

Sex determination, sexual development, and sex change in slipper snails

(in *EvoDevo* : Non-Model Species in Cell and Developmental Biology)

Maryna P. Lesoway and Jonathan Q. Henry

University of Illinois, Urbana-Champaign

Department of Cell and Developmental Biology

601 S Goodwin, Urbana, IL, USA 61801

## **Keywords**

*Crepidula*

environmental sex determination

genetic sex determination

primordial germ cells

sequential hermaphroditism

protandry

tributyltin

imposex

## **Abstract**

Sex determination and sexual development are highly diverse, and controlled by mechanisms that are extremely labile. While dioecy (separate male and female functions) is the norm for most animals, hermaphroditism (both male and female functions within a single body) is phylogenetically widespread. Much of our current understanding of sexual development comes from a small number of model systems, limiting our ability to make broader conclusions about the evolution of sexual diversity. We present the calyptraeid gastropods as a model for the study of the evolution of sex determination in a sequentially hermaphroditic system. Calyptraeid gastropods, a group of sedentary, filter-feeding marine snails are sequential hermaphrodites that change sex from male to female during their life span (protandry). This transition includes resorption of the penis and the elaboration of female genitalia, in addition to shifting from production of spermatocytes to oocytes. This transition is typically under environmental control, and frequently mediated by social interactions. Males in contact with females will delay sex change to transition at larger sizes, while isolated males will transition more rapidly at smaller sizes. This phenomenon has been known for over a century; however, the mechanisms that control the switch from male to female are poorly understood. We review here our current understanding of sexual development and sex determination in the calyptraeid gastropods and other molluscs, highlighting our current understanding of factors implicated in the timing of sex change and the potential mechanisms. We also consider the embryonic origins and earliest expression of the germ line, and the effects of environmental contaminants on sexual development.

*“Sex is the queen of problems in evolutionary biology.”*  
Graham Bell 1982

*“However, what is more interesting is the mollusc’s sex life.”*  
Monty Python’s Flying Circus, Episode 32, “The War Against Pornography”, 1972

## **Introduction**

The most intriguing questions in biology often focus on the fundamentals of life: food, relationships, and sex. Sex in biology has led to foundational questions of why sex exists and why most species have separate sexes, and has proven to be a fruitful area of research across evolution, development, and ecology. As intriguing as these questions are, the focus of most research has been on separate sexes, the dominant expression of sexuality in metazoans (Jarne and Auld 2006). Hermaphroditism, where individuals function as both male and female either at the same time (simultaneous hermaphroditism) or in series (sequential hermaphroditism) is less common as a reproductive strategy, occurring in approximately 5% of animals (Jarne and Auld 2006). Despite the relative rarity of hermaphroditism as a sexual strategy in animals, it is phylogenetically widespread, found in 70% of phyla (Policansky 1982; Jarne and Auld 2006). For example, sequential hermaphroditism is known from systems as diverse as reef fishes (e.g., wrasse, Robertson 1972; clownfish, Buston 2003), shrimp (Bauer 2006), and molluscs (Heller 1993), including the slipper snails, described below. These exceptions to the norm have stimulated exploration of how individuals determine optimal sexual roles and what factors influence timing and direction of sexual transitions. As hermaphroditism has emerged multiple times, this mode of reproduction is of interest for understanding the evolution of sex and repeated transitions between dioecy and hermaphroditism. Sequential hermaphroditism, in which an individual functions as different sexes through time, is particularly valuable as a system in which to explore sexual development in an evolutionary context.

While most groups have some level of sexual diversity, molluscs (e.g., snails, squid and octopus, bivalves, etc.) exhibit nearly as much diversity in their sexual types as they do in their body plan. As the second most speciose phylum after the arthropods, molluscs are widely distributed and often

commercially important. As members of the spiralian lophotrochozoans, which include over one third of recognized phyla (e.g., annelids, nemerteans, platyhelminths, etc.), molluscs develop via a stereotyped pattern of early cleavage. This shared developmental pattern allows cellular homologies in early development to be traced across large phylogenetic distances, and is a powerful tool for understanding the origins of body plan diversity. These features make molluscs an attractive system for exploring the evolution of sexual development. As in other animals, most molluscs are dioecious with separate sexes (around 75%, Auld and Jarne 2016). Many groups are hermaphroditic. This includes simultaneous hermaphroditism as in most pulmonates and opisthobranchs, where both sexes are expressed at the same time, and sequential hermaphroditism, as in many bivalves and certain gastropod groups, where an individual transitions from one sex to another (Fig. 1) (Heller 1993). Sequential hermaphroditism can be expressed as male to female sexual transition (protandry), as found in the calyptraeid gastropods, or as female to male transition (protogyny), which is common in other sex-changing animals such as fish (Robertson 1972; Buston 2003), but is not known to occur in molluscs (Heller 1993; Hoagland 1978). Some molluscs, namely bivalves, have alternating sexuality, where multiple sex changes and reversals in the same individual are possible (Coe 1943). It should be noted that the terminology surrounding sexual diversity in animals has historically been muddled (see Collin 2013). Here, we restrict the term “sex change” to a unidirectional, non-reversible change in sex and “sexual reversal” to repeated changes in sexual characters.

While sexual types in the molluscs are diverse, most of this diversity is limited to the gastropods and bivalves (Fig. 1) (see Collin 2013). Outside of these two classes, molluscs are almost exclusively described as dioecious with separate sexes, with few exceptions. For example, all aplacophoran solenogasters described to date are simultaneous hermaphrodites. Other exceptions include two members of one polyplacophoran genus with simultaneous hermaphroditism (*Lepidochitona*, Eernisse 1988), one case of potential sequential hermaphroditism in a cephalopod (*Ancistrocheirus lesueurii*, Hoving et al. 2006), and one case of simultaneous hermaphroditism in a monoplacophoran (*Micropilina arntzi*,

Haszprunar and Schaefer 1996). The infrequency of these exceptions does not rule out the possibility that there are more cases yet to be described. Molluscan phylogenetic relationships and outgroup comparisons suggest that dioecy is the ancestral state, with multiple independent origins of hermaphroditism (Fig. 1) (Ponder and Lindberg 1997; Kocot et al. 2011; Smith et al. 2011). Basal groups of gastropods and bivalves are dioecious, lending support to this hypothesis (Collin 2013). Thus, the evolution of molluscs includes repeated parallel emergence of different types of hermaphroditism, mainly restricted to a few groups within the gastropods and bivalves.

Hermaphroditism is thought to emerge under certain selective conditions. Theoretical models, most prominently sex allocation theory, have been used to explore how selection has led to the repeated evolution of hermaphroditism. Sex allocation asks how individuals and populations divide resources between male and female functions (Charnov 1982). In dioecious species with separate sexes, sex allocation determines the sex ratio (ratio of males to females) in a population. It can also be used to understand how individuals and populations balance costs of male and female function with reproductive output. In hermaphroditic species, sex allocation relates to the timing and direction of sex change in sequential hermaphrodites and the allocation of male and female reproductive resources in simultaneous hermaphrodites (Charnov 1982). The size-advantage model of sex allocation (Ghiselin 1969; Warner 1988) posits that changing sex should be advantageous if one sex has a higher reproductive output at larger sizes than the other sex. For example, larger males that produce more offspring relative to females of the same size should select for a transition from female to male (protogyny). Conversely, when larger females produce more offspring relative to males of comparable sizes, this should select for a transition from male to female (protandry) (Munday et al. 2006; Warner 1975; Warner et al. 1975). When conspecifics are scarce and/or widely scattered, it appears to be advantageous for an individual to perform both male and female functions simultaneously, thereby increasing reproductive output (Ghiselin 1969). Sedentary lifestyles, combined with patchy distribution and environmental heterogeneity is thought to select for sequential hermaphroditism (Wright 1988). Sequentially hermaphroditic molluscs such as

oysters and some gastropod groups, like limpets and slipper snails are often sedentary, reducing the chances of encountering a mate (Hoagland 1978; Wright 1988). Reproductive behavior does not appear to influence this, as sequential hermaphrodites reproduce via either free-spawning or copulation (Hoagland 1978), however, sexual reversals in molluscs appear to be associated with free-spawning (Collin 2013). While sequential hermaphrodites generally function as only one sex at a time, nearly all heterobranchs (opisthobranchs and the pulmonates) are simultaneous hermaphrodites with mutual insemination (Heller 1993; Jarne and Auld 2006). While marine opisthobranchs do not typically self-fertilize, it is common for terrestrial pulmonates to do so, and some species do so preferentially (Jarne and Auld 2006; Auld and Jarne 2016; Koene 2017). Simultaneous hermaphroditism, including the role of sex allocation and sexual selection, has been reviewed elsewhere (Auld and Jarne 2016; Nakadera and Koene 2013).

Within the molluscs, slipper snails (calyptraeid gastropods, Fig. 2), provide an excellent system for exploration of sequential hermaphroditism, including the molecular mechanisms and evolution of sex determination, sexual differentiation, and sex change. For more than a century, scientists have studied the sex lives of these animals. The influence of environment in sex determination makes the calyptraeid gastropods of particular interest. While all calyptraeids change sex from male to female (Fig. 2), different species show greater or lesser influence from the presence of conspecifics on the timing of sex change. New molecular techniques are opening non-model systems to mechanistic exploration, and the calyptraeids, with an abundance of information on sexual development and the ability to make comparisons between closely related species, make them an excellent model to study the development of sexual systems (Henry et al. 2017; Henry and Lyons 2016). While the underlying mechanisms of these interactions remain poorly known, new tools and techniques are increasing our ability to answer questions about all aspects of development in this group of animals (e.g., Henry et al. 2017). In this chapter, we will discuss sexual systems and sex determination in molluscs, with a focus on environmental sex determination in calyptraeids. This will include discussion of some known proximate mechanisms controlling sex determination in molluscs. We will also briefly discuss the earliest expression of sexual

development, the germline. We conclude with anthropogenic impacts on sexual development in molluscan populations, and suggest directions for future research.

### **Mechanisms of sex determination**

Sex determination, the developmental processes that produce male or female individuals or cells (eggs and sperm), is fundamental to questions of sex allocation (Beukeboom and Perrin 2014). Exploration of mechanisms of sex determination has led to the realization that diversity is the rule (Beukeboom and Perrin 2014; Bull 1983). The diversity of sex determination mechanisms has prompted a search for commonalities at multiple levels of biological organization, and has shown that there are at least some shared mechanisms. The lability of sex determination mechanisms and the action of selection in producing this variety is an exciting question. Well established model systems have taught us a great deal about how sex is determined; however, they are not able to fully account for the diversity of sexual systems. Increasing accessibility of genetic and molecular techniques in non-model organisms and the conceptual approach of evo-devo are powerful tools for understanding the diversity of sex allocation, sex determination, and the how the underlying mechanisms have been altered by selective forces.

While the mechanisms that produce male and female forms are staggeringly diverse, most mechanisms fall into two main categories, genetic sex determination and environmental sex determination. Interactions between environmental and genetic factors are the norm. Genetic sex determination (GSD) includes chromosomally determined sex and determination by multiple genetic factors. In chromosomally determined sex, either the male can be the heterogametic sex (i.e., XX/XY systems) or the female can be the heterogametic sex (i.e., ZW/WW systems), or males may lack a copy of one sex chromosome (i.e., XX/XO systems). Systems with sex determination under the control of multiple genetic factors may be oligogenic, with control by a few genes, or polygenic, with control by many genes of small effect. Environmental sex determination (ESD) includes sexual systems that are determined by external factors such as nutritional state, temperature, social structure, or some combination of environmental triggers.

This diversity has prompted a great deal of speculation about how and why diversity of sex determination has arisen (Bachtrog et al. 2014; Beukeboom and Perrin 2014; Bull 1983; Haag and Doty 2005), and prompted the search for underlying commonalities. Molluscs include examples of all types of genetic sex determination. However, despite the diversity of sex determination in molluscs, the mechanistic bases have only been explored in detail for relatively few species.

Much of our current understanding of genetic sex determination comes from studies of model organisms (e.g., *Drosophila*, Oliver and Clough 2012; *C. elegans*, Riddle et al. 1997; and mouse, Koopman et al. 1991). Evidence for the genetic control of sex determination in molluscs has come from chromosomal analyses, analysis of sex ratios in natural and experimental populations, and more recently transcriptomic and molecular expression studies. These studies are increasing our understanding of the diversity of molluscan sex determination, and started to hint at key players in the underlying gene regulatory networks. Many studies of sex determination in molluscs rely on the use of sex ratios to infer underlying mechanisms (see Yusa 2007). Sex ratios have the advantage of being relatively easy to measure, at least in species with obvious, external sexual characters. While the sex ratio approach has been useful, it has important caveats. Simple tallies of sexes in a given population can miss important influences on sex ratios such as differential mortality at various life stages, or broader population differences, and can miss cases of sequential hermaphroditism (Yusa 2007). While the best method of determining sexual patterns is to follow animals throughout the life cycle, this remains impractical for long-lived or rare species.

### ***Genetic Sex Determination in Molluscs***

Chromosomal studies have found heterogametic chromosomes in a number of gastropods, presumed to be sex chromosomes (reviewed by Thiriot-Quévrevre 2003). This includes the XX-XY system, with males as the heterogametic sex (i.e., *Viviparus spp.*, *Littorina saxitalis*, 2 neogastropod species), the XO-XX system where males lack a sex chromosome (e.g., *Neotricula aperta*), and the ZW-ZZ system, with

females as the heterogametic sex (e.g., *Viviparus spp.*). No sex chromosomes have been reported in either Pulmonata or Opisthobranchia (Thiriou-Quévroux 2003). Comparable studies of bivalves have also found no evidence of heterogamety (see Breton et al. 2017 for review). Many members of these groups are true hermaphrodites, expressing both male and female gametes at the same time, making chromosomally-based sex determination mechanisms unlikely. Karyotypes continue to be explored (e.g., García-Souto et al. 2018, *Littorina spp.*); however, these studies generally have been limited to chromosome number and description. The actual mechanisms of sex determination in relation to chromosomally expressed genes remains unexplored in molluscs.

Genetic control of sex allocation, and sex determination more generally has been best studied in bivalves, owing to their commercial importance and value in aquaculture (see Breton et al. 2017 for review). Controlled crosses in the Pacific oyster, *Crassostrea gigas*, suggest that sex determination is controlled by a single locus (Guo et al. 1998). Analysis of 86 families suggested the presence of a dominant male allele (M) and recessive protandrous hermaphroditic allele (F) (Guo et al. 1998). However, this two-locus model does not fully account for the ratios observed, and a three locus model proposes that FF produces true females, MM true males, and FM protandric females (Hedgecock and Hedrick 2010). Similar lineage experiments in the dioecious pulmonate, *Pomacea canaliculata*, have suggested that a small number of genes are required to produce the sex ratios observed in full- and half-sibling crosses (Yusa 2007; Yusa and Kumagai 2018).

Chromosomal and genomic information is generally lacking in the calyptraeid gastropods. Conklin (1902) reported 30 chromosomes (n=30) in maturing oocytes of *Crepidula plana*. The karyotype of *Crepidula unguiformis* is reported as n=17 (Libertini et al. 2009). Interestingly, the authors of the latter report a heteromorphic chromosome in approximately one third of the cases examined but doubt that this is a sex chromosome, stating unequivocally “the possibility that it may be linked to a sex determining mechanism is easily ruled out since hermaphrodite organisms cannot have sex-linked chromosomes” (Libertini et al.

2009, p. 112). Be that as it may, several aspects of calyptraeid sexual development and behavior likely have a genetic component. For example, juveniles and small males are typically highly motile, and become more sedentary as they increase in size and age. This behavioral shift includes changes in pedal gland morphology (Chaparro et al. 1998; Chaparro et al. 2001). These shifts in motility are linked to the initiation of the sexual transition (Coe 1935). However, inter-individual differences in behavior and motility of males in relation to the timing of sexual transition could be also under genetic control (Coe 1935). Indeed, some more sedentary males delay their transition to females sometimes for very lengthy periods (Coe 1935, 1938a, b). Lack of mechanistic understanding limits the conclusions that we can make about the genetic control of sex determination without further study. Chromosomal studies and multi-generation crosses, as described previously, could be used to demonstrate the genetic component of sex allocation in calyptraeids.

### *Environmental Sex Determination in Molluscs*

While no system can realistically be said to be under either exclusive genetic or environmental control, environmental factors can be the controlling factor in sex determination. For example, temperature is known to determine sex in turtles and other reptiles, with higher temperatures producing females and lower temperatures resulting in development of males (Bull and Vogt 1979; Miyagawa et al. 2018). Molluscan examples of ESD are found primarily in sequential hermaphrodites, which allocate resources to male or female function in response to environmental factors. Potential environmental influences include (but are not limited to) temperature, nutrition status, and social environment. For example, some patellogastropods (true limpets) change sex from male to female depending on population density (*Lottia gigantea*, Lindberg and Wright 1985). Detailed observations have also shown seasonal sex reversals are possible in limpets (*Patella ferruginea*, Guallart et al. 2013). The oyster, *Ostrea edulis*, and other bivalves also change and reverse sex in response to nutritional and temperature cues (Coe 1943; Breton et al. 2017).

All calyptraeids are protandric hermaphrodites, changing sex once from male to female (Fig 2). Fertilization is internal via copulation, so the sexual transition includes resorption of the penis, elaboration of a female genital papilla and associated reproductive structures, as well as a shift from production of spermatocytes to oocytes in the gonad (Fig. 2). While Conklin (1897) speculated that *C. fornicata* might be hermaphroditic, Orton's (1909) examination of the gonadal tissues was the first to confirm sequential hermaphroditism in *Crepidula*. Subsequent studies have confirmed this pattern in all members of the family (Coe 1936, 1938b, a; Collin 1995, 2000, 2006; Collin et al. 2005; Gould 1952; Gould 1917a, b, 1919; Hoagland 1978; Warner et al. 1996). While all individuals pass through a male phase before transitioning to female maturity, the precise timing of sexual development is influenced by the presence of conspecifics. Males associated with females delay their sexual transition, changing sex at a larger size relative to isolated males or males associated with other males (Coe 1938b; Collin 1995). This has been confirmed in multiple species (Coe 1935, 1936, 1938a, b; Ishiki 1936, 1938; Warner et al. 1996; Collin 2000, 2006; Collin et al. 2005). Empirical evidence of size at sex change in the calyptraeids has generally shown that individuals make decisions about transitions based on local environmental factors. These include population density, local mating population size and composition, as well as the age, size, and nutritional status of an individual.

One of the best studied examples in the calyptraeids is the Atlantic slipper snail, *Crepidula fornicata*, which famously forms stacks or chains of up to tens of animals (Fig. 3A). Small males are motile and are found at the top or along the sides of stacks, while large, mature females are sedentary and found at the bottom of a stack attached to an empty shell or other substrate (Coe 1936; Collin 1995). Reduced motility in larger males is thought to reflect reduced opportunity for copulation, however, paternity studies have shown that the male closest to the female in a stack has a reproductive advantage (Dupont et al. 2006; Proestou et al. 2008; Broquet et al. 2015). Furthermore, paternity is generally restricted to members of a stack (Dupont et al. 2006; Le Cam et al. 2009; Proestou et al. 2008). Stack or aggregate size, motility, and

mode of development are all associated with variation in sex ratio and size at sex change (Collin 2006). Adult behavior in other calyptraeid species varies, and adults of some species may be found forming stacks as in *C. fornicata*, others are more commonly found in isolation or in mating pairs (Fig. 3B), or in masses covering available substrate (Fig. 3C). These behaviors influence local mating populations, and therefore sexual transitions, as the size of a mating group influences size at sex change and the sex ratio of a population (Collin 2006). Experimental manipulation of *Crepidula norrisarium* also found evidence of social control of sex change in a large, direct-developing species restricted to limited habitats on the shells of other gastropods (Warner et al. 1996). When multiple hosts are available, males of *C. norrisarium* tend to move to hosts where females are present, thereby increasing their reproductive potential (Hobday and Riser 1998). Male reproductive success has been shown to directly influence timing of sexual transition in *Crepidula coquimbensis*, where males with more access to females and longer periods in copulatory positions showed increased levels of paternity and reduced growth rates and rates of sex change (Brante et al. 2016).

Not all calyptraeids appear to be influenced by the presence of conspecifics. For example, *C. convexa* are small, and brood their direct-developing offspring until they hatch as juveniles. Field and laboratory manipulations shows little influence of conspecifics on the timing of sex change (Coe 1938b; Hoagland 1978). Similarly, *Calyptraea chinensis* does not show any changes in the rate of male growth due to female presence or absence (Bacci 1951). Likewise, social environment is not the only factor that can influence the timing of sex change in calyptraeids. Decreased food availability appears to lengthen the transitional period at the expense of male function in *C. cf. marginalis* and *C. incurva* (Mérot and Collin 2012b; Mérot and Collin 2012a), while desiccation stress and the increased early mortality produces smaller females (Mérot and Collin 2012b).

These studies highlight the role of plasticity in response to social and environmental cues in controlling the timing of sex change. Considering sexual development and sex change as plastic responses to

environmental inputs provides us with a conceptual framework to ask and address these questions (Capel 2017). Few systems to date present examples of known mechanisms of environmentally mediated polyphenic shifts. One example is the nematode worm, *Pristionchus pacificus*, which produces either wide-mouthed, predatory morph or a narrow-mouthed, grazing morph in response to nutritional and social cues (Ragsdale et al. 2013). This developmental switch appears to be controlled at the site of the polyphenism by the activity of a sulfotransferase (SEUD-1), and NHR-40, with a threshold set by the opposing activity of a sulfatase (EUD-1) (Bui et al. 2018). The integration of multiple inputs (e.g., nutrition, social environment, etc.) in calyptraeid sex change suggests the integration of hormonal/neural signals with molecular switches based on certain thresholds. The availability of multi-species comparisons within the calyptraeids, and with species that show differences in the degree to which conspecifics influence sex change, will allow us to explore the commonality (or lack) of these environmentally mediated mechanisms.

### **Mechanisms of Sexual Differentiation**

While the role of conspecifics on the timing of sex change in many calyptraeids is well recognized, the proximate mechanisms controlling these changes have only recently begun to be described. Early workers suggested that females secreted a water-borne pheromonal cue that could act at a distance (Gould 1952; Gould 1917b, 1919) or that males were delaying their sexual transition in response to cues that required direct contact with females (Coe 1938a, 1942). However, recent experiments have revealed that social control of sex change is contact-mediated. In *C. fornicata* (Cahill et al. 2015) and *C. c.f. marginalis* (Carrillo-Baltodano and Collin 2015), animals were reared in conditions that allowed for the free flow of water and potential water-borne cues, and either allowed or blocked from direct physical contact. Males of *C. fornicata* were also exposed to clean, empty shells (Cahill et al. 2015; see also Coe 1938a), and males of *C. c.f. marginalis* were exposed to mucus and substrate that was previously in contact with conspecifics (Carrillo-Baltodano and Collin 2015). Males of both species were inhibited from

transitioning to female only by direct physical contact with females. While these experiments confirm the importance of physical contact in influencing the timing of sex change, the precise nature of the signal remains unknown, though Carrillo-Boltando and Collin (2015) speculate that signals during copulation may play a role in mediating this response (see also Coe 1938a).

Male development may be controlled by secretions of the pedal ganglion at the base of the right tentacle, adjacent to the penis outgrowth region (Le Gall and Streiff 1975; Le Gall 1981; Le Gall and Feral 1982; Féral et al. 1987). In a series of experiments Le Gall (1981) found that removal of the right tentacle will quickly result in tentacle regrowth. Similarly, if the penis is removed, it can be regrown. When both tentacle and penis are removed, the tentacle will regenerate first, followed by the penis. However, a second removal of the right tentacle prior to penis regrowth blocks its regeneration (Le Gall 1981). Removal of the pedal ganglion blocks regeneration of the penis, but not the tentacles (Le Gall 1981). Interestingly, the insertion of another pedal ganglion from another male individual of the same species (*C. fornicata*), or even the pedal ganglion from males of another species (*Calyptreaea*, *Littorina spp.*, *Buccinum spp.*, Le Gall 1981) is able to induce penis growth. Similar results were reported in *Calyptreaea chinensis* (Streiff 1966, 1967). This is reminiscent of the nervous control of optic gland secretions in octopus. Severing innervation to the optic gland initiates growth of the reproductive organs in males and females (Wells and Wells 1959), and similar neurohormonal controls may be shared among molluscs. The broad action of the pedal ganglion in regeneration and penis growth suggests a hormonal secretion. While a potential signaling compound has been partially purified (Le Gall et al. 1987), its identity remains unknown.

Despite these tantalizing glimpses of mechanisms controlling sex determination and sex change in the calyptraeid gastropods, the precise molecular and hormonal mechanisms remain elusive. However, a recent spate of transcriptomic studies in molluscs, primarily bivalves, have implicated several candidate genes, including conserved sex-determining genes. For example, in the oyster *Crassostrea gigas*, Zhang

and colleagues (2014) showed expression of several conserved sex determining genes, including *Cgdsx* (*C. gigas doublesex*) and *CgSoxH* (*C. gigas Sry-related HMG box*), which are expressed specifically in gonadal tissues (Zhang et al. 2014). Similar transcriptomic studies in other bivalves have suggested a number of conserved genes involved in sexual differentiation. This includes *dmrt* in *Pinctada margaritifera* (Teaniniuraitemoana et al. 2014), and *dmrt*, *SoxH*, and *FoxL2* in *Patinopecten yessoensis* (Li et al. 2016). More fine-grained approaches are beginning to identify novel candidates, and suggest important roles for neurotransmitters and long non-coding RNAs (*C. gigas*, Yue et al. 2018). Although their functions in sexual differentiation and sex change have yet to be confirmed, these studies provide a useful starting point. Further, the molecular components identified in these studies may also play important roles in sexual development and differentiation in the calyptraeid gastropods.

The role of broadly conserved components, such as DM-domain containing *dmrt-like* genes in gonadal tissues of molluscs is of particular interest. The transcription factor, *dmrt* (*doublesex/male-abnormal-3 related*), was first described in studies of the fruit fly, where alternately spliced forms of the *Drosophila melanogaster* transcript *dsx* (*doublesex*) lead to differentiation between male and female somatic forms (Burtis and Baker 1989; Hildreth 1965). *Dsx* contains a DM domain, which binds DNA. The discovery of DM-like domains in *Caenorhabditis elegans mab-3* (*male abnormal*), and their role in sex determination demonstrated that despite abundant variation in sex differentiation mechanisms, some commonalities are present (Raymond et al. 1998). DM-domains have subsequently been found to play a role in sex determination in vertebrates (e.g., *dmrt1* in mouse, Raymond et al. 2000), and throughout the metazoa (Picard et al. 2015), including lophotrochozoans (Chong et al. 2013). *Dmrt* is so far the only gene family identified with a conserved role in sex determination (Kopp 2012). While DM-domain containing *dmrt* homologues are found throughout the animal kingdom, its position within the hierarchy of sex-determining gene regulatory networks is highly variable (Kopp 2012). Exploring the role of this conserved sex-determining gene in a group with environmental sex determination will be a useful comparison to existing models of genetic sex determination.

## Developmental Origins of the Germ Line

The earliest expression of sexual development in the individual is the primordial germ cell. The germ line is either determined autonomously early in development as in the fly, or induced by surrounding cells later in development as in the mouse (Extavour and Akam 2003). It is thought that induction is the ancestral mode of germ cell determination in the Metazoa, and this has also been suggested for spiralian animals (Extavour and Akam 2003; Rebscher 2014). However, evidence increasingly suggests that many spiralian animals have autonomous determination of the germ line early in development (Rebscher 2014).

Spiralian animals (lophotrochozoans) share a highly conserved, stereotyped early cleavage pattern, referred to as spiral cleavage (Henry 2014). This pattern of early development allows for comparisons of cell lineages across the group and is a powerful tool for understanding how changes in early development have led to diversity in adult body plans. One of the best characterized cells in all spiralian animals is the 4d cell, which plays an important role in early embryonic organization, gives rise to much of the mesoderm, and is often the first cell to break the spiral cleavage pattern (Henry 2014). This cell arises from one of the 4 cell quadrants, the D quadrant (Fig. 4A) after the 3 earlier tiers of micromeres have been born. The 4d cell also appears to give rise to the primordial germ cells (Fig. 4, Table 1) (Rebscher 2014). Several lines of evidence have been used to identify the putative primitive germ cells (PGCs) in spiralian animals. Classical studies used the presence of distinct cytoplasmic markers and arrest of cell divisions to identify primordial germ cells. For example, dense aggregations of mitochondria are localized to the primordial germ cells of the bivalve *Sphaerium striatinum*, which are incorporated into the developing gonad (Woods 1931, 1932). Similarly, the primordial germ cells of the polychaete *Salmacina dysteri* were identified by their arrest of cell divisions in addition to their distinctive cytoplasmic contents and size (Malaquin 1925). In both these cases, the putative germ cells appear to arise from the 4d cell (Malaquin 1925; Woods 1931, 1932). Modern cell lineage studies, using long-lived vital dyes to trace the cell

lineage fates, have similarly highlighted cell populations that arrest division shortly after their birth and localize to positions where they are likely to contribute to the development of germ cells. For example, In *C. fornicata* and *C. convexa*, the origins of the PGCs have been traced to the 4d lineage. Cell behavior suggests that two daughter cells,  $2mL^{2,2}$  and  $2mR^{2,2}$ , are the source of the germ line in these calyptreaeids (Fig. 4B-C) (Lyons et al. 2012). These cells do not divide, and from their initial location on bilateral sides of the embryo, they migrate to a position adjacent to the intestinal rudiment, where they are ultimately relocated to the region where the gonads form, deep to the heart (Lyons et al. 2012).

In addition to cell lineage studies, widely conserved germ line markers (e.g., *vasa*, *piwi*, and *nanos*) have been used to define PGCs (Ewen-Campen et al. 2010). For example, *vasa* is initially expressed throughout embryos of *Tritia (Ilyanassa) obsoleta*, becoming progressively more concentrated in the 4d lineage, until it is finally restricted to the 3ML and 3MR teloblasts (Fig. 4) (Swartz et al. 2008). Likewise, *nanos* expression is initially widespread, becoming localized to the 4d lineage and persisting in the  $2mL^2/R^2$  cells (Rabinowitz et al. 2008). Expression of *vasa* and *nanos* in the abalone *Haliotis asinina* suggest that the primordial germ cells arise from the 4d lineage; however, expression is not restricted to the gonadal region during larval development in these equal-cleaving animals (Kranz et al. 2010). The authors suggest that the PGCs are not formed strictly by maternal preformation, but that induction is also involved. *Vasa* expression in the bivalve *C. gigas* is restricted to two clusters of cells that appear to arise from the 4d lineage and localize to the gonad after metamorphosis (Fabioux et al. 2004). *Cg-nanos-like* is restricted to bilateral expression in what appears to be the ML and MR cells of at gastrulation following ubiquitous expression in early development, supporting the 4d origin of the PGCs in *C. gigas* (Xu et al. 2018). A combination of lineage tracing, expression of conserved germ line markers, cell deletion, and cell behavior have been used to suggest the identity of putative PGCs in other spiralian, highlighting the diversity of PGC origins possible within the conserved spiral cleavage pattern. For example, the presumptive PGCs of the polychaete *Platynereis dumerilii*, are the first lineage to segregate from 4d (Fischer and Arendt 2013; Rebscher et al. 2007; Rebscher et al. 2012; Özpölat et al. 2017) (Fig. 4, Table

1). In contrast, clitellates such as the sludge worm *Tubifex tubifex* and the leech *Helobdella* show much later segregation of germ cells from 4d (Fig. 4, Table 1) (Kang et al. 2002; Oyama and Shimizu 2007; Gline et al. 2011; Cho et al. 2013; Kato et al. 2013). This variation, particularly evident in the annelids, may be related to regenerative capacity. For example, in the polychaete, *Capitella telata*, cell lineage data and expression of *vasa*, *piwi*, and *nanos* suggest that PGCs derive from 4d (Meyer et al. 2010; Dill and Seaver 2008; Giani et al. 2011). Cell deletion experiments show that while expression of the PGC markers *vasa*, *piwi* and *nanos* is lost in ablated embryos, juveniles regain *vasa* expression, and are able to regenerate the germline and produce offspring (Dannenbergh and Seaver 2018). Despite the diversity in primordial germ cell origins, these and other examples all point to 4d as the source of the germ line in spiralian (Fig. 4, Table 1) (Rebscher 2014). What remains lacking is an understanding of how germ cell origins differ (or remain the same) within more closely related groups, such as the calyptreae. Other outstanding questions include the timing of segregation of male and female functions in hermaphroditic animals. For example, in *Helobdella robusta*, the putative PGCs segregate relatively late in embryonic development (Fig. 4, Table 1). In these simultaneous hermaphrodites, *nanos* is differentially expressed at the site of the future testes, while *vasa* and *piwi* are expressed at the site of the future ovaries (Cho et al. 2013). Similar segregation of male and female germ cells may be possible in the sequential hermaphrodite *Crepidula*, where 2 PGCs are initially formed (Lyons et al. 2012), but it is not known if these cells eventually contribute to the production of either eggs or sperm, or both.

### **Environmental Contaminants and Sexual Development in Molluscs: Imposéx**

Adult reproductive structures remain understudied in molluscs, despite their widespread use as bioindicators in marine environments. In the 1970s, researchers began to recognize widespread abnormalities in marine mollusc populations. Shell growth abnormalities and fisheries collapses in commercially important bivalves in the European Atlantic (Evans et al. 1995) and penis growth in normally dioecious females, particularly in neogastropods (Blaber 1970; Jenner 1979; Smith 1971) were

linked to exposure to the organotin compound, tributyltin (TBT) (Smith 1981). TBT, called the most toxic compound ever knowingly introduced to the aquatic environment (Goldberg 1986), was widely used as a highly effective biocide in anti-fouling paints on ships and docks. The abnormal growth of male sexual characteristics in females without becoming functional males is termed imposex (“superimposed sex”) (Smith 1971), exemplified by the sentinel species, *Nucella lapillus* (Gibbs et al. 1987). Imposex can be measured on a scale determined by the extent of growth of the penis (Fig. 5) (Gibbs et al. 1987; Stroben et al. 1992) and by the extent of vas deferens growth (Gibbs et al. 1987). Imposex eventually blocks the female genital tract, resulting in sterility and in severe cases, death. Local efforts to ban TBT were initiated relatively quickly, and a worldwide ban on TBT has been in effect since September 2008 (see Sousa et al. 2014 for review). In some areas, this has proven to be an effective strategy for reducing TBT concentration in the environment and concomitant imposex rates (Schøyen et al. 2019). However, TBT continues to effect marine populations in some areas, particularly in non-signatory countries or where local regulations are lacking (e.g., Batista et al. 2016).

Since exposure to TBT and related compounds were first linked to imposex in molluscs, there has been interest in elucidating the underlying mechanisms. Proposed hypotheses fall into three major categories (Pascoal et al. 2013; Horiguchi 2017). The steroid hypothesis suggests that TBT increases the activity of androgenic steroidal hormones, increasing concentrations of androgens via interference with steroid metabolism (Bettin et al. 1996; Gooding et al. 2003; Ronis and Mason 1996). However, endogenous production of vertebrate steroid hormones in molluscs remains questionable, as suggested by the ready uptake of environmental hormones and the lack of conserved biosynthesis pathways in many invertebrates (see Horiguchi 2017; Scott 2018, for review). The neuroendocrine hypothesis suggests that TBT interferes with excretion of neuropeptides that control reproductive development (Féral and Le Gall 1983), such as APGWamide, which induces penis growth in *T. obsoleta* (Oberdörster and McClellan-Green 2000; Oberdörster and McClellan-Green 2002). However, the effect of APGWamide is weak compared to TBT, and does not induce imposex in other species, including *N. lapillus* (Castro et al.

2007). The retinoid hypothesis is the most promising mechanistic explanation to date, suggesting that TBT actively binds to the nuclear receptor, retinoid X receptor, RXR. TBT has been shown to bind specifically to RXR in molluscs (Nishikawa et al. 2004), and treatment with RXR agonists induces imposex in *N. lapillus* (Castro et al. 2007; Lima et al. 2011) and *Thais clavigera* (Nishikawa et al. 2004). Transcriptomic expression in *N. lapillus* exposed to TBT confirmed expression of RXR in imposex animals, and further suggested a role for peroxisome proliferator-activated receptors (PPAR) (Pascoal et al. 2013). Application of rosiglitazone, a PPAR agonist, induced imposex at levels comparable to TBT (Pascoal et al. 2013). Both RXR and PPAR belong to NR subfamily 1, and form homo- and heterodimers with each other and other members of the retinoic acid pathway, including the retinoic acid receptor, RAR. TBT is capable of binding to both RXR (Nishikawa et al. 2004) and PPAR (le Maire et al. 2009). While nuclear receptors such as RAR (Babonis and Martindale 2017) are broadly conserved across the Metazoa, care must be taken to understand their specific roles in molluscs and non-vertebrate animals. For example, molluscan RAR shows no binding activity to known agonists, although RAR-RXR heterodimers show changes in transcriptional activity in *in vitro* transactivation assays (André et al. 2019). In contrast, in the polychaete *P. dumerilii*, RAR binds retinoic acid and functions in neurogenesis, although this requires much higher concentrations than in chordates (Handberg-Thorsager et al. 2018).

The majority of studies on mechanisms of imposex induction have typically been carried out in temperate gastropod species with separate sexes. For example, *N. lapillus* is used as an environmental sentinel and experimental model for many TBT-imposex studies (as mentioned above), and *T. obsoleta* was among the first species where imposex was described (Jenner 1979; Smith 1981). Experimental work with these temperate species often requires long periods of study, and the effects of environmentally relevant doses of TBT can require months to be visible. In contrast, in sequentially hermaphroditic calyptraeid gastropods, the effects of TBT exposure can be seen within a few days (Fig. 5, MPL, pers. obs.), making them a useful model not only for testing the effects of potential reproductive disrupting toxins, but also for understanding the development of the reproductive organs in gastropods and molluscs more broadly.

The growth, resorption, and remodeling of the reproductive organs during the normal course of individual development (see Fig. 2) makes *Crepidula* ideal for laboratory manipulation of sexual differentiation. This includes functional testing of the signaling pathways implicated in reproductive development using environmental toxins such as TBT and related compounds. For example, the connection between TBT and retinoic acid signaling suggests a key role for nuclear receptors in regulating sexual differentiation in the calyptraeids and other molluscs.

## **Conclusion**

Sex determination is an interesting and important area of study for evolutionary developmental biology, and the calyptraeid gastropods are an excellent system to explore the evolution of sex determination and sexual development. As lophotrochozoans, they share with more than a third of known phyla a common pattern of early development, namely spiral cleavage, allowing direct comparisons of early development across large evolutionary distances. As molluscs, they are one example of sequential hermaphroditism, and provide comparative data with other examples of this type of development, such as bivalves. The calyptraeid gastropods provide a powerful system of related species with differences in timing of sex change and varying environmental influence on sex determination. Understanding the role of plasticity and other developmental mechanisms in the evolution of sequential hermaphroditism and ESD will benefit from multiple comparisons of closely related species as offered by the calyptraeids. In addition, the calyptraeids are amenable to experimental manipulation, including initiation of sexual development in the embryo, the effects of conspecifics and other environmental factors on sexual transitions, and the effects of industrial pollutants on sex determination and sexual development. The calyptraeids, using *C. atrasolea* as a central hub from which to explore development of these sequential hermaphrodites (Henry et al. 2017), as well as through making broader comparisons across the diversity of molluscan sexual systems (Collin 2013), show great promise in providing a better understanding the diversity of sexual systems.

## **Acknowledgements**

The authors acknowledge the invaluable support of the National Science Foundation (NSF) and were supported by NSF grant IOS-1558061 and NSF EDGE IOS-1827533 to JQH (JJH). MPL was supported by a Postdoctoral Fellowship from the Fonds de recherche du Québec - Nature et technologies (FRQ-NT), and thanks the Society for Developmental Biology for an Emerging Models Grant.

## References

- André A, Ruivo R, Fonseca E, Froufe E, Castro LFC, Santos MM (2019) The retinoic acid receptor (RAR) in molluscs: Function, evolution and endocrine disruption insights. *Aquat Toxicol* 208:80-89. doi:<https://doi.org/10.1016/j.aquatox.2019.01.002>
- Auld JR, Jarne P (2016) Sex and Recombination in Snails. In: Kliman RM (ed) *Encyclopedia of Evolutionary Biology*. Academic Press, Oxford, pp 49-60. doi:<https://doi.org/10.1016/B978-0-12-800049-6.00149-9>
- Babonis LS, Martindale MQ (2017) Phylogenetic evidence for the modular evolution of metazoan signalling pathways. *Philos Trans R Soc B-Biol Sci* 372 (1713):20150477. doi:[doi:10.1098/rstb.2015.0477](https://doi.org/10.1098/rstb.2015.0477)
- Bacci G (1951) L'ermafroditismo di *Calyptraea chinensis* L. e di altri Calyptraeidae. *Pubbl Stn Zool Napoli* 23 (1):66-90
- Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman T-L, Hahn MW, Kitano J, Mayrose I, Ming R, Perrin N, Ross L, Valenzuela N, Vamosi JC, The Tree of Sex Consortium (2014) Sex Determination: Why So Many Ways of Doing It? *PLOS Biology* 12 (7):e1001899. doi:[10.1371/journal.pbio.1001899](https://doi.org/10.1371/journal.pbio.1001899)
- Batista RM, Castro IB, Fillmann G (2016) Imposex and butyltin contamination still evident in Chile after TBT global ban. *Sci Total Environ* 566-567:446-453. doi:<https://doi.org/10.1016/j.scitotenv.2016.05.039>
- Bauer RT (2006) Same sexual system but variable sociobiology: evolution of protandric simultaneous hermaphroditism in *Lysmata* shrimps. *Integr Comp Biol* 46 (4):430-438. doi:[10.1093/icb/icj036](https://doi.org/10.1093/icb/icj036)
- Bell G (1982) *The Masterpiece of Nature: The Evolution and Genetics of Sexuality*. University of California Press, Berkeley
- Bettin C, Oehlmann J, Stroben E (1996) TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. *Helgoländer Meeresun* 50 (3):299-317. doi:[10.1007/bf02367105](https://doi.org/10.1007/bf02367105)
- Beukeboom LW, Perrin N (2014) *The evolution of sex determination*. Oxford University Press, USA, New York, NY
- Blaber SJ (1970) The occurrence of a penis-like outgrowth behind the right tentacle in spent females of *Nucella lapillus* (L.). *J Molluscan Stud* 39 (2-3):231-233
- Brante A, Quiñones A, Silva F (2016) The relationship between sex change and reproductive success in a protandric marine gastropod. *Sci Rep* 6 (29439):1-10. doi:[10.1038/srep29439](https://doi.org/10.1038/srep29439)
- Breton S, Capt C, Guerra D, Stewart D (2017) Sex determining mechanisms in bivalves. Preprints 2017060127. doi:[10.20944/preprints201706.0127.v1](https://doi.org/10.20944/preprints201706.0127.v1)
- Broquet T, Barranger A, Billard E, Bestin A, Berger R, Honnaert G, Viard F (2015) The size advantage model of sex allocation in the protandrous sex-changer *Crepidula fornicata*: Role of the mating system, sperm storage, and male mobility. *Am Nat* 186 (3):404-420. doi:[10.1086/682361](https://doi.org/10.1086/682361)
- Bui LT, Ivers NA, Ragsdale EJ (2018) A sulfotransferase dosage-dependently regulates mouthpart polyphenism in the nematode *Pristionchus pacificus*. *Nat Commun* 9 (1):4119. doi:[10.1038/s41467-018-05612-8](https://doi.org/10.1038/s41467-018-05612-8)
- Bull J, Vogt R (1979) Temperature-dependent sex determination in turtles. *Science* 206 (4423):1186-1188. doi:[10.1126/science.505003](https://doi.org/10.1126/science.505003)
- Bull JJ (1983) *Evolution of sex determining mechanisms*. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA
- Burtis KC, Baker BS (1989) *Drosophila* doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell* 56 (6):997-1010. doi:[https://doi.org/10.1016/0092-8674\(89\)90633-8](https://doi.org/10.1016/0092-8674(89)90633-8)
- Buston P (2003) Social hierarchies: Size and growth modification in clownfish. *Nature* 424 (6945):145-146

- Cahill AE, Juman AR, Pellman-Isaacs A, Bruno WT (2015) Physical and chemical interactions with conspecifics mediate sex change in a protandrous gastropod *Crepidula fornicata*. *Biol Bull* 229 (3):276-281. doi:10.1086/BBLv229n3p276
- Capel B (2017) Vertebrate sex determination: evolutionary plasticity of a fundamental switch. *Nat Rev Genet* 18:675. doi:10.1038/nrg.2017.60
- Carrillo-Baltodano A, Collin R (2015) *Crepidula* slipper limpets alter sex change in response to physical contact with conspecifics. *Biol Bull* 229 (3):232-242. doi:10.1086/BBLv229n3p232
- Castro LFC, Lima D, Machado A, Melo C, Hiromori Y, Nishikawa J, Nakanishi T, Reis-Henriques MA, Santos MM (2007) Imposex induction is mediated through the Retinoid X Receptor signalling pathway in the neogastropod *Nucella lapillus*. *Aquat Toxicol* 85 (1):57-66. doi:http://dx.doi.org/10.1016/j.aquatox.2007.07.016
- Chaparro OR, Bahamondes-Rojas I, Vergara AM, Rivera AA (1998) Histological characteristics of the foot and locomotory activity of *Crepidula dilatata* Lamarck (Gastropoda : Calyptraeidae) in relation to sex changes. *J Exp Mar Biol Ecol* 223 (1):77-91
- Chaparro OR, Pereda SV, Bahamondes-Rojas I (2001) Effects of protandric sex change on radula, pedal morphology, and mobility in *Crepidula fecunda* (Gastropoda : Calyptraeidae). *New Zeal J Mar Fresh* 35 (5):881-890
- Charnov EL (1982) The theory of sex allocation. Monographs in Population Biology, vol 18. Princeton University Press, Princeton, NJ
- Cho S-J, Vallès Y, Weisblat DA (2013) Differential expression of conserved germ line markers and delayed segregation of male and female primordial germ cells in a hermaphrodite, the leech *Helobdella*. *Mol Biol Evol* 31 (2):341-354. doi:10.1093/molbev/mst201
- Chong T, Collins JJ, Brubacher JL, Zarkower D, Newmark PA (2013) A sex-specific transcription factor controls male identity in a simultaneous hermaphrodite. *Nat Commun* 4:1814. doi:10.1038/ncomms2811
- Coe WR (1935) Sexual phases in prosobranch mollusks of the genus *Crepidula*. *Science* 81 (2110):570-572
- Coe WR (1936) Sexual phases in *Crepidula*. *J Exp Zool* 72 (3):455-477. doi:10.1002/jez.1400720306
- Coe WR (1938a) Conditions influencing change of sex in mollusks of the genus *Crepidula*. *J Exp Zool* 77 (3):401-424
- Coe WR (1938b) Influence of association on the sexual phases of gastropods having protandric consecutive sexuality. *Biol Bull* 75 (2):274-285
- Coe WR (1942) Influence of natural and experimental conditions in determining shape of shell and rate of growth in gastropods of the genus *Crepidula*. *J Morphol* 71 (1):35-51
- Coe WR (1943) Sexual Differentiation in Mollusks. I. Pelecypods. *Q Rev Biol* 18 (2):154-164. doi:10.1086/394673
- Collin R (1995) Sex, size, and position: A test of models predicting size at sex change in the protandrous gastropod *Crepidula fornicata*. *Am Nat* 146 (6):815-831
- Collin R (2000) Sex change, reproduction, and development of *Crepidula adunca* and *Crepidula lingulata* (Gastropoda : Calyptraeidae). *Veliger* 43 (1):24-33
- Collin R (2006) Sex ratio, life-history invariants, and patterns of sex change in a family of protandrous gastropods. *Evolution* 60 (4):735-745
- Collin R (2013) Phylogenetic patterns and phenotypic plasticity of molluscan sexual systems. *Integr Comp Biol* 53 (4):723-735. doi:10.1093/icb/ict076
- Collin R, McLellan M, Gruber K, Bailey-Jourdain C (2005) Effects of conspecific associations on size at sex change in three species of calyptraeid gastropods. *Mar Ecol-Prog Ser* 293:89-97
- Conklin EG (1897) The embryology of *Crepidula*: A contribution to the cell lineage and early development of some marine gasteropods. *J Morphol* 13 (1):1-226
- Conklin EG (1902) Karyokinesis and cytokinesis in the maturation, fertilization and cleavage of *Crepidula* and other Gasteropoda.

- Dannenberg LC, Seaver EC (2018) Regeneration of the germline in the annelid *Capitella teleta*. *Dev Biol* 440 (2):74-87. doi:<https://doi.org/10.1016/j.ydbio.2018.05.004>
- Dill KK, Seaver EC (2008) Vasa and nanos are coexpressed in somatic and germ line tissue from early embryonic cleavage stages through adulthood in the polychaete *Capitella sp. I*. *Dev Genes Evol* 218 (9):453-463
- Dupont L, Richard J, Paulet YM, Thouzeau G, Viard F (2006) Gregariousness and protandry promote reproductive insurance in the invasive gastropod *Crepidula fornicata*: evidence from assignment of larval paternity. *Mol Ecol* 15 (10):3009-3021. doi:[10.1111/j.1365-294X.2006.02988.x](https://doi.org/10.1111/j.1365-294X.2006.02988.x)
- Eernisse DJ (1988) Reproductive patterns in six species of *Lepidochitona* (Mollusca: Polyplacophora) from the Pacific Coast of North America. *Biol Bull* 174 (3):287-302. doi:[10.2307/1541955](https://doi.org/10.2307/1541955)
- Evans SM, Leksono T, McKinnell PD (1995) Tributyltin pollution: A diminishing problem following legislation limiting the use of TBT-based anti-fouling paints. *Mar Pollut Bull* 30 (1):14-21. doi:[https://doi.org/10.1016/0025-326X\(94\)00181-8](https://doi.org/10.1016/0025-326X(94)00181-8)
- Ewen-Campen B, Schwager EE, Extavour CGM (2010) The molecular machinery of germ line specification. *Mol Reprod Dev* 77 (1):3-18. doi:[doi:doi:10.1002/mrd.21091](https://doi.org/10.1002/mrd.21091)
- Extavour CG, Akam M (2003) Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development* 130 (24):5869-5884. doi:[10.1242/dev.00804](https://doi.org/10.1242/dev.00804)
- Fabioux C, Huvet A, Lelong C, Robert R, Pouvreau S, Daniel JY, Minguant C, Le Penec M (2004) Oyster vasa-like gene as a marker of the germline cell development in *Crassostrea gigas*. *Biochem Biophys Res Commun* 320 (2):592-598. doi:[10.1016/j.bbrc.2004.06.009](https://doi.org/10.1016/j.bbrc.2004.06.009)
- Féral C, Le Gall S (1983) The influence of a pollutant factor (tributyltin) on the neuroendocrine mechanisms responsible for the occurrence of a penis in the females of *Ocenebra erinacea*. International minisymposium on molluscan endocrinology. North Holland Publishing, Amsterdam
- Féral C, Le Gall S, Martin MC, Lengronne C (1987) The neuroendocrine mechanism responsible for sexual inversion of the gonad in the protandrous hermaphroditic mollusk, *Crepidula fornicata* L. *Gen Comp Endocr* 65 (3):432-438
- Fischer AHL, Arendt D (2013) Mesoteloblast-like mesodermal stem cells in the polychaete annelid *Platynereis dumerilii* (Nereididae). *J Exp Zool B Mol Dev Evol* 320 (2):94-104. doi:[10.1002/jez.b.22486](https://doi.org/10.1002/jez.b.22486)
- García-Souto D, Alonso-Rubido S, Costa D, Eirín-López JM, Rolán-Álvarez E, Faria R, Galindo J, Pasantes JJ (2018) Karyotype characterization of nine periwinkle species (Gastropoda, Littorinidae). *Genes* 9 (11):517
- Ghiselin MT (1969) The evolution of hermaphroditism among animals. *Q Rev Biol* 44 (2):189-208. doi:[10.1086/406066](https://doi.org/10.1086/406066)
- Giani VC, Yamaguchi E, Boyle MJ, Seaver EC (2011) Somatic and germline expression of piwi during development and regeneration in the marine polychaete annelid *Capitella teleta*. *EvoDevo* 2 (1):10. doi:[10.1186/2041-9139-2-10](https://doi.org/10.1186/2041-9139-2-10)
- Gibbs PE, Bryan GW, Pascoe PL, Burt GR (1987) The use of the dogwhelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *J Mar Biol Assoc UK* 67 (3):507-523. doi:[10.1017/S0025315400027260](https://doi.org/10.1017/S0025315400027260)
- Gline SE, Nakamoto A, Cho S-J, Chi C, Weisblat DA (2011) Lineage analysis of micromere 4d, a super-phylogenetic cell for Lophotrochozoa, in the leech *Helobdella* and the slugworm *Tubifex*. *Dev Biol* 353 (1):120-133. doi:<https://doi.org/10.1016/j.ydbio.2011.01.031>
- Goldberg ED (1986) TBT: An Environmental Dilemma. *Environment: Science and Policy for Sustainable Development* 28 (8):17-44. doi:[10.1080/00139157.1986.9928814](https://doi.org/10.1080/00139157.1986.9928814)
- Gooding MP, Wilson VS, Folmar LC, Marcovich DT, LeBlanc GA (2003) The biocide tributyltin reduces the accumulation of testosterone as fatty acid esters in the mud snail (*Ilyanassa obsoleta*). *Environ Health Persp* 111 (4):426-430. doi:[doi:doi:10.1289/ehp.5779](https://doi.org/10.1289/ehp.5779)
- Gould H (1952) Studies on sex in the hermaphroditic mollusk *Crepidula plana*: IV. Internal and external factors influencing growth and sex development. *J Exp Zool* 119:93-163. doi:[10.1002/jez.1401190107](https://doi.org/10.1002/jez.1401190107)

- Gould HN (1917a) Studies on sex in the hermaphrodite mollusc *Crepidula plana* I. History of the sexual cycle. *J Exp Zool* 23 (1):1-69
- Gould HN (1917b) Studies on sex in the hermaphrodite mollusk *Crepidula plana*. II. Influence of the environment on sex. *J exp Zool* 23 (1):225-250
- Gould HN (1919) Studies on sex in the hermaphrodite mollusc *Crepidula plana*. III. Transference of the male-producing stimulus through sea-water. *J exp Zool* 29:113-120
- Guellart J, Calvo M, Acevedo I, Templado J (2013) Two-way sex change in the endangered limpet *Patella ferruginea* (Mollusca, Gastropoda). *Invertebr Reprod Dev* 57 (3):247-253
- Guo X, Hedgecock D, Hershberger WK, Cooper K, Allen SKJ (1998) Genetic determinants of protandric sex in the Pacific oyster, *Crassostrea gigas* Thunberg. *Evolution* 52 (2):394-402. doi:doi:10.1111/j.1558-5646.1998.tb01640.x
- Haag ES, Doty AV (2005) Sex Determination across Evolution: Connecting the Dots. *PLOS Biology* 3 (1):e21. doi:10.1371/journal.pbio.0030021
- Handberg-Thorsager M, Gutierrez-Mazariegos J, Arold ST, Kumar Nadendla E, Bertucci PY, Germain P, Tomançak P, Pierzchalski K, Jones JW, Albalat R, Kane MA, Bourguet W, Laudet V, Arendt D, Schubert M (2018) The ancestral retinoic acid receptor was a low-affinity sensor triggering neuronal differentiation. *Science Advances* 4 (2):eaao1261. doi:10.1126/sciadv.aao1261
- Haszprunar G, Schaefer K (1996) Anatomy and phylogenetic significance of *Micropilina arntzi* (Mollusca, Monoplacophora, Micropilinidae Fam. Nov.). *Acta Zoologica* 77 (4):315-334. doi:doi:10.1111/j.1463-6395.1996.tb01276.x
- Hedgecock D, Hedrick PW (2010) Sex Determination: Genetic Models for Oysters. *J Hered* 101 (5):602-611. doi:10.1093/jhered/esq065
- Heller J (1993) Hermaphroditism in molluscs. *Biol J Linn Soc* 48 (1):19-42. doi:10.1111/j.1095-8312.1993.tb00874.x
- Henry JQ (2014) Spiralian model systems. *Int J Dev Biol* 58:389-401
- Henry JQ, Lesoway MP, Perry KJ, Osborne CC, Shankland M, Lyons DC (2017) Beyond the sea: *Crepidula atrasolea* as a spiralian model system. *Int J Dev Biol* 61 (8-9):479-493. doi:10.1387/ijdb.170110jh
- Henry JQ, Lyons DC (2016) Molluscan models: *Crepidula fornicata*. *Curr Opin Gen Dev* 39:138-148. doi:http://dx.doi.org/10.1016/j.gde.2016.05.021
- Hildreth PE (1965) Doublesex, a recessive gene that transforms both males and females of *Drosophila* into intersexes. *Genetics* 51 (4):659
- Hoagland K (1978) Protandry and the evolution of environmentally-mediated sex change: A study of the Mollusca. *Malacologia* 17 (2):365-391
- Hobday AJ, Riser KL (1998) The role of movement by *Crepidula norrisiarum* Williamson in altering reproductive potential. *J Exp Mar Biol Ecol* 225 (1):139-154. doi:https://doi.org/10.1016/S0022-0981(97)00218-9
- Horiguchi T (2017) Mode of Action of Organotins to Induce the Development of Imposex in Gastropods, Focusing on Steroid and the Retinoid X Receptor Activation Hypotheses. In: Horiguchi T (ed) *Biological Effects by Organotins*. Springer Japan, Tokyo, pp 199-219. doi:10.1007/978-4-431-56451-5\_9
- Hoving HJT, Roeleveld MAC, Lipinski MR, Videler JJ (2006) Nidamental glands in males of the oceanic squid *Ancistrocheirus lesueurii* (Cephalopoda: Ancistrocheiridae) – sex change or intersexuality? *J Zool* 269 (3):341-348. doi:doi:10.1111/j.1469-7998.2006.00093.x
- Ishiki H (1936) Sex-changes in Japanese slipper limpets, *Crepidula aculeata* and *Crepidula walshi*. *Journal of Science of Hiroshima University, Series B* 4:91-99
- Ishiki H (1938) Histological studies on the sexual organs during sex changes of *Crepidula aculeata* and *Crepidula walshi*. *Journal of Science of Hiroshima University, Series B* 6:103-113
- Jarne P, Auld JR (2006) Animals mix it up too: The distribution of self-fertilization among hermaphroditic animals. *Evolution* 60 (9):1816-1824. doi:doi:10.1111/j.0014-3820.2006.tb00525.x

- Jenner MG (1979) Pseudohermaphroditism in *Ilyanassa obsoleta* (Mollusca: Neogastropoda). *Science* 205 (4413):1407. doi:10.1126/science.472758
- Kang D, Pilon M, Weisblat DA (2002) Maternal and zygotic expression of a nanos-class gene in the leech *Helobdella robusta*: Primordial germ cells arise from segmental mesoderm. *Dev Biol* 245 (1):28-41. doi:https://doi.org/10.1006/dbio.2002.0615
- Kato Y, Nakamoto A, Shiomi I, Nakao H, Shimizu T (2013) Primordial germ cells in an oligochaete annelid are specified according to the birth rank order in the mesodermal teloblast lineage. *Dev Biol* 379 (2):246-257. doi:https://doi.org/10.1016/j.ydbio.2013.04.028
- Kocot KM, Cannon JT, Todt C, Citarella MR, Kohn AB, Meyer A, Santos SR, Schander C, Moroz LL, Lieb B, Halanych KM (2011) Phylogenomics reveals deep molluscan relationships. *Nature* 477 (7365):452-456. doi:10.1038/nature10382
- Koene JM (2017) Sex determination and gender expression: Reproductive investment in snails. *Mol Reprod Dev* 84 (2):132-143. doi:10.1002/mrd.22662
- Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for Sry. *Nature* 351 (6322):117-121. doi:10.1038/351117a0
- Kopp A (2012) Dmrt genes in the development and evolution of sexual dimorphism. *Trends Genet* 28 (4):175-184. doi:https://doi.org/10.1016/j.tig.2012.02.002
- Kranz AM, Tollenaere A, Norris BJ, Degnan BM, Degnan SM (2010) Identifying the germline in an equally cleaving mollusc: Vasa and Nanos expression during embryonic and larval development of the vetigastropod *Haliotis asinina*. *J Exp Zool B Mol Dev Evol* 314B (4):267-279
- Le Cam S, Pechenik JA, Cagnon M, Viard F (2009) Fast versus slow larval growth in an invasive marine mollusc: does paternity matter? *J Hered* 100 (4):455-464. doi:10.1093/jhered/esp007
- Le Gall S (1981) Etude expérimentale du facteur morphogénétique contrôlant la différenciation du tractus génital mâle externe chez *Crepidula fornicata* L. (Mollusque hermaphrodite protandre). *Gen Comp Endocr* 43 (1):51-62. doi:http://dx.doi.org/10.1016/0016-6480(81)90031-9
- Le Gall S, Feral C (1982) Experimental study of in vitro development of the male genital tract in *Crepidula fornicata* L (Protandric Hermaphrodite Mesogastropod). *Int J Invertebr Reprod* 5 (1):31-42
- Le Gall S, Feral C, Lengronne C, Porchet M (1987) Partial purification of the neuroendocrine mitogenic factor in the mollusk *Crepidula fornicata* L. *Comp Biochem Phys B* 86 (2):393-396
- Le Gall S, Streiff W (1975) Protandric Hermaphroditism in Prosobranch Gastropods. In: Reinboth R (ed) *Intersexuality in the Animal Kingdom*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 170-178. doi:10.1007/978-3-642-66069-6\_17
- le Maire A, Grimaldi M, Roecklin D, Dagnino S, Vivat - Hannah V, Balaguer P, Bourguet W (2009) Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. *EMBO Rep* 10 (4):367-373. doi:10.1038/embor.2009.8
- Li Y, Zhang L, Sun Y, Ma X, Wang J, Li R, Zhang M, Wang S, Hu X, Bao Z (2016) Transcriptome sequencing and comparative analysis of ovary and testis identifies potential key sex-related genes and pathways in scallop *Patinopecten yessoensis*. *Mar Biotechnol* 18 (4):453-465. doi:10.1007/s10126-016-9706-8
- Libertini A, Vitturi R, Gregorini A, Colomba M (2009) Karyotypes, banding patterns and nuclear DNA content in *Crepidula unguiformis* Lamarck, 1822, and *Naticarius stercusmuscarum* Gmelin, 1791 (Mollusca, Caenogastropoda) *Malacologia* 51 (1):111-118
- Lima D, Reis-Henriques MA, Silva R, Santos AI, Filipe C, Castro L, Santos MM (2011) Tributyltin-induced imposex in marine gastropods involves tissue-specific modulation of the Retinoid X Receptor. *Aquat Toxicol* 101 (1):221-227. doi:http://dx.doi.org/10.1016/j.aquatox.2010.09.022
- Lindberg DR, Wright W (1985) Patterns of sex change of the protandric patellacean limpet *Lottia gigantea* (Mollusca: Gastropoda). *Veliger* 27 (3):261-265

- Lyons DC, Perry KJ, Lesoway MP, Henry JQ (2012) Cleavage pattern and fate map of the mesentoblast, 4d, in the gastropod *Crepidula*: a hallmark of spiralian development. *EvoDevo* 3 (1):1-26
- Malaquin A (1925) La ségrégation, au cours de l'ontogenèse, de deux cellules sexuelles primordiales, souches de la lignée germinale, chez *Salmacina dysteri* (Huxley). *CR Acad Sci D Nat* 180:324-327
- Mérot C, Collin R (2012a) Effects of food availability on sex change in two species of *Crepidula* (Gastropoda: Calyptraeidae). *Mar Ecol Prog Ser* 449:173-181
- Mérot C, Collin R (2012b) Effects of stress on sex change in *Crepidula cf. marginalis* (Gastropoda: Calyptraeidae). *J Exp Mar Biol Ecol* 416-417:68-71. doi:<https://doi.org/10.1016/j.jembe.2012.02.012>
- Meyer NP, Boyle MJ, Martindale MQ, Seaver EC (2010) A comprehensive fate map by intracellular injection of identified blastomeres in the marine polychaete *Capitella teleta*. *EvoDevo* 1 (1):8. doi:10.1186/2041-9139-1-8
- Miyagawa S, Yatsu R, Iguchi T (2018) Environmental control of sex determination and differentiation in reptiles. In: Kobayashi K, Kitano T, Iwao Y, Kondo M (eds) *Reproductive and Developmental Strategies: The Continuity of Life*. Springer Japan, Tokyo, pp 367-390. doi:10.1007/978-4-431-56609-0\_18
- Munday PL, Buston PM, Warner RR (2006) Diversity and flexibility of sex-change strategies in animals. *Trends Ecol Evol* 21 (2):89-95. doi:<https://doi.org/10.1016/j.tree.2005.10.020>
- Nakadera Y, Koene JM (2013) Reproductive strategies in hermaphroditic gastropods: conceptual and empirical approaches. *Can J Zool* 91 (6):367-381. doi:10.1139/cjz-2012-0272
- Nishikawa J-i, Mamiya S, Kanayama T, Nishikawa T, Shiraiishi F, Horiguchi T (2004) Involvement of the Retinoid X Receptor in the development of imposex caused by organotins in gastropods. *Environ Sci Technol* 38 (23):6271-6276. doi:10.1021/es049593u
- Oberdörster E, McClellan-Green P (2000) The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta*. *Peptides* 21 (9):1323-1330. doi:[https://doi.org/10.1016/S0196-9781\(00\)00274-6](https://doi.org/10.1016/S0196-9781(00)00274-6)
- Oberdörster E, McClellan-Green P (2002) Mechanisms of imposex induction in the mud snail, *Ilyanassa obsoleta*: TBT as a neurotoxin and aromatase inhibitor. *Mar Environ Res* 54 (3-5):715-718. doi:[http://dx.doi.org/10.1016/S0141-1136\(02\)00118-6](http://dx.doi.org/10.1016/S0141-1136(02)00118-6)
- Oliver B, Clough E (2012) Genomics of sex determination in *Drosophila*. *Brief Funct Genomics* 11 (5):387-394. doi:10.1093/bfgp/els019
- Orton JH (1909) On the occurrence of protandric hermaphroditism in the Mollusc *Crepidula fornicata*. *P Roy Soc Lond B Bio* 81 (550):468-484
- Oyama A, Shimizu T (2007) Transient occurrence of vasa-expressing cells in nongenital segments during embryonic development in the oligochaete annelid *Tubifex tubifex*. *Dev Genes Evol* 217 (10):675-690. doi:10.1007/s00427-007-0180-1
- Özpolat BD, Handberg-Thorsager M, Vervoort M, Balavoine G (2017) Cell lineage and cell cycling analyses of the 4d micromere using live imaging in the marine annelid *Platynereis dumerilii*. *eLife* 6
- Pascoal S, Carvalho G, Vasieva O, Hughes R, Cossins A, Fang Y, Ashelford K, Olohan L, Barroso C, Mendo S, Creer S (2013) Transcriptomics and in vivo tests reveal novel mechanisms underlying endocrine disruption in an ecological sentinel, *Nucella lapillus*. *Mol Ecol* 22 (6):1589-1608. doi:10.1111/mec.12137
- Picard MA-L, Cosseau C, Mouahid G, Duval D, Grunau C, Toulza È, Allienne J-F, Boissier J (2015) The roles of Dmrt (Double sex/Male-abnormal-3 Related Transcription factor) genes in sex determination and differentiation mechanisms: Ubiquity and diversity across the animal kingdom. *CR Biol* 338 (7):451-462. doi:<https://doi.org/10.1016/j.crv.2015.04.010>
- Policansky D (1982) Sex change in plants and animals. *Annual Review of Ecology and Systematics* 13 (1):471-495

- Ponder WF, Lindberg DR (1997) Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. *Zool J Linn Soc-Lond* 119:83-265
- Proestou DA, Goldsmith MR, Twombly S (2008) Patterns of male reproductive success in *Crepidula fornicata* provide new insight for sex allocation and optimal sex change. *Biol Bull* 214 (2):192-200
- Rabinowitz JS, Chan XY, Kingsley EP, Duan Y, Lambert JD (2008) Nanos is required in somatic blast cell lineages in the posterior of a mollusk embryo. *Curr Biol* 18 (5):331-336. doi:10.1016/j.cub.2008.01.055
- Ragsdale EJ, Muller MR, Rodelsperger C, Sommer RJ (2013) A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* 155 (4):922-933. doi:10.1016/j.cell.2013.09.054
- Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D (2000) Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* 14 (20):2587-2595
- Raymond CS, Shamu CE, Shen MM, Seifert KJ, Hirsch B, Hodgkin J, Zarkower D (1998) Evidence for evolutionary conservation of sex-determining genes. *Nature* 391:691. doi:10.1038/35618
- Rebscher N (2014) Establishing the germline in spiralian embryos. *Int J Dev Biol* 58:403-411
- Rebscher N, Lidke AK, Ackermann CF (2012) Hidden in the crowd: primordial germ cells and somatic stem cells in the mesodermal posterior growth zone of the polychaete *Platynereis dumerillii* are two distinct cell populations. *EvoDevo* 3 (1):9. doi:10.1186/2041-9139-3-9
- Rebscher N, Zelada-González F, Banisch TU, Raible F, Arendt D (2007) Vasa unveils a common origin of germ cells and of somatic stem cells from the posterior growth zone in the polychaete *Platynereis dumerillii*. *Dev Biol* 306 (2):599-611. doi:https://doi.org/10.1016/j.ydbio.2007.03.521
- Riddle D, Blumenthal T, Meyer B, Priess J (1997) Introduction to *C. elegans*. In: Riddle D, Blumenthal T, Meyer B, Priess J (eds) *C. elegans* II. 2nd edition edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY),
- Robertson DR (1972) Social control of sex reversal in a coral-reef fish. *Science* 177 (4053):1007-1009. doi:10.1126/science.177.4053.1007
- Ronis MJJ, Mason AZ (1996) The metabolism of testosterone by the periwinkle (*Littorina littorea*) in vitro and in vivo: Effects of tributyl tin. *Mar Environ Res* 42 (1):161-166. doi:https://doi.org/10.1016/0141-1136(95)00069-0
- Schøyen M, Green NW, Hjermann DØ, Tveiten L, Beylich B, Øxnevad S, Beyer J (2019) Levels and trends of tributyltin (TBT) and imposex in dogwhelk (*Nucella lapillus*) along the Norwegian coastline from 1991 to 2017. *Mar Environ Res* 144:1-8. doi:https://doi.org/10.1016/j.marenvres.2018.11.011
- Scott AP (2018) Is there any value in measuring vertebrate steroids in invertebrates? *Gen Comp Endocr* 265:77-82. doi:https://doi.org/10.1016/j.ygcen.2018.04.005
- Smith BS (1971) Sexuality in the American mud snail, *Nassarius obsoletus* Say. *J Molluscan Stud* 39 (5):377-378
- Smith BS (1981) Tributyltin compounds induce male characteristics on female mud snails *Nassarius obsoletus* = *Ilyanassa obsoleta*. *J Appl Toxicol* 1 (3):141-144. doi:doi:10.1002/jat.2550010302
- Smith SA, Wilson NG, Goetz FE, Feehery C, Andrade SC, Rouse GW, Giribet G, Dunn CW (2011) Resolving the evolutionary relationships of molluscs with phylogenomic tools. *Nature* 480 (7377):364-367. doi:10.1038/nature10526
- Sousa ACA, Pastorinho MR, Takahashi S, Tanabe S (2014) History on organotin compounds, from snails to humans. *Environ Chem Lett* 12 (1):117-137. doi:10.1007/s10311-013-0449-8
- Streiff W Endocrinological study of determinism of sexual cycle in a protandric hermaphrodite mollusc *Calyptrea sinensis* (L.). In: *Annales d'endocrinologie*, 1966. vol 3 pp 385-&
- Streiff W Endocrinologic study of determinism of the sexual cycle in a protandrous hermaphroditic mollusk, *Calyptrea sinensis* (L.). II. Demonstration by in vitro cultivation of hormonal factors

- conditioning the development of the female tract. In: *Annales d'endocrinologie*, 1967. vol 4. p 461
- Stroben E, Oehlmann J, Fioroni P (1992) The morphological expression of imposex in *Hinia reticulata* (Gastropoda: Buccinidae): a potential indicator of tributultin pollution. *Mar Biol* 113 (4):625-636. doi:10.1007/bf00349706
- Swartz SZ, Chan XY, Lambert JD (2008) Localization of Vasa mRNA during early cleavage of the snail *Ilyanassa*. *Dev Genes Evol* 218 (2):107-113. doi:10.1007/s00427-008-0203-6
- Teaniniuraitemoana V, Huvet A, Levy P, Klopp C, Lhuillier E, Gaertner-Mazouni N, Gueguen Y, Le Moullac G (2014) Gonad transcriptome analysis of pearl oyster *Pinctada margaritifera*: identification of potential sex differentiation and sex determining genes. *BMC Genomics* 15 (1):491. doi:10.1186/1471-2164-15-491
- Thiriot-Quiévreux C (2003) Advances in chromosomal studies of gastropod molluscs. *J Molluscan Stud* 69 (3):187-202. doi:10.1093/mollus/69.3.187
- Warner RR (1975) The adaptive significance of sequential hermaphroditism in animals. *Am Nat* 109 (965):61-82. doi:10.1086/282974
- Warner RR (1988) Sex change and the size-advantage model. *Trends Ecol Evol* 3 (6):133-136. doi:https://doi.org/10.1016/0169-5347(88)90176-0
- Warner RR, Fitch DL, Standish JD (1996) Social control of sex change in the shelf limpet, *Crepidula norrisiarum*: size-specific responses to local group composition. *J Exp Mar Biol Ecol* 204 (1):155-167
- Warner RR, Robertson DR, Leigh EG (1975) Sex Change and Sexual Selection. *Science* 190 (4215):633-638
- Wells MJ, Wells J (1959) Hormonal control of sexual maturity in *Octopus*. *J Exp Biol* 36 (1):1-33
- Woods FH (1931) History of the germ cells in *Sphaerium striatinum* (Lam.). *J Morphol* 51 (2):545-595. doi:doi:10.1002/jmor.1050510208
- Woods FH (1932) Keimbahn determinants and continuity of the germ cells in *Sphaerium striatinum* (Lam.). *J Morphol* 53 (2):345-365. doi:doi:10.1002/jmor.1050530207
- Wright WG (1988) Sex change in the Mollusca. *Trends Ecol Evol* 3 (6):137-140. doi:https://doi.org/10.1016/0169-5347(88)90177-2
- Xu R, Li Q, Yu H, Kong L (2018) Oocyte maturation and origin of the germline as revealed by the expression of *Nanos*-like in the Pacific oyster *Crassostrea gigas*. *Gene* 663:41-50. doi:https://doi.org/10.1016/j.gene.2018.04.021
- Yue C, Li Q, Yu H (2018) Gonad transcriptome analysis of the Pacific oyster *Crassostrea gigas* identifies potential genes regulating the sex determination and differentiation process. *Mar Biotechnol* 20 (2):206-219. doi:10.1007/s10126-018-9798-4
- Yusa Y (2007) Causes of variation in sex ratio and modes of sex determination in the Mollusca - an overview. *Am Malacol Bull* 23 (1-2):89-98
- Yusa Y, Kumagai N (2018) Evidence of oligogenic sex determination in the apple snail *Pomacea canaliculata*. *Genetica* 146 (3):265-275. doi:10.1007/s10709-018-0017-z
- Zhang N, Xu F, Guo X (2014) Genomic analysis of the Pacific Oyster (*Crassostrea gigas*) reveals possible conservation of vertebrate sex determination in a Mollusc. *G3-Genes Genom Genet* 4 (11):2207-2217. doi:10.1534/g3.114.013904

**Figure 1. Phylogram of molluscan relationships.** After Collin 2013. Based on Kocot et al. 2011, and Smith et al. 2011.

**Figure 2. Generalized life cycle and external anatomy of calyptraeid gastropods.** A. Life cycle of sequentially hermaphroditic slipper snails. Females brood embryos, which in the case of direct developers, complete development to juvenile stages within the capsule. Embryos emerge either as juvenile snails (direct developers) or veliger larvae (indirect developers, not shown), which complete their development in the water column prior to settlement and metamorphosis. Juvenile snails first mature into males, producing sperm and grow an external penis. Males eventually resorb the penis, convert to oocyte production and produce female external genitalia. Timing of sexual transitions typically depends on environment. Relevant factors include age, nutrition, size, and presence/absence and sex of conspecifics. B. Ventral view of *Crepidula atrasolea* female. C. Ventral view of smaller *C. atrasolea* male. fg, food groove; fgp, female genital papilla; ft, foot; gd, gonoduct; in, intestine; lt, left tentacle; m, mouth; ma, mantle; oc, ocellus; os, ospharadium; p, penis; pp, propodium; rt, right tentacle; sh, shell. Scale bars represent 1mm.

**Figure 3. Social behavior and sexual dimorphism in calyptraeid gastropods.** A. Gregarious stacking as exemplified by *C. fornicata*. This stack contains at least 10 animals, anchored by an empty shell (0). The largest animals at the bottom of the stack are female (1, etc.), and smaller animals at the top of the stack are males (9, 10). B. Substrate-restricted animals such as *C. convexa* are typically found singly or as breeding pairs. In this case, three animals form a mating group, with a large female at the bottom (1) and two smaller males above (2, 3). C. Where substrate is not limiting, calyptraeids can cover all available hard substrate, as seen in this group of mainly *C. lessoni*. Note the presence of smaller animals (likely males, m) on top of much larger females (f). Scale bars in A and C represent 1 cm, 0.5 cm in B.

**Figure 4. Origins of primordial germ cells from the 4d cell lineage of selected molluscs and annelids, where sublineages of the teleoblasts have been reported.** A. Cartoon of the 25-cell stage in a generalized embryo of *Crepidula*. The 4d cell is indicated in blue. The relative positions of the four embryonic quadrants (A, B, C, D) are also shown. B. 4d sub-lineage of *C. fornicata* at the birth of the putative primordial germ cells, 2mL<sup>2.2</sup> and 2mR<sup>2.2</sup>. Some sister cells are also labeled including the teleoblasts 4ML and 4MR. Central dashed line indicates the plane of bilateral symmetry. The 4d cell has been injected with a fluorescent lineage tracer at the 25-cell stage (A) to permit visualization of the lineage. C-H. Origins of the primordial germ cells as reported in various spiralian embryos. Diagrams in C-H show only half of the bilaterally symmetric 4d lineages with primordial germ cell lineages represented in light blue, putative primordial germ cells in dark blue, other lineages in grey. Shown here are the gastropod molluscs C. *Crepidula fornicata*, D. *Tritia obsoleta*, the bivalve mollusc E. *Sphaerium striatinum*, the clitellate annelids F. *Helobdella austinensis*, and G. *Tubifex tubifex*, and the polychaete annelid H. *Platynereis dumerilii*. Dashed lines indicate continuation of lineages not detailed here. Cells with white outlines indicate cleavage arrest where reported. 4d cleavage nomenclature follows that used in Lyons et al. 2012. See Table 1 and references therein for further details.

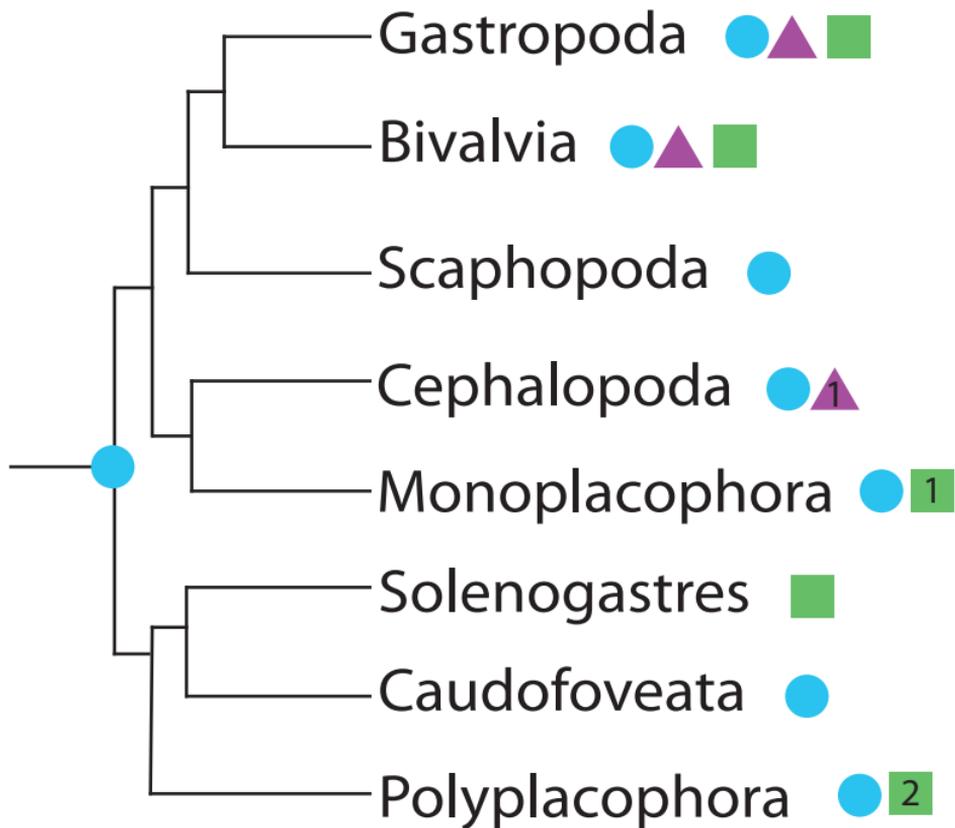
**Figure 5. The effects of tributyltin (TBT) exposure on *C. atrasolea*.** A. Adult females exposed to TBT show imposex, superimposed growth of male characters, most obviously the penis. B. Ventral view showing imposex female of *C. atrasolea* after 8 days of exposure to TBT. Note the presence of both the female genital papilla and a penis on the right side. C. Juvenile snails exposed to TBT initiate penis growth within a few days. TBT can induce accelerated penis development in juveniles immediately after hatching. Typical males grown in the laboratory show penis development after approximately 1 month. D. Ventral view of juvenile snail exposed to tributyltin for 8 days post hatching. Note the growth of the external penis on the right side of the neck. Comparable results are seen in other calyptraeids (not shown). Scale bars represent 1mm.



**TABLE 1. Origins, Specification and Behavior of the Primordial Germ Cells within Spiralian**

Phylum/Class	Species	Embryonic origins (#)	Specification/Behavior	References
<u>Annelida</u>				
Clitellata	<i>Helobdella robusta</i>	4d (16mL/R, 18-23mL/R)	Autonomous	Kang et al., 2002 Cho et al., 2013
	<i>Helobdella austinensis</i>			
	<i>Tubifex tubifex</i>	4d (10mL/R, 11mL/R)	Autonomous	Oyama et al, 2007 Gline et al., 2011 Kato et al., 2013
Polychaeta	<i>Capitella teleta</i>	4d (?)	Autonomous Can be regenerated	Dill and Seaver, 2008 Meyer et al., 2010 Giani et al., 2011 Dannenberg and Seaver, 2018
	<i>Platynereis dumerilii</i>	4d (1mL/R, 2mL/R) (4)	Autonomous Cleavage arrested	Rebscher et al., 2007, 2012 Özpolat et al., 2017
	<i>Salmacina dysteri</i>	4d (1mL/R)	----	Malaquin, 1925
<u>Mollusca</u>				
Bivalva	<i>Crassostrea gigas</i>	4d (ML/R)	Autonomous	Fabioux et al., 2004 Xu et al, 2018
	<i>Sphaerium striatinum</i>	4d (4ML/R, 4mL/R) (4)	Autonomous Cleavage arrested	Woods, 1931, 1932
Gastropoda	<i>Crepidula fornicata</i>	4d (2mL/R <sup>2.2</sup> ) (2)	Autonomous Cleavage arrested	Lyons et al., 2012
	<i>Haliotis asinina</i>	4d (ML/R)	Conditional	Kranz et al., 2010
	<i>Tritia obsoleta</i>	4d (2mL/R <sup>2</sup> or 3ML/R)	Autonomous	Swartz et al., 2008 Rabinowitz et al., 2008

Table modified from Rebscher, 2014. In all cases the germ line arises from the 4d lineage. The 4d sublineages are provided where this is known. Lineage nomenclature is based on that of Lyons et al. (2012). For some cases the initial number (#) of PGCs is provided. In a few cases it has been shown that the PGCs are initially cleavage arrested after they are born. These cells eventually undergo divisions much later during development or in the adult. Cases exhibiting early unequal spiral cleavages with early segregation of the germ line in the 4d lineage are assumed to have autonomous specification. *Haliotis asinina* exhibits early equal spiral cleavages and thus the germ line is argued to be conditionally specified. In some cases it is known that the germ line can be regenerated from other cell lineages, such as in the annelid *Capitella teleta* (Dannenberg and Seaver 2018).



- dioecy (separate sexes)
- ▲ sequential hermaphroditism (protandry and/or reversal)
- simultaneous hermaphroditism

