

Sex, Polyps, and Medusae: Determination and Maintenance of Sex in Cnidarians

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SUMMARY

Mechanisms of sex determination vary greatly among animals. Here we survey what is known in Cnidaria, the clade that forms the sister group to Bilateria and shows a broad array of sexual strategies and sexual plasticity. This observed diversity makes Cnidaria a well-suited taxon for the study of the evolution of sex determination, as closely related species can have different mechanisms, which allows for comparative studies. In this review, we survey the extensive descriptive data on sexual systems (e.g., gonochorism and hermaphroditism) and the plasticity of sex in various cnidarian taxa. Within Cnidaria, hydrozoans (e.g., *Hydra*, *Hydractinia*, and *Clytia*) are the best understood in regard to mechanistic determination and maintenance of sex, largely due to the discovery of the interstitial stem cells, which give rise to the germ cells. We also present a hypothesis for the evolution of the various sexual systems that are observed in *Hydra*. Finally, given the rapid advances in genome sequencing and editing, several exciting possible future directions for increasing our understanding of sex determination mechanisms in cnidarians are discussed.

Our ability to unravel the mechanisms of sex determination in a broad array of cnidarians requires a better understanding of the cell lineage that gives rise to germ cells.

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INTRODUCTION

The mechanisms that determine sex can evolve rapidly and thus vary widely among animal taxa (Bachtrog et al., 2014). In some animals such as the fruit fly, the mouse, and humans, genes on distinct sex chromosomes dictate the sex of the organism (Swain and Lovell-Badge, 1999; Salz and Erickson, 2014). However, there are a wide variety of sex determination mechanisms among animals, including environmental and social influences (Bachtrog et al., 2014), that do not rely on dimorphic chromosomes. Sex determination results in the realization of a phenotype with male or female characteristics, or with characteristics of both sexes, as in the case of hermaphrodites. Hermaphroditism occurs in approximately one-third of animals, excluding insects (Jarne and Auld, 2006). Both gonochorism

(exclusively male or female) and hermaphroditism have evolved multiple times independently in many taxa (Jarne and Auld, 2006; Kerr et al., 2011).

Cnidaria is a clade of gelatinous, primarily marine species with both gonochoristic and hermaphroditic forms. Cnidaria includes corals, sea anemones, and jellyfish, as well as a few freshwater species, such as *Hydra* (Fig. 1). Recent phylogenetic analyses provide strong support for Cnidaria as the sister clade to Bilateria (Ryan et al., 2013; Chang et al., 2015). The question of sex determination in this clade is inherently interesting for several reasons

Abbreviations: GSC, germ line stem cell; ISC, interstitial stem cell

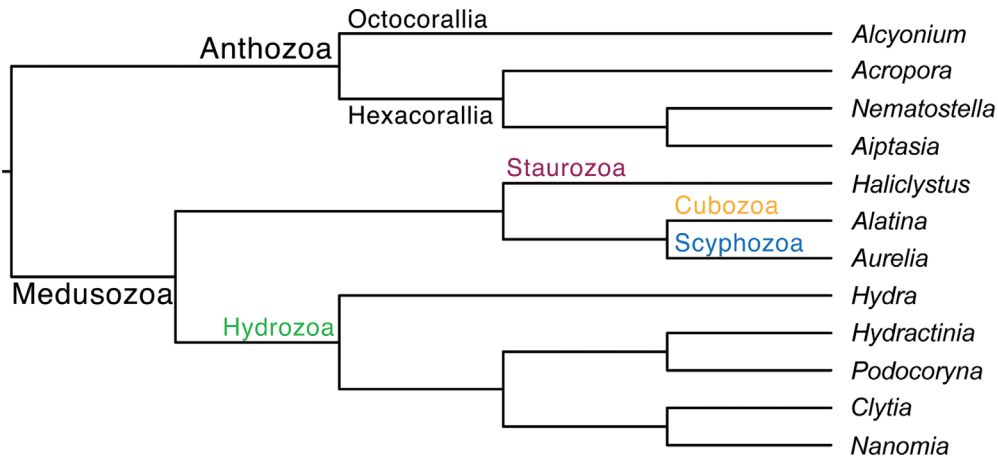


Figure 1. Phylogenetic relationship of cnidarian clades and genera discussed in this review. Relationships are based on Zapata et al. (2015).

including: (i) the use of diverse reproductive strategies; (ii) high plasticity of the sexual states is observed in several cnidarian taxa (Campbell, 1974; Fautin, 2002); and (iii) deciphering how sex is determined and maintained in cnidarians, which appears to occur in at least some species in the absence of dimorphic chromosomes, may further our understanding of ancestral sex determination mechanisms in animals.

Here we survey what is known about the establishment, maintenance, and stability of sex in cnidarians, and review our current understanding of the mechanisms that influence the realization of these phenotypes. Only a few cnidarian species—such as representatives of the hydrozoan genera *Hydractinia* and *Hydra*—have a long history in developmental biology research, which has enabled understanding of sex determination mechanisms

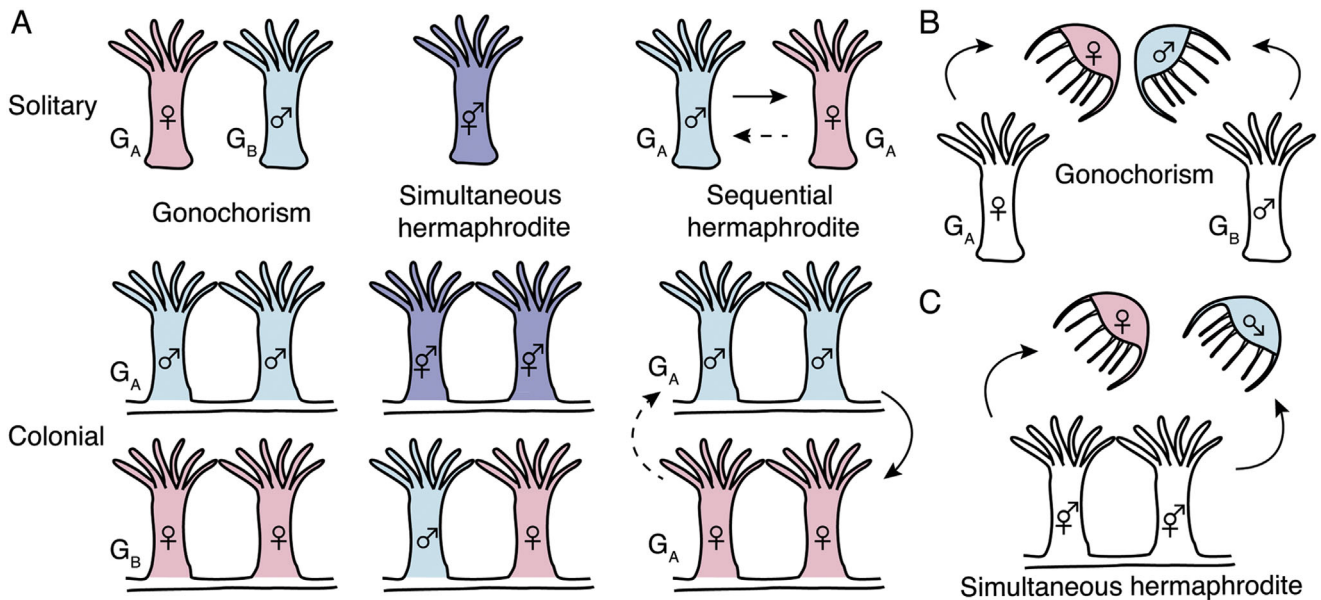


Figure 2. Sexual systems in Cnidaria. A zooid produces female (pink), male (blue), female and male (purple), or no gametes (white). **A:** Gonochorism and hermaphroditism in solitary and colonial cnidarians. Genets of gonochoristic species (G_A, G_B) stably maintain sex throughout their lifetime. Simultaneous hermaphrodites produce both male and female gametes at the same time. In colonial forms, gamete production of both sexes takes place either in a single zooid or in different zooids within the colony, depending on species. In sequential hermaphrodites, the sexual phenotype switches; this can be unidirectional or bidirectional. In some species a particular sex will always come first. **B:** Gonochorism in a species that has both a polyp and a medusa stage in its life cycle. The polyp gives rise asexually to a medusa, and gamete production takes place in the medusa. Different genets (G_A, G_B) produce gametes of a single sex. The medusa can be free-swimming or remain attached (gonophore). **C:** A hermaphroditic genet in which the asexual life stage can give rise to reproductive medusae of both sexes. G_A, genet A; G_B, genet B.

at the cellular level. Thus, these genera will dominate the mechanistic data presented here. Placing these detailed data into the context of sexual plasticity and sexual systems (gonochorism and hermaphroditism), within Cnidaria will be key to gain insights into the forces that drive the transitions between sexual systems and the evolution of sex determination.

TERMINOLOGY: GONOCHORISM AND HERMAPHRODITISM IN CNIDARIANS

Cnidarians are characterized by a tremendous diversity in lifecycles, morphology, and development. Some cnidarian species lead a sessile benthic lifestyle, while others are pelagic and inhabit the open water column. Many cnidarian life cycles are complex; they may include several life stages and alternating sessile (e.g., the polyp) and pelagic (e.g., the medusa) forms (Figs. 2 and 3). Sexual reproduction is often restricted to a particular stage within a life cycle, and this is usually the only stage at which the sexual identity of the organism can be determined by researchers. This limitation complicates the assessment of sexual identity in stages where primary (gametes) or secondary (gonadal structures) sexual characters are absent (Campbell, 1974).

Gamete-containing structures are commonly referred to as a gonad, even though they are not distinct organs, as found in other animals, instead arising in unspecialized regions as an individual reaches sexual maturity (Campbell, 1974). Gonads and gonophores of opposite sexes are often morphologically distinct (e.g., Nawrocki and Cartwright, 2013). Members of the cubozoan family Tripedaliidae, for example, present gonadal dimorphism, and include the only known cnidarians to display complex courtship behavior (Lewis and Long, 2005; Bentlage et al., 2010). Sexual dimorphisms that go beyond differences in reproductive structures are, to our knowledge, absent within Cnidaria.

In addition to sexual reproduction, most cnidarians can also reproduce asexually. The diverse mechanisms by which genetically identical individuals are generated include fission, laceration, budding, and parthenogenesis (Fautin, 2002; Bocharova and Kozevich, 2011). Analogous to examples in the plant world, individuals resulting from sexual reproduction have been termed “genets,” while asexually produced individuals have been termed “ramets” (see the *Terms Used Throughout This Review* sidebar) (Heyward and Collins, 1985; Karlson, 1991). Asexual reproduction in cnidarians may result in either a collection of solitary clones (e.g., sea anemones and *Hydra*) or a colony of physically attached clones (e.g., corals, siphonophores, *Hydractinia*, and *Clytia*) (Figs. 2 and 3). In the latter case, the budding process is not followed by separation, and thus a colony of genetically identical clones, or zooids, is formed.

Gonochoristic and hermaphroditic species have been identified among both solitary and colonial cnidarians. (Definitions of terms used to describe sexual systems in cnidarians are not always clear in the literature, so we define our use in the *Terms Used Throughout This Review*

Terms used throughout this review

Gastrozoid	Feeding zooid
Genet	Sexually produced genetic individual. Asexual reproduction gives rise to ramets. All ramets within a genet are genetically identical.
Gonochorism	One of two sexes is retained throughout the lifetime and individuals produce either eggs or sperm.
Gonophore	The life stage or zooid that bears the gametes. Can apply to any sexually reproductive life stage (e.g., medusa) or zooid, but is most often used to refer to derived medusae which remain attached to the parent polyp (gonozooid) and lack feeding and locomotory function.
Gonozooid	Non-feeding zooid in hydrozoan colonial systems that gives rise to free-swimming reproductive medusae or bear attached gonophores.
Interstitial cells (Hydrozoa)	Include interstitial stem cells (ISCs), progenitor cells that differentiate into gametes, and progenitor cells that differentiate into somatic cells of the lineage. These undifferentiated interstitial cells are morphologically indistinguishable. Differentiated somatic cells of the interstitial cell lineage include nematocytes (stinger cells), nerve cells, gland cells and in some species epithelial cells.
Interstitial stem cell (Hydrozoa)	Stem cell of the interstitial cell lineage. These are multipotent or totipotent in different hydrozoan species. ISCs give rise to the germ line and somatic cells.
Medusa	Usually free-swimming “jellyfish” stage in the life cycle. Reproductive stage.
Polyp	Sessile stage in many cnidarian life cycles that can be solitary or part of a colony; can bear gametes in species that lack a medusa stage in their life cycle.
Ramet	Asexually produced individual within a genet.
Sequential hermaphroditism	The genet is capable of both egg and sperm production. In solitary cnidarians, ramets frequently undergo switching of sexual phenotypes, but rarely produce both eggs and sperm at the same time. In colonial cnidarians, the reproductive zooids either produce egg or sperm (rarely both) at any given time, but this can change over time.
Simultaneous hermaphroditism	The genet is capable of both egg and sperm production. In solitary cnidarians a single ramet can simultaneously produce both eggs and sperm. In colonial cnidarians, the entire colony produces both eggs and sperm at the same time. An individual reproductive zooid in the colony is capable of making both eggs and sperm simultaneously or some reproductive zooids are capable of making eggs and other reproductive zooids are capable of making sperm.
Zooid	Individual body within a colonial system.

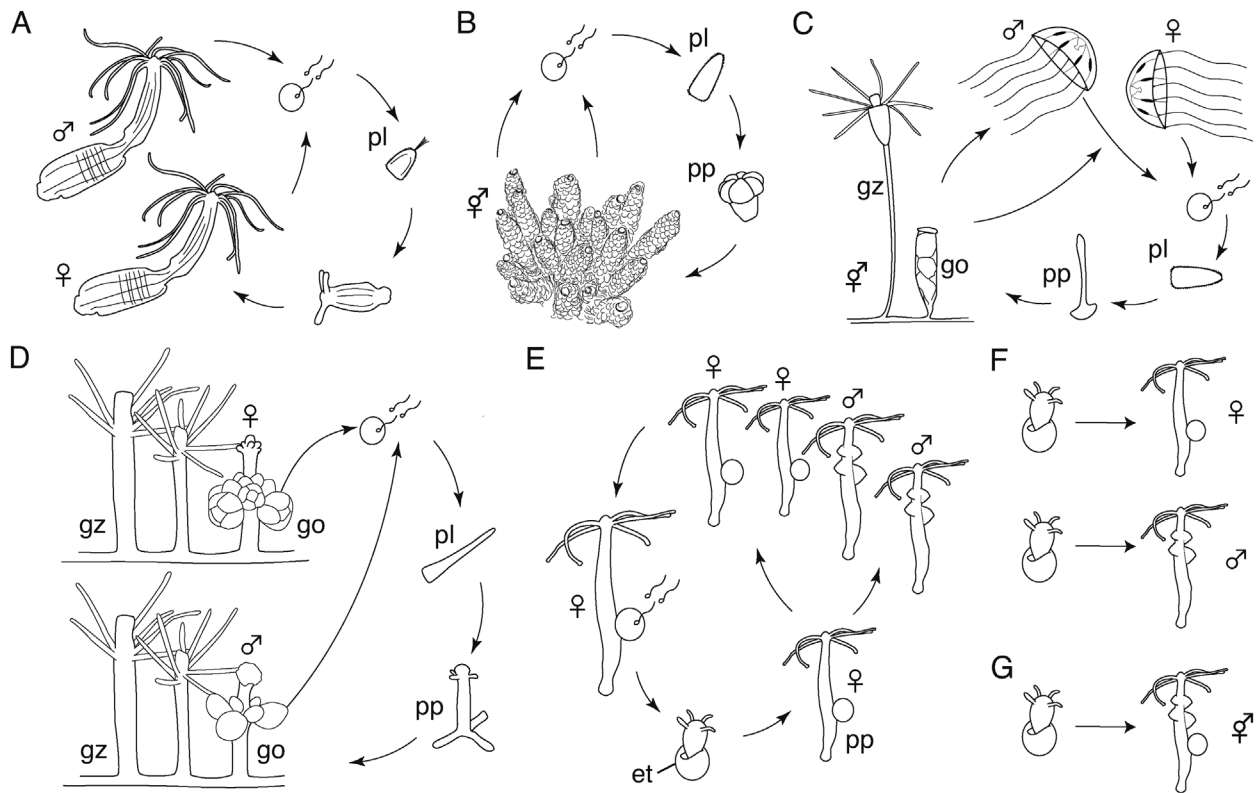


Figure 3. Cnidarian life cycles and sexual systems. **A:** *Nematostella vectensis*. A planula larva gives rise to a gonochoristic solitary polyp. **B:** *Acropora millepora*. A founder polyp gives rise to a colony consisting of identical hermaphroditic polyyps. **C:** *Clytia hemisphaerica* (hermaphroditic colony). A primary polyp gives rise to a gonochoristic or hermaphroditic colony consisting of physically attached gastrozooids and gonozooids. Gonozooids of hermaphroditic colonies release medusae of both sexes. **D:** *Hydractinia* spp. A primary polyp gives rise to a gonochoristic colony consisting of functionally specialized zooids including gastrozooids and gonozooids. **E:** *Hydra vulgaris* strain AEP, sequential hermaphroditism. The founder polyp and asexually generated daughter polyyps display a single sex. Stochastic change of sex leads to populations of male and female sexual phenotypes. **F:** *Hydra oligactis*, gonochorism. Primary polyp and asexual offspring are of a single sex that is usually stably maintained. **G:** *Hydra viridissima*, simultaneous hermaphroditism. Primary polyp and asexual offspring simultaneously produce male and female gametes. et, egg theca; gz, gastrozooid; go, gonozooid; pl, planula larva; pp, primary polyp.

sidebar). For both solitary and colonial forms, gonochorism occurs when ramets (solitary cnidarians) or reproductive zooids (colonial cnidarians) are stably male or female (Figs. 2A and 3A). Two types of hermaphroditism, simultaneous or sequential, exist (Fig. 2A): For genets with solitary clones, simultaneous hermaphroditism occurs when all ramets simultaneously produce eggs and sperm (Figs. 2A and 3G). Sequential hermaphroditism occurs when both male and female ramets are present in a single genet. This is usually caused by single ramets converting from male to female or vice versa (commonly seen in *Hydra*; described in detail below) (Figs. 2A and 3E). In colonial forms, if an entire colony produces both eggs and sperm (regardless of what each zooid is doing), we define this as simultaneous hermaphroditism (Figs. 2A and 3B). Sequential hermaphroditism, however, occurs when the colony changes sex over time (i.e., all reproductive zooids start as male and then become female) (Fig. 2A).

Many cnidarians have both a polyp and a medusa stage in their life cycle. Through asexual production, polyyps

often give rise to a medusa, the stage in which gamete formation takes place (Fig. 2B,C). The medusa can be free-swimming or remain attached as a gonophore (Fig. 3C,D); in the latter case, the medusa can be highly reduced (see Cartwright and Nawrocki, 2010). In gonochoristic species, a genet will produce medusae of a single sex (Fig. 2B). In some species genets can give rise to both male and female medusae, suggesting that the colony is hermaphroditic (Figs. 2C and 3C; also see below). Given the variety and diversity among cnidarian sexual systems, detailed studies of sexual determination mechanisms promise to uncover interesting phenomena.

CNIDARIAN SEXUAL SYSTEMS: MULTIPLE TRANSITIONS BETWEEN GONOCHORISM AND HERMAPHRODITISM

Studies conducted on cnidarians both in the field and in the laboratory have revealed a diversity of reproductive

strategies and variable sexual systems. Cnidaria is comprised of two large clades, Anthozoa and Medusozoa (Fig. 1). Only the polyp form exists in anthozoans (Figs. 2A and 3A,B), whereas medusozoans are characterized by a sexually reproductive medusa life stage (Figs. 2B,C and 3C,D), which has been secondarily lost in some medusozoan taxa (Fig. 3E–G). Extensive literature is available on anthozoan sexual systems, life strategies, and mating systems (Richmond and Hunter, 1990; Fautin, 2002), whereas stability-of-sex information that could shed light on sex determination mechanisms is less comprehensive. This is partly a consequence of the many observations made on specimens collected or observed in the field, making reassessment of the same individual(s) over time logistically challenging; consequently, long-term studies are still rare. While the larval mode of development varies greatly across anthozoans, fertilization often leads to the formation of a planula larva, which will metamorphose into a polyp (Fig. 3A,B). In addition, most anthozoan polyps can reproduce asexually to form new, solitary clones (e.g., sea anemones) (Fig. 3A) or colonies (e.g., corals) (Fig. 3B).

Anthozoa is split into two large clades: Hexacorallia and Octocorallia (Fig. 1). Hexacorals include, among others, sea anemones (Actiniaria) and stony corals (Scleractinia). Two well-studied sea anemones, in terms of development and life history, belong to the genera *Aiptasia* and *Nematostella*; both of these are generally gonochoristic in the lab (Hand and Uhlinger, 1992; Schlesinger et al., 2010), although there is one report that *Aiptasia diaphana* displays sequential hermaphroditism when clonally propagated under laboratory conditions (Schlesinger et al., 2010).

The reproduction of stony corals (Scleractinia) is well-studied due to the importance of these animals in coral reef ecosystems (Richmond and Hunter, 1990; Harrison, 2010). Stony corals are usually comprised of colonies with identical zooids. Hermaphroditic broadcast spawning is the most common mode of reproduction within scleractinian corals (Wallace, 1985; Richmond and Hunter, 1990; Carlon, 1999; McFadden et al., 2001; Harrison, 2010), although all sexual systems—gonochorism, sequential hermaphroditism, and simultaneous hermaphroditism—are found within this clade (Harrison, 2010; Kerr et al., 2011). The most comprehensive study on stony corals (>200 species) concluded that gonochorism is ancestral while hermaphroditism has independently evolved on at least three separate occasions (Kerr, 2005; Kerr et al., 2011). Long-term studies of *Stylophora pistillata* demonstrated that the reproductive state of a colony can bidirectionally change from simultaneously hermaphroditic to male and sterile, and that these changes may be triggered by environmental stressors or energy limitations (Rinkevich and Loya, 1987). Bidirectional sex change has also been reported for solitary, single-polyped mushroom stony corals (Loya and Sakai, 2008). The colony structure of corals in general allows for a subset of zooids to take on the task of reproduction; location within the colony appears to be an important factor in determining which zooids will undergo gametogenesis.

In some scleractinian species, the zooids found near the colony margins are less likely to reproduce and may instead serve protective functions whereas zooids inside the colony produce gametes (Soong and Lang, 1992). In the hermaphroditic species *Rhodactis rhodostoma* (Corallimorpharia), zooids on the margins are smaller and more likely to be male while internal zooids are larger and more likely to be female (Chen et al., 1995; Chadwick-Furman et al., 2000). When large female zooids from the center are transplanted to the margins, they decrease in size and turn into males (Chen et al., 1995). Taken together, these data suggest that the sex of individual zooids in certain coral colonies is influenced by spatial cues.

Octocorals are comprised of mostly colonial forms. In contrast to scleractinian corals, gonochorism is the predominant sexual system and hermaphroditism is rare in octocorals (Kahng et al., 2011). Yet, evolutionary transitions between different sexual systems can occur rapidly in some octocoral taxa—for example, the reproductively diverse genus *Alcyonium* (McFadden et al., 2001), in which gonochorism appears to be ancestral while hermaphroditism or parthenogenesis has evolved at least twice (Benayahu, 1991; McFadden et al., 2001). Sequential hermaphroditism has not been reported for octocoral colonies (Kahng et al., 2011).

Very limited information is available regarding sexual plasticity in three of the four medusozoan taxa: Staurozoa, Cubozoa, and Scyphozoa (Fig. 1). Cubozoan and scyphozoan life cycles generally include both a solitary polyp and a medusa stage, with gametogenesis occurring in the medusa. Findings thus far suggest that the vast majority of staurozoans, cubozoans, and scyphozoans are gonochoristic (i.e., polyps give rise to medusae of a single sex) (Fig. 2B) (Berrill, 1962; Arai, 1997; Morandini and Da Silveira, 2001; Straehler-Pohl et al., 2014), although sequential hermaphroditism has been reported in the scyphozoan *Chrysaora hyoscella* (Berrill, 1949).

Hydrozoans are diverse in terms of life cycle and colony and medusa morphology (Bouillon and Boero, 2000; Bouillon et al., 2006; Schuchert, 2006, 2010; Cartwright and Nawrocki, 2010). Examples of this diversity can be found in species that are frequently studied in evolutionary developmental biology. In contrast to most medusozoans, we know significantly more about the maintenance of sex within Hydrozoa due to a large body of research conducted in three genera: *Clytia*, *Hydractinia*, and *Hydra* (Figs. 3C–G and 4). *Hydra* is a solitary freshwater hydrozoan with a life cycle that lacks a medusa stage; fertilization and embryogenesis gives rise directly to a new polyp (Figs. 3E and 4I–M). By contrast, *Hydractinia* and *Clytia* are colonial hydrozoans. Whereas many colonial hydrozoans consist of identical zooids, functional specialization can be observed in several hydrozoan taxa; a stolon system or a stem often interconnects these specialized zooids. Both *Hydractinia* and *Clytia* colonies are composed of functionally specialized zooids. *Hydractinia* colonies are sessile and harbor feeding zooids (gastrozooids) and reproductive zooids (gonozooids), among others (Figs. 3D and 4A–C). *Hydractinia echinata* colonies are



Figure 4. Sexual phenotypes in *Hydractinia symbiolongicarpus* (A–C), *Clytia hemisphaerica* (D–H), and *Hydra* spp. (I–M). **A:** Male colony with gastrozooids and gonozooids. **B:** Close-up of female gonozooid with developing and mature gonophores. **C:** Close-up of male gonozooid with developing and mature (white) gonophores. **D:** Stolon system with gastrozooids. **E:** Close-up of gonozooids harboring developing medusae. **F:** Mature female medusa with four gonads. **G:** Close-up of female gonad. **H:** Close-up of male gonad. **I:** Simultaneous hermaphroditic *Hydra viridissima* with testis and egg. **J–M:** Sequential hermaphrodite *Hydra vulgaris* strain AEP displaying male (J) and female sexual phenotype (K). Close-ups of egg and forming egg (L) and testes (M). Scale bars, as indicated. de, developing egg; e, egg; fg, female gonophore; fgd, female gonad; mg, male gonophore; st, stolon; t, testis; go, gonozooid; gz, gastrozooid. Photo credits: Annalise Nawrocki (A), Matthew Nicotra (B–C), Tsuyoshi Momose (D–H).

described as gonochoristic; sex appears stable in the laboratory (Hauenschild, 1954; Müller, 1964). In contrast to *Hydra* and *Hydractinia*, the reproductive zooids of *Clytia hemisphaerica* colonies produce free-swimming medusae,

which is the sexual stage in the life cycle (Figs. 3C and 4D–H) (Houliston et al., 2010). Gametes form in gonads within the medusa; medusae are usually of a single sex, and the sex is stable in adult medusae.

SEX DETERMINATION IN HYDROZOANS

The Hydrozoan Interstitial Cell Lineage and Germ Line Stem Cells

The interstitial stem cell (ISC) was the first stem cell to be identified (Weismann, 1883), and now appears to be characteristic of hydrozoans (Frank et al., 2009; David, 2012). ISCs give rise to the germ line and to a variety of somatic cells, such as nerve cells, gland cells, nematocytes, and in some species, to epithelial cells; their somatic potential varies across taxa, and ISCs can be multi- or totipotent, depending on the species (Fig. 5) (Bosch and David, 1987; Bode, 1996; Plickert et al., 2012). The identification of this interstitial cell lineage has allowed for significant progress in the study of hydrozoan sex determination (Hauenschild, 1954; Müller, 1964, 1967; Tardent, 1985; Littlefield and Bode, 1986; Bosch and David, 1987; Littlefield, 1991; Nishimiya-Fujisawa and Sugiyama, 1993; Müller et al., 2004).

The sexual identity of ISCs is not completely understood. ISCs may have distinct sexual identities as male or female, ultimately giving rise to sperm or eggs, respectively. These two populations could coexist in one animal, with the interaction between these sexually distinct ISCs dictating the sex and sexual system used by the individual (see below). Alternatively, a single ISC may be able to give rise to both male and female gametes (Littlefield, 1994). Exploring how this occurs requires determining whether hydrozoan ISCs directly differentiate into germ cells or if a self-renewing, unipotent germ-line stem cell (GSC) intermediate exists as part of the interstitial cell lineage.

Several studies in *Hydra* demonstrate the existence of self-renewing male and female GSCs (Fig. 5A). *Hydra oligactis* polyps capable of making either eggs or sperm, but not somatic cells of the interstitial cell lineage, were obtained experimentally and determined to contain female and male GSCs, respectively. Long-term culturing of these animals demonstrated the self-renewing capabilities of these GSCs (Littlefield, 1991, 1985; Littlefield and Bode, 1986). Similar experiments revealed the existence of male and female GSCs in *Hydra magnipapillata* (Nishimiya-Fujisawa and Sugiyama, 1993, 1995). A single, multipotent ISC can also reconstitute the entire interstitial lineage, including germ cells, in *Hydra magnipapillata* (Bosch and David, 1987). Taken together, these data suggest that the multipotent ISC in *Hydra* retains the ability to give rise to new GSCs when necessary, but once created, the unipotent GSC is responsible for the majority of gamete production (Nishimiya-Fujisawa and Kobayashi, 2012).

Hydractinia gamete formation occurs in gonophores (highly reduced medusae) located on the reproductive polyps (i.e., gonozooids). In gonozooids, uncharacterized interstitial cells migrate from the ectoderm into the endoderm, where they are incorporated into the tissue that ultimately forms the gonophore (Weismann, 1892; Müller, 1964). This migration may be accompanied by a change from totipotent to unipotent germ cell fate (Mali et al., 2011). Mitotically active male germ cells are present in

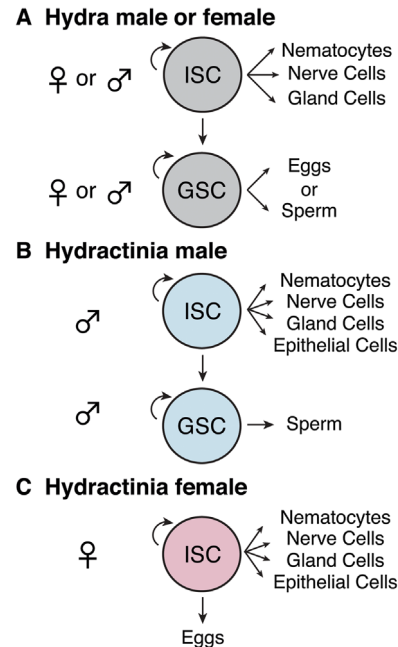


Figure 5. Interstitial cell lineage in *Hydra* and *Hydractinia*. **A:** In *Hydra*, the multipotent ISCs of the interstitial cell lineage gives rise to three somatic cell types (nerve cells, gland cells, nematocytes) and to the GSCs. Female and male GSCs are capable of self-renewal, and give rise to eggs or sperm. **B–C:** In *Hydractinia*, the ISCs are totipotent and capable of giving rise to all somatic cell types of the animal, including the epithelial cells of the endoderm and ectoderm. In *Hydractinia* males (**B**), cell proliferation suggests that GSCs may exist, whereas in *Hydractinia* females (**C**), it appears that oogenesis occurs in the absence of cell proliferation.

gonophores, suggesting that a male GSC population exists (Fig. 5B) (Müller, 1964). By contrast, interstitial cells in the gonophore appear to develop directly into oocytes, which may argue against a female GSC that continually produces oocytes (Fig. 5C).

Competing Cell Populations: The Presence of Male Germ Cells Represses Egg Differentiation

When ISCs and/or GSCs of different sex exist together in a single *Hydra* polyp (in sequential hermaphroditic and gonochoristic species), male germ cell fate is dominant over female germ cell fate. The occurrence of male and female cells within a single polyp can be due to sex switches in sequential hermaphrodites (see below) or can be generated experimentally. Grafting male (i.e., sperm-producing) and female (i.e., egg-producing) halves of *Hydra vulgaris* (formerly *Hydra attenuata*) polyps together always results in sperm-producing polyps. The interstitial cell lineage is required in this process, which demonstrates that the resulting sex is indeed a consequence of interstitial cell competition rather than a sex-determining influence of the epithelial cells of the host (Tardent, 1968). Further experiments performed in a closely related *Hydra magnipapillata*

strain suggest that the presence of male ISCs and/or GSCs leads to the production of a repressive signal that inhibits egg production, but does not repress the formation of other somatic cells of the interstitial cell lineage (Sugiyama and Sugimoto, 1985). In the gonochoristic species *Hydra oligactis*, introducing a male GSC into a female polyp leads to the loss of all female GSCs; whether or not complete GSC loss occurs in the sequential hermaphroditic species of *Hydra* is unclear (Littlefield, 1994).

Male sex is also dominant over female sex in *Hydractinia*. When male stolon grafts containing ISCs are inserted into a female colony, male gonozooids will appear in the host colony and the male sex will eventually take over the entire colony (Hauenschild, 1954; Müller, 1964). Chimeric colonies can be created either experimentally or occur naturally through fusion of histocompatible colonies of the opposite sex. Due to cell invasion, gonozooids at fusion borders harbor gonophores with both male and female gametes (Hauenschild, 1954). In this situation, the proliferative activity of male germ cells may result in a competitive advantage over female germ cells for the limited space in the forming gonophores, as oocytes appear to be displaced into the gastric cavity (Müller, 1964). The dominance of the male sex might be explained by different proliferation rates of the two germ-cell populations rather than by a repressive signal (Müller, 1964)—although this observation has been challenged by the recent collection of wild *Hydractinia* that make functional sperm, but also consistently harbor nonfunctional oocytes that do not reach maturity (Mali et al., 2011). Offspring of these field-collected colonies have the same phenotype, proving that the coexistence of both gametes is a heritable trait in these animals rather than the result of a recent fusion event. In addition, since oocytes were consistently found, differences in mitotic rates cannot always explain the dominance of the male sex; perhaps a repressive signal that keeps female oocytes from maturing, similar to what is observed for *Hydra*, is active instead (Mali et al., 2011). Ultimately, more studies need to be conducted in different *Hydractinia* strains and species in order to understand the interaction of opposite-sex interstitial cell lineages in the genus.

The Effect of Temperature on Sex Determination in Hydrozoans

Temperature changes can induce sex switching in the colonial hydrozoan *Clytia hemisphaerica*, for which both gonochoristic and hermaphroditic colonies have been reported (Carré and Carré, 2000). The reproductive zooids (gonozooids) of *Clytia* colonies give rise to free-swimming medusae that produce gametes (Fig. 4D–H) (Houliston et al., 2010). Hermaphroditic colonies give rise to both male and female medusae of a single sex, and sexes are largely stable in adult medusae—although the sex is influenced by temperature during early medusa development (Carré and Carré, 2000). When colonies are raised at higher temperatures (24°C), the majority of medusae released are female whereas those raised at lower temperatures (15°C) produce more male medusae. Medusae released at

high temperature, but then switched to a low temperature, develop as males. Therefore, both male and female populations of ISCs or GSCs may simultaneously exist in young medusae produced at higher temperatures (Carré and Carré, 2000). Male ISCs or GSCs do not influence the phenotypic sex unless the temperature is lowered, suggesting some female dominance over male development and/or a suppressive effect of high temperature on male development. On the other hand, young medusae produced at the lower temperature still develop predominantly into males, even when subsequently cultured at higher temperature (Carré and Carré, 2000). Therefore, low temperatures may lead to the complete inhibition of the female pathway or to the loss of female germ-line cells. These data suggest that male and female interstitial cells (ISCs or GSCs) are competing in *Clytia*, and that the outcome of this competition is dependent on temperature. It is unclear how conserved these mechanisms may be in other hydrozoans. Interestingly, in *Hydra oligactis*, a gonochoristic species with largely stable sex, culturing males at the higher-than-normal temperature of 22°C leads to a low level of male-to-female switching; this high temperature appears to inhibit spermatogenesis (Littlefield, 1986), which is consistent with the effect of temperature on *Clytia* sexual development.

Somatic Tissue Does Not Determine Sex in Hydrozoans, But Influences the Location of Gametogenesis

In bilaterians, the somatic cells of the gonad influence the sexual identity of the germ cells to various degrees. In extreme cases, such as frogs (Blackler, 1965) and fish (Shinomiya et al., 2002; Okutsu et al., 2006; Yoshizaki et al., 2010), the somatic cells entirely dictate the sexual identity of the germ cells; transplanted germ cells take on the identity of the host. In fruit flies (Steinmann-Zwicky et al., 1989) and mammals (Durcova-Hills and Capel, 2008), sexual identity of the somatic gonad and the germ cells must be the same for proper gametogenesis to occur. The somatic cells within *Hydra* and *Hydractinia*, by contrast, appear to have no influence on sexual identity. Grafting epithelialized males (i.e., males that lack the interstitial cell lineage) to normal females, or vice versa, has no effect on the sexual identity of the donor interstitial cell lineage in *Hydra* (Littlefield, 1984; Campbell, 1985). Similarly, introducing ISCs into opposite-sex *Hydractinia* colonies after irradiating host interstitial cells results in the host switching sex (Müller, 1964, 1967). Therefore, in at least these two hydrozoans, sex is cell-autonomously specified by the interstitial cell lineage, with no influence exerted by surrounding epithelial cells.

Epithelial cells within hydrozoan polyps do appear to have some influence over the location of gametogenesis. Testes usually develop more distally and the eggs more proximally on the body column of simultaneous hermaphroditic *Hydra* polyps (Fig. 4I) (Brien and Reniers-Decoen, 1950; Tardent, 1968). The epithelium therefore promotes a particular sex in a spatially defined manner,

suggesting the presence of epithelial signals that guide cells of a determined sex to specific locations. If these observations hold up for other hydrozoans, this model might help us understand phenomena in more complex colonies, such as the siphonophore *Nanomia bijuga*, a colonial hydrozoan with male and female gonozooids arranged in a highly organized spatial pattern (Totton, 1965). Molecular markers indicate localized growth zones in *N. bijuga* as the source of ISCs, which are incorporated into newly forming zooid buds within the growth zones (Siebert et al., 2015). The predictable arrangement of male and female zooids indicates that the somatic cells of the newly forming zooids influence whether male or female interstitial cells colonize a new reproductive zooids, or at least influence whether eggs or sperm are produced in a particular zooid.

Models for Sex Switching in Sequential Hermaphroditic *Hydra*

In sequential hermaphroditic *Hydra*, including many *Hydra magnipapillata* strains, a single *Hydra* polyp may give rise to both male and female ramets; some polyps produce eggs and others produce sperm, but a single polyp very rarely produces both simultaneously. This outcome is only possible if individual polyps switch sex. A switch from female to male is explained by the ability of male GSCs to repress female gametogenesis or to eliminate female GSCs (Littlefield, 1994; Nishimiya-Fujisawa and Kobayashi, 2012). Nishimiya-Fujisawa and Kobayashi (2012) proposed and provided evidence that a female polyp can switch to a “phenotypic male” if just one female GSC switches into a male GSC (Fig. 6A). Further support for this model comes from reports that phenotypic males start producing eggs every time the males are forced to re-specify their GSCs from multipotent ISCs. This is achieved by reestablishing the entire interstitial cell lineage from a single multipotent ISC or by regenerating an animal from a portion that does not have GSCs. These data demonstrate that the multipotent ISCs of phenotypic males are actually female (Bosch and David, 1986; Nishimiya-Fujisawa and Kobayashi, 2012). Female-to-male switching has also been observed in *Hydra oligactis*, likely due to a similar mechanism (Littlefield, 1991, 1994). Therefore, even in the largely gonochoristic *Hydra oligactis*, conditions are possible that lead to sex switching.

Nishimiya-Fujisawa and Kobayashi (2012) proposed that ISCs are always female, and that female GSCs spontaneously switch to male GSCs, thereby causing polyps in the colony to become phenotypically male; their model also suggests that true males do not exist (Fig. 6A). An alternative model is that ISCs can be either male or female, allowing a sequential hermaphroditic *Hydra* to be either male or female at birth; ramets in the population subsequently switch sex. If this were true, a mechanism for male polyps to turn female would have to exist; indeed, stable, lab-cultured male strains of *Hydra magnipapillata* have been used to demonstrate the likely existence of male ISCs (Bosch and David, 1986; Nishimiya-Fujisawa and

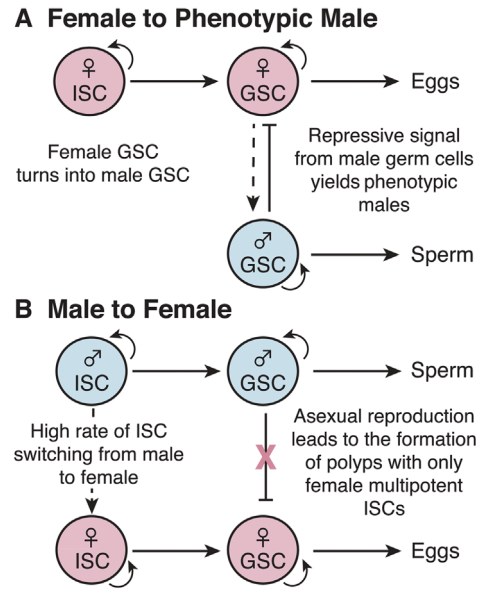


Figure 6. A model for cellular sex switching and the resulting sexual phenotype in sequential hermaphroditic *Hydra*. *Hydra* are born with either male or female multipotent ISCs, but sex switching at the cellular level leads to asexual progeny consisting of both male and female phenotypes. **A:** Female *Hydra* are born with ISCs that produce female GSCs that give rise to eggs. Occasionally, a female GSC will turn into a male GSC, which is capable of self-renewal, sperm production, and repression of egg production. The presence of a single male GSC can cause a switch from female into phenotypic male (after Nishimiya-Fujisawa and Kobayashi (2012)). **B:** *Hydra* born with male ISCs produce male GSCs that give rise to sperm. In sequential hermaphrodites, male ISCs switch to female ISCs at a relatively high rate (estimated at 1% per cell generation). These female ISCs are unable to produce eggs because of a repressive signal produced by male cells of the interstitial lineage. Due to the high rate of male to female ISC switching, an occasional bud is produced that contains only female ISCs, relieving repression by male GSCs and resulting in a female polyp.

Kobayashi, 2012). We currently favor the model that male ISCs do exist, and that switching from male to female likely occurs at the level of ISCs (Fig. 6B). Cloning of single multipotent ISCs from males of the strain ms-1 (male sterile-1) resulted in 92 polyps, each with the entire interstitial cell lineage reconstituted from a single multipotent ISC (Bosch and David, 1986). More than 70% of these ISCs cloned from male polyps, all of which were making testes before the experiment, were actually female and started producing eggs. Therefore, male polyps harbor a large number of female ISCs while maintaining ISCs that continue to give rise to sperm. When ISCs from males and females of this experiment were re-cloned, all of the female ISCs continued to produce eggs whereas 33% of the male ISCs started making eggs (Bosch and David, 1986). This can only be explained if male multipotent ISCs convert to female multipotent ISCs (Fig. 6B). A male-to-female ISC switching rate of 1% per cell generation was estimated from these observations (Bosch and David, 1986). A high rate of switching suggests that females arise from male colonies

during asexual propagation when new buds receive only female ISCs, thereby relieving the repression of the male germ cells and allowing eggs to be made (Fig. 6B). If a polyp becomes a female, it may revert to phenotypic male if a female GSC turns into a male GSC. Importantly, these proposed mechanisms of sex switching in *Hydra* are not proven, but do provide a strong framework for future experimentation. The identification of molecular markers to identify and track male and female cells using transgenic techniques is vital for definitively testing these models.

Transitions Between Hermaphroditism and Gonochorism in the Genus *Hydra*

The models discussed above (Fig. 6) demonstrate possible mechanisms underlying sex reversals in *Hydra magnipapillata* polyps, and can help us understand evolutionary transitions between different sexual systems (e.g., hermaphroditism to gonochorism) in different *Hydra* species (Fig. 7). We hypothesize that the presence of both male and female ISCs is ancestral in the genus *Hydra*. *Hydra* polyps are born either male or female, but we hypothesize that a low level of male-to-female switching (potentially at

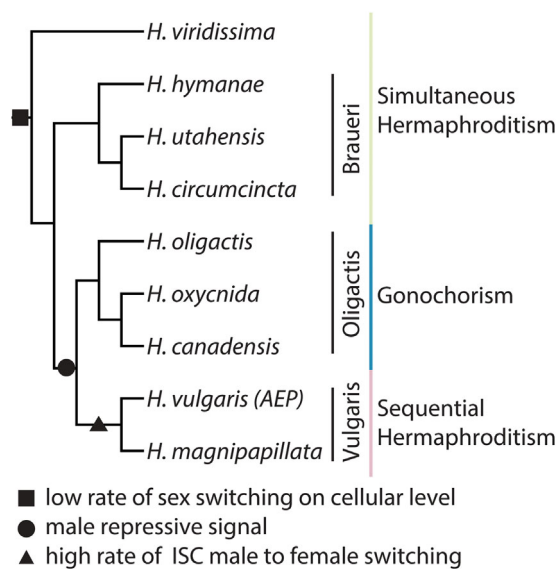


Figure 7. Hypothesis for evolutionary transitions in sexual systems in *Hydra*. Phylogenetic relationships after Martínez et al. (2010). *Hydra* male and female multipotent ISCs and/or GSCs can switch sexes; this seems to occur at a low rate in most *Hydra*, and may therefore be an ancestral character. This ability would allow for simultaneous hermaphroditism, which is observed in *Hydra viridissima* and the Braueri group. A male signal that represses egg formation was present in the most recent common ancestor of a clade that includes the Oligactis and Vulgaris groups. This caused the stabilization of sexual phenotypes still observable in the Oligactis group, which is largely stable gonochoristic. Finally, ISC switching from male to female at a high rate was present in the most recent common ancestor of the Vulgaris group. This feature allowed for male-to-female switches in the presence of a male repressive signal and for sequential hermaphroditism, which is observed in this clade (see Figure 6 for details).

the level of the ISCs) and female-to-male switching (potentially by the creation of male GSCs from female GSCs) occurred in the most recent common ancestor (Fig. 7). Under these conditions and in the absence of a male repressive signal, the coexistence of male and female cells results in simultaneous hermaphroditism, as is observed in *Hydra viridissima* (Brien and Reniers-Decoen, 1950; Stagni, 1966) and representatives of the Braueri group (*Hydra hymanae*, *Hydra utahensis*, and *Hydra circumcincta*) (Hyman, 1931; Hadley and Forrest, 1949; Tardent, 1968). The appearance of a male repressive signal (Fig. 7) in combination with the ancestral low rate of cellular sex-switching rates allowed for the appearance of gonochoristic phenotypes. Stable gonochorism is reported for species of the Oligactis group, such as *Hydra oxycnida* (Brien, 1961; Schuchert, 2010) and *Hydra canadensis* (Rowan, 1930; Hyman, 1931). Although these species are largely gonochoristic, *Hydra oligactis* exhibits very low levels of phenotypic sex switching when cultured at elevated temperatures (Hyman, 1930; Littlefield, 1986). Thus, a low level of switching still occurs in this group. By contrast, the ISCs of *Hydra magnipapillata* and *Hydra vulgaris*, both in the Vulgaris group, have acquired the ability to switch from male to female at a high rate (Bosch and David, 1986). This allows for sex change even in the presence of a male repressive signal and led to the establishment of sequential hermaphroditic phenotypes. Yet, a few *Hydra vulgaris* strains have been reported that resemble simultaneous hermaphrodites (Grassi et al., 1995), indicating that sequential hermaphroditism may have been secondarily lost. These models for *Hydra* phenotypic sex switching provide a framework for future experimentation.

POSSIBLE CONSERVED DOWNSTREAM MOLECULAR MECHANISMS FOR ESTABLISHMENT OF CNIDARIAN SEXUAL DETERMINATION

The upstream molecular mechanisms that control sex determination in animals are highly variable, but some commonalities have been found in the downstream mechanisms. In Bilateria, the DM domain-containing proteins are transcription factors with an apparently conserved role in specifying somatic sex-specific phenotypes (Matson and Zarkower, 2012). This DNA-binding domain was first identified as a conserved feature shared between the otherwise disparate *mab-3* (Male abnormal 3) in *Caenorhabditis elegans* and *doublesex* in *Drosophila melanogaster*, both of which are involved in sexual development (Raymond et al., 1998). In mice, the DM-domain containing *Dmrt1* (Doublesex and *mab-3* related transcription factor 1) is expressed in the genital ridge and is required for testis development (Raymond et al., 2000). Subsequent studies have found that the *DMRT* gene family has conserved roles in both sex determination and neurogenesis, including sex-specific neural phenotypes (Rideout et al., 2010).

Despite the apparently conserved role of DM domain-containing transcription factors in sex determination, how these genes are used in this capacity is considerably variable (Matson and Zarkower, 2012). Understanding their function in cnidarians could help us better understand the evolutionary history of this family of proteins. A phylogenetic analysis reveals that *DMRT* genes were present in the most recent common ancestor of Eumetazoa, a clade that includes most animal taxa (Wexler et al., 2014). Furthermore, *DMRT* gene families have undergone independent expansions in many clades, such that genes with well-defined functions (e.g., *doublesex* in *Drosophila* and *Dmrt1* in mice) are phylogenetically restricted and not orthologous (Wexler et al., 2014). The first study of a *DMRT* gene in cnidarians was conducted in the scleractinian coral *Acropora millepora* (Fig. 3B), revealing correlated expression of one DM-containing gene with gametogenesis; no sex-specific difference in expression could be determined (Miller et al., 2003). Functional analysis of a *Nematostella vectensis* DM-containing gene, *NvDmrtB*, demonstrated a role for it in neural cells, which suggests that *DMRT* genes have an ancestral function in neurogenesis (Bellefroid et al., 2013). An independent expansion of the *DMRT* gene family in cnidarians is likely, though, as *N. vectensis* has 11 family members, which is more than most species described so far (Parlier et al., 2013; Traylor-Knowles et al., 2015). Several of these genes have sex-specific expression, which offers the opportunity to explore possible mechanisms of sex determination in this species (Traylor-Knowles et al., 2015).

PERSPECTIVES

Multiple transitions between gonochorism and hermaphroditism have occurred in Cnidaria. Furthermore, sexual plasticity (i.e., changing sex) in response to external and internal cues in various taxa has been observed. This makes Cnidaria a fascinating group of animals for the study of both the upstream mechanisms and the various sexual systems utilized.

Currently, we know nothing about the genetic control of sex determination in cnidarians. ISCs and GSCs of certain hydrozoan species behave as male or female, but how this occurs at a genetic or epigenetic level is not known. In *Hydra*, the sex of interstitial cells can change, suggesting that sex is not hard-wired and is likely not linked to sex chromosomes. This conclusion is supported by karyotyping experiments in *Hydra*, which did not identify dimorphic chromosomes (Anokhin et al., 2010). One study using comparative genomic hybridization found evidence for sexually dimorphic chromosomes in the stony coral *Acropora solitaryensis* (Taguchi et al., 2014). In general, though, cytogenetic methods that look for gross differences in chromosome morphology may not be sufficient to identify heterogamety if the sex chromosomes are not highly divergent. Methodology such as restriction-site-associated DNA sequencing (RAD-seq) could be used to identify the presence or absence of

cnidarian sex-specific genes (Gamble and Zarkower, 2014). Organisms that appear to switch frequently between sexes likely do not use a genetic basis for sex; however, if RAD-seq did uncover genes or alleles associated with a particular sex, understanding how these are overridden during sex switching would be of particular interest.

The discovery of ISCs in hydrozoans has greatly advanced the study of germ cell function and their role in sex determination. Yet, the presence or absence of dedicated GSCs has not been determined for most hydrozoan species. Studies have attempted to use germ cell markers (e.g., *vasa*, *nanos*, and *piwi*) to understand the embryonic origins of the germ line in cnidarians (Extavour et al., 2005; Leclère et al., 2012), but it is likely that these markers do not definitively differentiate a dedicated germ lineage (i.e., primordial germ cells or germ-line stem cells) from multipotent stem cells with both somatic and germ line competency (Mochizuki et al., 2000, 2001; Seipel et al., 2004; Juliano et al., 2010, 2014; Millane et al., 2011). Definitively establishing the presence or absence of GSCs in cnidarians has broader implications for understanding germ line biology in animals, as GSCs allow for the segregation and protection of the germ line from the soma, a strategy widely observed in bilaterians (Juliano et al., 2010). A recent study presented evidence for the existence of a segregated germ line in a scleractinian coral: Using a high-throughput sequencing approach, high frequencies of somatic mutations were determined not to be passed on to offspring (Barfield et al., 2016).

Our ability to unravel the mechanisms of sex determination in a broad array of cnidarians requires a better understanding of the cell lineage that gives rise to germ cells. *Hydra* GSCs clearly exist, albeit unique molecular markers for them have not been identified so these cells cannot be followed during embryonic development, adult gametogenesis, or sex switching. Markers that differentiate between male and female interstitial cells, including GSCs, would be particularly useful for tracking by transgenic reporters (Wittlieb et al., 2006). Future work should aim to identify such markers, which will also aid in the discovery of similar cell types in other hydrozoans. The current revolution in single-cell analysis makes identification of subpopulations within lineages possible, and should be applied to this question (van Wolfswinkel et al., 2014). Whether or not general mechanisms learned in *Hydra* and *Hydractinia* are applicable to other cnidarians is unclear, though. It is also unknown if cnidarians outside of Hydrozoa contain a cell lineage analogous to the hydrozoan interstitial cell lineage; results to date suggest that they do not. To this end, specific markers of hydrozoan GSCs should aid in the identification of similar cell types in other cnidarians, and thus assist in the study of sex determination mechanisms across Cnidaria.

The number of laboratory models established to study molecular mechanisms in cnidarians is currently limited and likely creates bias in our analyses. Rapid advances in CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) technology for genome editing have made it possible and

sensible to expand the number of cnidarian models in which functional studies are performed (Ikmi et al., 2014). In addition, these methodologies will allow us to conduct more sophisticated analyses in well-established cnidarian models such as *Hydra*, *Hydractinia*, *Clytia*, and *Nematostella*. An important caveat to keep in mind as the number of species studied expands is that animals kept in laboratory culture may start to behave differently than their wild counterparts. For example, wild zebrafish have heterogametic sex chromosomes that are not found in the laboratory populations (Wilson et al., 2014). As mentioned above, studies of wild *Hydractinia* populations challenge results previously found in laboratory animals regarding the ability of a colony to harbor oocytes while undergoing spermatogenesis. These contradictions and exceptions are unavoidable since detailed studies of molecular function must take place in a controlled laboratory environment; nevertheless, they should be kept in mind, particularly when considering evolutionary implications. In addition to functional tests in the laboratory, animal biology should be studied more widely in the field to obtain a coherent picture of sex determination mechanisms in the context of other fundamental processes. Expanded and detailed studies of sex determination in cnidarians will teach us important lessons about how sex determination mechanisms evolve and about ancestral features of sex determination.

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