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Changes in behavior and brain immediate early gene expression in male threespined sticklebacks as they become fathers

Molly Kent[a,1], Alison M. Bell[b,*]

[a] Program in Neuroscience, University of Illinois, Urbana Champaign, United States
[b] School of Integrative Biology, Program in Neuroscience, Program in Ecology, Evolution and Conservation, Institute for Genomic Biology, University of Illinois, Urbana Champaign, United States

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ABSTRACT

Motherhood is a period of intense behavioral and brain activity. However, we know less about the neural and molecular mechanisms associated with the demands of fatherhood. Here, we report the results of two experiments designed to track changes in behavior and brain activation associated with fatherhood in male threespined stickleback fish (*Gasterosteus aculeatus*), a species in which fathers are the sole providers of parental care. In experiment 1, we tested whether males' behavioral reactions to different social stimuli depends on parental status, i.e. whether they were providing parental care. Parental males visited their nest more in response to social stimuli compared to nonparental males. Rates of courtship behavior were high in non-parental males but low in parental males. In experiment 2, we used a quantitative in situ hybridization method to compare the expression of an immediate early gene (*Egr-1*) across the breeding cycle – from establishing a territory to caring for offspring. *Egr-1* expression peaked when the activities associated with fatherhood were greatest (when they were providing care to fry), and then returned to baseline levels once offspring were independent. The medial dorsal telencephalon (basolateral amygdala), lateral part of dorsal telencephalon (hippocampus) and anterior tuberal nucleus (ventral medial hypothalamus) exhibited high levels of *Egr-1* expression during the breeding cycle. These results help to define the neural circuitry associated with fatherhood in fishes, and are consistent with the hypothesis that fatherhood – like motherhood – is a period of intense behavioral and neural activity.

1. Introduction

Motherhood is a period of intense behavioral and neural activation. Decades of studies have started to reveal the structure and organization of the maternal brain (Hillerer et al., 2014; Kinsley and Amory-Meyer, 2011; Lambert, 2012; Rilling and Young, 2014), the brain areas that are activated during mothering (Rocchetti et al., 2014) and the neural control of maternal care (Dulac et al., 2014). However, we know less about the neural mechanisms underlying the transition to fatherhood (Kentner et al., 2010).

Fishes are particularly good subjects for studying the neuroendocrine mechanisms involved in fathering because they exhibit tremendous diversity in reproductive mode, and paternal care is relatively common in fishes compared to other vertebrates (Smith and Wootton, 2016). Recent studies have begun to describe the dramatic neuroendocrine changes that accompany the transition to fatherhood in fishes, e.g. (DeAngelis and Rhodes, 2016; Pradhan et al., 2014; Stiver et al., 2015), and have suggested that isotocin and arginine vasotocin, like their mammalian homologs oxytocin and arginine vasopressin, are involved in regulating paternal behavior in fishes (Kleszczynska et al., 2012; O'Connell et al., 2012; Ripley and Foran, 2010). GnRH and the distribution of GnRH neurons in key brain areas such as the preoptic area of the hypothalamus are also key players that orchestrate reproductive behavior in fishes, e.g. (Burmeister et al., 2005; Scaggiante et al., 2004, 2006; Tubert et al., 2012), reviewed in (Chen and Fernald, 2008; Fernald, 2012; Maruska and Fernald, 2011).

Threespined stickleback fish are especially good models for studying fathering because male sticklebacks are the sole providers of parental care that is necessary for offspring survival, and their paternal behavior has been well studied in the field and in the lab. Male sticklebacks undergo dramatic changes in behavior and physiology during the reproductive cycle (Wootton, 1976, 1984), which is photoperiod-dependent (Hellqvist et al., 2008). For example, as day length increases, males become aggressive, defend territories and construct nests. Only upon completing their nest do males start to court females and display courtship behaviors such as the conspicuous zig zag dance. Males also

* Corresponding author.
E-mail address: alisonmb@life.illinois.edu (A.M. Bell).
[1] Present address: University of Richmond.

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advertise their parental abilities during courtship, e.g. by fanning, even when they do not have eggs in their nest (Candolin, 1997). After spawning, males provide parental care for the eggs in the form of territory defense and fanning. After the eggs hatch, certain paternal behaviors make an abrupt appearance: fathers become very active, chasing and retrieving their free-swimming fry (Stein and Bell, 2012). Males continue to defend their newly-hatched and vulnerable fry from predators. Fathers and their fry are intimately associated during this period, with many opportunities for sensory, especially tactile, interactions. Parenting is an energetically demanding period, yet it is necessary for reproductive success (Smith and Wootton, 1999). Interestingly, threespine sticklebacks exhibit greater sexual dimorphism in brain size than any other vertebrate (Kotrschal et al., 2012) and in sticklebacks populations in which males do not provide care, the sexual dimorphism in brain size is reversed (Samuk et al., 2014). These results are consistent with the hypothesis that the male stickleback brain has evolved in response to the cognitive demands of parenting (Kotrschal et al., 2012).

The reproductive cycle in male sticklebacks is marked by dramatic neuroendocrine changes. For example, GnRH and gonadotropins (Andersson et al., 1995; Hellqvist et al., 2004; Shao et al., 2015) as well as androgens (Hoffmann et al., 2008) change as males move through the breeding cycle, from establishing a territory to caring for offspring. In particular, levels of 11 keto-testosterone (11KT), a potent androgen, are high during the territorial and courtship phase but then drop when males are providing care (Mayer et al., 2004; Pall et al., 2005; Pall et al., 2002b). However, the drop in 11KT is not responsible for the increase in care (Pall et al., 2002a). Instead, other studies point to arginine vasotocin (AVT) (Kleszczynska et al., 2012) and prolactin as important players during the parental phase (Pall et al., 2004).

Here, we report the results of two experiments designed to track changes in behavior and neural immediate early gene expression (IEG) as male sticklebacks become fathers. In experiment 1, we compare behavior toward conspecifics and a model predator between parental and nonparental males. In experiment 2, we use in situ hybridization to track changes in the expression of an IEG (*Egr-1*) across different stages of the breeding cycle. IEG expression has been used to reveal brain areas important for behavior (Clayton, 2000; Fernald, 2012; Hillerer et al., 2014; Hofmann, 2010; Robinson et al., 2008), including those involved in fathering in rodent models (e.g. prairie voles (Northcutt and Lonstein, 2009), California mice (de Jong et al., 2009; Lambert et al., 2011)). IEG expression has also been used to track changes in brain activation in fishes (Burmeister et al., 2005; Butler and Maruska, 2016; Desjardins et al., 2015; Desjardins and Fernald, 2010; Desjardins et al., 2010; Harvey-Girard et al., 2010; Kress and Wullimann, 2012; Lau et al., 2011; Loveland and Fernald, 2017; Maruska et al., 2013a; Maruska et al., 2013b; O'Connell et al., 2012; O'Connell et al., 2013; Rajan et al., 2011; Yaeger et al., 2014). We focus on *Egr-1* expression in brain areas involved in the social behavior network (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011), a linked set of brain nuclei important for social behavior in vertebrates. We use a whole mount, quantitative in situ hybridization protocol that has been validated in several species and tissues (Bacharach et al., 2016; Long et al., 2016; McNeill and Robinson, 2015; Stapel et al., 2016; Tantirigama et al., 2016).

2. Methods

2.1. Animals

The three-spined sticklebacks were collected as juveniles from Putah Creek, California. Freshwater sticklebacks typically reproduce at one year of age, and breed several times during the spring-summer. Fish were maintained in the laboratory in 104 l tanks at approximately 16 °C under 8:16 h light/dark photoperiod until they became sexually mature. The water was filtered through particulate, UV, biological and charcoal filters. The fish were fed ad libitum with a mixture of bloodworms, brine shrimp and mysis shrimp daily.

Once nuptial coloration was observed, male sticklebacks were switched to a 16:8 h light/dark photoperiod at 20 °C, measured for length and housed individually in 9.5 l (36 × 21 × 18 cm) tanks with a refuge, an open plastic box (13 × 13 × 3 cm) filled with sand, and algae for nest building. Prior to the experiment, males were randomly assigned to one of five breeding stage conditions (territorial, courtship, tending eggs, tending fry, and post-fry). To induce spawning, females were added to the tanks 24 h after the male crept through his nest. If spawning did not occur, another female was introduced into the tank 12–24 h later. Males were observed every day to assure that they were providing parental care (fanning nest, hovering near nest, oxygenating the eggs). The experiment was carried out during summer 2011.

2.1.1. Experiment 1

In order to examine changes in behavior that occur as males become fathers, we compared the behavioral reaction of nonparental and parental males to three different stimuli: a male stickleback, a female stickleback and a model predator. Nonparental males were measured during the courtship stage (24 h after the male crept through his nest) and parental males were measured during the tending fry stage (three days after hatching).

In order to measure their behavioral reaction to a female stickleback, males were presented with a gravid female (potential mate) in a clear round bottom flask for 10 min. Five different gravid females were used as stimuli. To measure their behavioral reaction to a male stickleback, males were presented with a reproductive male (potential rival) in a clear round bottom flask for 10 min. Five different reproductive and nuptially-colored males were used as stimuli. To measure their behavioral reaction to a model predator, males were confronted with a model bird predator. The beak of a great blue heron (a predator that occurs in this population) was plunged into the tank every minute for 10 min. We recorded the number of zig zags (a conspicuous courtship behavior) and visits to the nest during each ten-minute observation period. Different individuals were measured in each condition, with the following final sample sizes: nonparental: female stickleback, $n = 9$; male stickleback, $n = 6$; model bird predator, $n = 7$; parental: female stickleback, $n = 7$; male stickleback, $n = 5$; model bird predator, $n = 6$.

2.1.2. Experiment 2

Males assigned to the territorial stage were sampled after the fish started but not yet completed a nest. Males assigned to the courtship stage were sampled within 24 h after creeping through the nest, a conspicuous behavior that marks the transition into the courtship stage. Males assigned to the tending eggs stage were removed three days after fertilization. Males assigned to the tending fry treatment were sacrificed three days after the fry hatched, when levels of parental behavior are high (Stein and Bell, 2012). Males assigned to the post-fry treatment were transferred to a new tank seven days after the fry hatched, when males typically begin to defend new territories, and were sacrificed 24 h later, after males had recovered from handling but had not yet started a new nest.

Males were sacrificed via decapitation between 1000 and 1400. The head was removed from the body just behind the operculum. The muscles at the base of the skull along with the skull were removed using rongeurs (FST, Foster City, CA, USA). The eyes were detached from the optic nerve using fine inverted scissors (FST). The brain (minus pituitary) was then placed in 4% paraformaldahyde (Sigma Aldrich, St Louis, MO, USA) made in phosphate buffered saline (PBS: Fisher Scientific, Fair Lawn, NJ, USA).

Twenty-four hours later, all brains were cleaned of dura, excess fibers that were attached to the brain after it was removed from the skull and miscellaneous tissues using a stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) to view the brain. Fine