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Review article

Rapid sex steroid effects on reproductive responses in male goldfish: Sensory and motor mechanisms

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ABSTRACT

Contribution to Special Issue on Fast effects of steroids.

Although we have learned a great deal about the molecular mechanisms through which sex steroids rapidly affect cellular physiology, we still know little about the links between those mechanisms and behavioral output, nor about their functional consequences in natural contexts. In this review, we first briefly discuss the contexts associated with rapid effects of sex steroids on reproductive behaviors and their likely functional outcomes, as well the sensory, motor, and motivational mechanisms associated with those effects. We then discuss our recent studies on the rapid effects of testosterone in goldfish. Those studies indicate that testosterone, through its aromatization and the subsequent activation of estrogen receptors, rapidly stimulates physiological processes related to the release of milt/sperm through likely influences on motor pathways, as well as behavioral responses to female visual stimuli that may reflect, in part, influences on early stages of sensory processing. Such motor and sensory mechanism are likely important for sperm competition and mate detection / tracking, respectively, in competitive mating contexts. We also present preliminary data on rapid effects of testosterone on responses to pheromones that may not involve estrogen receptors, suggesting a dissociation in the receptor mechanisms that mediate behavioral responses in different sensory modalities. Lastly, we briefly discuss the implications of our work on unresolved questions about rapid sex steroid neuromodulation in fish.

Although a primary function of sex steroid hormones is to sculpt neural pathways through transcriptional regulation, it is now clear that steroids can also rapidly modulate those circuits in ways that influence ongoing social interactions. Such rapid influences are associated with the activation of membrane steroid receptors that directly affect cell physiology and, in some cases, behavior. While our understanding of the molecular mechanisms associated with rapid steroid effects on cell physiology is increasing rapidly, we are only just beginning to determine how those mechanisms modulate behavioral output. Furthermore, we still know little about the functional consequences of rapid steroid effects in naturalistic settings. In this article, we first briefly review the contexts in which sex steroids produce rapid influences on social behaviors related to reproduction, which leads to the prediction that rapid steroid signaling mechanisms help animals adjust ongoing behaviors to changing social contexts and, ultimately, increase reproductive competitiveness. We then review what is known about the neural mechanisms underlying rapid behavioral effects of sex steroids, including rapid influences on brain systems related to sexual motivation, motor output, and sensory processing, which highlights that we know the least about rapid steroid influences on early stages of sensory processing. Finally, we summarize our own work on the rapid effects of testosterone (T) and estradiol (E2) on behavioral and physiological responses associated with reproduction in male goldfish, emphasizing what those studies suggest about the motor and sensory mechanisms through which sex steroids may facilitate reproductive success in competitive mating contexts.

1. Context for rapid steroid influences on reproductive behavior

Social stimuli, including sexual stimuli, produce fluctuations in circulating steroids in a wide range of vertebrates (reviewed in Goymann, 2009; Nyby, 2008; Oliveira et al., 2002). One important function associated with such fluctuations is to alter behavioral tendencies in future social interactions, which occur well after hormone levels have returned to baseline, likely through traditional genomic mechanisms (Gleason et al., 2009; Oliveira et al., 2009; Oyegbile and Marler, 2005; Trainor et al., 2004). However, we have begun to identify additional functions for socially induced steroid fluctuations, in

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particular their ability to enhance behavioral and physiological processes – likely through non-genomic mechanisms – that may rapidly promote mating success during social interactions that occur coincident with the surges. In an earlier review of data on socially induced, "reflexive" T surges in rodents, Nyby (Nyby, 2008) highlighted three key findings that support such functions. First, pre-exposure to female stimuli induce peripheral T surges in males and enhance copulatory behaviors that follow soon thereafter (de Jonge et al., 1992; James and Nyby, 2002). Second, exogenous T surges, at least in gonadally intact males, rapidly mimic the effects of female stimuli on those behaviors (James and Nyby, 2002; Malmnas, 1977). Third, aromatase inhibition rapidly decreases copulatory behaviors (Taziaux et al., 2007). Together, these findings led Nyby to conclude that endogenous surges of circulating T mediate the behavioral responses induced by female stimuli through estrogenic neurosteroid mechanisms. As he noted, the ability to display reproductive behaviors is not dependent on the surges. Instead, T surges facilitate processes, such as male courtship behavior, mounting of females, and sperm ejaculation, that are likely to promote reproductive success in naturalistic contexts. Thus, the importance of socially induced hormone fluctuations may not always be apparent in simplified laboratory tests, much as the importance of circulating steroids in primate sexual behavior was not apparent in simple pair tests, but emerged in tests done in naturalistic, group contexts (reviewed in Wallen, 2001). Those classic studies demonstrated that sex steroids are actually critical for the expression of reproductive behavior in primates in such contexts, particularly in social subordinates, for which sexual behavior is risky and requires a high level of steroid-dependent motivation to initiate. Although those studies did not directly address whether fluctuations in steroid levels can rapidly influence behavior, they pointed out how critical context can be for assessing the function of sex steroids, including, in all likelihood, their rapid effects. In competitive mating contexts, in particular, the rapid activation of motivational or arousal processes that promote reproductive behaviors and/or decrease the time it takes to complete them would likely enhance reproductive success. So too would the rapid activation of mechanisms that help animals detect potential mates in their natural environment, that enhance their own attractiveness to potential mates, and that increase paternity once mating occurs through behavioral or physiological tactics that enhance fertilization. Thus, it is likely that sex steroids rapidly modulate many of the same motivational, sensory and motor systems already shaped by genomic steroid mechanisms, thereby allowing their output to be adjusted in relation to social context and thus avoiding, as Wingfield originally proposed (Wingfield et al., 1990, 2001), the costs associated with sustaining maximal levels of sex steroids, particularly T.

Importantly, rapid steroid mechanisms that facilitate behaviors likely to enhance reproductive success are not unique to rodents or even mammals. For example, in male toadfish the vocalizations of other males induce rapid surges of circulating 11-ketotestosterone (KT), a potent, non-aromatizable androgen in fish, and increase call rates in males. Injection of exogenous KT, like exposure to male vocalizations, also increases call rates within 30 min (Remage-Healey and Bass, 2005). Increased call rates likely facilitate reproductive success in competitive mating contexts by helping repel competitors from a male's territory and/or attracting females to it. In contrast, in birds the peripheral surges of sex steroids that occur in response to social stimuli have not been widely linked to rapid effects on territorial or reproductive behaviors (Goymann et al., 2015), though it should be noted that few studies have measured rapid effects of peripheral T surges on reproductive/courtship behaviors. However, social stimuli can also alter levels of brain neurosteroids independently of changes in circulating steroids in several avian species (Charlier et al., 2011; Cornil et al., 2009; Remage-Healey et al., 2008), and those fluctuations do play a role in rapid behavioral regulation. For instance, estradiol (E2) produced in the preoptic area rapidly stimulates sexual behavior in Japanese quail (reviewed in Cornil et al., 2013), and E2 elevations in the auditory forebrain rapidly affect song preferences potentially important for reproduction in zebra finches (Remage-Healey et al., 2010). Local elevations in E2 in these species depend on rapid changes in aromatase activity and/or subsequent, activity-dependent E2 release (Balthazart et al., 2006; Cornil et al., 2006a, 2005; Remage-Healey et al., 2011). Socially-induced elevations of neurosteroids also play a role in the rapid regulation of aggression outside of the breeding season in some birds and rodents (reviewed in Heimovics et al., 2015), and similar, rapid neurosteroid mechanisms may be operative in some fish. The production of KT in the brain has been causally linked to the expression of parental behaviors in bluebanded gobies (Pradhan et al., 2014a), and socially induced, rapid changes in brain neurosteroid production have been described (Black et al., 2005; Lorenzi et al., 2012), though these changes have not yet been linked to rapid behavioral regulation (Black et al., 2011). Together, studies across taxa make it clear that rapid steroid signaling mechanisms are a fundamental reproductive regulatory mechanism in vertebrates, but whether such mechanisms are activated by socially induced elevations of circulating steroids, local elevations of neurosteroids in discrete brain areas, or a combination of both likely varies by species and/or social context. For example, the need to coordinate and sustain central and peripheral responses for a period of prolonged social contact may favor rapid influences activated by changes in circulating steroids. On the other hand, in some species and/or contexts, systemic elevations of circulating steroids are disadvantageous, either because they activate off-target pathways that promote inappropriate responses or because responses to particular social cues must be immediate but then suppressed as the context quickly changes. In these cases, mechanisms favoring local changes in neurosteroid production may be predominant.

2. The neural mechanisms that link rapid steroid physiology to behavior

Although it is now clear that sex steroids rapidly alter cellular physiology and reproductive behavior, we know little about the relationship between the two. That is, how do rapid effects on cell physiology translate into behavioral output? In principle, steroids could influence a number of brain processes critical for the expression of reproductive behavior, from early stages of stimulus detection to sexual motivation to motor output critical for behavioral expression. Work in several species has demonstrated that sex steroids can rapidly influence motivational processes in sociosexual contexts. In male rats and mice, elevations of T and E2 decrease the latency for males to initiate copulation (James and Nyby, 2002; Malmnas, 1977), and in Japanese quail, elevations of E2 in the preoptic area rapidly increase several appetitive responses towards sexual stimuli that, like latency to copulate, are considered measures of sexual motivation (Cornil et al., 2006b; de Bournonville et al., 2016; Seredynski et al., 2013). There is also strong evidence that steroids can modulate social behavior through non-genomic influences on motor output pathways. The rapid effects of KT on vocal output in Gulf toadfish mentioned earlier are associated with rapid increases in neuronal activity in the hindbrain neurons that drive calling behavior (Remage-Healey and Bass, 2006a). KT, T and E2 can similarly influence these hindbrain motor circuits in the related plainfin midshipman (Remage-Healey and Bass, 2004a, 2004b, 2007).

Sex steroids may also change behavioral responses to conspecifics by rapidly influencing sensory processing mechanisms. In zebra finches, E2 rapidly alters neural responses to song in auditory regions of the forebrain (Remage-Healey et al., 2010, 2012), as well as the functional connectivity between those regions and areas that integrate auditory input into behavioral responses (Pawlisch and Remage-Healey, 2015). These neural effects are associated with enhanced behavioral selectivity for particular songs, which may influence reproductive success in some contexts. It is also possible that sex steroids rapidly modulate peripheral stages of sensory processing, though steroid influences on ascending sensory responses to social cues documented thus far have primarily

Table 1
Summary of sites of GPER/GPR30 expression in goldfish and a comparison with sites of reported aromatase, ERα and/or ERβ1/2 expression in teleost fish. Abbreviations: +, presence; −, absence or not reported; N/A, data not available. Table modified from Mangiamele et al., 2017. Reference guide: {1, 2, 3, (Mangiamele et al., 2017); 4, (Gelinas and Callard, 1997a); 5, (Tchoudakova and Callard, 1998); 6, (Forlano et al., 2001); 7, (Goto-Kazeto et al., 2004); 8, (Menuet et al., 2003); 9, (Forlano et al., 2001); 10, 11, (Munchrath and Hofmann, 2010); 12, (Fergus and Bass, 2013a); 13, (Tchoudakova et al., 1999); 14, (Zempo et al., 2013)}.

Anatomical Location	GPR30	Aromatase	ERα and/or ERβ1/2
Primary Visual Pathways ***From Springer and Gaffney, 1981; Yamamoto and Ito, 2008; Northcutt, 2006; Saidel et al., 2001***	Mangiamele et al. (2017): RT-PCR (1), *in situ*/mRNA (2); IHC/protein (3)	Enzyme in goldfish (4); mRNA in goldfish (5); mRNA transcript and enzyme in midshipman fish (6); mRNA in zebrafish; (7); mRNA and/or protein in trout (8)	ERα mRNA and/or protein in trout (8); ERα mRNA in midshipman fish (9); ERα mRNA or protein in cichlid (10); ERβ (subtype unspecified) mRNA or protein in cichlid (11) ERβ1/ERβ2 protein in midshipman fish (12) ERβ (subtype unspecified) mRNA in goldfish (13); ERα, ERβ1, and ERβ2 mRNA in medaka (14)
Retina	+ (1)	+ (4, 5, 7)	+ ERβ only (13)
Optic tract (OPT)	+ (2)	+ (6, 7)	- (10,11)
Optic tectum (OT)	+ (1,2)	+ (4, 8), but not (6)	+ (10,11), but not (14)
Dorsal accessory optic nucleus (NA-OD)	+ (2)	-	-
Central pretectal nucleus (CPN)	+ (2)	-	-
Parvocellular superficial pretectal nucleus	-	-	-
Dorsal accessory optic nucleus (NA-OD)	+ (2)	-	-
Suprachiasmatic nucleus (SCN)	+ (2)	+ (4,6,8)	- (9, 10, 11, 12)
Preoptic area (PPa, PM)	+ (2,3)	+ (4,6,7)	+ (8, 9, 10, 11, 14), + but not PM (12)
Ventrolateral nucleus of the torus semicircularis (retinal target)	-	+ (7)	N/A
Anterior tuberal nucleus (aTn; retinal target)	-	+ (4,7)	+ (9, 10, 11), + ERβ2 only (12)
Dorsal thalamus	-	+ (4,6)	-
Secondary Visual Pathways (Tectal, Thalamic and Preglomerular Targets or Visually-responsive areas without direct retinal input)			
Dorsolateral tegmentum (tectal efferent target)	Diffuse (2)	-	-
Horizontal commisure (HC) (Tectal efferent pathway)	+ (2)	-	-
Medial / Dorsal / Central regions of the dorsal telencephalon (preglomerular/ thalamic targets)	-	+ (4,7, 8)	+ (9, 10, 11) Note: ERβ cytosolic expression
Reticular formation	-	+ (4, 7)	-
Nucleus isthmi	-	-	-
Magnocellular superficial pretectal nucleus	-	-	-
Social Behavior Network Nuclei (olfactory targets are indicated)			
Lateral nucleus of the ventral telencephalon (Vl; medial olfactory tract target)	-	+ (6,7)	+ ERβ only (11)
Supracommissural nucleus of the ventral telencephalon (Vs; medial olfactory tract target)	-	+ (4,7)	+ (9, 10, 11 ,14), + ERβ2 only (12)
Ventral nucleus of the ventral telencephalon (Vv)	-	+ (4,7, 8)	+ (9, 10, 11), + ERβ2 only (12), + ERβ1 only (14)
Preoptic area (PPa, PM, PG) (medial olfactory tract and retinal target)	+ (2,3)	+ (4,6,7, 8)	+ (8, 9, 10, 11, 14), + but not PM (12)
Anterior tuberal nucleus (also a retinal target; see above)	-	+ (4,7)	+ (8, 9, 10, 11), + ERβ2 only (12)
Ventral tuberal nucleus	-	+ (4,7)	+ (10,11,14, but not 9)
Periaqueductal gray (PAG)	+ (2,3)	+ (4,6,7)	+ (10,11, but not 9), + ERβ2 only (12)
Additional Sites of Expression			
Saccule (auditory)	N/A	N/A	+ (12)
Olfactory bulb	-	+ (7)	+ (10,11), but not (14)
Posterior division of dorsal telencephalon (Dp, lateral olfactory tract target)	-	+ (7)	- (10,11)
Inferior hypothalamus	-	+ (7)	+ (10,11)
Cerebellum	-	+ (7)	-
Valvula cerebellum cerebelli (VC)	+ (2)	+ (4)	N/A
Reticulospinal neurons (MLF)	Diffuse (2)	+ (4)	+ ERα only (14)
Midbrain tegmentum	Diffuse (2)	+ (6)	Diffuse (10,11)
Torus semicircularis (auditory)	-	+(8), but not (6)	- (9, 14), + (10,11), +ERβ2 only (12)
Periventricular posterior nucleus (NPPv)	N/A	N/A	+ (14)

been associated with chronic manipulations that likely work through genomic mechanisms. For example, slow-release androgen implants shift electrosensory responsiveness of primary afferents to frequencies characteristic of the electric fields produced by conspecifics in stingrays, which likely helps males locate cryptic females during the mating season (Sisneros and Tricas, 2000). Both T and E2 also enhance responsiveness of saccular afferents to frequencies associated with male vocalizations in plainfin midshipman (Sisneros et al., 2004; Sisneros and Tricas, 2000). Similarly, chronic androgen manipulations selectively enhance early sensory responses to a sex pheromone in several cyprinid fish; three weeks of daily methyltestosterone treatments increase electro-olfactogram responses of the olfactory epithelium to the ovulatory pheromones that elicit courtship in that family (Belanger et al., 2010). Evidence that steroids may have non-genomic effects on primary sensory neurons and/or structures associated with early stages of sensory detection is just beginning to emerge. Membrane-bound steroid receptors, such as G protein-coupled estrogen receptors (GPER/GPR30), have been identified in fish retina and visual system (Friesen et al., 2017; Mangiamele et al., 2017) and the hair cells of the lateral line in aquatic frogs (Hamilton et al., 2014). The classical sex steroid receptors ERα, ERβ, and the androgen receptor (AR) are present in primary sensory neurons and/or in structures associated with early stages of sensory detection and processing in numerous species (e.g., see Table 1 for a summary of ERα and ERβ distribution in teleost fish visual system). These include the vomeronasal sensory neurons of mice (Cherian et al., 2014a, 2014b), the inner ear of plainfin midshipman (Fergus and Bass, 2013b; Forlano et al., 2005, 2010) and zebra finches (Noirot et al., 2009), the retina of rats (Cascio et al., 2007), and the olfactory bulbs of rats and several fish species (Gelinas and Callard, 1997a; Maruska and Fernald, 2010; Mitra et al., 2003; Portillo et al., 2006). However, we do not yet know if these receptors can be trafficked to neuronal membranes in those tissues as they can be in others. We also do not yet know if sex steroids can rapidly modulate sensory processes that facilitate reproduction. Recent studies in mice have shown that E2 can rapidly decrease electrophysiological responses evoked by non-social odorants in main olfactory receptor neurons (Kanageswaran et al., 2016), as well as responses evoked by conspecific urine and the sulfated steroids in it in vomeronasal sensory neurons (Cherian et al., 2014a), though the social significance of either finding has not yet been resolved.

3. Endocrine contexts for rapid steroid effects in goldfish

Our work uses the domesticated comet goldfish (*Carassius auratus*) to investigate the mechanisms through which sex steroids produce rapid effects on reproductive behavior. Goldfish males engage in a scramble competition for access to ovulated females. Females typically ovulate in the early morning, and subsequent spawning therefore occurs in dim light conditions (reviewed in Kobayashi et al., 2002). When they spawn, many males will chase, nudge and spawn repeatedly with individual females. Although detailed field work is not available on mating in the wild Crucian carp species from which goldfish were derived (Komiyama et al., 2009), carp respond to the same sex pheromones (Bjerselius et al., 1995; Lim and Sorensen, 2012; Olsen et al., 2006) and are, like goldfish, batch spawners {females ovulate several times each mating season and, presumably, mate with multiple males each time (Aho and Holopainen, 2000)}. Thus, the goldfish promiscuous mating system is likely not a function of domestication, though selective breeding pressures associated with domestication have undoubtedly modified some characteristics.

As in males of many other vertebrate species, exposure to female sexual stimuli affects levels of circulating steroids in male goldfish. However, whereas agonistic cues from other males often increase 11-ketotestosterone (KT) or KT and T in male teleosts (Remage-Healey and Bass, 2005; Saraiva et al., 2017; Sessa et al., 2013; Teles and Oliveira, 2016; von Kuerthy et al., 2016; Antunes and Oliveira, 2009; Dijkstra et al., 2012; Oliveira et al., 1996), female sexual stimuli increase T, but not KT, in male goldfish (Kobayashi et al., 1986). Those T surges occur subsequent to elevations of gonadotrophins (GtH), which are themselves induced by the pre-ovulatory pheromone 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (17,20BP) that is released by females into the water beginning 7–10 h prior to ovulation (Sorensen et al., 1989a; Stacey et al., 1989). This pheromone thus acts as a context cue indicating that a spawning opportunity is imminent, though it is not yet clear how the elevations of GtH it induces presumably lead to selective increases in T, but not KT. At ovulation, females begin releasing a mix of prostaglandins (chief among them prostaglandin F2α and 11-keto-prostaglandin F2α, hereafter collectively referred to as PGFs) that rapidly elicit courtship/following behaviors in males (Appelt and Sorensen, 2007; Sorensen et al., 1988b, 1989a).

In the context of understanding rapid steroid effects, the fact that T surges are the predominant physiological response to female stimuli that predict spawning is noteworthy. Androgen receptors (ARs) in some fish have higher affinities for KT than T and/or can be more strongly transactivated by KT (Bain et al., 2015; Olsson et al., 2005; Todo et al., 1999), though in many species, including goldfish, the reverse is true or the ARs are similarly responsive to both androgens (Braun and Thomas, 2004; de Waal et al., 2008; Sperry and Thomas, 1999; Takeo and Yamashita, 2000). Nonetheless, KT typically has more potent, long-term effects on male-typical behaviors. For example, in goldfish, chronic KT implants, but not T implants, stimulate male-typical courtship in females, though only fish treated with both androgens showed full levels of male-typical courtship in that study (Stacey and Kobayashi, 1996). Though KT has more potent chronic effects, the fact that T responds to sexual stimuli suggests that T may act rapidly upon circuits that develop in response to chronic KT to support male-typical behaviors, possibly amplifying their activity in reproductive contexts through non-genomic mechanisms. Furthermore T, but not KT, can be aromatized into E2, suggesting that any such rapid modulation may involve the activation of membrane estrogen receptors, as discussed earlier occurs in many birds and mammals. Aromatase is expressed in numerous regions of the goldfish brain (see Table 1), including several nodes of the Social Behavior Network (SBN), a group of interconnected, primarily forebrain structures conserved across vertebrates that respond to sex-steroids and modulate various social behaviors (Goodson and Kabelik, 2009; Newman, 1999; O'Connell and Hofmann, 2012). Importantly, aromatase is also expressed in ascending sensory pathways critical for helping male goldfish detect and orient towards ovulating females, particularly the olfactory and visual systems, as well as in a hindbrain premotor region and in the gonads (Callard et al., 1993; Gelinas and Callard, 1993, 1997b; Pasmanik and Callard, 1985). Brain estrogens are produced primarily in radial glial cells and mediate neurogenic functions related to brain growth, sexual differentiation, and/or injury repair in teleosts (reviewed in Pellegrini et al., 2016; Xing et al., 2014). However, E2 produced in and released by those cells or neurons (see further discussion below) following socially induced elevations of T could rapidly modulate, in parallel, numerous reproductive processes, including behavior, through estrogen receptor-mediated mechanisms. In particular, the anatomical distribution of aromatase suggests it could produce estrogens that influence the processing of sensory cues critical for the detection of female stimuli, the motivation to approach them, and/or the downstream motor pathways associated with the generation of behavioral and/or physiological responses towards them. Together, such estrogenic influences may increase the likelihood that a male can successfully locate, maintain proximity to, and ultimately fertilize the eggs of an ovulating female in competitive mating contexts like those that occur in goldfish and related carp.

4. Rapid T/E2 effects on goldfish physiology: potential motor mechanisms

One way that T surges induced by female stimuli may increase

mating success in competitive mating environments is via rapid effects on the neuro-motor mechanisms that underlie mating, particularly those that facilitate sperm output and release. For instance, in males of several species, even short-term exposure to female stimuli results in rapid increases in ejaculate volume and quality that could be mediated, in part, by the T surges that occur in response to those stimuli (Cornwallis and O'Connor, 2009; Jeannerat et al., 2017). To explore that possibility, we assessed the ability of T and E2 to rapidly influence amounts of milt (analogous to seminal fluid) and sperm in goldfish. The pre-ovulatory pheromone 17,20 BP not only increases GtH release in males and likely, as a result, mediates the T surges induced by females, but also increases amounts of expressible milt (Zheng and Stacey, 1996, 1997). The post-ovulatory pheromone PGF2α also increases milt volume within 30 min of exposure, although it appears to do so through a gonadotropin-independent mechanism and is highly dependent upon social context, as it only occurs in males that are grouped with other males (Sorensen et al., 1995, 1988a, 1989b; Zheng and Stacey, 1997). We reasoned that if the effects of female preovulatory pheromones on milt, particularly htose assoicated iwth GtH mechanisms, are mediated by peripheral increases in T, then elevations of T would acutely increase amounts of expressible milt in isolated males. Indeed, we found that injecting males intraperitoneally with T at a dose that causes elevations within the physiological range typically induced by female stimuli increased ejaculate volume and sperm density within 1 h of injections relative to fish injected with saline (Mangiamele and Thompson, 2012). Furthermore, aromatase inhibition blocked that effect, as did both low (1.5 μg) and high (15 μg) doses of ERβ and ERα antagonists, while T's effects were mimicked by intraperitoneal administration of E2:BSA. Thus, our results indicate that T, via its conversion to E2 and the activation of membrane-bound estrogen receptors, can have rapid effects on physiological processes that directly impact reproductive output. Further, they imply that both membrane-bound versions of ERα and ERβ are necessary, perhaps through a mechanism involving heterodimerization between the two. ER heterodimers, as well as homodimers, have been detected in plasma membranes (Guo et al., 2005; Razandi et al., 2004). Nonetheless, the possibility for cross-reactivity of ER-specific antagonist drugs makes that interpretation tentative. Rapid steroid effects on ejaculate volume and sperm density are potentially critical for increasing reproductive success in goldfish, particularly in the context of a mating system in which multiple males court and spawn with a single female when she ovulates. Indeed, pre-exposure to 17,20BP, which as mentioned likely drives the T surge in males, increases paternity in a competitive mating paradigm (Defraipont and Sorensen, 1993; Zheng et al., 1997), perhaps in part through rapid T/E2 influences on the amounts of milt and sperm released during spawning. Together, these results are important because they demonstrate that rapid estrogenic signaling could play a critical role in male reproductive success by enabling males to quickly modulate their reproductive physiology and increase their chances of fertilizing a female's eggs in response to changes in the social environment that predict impending mating opportunities.

The mechanism mediating this rapid increase in expressible milt is currently unknown, but could involve rapid T/E2 effects on motor systems, either directly on the smooth muscle contractions associated with the expression of milt and sperm from seminiferous tubules (Demski and Hornby, 1982; Weisel, 1949) or on the neural systems that control those contractions. Whether sex steroids influence the contractile properties of the muscles that release sperm in teleosts is not known, however, rapid (within 6 min) muscle contractions in response to T have been documented in the rat seminiferous tubules and bulbospongiosus muscle (Farr and Ellis, 1980; Sachs and Leipheimer, 1988). Thus, it is possible that a similar mechanism could facilitate the rapid release of milt from the ducts during spawning in goldfish following T's local conversion to E2. Alternatively, T/E2's effects on milt and sperm could reflect modulation of central motor pathways. Neural stimulation at several sites in a brain-gonad pathway, which includes the preoptic area, brainstem, and rostral spinal cord, induces contraction of the sperm ducts in goldfish (Demski and Dulka, 1984; Dulka and Demski, 1986). This raises the possibility that socially induced surges in T result in local elevations of E2 that increase the excitability of cells within those circuits and leads to the elevations of milt output that occur in reproductive contexts (Kyle et al., 1985; Liley et al., 1986, 1993; Rouger and Liley, 1993; Stacey et al., 2001). Either way, our results indicate that the neuro-motor system underlying sperm release is modulated via a non-genomic estrogenic mechanism.

5. Rapid T/E2 effects on goldfish visual responses: potential sensory mechanisms

Another way through which rapid steroid mechanisms may facilitate male behaviors that increase reproductive success is by increasing sensory responsiveness to female stimuli. As already mentioned, sex pheromones, particularly PGFs, play a predominant role in eliciting male courtship. However, visual cues also play a role. Males can court when anosmic, albeit at reduced levels, and will preferentially follow ovulating versus non-ovulating females (Partridge et al., 1976). Reproductively active males also prefer to spend more time near females than males in two-choice preference tests in which only visual cues are available (Thompson et al., 2004). Thus, goldfish males use visual cues, in addition to olfactory cues, to help localize and orient towards potential mates. Indeed, it is likely that a chemical “trail” from a female goldfish, moving quickly in still water while releasing PGFs and potentially being followed/courted by multiple males, would be difficult to follow without a male being able to visually localize the source and return to the chase whenever contact with the odorant is lost. It would, at the very least, provide a significant advantage to be able to track the female source during scramble competitions for access to females. It is not yet clear what visual cues males use to discriminate sex and identify ovulating females. Ovulating females could display unique swimming patterns, and/or there may be sexually dimorphic ultraviolet reflectance patterns in goldfish, like in other teleosts, that are used for mate-choice and intra-male communication (Boulcott et al., 2005; Cummings et al., 2003; Rick and Bakker, 2008). Although goldfish are not sexually dimorphic in our visible color spectrum, male and female goldfish do have receptor cells that are maximally sensitive to light in the ultraviolet range (360 nm) (Bowmaker et al., 1991; Palacios et al., 1998), indicating that UV information can be detected.

In light of extensive localization of aromatase in the ascending visual pathway, from the retina to the optic tectum and preoptic area, both of which receive direct visual input in goldfish (Springer and Gaffney, 1981)), we hypothesized that elevations of T that mimic the surges typically induced by sexual stimuli would rapidly enhance behavioral responses to female visual cues. Furthermore, we predicted that any such influences would be dependent upon the aromatization of T to E2. To test those hypotheses, we set up an environment that prevented olfactory communication so that endogenous T surges induced by female pheromones would not occur and so we could measure male approach responses towards just the visual stimuli of females. Intraperitoneal injections of T increased the time that males spent in proximity to the visual cues of females, relative to the time spent by vehicle-injected males, in tests completed within 1 h of the injections, but did not have the same effect on responses towards stimulus males (Lord et al., 2009). Pre-injecting males with an aromatase inhibitor blocked T's effects, and injections of E2, at the same dose, also rapidly increased the time spent near the visual cues of females. Thus, like the rapid effects of T on expressible milt/sperm, its enhancement of behavioral responses to the visual cues of females works through an estrogenic mechanism, though we have not yet determined the receptor(s) that mediate the behavioral effects.

T/E2 could rapidly stimulate approach responses to the visual cues of females by enhancing the ability to detect them, possibly through peripheral influences in the retina, and/or by increasing sexual

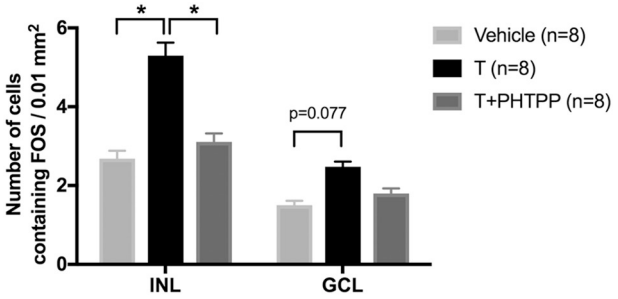

Fig. 1. Mean (+SEM) number of cells containing FOS in the INL and the GCL in retinas of males injected with T + PHTPP, T, and vehicle. Retinas from males injected with T contained significantly more FOS-ir cells than did retinas from males injected with T + PHTPP ($p = 0.01$) and vehicle ($p = 0.003$) in the INL. The overall difference across the three groups in the ganglion cell layer was not significant ({$F(2,21) = 2.78$, $p = 0.09$}. Figure reprinted from Yue et al., 2018. * indicates significant differences between groups in Tukey corrected pairwise comparisons following a significant main effect of treatment condition in an ANOVA.

motivation. To explore the first possibility, we measured acute effects of T on retina responses to visual stimuli using FOS immunohistochemistry. In these experiments, we housed males in very dim light conditions overnight (approximately 0.002 μW) in a small test chamber in which they had little room to move, with separate stimulus chambers on all four sides, then injected them with T or vehicle early the next morning. We also included a group of fish injected with T and the ERβ receptor antagonist, 4-[2-Phenyl-5,7-*bis*(trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]pheno (PHTPP). Forty-five minutes later, the lights were turned up and stimulus females were added to the four stimulus chambers. Thus, no matter which direction the male oriented in the central chamber, a female visual stimulus would have been present, nearby, in the visual field of both eyes. Ninety minutes after adding the females, males were removed, euthanized, and one retina from each fish was processed for FOS immunohistochemistry. It takes at least 60 min after stimulation onset for elevations of FOS protein to become detectable in fish brains (Okuyama et al., 2011), and levels typically peak by 90 min post-stimulation onset in most vertebrates; therefore, our measurement of stimulus-induced FOS should have reflected visual responses generated during the 45–75 min window after T or vehicle injections. Although not entirely within the hour often used as a cut-off for inferring non-genomic steroid modulation (Balthazart and Ball, 2006), differences between the T- and vehicle-injected groups in that window would nonetheless reflect an acute, relatively rapid effect of T on visual responses.

We found that fish injected with T had significantly more FOS-immunoreactive (FOS-ir) cells in the inner nuclear layer, but not in the ganglion cell layer, than fish injected with vehicle, though a similar trend was evident between the groups in the ganglion cell layer. In fish injected with T and PHTPP, FOS-ir did not differ from vehicle-treated controls, indicating that the effect of T on retina cells is likely mediated via ERβ (Yue et al., 2018; see Fig. 1, reprinted from the origianl manuscript). We cannot yet say if the ability of T to enhance retina responses to visual cues that include females depends on its conversion to E2 and the exclusive activation of ERβ, or if, like E2's effects on milt/sperm, they depend on both ERα and ERβ receptor types because initial tests with an ERα antagonist were inconclusive. We have also observed that GPER/GPR30 is expressed in the goldfish retina (Mangiamele et al., 2017), so that membrane estrogen receptor could be involved too. Because we have not yet conducted experiments with aromatase inhibition, it also remains possible that direct actions on androgen receptors could play some role.

Our FOS experiments suggest that T/E2 acutely modulates the activity of retina cells, but it still remains to be determined whether steroids selectively enhance the processing of stimulus features associated with females or enhance retina sensitivity more generally. To further explore this issue, we also measured the acute effects of T on electrophysiological retina responses to pulses of light across a range of intensities. Specifically, we compared the amplitude of the b-wave, a field potential generated by the retina that is typically used a measure of visual sensitivity in many species, including goldfish (Nussdorf and Powers, 1988). In that experiment, intramuscular T-injections rapidly (within 20 min) increased the amplitude of the b-wave in anaesthetized fish, but only to low-intensity pulses of visible light (Yue et al., 2018). Similar to what we observed in the FOS experiment, the ERβ antagonist tended to block T's effects at the lower intensities, and it significantly decreased b-wave amplitudes, relative to pre-injection baseline responses, at higher intensities. Together, these experiments suggest that T/E2 rapidly modulate general retina sensitivity, which thus amplifies responses to salient visual stimuli in the environment, including potential mates, though we cannot yet rule out selective effects of T on FOS responses to stimulus features unique to females. Such a generalized mechanism may enhance the ability of males to detect, orient towards, and/or maintain proximity to females in the dim light conditions typically associated with spawning. However, we have not yet been able to detect T effects on retina ganglion cells, which relay visual information to the rest of the brain, so it is particularly important for future experiments to determine if such influences might be detectable in more naturalistic conditions, perhaps in dimmer light characteristic of early morning when spawning normally occurs. It will also be critical to directly test the behavioral relevance of T/E2 influences on retina processing. In particular, we hope to determine if T/E2 rapidly decreases the visual detection thresholds of males, which would facilitate approach responses in dim light conditions in which goldfish usually spawn.

The ERβ gene is expressed in goldfish retina (Tchoudakova et al., 1999), though it will now become important to determine the cells in which the protein is produced, as well as to determine if it is trafficked to cellular membranes. The activity of ON bipolar cells is believed to be the major contributor to the b-wave amplitude of the electroretinogram (Dong and Hare, 2002), which was increased by T injections. This result, combined with the finding that FOS-ir increased in the inner nuclear layer of fish injected with T, suggests that E2 produced locally by aromatase may modulate the activity of bipolar cells, directly or indirectly, leading to changes in visual sensitivity. However, other studies have found that amacrine cells and Muller glia cells also contribute to the b-wave (Awatramani et al., 2001), raising the possibility that estrogens generated from T could also affect visual responses, including b-wave amplitude, via direct effects on those cells. Determining which, if any, of those cell types express ERβ, as well further testing the effects of an ERα and GPER/GPR30 antagonist, will help us clarify how T induces its acute effects on visual processing in the retina. Of course, it also remains possible that T's influences on retina processes are not mediated by direct conversion to E2 within the retina, but rather in other brain areas that send projections to the retina.

6. Potentially rapid T effects on goldfish olfactory responses

We are only just beginning to test the effects of T on behavioral responses to sex pheromones, but our preliminary data suggest it may also rapidly enhance responses towards the ovulatory PGFs that elicit following and courtship. In these tests, fish were habituated individually in test tanks, then injected with T (4 μg/fish) or vehicle and placed back in the tank. Thirty minutes later, ethanol was infused into a top corner of the tank for 15 min (baseline), followed by 15-minute infusions of either ethanol or PGF2alpha dissolved in ethanol (10^{-8}, 10 mL/min). Time spent in the quadrant in which the substances were infused was recorded, and the differences between the time spent in the quadrant during the baseline and test periods was compared across the groups. T injections significantly increased the time spent in the infusion quadrant after pheromone was added during the test period, but not after ethanol was added (see Fig. 2A, unpublished data). In a test

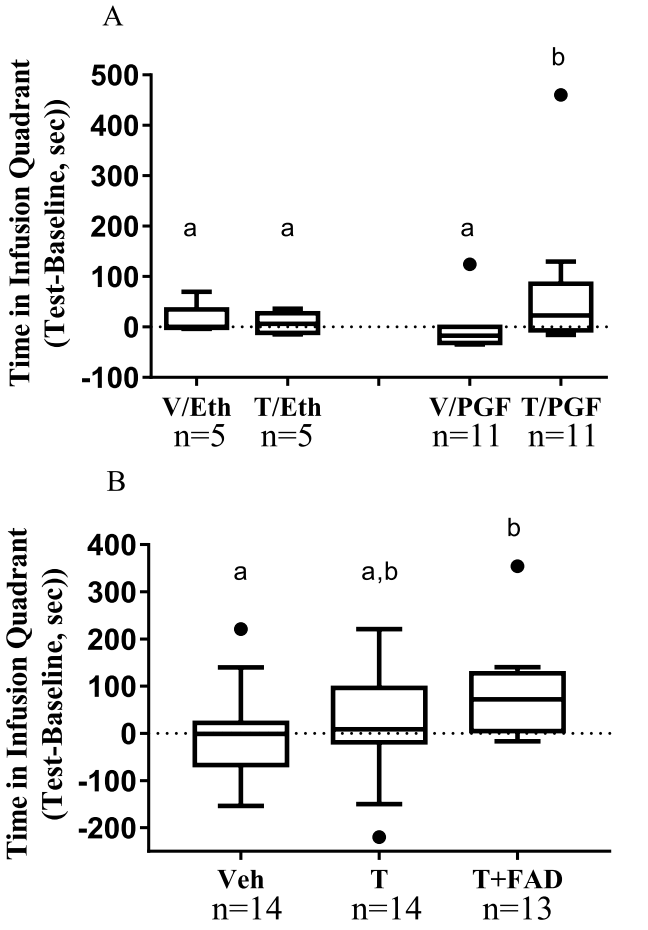

Fig. 2. Preliminary data showing the median and Tukey distributions of time spent in the quadrant into whichPGF2α dissolved in 95% ethanol (10^{-8}; 10 mL/min) or 95% ethanol (Eth) were infused during a 15 min test period minus the time spent in the same quadrant during a 15 min baseline while Eth was infused. Milting males were injected intraperitoneally with 100 μL of T (4 μg/fish) or vehicle (V), then placed in a 20 gal test tank. After 30 min, a 15-min baseline was conducted while infusing control solution (Eth) into the tank and time in the upper quadrant of the tank where the Eth was infused was recorded. In two groups, only Eth was infused during the test period (V + Eth and T + Eth fish); in V + $PGF_{2\alpha}$ and T + $PGF_{2\alpha}$ fish, PGF2α was infused ruing the test period. Difference scores (test period minus baseline) were compared with non-parametric Mann-Whitney *U* tests because data were not normally distributed; there was an overall difference across groups (chi square = 8.36, p = 0.04); follow up tests revealed that differences between V and T groups in the Eth only, control trials were not significant; differences between V and T in the PGF2α condition were (U = 22, z = −2.5, p = 0.01, indicated by *, A). In a test later in the summer when most fish were not milting, males were injected with V, T or T + FAD (approximately 8 mg/kg, per (Remage-Healey and Bass, 2007)) and the same test was performed. A Kruskal Wallis across all test groups indicated a significant difference (chi squared = 6.24, p = 0.04); follow up tests revealed that T + FAD fish spent significantly more time in the test quadrant than V fish (U = 50.5, z = −1.98, p = 0.047). Signiant differences between groups indicated by different letters above the box-plats.

conducted slightly later in the breeding season when most fish were no longer milting, T did not have the same effect, suggesting that sensitivity to the pheromone and/or to the rapid effects of T declines as fish come out of reproductive condition. However, aromatase inhibition with FAD, when coupled with T, quite surprisingly *increased* the time spent in proximity to the pheromone (see Fig. 2B, unpublished data). We cannot resolve whether FAD exclusively drove that result or if it required FAD and T because we did not have a FAD only group in that experiment. FAD treatments do increase levels of endogenous T (Lord et al., 2009), so one possibility is that the T injections alone did not produce high enough levels of T to overcome the seasonal shift in responsiveness to the pheromone and/or to T, but that combined FAD and T injections did. Most importantly, this preliminary result suggests, contrary to our expectations, that the rapid activation of estrogen receptors is unlikely to stimulate behavioral responses to the pheromone, as circulating and local E2 levels should have declined in the FAD group. Thus, T may rapidly enhance responses to sex pheromones through a different receptor mechanism – potentially one mediated by androgen receptors – than the estrogen receptor mechanisms that mediate its rapid effects on visual processing and milt expression. We are currently repeating our olfactory tests with 11-ketoprostaglandin F2α, the most potent PGF molecule in the pheromone blend (Sorensen et al., 1988b). We are also using infusion rates that more closely mimic female release patterns and adding controlled visual stimuli {(female models/aquatic vegetation, as per (Appelt and Sorensen, 2007)}. Testing the rapid effects of T on responses to isolated visual and olfactory stimuli has enabled us to begin elucidating specific mechanisms through which sex steroids affect behaviors related to courtship, and possibly to identify sensory-specific dissociations in how T rapidly affects responses to different stimuli. However, it is now critical to learn how those different mechanisms interact in more naturalistic settings, as well as if they do confer a reproductive advantage in competitive reproductive contexts.

7. Unanswered questions about rapid steroid neuromodulation in fish

The effects of T that we described on milt, visual approach, and retina responses to visual stimuli were mediated by estrogen receptor mechanisms. These results indicate that local estrogen synthesis may be important in coordinating rapid physiological and behavioral responses to salient sociosexual signals in goldfish, however, direct evidence of where and when estrogen synthesis occurs to mediate these rapid effects is still lacking. The best evidence to date is the co-localization of aromatase and estrogen receptors (both the "classical" ERα/β and GPER/GPR30) within key regions of the fish central nervous system – including the retina, preoptic area, and most of the Social Behavior Network (see Table 1) – that control and modulate behavioral responses to sexual cues. Aromatase has been localized in axon terminals in several other vertebrate groups (Jakab et al., 1993; Naftolin et al., 1996; Remage-Healey et al., 2009; Saldanha et al., 2000), indicating that E2 can be produced locally at synapses, where it can exert rapid effects on cell physiology and thus behavior through mechanisms analogous to those of neurotransmitters/neuromodulators. However, it is not yet clear if aromatase is likewise expressed in neurons in teleosts. There are at least two aromatase gene variants in fish, Cyp19a1a (aromatase-a) and Cyp19a1b (aromatase-b), the latter of which is the predominant form expressed in brain, primarily, if not exclusively, in radial glial cells (Forlano et al., 2001; Jeng et al., 2012; Menuet et al., 2003; Pellegrini et al., 2007; Takeuchi and Okubo, 2013; Tong et al., 2009). However, radial glial cells in fish are heterogeneous, and not all of their functions directly relate to their roles as progenitor cells (Lyons and Talbot, 2014). Some may, like mammalian astrocytes, affect synaptic functions, either through influences on ionic and/or neurotransmitter concentrations at local synapses or by releasing neuroactive substances themselves (Dallerac et al., 2013). They could even release neuroactive estrogens that affect local synapses or that influence neurons across distances typical of volumetric synaptic transmission mechanisms. If any of the rapid behavioral and/or physiological effects of E2 in goldfish, as well as in other teleost species in which they have been observed such as plainfin midshipman (Remage-Healey and Bass, 2004b, 2007), depend on estrogens produced centrally by glial cells, then it would indicate an important difference in how rapid estrogen signaling in the brain is achieved between teleosts and tetrapods. However, some species, including goldfish, are reported to have aromatase-b expression in non-glial, potentially neuronal populations (Chaube et al., 2015;

Gelinas and Callard, 1993, 1997b; Menuet et al., 2003), though definitive neuronal markers were not used in those studies and questions about antibody specificity have been raised (Pellegrini et al., 2016). Additionally, aromatase-a can be expressed in the nervous system of some fish, albeit at lower levels than aromatase-b, though in goldfish retina the reverse is true (Barney et al., 2008; Chaube et al., 2015; Kwon et al., 2001; Tchoudakova and Callard, 1998; Toffolo et al., 2007), and we know nothing about the cell types in which it is expressed. Thus, it remains possible that some aromatase gene variants may be expressed in neurons in some species and/or social contexts, and if so the E2 produced in such cells may rapidly modulate neuronal physiology and behavior. Of course, it also remains possible that the rapid effects of estrogens on visual approach and milt physiology in goldfish are solely a function of estrogens produced peripherally in the retina and gonads, respectively. Future work should address the important question of where the E2 synthesis that rapidly influences behavior occurs.

Our findings in goldfish also highlight the likelihood that rapid T/E2 effects are mediated via multiple receptors, perhaps acting synergistically or simultaneously to modulate cellular physiology. As stated above, T/E2-induced changes in milt volume appear to depend on both ERα and ERβ, and it is possible that multiple ERs are required for T-mediated retina responses as well. In fact, the available evidence suggests that co-localization of ERα and ERβ subtypes and GPER/GPR30 is widespread across the fish brain, particularly in primary sensory and multisensory regions and in the Social Behavior Network (Table 1), though it is not clear whether different ERs are always expressed in the same cells. Little work has been done in fish to address the important issue of whether rapid effects depend on or are enhanced by estrogen receptor crosstalk, but clearly the potential for such crosstalk exists. Several studies in mammals have demonstrated that agonists of GPER and ERα or ERβ have similar effects on behavior, indicating either some redundancy in E2 signaling mechanisms (Lymer et al., 2017), or that these pharmacological tools are non-specific in their effects on multiple ERs. On the other hand, studies in which antagonists for multiple receptors each block E2 effects, as we observed for increases in milt induced by T/E2 in goldfish, indicate that some rapid steroid effects may depend on multiple receptors acting in parallel on the same signaling pathway (reviewed in Hadjimarkou and Vasudevan, 2018), and/or on heterodimer complexes in the membrane (Guo et al., 2005; Razandi et al., 2004). More work needs to be done to determine how rapidly-modulated social responses and their underlying neural processes are affected by crosstalk between ERs.

Although the majority of rapid steroid signaling work across vertebrates has focused on estrogenic mechanisms, work in teleost fish is beginning to show that non-genomic AR mechanisms also play dynamic roles in reproductive regulation. A novel G-protein coupled receptor in the zinc transport family, ZIP9, has recently been identified in Atlantic croakers that binds androgens, mediates rapid effects of T on apoptosis in follicles, and is expressed in the brain (Berg et al., 2014), though its central functions remain unknown. Additionally, in male midshipman, the rapid effects of T (in sneaker males) and KT (in territorial males) are both blocked by a classical AR antagonist, suggesting ARs may be trafficked to membranes in those neurons (Remage-Healey and Bass, 2006b, 2007). KT, which cannot be aromatized, also rapidly stimulates paternal behavior in male gobies (Pradhan et al., 2014b). If our ongoing work in goldfish confirms that T rapidly enhances behavioral responses to sex pheromones through androgen receptor mechanisms, then it would add weight to the importance of such mechanisms in the regulation of social behavior. Furthermore, by showing a dissociation in the estrogen- and androgen-receptor mechanisms that mediate rapid T effects on visual and olfactory responses, respectively, it would demonstrate that T specifically affects sensory processes and not a central state of sexual motivation that uniformly affects “downstream” responses to sensory stimuli processed in different sensory modalities. It will become increasingly important in goldfish, as well as other teleosts, to characterize the AR mechanisms that mediate rapid androgen effects further, as work in this group of vertebrates is at the forefront of our efforts to understand their roles in social regulation.

8. Conclusions

In recent years the field of neuroendocrinology has greatly advanced our understanding of the molecular and cellular mechanisms through which sex steroids rapidly modulate social functions, but less progress has been made on how those mechanisms may influence behavior in naturalistic contexts. Our work in goldfish is beginning to suggest that sex steroids can rapidly influence early stages of sensory processing and motor output related to reproduction, but we too have focused much of our attention on those mechanisms in simplified laboratory conditions. We now hope to begin testing hypotheses about how those rapid influences may promote adaptive responses that enhance reproductive success in competitive mating environments. Though some large unanswered questions remain about the specific modes of rapid T/E2 actions on the fish nervous system (i.e., Where is E2 locally produced? What steroid receptors/complexes are involved in generating rapid behavioral effects?), we anticipate that what we have learned about how T/E2 may influence sensory and motor mechanisms in goldfish will help us predict how those mechanisms may operate in naturalistic environments, and that we will ultimately be able to determine the functional consequences of those mechanisms on reproductive success.

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