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Research Article

A case of hermaphroditism in the gonochoristic sea urchin, *Strongylocentrotus purpuratus*, reveals key mechanisms of sex determination†

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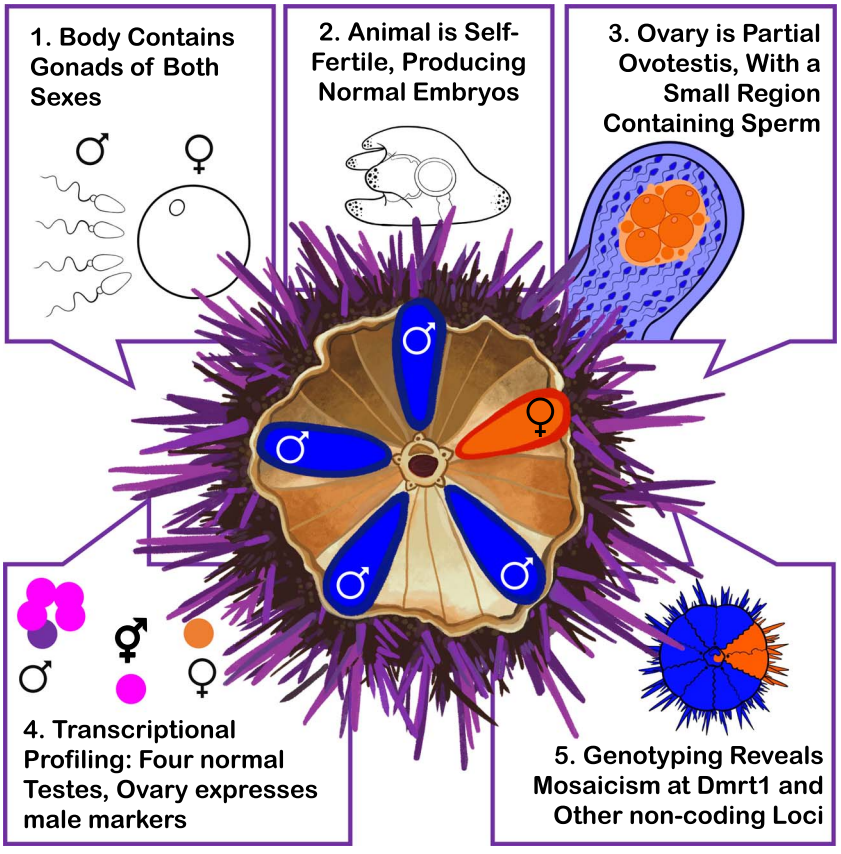
Abstract

Sea urchins are usually gonochoristic, with all of their five gonads either testes or ovaries. Here, we report an unusual case of hermaphroditism in the purple sea urchin, *Strongylocentrotus purpuratus*. The hermaphrodite is self-fertile, and one of the gonads is an ovotestis; it is largely an ovary with a small segment containing fully mature sperm. Molecular analysis demonstrated that each gonad producedviable gametes, and we identified for the first time a somatic sex-specific marker in this phylum: Doublesex and mab-3 related transcription factor 1 (DMRT1). This finding also enabled us to analyze the somatic tissues of the hermaphrodite, and we found that the oral tissues (including gut) were out of register with the aboral tissues (including tube feet) enabling a genetic lineage analysis. Results from this study support a genetic basis of sex determination in sea urchins, the viability of hermaphroditism, and distinguish gonad determination from somatic tissue organization in the adult.

Summary Sentence

A self-fertile hermaphroditic *S. purpuratus* sea urchin containing four testes and one ovary/ovotestis is described; the results support a hypothesis of genetic sex determination in this species.

Graphical Abstract



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Highlights

- A hermaphrodite sea urchin is described containing four testes and one ovary/ovotestis
- The hermaphrodite is self-fertile
- The ovotestis is largely an ovary with a small segment containing sperm
- The Doublesex ortholog, DMRT1, was found to be a sex-specific marker in *Strongylocentrotus purpuratus*
- These results support a genetic basis of sex determination in sea urchins
- We find that gonad determination is distinguishable from other somatic tissue organization in the adult

Keywords: sea urchin, ovary, testis, ovotestis, hermaphrodite, hermaphroditism, sex determination

Introduction

Reproduction is a defining quality of life, and a fundamental cell-fate decision for a developing embryo is the determination of mating type or of sex. Although sex as male and female appears binary in most metazoans, no molecular definition has been offered that is universal for defining sex. Instead, we rely on here the production of a motile gamete, a sperm cell, as a male; and production of a larger, non-motile gamete, the egg, as a female. The environment within which these gametes develop is provided by highly specialized somatic cells, non-germline in origin, that often develop from a bipotential precursor referred to as the "bipotential gonad" [1, 2]. Cellular signals in the embryo, larvae, or early adult direct this bipotential gonad to develop into an organ that will support the development of sperm (the testis) or eggs (the ovary), the aforementioned process referred to as sex determination [3]. Because functional sperm and eggs are highly specialized cell types, such signals must be unique to each sex, running the risk of infertility if they are abnormally regulated [4, 5]. Signals for sex determination in the gonad are often genetic: inherent to the individual and present in its genome [6–8]. For example, in mammals genetic sex determination relies on X/Y sex chromosomes to determine the fate of the bipotential gonad [9]. Alternatively, the gonad and/or its precursor may instead respond to signals in the environment that direct sex determination [10]. Such is the case in reptiles that rely on the ambient temperature during embryonic development for sex determination, or in many fish, even as adults, which change the sex of their gonads in response to external cues [11]. Many commonly studied sexually reproducing species are gonochoristic: either male *or* female; and such is the case in the species of the purple sea urchin, *Strongylocentrotus purpuratus* (Sp).

Many sexually reproducing species are hermaphroditic. Hermaphrodites may be sequential (switching from male to female or vice versa) like the clownfish [12] or simultaneous (both male and female) like gastropod snails and slugs of the orders Opisthobranchia and Pulmonata [13]. Hermaphroditic animals may also have separate gonads (ovary and testes) within their body or gonads containing both tissue types in a single organ called an ovotestis. Furthermore, certain species may even include hermaphrodites as a third sex, as observed in some nematode species [14, 15]. Regardless of whether the animal is gonochoristic or hermaphroditic, the process of sex determination must be accomplished, directed by the action of sex chromosomes, genetic factors, paracrine signaling molecules, or sex hormones [4, 6, 16, 17]. To further complicate the molecular mechanisms of sex determination, sex determination in both gonochoristic and hermaphroditic species may be influenced by diet, environment, behavior, and hormones, and is further compounded by toxins and pollutants, all in addition to the genes which act directly on the fate of the bipotential gonad [18]. To this end, we may often assume that an animal is simply male *or* female based on a single chromosome or a gene locus. However, such is not the paradigm in nature, and we must account for such extreme diversity and complexity when studying sex determination, in even seemingly "cut-and-dried" gonochoristic animals, like the purple sea urchin.

Thus far, it has been hypothesized that sea urchins (echinoids) have a genetic basis of sex determination. This conclusion stems largely from an experiment in which cells of an embryo at the two-cell stage were separated, cultured individually, and they each developed into separate larvae with normal physiology [19]. The resultant adult urchins from each half embryo, however, were always of the same sex [19]. The observation of a genetic basis of sex determination was also supported by karyotyping. A few chromosome spreads performed in echinoid species, notably even *S. purpuratus*, imply that the male urchin in this species may be the heterogametic sex, although no definitive male-specific chromosomal loci have been identified [20, 21]. Therefore, nothing conclusive has been established thus far as to whether echinoids have a chromosomal, autosomal, or gene-regulatory sex determination mechanism.

Furthermore, it is known that several species of sea stars (asteroids) may be sequential or simultaneous hermaphrodites, having both ovary and testis tissue within the body of the same animal. Recently, it was reported in *Acanthaster planci* (crown of thorns starfish, or COTS) that both ovary and testis markers were present in each gonad, suggesting a tendency toward hermaphroditism more commonly than previously expected [22]. COTS individuals, commonly thought to be a gonochoristic species, have a relatively high incidence of hermaphroditism [22]. However, hermaphroditism is rare in echinoids (sea urchins), and most echinoid genera are gonochoristic [23, 24]. Booloottian and Moore (1956) estimated that a hermaphrodite occurs between 1:500 and 1:3000 in various gonochoristic sea urchin species [24]. Furthermore, J.S. Pearse and R.A. Cameron discuss the discrepancies between 1:500 and 1:3000 chances of hermaphroditism in gonochoristic urchins, and hypothesize that adverse seasonal conditions may influence the incidence of hermaphroditism [23]. Perhaps turbulence, physiological stress, or nutrition may have an effect on normal genetic sex determination, producing abnormally high numbers of hermaphrodite urchins due to intense stress during early cleavage and embryonic development. Regardless of the probable cause, in our laboratory we receive approximately ~1200 *S. purpuratus* yearly, and in the past 5 years (2018–2022) we have observed a single hermaphrodite Sp. This puts our estimates of the incidence of hermaphroditism in

this animal in the 1-in-5000 range. Whatever the cause, the true incidence of hermaphroditism in gonochoristic echinoderms is unclear, but through firsthand experience, we have observed that it is incredibly rare to find a visually evident hermaphrodite sea urchin.

Here, we document a hermaphroditic purple sea urchin, *S. purpuratus*, the results of which support the occurrence of hermaphroditism, of a partial ovotestis, and of a genetic basis for sex determination in this animal. Leveraging several new markers of sex in this animal, we also reveal new basic insights into how the adult body plan may be constructed.

Materials and methods

Dissection

All dissections were performed on ice, in sterile filtered artificial seawater (ASW) prepared at 34 ppt salinity (Instant Ocean, Cat# 138510). Gut segments, tube feet, and other somatic tissues were set aside and frozen at −80°C for further analyses. While dissecting individual gonads, sperm and eggs were collected for crosses. The resultant embryos of each of these crosses are stored at −80°C for any future studies.

Culture

All larval cultures were performed following the crosses outlined in supplemental figure 8, and larvae were grown published previously [25].

Imaging/antibodies

Tissues were fixed in 4% paraformaldehyde in filtered ASW (Paraformaldehyde, Electron Microscopy Sciences, Cat #50-980-487) for 1h at room temperature. After fixation, tissues were rinsed in phosphate buffered saline (PBS) and processed through a sucrose gradient for embedding in OCT (Optimal Temperature Cutting medium, Fisher, Cat #23-730-571). OCT-embedded tissues were sectioned into 20-μM sections on Leica Cryostat (Model #CM3050 S). Sectioned tissues were allowed to dry at room temperature.

Immunohistochemistry was performed as follows: 10-min rehydration (PBS), 10-min permeabilization (PBST) three times, 45-min blocking (PBST + 4% sheep serum), overnight primary antibody application (in PBST + SS at 4°C), 10-min washing (PBST) three times, 1-h secondary antibody application (in PBST + SS at room temperature), and 10-min washing (PBST) four times.

Tissue was treated with the following antibodies at the specified dilutions: 1:300 rabbit anti-Vasa C terminus antibody (custom made, generated in rabbit against Sp antigen), 1:50 mouse monoclonal anti-Hyalin antibody (custom made, generated in mouse against *S. purpuratus* antigen), 1:300 rabbit anti-SoxE antibody (custom made against *S. purpuratus* SoxE), 1:1000 mouse monoclonal anti-tubulin antibody (Abcam, Cat# ab7291), 1:1200 rabbit anti-bindin antibody [26, 27] (custom made against *Hemicentrotus pulcherrimus* bindin), 1:500 Alexa Fluor 488 anti-rabbit secondary antibody (Invitrogen, Cat# A11008), and 1:500 Alexa Fluor 594 anti-mouse secondary antibody (Invitrogen, Cat# A32742).

All fluorescent images were produced on the Nikon Spinning Disk AX-Series Confocal microscope using 20× dry, as well as 40× and 60× oil-immersion lenses.

Quantitative polymerase chain reaction

For each sample analyzed, RNA was isolated using Trizol reagent. $N = 3$ unrelated ovaries and $N = 3$ unrelated testes were used for the unrelated control data points. Isolated RNA (300 ng) from each sample was used to generate cDNA with Maxima First Strand reagents (Cat# K1641). qPCRs were performed as published previously (see Supplementary Table S1 for primer sequences). All gene expression is normalized to a ubiquitin control [25].

SNP analysis

Single nucleotide polymorphisms (SNPs) from the Nodal promoter, SoxE promoter, and an intergenic region corresponding to Scaffold# NW_022145604.117136530.0.17199660 (Echinobase S. purpuratus v 5.0) were obtained via PCR amplification using GoTaq Green Mastermix reagents (Cat# Promega M7822). See Supplementary Table S1 for primer sequences used. Nodal promoter sequences were identical to those published previously [25].

All PCR products were sent for sequencing to Azenta Genewiz using Fw and Rev primers for bidirectional sequencing (https://www.genewiz.com/en). All analyses of SNPS were performed using Clustal Omega alignment software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

We present the first report of its kind, detailing the anatomy, molecular biology, and physiology of a hermaphrodite sea urchin. We provide data and a hypothesis that sex determination in this species is at least, in part genetic, and discuss the revelation of molecular markers of sexual differentiation in the purple sea urchin.

Physical description of the hermaphrodite

On 29 March 2022, we received a shipment of *S. purpuratus* (Sp) from Marinus Scientific collected at the Los Angeles Harbor Lighthouse break wall (Supplementary Figure S1), approximately 33°42′19.5″N 118°16′20.7″W (33.705416, −118.272418). Within the shipment of normal animals was a single sea urchin spawning both eggs and sperm (Supplementary Figure S1).

External features of the hermaphrodite are as follows: penta-radial symmetry, spines, coloration, gonopores, and madreporite appeared unremarkable (Supplementary Figure S1). Echinoids have penta-radial symmetry, and simple lines delineating segments of the test are clearly visible on the outside, inside, and internal anatomy of the animal. For naming purposes, segments were oriented (Figure 1) starting with the first segment containing the madreporite, designated as "♀1." The only apparent physical abnormality observed was a slight difference in the animals' locomotion, visible as tube feet activity (Supplemental video). It was interesting to note that segments ♀1, ♀2, and ♀4 were, in general, more active while segments ♀3 and ♀5 had little to no activity, and no tube feet were extended or motile (Supplemental video).

Upon opening the animal, the mouth and esophagus appeared normal, and five individual segments of the gut were each collected corresponding to each of the five internal body segments (Supplementary Figures S2 and S3). After removing the gut and inspecting the gonads, we identified four large gravid testes corresponding to body segments 1, 3, 4, and 5 (Figure 1). These are further referred to as gonads ♀1, ♀3, ♀4, and ♀5, or simply "the four testes." One gravid ovary corresponding to segment ♀2 was observed and was noticeably smaller than the four testes (Figure 1). It is important to note that absolute size of the gonad does not correspond to gravidity, and regardless of their size, these

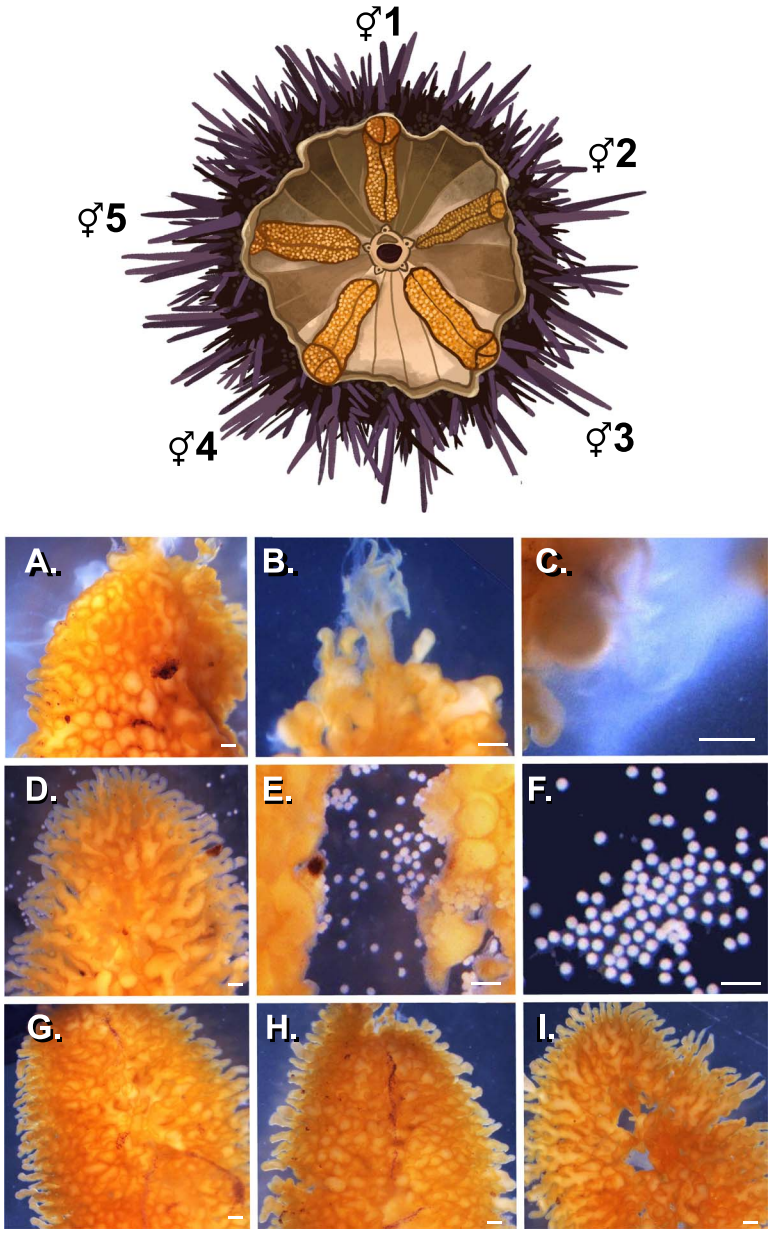


Figure 1. Physical description of hermaphrodite *S. purpuratus* (scale bar = 200 μM). (A) Darkfield image of gonad ♂1, a normal testis. (B) Close-up of lobes of gonad ♂1, a testis, note presence of sperm. (C) Seawater around gonad ♂1 containing viable, swimming sperm. (D) Darkfield image of gonad ♂2, appearance is that of a normal ovary. (E) Close-up of lobes of gonad ♂2, note presence of mature, unfertilized eggs. (F) Darkfield image of gonad ♂2 and associated unfertilized eggs. (G– I) Darkfield image of gonads ♂3, ♂4, and ♂5. Each of these were normal testes, indistinguishable from ♂1.

five gonads were all considered to be at the mature stage III in the reproductive cycle. Following immunolabeling, we discovered a small portion of the ovary (♂2) that contained male stem cells (Figure 3) and sperm (Supplementary Figure S4), indicating a partial ovotestis. Throughout this report, we will refer to gonad ♂2 as the ♂2 ovary when discussing the bulk exclusively egg-producing portion of the organ, or the ♂2 ovotestis when referring to the smaller portion with both egg- and sperm-producing cells. Following extensive imaging and molecular analyses, each of the four testes were never found to contain eggs, oocytes, or oogonia (Figure 4).

The hermaphrodite produced both eggs and sperm and was self-fertile

While the four testes were significantly larger than the single ovary, no difference in gravidity was observed; all five gonads contained mature, viable gametes. Sperm from each of the four hermaphrodite testes were crossed with normal eggs from an unrelated control female (Figure 2I–L), and all crosses yielded the same result: normal embryonic development through day 4. Viable non-fertilized eggs were collected from the dissection of gonad ♂2, and these eggs were crossed with sperm from each of the four testes ♂1, 3, 4, and 5 (Figure 2), and with normal sperm from an unrelated control male (Figure 2). All crosses produced normal embryos. We tested self-fertility all four ways and observed that the animal was self-fertile, and it too resulted in normal developing embryos, at least through 4-day larvae (Figure 2; Supplementary Figure S8 Summary). Individual crosses corresponding to each gonad are not shown, rather a summary is presented as every cross was successful, with no observable difference in fertilization speed or efficiency, and all crosses resulted in phenotypically

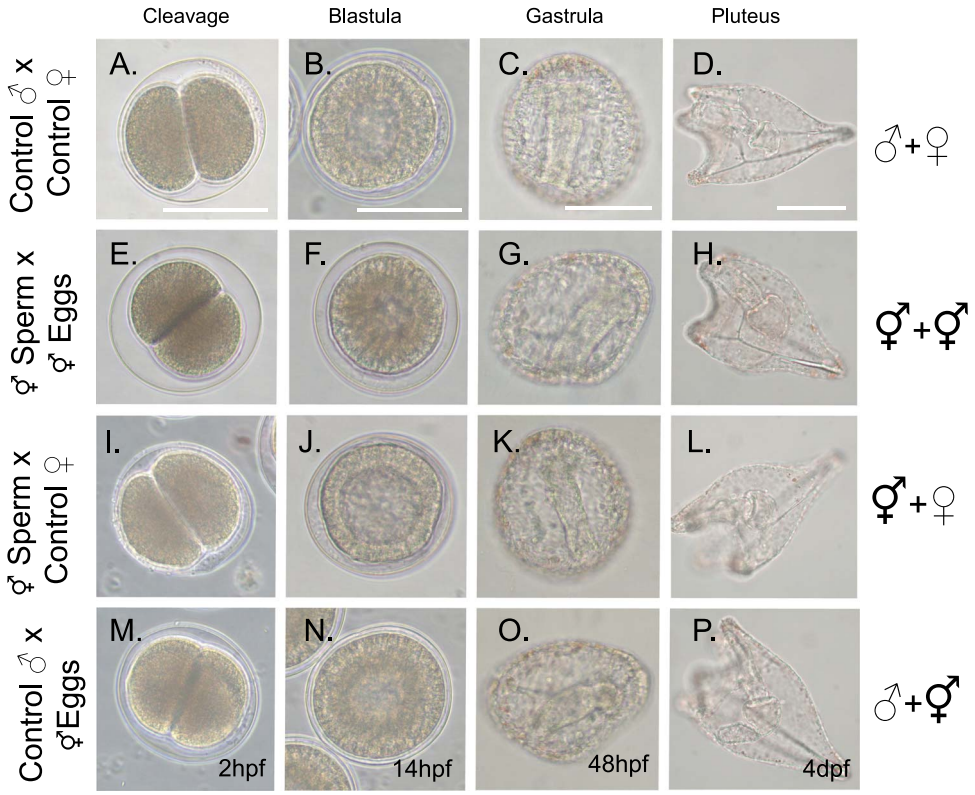


Figure 2. Summary of crosses (scale bar = 100 μM). (A–D) Normal development of Sp embryos using control sperm and control eggs from unrelated male and female urchins. Timepoints embryos were imaged and collected correspond to first cleavage (2hpf), blastula (14hpf), late gastrula (48hpf), and larval stage (4dpf). (E–H) Normal development of embryos from hermaphrodite self-fertilization, sperm from testis and ovary of the same animal were crossed, yielding normal development through day 4. Images are a summary of data; for a full table of crosses, see Supplementary Figure S8. (I–L) Normal development through day 4 from crossing hermaphrodite sperm with control unrelated female eggs. (M–P) Normal development through day 4 from crossing hermaphrodite eggs with unrelated control male sperm. All crosses yielded unremarkable embryos with normal developmental features.

normal embryos (Figure 2). For a full table of crosses, as well as a video of self-fertilization, please refer to Supplementary Figure S7. These crosses demonstrated that the eggs and sperm from within the same animal were self-fertile (Figure 2E–H), and that they were further compatible with eggs and sperm from unrelated animals, all producing normal and viable embryos and larvae out to day 4, the final day of observation.

Histology reveals a partial ovotestis in the hermaphrodite urchin

Several sections of each gonad were made following fixation and staining for microscopy. Each of the testes, ⚥1, ⚥3, ⚥4, and ⚥5, appeared unremarkable and were directly comparable to control male gonads from unrelated sea urchins in terms of morphology, structure, sperm content, and cellular organization (Figure 4, Supplementary Figure S6). FM1-43 staining of the membranes and phalloidin staining of the actin network revealed normal morphology of sperm within the lumen of the four testes (Figure 4). Bindin and tubulin [27] staining also appeared unremarkable in the four hermaphrodite testes, sperm had normal tubulin + flagella, and bindin puncta were present (Figure 3F–I). Interestingly, the ovotestis portion of gonad ⚥2 also displayed bindin and tubulin expression similar to that of the control testis (Figure 3J); however, an oocyte was also visible (Supplementary Figure S6C). The ovary portion of ⚥2 did not stain positive for bindin puncta, nor were tubulin + sperm visible (data not shown). Further immunostaining for male-specific markers demonstrated that a subpopulation of male-specific outer epithelial stem cells (SoxE+) were visible in the testes ⚥1, 3, 4, and 5, and these SoxE+ cells were comparable to those of a control male testis (Supplementary Figure S6).

In the ovary, oogonia, oocytes, and eggs are visible with different combinations of antibody stains. Very small and early stage oogonia are identified by their dense cytoplasmic expression pattern of the protein Vasa, a germline stem cell marker [28, 29]. Further oocytes and eggs may then be distinguished by their expression of the hyalin protein, a component of the egg cortical granules and apical lamina [30]. Vasa+ oogonia are solidly cytoplasmic stained for the Vasa protein and lack Hyalin expression. Oocytes have scattered Vasa+ cytoplasmic signal but contain varying amounts of cytoplasmic Hyalin protein, based on meiotic progression stage. Mature eggs only have sparse punctate Vasa granules and a corona of Hyalin protein, reflecting its storage within the cortical granules. All three of these staining patterns were visible in gonad ⚥2 and are clearly visible in a normal stage III female ovary (Figure 3A and B). Gonad ⚥2 mainly contained normal oocytes (Figure 3C) and an unremarkable ovarian morphology, directly comparable to that of unrelated control females (Figure 3A–D).

Throughout the initial dissection and gamete collection for crosses, gonad ⚥2 was concluded to be a normal ovary as no sperm were visible (Figure 1E). Further histological analyses of gonad ⚥2, however, revealed small gonadal lobes containing both eggs and sperm (Supplementary Figure S4), indicating

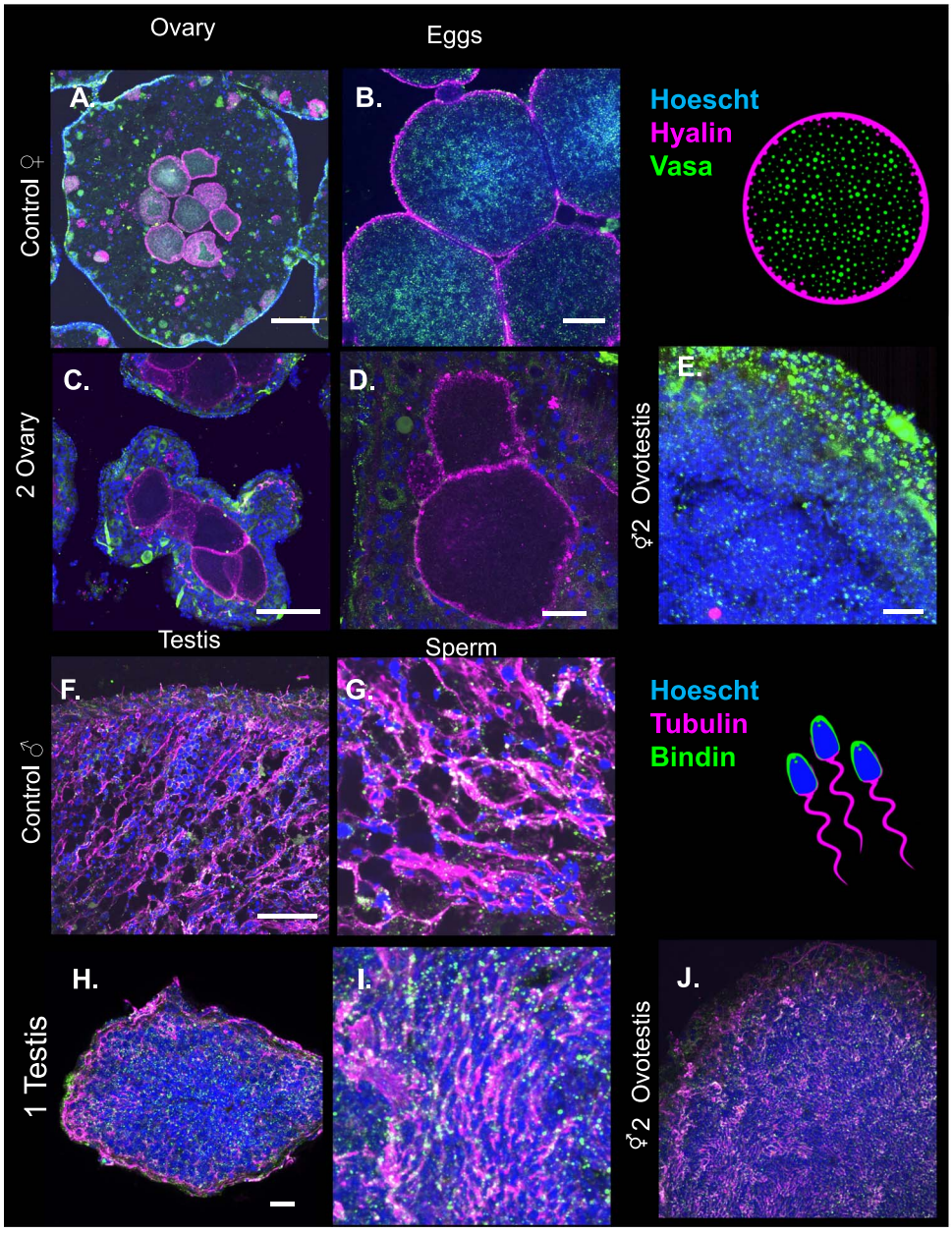


Figure 3. Immunofluorescence of hermaphrodite gonads. (A) Normal control ovary with Hyalin− and Vasa− localization. Vasa highlights the oogonia and early oocytes with intense green cytoplasmic stain, and a cluster of mature Hyalin+ eggs is clearly visible in the center of the gonad lobe (scale bar = 100 μM, inset = 20 μM). (B) 60× image of Hyalin+ mature eggs in a control ovary. (C) Hermaphrodite gonad ♀2, ovary Hyalin− and Vasa− localization. Several large, Hyalin+ eggs are clearly visible. (D) Close-up of Hyalin+ mature eggs in ovary portion of hermaphrodite gonad ♀2. (E) Inset showing Hyalin− and Vasa− localization in the ovotestis portion of hermaphrodite gonad ♀2, note distinct lack of not only Hyalin+ eggs but oogonia and early oocytes are not visible as well. This region was noted to contain sperm. (F) Bindin− and tubulin− localization in a normal control testis. Bindin puncta are visible in green at the tip and collar of the sperm, while tubulin marks the sperm flagella. (G) Inset showing cluster of spermatozoa with highlighted sperm tails. (H) Bindin and tubulin localization in hermaphrodite gonad ♀1, a normal testis. Localization is indistinguishable from the control. (I) Inset showing tubulin flagella of normal sperm in hermaphrodite gonad ♀1. (J) Bindin and tubulin localization in ovotestis portion of hermaphrodite gonad ♀2, appearing indistinguishable from localization in control male.

that gonad ♀2 is at least a partial ovotestis; this finding was supported by regions of the ovotestis that did not have Vasa+ oogonia nor Hyalin+ eggs present (Figure 3E). Gonad ♀2, on the whole, was composed of primarily ovarian lobes containing oogonia, numerous mature, unfertilized oocytes, visible by Vasa and Hyalin staining patterns; interspersed with indeterminate lobes containing disorganized oogonia and yolk (Supplementary Figure S5). Seldom were sperm-containing lobes observed, and only one small region was found to be a true ovotestis (Supplementary Figure S4).

The scant ovotestis regions found in ♀2 contained somewhat organized spermatogonial stem cells and normal looking sperm (Figure 5A and B). The ovotestis region was contained within three gonadal lobes, each connected with the same channel. Thus, some sperm strayed into an ovarian lobe and some eggs in a testis lobe (Figure 5). We noted that several, mature, unfertilized eggs were mixed within the lumen of the same gonad with sperm and indicated to us that the sperm and eggs contained within the partial ovotestis were either non-viable, under a self-fertilization block, or that the

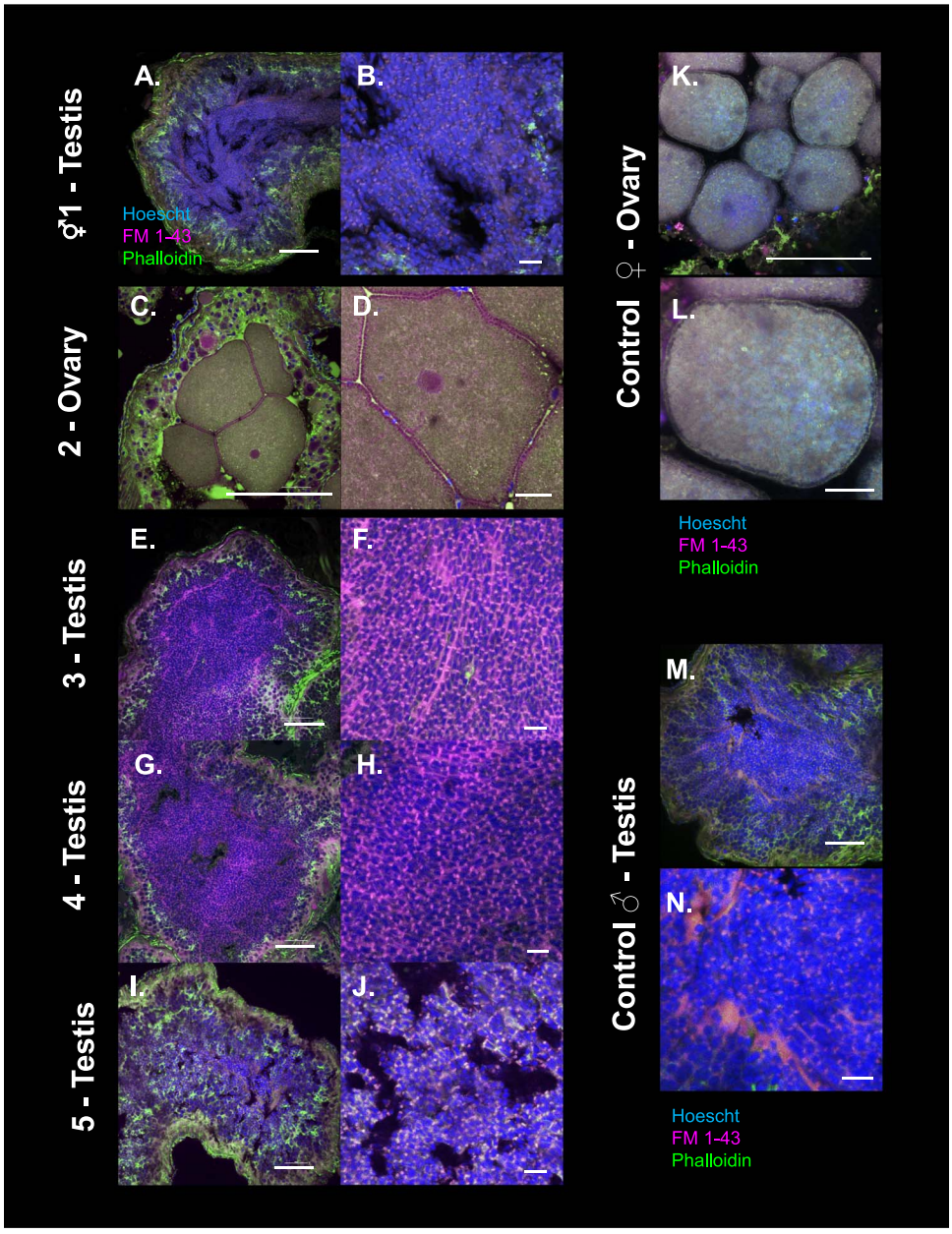


Figure 4. Fluorescent imaging of hermaphrodite gonads (scale bar = 100 μM, inset = 20 μM). (A–B) Hermaphrodite gonad ♂1, normal sperm (scale bar = 20 μM). (C, D) Hermaphrodite gonad ♀2, normal eggs. (E, F) Hermaphrodite gonad ♂3. (G–H) Hermaphrodite gonad ♂4. (I–J) Hermaphrodite gonad ♂5. (K, L) Control ovary. (M, N) Control testis.

environment within the ovotestis region was incompatible with sperm activation and/or fusion with the egg (Figure 5C and D). No functional tests were made with these gametes from the ovotestis region since this co-occurrence of both gametes was not identified until post-fixation, histological examination.

Ovary/ovotestis is genetically distinct from testes of the same animal at the Dmrt1 locus

Dmrt1, the echinoderm ortholog of Doublesex-and-mab3, is a gene responsible for male sex determination across numerous species [31–34]. *Strongylocentrotus purpuratus* has four Dmrt orthologs in the genome, Dmrt1, DmrtA2, Dmrt2, and Doublesex-Like (Dsx-L). Dmrt1 is of great interest as it is first expressed at late gastrulation, and then most abundantly in the testes of adult males (data not shown). Importantly, there is a small genetic difference in the Dmrt1 locus between male and female Sp urchins (Supplementary Figure S3). When performing PCR from genomic DNA, primers made for the Dmrt1 exon 2 did not amplify in female genomic DNA samples. In our hands, we have used this genomic difference in the Dmrt1 locus as a simple way to genotype male and female adult urchins (Supplementary Figure S3). The genetic difference in the Dmrt1 locus does not extend to the entire coding region of the Dmrt1 gene itself, however, and the genomic regions up- and down-stream of the Dmrt1 locus are both identical in male and female urchins (Supplementary Figure S3). Our first investigation of whether the four testes were genetically distinct from the ovary/ovotestis was to perform a sexing PCR with Dmrt1

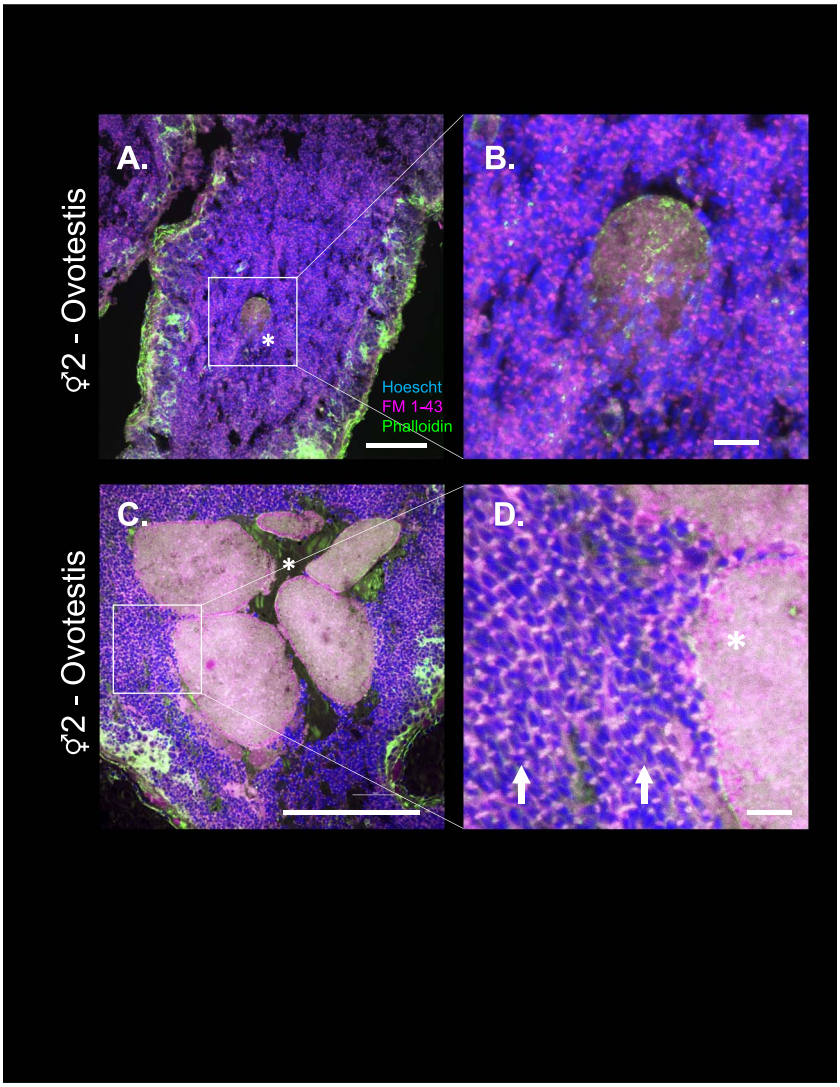


Figure 5. Fluorescent imaging of hermaphrodite ovotestis (scale bar = 100 μM, inset = 20 μM). (A, B) Partial ovotestis in a lobe of hermaphrodite gonad ♂2, where all morphology appears to be that of a normal testis; however, a single egg is present among spermatozoa (asterisk). (C) Partial ovotestis in hermaphrodite gonad ♂2, a single lobe where a normal looking nest of mature eggs is present; however, all somatic structures resemble that of a testis. (D) Close-up of the partial ovotestis showing spermatozoa with triangular heads (arrowheads) next to a mature egg within the same lobe of the ovotestis.

exon 2 genomic primers against individual genomic DNA samples collected from the five gonads. The testes (♂1, 3, 4, and 5) all had the expected PCR product size of Dmrt1 exon 2 as do the control testis samples, while in gonad ♂2, the ovary/ovotestis lacked the locus amplification as observed in control female ovary samples (Figure 6B). Doublesex-like, or DsxL, another Doublesex ortholog in the same gene family, was used as a genomic DNA control because the DsxL gene product and gene family are similar to Dmrt1. Note that there is no sex difference at the DsxL locus (Figure 6A). The same genetic difference observed between normal, unrelated female and male Sp urchins was thus observed to occur within the same animal, leading us to conclude that there was a genomic sex difference in this hermaphrodite *S. purpuratus* (Figure 6).

We further analyzed non-gonadal tissues of this hermaphrodite as well, including tube feet, test, gut, and spines. All segments were numbered ♂1–5 based on their body segment defined by the gonads (madrepore is segment 1), and tissue samples from the outside and inside were collected to test genetic composition of each tissue. Remarkably, the DMRT-segment identity of these samples, both the aboral tube feet samples and the gut, did not match the segment identity of the corresponding gonads. For example, tube feet collected from the aboral surface (A1–5) had a male Dmrt1 PCR product, which means that A2 did not match the segment identity. Segment A5 had no Dmrt1 amplification, and segment A1 had an unclear result, once again, not matching the testes, ♂1 and ♂5, inside the animal corresponding to these aboral segments. A final strange observation was that the majority of the gut samples (G2–5) had the same Dmrt1 PCR result as control female genomic DNA, indicating that the majority of gut tissue had similar genetic composition to the ovary/ovotestis and that of a genetic female (Supplementary Figure S3). The gut in this animal does have some pentaradial asymmetry, although the majority of the gut appears to be from the same tissue lineage as gonad ♂2 (Supplementary Figure S3).

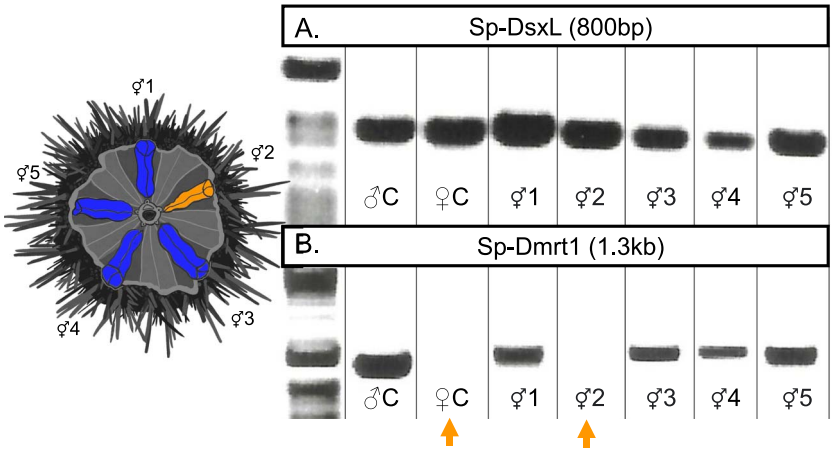


Figure 6. Hermaphrodite has genetic differences at the Dmrt1 Locus. (A) PCR genotyping control, primers to exon 1 of a doublesex family gene, Doublesex-Like (DsxL), amplify in both male and female control DNA (lanes 1 and 2, respectively), as well as in each of the five gonads of the hermaphrodite Sp. (B) PCR genotyping of Dmrt1 exon 2 yields a genetic difference in control male and female DNA, where the product amplifies in male DNA but not in female DNA. This same difference is observed across the five hermaphrodite gonads; note the absence of the PCR product in the lane corresponding to gonad ♀2, the ovary, identical to the result in the control female DNA lane.

These genotyping analyses not only demonstrate that the ♀2 ovary/ovotestis had a genetic difference in the Dmrt1 exon, comparable to a control female Sp, but also that male or female genotypes of other body tissues did not correlate to the same segment as the gonad, implying that the adult gonads come from a distinct lineage in development than the somatic tissues of gut and tube feet.

Gene expression profiles support the conclusions of hermaphroditism and partial ovotestis

Gene expression profiling was conducted on the five gonads of the hermaphrodite. Two oocyte-selective transcripts (ovoperoxidase: Opo, and Nanos2) and three sperm-selective transcripts (Catsper, SoxH, and Seawi) were used to construct gene expression matrices for female- and male-gene expression analyses. The egg-selective transcript ovoperoxidase (Opo) [35, 36] and the early oocyte marker Nanos2 are sometimes detectable in some testis samples [37]. However, the sperm-specific male genes, Catsper2 and Catsper3 sperm cation channel transcripts, are never detected in phenotypically normal ovary samples [38, 39]. The genes Seawi and SoxH were also used as male gene expression parameters, as they have distinct sexually dimorphic gene expression. Expression levels of seawi are between 100- and 1000-fold enriched in testis compared to ovary in this animal, while SoxH is also highly enriched in the testes and is thought to be a male stem-cell regulator [40].

Starting with control ovaries and testes, profiles of each hermaphrodite gonad sperm (male-) and oocyte (female-) gene expression were all analyzed using individual quantitative PCRs. In gonad ♀2, the expression of the oocyte transcripts, Opo and Nanos2, as well as the spermatogonia transcripts, SoxH and Seawi, were unremarkable and directly comparable to that of a control female (Figure 7A). The converse was also evident as ♀1, 3, 4, and 5 (the hermaphrodite testis samples) all shared very similar gene expression profiles to the control testis. It is important to note that the two sperm transcripts, Catsper2 and Catsper3, are low but detectable (~1000-fold lower than in a testis) in the ♀2 ovary/ovotestis sample (Figure 7A). This result indicates that the ovary was in fact a partial ovotestis, with bona fide expression of two sperm-specific transcripts. In addition, the expression of mRNA for these two Catsper sperm-specific cation channels has not been detectable (data not shown), confirming our observation of the partial ovotestis. This result is further strengthened after performing principal component analysis (PCA) on the gene expression matrix (Figure 7B), where individual expression profiles are clustered in two dimensions based on covariance. It was visible in the PCA plot that gonad ♀2 gene expression clusters away from other gonads from the same animal at a clear midpoint between control female ovary and control male testis gene expression, while the other four ♂ testes cluster strongly with normal control male testis gene expression and each other.

SNP analysis supports the hypothesis of mosaicism in constructing this hermaphrodite

Finally, we sought to determine whether or not the genetic difference observed in the Dmrt1 locus extended to other regions of the genome. The presence or absence of the Dmrt1 PCR product gave us insight into the genetic sex of the segments but did not give further insight into mosaicism of the whole animal. For this test, we randomly selected non-coding regions of the genome, an intergenic region as well as the promoters of the Nodal and SoxE genes, for genotyping analysis (Supplemental tables; Figure 8 and Supplementary Figures S9 and S10).

These regions were amplified and sequenced, and SNPs at each location were highlighted after performing a multiple sequence alignment (Figure 7A). A total of the validated SNPs in non-coding regions were tallied and compared across the five gonads of the hermaphrodite, an unrelated Sp male, and an unrelated Sp female. Although we only tested several kilobases of sequence, we found that the overall number of SNPs in unrelated individuals varies, but all four testes within the hermaphrodite had very similar, if not identical, SNPs at the loci sequenced. Gonad ♀2, however, had significantly different SNPs and at different loci. In considering how the genotype of this hermaphrodite was binary, these data support the hypothesis that this animal was a mosaic, perhaps forming from a fusion of two distinct embryos or larvae.

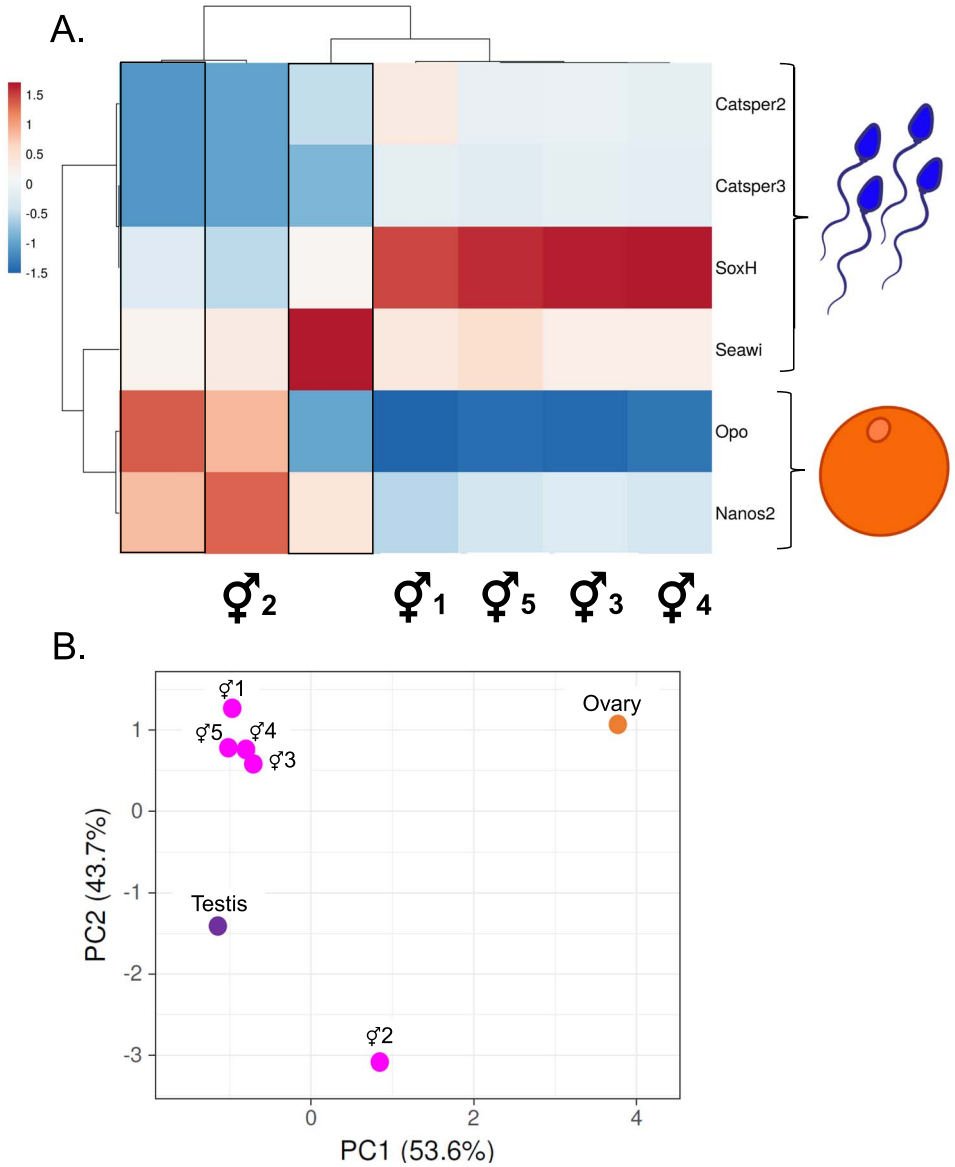


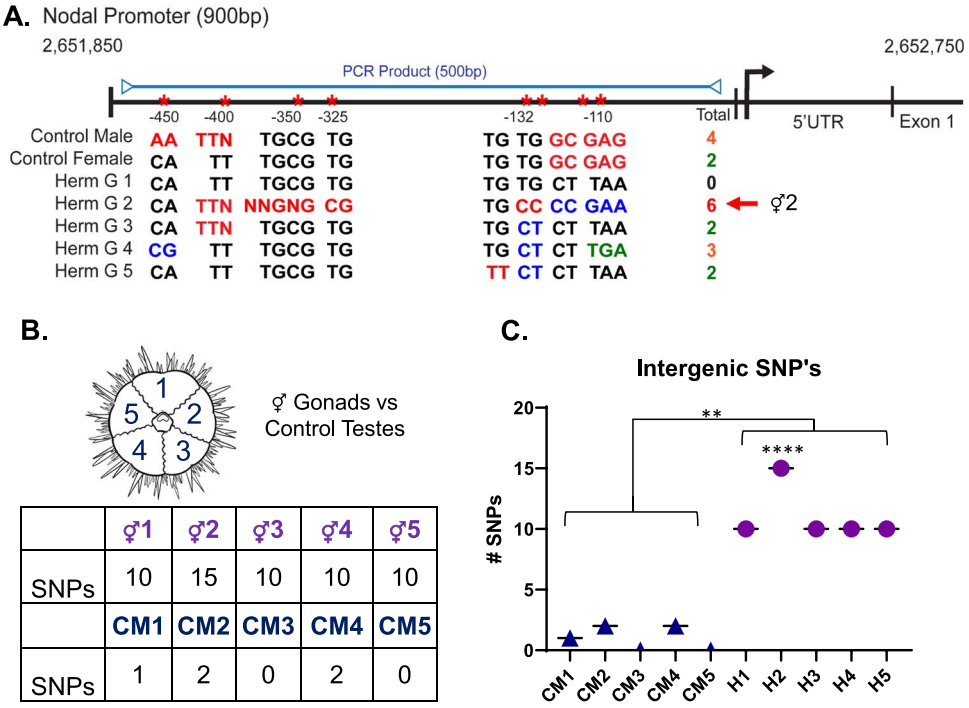
Figure 7. Differential gene expression profiling. (A) Heatmap of gene expression. Each square represents relative expression of genes on the *Y*-axis normalized to ubiquitin control, with sperm-related and male-specific transcripts at the top and oocyte-specific transcripts at the bottom. Control male and control female testis and ovary expression patterns are highlighted with a box. (B) PCA plot of gene expression values from individual qPCR results, hermaphrodite gonad expression profiles are shown in pink, control male is purple, and control female is orange.

Discussion

Our results are correlational but indicate that this hermaphrodite *S. purpuratus* is most likely the result of a chimeric individual, one male and one female fused during development (Figure 9). Our genomic PCR results support the contention that sea urchins have a genetic basis of sex determination, wherein males and females are genetically distinct. This was evidenced by both Dmrt1 exon sexing PCR results, and from several SNP genotyping experiments from non-coding loci. Together, these data support two genetically distinct male and female segments, indicating that this hermaphrodite *S. purpuratus* was most likely a mosaic animal (Figure 9). Furthermore, we can assume that if sex were to be determined by environmental cues, such as diet or temperature, we would not expect to see two different and distinct gonad sexes contained within the body of the same animal.

Our genetic results, however, are further complicated by the existence of the partial ovotestis in gonad ♀2 (Figure 5, Supplementary Figure S4). Integrating the genomic results, the expression profiling, gamete crosses, and imaging analyses, we hypothesize that the partial testis region of ♀2 originated from cells of the same genetic composition; however, we have not tested the testis tissue found in gonad ♀2 for presence or absence of the Dmrt1 exon, due to ♀2 not being identified as an ovotestis until after dissection. Functionally, however, throughout dissection and fertilization experiments, the apparent inability of any sperm from the ovary/ovotestis to fertilize viable eggs from the same tissue led us to consider some possible explanations:

1. The ovary/ovotestis was genetically identical throughout, and there is a self-fertilization block at work preventing



	♀1	♀2	♀3	♀4	♀5
SNPs	10	15	10	10	10
	CM1	CM2	CM3	CM4	CM5
SNPs	1	2	0	2	0

Figure 8. (A) Map of the Nodal promoter region sequenced for genotyping analysis; primers are shown as blue arrows, and SNP locations are highlighted with red asterisks. Numbers refer to distance (in bp) from the Transcription Start Site (0) which precedes the 5′UTR region, denoted after the arrow. In descending order, genotype at each SNP location for a control male DNA sample, a control female DNA sample, and each of the five hermaphrodite gonads. Alternative genotypes (differing from the majority genotype at each SNP location) are highlighted in different colors. (B) SNP analysis of intergenic region in five unrelated testis samples from a control male. Primers were generated to an intergenic locus at random, producing a 750-bp product. The SNPS within this 750-bp region were quantified and totaled. (C) Analyses of variance of SNPs from intergenic region. Each of the five control male testes SNPs were compared to those in the five hermaphrodite gonads, asterisks represent *P*-values. For reference, a full table of all pairwise comparisons is given in the supplement, with lowest *P*-values (four asterisks, $P < 0.0001$) highlighted in yellow.

the sperm from fertilizing eggs of the same gonad with the same genome.

2. The sperm produced were non-viable due to an incorrect niche (such as ovary hormones, ovary-specific signals) and therefore were never seen swimming within the wells of the dissection plate and, thus, did not fertilize eggs.
3. The sperm was viable, but unable to activate eggs due to a lack of testis signals, as observed in *Caenorhabditis remanei* females in which the tra-2 gene was targeted by RNA interference; the sperm they produced required an outside activation signal to become motile and fertilize oocytes [41].

Regardless of the reason(s) for ♀2 having non-viable sperm in fertilization experiments, we must consider the existence of an additional, unknown sexual differentiation signal which initiated the partial testis differentiation in a small region of this ovary (Supplementary Figure S4). While we do not have data supporting nor excluding these claims, we surmise that either signaling molecules or hormonal cues from the neighboring testes may have trans-fated a small region of this ovary into a testis. We also cannot exclude the possibility that gonad ♀2 had a small fragment that was genetically male.

In all, we find it important to note that this "single gonad containing two sexes, and producing two gametes" morphology appears strikingly similar to the asteroid ovotestis images recently reported in hermaphrodite crown-of-thorns sea star individuals [22]. Also similar were the gene expression results, where gonad ♀2 expressed some male- and sperm-specific transcripts, while a control female ovary did not. Unlike asteroid relatives, however, fertilization did not occur within this ovotestis as has been reported for hermaphroditic species of sea star [42, 43]. Our working hypothesis is that *S. purpuratus* are gonochoristic animals, and having adults of separate sexes is, and continues to be, a paradigm for reproduction in this species. An individual with two sexes in one body is a very rare finding, most likely due to a genetic perturbation, leaving environmental sex determination an unlikely possibility in this species.

Our mosaic SNP and genotyping results raise further questions about the fates of cell lineages during metamorphosis of Sp sea urchins. The PCR results from the gut and other body tissues (aboral side tube feet) appeared to have originated from genetically distinct, i.e., separate lineages, during embryonic development as the gonads within the body of the animal (Supplementary Figure S3). The gut itself was more "female" (Dmrt1−) than "male" (Dmrt1+) indicating that most of the gut in this animal had originated from cells of the same lineage as gonad ♀2 (Supplementary Figure S3).

Overall, the apparent non-overlapping origin of external body wall tissues and gonads means that the animal does not metamorphose into exactly correlative segments as was previously hypothesized [44]. Instead, the adult body appears to be formed by an outer/inner layer of segments that develop into the outer structures of the animal and the inner viscera during the process of metamorphosis [45, 46]. To further complicate the data, the pigment gene PKS was partially

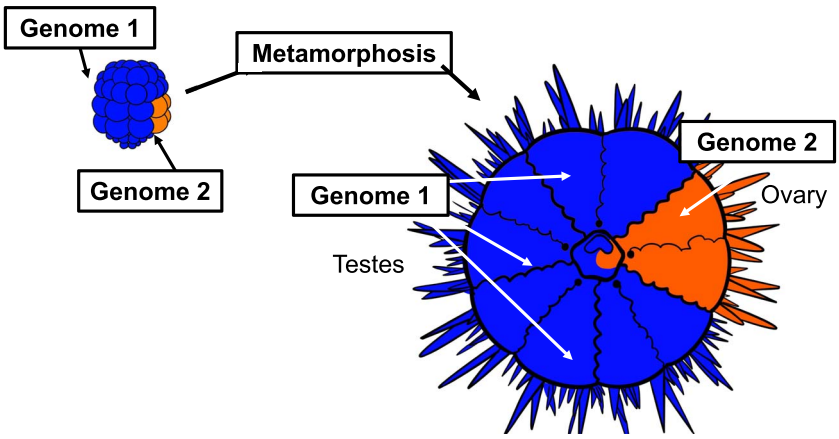


Figure 9. We hypothesize that the hermaphrodite is a mosaic. Integrating expression profiles, the Dmrt1 genomic difference, and the SNP profiles, we hypothesize here that at some point during development, chimeric embryo composed of two different genomes developed into this animal. At some point during metamorphosis, a small region of the soma developed female and thus produced a single ovary. It is still unclear exactly what mechanisms underlie this phenotype.

inactivated in *Hemicentrotus pulcherrimus* embryos, and when grown to adults, the outer segments of the animal were mosaic in pigment identity suggesting a segment-based organization for the external tissues of the adult [47]. The experiments performed in *H. pulcherrimus* urchins demonstrate how we can now test whether the outer (pigment or not mutation) tissues may or may not be in register with the same mutations in internal tissues [47]. Multiple such F0 mutations using CRISPR-Cas9 are now in reach for such detailed lineage assessment to further test the "segment register" model of development in these radial animals, and we now have some evidence to support an inner–outer segmentation of the adult body plan during metamorphosis [44, 48, 49].

While only from a single individual, our data yield more support for a genetic means of sex determination in *S. purpuratus* urchins. We believe these results support a multiple-loci system, where small regions of sex-determining genes differ between male and female individuals, together driving sex determination [50]. In addition, we have yet to identify any single large swath of genomic sequence, chromosomal or not, that is unique to one sex and not the other [51]. Due to the small size of the genetic difference, it appears that Dmrt1 regulation may be key here, as cutting-edge research is asking whether Doublesex genes are a male-determining factor in echinoderms as they are in other species [52]. We cannot yet conclude from this study that the gene Dmrt1 is responsible for sexual differentiation, as sex determination in all animals requires a concert of dozens, if not hundreds, of genes and signals [1, 5, 53], and we have yet to perform a conclusive Dmrt1 KO in Sp urchins. We hypothesize that the testis region of gonad ♂2 may have been a result of a male genetic pathway being activated, or female pathway inactivated, which has been known to cause varying degrees of sex reversal [54]. For example, in *Caenorhabditis elegans*, simple modulation of the genes TRA-1, TRA-2, and swm-1 can produce functional hermaphrodites with a "female" genome [55, 56]. From our mosaic analyses, however, it appears that this animal as a whole contained two genomes and therefore was able to generate functional gonads of separate sexes within the same body. Hopefully, future studies building on the observations documented here, and including CRISPR-Cas9 knockout of doublesex family genes, may yield interesting insights into the functional pathways governing sex determination in echinoderms.

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Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest: The authors have declared that no conflict of interest exists.

Data availability

The data underlying this article are available in the article and in its online supplementary material. Data are openly available directly to researchers and/or will be available in a public repository that issues datasets with DOIs.

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