A review of the molecular mechanisms of monogonont rotifer reproduction

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Abstract Most animals use proteins, peptides, steroids, eicosanoids, or amino acid derivatives as chemical signals, along with receptors, secondary messengers, transduction systems, and transcription factors to finely control reproduction. Many protostomes have complex endocrine systems with vertebrate-like sex steroid receptors, but some are unresponsive to vertebrate sex steroids. Others are responsive to estrogen and testosterone, but the effects are mediated through non-estrogen receptor pathways. In this article, I review the pheromones that rotifers use to synchronize reproduction and rotifer response to waterborne vertebrate steroid hormones. I also describe the impact on rotifer reproduction of endocrine disruptors that mimic androgens and their antagonists. A fraction of the brachionid transcriptome is surveyed for candidates involved in endocrine signaling systems and genes are identified that are putatively involved in steroidogenesis and oocyte maturation. We use the new technique of RNAi in rotifers to selectively knock down gene expression and identify the functional roles of genes involved in the regulation of reproduction.

Introduction

Reproduction is a key biological process impacting the abundance of organisms, their persistence in communities, their fate in competition, their resistance to predation and parasites, and their role in energy flow. The regulation of reproduction also is a critical element in the fitness of individuals. The timing of reproduction so that it occurs in favorable environments, the adjustment for optimal levels of reproductive effort, and maximization of resource use all have important effects on the evolutionary persistence of populations. This makes the mechanisms regulating reproduction of great interest in population biology.

The mechanisms by which animals regulate reproduction reflect the enormous diversity in the animal kingdom, but some themes are apparent. Chemical signaling in reproduction typically involves sources, signals, receptors, secondary messengers, transduction systems, and transcription factors. Endocrine systems employing a variety of hormones are commonly observed, most often proteins, peptides, steroids, eicosanoids, or amino acid derivatives (Heyland et al., 2004). There are at least two types of receptors:

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membrane bound proteins that are linked to signal transduction pathways, or free proteins in the cytoplasm or nucleus that bind to steroids (Tsai & O'Malley, 1994; Escriva et al., 2000). Pheromones are another important element in reproductive systems (Wyatt, 2003), typically synchronizing reproductive activity through processes like quorum sensing (Kubanek & Snell, 2008).

Complex endocrine systems are phylogenetically ancient (Stoka, 1999; Bertrand et al., 2004). Bilaterian animals share receptors for many steroids, retinoids, and other hormones belonging to the superfamily of nuclear receptors (NR). NR are ligand-activated transcription factors regulating many aspects of reproduction, development, and homeostasis, and often play a key role in regulating gene expression. About 25 NR genes were present in Urbilateria, which are more than 400 mya (Bertrand et al., 2004). Derived endocrine systems evolved in different major lineages by gene duplication and loss. Steroid and thyroid receptors were present in Urbilateria (Thornton et al., 2003) and steroid hormone regulation of reproduction is widespread among extant bilateria animals.

Sex steroid signaling systems also are widespread among invertebrates (Kohler et al., 2007). Cephalochordates have a sex steroid signaling systems similar to vertebrates. Echinoderms respond to vertebrate sex steroids in a comparable manner as vertebrates, and possess androgen and estrogen receptors. Many protostomes have vertebrate-like sex steroid receptors, but some are unresponsive to vertebrate sex steroids (Lafont & Mathieu, 2007). Several molluscs are responsive to estrogen and testosterone, but effects may be mediated through non-estrogen receptor pathways (Lafont & Mathieu, 2007). Some crustaceans have retained features of estrogen and androgen signaling pathways. However, the genome of *Caenorhabditis elegans* does not contain any orthologs of vertebrate nuclear sex steroid receptor genes (Novillo et al., 2005). Annelids produce and respond to estrogens (Garcia-Alonso & Rebscher, 2005; Garcia-Alonso et al., 2006) and they possess estrogen receptors that bind estrogen with high affinity and activate transcription upon exposure to low concentrations of estrogen (Keay & Thornton, 2009). Every invertebrate taxon has probably evolved its own distinct steroid signaling system derived by modifying ancestral steroid receptors (Thornton et al., 2003).

In this review, I describe what is known about the pheromones that rotifers use to synchronize reproduction, the responses of rotifers to vertebrate hormones, and how endocrine disruptors, especially those that mimic androgens, impact rotifer reproduction. I further survey receptors in the rotifer transcriptome for candidates involved in endocrine signaling systems, identify genes putatively involved in steroidogenesis, and describe how RNAi knockdown of their expression impacts mictic reproduction.

Rotifers

Pheromones

There is abundant evidence that rotifers use chemical signals to regulate their reproduction. In the monogonont *Brachionus*, the switch from asexual to sexual (mictic) reproduction is triggered by compounds that rotifers excrete into the medium (Gilbert, 1963; Stelzer & Snell, 2006). Recently, one has been characterized in *B. manjavacas* as a mixis inducing protein (MIP) (Snell et al., 2006). This process is similar to the quorum sensing (Kubanek & Snell, 2008) that has been so well characterized in bacteria (Miller & Bassler, 2003). Male mating behaviour likewise is triggered by an external signal, a surface glycoprotein called the mate recognition pheromone (MRP) detected by contact chemoreception when a *Brachionus* male encounters a conspecific female (Snell et al., 1995; Snell & Stelzer, 2005). The MRP gene has been identified from a family of genes that encode similar proteins and its potential for rapid evolution has been elucidated (Snell et al., 2009). The MRP gene begins with a short signal peptide region and is followed by three nearly perfect repeats of 87 amino acids. Each repeat contains three asparagines that are potential sites for N-glycosylation, and the processed MRP protein has no significant similarity to any sequence in GenBank/EMBL/DDJ databases in BLASTX and BLASTN searches. Sequence analysis of the MRP gene has been performed on clonal isolates from the *B. plicatilis* species complex, and phylogenies compared to those obtained with COI and ITS genes (Gribble et al., this symposium).

Progress has been made in identifying the MRP receptor in males. A survey of receptors in the *B. manjavacas* male transcriptome has revealed a

mannose receptor that has become a prime candidate for the MRP receptor. It is significant because males have a limited repertoire of receptors since they do not feed, and their primary activity is finding and copulating with conspecific females. The MRP is glycoslyated, and oligosaccharides are important in mate recognition (Snell et al., 1995), so the MRP receptor is expected to recognize oligosaccharides. In addition to the lectin-like nature of the MRP receptor, it is known that plant lectins with mannose/glucose affinity bind to the MRP and localize to the corona of females (Snell & Nacionales, 1990). Mannose is thought to be an important component of MRP oligosaccharides (Snell et al., 1995), making a mannose receptor a particularly attractive candidate for the MRP receptor.

The ability to bind mannose makes it likely that the MRP receptor is some type of mannose receptor, a well-characterized class of receptors involved in a variety of cellular recognition phenomena. Mannose receptors are C-type lectins with natural ligands of glycoproteins and microbial glycans (East & Isacke, 2002). They are found in abundance on human macrophages where they facilitate the uptake of glycosylated antigens by endocytosis. A polyclonal antibody is commercially available, raised against a human mannose receptor antigen, and it cross reacts with chimpanzee, Rhesus monkey, mouse, and rat proteins. The human mannose receptor antibody also cross reacts with rotifers, binding to the coronas of males but not females (Fig. 1). Additional experiments are necessary to confirm the functional significance of a mannose receptor in male *B. manjavacas*, like a western blot to compare antibody binding to male and female proteins and demonstrate that mannose receptor antibody binding to males inhibits mate recognition. The genetic characterization of the MRP signal and its receptor represents a major advance in the understanding of pheromone signaling by aquatic invertebrates and will make rotifers a premier model for its investigation.

Like MRP, the mixis induction protein (MIP) signal that triggers the switch from asexual to sexual reproduction is believed to interact with cell surface receptors, but these are as yet uncharacterized in rotifers. This type of signal transduction is well known in other invertebrates (Krieger & Breer, 1999), but we are just beginning to probe these pathways in rotifers. Also uncharacterized are the rotifer signal transduction pathways that are likely regulated by these
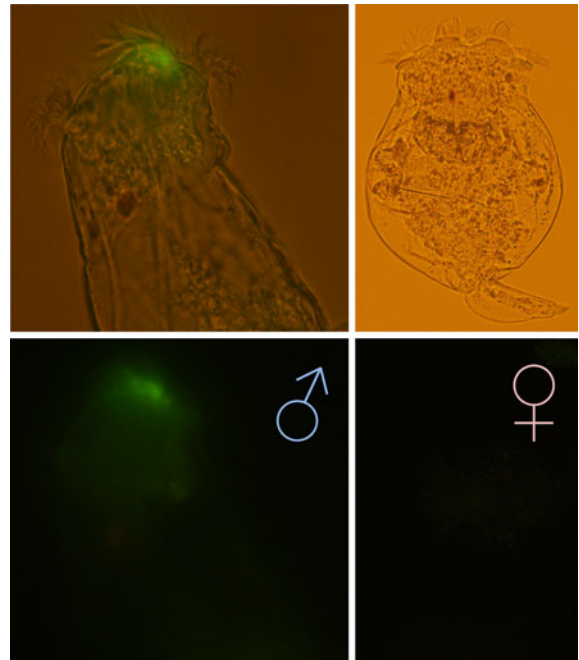

Fig. 1 Binding of the mannose receptor (MR) antibody to male and female *B. manjavacas*. Photomicrographs are the same individuals with (*top*) and without (*bottom*) white light. Male—×100, female—×25. Localized fluorescence represents the binding of a fluorescein-labeled secondary antibody to the primary MR antibody

receptors and the role that hormones play in this regulation.

Rotifer hormones

No rotifer hormones have been described, but brachionids are known to respond to a variety of vertebrate hormones (Gallardo et al., 1997, 1999, 2000). These authors investigated the effects on rotifer sexual female production and body size of vertebrate growth hormone, human chorionic gonadotropin, 17β-estradiol, and triiodothyronine, as well as the insect steroid 20-hydroxyecdysone and juvenile hormone. Effects of neurotransmitters 5-hydroxytryptamine (serotonin) and gamma aminobutyric acid (GABA) also were investigated. *Brachinous plicatilis* in batch cultures had 30% higher mixis rates than controls when exposed to 0.05 and 0.5 mg l^{-1} of juvenile hormone, but at concentrations of 50 mg l^{-1} mixis rates significantly decreased compared to controls (Gallardo et al., 1997). Gallardo et al. also reported that exposure to 50 mg l^{-1} 17β-estradiol

increased the mixis rate by 2-fold compared to controls. Gallardo et al. (2000) reported a doubling of the mixis rate in individual rotifer cultures from 4% in controls to 8% in the F2 generation of females exposed to 5 and 50 mg l^{-1} juvenile hormone.

More recently, the effects of progesterone on rotifers have been explored (Snell & DesRosiers, 2008). When *B. manjavacas* females were exposed to progesterone up to 10 mg l^{-1} (32 μM) in 15 ppt artificial seawater, there was no significant effect on asexual reproduction (*r*). However at 14 mg l^{-1}, *r* became strongly negative. At 5 mg l^{-1} progesterone enhanced resting egg production 3.9-fold over controls, but the enhancement disappeared at progesterone concentrations >10 mg l^{-1}. Progesterone significantly increased the fraction of mictic daughters, but not the males or resting eggs produced per mictic female. Exposure of males to 4 mg l^{-1} progesterone did not significantly increase circling behavior, but increased copulation rates 2.1-fold over controls. Females exposed to 4 mg progesterone l^{-1} during resting egg formation experienced a 1.8-fold increase in percentage of resting eggs hatching. Progesterone exposure significantly enhanced resting egg production in *B. manjavacas* and *B. ibericus*, but not *B. plicatilis* and *B. calyciflorus*. Progesterone is present in *B. manjavacas* biomass at 3–4 ng/g dry mass, which is comparable to the 2 ng/ml typical in mammalian blood (Stout et al., 2010). Waterborne exposures to progesterone were used in our experiments because they were the most practical. With this route of exposure, we cannot be sure about the actual dose to the rotifers because of uncertainties in ingestion, absorption, assimilation, and excretion. It is likely that much of the progesterone in the waterborne exposure (3–30 μM) never entered the test animals, but we do not know how much. Nevertheless, all of these studies demonstrate the responsiveness of rotifers to vertebrate hormones and suggest that as yet uncharacterized endogenous steroids probably play a central role in regulating rotifer reproduction.

If rotifers are like other invertebrates (Kohler et al., 2007), reproductive hormones bind to steroid receptors, either associated with cell membranes or localized in the nucleoplasm. These steroid receptor complexes can either trigger signal transduction pathways or act as transcription factors initiating gene expression. Although little is known about these processes in rotifers, it is possible to piece together a framework of mechanisms known from other invertebrates that can serve as a working hypothesis to direct research on rotifer endocrine systems.

Endocrine disruptors

Another line of evidence which supports the idea that steroid hormones control rotifer reproduction comes from invertebrate endocrine disruption studies (DeFur et al., 1999). Snell & Joaquim-Justo (2007) reviewed the effects of several putative endocrine disruptors on rotifers. Some pharmaceuticals, pesticides, and organic compounds are sufficiently similar to hormones that they interfere with normal endocrine signaling in aquatic animals (Guillette et al., 1996; Mathiessen & Gibbs, 1998). In *Brachionus calyciflorus*, for example, exposure to nonylphenol (estrogen agonist), testosterone (androgen agonist), and flutamide (androgen antagonist), all had significant effects on reproduction (Preston et al., 2000). Flutamide effects were significant two orders of magnitude lower than the no observed effect concentration (NOEC) for asexual reproduction. In contrast, 17α-ethinylestradiol and nonylphenol, two estrogenic contaminants common in surface waters, had little effect on *B. calyciflorus* sexual reproduction until concentrations reached close to asexual NOEC values (Radix et al., 2002). Joaquim-Justo (this symposium) has shown that the anti-androgens fenitrothion and cyproterone acetate altered the number of resting eggs produced per *B. calyciflorus* female at concentrations as low as 50 μg l^{-1}. This is 20–40 times lower than the asexual NOECs for these chemicals. Curiously, fenitrothion depressed resting egg production, whereas cyproterone acetate enhanced it by increasing the fertilization rate and average number of resting eggs produced per fertilized female. All of these observations suggest that sexual reproduction in brachionids is more sensitive to disruption by androgens and their antagonists than to estrogens. Exposure to progestins like progesterone also seems to have significant effects on brachionid sexual reproduction (Snell & DesRosiers, 2008).

Rotifer transcriptomes and the search for receptors

Data from a variety of *B. plicatilis* sequencing projects are now becoming available in public databases (Suga et al., 2007; Denekamp et al., 2009; Mark Welch et al.,

this symposium). These databases provide a rich resource to search for elements of an endocrine signaling system in the rotifer genome. The presence of genes for hormone receptors, steroidogenesis enzymes, and elements of metabolic pathways relevant to reproduction will provide insight for researchers to probe the molecular mechanisms regulating rotifer reproduction. An example of such an approach can be seen with the discovery of a rotifer progesterone receptor (Snell & DesRosiers, 2008). A search of the *B. manjavacas* GMOD database (http://gmod.mbl.edu, Mark Welch et al., this symposium) detected contig 1830 that encodes part of a 600-nucleotide gene with strong similarity to the progesterone receptor membrane component 1 of the sea urchin *Strongylocentrotus purpuratus* (XP 783332),with an E-value of $5E^{-43}$ and 51% identity. The presence of a membrane-associated progesterone receptor in rotifers suggests that a progesterone-like ligand may play a role in regulating reproduction. Progesterone is a steroid molecule that plays a key role in reproduction of a variety of vertebrates (Graham & Clarke, 1997). Interestingly, an estrogen receptor has not been found in the rotifer transcriptome and there is only weak evidence for the presence of an androgen receptor. It should be noted, however, that sequencing of the rotifer transcriptome is not complete, so that these types of receptors may yet be discovered.

Finding a progesterone receptor in the rotifer transcriptome motivated a series of experiments exploring the effects of progesterone on several aspects of amictic and mictic reproduction (Snell & DesRosiers, 2008). The tissue distribution of the progesterone receptor in *B. manjavacas* females and males also has been described, and it has been demonstrated that this receptor binds mammalian progesterone (Stout et al., 2010). The rotifer progesterone receptor is localized in the ovaries, vitellarium (yolk gland), oviduct, and eggs of females, and in the seminal vesicle, rudimentary gut, and sperm duct of males. Receptor binding is specific for progesterone; steroids with similar structures did not bind. The phylogeny of the membrane-associated progesterone receptor (MAPR) has been investigated (Smith et al., this symposium). Phylogenetic relationships among clades of the *B. plicatilis* cryptic species complex based on this gene are compared to those based on COI gene sequences. Analysis of ratios of nonsynonymous to synonymous nucleotide substitutions (d_N/d_S) by

Smith et al. reveals that MAPR is under strong purifying selection.

Oocyte maturation pathway

The maturation of oocytes in many animal species is tightly orchestrated through signal transduction pathways (Schmitt & Nebreda, 2002). An important one of these is a progesterone-mediated pathway that includes a progesterone receptor gene, a heat shock 90 gene, and a mitogen-activated protein kinase (MAPK) gene (Kyoto Encyclopedia of Genes and Genomes, KEGG, www.genome.ad.jp/en/gn_kegg.html, ko04914). In rotifers, oocyte maturation occurs sequentially, initiated by the filling of eggs with yolk produced by the vitellarium (Gilbert, 1983). Egg development in utero is a critical time, when the reproductive mode of daughters (asexual or sexual) is determined (Snell et al., 2006). Exposure to waterborne progesterone has an impact on rotifer reproduction (Snell & DesRosiers, 2008), so we hypothesized that suppressing the expression of genes in the oocyte maturation pathway might produce recognizable reproductive phenotypes.

RNA interference (RNAi) has revolutionized the study of gene expression in a variety of organisms, enabling experimentation with many species that are not amenable to traditional gene expression analysis (Fire et al., 1998; Whangbo & Hunter, 2008). RNAi is phylogenetically ancient and ubiquitous throughout eukaryotes (Cerutti & Casas-Mollano, 2006; Shabalina & Koonin, 2008), so we expected this pathway to be present in rotifers. We have developed RNAi techniques for gene transcript suppression in rotifers and have employed them in the analysis of rotifer genes putatively involved in the oocyte maturation pathway (Snell et al., 2010). Transfection of rotifers with heat shock protein 90 (hsp90), a progesterone receptor (MAPR), or mitogen-activated protein kinase (MAPK) dsRNA, all significantly increased the mean proportion of non-reproductive females in the treated populations relative to controls. For females transfected with hsp90 dsRNA there was a 25.7% increase in non-reproductive daughters, and a 20.1 and 26.9% increase for MAPR and MAPK genes, respectively. The mean rates for non-reproductive daughter production in the RNAi treatments were 2–2.4 times higher than the rate in the controls. These data suggest that the hsp90, MAPR, and MAPK genes in rotifers play a role in reproduction

and that RNAi knockdown of their expression increases the frequency of non-reproductive offspring. I further hypothesize that the process of oocyte maturation is conserved in rotifers and regulated by steroids through signal transduction pathways similar to those of vertebrates.

Steroid metabolism genes

If rotifers use steroids to regulate their reproduction, enzymes for steroid biosynthesis are expected to be present in their transcriptome. We have searched available sequences in the rotifer transcriptome (Suga et al., 2007; Denekamp et al., 2009, Mark Welch et al., this symposium) and identified six genes with products whose putative function is steroid biosynthesis (Table 1). One is cytochrome P450 4vC (P450) that is a monooxygenase/oxidoreductase with metal ion and heme binding capability (GenBank ES467780). A second is estradiol 17-β dehydrogenase 12 (17-β hydroxysteriod dehydrogenase 12) (Est) that converts estrone to 17β-estradiol (Brown et al., 2007). A third is sphingolipid delta 4 desaturase/c-4 hydroxylase protein des2 (Sph) that is involved with membrane lipid metabolism and sphingolipid biosynthesis (Mizutani et al., 2004). If these enzymes play key roles in rotifer reproduction, RNAi knockdown of their gene expression should reduce reproduction.

Sexual reproduction is one of the most sensitive bioassays available for rotifers (Preston et al., 2000), so we examined the effect on mixis rate of transfection with dsRNA from these three genes (Fig. 2). Mixis rates are always zero in brachionid resting egg hatchlings that reproduce exclusively asexually. Mictic daughters are typically observed in the F1 of our strain of *B. manjavacas*, but at a frequency of <5%. Mixis rate usually increases in subsequent generations (Gilbert, 2002), reaching a maximum of about 30–40%, depending on the species (Serra et al., 2005). In a mixis bioassay where single F1 females are cultured in 0.5 ml and auto-induce mixis, we observed an average of 4% mictic daughters in the controls transfected with PBS buffer. In the F2 generation, this increased to 12% at 72 h and 17% at 96 h. In comparison, we observed only 1–2% in F1 offspring of mothers transfected with dsRNA of one of the three steroidogenesis enzyme genes (P450, Est, Sph). Mixis was maximally suppressed in the F2 generation at 72 h where rates were 0, 1, and 3%,

Table 1 Genes potentially involved in steroidogenesis in *B. manjavacas*

Gene	Identifier	Function	PCR primers (5′–3′)	dsRNA size (bp)	SwissProt E-value
Cytochrome P450 4vC (P450)	ES467780 (GenBank)	Enzyme activities involved in steroidogenesis	for:TTGATGCCAGTAGCTACG rev:GTTGTTACAGAAAGTCTG	460	4.E−37
Estradiol 17-β dehydrogenase 12 (EST)	Orf 2348 (Bm)	Catalyzes the interconversion of testosterone, androstenedione, estradiol, and estrone.	for:TTTCAATACTTTGGCCTGTTCA rev:ACGCCAAGCATCATGTTAAA	875	5.E−77
Sphingolipid delta 4 desaturase/c-4 hydroxylase (SPH)	Orf 18166 (Bm)	Important signaling molecule triggering the entry into both meiotic and spermatid differentiation pathways of *Drosophila* spermatogenesis	for:AGAATCGCAGCGCAATTAGT rev:ATCTGATGCTTGAGCCCATT	858	1.E−113
Estrogen receptor binding protein (ERB)	contig 2270 (Bp male)	Enhances the activity of estrogen receptor	for:CGAGTTGGATACCGGGTGTA rev:TGTTTTTAGATTGCTTTTGTTCTTG	551	2.E−35
Sterol O-acyltransferase 1 (SOA)	contig 2490 (Bp male)	Facilitates lipoprotein assembly and dietary cholesterol absorption, acts as a acyltransferase and ligase	for:TGCATCCGAAAAGTTGATGA rev:GCAACCACATAACAAGGCAAG	567	2.E−16
Steroid reductase (SR)	contig 2807 (Bm EST)	Catalyzes the 5α-reduction of several animal steroid substrates	for:AAAAATGGAATTAACGCTTTTCA rev:GGACACCAGTAATAATGATTTCAA	739	2.E−41

Fig. 2 Effect of RNAi knockdown of steroidogenesis genes on mictic reproduction. The X-axis is the type of dsRNA used in the transfection. PBS is the phosphate-buffered saline control; the full gene names are listed in Table 1. The Y-axis is the percent of transfected females producing F1 or F2 mictic offspring. *Vertical lines* indicate standard error
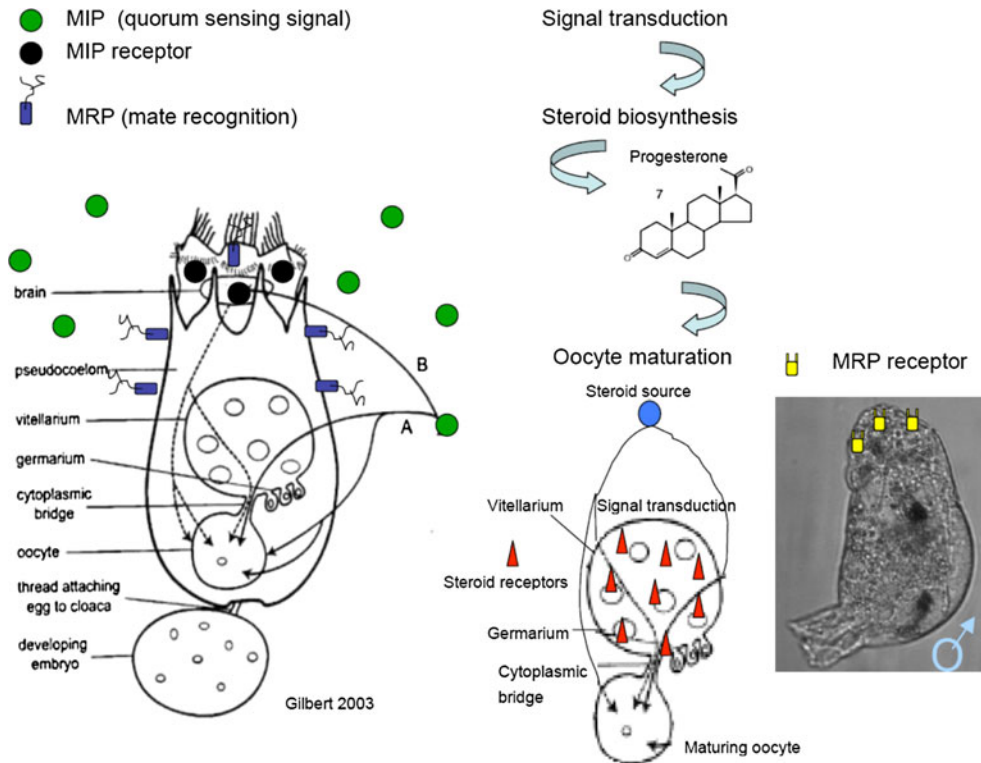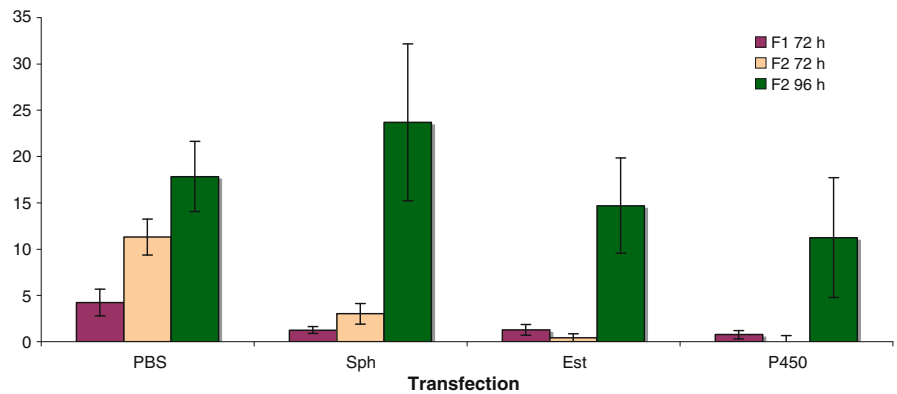

Fig. 3 Cartoon of pheromone and hormone signaling in brachionid rotifer reproduction. Females secrete the MIP protein that accumulates in the medium. Females have MIP receptors in their coronas that regulate a signal transduction pathway that triggers mictic reproduction. Female reproduction is controlled internally by steroid hormone signals and receptors that regulate oocyte maturation and embryo development. The body surface of females also is covered with a glycoprotein called the MRP. Males detect this protein with MRP receptors in their coronas which trigger mating behavior

respectively. All were significantly lower than control mixis by ANOVA and Dunnett's test ($P = 0.007$, 0.003, 0.032 for P450, Est, Sph, respectively). The suppressive effect of maternal transfection disappeared by 96 h when mixis rates of transfectants, and controls again were not significantly different. These data suggest that reduced expression of enzymes putatively involved in steroid

biosynthesis can suppress mixis compared to controls. The functional role of steroids in initiating and regulating the progression of mixis remains to be described.

Current model of rotifer endocrine signaling

A model of rotifer endocrine signaling is presented in Fig. 3. Genes critical for sexual reproduction have been identified, like the MRP on females and its putative receptor on males that initiates mating behavior. Similar approaches are being employed to identify the genes for the secreted mixis inducing protein MIP and its receptor on females. Rotifers regulate other aspects of their reproduction through an endocrine system that uses oxidized sterols similar to most other animals. Transcriptome surveys have shown that steroid receptors, biosynthetic enzymes, and signal transduction pathway elements are present in brachionids. There is strong evidence that steroids similar to progesterone and androgens are employed as regulatory signals, whereas estrogens seem less important. RNAi knockdown of various steroid receptors or biosynthetic enzymes suppresses either amictic or mictic reproduction, or both. These genes are being used in phylogenetic analyses to more finely resolve cryptic species and to better understand their evolutionary dynamics, and to comprehend the evolutionary forces responsible for the origin and maintenance of sex.

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