present. Fish in this stage were collected on Days: 3, 5, 7, and 12.

Stage O_3: Initial Spermatogenic proliferation

Stage O_3 was made up of individuals in the upper half of Cluster 5 in the k-means cluster plot (above the red line in Fig. 3). These individuals had a greater proportion of the gonad made up of spermatogenic tissue than the previous two stages and o-phase control fish. Stage O_3 ($n = 4$) was characterized as having high levels of atresia and between 4% and 10% (average: 7.2%) of the gonad consisting of spermatogenic tissue (see Fig. 4c). Spermatogenic tissue was arranged into several spermatogenic lobules. Two out of the four individuals also had spermatozoa present within the lobular lumina. Previtellogenic oocytes were present in all individuals but no vitellogenic oocytes were present. Individuals in stage O_3 were collected on Days 8 and 10.

Stage O₄: Continued Spermatogenic growth

Fish in Stage O₄ were found in Clusters 3 and 4 in the k-means cluster plot (Fig. 3). Stage O₄ individuals ($n=4$) had gonads contained mature sperm and increased proportions of spermatogenic tissue compared to stage O₃ individuals, with a gonad made up of 9%–27% (average: 15%) spermatogenic tissue (Fig. 5a). Individuals in this stage also had a high portion of oogenic tissue (in the form of previtellogenic oocytes) and a lower percentage of atretic tissue when compared to stage O₃

individuals. Individuals in this stage were collected on Days 10, 11, 12, and 14.

Stage O₅: Advanced Spermatogenic growth

The Stage O₅ individuals were found in Cluster 6 in the k-means cluster plot (Fig. 3) and had a higher proportion of spermatogenic tissue on average than the stage O₄ individuals (as indicated in the k-means cluster plot). There was also a larger amount of sperm within the seminiferous lobules when compared to Stage O₄. There was a higher proportion of atretic tissue and a

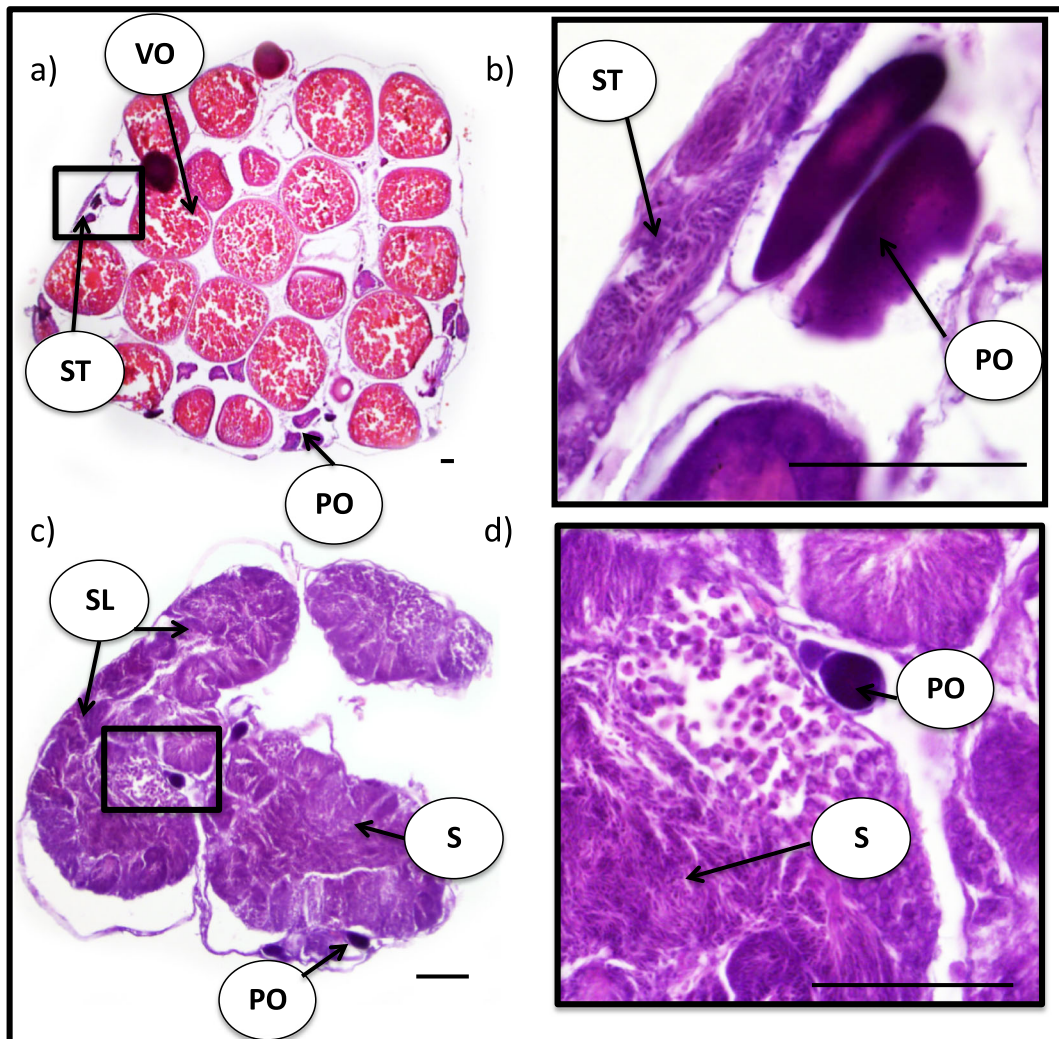
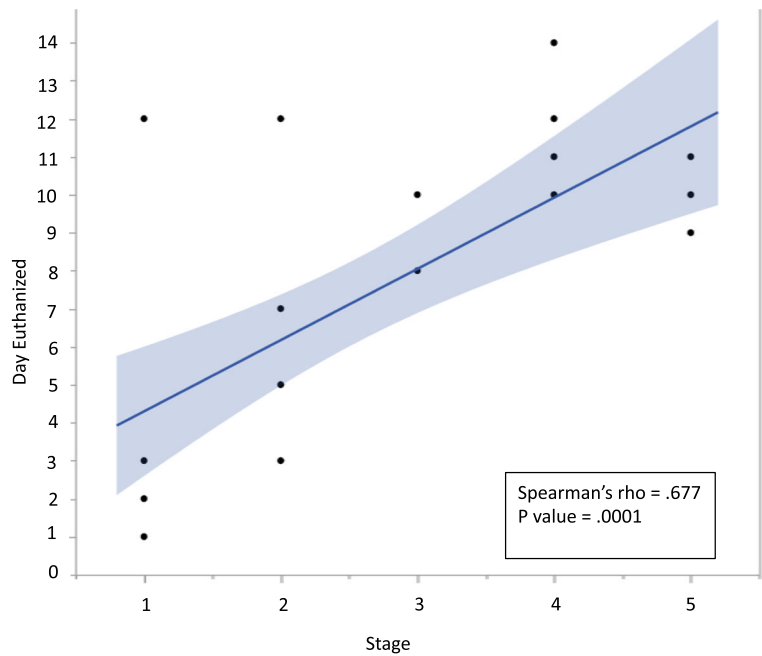


Fig. 1 Cross sections of the gonad of a representative o-phase (a, b) and s-phase (c, d) *Lythrypnus dalli*. **a** Section of an entire o-phase gonad with vitellogenic oocytes (VO), some previtellogenic oocytes (PO) and a small portion of spermatogenic tissue (ST). **b** Detail of region outlined with a black box in (a). **c** Section of an entire s-phase

gonad made up almost entirely of seminiferous lobules (SL) and exhibiting a large quantity of spermatozoa in the lobular lumina (S). **d** Detail of region outlined with a black box in (c) showing sperm (S) and a pre-vitellogenic oocyte (PO) in close proximity. All scale bars are 50 μm

Fig. 2 Correlation between the day individuals were euthanized (Y-axis) and the transitional stage (O_1 to O_5) they were placed into (X-axis). Spearman’s rho is 0.677 and *p* value is 0.0001



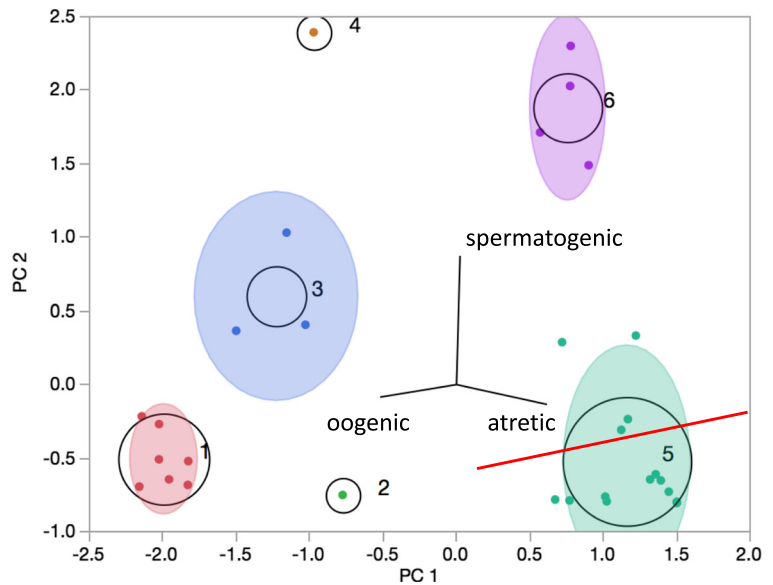
lower proportion of oogenic tissue when Stage O_4. The gonad of O_5 individuals (n=4) was made up spermatogenic lobules containing large amounts of sperm, as well as atretic tissue and a small number of previtellogenic oocytes (Fig. 4). The proportion of spermatogenic tissue ranged from 20 to 28% (average: 23%) of the gonad. Fish placed in this stage came from Days 9, 10, and 11.

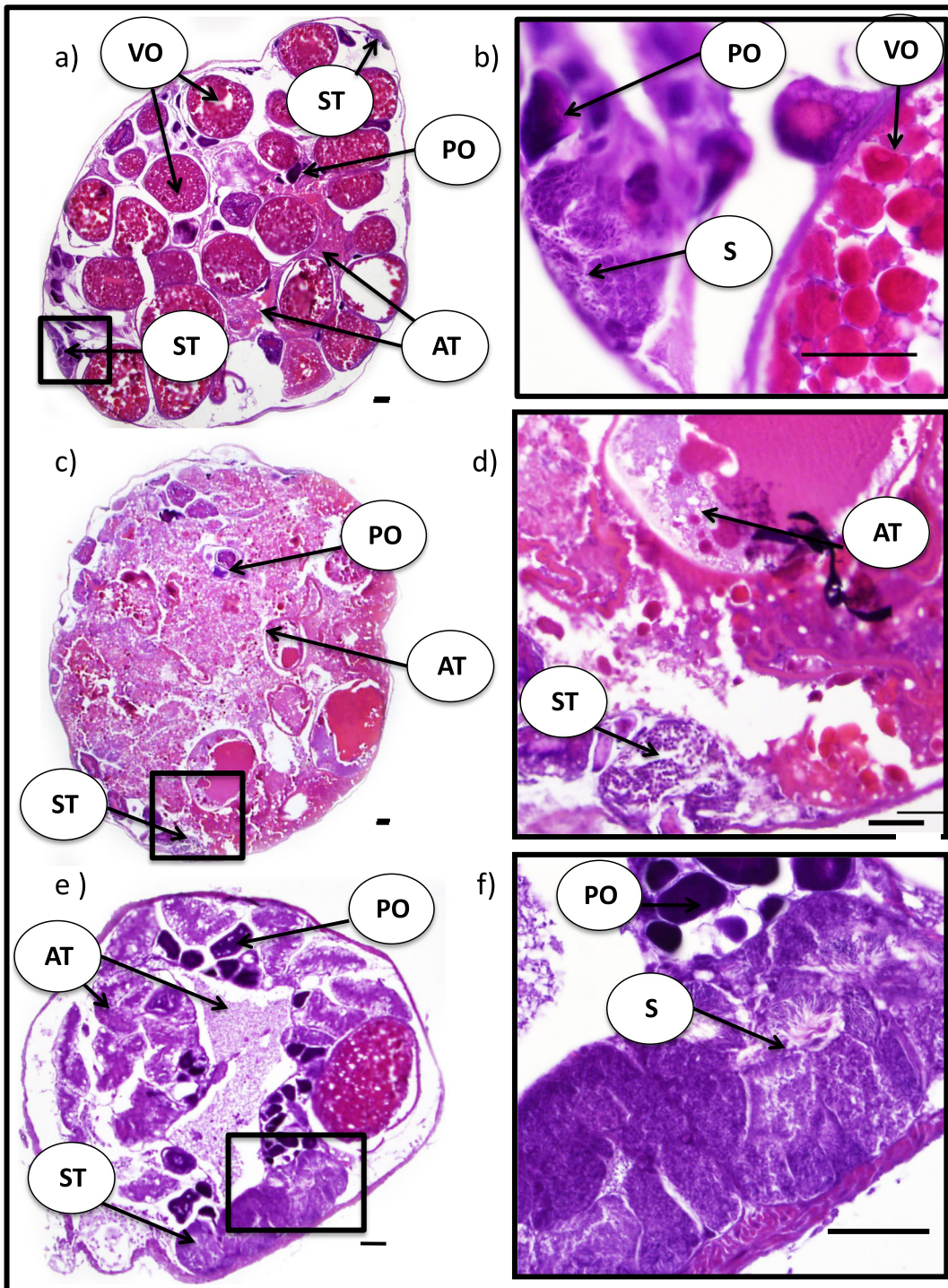
Discussion

A summary of gonadal changes during sex change

The gonads of all *Lythrypnus dalli* transiting from o-phase to s-phase contained oogenic tissue. Twenty-four (82%) of the examined fish had gonads containing both spermatogenic and oogenic tissues (i.e., an ovotestis). In

Fig. 3 K Mean Cluster biplot, distinguishing six groups based on three axes, the mean value of oogenic, spermatogenic and atretic tissue present. The red line bisecting Cluster 5 post-hoc represents a division based on a difference in spermatogenic tissue, when compared to control fish





the remaining five transitioning fish, no visible spermatogenic tissue was present. In this study the process of transitioning from producing one gamete type to the other was, broadly speaking, best characterized by a

change in the proportion and maturity of tissue types in the gonad. In *L. dalli*, the process of changing from ova production to sperm production started with the breakdown of vitellogenic oocytes (i.e., atresia). This

Fig. 4 This figure shows transitional stages 1–3. The image on the left shows an entire cross section of the gonad and the images on the right show a magnification of a specific region of that section (outlined with a black box). All scale bars are 50 μm . **a, b** These shows a stage O₁ individual. **a** The gonad is made up predominantly of vitellogenic oocytes (VO) with some previtellogenic oocytes (PO), some atretic tissue (AT) and a small portion of spermatogenic tissue (ST). **b** A magnification of the region outlined in the black box in (a), clearly showing the presence of spermatozoa (S). **c–d** These are representative images for stage O₂ individuals. **c**. The gonad is made up predominantly of atretic tissue (AT) with some previtellogenic oocytes (PO), and some spermatogenic tissue (ST). **d** A magnification of the region outlined in the black box in (c). **e–f** These images shows a stage O₃ individual. **e** The gonad is made up of some atretic tissue (AT), previtellogenic oocytes (PO), and spermatogenic tissue (ST). **f** A magnification of the region outlined in the black box in (e) showing the presence of spermatozoa (S)

process continued until no intact, vitellogenic oocytes remained and the gonad was almost entirely made up of atretic tissue accompanied by a small portion of both pre-vitellogenic oocytes, and with some spermatogenic tissue. From this point spermatogenic tissue began to proliferate and expand into the gonad from both the dorsal and ventral regions. By the end of the transition, the gonad predominantly comprised spermatogenic tissue with sex cells in various stages of development and was accompanied by a small number of previtellogenic oocytes.

Our finding of the larger individual being the one to initiate sexual transition (28 out of 29 pairs), is consistent with previous work in which the larger individual in a group of o-phase fish was, on average, the one to initiate sexual transition (St. Mary 1994). In the case of one experimental group in which both individuals started to transition (size differential = 4 mm) there was no obvious indicator as to why the smaller individual also initiated a shift in gonad morphology.

Allocation to active oogenic and spermatogenic tissue

In a study of wild caught *L. dalli* 30% had both vitellogenic oocytes and spermatozoa simultaneously present, even in fish with higher proportions of spermatogenic tissue (St. Mary 1993). This differed from our experiment in that vitellogenic oocytes were only seen in o-phase and Stage O₁ transitional fish; they were never present in later stage transitional fish or s-phase fish. In contrast, the presence of spermatozoa was seen in all transitional stages and in both o-phase and s-phase control fish.

In St. Mary (1994), a strong correlation was found between the proportion of spermatogenic tissue present in the gonad and spawning success. Individuals acting as the sperm-producing individual during spawning but having less than 24% of the gonad comprising spermatogenic tissue had no spawning success in terms of acquiring and fertilizing eggs. This suggests that although many experimental individuals in o-phase and stages O₁ - O₃ in our study had spermatozoa present in the gonad, it is unlikely that these individuals were capable of producing a clutch of fertilized eggs successfully under natural conditions.

Comparison to other sex changing fish species

In *L. dalli*, the process of breaking down ovigerous tissue, reabsorbing maturing oocytes, and initiating the proliferation of spermatogenic tissue type is similar to that described in reports of changes in gonad morphology among protogynous fishes (Nakamura et al. 1989; Shapiro et al. 1993; Bhandari et al. 2003; Sundberg et al. 2009). In contrast, *L. dalli* differ from protogynous species in maintaining spermatogenic and oogenic tissue in the gonad before, during, and after shifting sexual function. In protogynous fish species that have been examined, only one tissue type is present after transitioning (Nakamura et al. 1989; Hattori and Yanagisawa 1991; Shapiro et al. 1993; Bhandari et al. 2003; Sundberg et al. 2009; Law and de Sadovy Mitcheson 2017). As protogynous species do not have the capacity to reverse sexual function back to ova production, there is no need to retain oocytes. When comparing *L. dalli* to the only other bidirectional hermaphroditic fish species described in similar detail, *Eviota epiphanes* (Maxfield and Cole 2018), we see both similarities and differences. The overall process of the breakdown of vitellogenic oocytes and proliferation of spermatogenic tissue is similar between the two species. They were also similar in that oocytes in various developmental stages were present in the gonads of all fish, including o-phase, s-phase and all stages of transition. The majority (82%) of *L. dalli* examined here maintained spermatogenic tissue at all stages of transition as well as when in o-phase and s-phase. The incidence in *E. epiphanes* was much lower, only 36% of individuals in transition and in o-phase and s-phase had spermatogenic tissue present (Maxfield and Cole 2018). This difference in allocation to spermatogenic tissue between species may be do to differences in their social

structure and related to the microhabitat specialization and high sight attachment exhibited by *Eviota* species (St. Mary 1993; Munday et al. 2010; Tornabene et al. 2013b).

Another difference of note regarding the transition process between *L. dalli* and *E. epiphanes* is how many individuals in a same-phase pair initiate gonadal transition when first established. In *E. epiphanes*, both individuals began to transition in half (16 of 33) of same-

phase pairs examined (Maxfield and Cole 2018). In contrast, with *L. dalli* only one of 29 same-phase pairs (<3%) had both individuals begin to transition. This notable difference in transition initiation may reflect differences life history characteristics. The relative life span of *E. epiphanes* (estimated to be a maximum of 60 days; Grant 2013) is much shorter than that of *L. dalli* (estimated to be 1.5–2 years, Wiley 1976). There may therefore be greater consequences for missing out on the

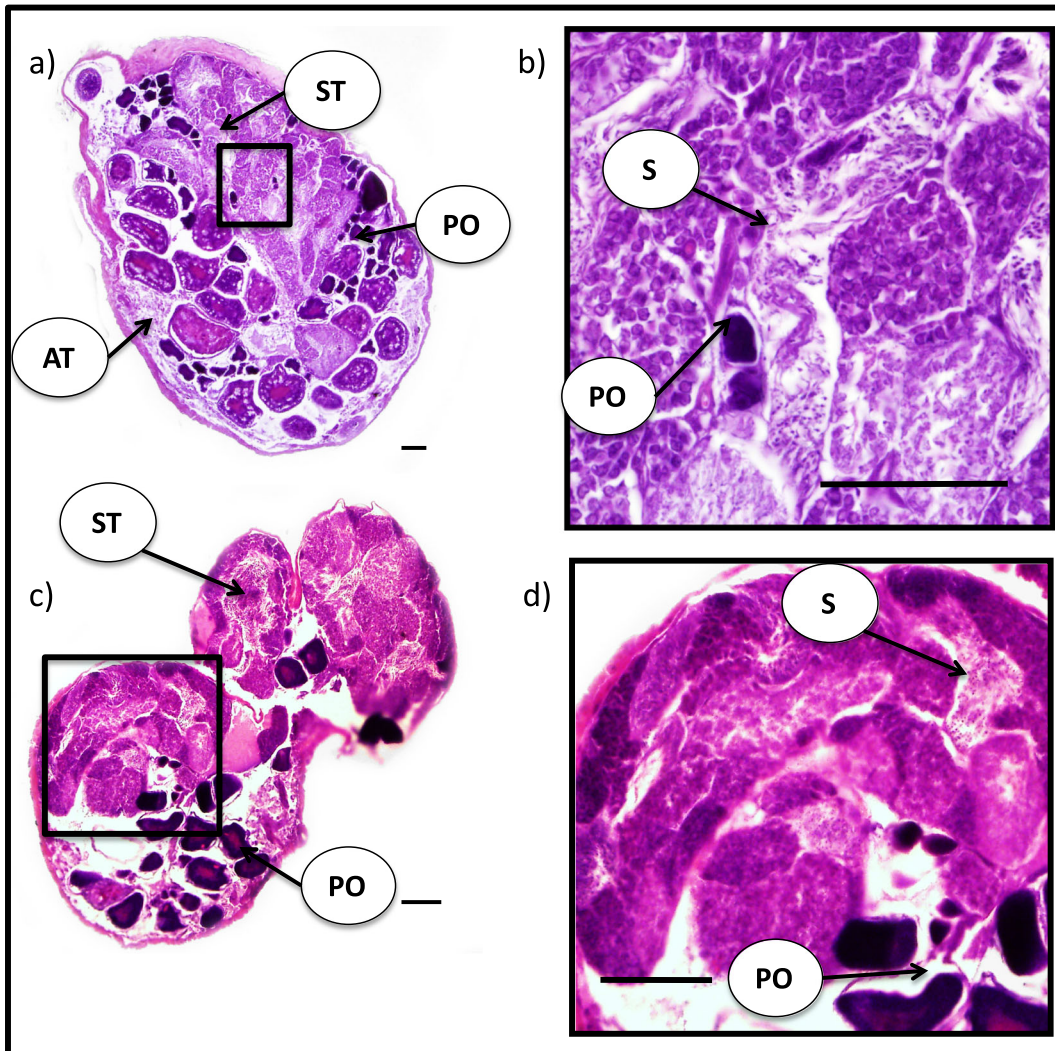


Fig. 5 This figure shows transitional stages 4 and 5. The image on the left shows an entire cross section of the gonad and the images on the right show a magnification of a specific region of that section (outlined with a black box). All scale bars are 50 μm . **a**, **b** These are a representative image for stage O_4 individuals. The gonad is made up predominantly of previtellogenic oocytes (PO) and spermatogenic tissue (ST), with some Atretic tissue (AT)

present. **b** A magnification of the region outlined in the black box in (a), showing the presence of spermatozoa (S). **c**, **d** These images represent a stage O_5 individual. **c** The gonad is primarily spermatogenic tissue (ST) and some previtellogenic oocytes (PO). **d** A magnification of the region outlined in the black box in (c), showing the presence of an abundance of spermatozoa (S)

opportunity to produce sperm for the short-lived *E. epiphanes*. *Eviota epiphanes* is collected at roughly a 1:2 (s-phase:o-phase) ratio, which suggests the possibility of a harem social structure (Cole 1990). If they do live in harems, sperm producers would have significantly increased opportunities for embryo production compared to the individual females within the harem. Therefore, when given the opportunity to change sexual function in the absence of an s-phase individual, it may be worth the cost in lost time and energy to attempt to transition.

While our work does not directly address Cole's (2010) hypothesis that the ability to change sex evolved independently in these two lineages, it does illustrate the differences in life history and possibly social structure that may have played a role in shaping the evolution of differences in gonad morphology and timing during the process of transition.

In conclusion, we found no differences in transitional gonad morphology between *L. dalli* and *E. epiphanes* based on the criteria of this study. The process of breaking down oogenic tissue and the proliferation of spermatogenic tissue during a sexual transition is similar between the two species and similar to what is observed in protogynous sex changers. We did however observe striking differences in the process of transition, in the timing it took each species to initiate transition, the timing to the first fertilized eggs, when tailed sperm first appear, and the number of individuals within a pair to initiate transition. We hypothesize that many of these differences are due to differences in social structure and life history between these two species.

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Conflict of interest The authors declare that they have no conflict of interest.

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