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RESEARCH ARTICLE

Variation in sexual brain size dimorphism over the breeding cycle in the three-spined stickleback

Séverine D. Buechel[1,‡], Kristina Noreikiene[2,3], Jacquelin DeFaveri[2], Elisavet Toli[2,4], Niclas Kolm[1,*] and Juha Merilä[2,*]

ABSTRACT

Snapshot analyses have demonstrated dramatic intraspecific variation in the degree of brain sexual size dimorphism (SSD). Although brain SSD is believed to be generated by the sex-specific cognitive demands of reproduction, the relative roles of developmental and population-specific contributions to variation in brain SSD remain little studied. Using a common garden experiment, we tested for sex-specific changes in brain anatomy over the breeding cycle in three-spined stickleback (*Gasterosteus aculeatus*) sampled from four locations in northern Europe. We found that the male brain increased in size (ca. 24%) significantly more than the female brain towards breeding, and that the resulting brain SSD was similar (ca. 20%) for all populations over the breeding cycle. Our findings support the notion that the stickleback brain is highly plastic and changes over the breeding cycle, especially in males, likely as an adaptive response to the cognitive demands of reproduction (e.g. nest construction and parental care). The results also provide evidence to suggest that breeding-related changes in brain size may be the reason for the widely varying estimates of brain SSD across studies of this species, cautioning against interpreting brain size measurements from a single time point as fixed/static.

KEY WORDS: Brain anatomy, Courtship behaviour, Paternal care, Phenotypic plasticity, Sexual dimorphism, *Gasterosteus aculeatus*

INTRODUCTION

The evolution of brain morphology has received much attention (e.g. Aiello and Wheeler, 1995; Benson-Amram et al., 2016; Gonda et al., 2013; Kotrschal et al., 2013; MacLean et al., 2014; Ruiz-González et al., 2009; Striedter, 2005). Although the structural architecture of the brain varies extensively between sexes (Breedlove, 1992; Hoekzema et al., 2017; Nottebohm, 1981; Smith et al., 1997), only a handful of cases of sexual size dimorphism (SSD) in relative brain size (i.e. brain size corrected for body size) have been reported (Garamszegi et al., 2005; Herczeg et al., 2015, 2014; Kotrschal et al., 2012a; Samuk et al., 2014). This is somewhat surprising given that sex-specific selective pressures have produced numerous other kinds of morphological and behavioural dimorphisms. Given that variation in brain (or brain region) size is likely associated with variation in cognitive abilities (Benson-Amram et al., 2016; Buechel et al., 2018; Kotrschal et al., 2013, 2015; Lázaro et al., 2018; MacLean et al., 2014; but see Chittka et al., 2012), it would be reasonable to expect that brain anatomy would also reflect different cognitive challenges faced by the two sexes. Hence, the male and female brain should differ in size if the sexes face consistently different cognitive demands. In particular, reproductive role division has produced a number of sex-specific behaviours (Andersson, 1994) that are expected to place distinct cognitive demands on males and females. In support of this, positive relationships have been found between brain size and the accuracy of female mate choice performance (Corral-López et al., 2017), the complexity of male courtship behaviours (Day et al., 2005; Lindsay et al., 2015; Madden, 2001) and the degree of parental investment (Gittleman, 1994; Gonzalez-Voyer et al., 2009; Herczeg et al., 2014; Kotrschal et al., 2012a; Samuk et al., 2014). Moreover, theory predicts that the sex that makes a greater investment into reproductive behaviours is the one with enhanced cognitive abilities, and thus, the sex with the larger brain or brain regions (Jacobs, 1996).

Sticklebacks form one rare example of a species displaying extensive male-biased SSD in the brain (but see Samuk et al., 2014) that has been attributed to their distinct and sex-specific reproductive behaviours (Kotrschal et al., 2012a; Herczeg et al., 2014). It is the male that builds and defends an elaborate nest, courts females and cares for offspring, whereas the female's role in reproduction is restricted to choosing a male with a nest to lay her eggs in. Interestingly, recent studies indicate that the degree of SSD in the stickleback brain can vary from 4% to more than 20% depending on the population studied (Herczeg et al., 2015; Kotrschal et al., 2012a; Samuk et al., 2014; Toli et al., 2017). However, it is unclear whether these widely varying estimates of brain SSD reflect genetic differences between populations, or whether they are attributable to developmental plasticity; if brain SSD exhibits breeding cycle dependent developmental plasticity, heterogeneity in timing of sampling in relation to breeding cycle in different populations could explain among-population variation in brain SSD (see Herczeg et al., 2015). Although a genetic component to brain size SSD has been demonstrated in a common garden experiment (Toli et al., 2017), there are good reasons to assume that a developmental component to SSD variation in the stickleback brain may also be important. First, the brain is amongst the most energetically expensive organs to develop and maintain, and adaptive phenotypic plasticity in brain anatomy can therefore be expected. Second, fishes in general have indeterminate growth of neural cells that may facilitate lifelong neural adaption to cognitive demands (Zupanc, 2001, 2006), and the stickleback brain has been shown to react to environmental stimuli

[1]Department of Zoology/Ethology, Stockholm University, Svante Arrhenius väg 18B, 10691 Stockholm, Sweden. [2]Ecological Genetics Research Unit, Organismal and Evolutionary Biology Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, PO Box 65, FI-00014 Helsinki, Finland. [3]Chair of Aquaculture, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Kreutzwaldi tn. 46, 51006 Tartu, Estonia. [4]Molecular Ecology & Conservation Genetics Lab, Department of Biological Applications & Technology, University of Ioannina, 45110 Ioannina, Greece.
*Shared senior author contribution

‡Author for correspondence (severine.buechel@zoologi.su.se)

S.D.B., 0000-0002-2385-2973; K.N., 0000-0001-7529-4902; N.K., 0000-0001-5791-336X; J.M., 0000-0001-9614-0072

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strongly and rapidly (Herczeg et al., 2015; Park et al., 2012). Third, and perhaps most important, one can expect a developmental component to brain SSD because the high cognitive demands that are associated with reproduction also change over the breeding cycle.

Using a common garden experiment, we tested for sex-specific developmental changes in brain anatomy in four populations of the three-spined stickleback (*Gasterosteus aculeatus*) sampled over their breeding cycle. To this end, we sampled laboratory-reared F_1-generation males and females from four northern European locations at three time points: before sexual maturity (non-breeding), at the onset of sexual maturity (pre-breeding), and after a 1-week mating period, when females had laid eggs and males cared for eggs (breeding). Relative brain masses and the volumes of six different brain subregions were compared between the sexes at each time point. In comparison to field sampling across the breeding cycle, this experiment conducted under controlled environmental conditions allowed us to evaluate to what degree variation in brain SSD is attributable to developmental, sex-specific or population-specific sources. As to the latter, large population differences that are constant over all three sampling points would provide evidence for the importance of fixed differences in SSD among populations, and support the hypothesis that the large variance in brain SSD among stickleback populations observed in earlier studies (Herczeg et al., 2015; Kotrschal et al., 2012a; Samuk et al., 2014; Toli et al., 2017) is mainly attributable to population differentiation (see Toli et al., 2017). On the contrary, constant (parallel) changes in brain SSD over different sampling periods in all replicate populations would provide strong evidence for a developmental component to brain SSD, and indicate that the large variance in brain SSD observed in earlier studies (see above) could owe to differences in timing of sampling relative to the breeding cycle. Furthermore, we also investigated how variation in brain anatomy was associated with the variation in expression of breeding-related behaviours. We did this by comparing the size of the brain and different brain subregions between males that built a nest and those that did not; between males that engaged in paternal care of eggs and those that did not; and between females that had laid eggs in the male's nest and those that did not. Under the assumptions that nest building (Day et al., 2005; Madden, 2001), paternal care (Herczeg et al., 2014; Kotrschal et al., 2012a) and mate choice (Boogert et al., 2011; Corral-López et al., 2017) are cognitively demanding behaviours, and that the stickleback brain is plastic, one would expect to see an association between the expression of the respective reproductive behaviour and variation in brain size and/or size of particular brain subregions.

MATERIALS AND METHODS

Origin and rearing of sticklebacks

Individual three-spined sticklebacks (*Gasterosteus aculeatus* Linnaeus 1758) used in this experiment were F_1-generation full-sibs, which allowed us to contrast the SSD and account for the genetic background. Parental fish were caught during June 2015 from Sylt, Germany (S), Mariager, Denmark (D), Kotka, Finland (K), and Oulu, Finland (O) (Fig. 1). Ten full-sib families per location were created using artificial fertilization, either in the field (S, D) or in the laboratory facilities at the University of Helsinki, Finland (K, O), during 6 June to 5 July 2015. Eggs were kept in Petri dishes until larvae hatched. After hatching, larvae were kept in 0.5 litre containers and fed *Artemia salina* nauplii twice daily for 1 week. Fry were then transferred to 1.2 litre tanks in Allentown zebrafish racks (Allentown, San Diego, CA, USA). After 1 to 2 months, each family was divided in half and each sibling group (consisting of ca. 20 individuals) was transferred to 5 litre tanks randomly distributed among four different zebrafish racks. Chopped chironomid larvae were slowly introduced with *A. salina* until the fish could feed on whole chironomids, provided twice daily *ad libitum*.

The experiments were approved by the Finnish National Animal Experiment Board under license PH1236A, and all used methods were carried out in accordance with approved guidelines.

Sampling protocol

We sampled fish at three time points: non-breeding (5–6 months of age), pre-breeding (following 1 month of overwintering conditions, which included a short photoperiod of 6 h:18 h light:dark and low temperature of 10°C; 8–9 months of age) and breeding (following exposure to 24 h:0 h light:dark conditions at 18°C; 10–11 months of age; Fig. 1). During the first two sampling points, individual sex identification *a priori* was not possible, so sampling effort was increased to three to four randomly chosen individuals per family. A pectoral fin clip was taken during these sampling periods for subsequent molecular sexing using the method described in Toli et al. (2016). For the breeding sampling period, a mature male and female (as confirmed by visual inspection of male coloration and the presence of ovulated eggs in females) from separate families within each population were selected and transferred together to 38 litre aquaria (40×30×24 cm, length×height×width), each equipped with aerators, 0.10 litre plastic boxes filled with sand for nesting substrate, and similar quantities of filamentous algae and 7-cm-long sewing threads for nesting material. Some males started building nests minutes after transfer to breeding aquaria, while others took up to 24 h to initiate the process or failed to build nests. Adjacent aquaria were separated with opaque plastic dividers as visual barriers. Breeding fish were fed twice per day with chironomid larvae. In cases where females died owing to unknown reasons, they were replaced by a sibling female and the change was marked. Each pair was killed after 7 days, i.e. before fry had hatched. Nests were deconstructed and (if present) eggs were removed in order to determine their developmental stage following Swarup (1985). The presence of nest, eggs in the nest and their developmental stage were used as proxies for breeding-related behaviour. After each pair was removed, aquaria were rinsed with alcohol, filled with fresh tap water and new nesting material, and allowed to settle for 24 h before introducing the next pair. At the end of the experiments, fish were over-anaesthetized with MS222 (tricaine methane sulphonate), weighed to the nearest 0.001 g, and measured to the nearest 0.01 cm for total and standard lengths (from the tip of the mouth to the tail base). Processed fish were fixed for 5 days in a phosphate-buffered saline (PBS) solution containing 4% paraformaldehyde and 2.5% glutaraldehyde. Fish were then washed twice with PBS and kept in PBS at +4°C until brain dissections were conducted.

Dissections and analysis of brain anatomy

For the analysis of population-specific changes in brain anatomy over the breeding cycle, 240 male and female sticklebacks were selected for further processing. This sub-sample included one male and one female from each family (n=10) and population (n=4) for each of the three sampling points (i.e. n=120 males and 120 females in total; Fig. 1). All fish from the breeding sample fulfilled the following criteria that are necessary for successful reproduction: all males had built a nest and also had eggs in their nest to care for, and all females had laid eggs when offered a nest. The formalin-fixed and dissected brains were stored in phosphate buffer and weighed to

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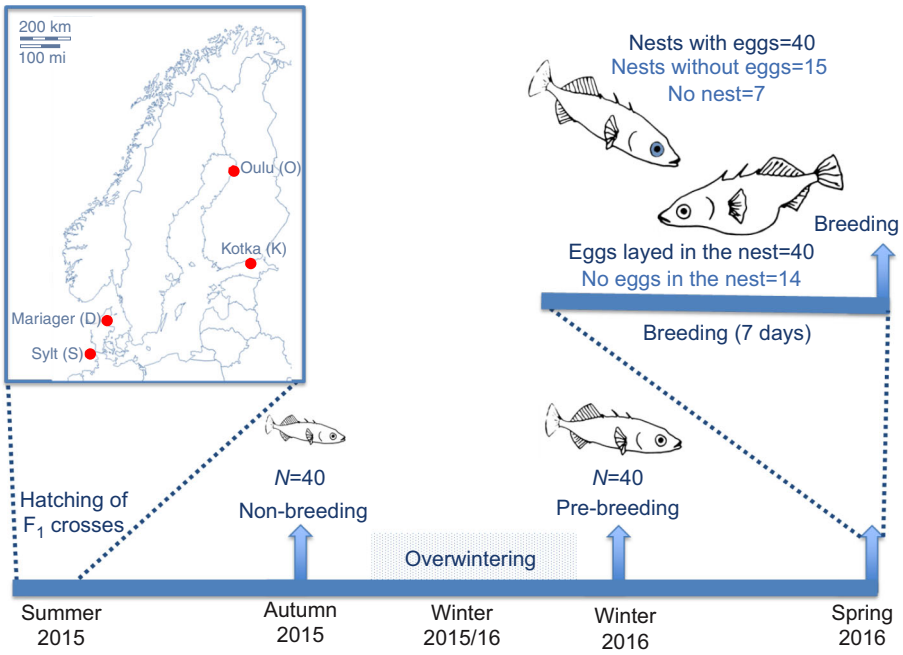

Fig. 1. Experimental set-up. Male and female sticklebacks were sampled from four different populations in northern Europe in summer 2015, and 10 full-sib families per location were created using artificial fertilization. One male and one female per family and location (n=40) were sampled for subsequent brain size analysis at each of three sampling points (non-breeding, pre-breeding and breeding) over the breeding cycle. Note that visually mature males (blue eyes) and females (presence of eggs in reproductive tract) were kept together for breeding during a period of 7 days during which the expression of three different reproductive behaviours (nest building, paternal care, egg laying) was scored. For details, see Materials and Methods.

the nearest 0.001 mg. The volume of six different brain subregions (*viz*. olfactory bulb, telencephalon, optic tectum, cerebellum, dorsal medulla and hypothalamus) were quantified as described in Kotrschal et al. (2012b). Briefly, we used a dissection microscope (LAS EZ version 3.0, Leica Microsystems, Switzerland) to obtain four digital images per brain (*viz*. ventral view, dorsal view, right and left side views). The length (L), width (W) and height (H) of the respective brain subregion were measured with ImageJ (http://rsb.info.nih.gov/ij/) and used to estimate the volume (V) of each brain subregion using the ellipsoid model $V=(L\times W\times H)\pi/6$ (Van Staaden et al., 1995). For bilateral subregions (*viz*. optic tectum, the telencephalon and the olfactory bulb), both sides were measured and the total volume was estimated as the sum of the two bilateral subregions. To analyze how brain anatomy is associated with the expression of breeding-related behaviours, an additional 36 fish in breeding condition were dissected. These samples included males that did not build nests (n=7), females that did not lay eggs in the nest (n=14) and males that did not have eggs in their nests (n=15). We checked all females that had not laid eggs in the nest for the presence of overripe eggs in their reproductive tract (Roufidou et al., 1995). No female was found to be overripe, which suggests that they must have laid eggs most likely elsewhere in the breeding tank (but see Discussion). All dissections were performed by the same person (S.D.B.) and all quantifications were performed blind in respect to the sampling point, family and sex.

Statistical analyses

We tested for sex-specific changes in brain size and anatomy over the breeding cycle using a general linear mixed model (LMM) approach as implemented in the lmerTest package (http://cran.r-project.org/package=lmerTest) in R (version 3.3.3; https://www.r-project.org/). Relative differences in brain size (response variable) were analysed by fitting a model on log-transformed brain mass measures using sex, the population of origin (S, D, K and O) and the sampling point (*viz*. non-breeding, pre-breeding and breeding) as fixed factors, the log-transformed standard length (SL) as a covariate and the family within the population as a random factor. Note that we modelled all possible two-way interactions, but for ease of interpretation, only the biologically relevant three-way interaction (sex×population×sampling point) was included: this interaction tests for population differences in the increase in relative SSD in the brain between sampling points [lme4 syntax for R, full model: brain mass ~sex+sampling+SL+population+sex:sampling+sampling:SL+population:sampling+sex:SL+sex:population+population:SL+sex:population:sampling+(1|family in population); lme4 syntax for R, final model: brain mass~sex+sampling+SL+population+sex:sampling+population:sampling+(1|family in population)]. Sex-specific differences in relative brain size between the sampling points were analysed *post hoc* using the lsmeans package in R (Lenth, 2016). Population-level differences in relative SSD in the brain within each sampling point were analysed by pairwise comparisons of sex×population interaction terms. A non-significant interaction term was interpreted to indicate a lack of difference in SSD between two populations. Variation in absolute brain and body size over the breeding cycle was analysed by fitting analogous models on male and female brain mass and body size (same fixed and random factors as used in the model for brain mass differences, but no covariate was included).

Differences in relative brain subregion volumes (*viz*. telecephalon, optic tectum, olfactory bulb, hypothalamus, cerebellum and dorsal medulla) were analysed using six identical models on log-transformed brain subregion volumes (response variables) including sex, the population of origin (S, D, K and O) and sampling point (*viz*. non-breeding, pre-breeding and breeding) as fixed factors, the log-transformed brain mass (minus the volume of the respective brain subregion) as a covariate, and the family within the population as a random factor [lme4 syntax for R, full model: brain subregion~sex+sampling+population+co-variate+sex:sampling+population:sampling+population:sex+population:sex:sampling+(1|family in population); lme4 syntax for R, final model: brain subregion~sex+sampling+population+covariate+(1|family in population), for all brain subregions except for the dorsal medulla, for which the full model was also the final model]. To analyse relative brain subregion volumes, we controlled given brain subregion volume for total brain mass minus the volume of the region of interest. Otherwise the total brain mass would be confounded by the respective region of interest

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Table 1. General linear mixed model results for three-spined stickleback brain mass, brain subregion volumes and body size over three sampling points

Trait	Sex	Population	Sampling point	Covariate	Sex×Sampling point	Sex× Population	Population× Sampling point	Sex×Population× Sampling point
Brain mass	170.46 (1,194)***	8.34 (3,39)***	42.12 (2,195)***	479.29 (1,202)***	4.96 (2,177)**	1.24 (3,178)	4.09 (6,179)***	1.39 (6,176)
Telencephalon	72.00 (1,180)***	1.73 (3,40)	37.82 (2,193)***	408.33 (1,198)***	1.96 (2,177)	0.72 (3,180)	0.67 (6,178)	0.98 (6,176)
Optic tectum	2.61 (1,177)	1.16 (3,32)	0.80 (2,192)	543.03 (1,173)***	1.16 (2,171)	0.26 (3,174)	1.55 (6,175)	0.92 (6,170)
Cerebellum	5.92 (1,208)*	10.09 (3,40)***	10.80 (2,208)***	352.87 (1,208)***	0.14 (2,180)	0.35 (3,183)	1.43 (6,184)	0.12 (6,179)
Dorsal medulla	0.13 (1,186)	3.42 (3,33)*	1.99 (2,189)	145.89 (1,136)***	1.93 (2,178)	1.23 (3,183)	0.53 (6,181)	2.36 (6,177)*
Olfactory bulb	1.76 (1,181)	1.60 (3,37)	4.62 (2,191)*	63.58 (1,168)***	0.86 (2,176)	0.13 (3,178)	1.99 (6,179)	0.71 (6,175)
Hypothalamus	1.27 (1,209)	1.51 (3,40)	0.62 (2,208)	194.28 (1,209)***	0.14 (2,209)	0.77 (3,209)	0.52 (6,179)	1.25 (6,209)
Body size	53.51 (1,178)***	2.39 (3,40)	156.95 (2,178)***		0.17 (2,177)	0.61 (3,178)	0.80 (6,178)	0.62 (6,177)

Shown are the *F*-statistics of log-transformed brain mass, body size (standard length) and brain subregion volumes with degrees of freedom in parentheses. The significance level is given in (*P<0.05, **P<0.01, ***P<0.001). The three-way interaction indicates significant population differences in brain sexual size dimorphism over the three sampling points. Note that the covariate for brain subregions is calculated as brain mass (mg) minus the volume of the respective brain subregion.

(note that fixed brains have a density very close to 1, such that 1 g equals 1 ml; volume and mass can therefore be used interchangeably to a great extent). Pairwise differences between significant model terms were tested *post hoc* using lsmeans (R package lsmeans; Lenth, 2016).

To analyse the associations between relative brain size (response variable) and breeding-related behaviours (explanatory variables), we performed three LMMs on log-transformed brain mass values for different reproductive behaviours (*viz*. nest building, egg laying in the nest and paternal care). These models used the presence/absence of the respective behaviour as a fixed factor, the log-transformed SL as a covariate and the population of origin as a random factor [lme4 syntax for R, full and final models: brain mass∼presence/absence behaviour+SL+(1|population)]. Underlying brain subregion volumes were tested using six LMMs for each behaviour (one model per brain subregion) [lme4 syntax for R, full and final models: brain subregion volume∼presence/absence behaviour+covariate+(1|population)]. Potential differences in condition between individuals that showed versus did not show a certain behaviour were tested using body size as a response variable, the respective behaviour (*viz*. nest building, egg laying in the nest, paternal care) as a fixed factor, the log-transformed SL as a covariate and the population of origin as a random factor [lme4 syntax for R, full and final models: body mass∼presence/absence behaviour+SL+(1|population)]. Model selection was performed backwards (Crawley, 1993) by stepwise elimination of terms based on Akaike's information criterion (ΔAIC>2), with the final model being confirmed by automated model selection using the R glmulti package (Calcagno and de Mazancourt, 2010).

RESULTS

Across all populations, males had larger brains than females, both in absolute terms (LMM on brain mass, sex: $F_{1,177}$=7.37, P=0.007) and when controlling for body size (LMM on brain mass, sex: $F_{1,194}$=170.46, P<0.001; LMM on body size; sex: $F_{1,178}$=53.51, P<0.001; males n=108, 36.22±0.38 mm versus females n=105, 38.48±0.38 mm; Table 1, Fig. 2, note that males were smaller than females). This pattern was consistent across all sampling points (Fig. 2). Although an increase in relative brain size towards breeding was observed in both sexes (LMM on brain mass, sampling point: $F_{2,195}$=42.12, P<0.001; pairwise brain mass comparisons of non-breeding versus pre-breeding, males: t=−6.02, d.f.=193.7, P<0.001; females: t=−3.35, d.f.=190.5, P=0.012; pre-breeding versus breeding, males: t=−5.34, d.f.=185.2, P<0.0001, females: t=−3.8, d.f.=186.3, P=0.002; Table 2, Fig. 2), the increase in male brain size exceeded that of females (LMM on brain mass, sex×sampling: $F_{2,177}$=4.96, P=0.008; Table 1). This sex-specific increase in relative brain size resulted in an increase in relative brain SSD from 10.4% in non-breeding to 19.8% in breeding conditions (means calculated over all populations; Table 2).

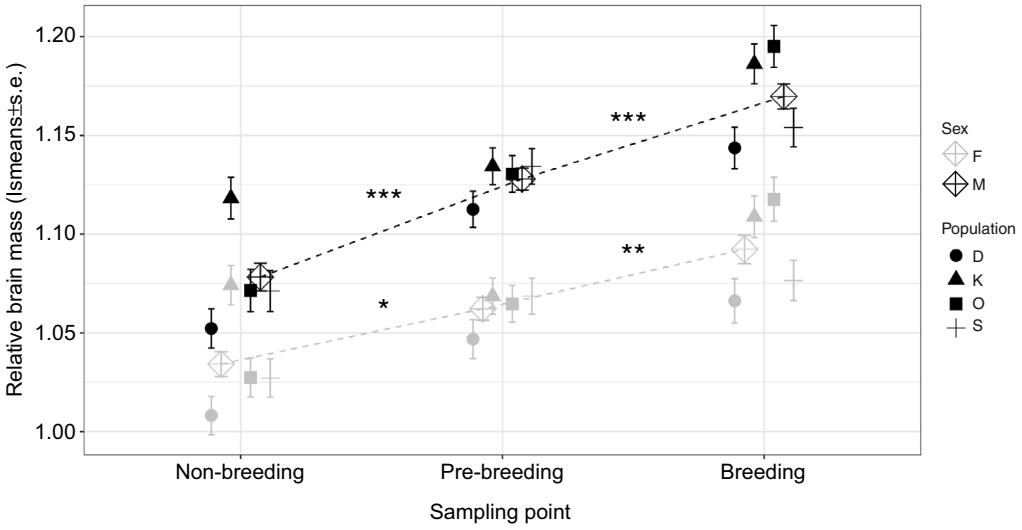

Fig. 2. Relative brain size of male and female sticklebacks over the breeding cycle. Shown are the least square means (±s.e.m.) for males (black solid symbols) and females (grey symbols) from four populations over three sampling points. Open diamonds indicate the population means for males (black) and females (grey). Least square means were obtained from a model that used sex, sampling, population of origin and the interaction terms as fixed factors, brain mass corrected for body size as covariate and the family within the population as random factor (see Materials and Methods). Asterisks (*P<0.05, **P<0.01, ***P<0.001) indicate the significance level of pairwise comparisons between sampling points for males and females.

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Table 2. Sexual size dimorphism (SSD) in the three-spined stickleback brain measured over three sampling points during the reproductive cycle

	Non-breeding			Pre-breeding			Breeding		
Population	Males	Females	SSD (%)	Males	Females	SSD (%)	Males	Females	SSD
Kotka	13.1±1.0	11.9±1.0	10.1	13.6±1.0	11.7±1.0	16.2	15.4±1.0	12.9±1.0	19.4
Sylt	11.7±1.0	10.6±1.0	10.4	13.6±1.0	11.7±1.0	16.2	14.3±1.0	11.9±1.0	20.2
Mariager	11.3±1.0	10.2±1.0	10.8	13.0±1.0	11.1±1.0	17.1	13.9±1.0	11.6±1.0	19.8
Oulu	11.8±1.0	10.7±1.0	10.3	13.5	11.6±1.0	17.1	15.7±1.0	13.1±1.0	19.8

Shown are back-transformed least square means±s.e.m. from a general linear mixed model (LMM) on log-transformed brain mass (mg) of male and female stickleback (see Materials and Methods for model details). Percentage of difference between sexes (SSD) are calculated as [(higher value–lower value)/lower value]×100. Note that the magnitude of brain SSD did not differ among populations within sampling points [P>0.1 for all pairwise interactions (sex×population) within each sampling point].

Even though brain–body allometry did not differ between the sexes (LMM on brain mass, sex×body size: $F_{1,194.32}$=0.11, P=0.744), it is noteworthy that the male and female allometry differed in their intercepts for the different sampling points (Fig. 3; for intercept differences, see pairwise comparisons between sampling points for males and females given above), but not in their slopes [LMM on brain mass, sampling×body size×sex (compares the interaction between all six slopes): $F_{2,191.61}$=0.05, P=0.952]. This suggests that the brain size of males and females did not increase linearly with body size, but passed through phases of rapid growth upon transition from one sampling point to another.

Even though there were population differences in relative brain size when tested across all sampling points (LMM on brain mass, population: $F_{3,39}$=8.34, P<0.001; Table 1, Fig. 1), male relative brain size exceeded that of females in all populations and at all three sampling points (Fig. 2, Table 2). As such, all four populations showed a similar increase in brain SSD across the sampling points (LMM on brain mass, sex×population×sampling: $F_{6,176}$=1.39, P>0.1; Table 1). The resulting brain SSD did not differ among populations at any sampling point [Table 2, all pairwise SSD comparisons (population×sex interaction terms) within each sampling point were non-significant].

Four brain subregions (*viz*. telecephalon, cerebellum, olfactory bulb and dorsal medulla) showed statistically significant volume changes between the sexes, populations and/or over the sampling points (see Fig. 4 and Table 1 for F-statistics and significance levels). The telencephalon was larger in males than in females, but it increased for both sexes upon transition to sexual maturity (sex: $F_{1,180}$=72.00, P<0.001; sampling: $F_{2,193}$=37.82, P<0.001). This pattern did not differ between populations (population: $F_{3,\ 40}$=1.73, P>0.1). Even though males and females from some populations had overall larger cerebelli than in other populations (population: $F_{3,40}$=10.09, P<0.001), relative cerebellum size was larger in females than in males, and it decreased in volume in both sexes and in all four populations in the pre-breeding condition (note that the cerebellum regained its initial non-breeding size during breeding; sex: $F_{1,208}$=5.92, P=0.02; sampling: $F_{2,208}$=10.80, P<0.001; population×sampling: $F_{6,184}$=1.43, P>0.01). There were no differences in olfactory bulb volume between the sexes and populations (sex: $F_{1,181}$=1.76, P>0.1; population: $F_{3,37}$=1.60, P>0.1), but the olfactory bulb volume decreased between the pre-breeding and the breeding conditions (sampling: $F_{2,191}$=4.62, P=0.01). The dorsal medulla volume showed population-specific patterns over the breeding cycle (sex×population×sampling: $F_{6,177}$=2.36, P=0.03).

Nest-building males had an average of 7.5% larger brains than non-nest building males (LMM on brain mass, $F_{1,52}$=5.77, P=0.02), but there were no significant differences in relative brain subregion volumes between nest-builders and non-nest-builders (Table 3). There was no difference in relative brain size between males that had eggs in their nest (and hence did engage in paternal care) and those that did not (LMM on brain mass, $F_{1,43}$=1.23, P>0.1); however, the

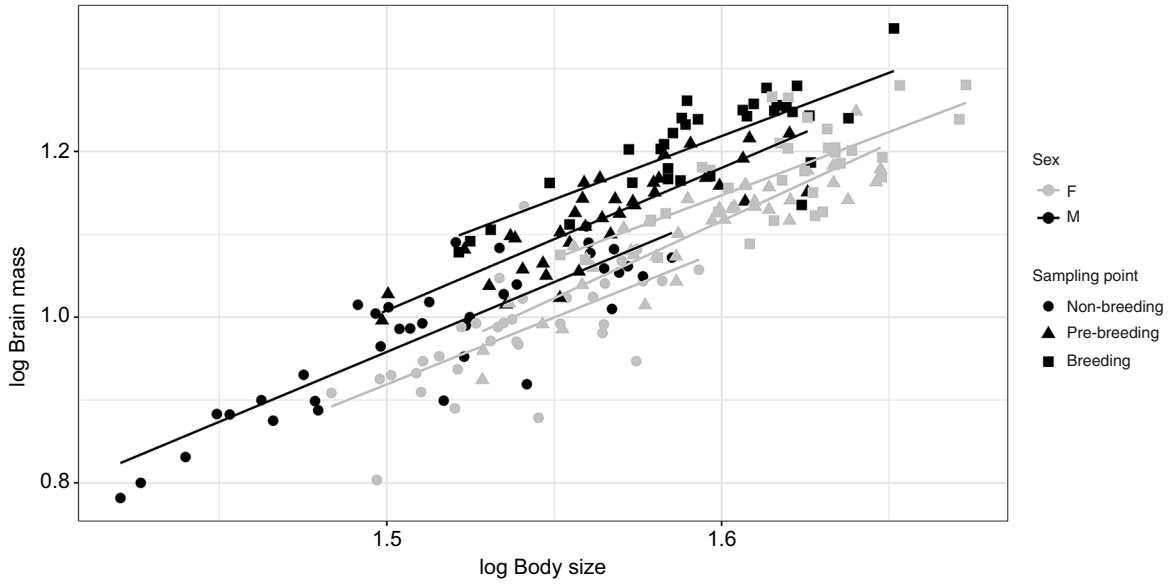

Fig. 3. Brain–body allometry for male and female sticklebacks over three different sampling points. Shown is the brain–body allometry for males (black solid symbols) and females (grey symbols) from four populations at three sampling points (different shapes) over the breeding cycle.

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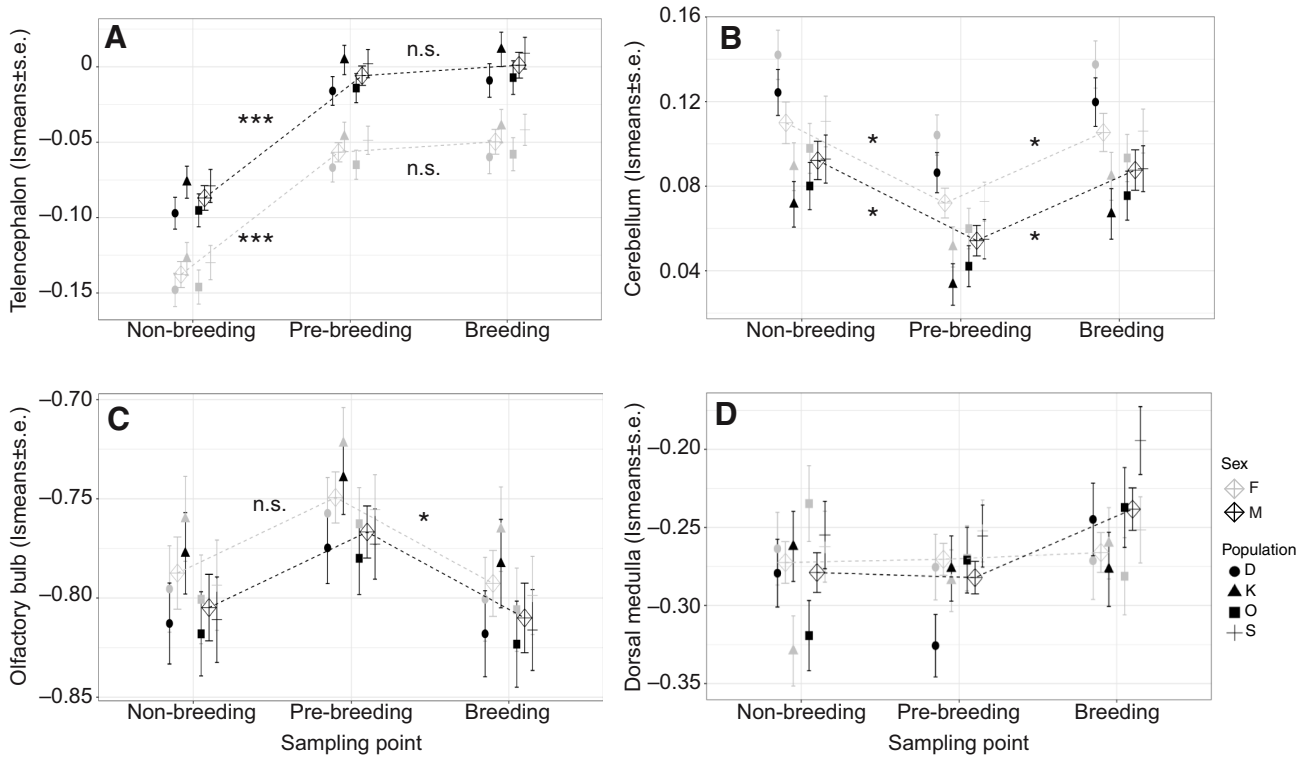

Fig. 4. Brain subregion volumes of male and female sticklebacks that showed statistically significant size changes over the breeding cycle. Shown are the least square means (±s.e.m.) of (A) the telencephalon, (B) the cerebellum, (C) the olfactory bulb and (D) the dorsal medulla for males (black solid symbols) and females (grey solid symbols) from four populations over three sampling points. Open diamonds indicate the population means for males (black) and females (grey; see Materials and Methods for model details). Asterisks (n.s., not significant; *P<0.05, ***P<0.001) indicate the significance level of relevant pairwise *post hoc* comparisons between sampling points for males and females.

hypothalamus was 29% larger in males with eggs in their nest than those without (Table 3). Nest-laying and non-nest-laying females did not differ in relative brain size (LMM on brain mass, $F_{1,44}$=0.82, P>0.1); however, nest-laying females had 7.5% larger optic tecti, 28.5% larger hypothalami and 12% smaller cerebelli as compared with non-nest-laying females (Table 3). The overall condition (Table 3, body mass in relation to SL) did not differ between individuals that expressed a certain behaviour versus individuals that did not show this behaviour.

DISCUSSION

We tested for sex-specific changes in brain anatomy over the breeding cycle of the three-spined stickleback, and in particular the prediction that the cognitive demands of breeding drive a stronger developmental increase in male than female brain size. In fact, we found that brain size increased in both males and females throughout the experiment, but the male brain increased significantly more than the female brain. These findings have two important implications. First, they are consistent with the idea that the sex that makes greater investment into reproduction, which in the case of sticklebacks is the male, also invests more into energetically expensive brain tissue. This finding corroborates the idea that cognition is important for reproduction. Second, the finding that not only male brain size, but also female brain size increases towards breeding supports the view that in addition to male courtship and paternal care, female reproduction, which includes comparing and choosing a potential mate, as well as the actual mating act, is also cognitively demanding (Boogert et al., 2011; Corral-López et al.,

Table 3. General linear mixed model results for the expression of breeding-related behaviours

Trait	Nest building: males	Nest laying: females	Paternal care: males
Sample size	*y*=48 vs *n*=7	*y*=32 vs *n*=14	*y*=32 vs *n*=15
Brain mass	5.77 (1,52)* diff=7.5%	0.82 (1,44)	1.23 (1,43)
Telencephalon	0.53 (1,54)	0.71 (1,43)	2.33 (1,42)
Optic tectum	0.47 (1,52)	7.00 (1,45)* diff=7.5%	0.31 (1,42)
Cerebellum	0.01 (1,51)	5.70 (1,44)* diff=−12%	2.06 (1,41)
Dorsal medulla	1.14 (1,54)	2.94 (1,43)	1.52 (1,42)
Olfactory bulb	0.11 (1,54)	3.0 (1,43)	1.52 (1,42)
Hypothalamus	0.47 (1,54)	13.80 (1,45)*** diff=28.5%	10.01 (1,46)** diff=29%
Condition	0.88 (1,55)	0.00 (1,42)	0.22 (1,47)

Shown are the *F*-statistics of log-transformed brain mass and brain subregion volumes with degrees of freedom in parentheses. The significance level is given by asterisks (*P<0.05, **P<0.01, ***P<0.001). Percentage of difference between the presence (*y*)/absence (*n*) of the respective behaviour is calculated as [(higher value–lower value)/lower value]×100 using back-transformed least square means. Positive values indicate a larger brain size or subregion volume for the group that shows the respective behaviour, whereas negative values indicate that the brain subregion is smaller if individuals show a behaviour.

2017). How do we know that the observed sex differences in brain anatomy are driven by the sex-specific cognitive challenges of reproduction, rather than by other selective forces that may act differently on the male and female phenotype? The strongest argument to support this interpretation is the distinct pattern of brain–body allometry (overlapping body sizes, but different intercepts among sampling time points) observed over the breeding cycle. This pattern shows that the stickleback brain does not grow monotonously along with body size as fish grow older and larger over the breeding cycle, but that both the male and female brain increase rapidly in size upon transition to sexual maturity and breeding, although to different degrees. Another supporting argument comes from Samuk et al. (2014), who report reversed SSD (i.e. female-biased SSD) in the brain of white sticklebacks, which, in contrast to common three-spined sticklebacks (uniparental paternal care), do not engage in parental care at all.

The sex-specific increase in brain size over the breeding cycle was similar in all four replicate populations used in this study, confirming the ubiquity of a developmental component to brain SSD. This finding also excludes geographic variation as the unique source for the observed between-population variation in the degree of brain SSD (Herczeg et al., 2015; Kotrschal et al., 2012a; Samuk et al., 2014; Toli et al., 2017). In essence, we conclude that studies using snapshot sampling for brain size measurements from different populations at different time points over the reproductive season need to be interpreted with caution. This is not only because of the heterogeneity introduced by sampling in different time points relative to breeding cycle, but also because populations might differ in how brain size changes in relation to breeding cycle. Whether these breeding-related brain size changes are purely plastic and thus reversible, similar to the post-breeding brain size shrinkage in shrews (Dechmann et al., 2017), or result from genetically determined developmental patterns would require study of sex-specific brain size changes over more than one reproductive cycle. As our experiments were terminated before the end of the breeding phase, we do not know whether three-spined sticklebacks also go through seasonal brain size shrinkage. Nevertheless, this seems likely because they are known to have the potential to rapidly decrease size of brain parts, for example in response to transfer to captivity (Park et al., 2012).

We can only speculate about the underlying cognitive selection pressures that may have caused the telencephalon, the cerebellum, the olfactory bulb and the dorsal medulla to change in volume over the three sampling points. The telencephalon is the cognitive centre of the brain, which, together with the cerebellum, is also responsible for different aspects of learning and memory (Kaplan and Aronson, 1969; López et al., 2000; Rodríguez et al., 2005; Rodríguez et al., 2002). That the male and female cerebellum and telencephalon both increased in volume towards breeding may indicate that similar cognitive aspects, in terms of the subregion location of these functions, are important for male and female reproductive behaviours (e.g. locating, comparing/remembering and choosing a suitable mate). It is important to note that the telencephalon was the only brain subregion that was larger in males than in females at all three time points measured. This finding suggests that it is the telencephalon that drives the differences in relative brain size between male and female sticklebacks. Evidence for the importance of the telencephalon in male stickleback breeding is also provided by Kent and Bell (2018), who noted that expression of an immediate early gene (*Egr-1*), strongly associated with elevated levels of parental behaviour, was highest in this subregion. The importance of the telencephalon for unipaternal care could be tested through comparisons with white sticklebacks, which do not engage in parental care (Jamieson et al., 1992). The olfactory bulb, which is important for olfaction, also changed in volume in a sex-synchronized manner. This finding suggests that olfaction is important for both sexes during breeding. In a reproductive context, olfaction indeed is important in identification of mates (Rafferty and Boughman, 2006) and their quality (Milinski and Bakker, 1990) as well as the reproductive state they are in (Häberli and Aeschlimann, 2004; Sørensen and Scott, 1994; Waas and Colgan, 1992). As sexual pheromones are used to synchronize reproductive behaviours (Dulka et al., 1987), the ability to detect them should also become important for both sexes in the given reproductive period. Finally, we abstain from functional interpretations for the observed patterns of population-specific volume changes in the dorsal medulla because it is a brain subregion that has multiple functions (Nieuwenhuys, 1982).

The comparison of brain anatomy between nest-building and non-nest-building males revealed that the males failing to build a nest had smaller brains than those that did build nests. This provides yet another reason to infer that the male brain adjusts to the high cognitive demands of breeding (courtship in this case) by increasing in size over the reproductive season. All six brain subregions increased concordantly in size in nest-building males, and might thus be important in controlling male reproductive behaviours. However, we found no difference in relative brain size between males that did and did not engage in parental care, and hence, the stickleback brain does not appear to show a marked response to the presence or absence of parental care. Instead, we found that the hypothalamus was larger in males that had eggs to care for than in males that did not. It is possible though that our last sampling point was too early to detect any changes related to parental care. In a recent study, Kent and Bell (2018) demonstrated that expression of *Egr-1* peaked after male sticklebacks had spent 3 days tending to fry (i.e. after eggs had hatched). Hence, as we only sampled during the egg-tending phase before hatching, there might not have been enough brain activation yet to induce changes in brain size.

Concerning female brain anatomy and reproductive behaviour, we found no difference in brain size between females that did lay eggs in the male's nest and those whose eggs were not in the nest. However, there were more subtle differences in the size of brain subregions: females without eggs in their nests had a 7.5% smaller optic tectum and a 28.5% smaller hypothalamus, but a 12% larger cerebellum as compared with females who had laid eggs in the nests. Our experimental design does not allow us to infer why some females did not lay their eggs in the nests. Nonetheless, the observed differences in the size of several brain subregions between females laying and not laying eggs in nests are associated with female fitness at least in the used laboratory setting. Whether the observed associations between size of different brain subregions and breeding success are driven by some mechanisms of mate choice (Bakker, 1986; Kraak and Bakker, 1998; Roufidou et al., 1995; Rowland, 1982) or other factors (Manica, 2002) will have to be determined in future investigations.

In conclusion, our findings demonstrate a significant developmental component to sexual size dimorphism in the stickleback brain, and corroborate the idea that the sex-specific cognitive demands for reproduction are likely drivers behind this developmental plasticity. Moreover, the consistent sex-specific changes in brain anatomy across the four replicate populations tested suggest that the large variance in the degree of brain SSD in earlier studies of three-spined sticklebacks could be explained by temporal sampling heterogeneity, as hypothesized by Toli et al. (2017). Furthermore, should developmental plasticity in brain anatomy turn

out to be taxonomically widespread, results of earlier studies using temporally heterogeneous sampling relative to species' breeding cycles (e.g. interspecific comparative studies of brain size variation) could be blurred, or even confounded by this plasticity. Hence, further studies testing for developmental and seasonal plasticity in brain size and anatomy on a wider range of taxa are warranted.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.K., J.M.; Methodology: S.D.B., K.N., J.D., E.T., J.M.; Validation: S.D.B., J.D.; Formal analysis: S.D.B.; Resources: N.K., J.M.; Data curation: S.D.B., K.N., J.D., E.T.; Writing - original draft: S.D.B., N.K., J.M.; Writing - review & editing: S.D.B., K.N., J.D., E.T., N.K., J.M.; Visualization: S.D.B.; Supervision: J.M., N.K.; Project administration: N.K., J.M.; Funding acquisition: K.N., J.D., N.K., J.M.

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Data availability
All data have been deposited in the Dryad Digital Repository (Buechel et al., 2019): dryad.0gm2pm3.

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