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

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A R T I C L E   I N F O

Keywords:
Fatherhood
Immediate early gene
Sociogenomics
Sticklebacks
Social behavior network

A B S T R A C T

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 depend 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 (Kettenre et al., 2016).

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. (Burmister 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 (Helleqvist 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
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 stickleback populations in which males do not provide care, the sexual dimorphism in brain size is reversed (Samak 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 (Hoffman 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) (Kleszczyńska 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% paraformaldehyde (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
forces and fine inverted scissors were used for removal of all excess. Once the brains were clean they were placed in 100% methanol stored at −20 °C until being processed through the in situ hybridization protocol.

The final sample sizes in each condition were: territorial (n = 9), courtship (n = 8), tending eggs (n = 6), tending fry (n = 4) and post-fry (n = 4). Fewer males were sampled in the later stages because not all males mated and/or successfully reared eggs or fry.

2.2. In situ hybridization

To quantify Egr-1 expression, we modified an mRNA in situ hybridization protocol for insects to analyze differential expression in whole mount brains (McNeill and Robinson, 2015). This method combines whole mount protocols that use bright field microscopy with a fluorescence microscopy protocol (Raj and Tyagi, 2010; Raj et al., 2008). Stellaris® (Biosearch Technologies, Petaluma, CA, USA) mRNA in situ hybridization probe sets comprised 48 DNA sequences, where each 20 bp sequence was attached to a single Quasar fluorophore. Probes are highly specific because probe sequences designed in the antisense direction bind in series along the targeted mRNA transcript. We designed antisense probe sets against Egr-1 using an online tool available through BioSearch (Supplementary Table 1).

Brains were prepared for hybridization through sequential dehydration from 100% methanol into PBS (Fisher) with Triton-X (Promega, USA) mRNA in situ hybridization probe sets comprised 48 DNA sequences, where each 20 bp sequence was attached to a single Quasar fluorophore. Probes are highly specific because probe sequences designed in the antisense direction bind in series along the targeted mRNA transcript. We designed antisense probe sets against Egr-1 using an online tool available through BioSearch (Supplementary Table 1).

Brains were prepared for hybridization through sequential rehydration from 100% methanol into PBS (Fisher) with Triton-X (Promega, Madison, WI, USA). Next, brains were treated with 2 μg/ml proteinase K (Invitrogen, Carlsbad, CA, USA) for 20 min. Proteinase K was used to increase signal that may have been lost due to parafomaldehyde exposure. Brains were then placed in 2 mg/ml glycine for 20 min to stop the proteinase K reaction. The brains were then rinsed in 4% paraformaldehyde (Sigma Aldrich) for 20 min and treated with 2 mg/ml glycine plus 75 mM ammonium acetate. Next, brains were incubated in prehybridization solution containing 2 × Saline sodium citrate buffer (SCC) (Fisher), 15% formamide (Fisher), 1% blocking solution (Roche, Indianapolis, IN), 0.5% 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS) (Fisher), 0.1% Triton-X (Fisher), 1 mg/ml yeast tRNA (Promega), 5 mM EDTA, 100 mg/ml Dextran Sulfate (Fisher) and 1 × Denhardt's solution (Fisher) at 37 °C for four hours. Brains were incubated overnight at 37 °C in fresh prehybridization solution with Stellaris probes (table S1) tagged with Quasar 570 (BioSearch) at a concentration of 1:100.

Brains were put through sequential stringency (15% formamide, 2 × SCC, 0.1% Triton-X, 100, 0.5% CHAPS) washes to remove excess probe. Washes were diluted with 2 × SCC ending in a 1:3 dilution of wash to SCC. After the stringency washes, the brains were gradually dehydrated into 100% methanol and cleared for imaging with 100% methyl salicylate (Sigma Aldrich). The brains were then covered to prevent photobleaching. After dehybridization the brains were submerged in a 1 mM solution of DAPI dissolved in methanol.

2.3. Imaging

The whole mount brains were processed on an automated LSM 710, confocal microscope (Carl Zeiss Microimaging Inc., Jena, Germany; NY, USA) from the dorsal to ventral side. Brains were focused on the microscope and then 12 tile images (4 × 3) were taken to allow for the entire brain to be analyzed. The overall thickness of the brain was also determined and then 7 μm sections were imaged by exciting fluorophores with a 555 nm laser line. The tiles and sections were compiled together to give a complete image of the whole brain. Pictures were taken and processed using software provided by Zeiss (Zeiss, 2010).

2.4. Analysis

Images were analyzed using NIH software, ImageJ. Whole brain images were first used to measure thickness and then key anatomical landmarks were identified, such as the anterior and posterior commissures. The two commissures were used to determine the location of brain areas. There is no brain atlas for the stickleback brain, therefore brain atlases from other teleost species as well as previous immunohistochemical studies in sticklebacks were used to locate putative brain areas within the social behavior network (Burmeister et al., 2009; Cerda-Reverter et al., 2008; Cerda-Reverter et al., 2001a, 2001b; Ekstrom, 1994; Ekstrom et al., 1995; Ekstrom et al., 1992; Ekstrom et al., 1985; Ekstrom and Ohlin, 1995; Ekstrom et al., 1986; Ekstrom and Van Veen, 1984; Honkanen and Ekstrom, 1991; O’Connell and Hofmann, 2011; Peter et al., 1975; Wullimann et al., 1996).

For example, before scanning each brain for Egr-1 and DAPI, brains were measured on the z-plane to determine initial whole brain thickness. All brains were scanned on the confocal microscope in the dorsal to ventral direction to ensure similar patterns of expression across all scans. Brain section images were taken every 7 μm. Before a brain area of interest was located, a second measurement of thickness was taken. The number of images were counted from first visualization of DAPI until no signal was present. The number of images was then multiplied by 7 to give a total μm thickness of the brain and compared to the initial number. If the measurements were incorrect the landmarks were used to confirm the correct location. When a brain area such as the anterior tuberal nucleus/ventral medial hypothalamus (aTn/VMH) was located by determining distance from the anterior commissures and overall thickness of the brain compared to published brain atlases (Burmeister et al., 2009; Cerda-Reverter et al., 2008; Cerda-Reverter et al., 2001a, 2001b; Ekstrom, 1994; Ekstrom et al., 1995; Ekstrom et al., 1992; Ekström et al., 2001; Ekstrom et al., 1985; Ekstrom and Ohlin, 1995; Ekstrom et al., 1986; Ekstrom and Van Veen, 1984; Honkanen and Ekstrom, 1991; O’Connell and Hofmann, 2011; Peter et al., 1975; Wullimann et al., 1996) an additional landmark, the ventricle, was used to confirm the correct location. Approximately 2 mm from where the DAPI signal was first observed, the aTn/VMH can be observed surrounding the ventricle. Once the correct section was located within the z-plane, mean gray value (MGV) measurements for Egr-1 expression were taken. A total of 35 μm of tissue was averaged to ensure a relative MGV for each brain area like the aTn/VMH.

Egr-1 expression was measured in brain areas important for the social behavior network (the fish name is indicated first followed by the putative mammalian homolog): ventral tuberal/anterior hypothalamus (VT/AH), medial dorsal telencephalon/basolateral amygdala (Dm/AMY), ventral pallium/bed nucleus of the stria terminalis_medial amygdala (VP/BNST), lateral part of dorsal telencephalon/hippocampus (DI/HC), ventral part of the ventral telencephalon/ lateral septum (Vv/LS), dorsal part of the ventral telencephalon/nucleus accumbens (Vd/NA), periaqueductal gray (PAG), preoptic area parvo-cellular (POAp), anterior tuberal nucleus/ventral medial hypothalamus (aTn/VMH) and posterior tuberculum/ventral tegmental area (TPp/VTA) (Fig. 1). We also measured Egr-1 expression in brainstem (located behind the cerebellum where cell bodies were present) as a control brain area that is unlikely to be involved in fathering.

Once a brain area was located, it was measured for mean gray value (MGV), a measurement of optical density (Rasband, 2011). MGV is calculated as MGV of pixels in region of interest divided by the number of pixels. To control for fluorescent signal background, a second MGV (control) was also taken in an area on the section where no staining occurred (i.e. ventricle). The second MGV was subtracted from the first to account for possible bleed-through of signal between sections. Because the optical sections were only 7 μm thick, there was a chance that a key part of an area might have been missed in a single section. Therefore, the same brain area was measured in five consecutive optical sections and the average of the five sections was computed for each area. In situ hybridization, imaging and analysis of the samples were carried out blind with respect to treatment and processed in a random order between October and April 2012.

To evaluate the effectiveness of the in situ protocol for detecting
Differences in brain activation, Egr-1 expression was compared in brains taken during day vs night using both the in situ protocol and qPCR (whole brain). Egr-1 expression was higher during the day according to both the in situ protocol (Supplementary Fig. 1) and qPCR (Supplementary Fig. 2). Further inspection of the ISH brains showed higher expression in specific brain areas associated with visual processing (Supplementary Fig. 1b). Further validation studies compared a sense to antisense probe (Supplementary Fig. 3), a probe from a different species (Supplementary Fig. 4) and used a Northern blot (Supplementary Fig. 5) to confirm that the probes for Egr-1 were specific.

2.5. Data analysis

We used generalized linear models (Poisson distribution) to test for the effect of stimulus (male, female, predator), parental status (non-parental, parental) and their interaction on zig zags (plus 1 to account for zeros) and number of visits to the nest followed by post-hoc tests based on marginal means. We report the range of Cohen’s d estimates for significant pairwise comparisons. General linear models were used to test for differences in Egr-1 expression across breeding stages within each brain area. Post hoc comparisons were made using the LSD test. Within each brain region we estimated Cohen’s d between each stage relative to the tending fry stage. Model fit was assessed by visual inspection of the residuals. Figures show controlled mean gray value (MGV) ± one standard error of the mean (S.E.M.). Statistical analyses were carried out in SPSS version 24.

All procedures were carried out under IACUC approval by the University of Illinois Urbana-Champaign IACUC (#12118) and conform to NIH standards for animal welfare. Fish were collected under collecting permit # SC-3310 to AMB from California Fish and Game.

3. Results

3.1. Experiment 1

Males adjusted their courtship behavior depending on their stage in the breeding cycle. Nonparental males exhibited high rates of courtship behavior (the zig zag display) toward a female stickleback, but courtship behavior was almost entirely absent in parental males (Fig. 2a, Stimulus Wald Chi-Square = 61.3, P < 0.0001, Parental status Wald Chi-Square = 7.1, P = 0.008, Stimulus x Parental status Wald Chi-Square = 22.2, P < 0.0001, n = 40; Cohen’s d pairwise estimates ranged from 1.24–1.29). Rates of nest visitation also depended on parental status (Fig. 2b, Stimulus Wald Chi-Square = 5.5, P = 0.064), Parental status Wald Chi-Square = 16.7, P < 0.0001, Stimulus x Parental status Wald Chi-Square = 2.8, P = 0.25, n = 40; Cohen’s d pairwise estimates ranged from 0.89–1.2). Overall, parental males visited the nest more compared to nonparental males, and rates of nest visitation were particularly high when males were presented with a female stickleback and a model predator.

3.2. Experiment 2

Egr-1 expression was consistently highest while males were tending fry compared to the other stages (Fig. 3). Cohen’s d effect size estimates comparing each stage to the tending fry stage ranged from 0.26–4.58. This result was confirmed by visual inspection of individual brain regions (example in Supplementary Fig. 6). The expression patterns within each brain region suggest that in general, Egr-1 expression increased as males proceeded through the breeding stages but then dropped to levels comparable to those at the beginning of the breeding cycle, i.e. the territorial stage, after parenting (Fig. 3).

In contrast, Egr-1 expression in a brain area outside the social behavior network (brain stem) did not differ across stages (F4,26 = 1.63, P = 0.129, Supplementary Fig. 7). Moreover, there were no differences in the expression of Egr-1 in this brain region across different stages.
in DAPI staining in the optic tectum (where penetration was greatest) across stages (F1,26 = 0.16, P = 0.958). The Egr-1 ISH and DAPI staining were performed on the same tissue and scanned on the microscope at the same time with two different channels for DAPI and the Egr-1 fluorescent tag, 461 and 567 respectively. These results suggest that the observed differences in were in fact due to Egr-1 expression and not due other confounds, e.g. batch differences between runs, bleed-through of staining, etc. To assess the specificity of the probe, we also compared Egr-1 expression between day and night in medial dorsal telencephalon/basolateral amygdala and lateral part of dorsal telencephalon/hippocampus, two areas not likely to be sensitive to day-light. Neither comparison was statistically significant (medial dorsal telencephalon/basolateral amygdala: t16 = 1.567, p = 0.137, lateral part of dorsal telencephalon/hippocampus: t16 = 0.404, p = 0.179, Supplementary Fig. 1c).

Overall, levels of Egr-1 expression were particularly high in the lateral part of dorsal telencephalon/hippocampus, with some subtle differences between different brain areas across stages (Fig. 3). For example, during the territorial stage, Egr-1 expression also tended to be high in the anterior tuberal nucleus/ventral medial hypothalamus. Once males entered the courtship stage, Egr-1 expression tended to increase in the ventral pallium/bed nucleus of the stria terminalis medial amygdala and dorsal part of the ventral telencephalon/nucleus accumbens. When males were tending eggs, expression tended to increase in all measured brain areas except ventral part of the ventral telencephalon/lateral septum, and then expression in all brain areas increased dramatically while males were caring for fry. When males were no longer caring for offspring (i.e. during the post-fry stage), Egr-1 expression dropped, although it tended to remain high in the lateral part of dorsal telencephalon/hippocampus, medial dorsal telencephalon/basolateral amygdala and anterior tuberal nucleus/ventral medial hypothalamus relative to other brain areas (Fig. 3).

We did not detect any differences in body size among males assigned to the different breeding stages (standard length F1,24 = 0.75, P = 0.57).

4. Discussion

Becoming a parent involves dramatic changes in brain, physiology and behavior. Detailed studies of the transition to motherhood have shown that hormonal changes during pregnancy and parturition cause structural remodeling of the brain, which is involved in mothers’ attraction to and responsiveness to her offspring (Pereira and Ferreira, 2016). Importantly, similar neural circuitry and systems are recruited in the service of fathering and in adoptive parents. Indeed, while fathers do not typically undergo pregnancy and birth (but see, for example “pseudopregnancy” in seahorses (Roth et al., 2012) and crop milk production in birds (Shetty et al., 1991)), fathers do often undergo changes that serve the same function, namely to successfully rear offspring, e.g. building a nest, defending the nest, providing care, responding to the changing needs of their offspring, etc. Indeed, functional MRI studies in humans show that similar brain areas are activated in mothers and fathers while they care for their offspring (Abraham et al., 2014; Rilling, 2013). However, we know less about the neural mechanisms associated with fatherhood in particular. Most of the animal models for fatherhood exhibit biparental care (e.g. African striped mice (Schradin et al., 2013), cichlids (O’Connell et al., 2012), California mice (Perea-Rodriguez et al., 2015), dart frogs (Schulte and Summers, 2017), degus (Gos et al., 2014), hamsters (Brooks et al., 2005)), and in biparental systems it can be difficult to tease out the effects of joint-caregiving from fatherhood per se (but see (DeAngelis et al., 2017) and (O’Connell et al., 2012)).

Results of experiment 1 suggest that males fine-tune their behavioral reactions to social stimuli depending on their parental status. Previous studies have suggested that although male sticklebacks will mate multiply within a given breeding attempt, they usually stop courting females once they have eggs in their nest (Kraak et al., 1999). Our behavioral results are consistent with this observation: males finely tuned their conspicuous courtship behavior depending on their parental status – only nonparental males performed zig zags. We also found that males modulated their nest-directed activities depending on their parental status and the immediate social context: parental males visited their nests more than nonparental males, and rates of nest visitation were particularly high when they were presented with a female stickleback and a model predator. Both female sticklebacks and birds are known to be nest predators (Bellesiles et al., 1990; Bellesiles and Fitzgerald, 1993; Defrajont et al., 1992; Fitzgerald, 1991, 1992; Fitzgerald et al., 1992; Foster, 1988; Largiader et al., 2001) therefore increased nest visitation rate in the presence of a female stickleback and a model bird predator could reflect increased nest defense.

The results from Experiment 2 suggest that dramatic changes in brain IEG expression accompany the transition between the nonparental and parental phases. Egr-1 expression increased as males started to provide care, and was especially high after the eggs hatched. This result parallels a study of biparental California mice, where exposing fathers to pup stimuli increased IEG expression in key brain areas that are involved in processing emotional stimuli such as the lateral habenula, caudal dorsal raphe nucleus (de Jong et al., 2010). We infer that the increase in Egr-1 expression in this study reflects the growing behavioral demands of caring for active offspring. For example, once the eggs hatch, fathers constantly engage in reciprocal
Fig. 3. Egr-1 expression across breeding stages within each brain area. (a) anterior tuberal nucleus/ventral medial hypothalamus (vTn/VMH), (b) lateral part of dorsal telencephalon/hippocampus (DI/HC), (c) medial dorsal telencephalon/basolateral amygdala (Dm/AMY), (d) periaqueductal gray (PAG), (e) preoptic area parvocellular (POAp), (f) posterior tuberculum/ventral tegmental area (TPp/VTA), (g) dorsal part of the ventral telencephalon/nucleus accumbens (Vd/NA), (h) ventral pallium/bed nucleus of the stria terminalis medial amygdala (Vs/BNST) (i) ventral tuberal/anterior hypothalamus (vTn/AH) and (j) ventral part of the ventral telencephalon/lateral septum (Vv/LS). Different letters indicate statistically significant differences between brain areas according to post-hoc tests.
behavioral interactions with their fry, and fry provide multiple types of sensory information. Fathering fry is particularly demanding as fathers must adjust their caregiving to match the physiological and behavioral needs of their free-swimming offspring.

It is also noteworthy that Egr-1 expression in fathers’ brains returned to ‘baseline’, pre-reproductive levels after they successfully reproduced. This result is consistent with studies of maternal care which have shown that brain IEG expression increases during care, and drops when offspring are absent (reviewed in (Stack and Numan, 2000)). The fact that Egr-1 expression dropped after breeding suggests that the high levels of Egr-1 expression observed while males were caring for offspring does not simply reflect the effects of season, maturation, age or experience. This pattern is intriguing given that male sticklebacks typically breed more than once during the breeding season, and there is widespread evidence that parenting experience permanently changes the brain and behavior of mothers (‘once a mother always a mother’, reviewed in (Stolzenberg and Champagne, 2016)). Further investigations of changes in neural IEG expression as a function of parenting experience in male sticklebacks is a promising future direction.

In experiment 2, Egr-1 expression changed over a relatively long, approximately 10 day period. These results are consistent with the literature showing that IEG expression can vary over both relatively short (minutes) and long (days) timescales. Other studies have documented similar constitutively different levels of IEG expression between relatively long-lasting states, e.g. between times of day (Kornhauser et al., 1996; Moffatt et al., 1995; Prosser et al., 1994) and stages of estrus (Lloyd et al., 1994; Nappi et al., 1997; Slade and Carter, 2000). Our results suggests that our in situ protocol is capable of tracking these quantitative changes in gene expression.

4.1. Egr-1 expression in particular brain areas

One somewhat surprising result from this study is that Egr-1 was expressed in many nodes within the social behavior network, rather than just a few. One possible explanation for this finding is that paternal care in sticklebacks involves elements not only of providing care, but also of aggression toward intruders and predators, and mating (because males continue to court females after they mate). We speculate that there was IEG expression in so many nodes of the social behavior network in male sticklebacks during the reproductive cycle because their behavior during this period is multidimensional. Indeed, it is possible that some of the changes in Egr-1 expression could be due to changes in activity or swimming, rather than attributable to the effects of parenting per say.

That being said, IEG expression was especially high in certain nodes within the social behavior network. For example, our results suggest that the lateral part of dorsal telencephalon/hippocampus had high levels of IEG expression in male sticklebacks throughout the breeding cycle. High levels of IEG expression in the lateral part of dorsal telencephalon/hippocampus could reflect the importance of cognitive
processes, especially spatial cognition, for territorial males. Learning and memory are especially important for territorial animals (Stamps and Krishnan, 2001), which need to learn the spatial boundaries of their territory and engage in repeated interactions with their neighbors as they learn the boundaries of their territory and how to detect and repel intruders (Peeke, 1969; Peeke and Veno, 1973). In rodents, both mothers (Kinsley and Lambert, 2006; Levy et al., 2011; Pavluski et al., 2016; Ruscio et al., 2008) and fathers (Gasper et al., 2011; Lambert et al., 2011) undergo changes in hippocampal-mediated plasticity. A promising research direction is to examine the effect of experience as a parent on neurogenesis, hippocampal plasticity and performance on cognitive tasks in male sticklebacks.

The preoptic area is often associated with maternal, paternal and biparental behaviors in vertebrates (Alger et al., 2009; Bales and Saltzman, 2016; Buntin et al., 2006; de Jong et al., 2009; Gammie, 2005; Lee and Brown, 2002, 2007; Numan, 1974, 1986). For example, the preoptic bed nuclei of the stria terminalis is involved in the switch from infanticidal to paternal behavior (Tsuneoka et al., 2015) and the VMH projects directly to the dorsal telencephalon/hippocampus in teleosts (O’Connell and Hofmann, 2011) therefore we have shown increases in IEG expression in mothers (Ga, 2011) undergo changes in hippocampal-mediated plasticity. A

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lateral septum, its expression was not quite as high as expected based on other studies. For example, cfos expression was higher in the lateral septum of fathers vs non-fathers in cichlids (O’Connell et al., 1999). The VMH projects directly to the dorsal telencephalon/hippocampus in teleosts (O’Connell and Hofmann, 2011) therefore we have shown increases in IEG expression in mothers (Ga, 2011) undergo changes in hippocampal-mediated plasticity. A


