

METHODS

Study Species: Courtship & Paternal Care

During the breeding season, male threespined stickleback build nests consisting of sand and algae glued together with secretions from their kidneys. Males attract females to spawn in their nests and defend their breeding territory from intruders and predators. During courtship, males perform a conspicuous zigzag dance where they dart rapidly back and forth in front of an approaching female. Males also attempt to lead females to their nest where males poke at the nest opening and fan it with their pectoral fins, as they would in paternal care, regardless of whether there are eggs in the nest. There is mutual mate choice and females show a variety of preference behaviours such as ‘head-ups’ where she directs her distended abdomen towards a male, ‘follows’ where she closely follows a male towards his nest and ‘inspections’ where she inserts her head into the nest. If a female chooses a male, she lays her entire clutch of eggs in the nest, after which the male follows and fertilizes the eggs.

As the embryos develop and after they hatch, fathers are the sole providers of the care necessary for offspring survival. During the ~6-day incubation period, the male ‘fans’ (oxygenates) the embryos with his pectoral fins, removes dead eggs and debris, and defends the territory from predators. After the eggs hatch, the male tends his offspring for ~7 days by fanning the nest and fry as well as retrieving fry that stray from the nest in his mouth and spiting them back into the nest. During the entire parenting period, males also guard their nest from a suite of embryo and fry predators which include sculpin and other sticklebacks [1-4]. Sticklebacks from our California population typically live for one year but have multiple breeding attempts during the mating season. Males will also mate with multiple females even within a single breeding

24 attempt. Studies have found that males adjust their reproductive investment as the mating season
25 progresses [6]. See [1] for details on stickleback breeding and paternal care.

26

27 *Collection & Maintenance*

28 Sticklebacks were collected from Navarro River, CA, before the mating season (collected
29 as adults in April 2012 and as juveniles in November 2011) and transported by air to the
30 University of Illinois. Collected fish were visibly free from external parasites. Piscivorous
31 predators such as Prickly sculpin (*Cottus asper*) are present in the Navarro population. At small
32 sizes, sculpins are primarily a threat to nests and juvenile stickleback although small adults can
33 also be captured [2-4]. At large sizes, sculpins are a threat to adult sticklebacks [4].

34

35 Fish were maintained at 20.6°C on a summer photoperiod schedule (16L:8D) and water
36 was cleaned in all tanks via a recirculating flow-through system with particulate, biological and
37 UV filters (Aquaneering, San Diego, USA). All fish were fed a slurry of frozen food
38 (bloodworms, spirulina brine shrimp and Cyclopeez®) daily and females received additional live
39 and frozen bloodworms to favour egg formation.

40

41 *Female Predator Exposure*

42 We randomly assigned females to either a predator-exposed or unexposed (control)
43 treatment tank with 10 females per 37.8 L tank (36 x 33 x 24 cm, length x width x height).
44 Within each tank, females were individually marked by a combination of spine clips thus we
45 could individually identify females from each tank (female identity was included as a random
46 effect in the analyses). Each female housing tank (N = 10 females per tank) was covered with

47 opaque plastic on all sides with a small closable “window” to allow us to monitor female
48 reproductive state with minimal disturbance. Tanks were checked daily for gravid females.

49

50 We chased females in predator-exposed tanks for 45 sec once a day with a 10 cm realistic
51 rubber model of a natural predator, the Prickly sculpin (*Cottus asper*), attached to a stiff metal
52 rod. We moved the model along the bottom and made lunges at each of the females, similar to
53 the predator's behaviour. During the daily predator exposure, we aimed to lunge at each of the 10
54 females in the tank at least once. In a separate pilot study, we found that being chased by a model
55 sculpin (details below) was an effective stressor to adults and elevated plasma cortisol levels in
56 adults (unexposed adults: 15.5 ± 3.3 ng/ml, N = 17; adults 30 min after exposure to the sculpin
57 model: 28.3 ± 6.0 ng/ml, N = 24). Females were chased at a random time each day so predator
58 exposure was unpredictable. Females in the unexposed treatment were undisturbed. Females
59 experienced their treatments for 32 ± 2 days on average but total treatment time was variable
60 depending on when females became gravid (range: 3 – 77 days). Importantly, there was no
61 significant association between treatment time and courtship behaviour (Table S1).

62

63 *Courtship & Breeding*

64 Males from the same population were housed singly in 9.5 L tanks (34 x 18 x 23 cm)
65 with gravel, one artificial plant and a plastic nest box with fine sand as well as filamentous algae
66 for nest building. As would occur in the field, males could see nearby males (i.e. competitors
67 [6]), but to reduce the perceived threat of territorial intrusion, male tanks were separated from
68 each other by at least 20 cm and the bottom half of the tank (where the nest was located) was
69 covered with black fabric. Within their individual tanks, males were shown a gravid ‘stimulus’

70 female in a glass flask every two days to encourage them to build a nest. These stimulus females
71 were not from our female treatment tanks and were not used in any behavioural assay.

72

73 When a female was gravid (distended abdomen with an egg almost protruding from her
74 gonopore), she was paired with a male that had completed a nest and ‘crept through’. Male
75 numbers were drawn out of a cup and paired randomly with either a predator-exposed or
76 unexposed female. Importantly, female predator exposure treatments did not differ significantly
77 in female body weight before spawning or clutch weight (Table S2). In the evening (between
78 8pm and 10pm), the female was gently blotted with a paper towel, weighed, and added to a male
79 tank. The pair was left together overnight in the male’s tank and could interact for several hours
80 before the lights turned off at 11pm and after the lights turned on in the morning at 6am. We did
81 evening pairings to reduce male aggression and minimize stress and damage to the female. For 5
82 minutes after the male noticed the female in the tank, we recorded male behaviour directed at the
83 female (number of zigzags) and directed at the nest entrance (number of pokes and fanning
84 bouts) as well as total female preference behaviours (sum of all head-ups, follows and nest
85 inspections).

86

87 The next morning (between 8am and 10am), the female was removed, weighed and
88 visually inspected for spawning. After a courtship trial, females were returned to their home tank
89 regardless of whether they had successfully spawned thus females were potentially reused
90 throughout the experiment. Spawning was confirmed by either visible eggs in a male’s nest
91 and/or the female was deflated and had lost at least 0.15g overnight. However, not all courtship
92 assays resulted in a successful spawn and we examined whether female treatment affected her

93 likelihood to spawn using logistic regression. In this analysis, we only included males once
94 (either their first spawn or first courtship bout) resulting in courtship assays for 109 different
95 males. There was no significant effect of female predator exposure on the likelihood that a
96 courtship assay would result in a successful spawn (Logistic regression: Wald $X^2 = 0.5971$, $P =$
97 0.4397). Trials without spawns could be due to females having immature eggs, or female or male
98 choice. To standardize female reproductive state and ensure they all had mature eggs, we
99 restricted our data to trials with successful spawns. For these spawnings, we estimated clutch
100 weight by calculating the difference in female body mass pre- and post-spawning. In this
101 analysis, we only included males once, on their first spawn, resulting 67 spawns for different
102 males, but some females were reused as they became gravid (48 unique females).

103

104 Before a female was introduced for the courtship assay, male throat colour was scored
105 using a crude scale ranging from 1 (pale pink on chin) to 4 (vivid red on chin and cheeks) before
106 the female was added. Male standard length was also measured after completion of the
107 experiment to minimize disturbance. Not all males had their throat colour scored thus sample
108 sizes are slightly lower for these analyses.

109

110 After a spawning and removing the female, the nest box containing a male's nest was
111 removed from the tank. For part of a different study (McGhee & Bell, *in preparation*), half of the
112 embryos were removed and reared as orphans. Nests were returned to fathers in <1 minute. The
113 remaining half of the embryos were returned with the nest and reared by their genetic father.
114 Paternal care in this study was measured on these father-reared offspring in their genetic father's
115 nest. Tanks with fathers and nests of father-reared offspring had their water flow turned off for 3

116 days with several drops of methylene blue added to prevent fungus in the eggs. Partial water
117 changes were made every day and on the 4th day, water flow was resumed on all tanks.
118 Throughout the parenting period while fathers were rearing offspring (embryos and fry), daily
119 feedings were increased to twice a day with additional frozen bloodworms.

120

121 *Paternal Care*

122 We made behavioural observations on a subset of males from these 67 spawns that were
123 successful in rearing offspring to the fry stage. There was no significant effect of female predator
124 exposure on the likelihood that a male on his first spawn would successfully rear offspring to fry
125 (Logistic regression: Wald $X^2 = 0.2588$, $P = 0.6109$, $N = 13$ males). We observed paternal care
126 for 5 minutes on 4 days (at 10am-12pm), with two observations occurring before embryos had
127 hatched (days 3 and 4 after fertilization), and two observations occurring after hatching when
128 offspring were fry (days 7 and 8 after fertilization). We recorded and summed (1) the number of
129 nest pokes and fanning bouts directed at the nest entrance and (2) the number of nest visits (i.e.
130 father hovering within a body length of nest) and offspring retrievals (i.e. father put an offspring
131 in his mouth and released it into nest). We kept these two types of paternal care behaviours
132 separate since males often hover above the nest box and circle the area (i.e. guarding or
133 retrieving fry) during a nest visit, but do not necessarily perform nest pokes or fans. Nests were
134 observed behind a blind several feet away. Observers were blind to maternal treatment.

135

136 *Statistical Details*

137 For the two mixed models examining how male courtship was affected by female
138 predator exposure, we included female treatment as a fixed effect and female identity as a

139 random factor in all models. We included total female preference behaviour during the 5 minute
140 assay as a single covariate in all analyses. Interactions with the covariate were removed as they
141 were nonsignificant and their exclusion improved model fit based on AIC values. We did not
142 include initial female mass as a covariate because it was uncorrelated with any male or female
143 behaviour (Spearman $r = -0.03$ to 0.04 , $P > 0.5$). We tested for the significance of female identity
144 with log-likelihood tests. Regardless of its statistical significance, the effect of female identity
145 was always retained in the models. All behavioural data was natural log-transformed after adding
146 1 to the data to account for any zero values. We specified REML estimation and estimated the
147 degrees of freedom with the Satterthwaite method. We validated model assumptions by visually
148 examining residuals.

149

150 Since males were measured four times during parenting, we used repeated measures
151 analyses for paternal care behaviours with father identity as the repeating subject through time
152 (days 3,4,7,8). Maternal predator exposure and day, as well as their interaction, were included as
153 fixed effects. Interactions were retained despite being nonsignificant as their inclusion improved
154 model fit based on AIC values. We specified an autoregressive covariance structure (type =
155 ar(1)) for the repeated measures of fathers based on model comparisons using AIC and estimated
156 degrees of freedom using the Satterthwaite method. We tested for the significance of the random
157 effect of male identity (i.e. family identity) with log-likelihood tests. Residuals were examined to
158 assess model assumptions and decide on appropriate data transformations. Nest pokes and
159 fanning bouts were natural log-transformed after adding 1 to data, and nest visits and retrievals
160 were untransformed.

161

162 For both the full courtship data set and the parental care subset, we examined whether
163 differences between female treatments might be associated with other differences. We used
164 mixed models to compare female treatments in male standard length, male throat colour, female
165 mass before spawning, clutch mass, and in the case of courtship, number of female preference
166 behaviours, including female identity or her particular housing tank as random effects as
167 appropriate. For female size-related traits in the courtship dataset, we included female housing
168 tank as a random effect. For female preference behaviour in the courtship dataset, we included
169 female identity as a random effect. We tested for the significance of random effects with log-
170 likelihood tests. Female mass and clutch mass were natural log-transformed and female
171 preference behaviour was natural log-transformed after adding 1 to the data to account for any
172 zero values. We also compared female treatments in male throat colour score (1-4), using a
173 generalized linear mixed models specifying a multinomial (ordered) response distribution and a
174 cumulative logit link with maximum likelihood estimation. Since male throat coloration and
175 female clutch size might be related to paternal care, we tested for correlations between these
176 traits and paternal care on each day using Spearman correlations.

177

178 All analyses were conducted with SASTM version 9.3.

179

180

RESULTS

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For the courtship assays, neither male size nor male throat colour score differed significantly between female treatments (Table S2, male standard length: $F_{1, 61} = 2.63$, $P = 0.1097$; male throat colour: $F_{1, 60} = 2.10$, $P = 0.1526$). Neither female mass before spawning nor the mass of the spawned clutch (prior to our clutch manipulation), were affected by female

185 treatment or female housing tank (female mass: $F_{1, 11.7} = 0.09$, $P = 0.7748$, random effect of
186 female housing tank: $X^2 = 1.5$, $P = 0.2207$; clutch mass: $F_{1, 12} = 0.02$, $P = 0.8884$, random effect
187 of female housing tank: $X^2 = 0.9$, $P = 0.3428$). Female preference behaviour did not differ
188 significantly between predator-exposure treatments (Table S2; $F_{1, 38.4} = 1.08$, $P = 0.3044$, random
189 effect of female identity: $X^2 = 0.6$, $P = 0.4386$).

190

191 For the subset of individuals used in the paternal care part of the study, neither male size
192 nor male throat colour score differed significantly between female treatments (Table S3, male
193 standard length: $F_{1, 14} = 2.17$, $P = 0.1630$; male throat colour: $F_{1, 11} = 3.23$, $P = 0.0999$).

194 Similarly, female mass before spawning and the mass of the spawned clutch (as determined by
195 the difference in female mass before and after spawning) were not significantly affected by
196 female treatment (Table S3, female mass: $F_{1, 14} = 1.00$, $P = 0.3336$; clutch mass: $F_{1, 14} = 3.25$, $P =$
197 0.0931). Neither type of paternal care was significantly associated with male throat coloration
198 (Spearman $r = -0.43$ to 0.09 , $P > 0.05$, $N = 15$) or initial (before manipulation) female clutch size
199 (Spearman $r = -0.49$ to 0.38 , $P > 0.05$, $N = 16$) when examining each day separately. Patterns of
200 courtship behaviour for this smaller subset (Table S3) were similar to the larger courtship data
201 set (Table S2).

202

203

204 Table S1. (A) Mean number of days (\pm standard errors) females had been in their respective
 205 treatments before being courted by a male and (B) Spearman rank correlations (P-values in
 206 parentheses) between days in treatment and courtship behaviour.

207

| Trait | All females N = 67 | Unexposed female N = 37 | Predator- exposed female N = 30 |
|--|-------------------------------|--|--|
| <i>A)</i> Mean number of days in treatment (\pm SE) | 31.6 (\pm 2.3) | 30.8 (\pm 3.6) | 32.2 (\pm 3.1) |
| <i>B)</i> Number of zigzags | -0.104 (0.4031) | -0.175 (0.3005) | 0.082 (0.9657) |
| Number of nest pokes and fanning bouts | -0.060 (0.6311) | -0.146 (0.3889) | 0.012 (0.9489) |
| Number of female preference behaviours | -0.096 (0.4370) | -0.262 (0.6896) | 0.096 (0.6152) |

208

209 Table S2. Means \pm standard errors of female and male traits for successful spawns (N = 67
 210 unless otherwise indicated).
 211

| Trait | Unexposed female N = 37 | Predator-exposed female N = 30 |
|--|------------------------------------|---|
| <i>Female traits:</i> | | |
| Mass (g) | 1.15 ± 0.03 | 1.13 ± 0.03 |
| Clutch mass (g) (N=66) | 0.23 ± 0.01 | 0.23 ± 0.01 |
| Number of preference behaviours | 4.3 ± 0.8 | 6.6 ± 1.2 |
| <i>Male traits:</i> | | |
| Standard length (mm) (N=63) | 39.4 ± 0.4 | 40.5 ± 0.6 |
| Throat colour score (N=64) | 2.6 ± 0.2 | 3.0 ± 0.2 |
| Number of zigzags | 12.0 ± 2.2 | 5.2 ± 1.2 |
| Number of nest pokes and fanning bouts | 7.5 ± 1.5 | 6.6 ± 1.7 |

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213

214 Table S3. Means \pm standard errors of female and male traits for fathers rearing offspring from
 215 predator-exposed and unexposed mothers (N = 16 unless otherwise indicated).

216

| Trait | Unexposed mother N = 8 | Predator-exposed mother N = 8 |
|--|---------------------------|----------------------------------|
| <i>Female traits:</i> | | |
| Mass (g) | 1.13 \pm 0.06 | 1.27 \pm 0.12 |
| Clutch mass (g) | 0.21 \pm 0.01 | 0.27 \pm 0.03 |
| Number of preference behaviours (N=15) | 8.1 \pm 1.3 | 4.4 \pm 1.6 |
| <i>Male traits:</i> | | |
| Standard length (mm) | 39.4 \pm 0.6 | 41.1 \pm 1.0 |
| Throat colour score (N=15) | 2.5 \pm 0.5 | 3.6 \pm 0.3 |
| Number of zigzags (N=15) | 12.4 \pm 3.2 | 4.9 \pm 2.1 |
| Number of nest pokes and fanning bouts (N=15) | 9.5 \pm 1.8 | 5.6 \pm 1.3 |

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