Retinoids promote penis development in sequentially hermaphroditic snails

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Abstract

Sexual systems are surprisingly diverse, considering the ubiquity of sexual reproduction. Sequential hermaphroditism, the ability of an individual to change sex, has emerged multiple times independently across the animal kingdom. In molluscs, repeated shifts between ancestrally separate sexes and hermaphroditism are generally found at the level of family and above, suggesting recruitment of deeply conserved mechanisms. Despite this, molecular mechanisms of sexual development are poorly known. In molluscs with separate sexes, endocrine disrupting toxins bind the retinoid X receptor (RXR), activating ectopic male development in females, suggesting the retinoid pathway as a candidate controlling sexual transitions in sequential hermaphrodites. We therefore tested the role of retinoic acid signaling in sequentially hermaphroditic *Crepidula* snails, which develop first into males, then change sex, maturing into females. We show that retinoid agonists induce precocious penis growth in juveniles and superimposition of male development in females. Combining RXR antagonists with retinoid agonists significantly reduces penis length in induced juveniles, while similar treatments using retinoic acid receptor (RAR) antagonists increase penis length. Transcripts of both receptors are expressed in the induced penis. Our findings therefore show that retinoid signaling can initiate molluscan male genital development, and regulate penis length. Further, we show that retinoids induce ectopic male development in multiple *Crepidula* species. Species-specific influence of conspecific induction of sexual transitions correlates with responsiveness to retinoids. We propose that retinoid signaling plays a conserved role in molluscan male development, and that shifts in the timing of retinoid signaling may have been important for the origins of sequential hermaphroditism within molluscs.

Introduction

Sexual systems and sex determination mechanisms are highly diverse. While most species have separate sexes (dioecy), approximately 5% are hermaphroditic (monoecy), expressing both sexes in the same individual simultaneously, sequentially, or in some combination thereof (Jarne and Auld, 2006). Hermaphroditism is widely, but unevenly distributed across the animal kingdom. Meanwhile, potential

selective pressures for hermaphroditism are widespread (Charnov, 1982; Ghiselin, 1969; Warner, 1975). Sequential hermaphrodites express both male and female reproductive characters in the same individual at different times, often under environmental control (Lesoway and Henry, 2019). The evolution of sequential hermaphroditism requires not only the activation of developmental modules for both sexes within a single individual, but also regulatory control of the transition from one sex to the other. Understanding the regulation of sexual development in sequential hermaphrodites will provide a useful model for resolving the paradox underlying sexual diversity.

Molluscs have frequently been used as models for the evolution of sex determination, owing to the repeated evolution of hermaphroditism and their unique sexual behaviors and anatomies (Auld and Jarne, 2016; Heller, 1993; Ponder et al., 2020). Separate sexes are thought to be ancestral in molluscs (Lesoway and Henry, 2019; Ponder et al., 2020), and estimates of hermaphroditism are as high as 40% (Heller, 1993; Jarne and Auld, 2006). In many molluscan families, including the calyptraeid gastropods, all members are hermaphroditic, suggesting either that sequential hermaphroditism is deeply conserved, or that developmental constraints play some role (Collin, 2018; Leonard, 1991, 2013). Frequently used as models of early development (Henry et al., 2017; Henry and Lyons, 2016), calyptraeids were first confirmed as sequential hermaphrodites over a century ago (Orton, 1909). All members of this family of sedentary, marine, filter-feeding snails, have internal fertilization and brood their young (Henry et al., 2010). Individuals develop first into males with an external penis, followed by a transitional period of penis resorption, elaboration of female external genitalia, and a shift from spermatogenesis to oogenesis, to become functional females (protandry) (**Fig. 1A**) (Lesoway and Henry, 2019). Sex determination is well studied in the Atlantic slipper snail, *Crepidula fornicata*, which form stacks or chains of animals with large females found at the bottom and smaller males located at the top of the chain (Coe, 1936; Collin, 1995, 2000, 2007; Collin et al., 2005). The timing of sexual transition is environmentally determined by a combination of nutrition, size, and social factors (Coe, 1938; Collin, 1995, 2000). For example, the presence of large females inhibits the transition of males into functional females, and direct physical contact has been shown to mediate social control of sex change (Cahill et al., 2015; Carrillo-

Baltodano and Collin, 2015). Similar interactions are important in most calyptraeids (Coe, 1938, 1942; Collin, 2007; Hoagland, 1978). However, the molecular pathways controlling sexual differentiation and sex change in the calyptraeids are generally unknown.

One promising window into the sexual development of calyptraeids is provided by the disruptive effects of the organotin compound, tributylin (TBT). In the 1970s and 80s, commercially important shell fisheries began to show high levels of mortality, shell abnormalities, and imposex, which is the superimposition of male sexual characters in female animals (Evans et al., 1995; Smith, 1971). Tributyltin, used at the time as an anti-fouling agent, was determined to be the cause of these sexual abnormalities (Smith, 1981; Sousa et al., 2014). The ability of tributyltin to induce imposex in many gastropod species suggests that it acts through a conserved signaling pathway.

Several hypotheses have been proposed to explain the endocrine disrupting effects of tributyltin. The strongest available evidence supports activation of the retinoid pathway via retinoid X receptor (RXR) binding (Horiguchi, 2006, 2017; Lesoway and Henry, 2019; Pascoal et al., 2013). RXR is a member of the nuclear receptor (NR) superfamily (Evans and Mangelsdorf, 2014). In vertebrates, RXR (NR2B) functions as a dimerizing partner with other nuclear receptors (e.g., retinoic acid receptor, RAR, NR1B; peroxisome proliferator-activated receptor, PPAR, NR1C) and other co-factors, initiating downstream transcriptional activity **(Fig. 1B)** (Germain et al., 2006b). Most nuclear receptors are activated via binding to hydrophobic ligands such as retinoic acid, steroid hormones, thyroid hormones, and fatty acids (Mazaira et al., 2018). The primary binding partner of RXR is RAR, which binds to retinoic acid (RA). RA is synthesized from retinol (vitamin A) by a series of enzymatic reactions (**Fig. 1B**). Retinoic acid is transported into the nucleus where it binds RAR-RXR dimers bound to retinoic acid response elements (RAREs) in the genome that regulate downstream activity. Retinoic acid levels are controlled by the cytochrome P450 26 subfamily enzyme (Cyp26), which metabolizes retinoic acid, regulating developmental activity (Rhinn and Dollé, 2012). Retinoic acid has previously been implicated in regulation of genital tubercle formation in mice (Liu et al., 2012; Ogino et al., 2001), and RXR, RAR and other components of the retinoid signaling pathway are highly conserved (Albalat, 2009).

To better understand mechanisms of sexual development in the sequentially hermaphroditic calyptraeids, we used drugs targeting different components of the retinoic acid pathway to test the role of retinoids in the initiation of male genital development. Drugs were used both alone and in combination with tributyltin (**Fig. 1B**). We also visualized mRNA expression of the receptors *RXR* and *RAR*, as well as *Cyp26*, the metabolizing enzyme for retinoic acid, in TBT-exposed juveniles of *C. atrasolea*. We then asked if tributyltin induces penis growth in mature females of four calyptraeid species. Our results demonstrate a role for the retinoic acid pathway in male genital development of these sequential hermaphrodites.

Results

Exposure to agonists of RXR and RAR induce penis growth

To determine the abilities of retinoids to induce penis development, we tested the effects of retinoid agonists on juvenile snails. Juveniles of *C. atrasolea*, exposed to moderate levels of the presumed RXR agonist, TBT (200ng Sn/ml; **Table 1**), show induction of penis growth within a few days. Penis length is significantly increased compared to DMSO controls (Kruskal-Wallis $\chi^2 = 144.43$, df = 9, $p<0.0001$, **Fig. 1C; Fig. 2A-B**). Similarly, juveniles exposed to all-trans retinoic acid (ATRA), which can bind to both RXR and RAR, show significantly increased penis length relative to DMSO controls (**Fig. 1C; Fig. 2C)**. Penis length in ATRA-induced juveniles is increased compared to TBT treatments, although this difference is not statistically significant (**Fig. 1C**). While exposure to the pan-RAR agonist TTNPB significantly increases penis length in juveniles compared to DMSO controls, the effect is less pronounced than treatment with either TBT or ATRA (**Fig. 1C; Fig. 2D**).

Blocking retinoic acid synthesis, RXR reduces the effects of tributyltin

To further examine the effects of retinoic acid on male genital development, juveniles of *C. atrasolea* were treated with antagonists of retinoic acid signaling, targeting different components of the retinoic acid signaling pathway. Animals were treated either with a drug that blocks receptor activity

(pan-RXR antagonist UVI-3003), or with drugs to block retinoic acid synthesis by targeting the enzymes that synthesize retinoic acid, RALDH and RDH (citral), or RALDH alone (disulfiram) (**Fig. 1D**).

Animals exposed to the RXR antagonist UVI-3003 alone show minor but non-significant increases in penis length (**Fig. 1D**). Citral and disulfiram treatments alone do not induce penis growth (data not shown). As expected if tributyltin is an RXR agonist, exposure to both tributyltin and the RXR antagonist UVI-3003 significantly reduces juvenile penis length compared to treatment with tributyltin alone (**Fig. 1D**). Combining tributyltin treatment with either citral or disulfiram, blocking retinoic acid synthesis, significantly reduces penis lengths compared to treatment with tributyltin alone (**Fig. 1D**). Furthermore, combined treatments of tributyltin with citral or UVI-3003 do not significantly increase penis lengths compared to DMSO control treatments (**Fig. 1D**).

Exposure to RAR antagonist increases penis length

In contrast to the experiments described above, treatment of *C. atrasolea* juveniles with the RAR-ß antagonist LE-135 significantly increases penis length compared to DMSO controls (**Fig. 1E**). Penis length in LE-135 treated animals is not significantly different from penis length of animals treated with tributyltin (**Fig. 1E**) or TTNPB (**Fig. 1C**). Combining tributyltin with LE-135 does not reduce penis length compared to treatment with tributyltin alone, but rather shows increased penis lengths compared to animals treated with the RAR agonist TTNPB, and are not statistically different from treatments with ATRA (**Fig. 1C**).

Expression of retinoid receptors, RAR and RXR, and retinoic acid metabolizing enzyme, Cyp26 in TBT induced penis development

Both *RAR* and *RXR* receptor mRNAs are expressed in immature juveniles of *C. atrasolea*. In DMSO treated animals, *RAR* expression is mainly visible in the gill filaments, while *RXR* appears to be mainly expressed in the nervous system (**Fig. 3A, B**). When animals are treated with tributyltin, both *RAR* and *RXR* expression are primarily visible in the developing penis (5-8 days of treatment, **Fig. 3D, E**).

Retinoic acid is regulated in a tissue specific manner by Cyp26, a hydroxylase that converts RA into metabolites such as 4-hydroxy-retinoic acid (**Fig. 1B**). As retinoic acid is difficult to visualize directly, Cyp26 is often used as a readout of retinoic acid signaling (Rhinn and Dollé, 2012; White et al., 1996). In control animals of *C. atrasolea*, *Cyp26* is most obviously expressed in a ring around the mantle, in the body wall, and in the digestive gland (**Fig. 3C**). After treatment with tributyltin, *Cyp26* expression is also evident in the penis (**Fig. 3F**).

Alignment of *C. atrasolea* RAR and RXR sequences with human and other molluscan protein sequences shows 54% identity with human RAR-ß, and 80% identity with human RXR-α (**Fig. 4**). Of the predicted amino acid residues in the ligand binding domain that are thought to confer binding ability, *C. atrasolea* RXR shares all but one of the residues in common with human RXR sequence (**Fig. 4A**), while *C. atrasolea* RAR shares 13/25 of these residues in common with human RAR sequence (**Fig. 4B**). Predicted protein sequences for *C. atrasolea* cluster with known RAR and RXR sequences (**Fig. S1A**), as do predicted protein sequences for Cyp26 (**Fig. S1B**).

Retinoid agonists initiate abnormal penis growth in calyptraeid females

The timing of sexual transitions in most calyptraeids is regulated by social environment, but not all species respond to conspecifics (**Fig. 5A**). To test the role of differing levels of social control on the ability of retinoids to induce penis development, we compared the effects of retinoid agonists in multiple calyptraeid species with differing abilities to respond to conspecific signals. Mature females of *C. atrasolea, C. fornicata, C. convexa, and C. plana* exposed to moderate levels of TBT (200ng Sn/ml; **Table 1**) showed induction of penis growth within a few days (*C. atrasolea*) to weeks (temperate species) (**Fig. 5B-E**). This is true of mature females injected with TBT (*C. fornicata*, 91%; *C. plana*, 100%; *C. convexa*, 56%; **Fig. 5C-E**), and females bathed in TBT (*C. atrasolea*, 92%; **Fig. 5B**). Comparisons of TBT-treated animals to DMSO controls indicate significant increases in the proportion of animals with penis development in treated individuals for all species observed (**Fig. 5B-E; Table 2**). Although females of *C. plana* and *C. convexa* have high proportions of penis presence in DMSO-treated control animals

(*C. plana*, 57%; *C. convexa*, 21%; **Fig. 5C, E; Table 2**), TBT-treated animals showed significant increases in the proportion of animals with penis presence (*C. plana*, 100%; *C. convexa*, 56%; **Fig. 5C, E; Table 2**). Although DMSO is generally considered to be inert, even low levels can induce biological activity, potentially by altering gene expression and methylation patterns (Schirling et al., 2006; Verheijen et al., 2019). Untreated controls of these species also show relatively high rates of penis incidence (*C. plana*, 31%; *C. convexa*, 9%; **Fig. 5C, E; Table 2**), suggesting these species may be more sensitive to non-specific inductive effects. Adult females of *C. fornicata* also show a significantly increased proportion of females with penis development in response to ATRA (100%) and the RAR-specific ligand TTNPB (88%) (**Fig. 5D; Table 2**), as do *C. atrasolea* juveniles exposed to ATRA (95%) and TTNPB (15%; **Fig. 5B; Fig. 2, Table 2**).

Discussion

We demonstrate that multiple components of the retinoic acid signaling pathway are active during male genital development in sequentially hermaphroditic calyptraeid gastropods. Exogenous retinoids and tributyltin, an RXR-agonist, induce penis growth and increase penis length in *C. atrasolea*, while drugs that antagonize RXR (UVI-3003) or interfere with the production of retinoic acid (citral and disulfiram) significantly reduce tributyltin's effects. Surprisingly, treatments with both RAR agonists (all-trans retinoic acid, TTNPB) and a specific β-RAR antagonist (LE-135) result in increased penis length. In all species tested, tributyltin induces imposex in mature females. The ability to alter the timing of penis induction and penis length via pharmacological means points to a potential role for retinoids in the evolution of sexual development and hermaphroditism in calyptraeids and other gastropods.

Retinoids and Imposex

Tributyltin has previously been shown to bind human RXR, PPAR and RXR-PPAR dimers (Harada et al., 2015; le Maire et al., 2009; Nishikawa et al., 2004), and association of RXR with tributyltin-caused imposex is well documented in molluscs. For example, in *Nucella lapillus*, a gastropod

with separate sexes, tributyltin exposure alters expression of RXR in tissue and sex-specific patterns (Lima et al., 2011), and upregulates global RXR transcription (Pascoal et al., 2013). Natural populations of *Tritia* (=*Ilyanassa*) *obsoleta*, show increasing RXR transcript levels with seasonal onset of both male and female reproductive recrudescence (Sternberg et al., 2008). RXR protein expression also correlates with imposex susceptibility; Argentinan populations of *Buccinanops globulosus* show high rates of imposex and high levels of RXR protein in the vas defens, while the co-occurring *Trophon geversianus* show low rates of imposex and low levels of RXR protein in vas deferens (Giulianelli et al., 2020). The purported natural ligand for RXR is 9-cis retinoic acid, which binds to both RAR and RXR receptors (Germain et al., 2006a, b). Exposure to 9-cis retinoic acid induces imposex in both *N. lapillus* (Castro et al., 2007) and *Reishia (=Thais) clavigera* (Nishikawa et al., 2004), and transfection assays show transcriptional activation on exposure to this and other RXR-specific ligands in the pulmonate gastropod *Biomphalaria glabrata* (Bouton et al., 2005). Penis development and imposex appears to be mediated by RXR in both separate-sexed gastropods and sequential hermaphrodites (current study).

Nuclear receptors, including RXR and RAR, are evolutionarily conserved (Babonis and Martindale, 2017; Bridgham et al., 2010). However, previous work indicates that molluscan RARs in several species have lost the ability to bind retinoids (André et al., 2019; Gutierrez-Mazariegos et al., 2014). In contrast, we found that exposure to RAR agonists and a specific RAR antagonist results in increased penis length in *C. atrasolea*. While the natural ligand of RAR is thought to be ATRA (Germain et al., 2006a), it can also bind to RXR (Mangelsdorf et al., 1990; Tsuji et al., 2015), including molluscan RXRs (André et al., 2019). In addition, ATRA and 13-cis retinoic acid (but not 9-cis retinoic acid) are present in adult tissues of *N. lapillus* (Gutierrez-Mazariegos et al., 2014), and RAR-RXR dimers can bind to retinoic acid response element DNA motifs in *R. clavigera*, (Urushitani et al., 2013). In the bivalve *C. gigas*, embryonic exposure to ATRA produces significant developmental effects (Vogeler et al., 2017), *Cg-RAR* and *Cg-RXR* expression in adults increases on exposure to ATRA, and Cg-RAR protein localizes to the nucleus and can heterodimerize with Cg-RXR (Jin et al., 2021). While the precise mechanism of

action remains unclear, RAR does play a role in molluscan development, and is implicated in calyptraeid penis development.

Conservation of specific amino acid residues in the ligand binding domain of RAR has been used as an indirect measure of binding ability (**Fig. 4**). For example, RAR of the polychaete annelid *Platynereis dumerilli* is functional, binds to various retinoids, and retains 20/25 binding residues (Handberg-Thorsager et al., 2018). RAR of the gastropod *Lymnaea stagnalis* retains 16/25 binding residues and is not thought to bind retinoids directly, though ATRA exposure can induce growth cone turning (Carter et al., 2015; Johnson et al., 2019). *C. atrasolea* RAR retains only 13/25 binding residues, suggesting that RAR does not bind to RAR-specific ligands (**Fig. 4**). In *N. lapillus*, the RAR ligand binding domain binds to corepressor proteins (Gutierrez-Mazariegos et al., 2014), and in *P. vulgata* RAR and RXR are individually able to promote transcription, while RAR-RXR heterodimers are not, suggesting a role for RAR in co-repressor release (André et al., 2019). *C. atrasolea RAR* mRNA expressed in the induced penis (**Fig. 3D**) may function by regulating corepressors to mediate transcription. This hypothesis would also explain the observed reduction in penis length caused by blocking the enzymes that produce retinoic acid in the presence of TBT (citral and TBT, disulfiram and TBT, **Fig. 1D**). Precise determination of the binding affinities of molluscan retinoid receptors, the levels of endogenous retinoids, and interactions with other members of this pathway will be necessary to understand the mechanisms of male genital induction in calyptraeids and other molluscs.

Connecting external and internal signals in calyptraeid sex determination

Nuclear receptors have evolved from an ancestral role in binding to fatty acids (Bridgham et al., 2010) and play important roles in a variety of life history transitions in other organisms. For example, the initiation of dauer larva formation in *C. elegans* is mediated by the nuclear receptor DAF-12, which acts as a switch between dauer formation and reproduction by integrating complex neural, nutritional, and metabolic inputs (Antebi, 2015). Genome analyses have identified 43 NRs in the bivalve *Crassostrea gigas* (Vogeler et al., 2014), and 39 and 33 NRs in the gastropods *B. glabrata* and *Lottia gigantia*,

respectively (Kaur et al., 2015), suggesting as yet unknown NR functions in molluscs. Indeed, unbiased sampling of transcriptional responses to tributyltin in separate-sexed molluscs has implicated the peroxisome proliferator-activated receptor (PPAR, NR1C) and a specific PPAR-γ agonist induces imposex in *N. lapillus* (Pascoal et al., 2013). Additionally, *PPAR* and *RXR* transcripts are differentially expressed between male and female tissues of *C. gigas* (Vogeler et al., 2016). Vertebrate studies link PPAR signaling to metabolism and lipogenesis (Feige et al., 2006), which, if similar roles exist in gastropods, could link nutritional states to sexual development. Altogether, the importance of nuclear receptors in integrating environmental signals is likely to be important for controlling life history transitions in the calyptraeids and other molluscs.

While our results show that retinoids can induce growth of male genitalia in calyptraeids, retinoid activity is unlikely to initiate male sexual development. Gould (1917) indicates that penis development is not seen until after the gonad begins spermatogenesis, suggesting that masculinizing signals may result from sperm maturation. Hormonal initiation of penis development has been suggested; however, steroidal sex hormone receptors (i.e., androgen or estrogen receptors) appear to be absent in molluscs (see (Horiguchi, 2017) for review). Initiation and maintenance of penis development in calyptraeids appears to be modulated by neural factors, as removal of the ganglia adjacent to the site of penis growth in *C. fornicata* blocks tributyltin activity (Le Gall and Feral, 1982). The shift of *RXR* transcript expression from the nervous system to the developing tissues of the penis in *C. atrasolea* (**Fig. 3B, E**) supports neural involvement, and similar shifts in transcript expression are reported for *N. lapillus RXR* (Lima et al., 2011). Downstream effectors are likewise unknown in molluscan male genital development. Expression of patterning molecules like *Sonic hedgehog* in male genitalia of species from *Drosophila* (Sanchez et al 2001) to mouse (Perriton et al 2002) and skate (O'Shaughnessy 2015) suggest a starting point for further investigation.

Tributyltin, imposex, and reproductive development in sequentially hermaphroditic calyptraeids

Reports on the occurrence of imposex in calyptraeids are mixed, which has led some to suggest that sexual development in sequential hermaphrodites is regulated by different developmental pathways from dioecious species (e.g., Cledón et al., 2016). No evidence of imposex was reported in *Bostrycapulus odites* (=*Crepidula aculeata*) collected from highly contaminated sites, though reduced retention of pollutants by hard substrates may have limited exposure (Bigatti et al., 2009). Imposex was reported in *Bostrycapulus calyptraeiformis* and *Crepidula* cf. *nivea* exposed to seawater from the highly-trafficked entrance to the Panama Canal, although the identity of the inductive agent was not determined (Li and Collin, 2009). We show here that tributyltin induces imposex in females of four species of *Crepidula* (**Fig. 5; Table 2**), with responses varying across species. Females of *C. convexa* show relatively low incidence of penis formation on exposure to tributyltin compared to other species (*i.e.*, 56% compared to 91-100%, **Fig. 5B-E**). In the solitary *C. convexa*, the timing of sexual transitions is not strongly influenced by the presence of conspecifics (Hoagland, 1978), and females retain the penis well into maturity (Coe, 1936). The level of gregariousness has been suggested to correlate with the level of social influence over sex determination (Hoagland, 1978) (**Fig. 5A**), suggestive of an alternate mechanism controlling reproductive transitions in species with reduced conspecific-based sex determination.

Animals exposed to high levels of tributyltin exhibit diverse reproductive impacts. Here, we show that tributyltin exposure and interference with retinoid signaling not only induces penis development, but appears to alter penis length (Fig. 1C-E). Gonadal-level effects are reported in other molluscs, including reduced oogenesis and initiation of spermatogenesis (Alzieu, 2000). Comparison of normal reproductive development to pharmacologically induced reproductive characters will be useful for understanding how reproductive tissues are formed. Reproductive development has been previously documented in calyptraeids (Coe, 1936; Giese, 1915; Gould, 1917), though developmental details are lacking. Both male and female germ cells are present in the gonad in young juveniles (Gould, 1917), and in males during seasonal gonadal changes in *C. fornicata* (Beninger et al., 2010). The calyptraeid penis is muscular, with an external ciliated sperm groove (Giese, 1915; Kleinsteuber, 1913). Detailed understanding of the

development of the male and female external genitalia and the ovotestis in *Crepidula* will be necessary to understand the basis for transitions in reproductive characters.

Evolution of sequential hermaphroditism

Sexual state is increasingly recognized as flexible and dynamic. Detailed examinations of sexual development highlight the plasticity of sexual expression via multiple levels of regulatory control (Zhao and Yao, 2019). Indeed, cross-sexual transfers are a well-known source of phenotypic novelties (West-Eberhard, 2003). Even in chromosomally determined systems, somatic and germ cell sex can be changed by disruption of a single gene (*e.g.*, tra-1 in *C. elegans,* Sox9 in mice (Williams and Carroll, 2009)). In the development of separate males and females, early signals for one sex typically inhibit development of the opposite sex, and in some cases initiate removal of tissue progenitors of the opposite sex (**Fig. 6**) (Zhao et al., 2017; Zhao and Yao, 2019). Control of sexual expression may be similar in sequential hermaphrodites, with shifts in timing of initiation and integration of signals for genital resorption and tissue development to allow for the expression of both sexes in sequence (**Fig. 6**). Alterations in timing of sexual expression represent important components of the evolution of sequential hermaphroditism (**Fig. 6**). Additionally, for animals with an intromittent organ, penis length and shape evolves rapidly, with important consequences for reproductive success (see Hoskin et al 2019 for review). The relative ease of altering the timing and size of male genitalia in a sequential hermaphrodite as shown here may have implications for the repeated evolution of hermaphroditism in sexually diverse molluscs.

Materials and Methods

Animal collection

Juvenile and adult females of *C. atrasolea* were sampled from a laboratory colony of snails maintained at the University of Illinois at Urbana-Champaign. Details of their culture conditions are as described previously (Henry et al., 2020; Henry et al., 2017). Adult females of *C. fornicata*, *C. plana*, and *C. convexa* were collected from wild populations around Woods Hole under a scientific collection permit

from the Commonwealth of Massachusetts Division of Marine Fisheries. Adult females of *C. fornicata* and *C. plana* were also provided by the Marine Resources Centre of the Marine Biological Laboratory (MBL) at Woods Hole, MA. Animals were maintained in natural flowing seawater at the MBL under ambient food and temperature conditions prior to experimental use.

Juvenile Treatments

Juveniles of *C. atrasolea* were collected from brooding females of the laboratory population described above. Hatchlings were separated into treatment and control groups of 5-15 juveniles per treatment. Broods from multiple females were randomized across treatments, and at least two replicates per treatment were performed. All animals were maintained in 35x10mm petri dishes filled to the brim with 0.22µm filtered Reef Crystals Instant Ocean Sea Water (Spectrum Brands, Inc. Blacksburg, VA) and fed $1x10^6$ cells/ml of Phytofeast daily. Food and sea water were changed daily. Drugs were added to culture dishes daily at the concentrations indicated in **Table 1**, for 5-8 days. Several concentrations of drugs were tested initially to determine dosages that induced a response without significant mortality (data not shown). At the end of the treatment period, animals were relaxed, scored for penis development, imaged, and fixed, as described below. Penis and shell length were measured from images using ImageJ (v.2.0.0-rc-69/1.52p) (Rasband, 1997-2018). Shell length was measured along the longest axis of the shell. Penis length was measured freehand in ImageJ, from the middle of the base of the penis to the tip, as indicated in **Figure 2**.

Adult treatments

Individual adult females of *C. fornicata*, *C. plana*, and *C. convexa* were transferred to 350ml plastic cups and maintained in bag-filtered (10µm porosity high-flow polyester filter bag, Cole-Parmer, Vernon Hills, IL, Item # EW-01524-82) natural seawater at the MBL at room temperature (~20°C). Experimental animals were fed approximately $1x10^6$ cells/ml of *Isochrisis galbana-T* or Phytofeast (Reed Mariculture Inc., Campbell, CA) daily, and water was changed every other day. Field collected animals

were acclimated for 24-48 hours before initiating treatments. Stock solutions of all drugs used were prepared in DMSO to concentrations reported in **Table 1**. Drugs were injected directly into the foot at a concentration of approximately 1µg of drug per gram adult body weight using a 5µl glass syringe (Hamilton, Reno, NV, Part No. 7633-01) with a 34-gauge needle (Hamilton, Reno, NV, Part No. 207434). Control animals were injected with a comparable volume of DMSO. Animals were injected twice, at least one week apart. Mortality was high for injection treatments, primarily due to difficulty animals had in attaching to the substrate for feeding. Treated adult females were scored for penis growth after 1 week (*C. atrasolea*) or 2-4 weeks (other species), relaxed using a 1:1 mixture of sea water and 7.5% $MgCl_2$, followed by addition of a saturated chloretone-seawater (Sigma-Aldrich, Cat. No. 112054) solution. Relaxed animals were fixed in 4% paraformaldehyde overnight at 4°C, rinsed in phosphate buffered saline (PBS), and transferred to 100% methanol for storage at -80°C. Non-treated adult females were scored post-fixation for penis presence. Adult females of *C. atrasolea* were treated as described for juveniles above.

Cloning and in situ hybridization

PCR primers were designed based on sequence data from a *C. atrasolea* developmental transcriptome (see Henry et al., 2017, NCBI TSA GFWJ00000000.1). Primers for *C. atrasolea* PCR amplification and cloning were: RAR forward 5'-ATGGGCATGTCCAAAGAAGCGGTG-3' and reverse 5'-TCCTGTCGGTCCAGCATCTCTTTCAC-3'; RXR forward 5'-GACGGGGAGGTGGAGAGCAC-3' and reverse 5'-CAGACCCTTGGCATCAGGATTG-3'; and Cyp26 forward 5'-AGGTGTACAAGACTCACATTCTGG-3' and reverse 5'-GTTCCTGGCCAAAAGATACAAGAG-3'. GenBank accession numbers for *C. atrasolea* sequences: RAR, MZ020963; RXR, MZ020964; Cyp26 MZ020965.

RNA was extracted from whole embryos of *C. atrasolea* using TriZol (Life Technologies, Grand Island, NY), following the manufacturer's protocol, which was used to prepare cDNA (iScript cDNA Synthesis kit, BioRad, Hercules, CA). Fragments of each gene of interest were cloned into pGEMt-Easy

vector, and validated by sequencing at the University of Illinois Core Sequencing Facility (Urbana, IL). Human, mouse, and molluscan sequences for RAR, RXR and CYP26 were aligned using ClustalOmega. Alignments were adjusted for readability using Jalview (V.2.11.0). Digoxigenin (DIG)-labeled antisense and sense *in situ* probes were prepared from PCR products of cloned template DNA, as described previously (Osborne et al., 2018). *In situ* hybridization of drug treated juveniles of *C. atrasolea* was performed as described previously (Osborne et al., 2018).

Phylogenetic Analyses

Phylogenetic trees (**Fig. S1**) were constructed using protein translations of previously identified sequences, sequenced fragments and transcriptomic data for *C. atrasolea*. Accession numbers are reported in **Table S1**. MEGAX software (v10.1.7, 1993-2020) was used to realign sequences and construct neighbor joining trees (1000 replicates) and maximum likelihood trees (500 replicates).

Statistical analyses

Penis length was measured directly in *C. atrasolea* juveniles treated with agonists alone, antagonists alone, TBT + antagonist and DMSO (control). Average penis lengths were not normally distributed, and were compared using the non-parametric Kruskal-Wallis test, tested post-hoc by the Dunn test. Penis length was not measured for adult females. Rather, adult females were scored for penis presence or absence in treated (TBT, ATRA, TTNPB) and DMSO control animals, and compared by chi-square test. Presence/absence penis counts were combined for adult and juvenile treatments of *C. atrasolea*. Unlike females of the other species tested, females of *C. convexa* retain the penis well after transitioning to functional females (Coe, 1936), therefore penis length relative to tentacle length was used as a proxy for penis presence/absence for this species only. Individual females of *C. convexa* with a penis longer than the adjacent tentacle were scored as present. Penis presence/absence in non-treated animals is also reported. All statistical tests were performed in R version 3.6.1 (2019-07-05).

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Figure Legends

Figure 1. The retinoic acid pathway, drug targets, and their effects on penis length in juveniles of the sequentially hermaphroditic snail *C. atrasolea*. A. Typical development of sequentially hermaphroditic calyptraeid snails. Immature juveniles mature first into males with a penis, followed by a transitional period of penis resorption, elaboration of external female genitalia, and maturation into a functional female. **B.** Retinoic acid (RA) is synthesized from an exogenous precursor, retinol (RO, Vitamin A). Progressive oxidation reactions by retinol and retinaldehyde dehydrogenases (RDH, RALDH) produce retinoic acid (RA) from retinol (RO) and retinaldehyde (RE) precursors. Transporters (CRABP) move RA into the nucleus and may aid in localization. Retinoic acid receptor (RAR) and retinoid X receptor (RXR) dimers are typically bound to retinoid acid response elements (RAREs) in the genome and associated with repressors that inhibit transcription. RA binding to RAR induces conformational changes that releases co-repressors, recruits co-activators, and activations downstream transcription. RA levels are controlled by the RA metabolizing Cyp26 enzyme. Drugs used in the current study target retinoic acid synthesis (citral, disulfiram) and retinoid receptor activity (ATRA, TBT, TTNPB, UVI-3003, LE-135). **C-E.** Box and whisker plots of penis length following drug treatments. All individuals measured are indicated as points, and total number of individuals measured (n) is indicated above each boxplot. The heavy central line indicates the mean penis length, upper and lower bounds are 25% intervals, whiskers indicate 1.5x interquartile range. Letters above plot indicate statistical significance as compared post hoc by the Dunn test. Treatments with the same letter are not statistically different from one another. Green ovals indicate agonists, red boxes indicate antagonists.

Figure 2. Retinoid receptor agonists induce premature penis development in juveniles of *C. atrasolea*. Juvenile *C. atrasolea* initiate precocious penis development in response to treatment with tributyltin (TBT) (B), all-trans retinoic acid (ATRA) (C), and the RAR agonist TTNPB (D), but not DMSO control treatment (A). White dotted lines show approximate penis measurement paths. Animals

are shown in ventral view with anterior towards the top of the image. ft, foot; lt, left tentacle; m, mouth; ma, mantle; p, penis; rt, right tentacle. Scale bar = 500 μm.

Figure 3. Expression of *RAR*, *RXR*, and *Cyp26* mRNA in juveniles of *C. atrasolea* exposed to DMSO control or treated with TBT. A. DMSO-control animals show faint expression of *RAR* primarily in the gill filaments. B. *RXR* expression is visible primarily in the nervous system of DMSO control animals. C. *Cyp26* is expressed in a ring around the mantle edge, the edge of the body wall and in the digestive gland of DMSO control animals. D, E. When treated with tributyltin, *RAR* and *RXR* expression is primarily seen in the induced penis. F. Likewise, *Cyp26* expression is visible in the penis of TBT-treated animals, and is retained in the mantle and digestive gland. ft, foot; gf, gill filaments; lt, left tentacle; ma, mantle; p, penis; rt, right tentacle. Scale bar = 250 μm.

Figure 4. Predicted protein sequence alignment of ligand binding domains of RXR and RAR. Clustal Omega alignments of protein residues that are predicted to be important for ligand binding in RXR (A) and RAR (B) based on vertebrate models are indicated in pink. Increasing percent sequence identity is indicated by increasingly darker blue, as indicated by the legend.

Figure 5. Tributyltin and retinoic acid receptor agonists induce ectopic penis growth in mature females of *Crepidula spp*. A. Simplified cladogram of the calyptraeid genus *Crepidula* indicating previously reported social behavior and experimentally determined extent of social control of sex change (Carrillo-Baltodano and Collin, 2015; Coe, 1953; Collin, 2000; Collin et al., 2005; Hoagland, 1978; Warner et al., 1996). Increased rate of social interaction is thought to increase social control of sex change (Hoagland, 1978). Species used in the current study are underlined. B–E. Mature females of *C. atrasolea* (B), *C. plana* (C), *C. fornicata* (D), and *C. convexa* (E), initiate penis growth in response to either water-borne exposure (B) or injection (C-E) of tributyltin (TBT). All adult females of *C. fornicata*, *C. plana*, and *C. convexa* and adults and juveniles of *C. atrasolea* treated with TBT or retinoic acid receptor

agonists (ATRA, TTNPB) show a significantly increased proportion of individuals with penis growth when compared to control DMSO treated animals. Animals are shown in ventral views, with anterior towards the top. The percentage of individuals with induced penis growth is indicated above bars, and the number of animals per treatment (n) is indicated below. Level of significance in change in penis presence compared to DMSO-treated controls is indicated by asterisks. * $p<0.5$, *** $p <<< 0.0001$. an, anus; ft, foot; fgp, female genital papilla; lt, left tentacle; m, mouth; p, penis; rt, right tentacle. Scale bar B, E = 250μm; C, D = 415μm.

Figure 6. Comparison of development between separate sexes and sequential hermaphrodites. All individuals have the potential to produce either male or female characters (top). Precise timing and mechanisms of sex determination varies, but in species with separate sexes is primarily via genetic or chromosomal mechanisms. Sex determination in sequential hermaphrodites including the calyptraeid gastropods is typically under greater control by environmental influences. In both cases, induction of one sex is coupled with regression and inhibition of characters of the other sex. In sequential hermaphrodites, a second round of inductive and inhibitory events cause regression of the first sex and development of the second sex. The ability of endocrine disruptors, including tributyltin, to induce ectopic male characters in females demontrates the flexibility of the sexual system. In sequential hermaphrodites, the same drugs accelerate male development in juveniles and induce imposex in mature females, implicating retinoids in the control of male sexual development. Altered timing of sexual induction produced by artificial means suggests heterochronic shifts in timing of sexual development as important factors in the evolution of sequential hermaphroditism, including earlier induction of the first sex, inhibition of the first sex, and induction of the second sex.

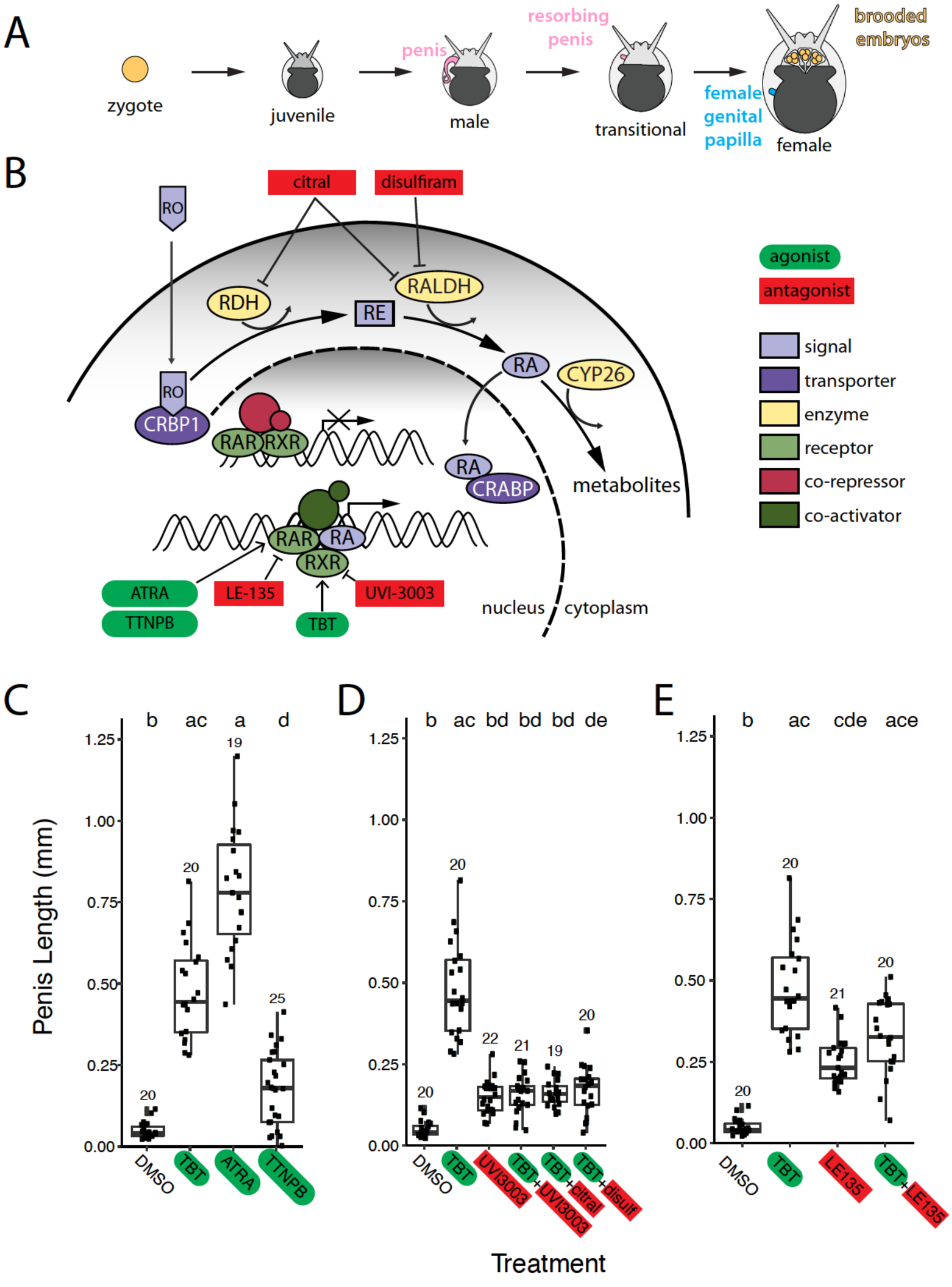
Tables

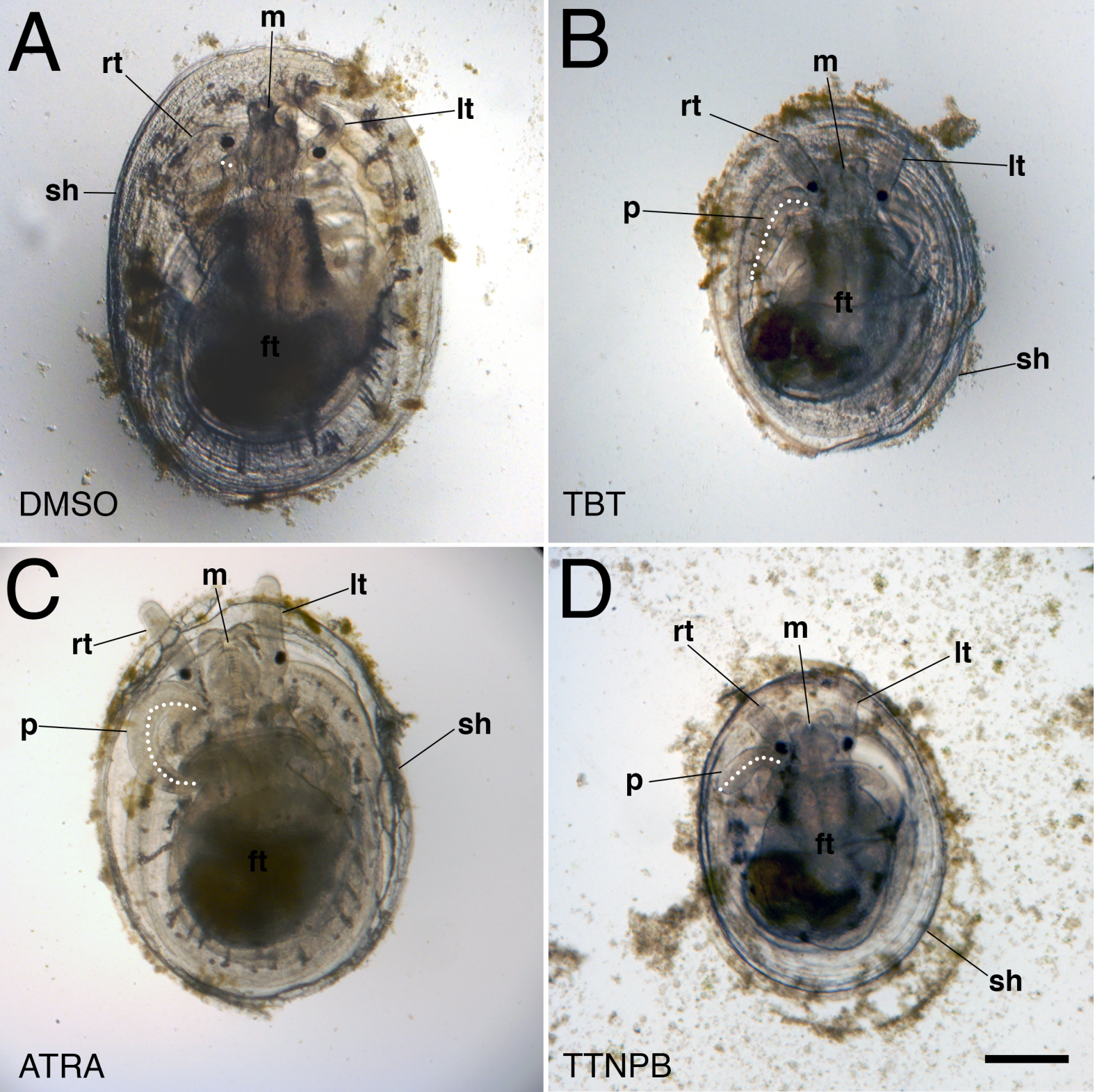
Table 1. Drug concentrations for injection and bath experiments. Dash indicates treatment not done.

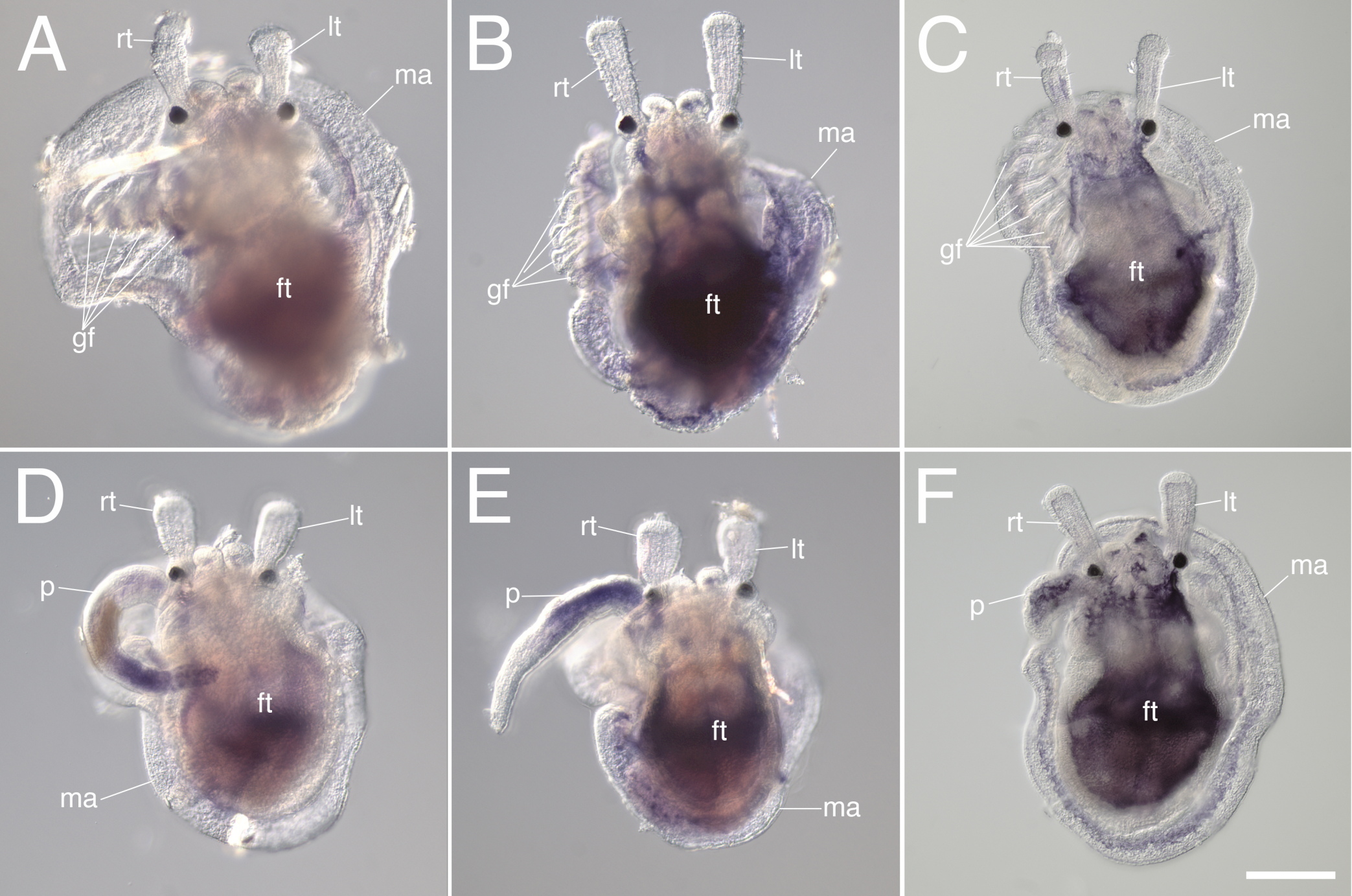
Drug	Working Concentration for Injections (1µg/g body weight)	Working Concentration for Bath Treatments	Target / Action
DMSO	volumetric equivalent	volumetric equivalent	carrier, negative control
TBT	4.6 mM	4.6 pM (=200ngSn/µl)	likely RXR agonist
ATRA	3.3 mM	1µM	RAR, RXR ligand
TTNPB	2.9 mM	1µM	pan-RAR agonist
LE-135	-	1µM	RAR-ß antagonist
UVI-3003	-	1µM	pan-RXR antagonist
citral	-	1µM	RDH, RALDH inhibitor
disulfiram	-	1µM	RALDH inhibitor

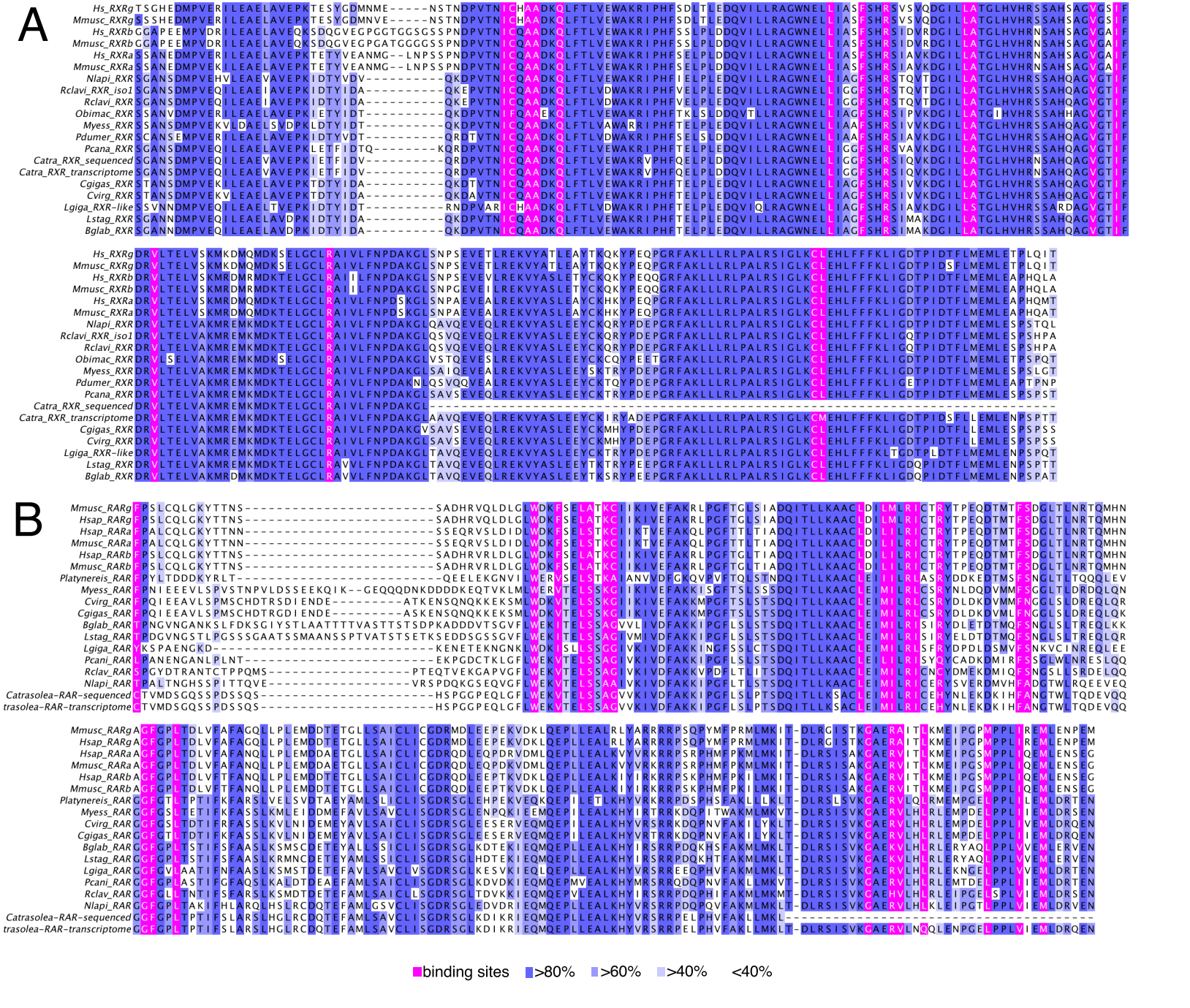
Table 2. Effects of retinoid agonists on penis induction. Note that for individuals of *C. convexa*, penis presence is determined by length relative to the length of the adjacent tentacle. See text for details.

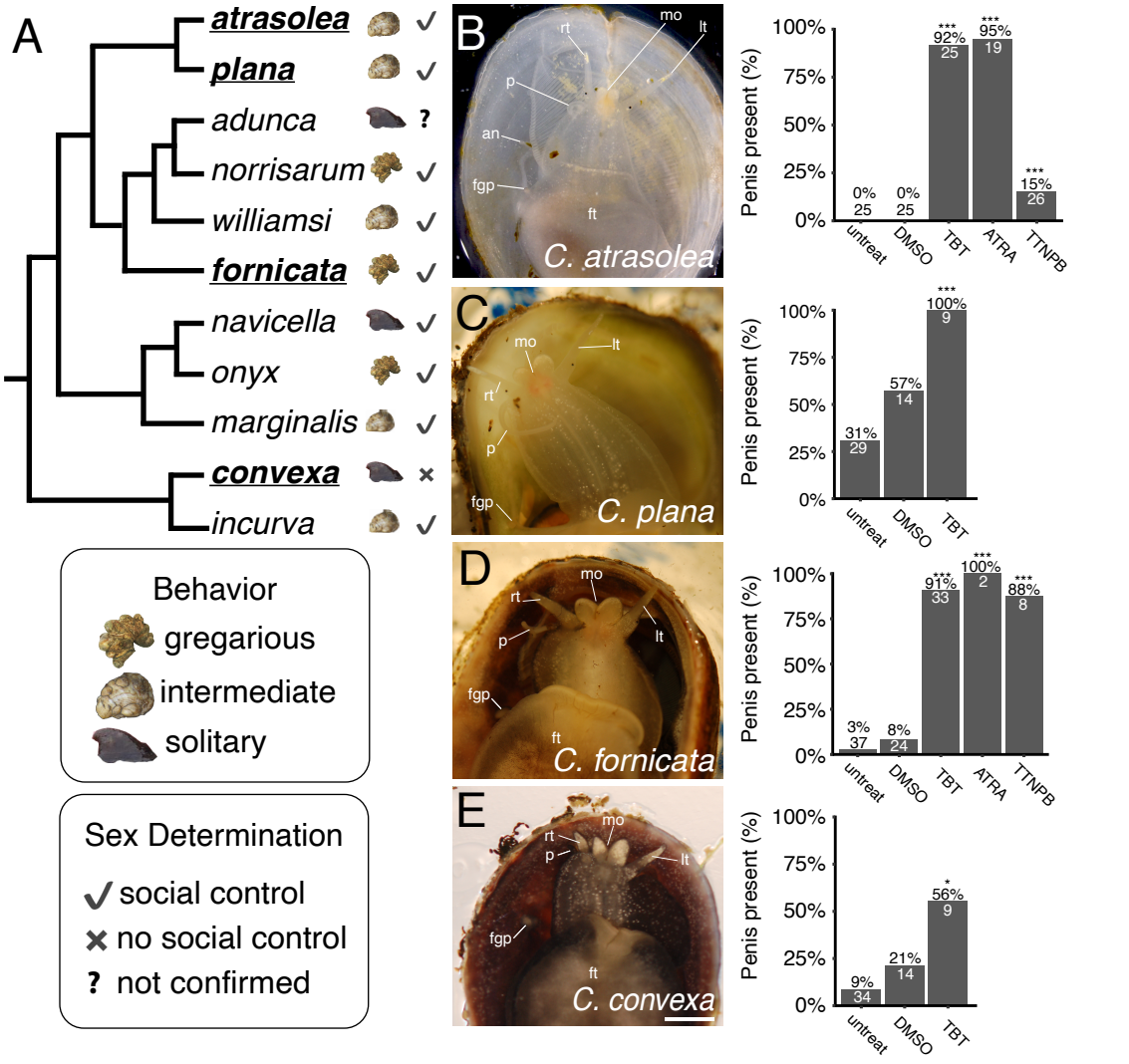
Species	Stage	Treatment	total (n)	with penis	without penis	% with penis	χ^2	df	p
C. atrasolea	**female**	DMSO	5	0	5	0			
		TBT	5	5	0	100	inf	1	**0**
		none	5	0	5	0	-	-	-
	juvenile	DMSO	20	0	20	0			
		TBT	20	18	2	90	inf	1	**0**
		ATRA	19	18	1	95	inf	1	**0**
		TTNPB	26	4	22	15	inf	1	**0**
		none	20	0	20	0	-	-	-
C. fornicata	**female**	DMSO	24	2	22	8			
		TBT	33	30	3	91	294.57	1	**<0.0001**
		ATRA	2	2	0	100	22	1	**<0.0001**
		TTNPB	8	7	1	88	65.64	1	**<0.0001**
		none	37	1	36	3	-	-	-
C. plana	**female**	DMSO	14	8	6	57			
		TBT	9	9	0	100	6.75	1	**0.0094**
		none	29	9	20	31	-	-	-
C. convexa	**female**	DMSO	14	3	11	21			
		TBT	9	5	4	56	6.23	1	**0.013**
		none	34	3	31	9	-	-	-

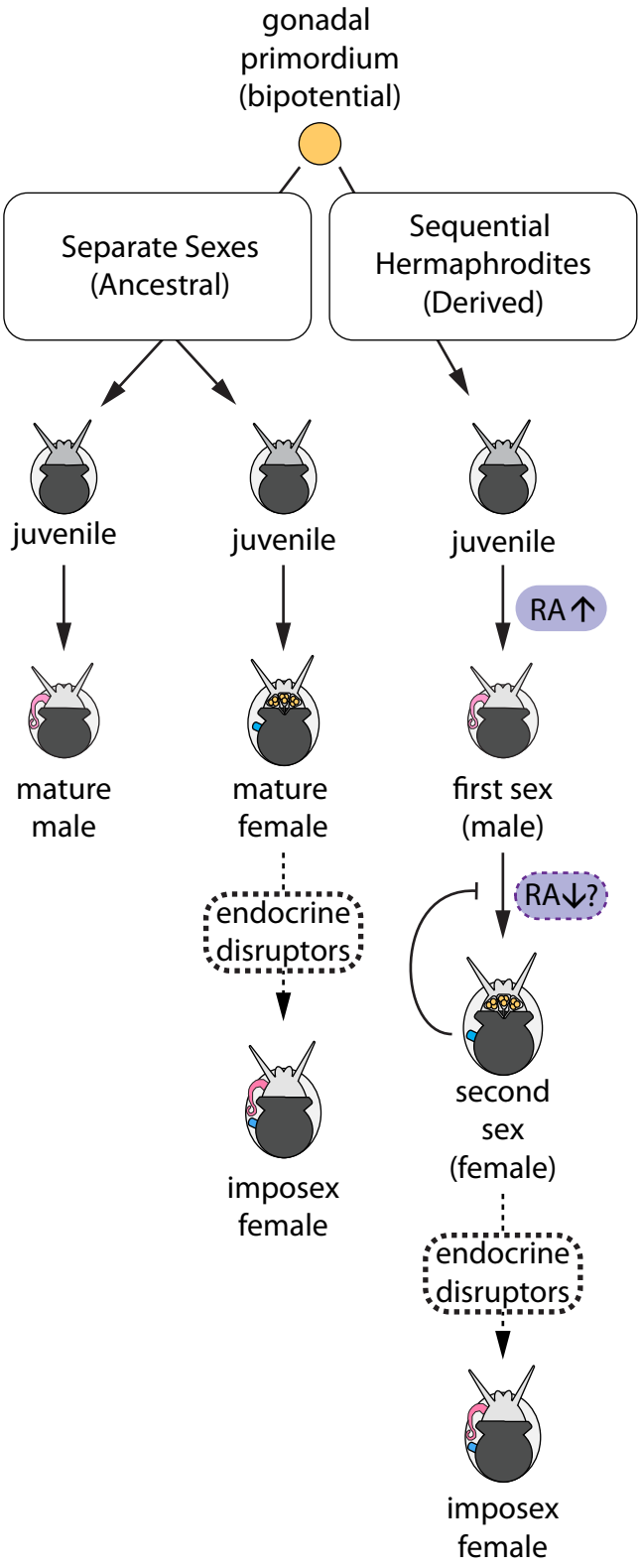

A
zygote
juvenile
penis
male
resorbing penis
transitional
female genital papilla
female
brooded embryos
B
RO
citral
disulfiram
RDH
RALDH
RE
RA
CYP26
metabolites
RO
CRBP1
RAR
RXR
RA
CRABP
RAR
RA
RXR
ATRA
TTNPB
LE-135
TBT
UVI-3003
nucleus
cytoplasm
agonist
antagonist
signal
transporter
enzyme
receptor
co-repressor
co-activator
C
D
E
Penis Length (mm)
Treatment
DMSO
TBT
ATRA
TTNPB
UVI3003
TBT+UVI3003
TBT+citral
TBT+disulf
LE135
TBT+LE135


A
m
rt
lt
sh
ft
DMSO
B
m
rt
lt
p
ft
sh
TBT
C
m
lt
rt
p
sh
ft
ATRA
D
rt
m
lt
p
ft
sh
TTNPB


A
rt
lt
ma
ft
gf
B
rt
lt
ma
gf
ft
C
rt
lt
ma
gf
ft
D
rt
lt
p
ft
ma
E
rt
lt
p
ft
ma
F
rt
lt
ma
p
ft


A
Hs_RXRg
Mmusc_RXRg
Hs_RXRb
Mmusc_RXRb
Hs_RXRa
Mmusc_RXRa
Nlapi_RXR
Rclavi_RXR_iso1
Rclavi_RXR
Obimac_RXR
Myess_RXR
Pdumer_RXR
Pcana_RXR
Catra_RXR_sequenced
Catra_RXR_transcriptome
Cgigas_RXR
Cvirg_RXR
Lgiga_RXR-like
Lstag_RXR
Bglab_RXR
B
Mmusc_RARg
Hsap_RARg
Hsap_RARa
Mmusc_RARa
Hsap_RARb
Mmusc_RARb
Platynereis_RAR
Myess_RAR
Cvirg_RAR
Cgigas_RAR
Bglab_RAR
Lstag_RAR
Lgiga_RAR
Pcani_RAR
Rclav_RAR
Nlapi_RAR
Catrasolea-RAR-sequenced
trasolea-RAR-transcriptome
binding sites
>80%
>60%
>40%
<40%


A
atrasolea
plana
adunca
norrisarum
williamsi
fornicata
navicella
onyx
marginalis
convexa
incurva
Behavior
gregarious
intermediate
solitary
Sex Determination
social control
no social control
not confirmed
B
C. atrasolea
C
C. plana
D
C. fornicata
E
C. convexa
mo
rt
lt
p
an
fgp
ft
Penis present (%)
100%
75%
50%
25%
0%
untreat
DMSO
TBT
ATRA
TTNPB
0%
25
92%
25
95%
19
15%
26
31%
29
57%
14
100%
9
3%
37
8%
24
91%
33
100%
2
88%
8
9%
34
21%
14
56%
9


gonadal primordium (bipotential)
Separate Sexes (Ancestral)
Sequential Hermaphrodites (Derived)
juvenile
juvenile
juvenile
RA↑
mature male
mature female
first sex (male)
endocrine disruptors
RA↓?
second sex (female)
endocrine disruptors
imposex female
imposex female

Supplemental Data

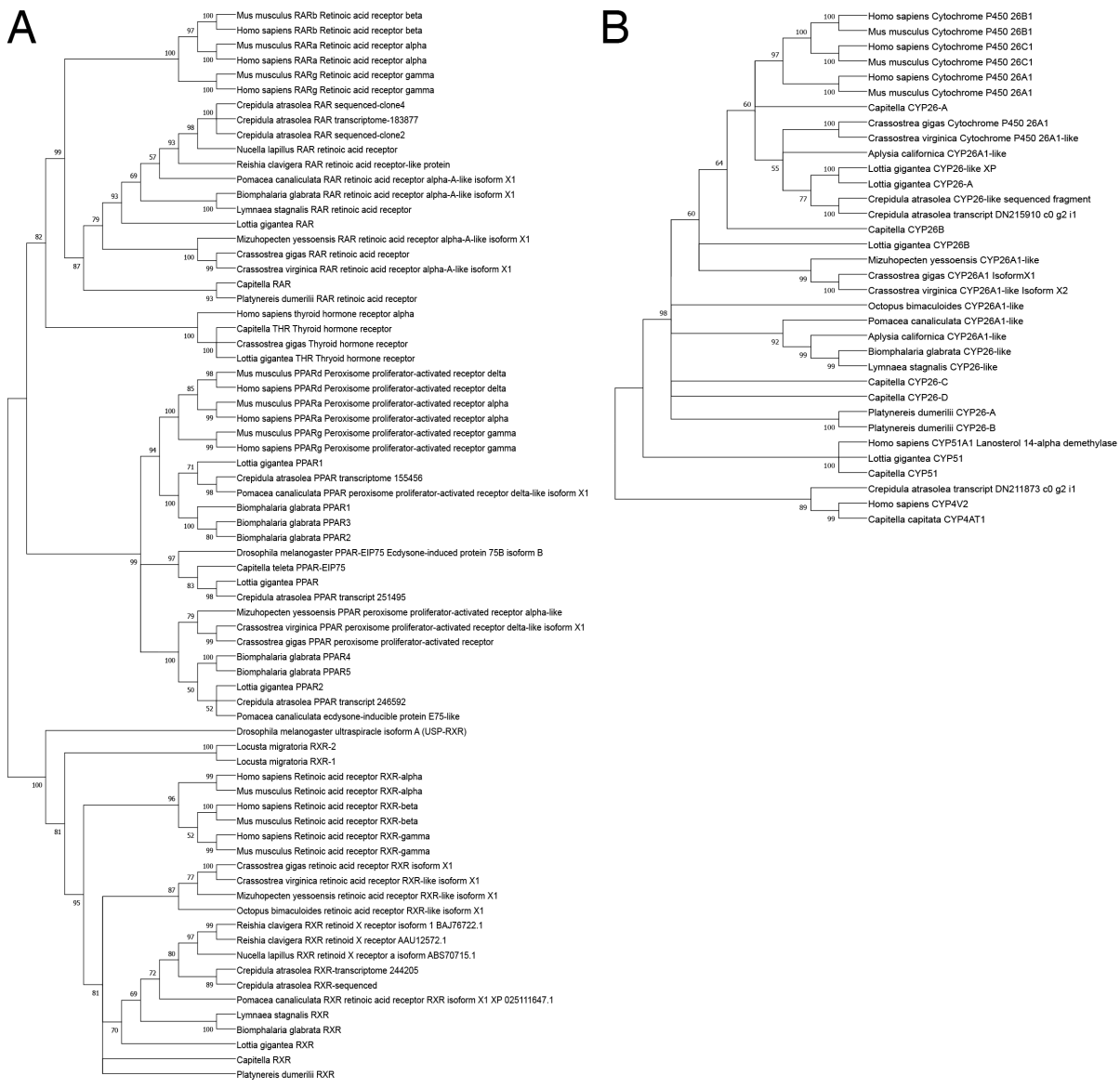


Figure S1. Maximum likelihood bootstrap consensus trees (500 replicates) for (A) the nuclear receptors retinoic acid receptor (RAR), retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR) and thyroid hormone (TH); and (B) the cytochrome p450 oxidases Cyp26, Cyp51, and Cyp4.

Table S1. Accession numbers for sequences used to build phylogenetic trees.

	Species	RAR	RXR	PPAR	TH	Cyp26	Cyp51	Cyp4
Deuterostomes	*Homo sapiens*	RARa P10276 RARb P10826 RARg P13631	RXRa P19793,NP_002948.1 RXRb P28702 RXRg P48443	PPARa Q07869 PPARd Q03181 PPARg P37231	BAH02277.1	Cyp26a O43174 Cyp26b Q9NR63 Cyp26c Q6V0L0	Q16850	Q6ZWL3
	Mus musculus	RARa P11416 RARb P22605 RARg P18911	RXRa P28700 RXRb P28704 RXRg P28705	PPARa P23204 PPARd P35396 PPARg P37238		Cyp26a O55127 Cyp26b Q811W2 Cyp26c B2RXA7		
Ecdysozoa	*Drosophila melanogaster*	-	NP_476781.1 *(ultraspiracle)*	NP_730321.1 (*EIP75)*	-	-	-	-
	Locusta migratoria		AAQ55293.1 AAF00981.1					
Lophotrochozoa, Annelida	*Platynereis dumerilii*	AVR59236.1	AVR59237.1			AVR59240.1 AVR59239.1		
	Capitella teleta	jgi|Capca1|168520	jgi|Capca1|164614	ELU11745.1	jgi|Capca1|167148	jgi|Capca1|212322 jgi|Capca1|150007 jgi|Capca1|95922 gi|Capca1|129163	jgi|Capca1|173561	AAS87604.1
Lophotrochozoa, Mollusca, Bivalvia	*Crassostrea gigas*	A0A1W6JGS2	XP_011434492.1	ARM65371.1	AKE80988.1	XP_011436768.1 XP_011433806.1		
	Crassostrea virginica	XP_022324320.1	XP_022322314.1	XP_022327518.1		XP_022288365.1 XP_022344876.1		
	Mizuhopecten yessoensis	XP_021341358.1	XP_021373595.1	XP_021370914.1		XP_021375591.1		
Lophotrochozoa, Mollusca, Gastropoda	*Pomacea canaliculata*	XP_025086531.1	XP_025111647.1	XP_025110191.1 XP_025110659.1		XP_025095355.1		
	Lymnaea stagnalis	ADF43963.1	Q5I7G2			KF669878.1		
	Biomphalaria glabrata	XP_013085550.1	Q8T5C6	BGLB000113-RA BGLB000113-RB BGLB000113-RC BGLB004656-RA BGLB004656-RB		XP_013083593.1		
	Aplysia californica					XP_005098124.1 XP_012935038.1		
	Lottia gigantea	XP_009050765.1	jgi|Lotgi1|206562	jgi|Lotgi1|174409 jgi|Lotgi1|238472 XP_009045016.1	jgi|Lotgi1|224598	XP_009054356.1 jgi|Lotgi1|232238 jgi|Lotgi1|111018	jgi|Lotgi1|205907	
	Reishia clavigera	BAN82614.1	BAJ76722.1 AAU12572.1					
	Nucella lapillus	AIB06349.1	ABS70715.1					
	Crepidula atrasolea	Catrasolea-RAR-like-clone2 Catrasolea-RAR-like-clone4 (sequenced, partial) Catra_DN183877_c0_g2_i1	Catra_RXR-like (sequenced, partial) Catra_DN244205_c7_g14_i2	Catra_DN246592_c1_g1_i8 Catra_DN251495_c7_g15_i5 Catra_DN155456_c0_g2_i1		Catra_CYP26-like (sequenced, partial) Catra_DN215910_c0_g2_i1		Catra_DN211873_c0_g2_i1
Lophotrochozoa, Mollusca, Cephalopoda	*Octopus bimaculoides*		XP_014769191.1			XP_014788587.1		