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Effects of maternal stress on egg characteristics in a cooperatively breeding fish

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ABSTRACT

Elevated stress experienced by a mother can compromise both her own reproductive success and that of her offspring. In this study, we investigated whether chronically stressed mothers experienced such effects in cooperatively breeding species, in which helpers at the nest potentially compound the negative effects of maternal stress. Using *Neolamprologus pulcher*, a group-living cichlid fish from Lake Tanganyika, we observed the effects of experimentally increased stress on female reproductive success (measured as inter-spawn interval, and number of eggs) as well as egg characteristics including egg size and cortisol concentrations. Stress levels were manipulated by repeated exposure to the acute stresses of chasing and netting. Stressed females had longer inter-spawn intervals and laid fewer, smaller eggs. Although no significant differences in egg cortisol concentrations were detected between control and stressed females, egg cortisol concentration fell between spawns in control but not in stressed fish. No effect of helper number was detected for any parameter examined, except there appeared to be less change in egg cortisol content in groups with helpers present. Our results suggest that stress imposes fitness costs on breeding females, and social regulation of a dominance hierarchy does not appear to exacerbate or alleviate the negative effects of maternal stress.

1. Introduction

Stress, resulting from sudden perturbations (acute) or from constant challenges (chronic) in the environment, induces a suite of important physiological and behavioural responses. Natural selection has shaped stress responses to enhance an organism's ability to cope with the stressor and to eventually regain homeostasis (Chrousos 1998; Charmandari et al. 2005). A key component of the physiological stress response is the mobilization of glucocorticoid (GC) stress hormones (cortisol or corticosterone) via activation of the hypothalamic-pituitary-adrenal (HPA, in tetrapods; in fish the HPI or hypothalamic-pituitary-interrenal) axis. The ability to mount an appropriate stress response is generally considered beneficial. However, a prolonged elevation of GCs in response to long-term exposure to stressors can have detrimental effects on an individual, including reductions in growth and immune function, and the suppression of reproduction (Chrousos, 1998; Wendelaar Bonga 1997; Charmandari et al. 2005; Schreck 2010). Stressful conditions experienced early in life (or in utero) may have profound impacts later in life (Contreras-Sánchez et al. 1998; Rondó et al. 2003; Hayward and Wingfield 2004; Ostrand et al. 2004; Sloman 2010). In this study, we used a cooperatively breeding fish species to explore whether stress experienced by breeding females projects onto the next generation. We examined the effects of chronic maternal stress (induced by repeated exposure to an acute stressor) on female reproductive rates, fecundity, and egg characteristics.

To our knowledge, the potential fitness costs of maternal stress have not yet been experimentally manipulated in cooperatively breeding species. Cooperative breeders are group-living species, in which subordinate group-members forgo reproduction to help raise the offspring of dominant individuals. We predicted that the effects of maternal stress on offspring number and quality would be exaggerated in social cooperative breeders due to the energy requirements and challenge of maintaining dominance over subordinates in the social group (see Goymann and Wingfield 2004). To date, the impact on reproductive success of maternal stress during gestation has been studied in several species that are not cooperative breeders. For example, maternal stress results in lowered birth-weight in human, *Homo sapiens*, infants (Rondó et al. 2003), slower juvenile growth rates in Japanese quail (*Coturnix coturnix japonica*, Hayward and Wingfield 2004), smaller egg clutches in moor frogs (*Rana arvalis*, Räsänen et al., 2005), and smaller eggs and young in rainbow trout (*Oncorhynchus mykiss*, Campbell et al. 1992; Contreras-Sánchez et al. 1998).

To address the impact of maternal stress on reproductive fitness in a cooperative breeder, we used the cichlid fish, *Neolamprologus pulcher*, endemic to Lake Tanganyika, Zambia. *N. pulcher* live in highly social groups organized into linear dominance hierarchies based on size, with a breeding male and female pair at the top of the hierarchy and 1-20 subordinate helpers (Taborsky and Limberger, 1981;

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Balshine et al. 2001; Heg et al. 2005). The dominant breeders remain in their position for 3-12 months (Stiver et al. 2004) and subordinates rarely breed in the wild but can eventually gain dominance and breeding status, via inheritance or take-over (Fitzpatrick et al. 2008, Stiver et al. 2009). *N. pulcher* groups live in communally defended rocky territories clustered together into subpopulations at 3-45 metres depth (Stiver et al. 2008). In these territories, female breeders may be exposed to a number of potential environmental and social stressors. First, breeders often experience elevated predation pressure and frequently defend their young against predators (Balshine et al. 2001). Second, individuals constantly need to vigorously defend their territories against encroaching, neighbouring conspecifics as well as heterospecific space competitors (Desjardins et al. 2008). Third, dominant breeders must police subordinates to control their reproduction (Goymann and Wingfield, 2004; Fitzpatrick et al. 2008). Exposure to such stressors has the potential to repeatedly raise GC levels and therefore could have fitness implications for both breeding females and their offspring.

We made a series of predictions for the effects of maternal repeated stress on reproductive fitness in *N. pulcher* based on findings in the literature. One, we predicted that stressed breeder females would take longer to reproduce. In rainbow trout, both chronic and acute stress caused delays in ovulation and spawning (Campbell et al. 1992; Contreras-Sánchez et al. 1998). Two, we predicted that stressed fish would lay fewer, smaller eggs, as chronic stress was found to reduce egg mass in rainbow trout (Campbell et al. 1992; reviewed in Schreck et al. 2001). Three, we predicted that cortisol levels would be higher in the eggs of stressed compared to those of unstressed (control) females. Similar findings of maternal transfer of GCs have been observed in egg yolk of Japanese quail (Hayward and Wingfield 2004) and in the eggs of coho salmon, *Oncorhynchus kisutch* (Stratholt et al. 1997). A final prediction stems from the novel aspect of our study. We predicted that the presence of helpers would increase the stress experienced by breeder females and therefore that females with more helpers would exhibit a heightened response to an experimental stressor. This notion is supported by work in which female damselfish (*Pomacentrus amboinensis*, a coral-dwelling social fish), in environments with multiple conspecifics exhibited higher cortisol levels and consequently hatched smaller juveniles than those that were allowed to breed in isolation (McCormick 2006). Goymann and Wingfield (2004) predicted that there would be greater physiological stress (termed allostatic load) for dominant individuals in species where acquiring and maintaining dominance is difficult. In *N. pulcher*, few individuals manage to attain a breeding position and individuals that do so must regularly assert their dominance to maintain their position (see Mileva et al. 2009 for a full allostatic load calculation for *N. pulcher*). Thus we predicted that under normal control conditions, breeding females with helpers or with many helpers would lay eggs containing higher cortisol levels than females without helpers or with few helpers. We also aimed to investigate the effects of helper presence on egg size in stressed females.

2. Methods

2.1. Fish husbandry and housing

All fish (*Neolamprologus pulcher*, Cichlidae) used in this experiment were held at McMaster University, Hamilton, Ontario, Canada. They were either descendants of wild-caught breeding pairs captured in 2002, or were wild-caught breeding pairs from early 2008, captured at the southern tip of Lake Tanganyika. Twenty stable social groups, ones in which spawning had occurred prior to the beginning of the experiment, were chosen for use in this experiment. Each social group was housed in a 189 L tank and groups consisted of a dominant breeding male and female with 0-4 subordinates of either sex (7 groups had no helpers; 2 groups had 1 helper; 5 groups had 2 helpers; 1 group had 3 helpers; and 2 groups had 4 helpers). Social groups had well-established dominance hierarchies. In addition to the social group, the 20 tanks contained a ~2 cm layer of sand, a water heater, a thermometer, two mirrors, and two flowerpot halves (to deposit eggs on), and was aerated using two foam filters. Water temperature was held at 26 ± 2 °C and the light: dark cycle was maintained at 13:11 h throughout the experiment. Each group was fed *ad libitum*, Nutrafin basix Large Flake commercial cichlid food once daily in the morning. To ensure that sufficient food was provided, an excess of flakes was left on the tank bottom for at least 1 hour before removal.

All fish husbandry protocols, as well as experimental procedures used for chasing and netting fish and egg sampling (see below), were reviewed and approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol # 06-10-59) and adhere to the animal handling guidelines specified by the Canadian Council for Animal Care.

2.2. Experimental setup and egg collection

In each tank, the two flowerpot halves on which the fish could spawn were lined with a sheet of roughened flexible transparent plastic; note that roughened acetate was provided after smooth acetate proved to be an inappropriate spawning substrate (see Discussion). Tanks were monitored daily for spawning and once spawning was noted, tanks were assigned to either the "stressed" or "control" treatment in an alternating fashion. This first spawning day was considered Day 1 of the experiment. On this day, the plastic sheet was removed, and eggs were dried carefully with kimwipes and photographed together with a ruler, using a digital SLR camera (Canon EOS Rebel 300D). The eggs were then gently scraped off the plastic surface, weighed to the nearest 0.001 g, and stored at -80 ° C for later analysis of egg cortisol concentrations (note that the small size of *N. pulcher* precludes collection of non-terminal blood samples for cortisol analysis – hence the emphasis on egg cortisol concentrations as a proxy for plasma cortisol concentrations in females). The breeding female and male were also caught on Day 1, and measured for standard length and body mass (Table 1).

The ten breeding females assigned to the control treatment were not subjected to any stressor other than the regular weekly tank cleanings, and were simply fed daily. The ten breeding females assigned to the "stressed" treatment were subjected to a daily stressor for up to 80 days in addition to their weekly cleaning and daily feeding regimes. These "stressed" females were chased with a net for 2-5 min twice per day for 50 days (once in the morning before 12 pm, and once in the afternoon, allowing more than 3 h between chasing events). By the end of 50 days no spawning had occurred, and so the stressor was therefore changed to netting for 10 min, once daily for

Table 1
Morphological characteristics, body condition and specific growth rate of control and stressed females at 1st and 2nd spawn.

Variable	Control females (mean ± se)	Stressed females (mean ± se)
Body mass at 1st spawn (g)	8.95 ± 0.55	8.35 ± 0.54
Body mass at 2nd spawn (g)	9.48 ± 0.66	8.07 ± 0.59*
SL at 1st spawn (cm)	6.64 ± 0.14	6.39 ± 0.10
SL at 2nd spawn (cm)	6.83 ± 0.15	6.52 ± 0.14
Fulton's K at 1st spawn	3.04 ± 0.08	3.17 ± 0.08
Fulton's K at 2nd spawn	2.95 ± 0.092	2.89 ± 0.11
Specific growth rate (% Δ mass per day)	0.10 ± 0.07	-0.06 ± 0.03*

SL refers to the standard length and is a commonly used length metric in fisheries research in which body length minus the tail is determined. Fulton's K refers to an index of body condition; the calculation of this variable and specific growth rate is detailed in the text. Asterisks denote a significant difference between control and stressed females ($p<0.05$, see text for details of statistical tests used).

30 days. If females had still not spawned by this point the stressor was further reduced to netting 3 times per week for 10 min. In the end, despite the continued reduction in stressors, only 7 of the 10 stressed females spawned twice and hence three tanks were excluded from further analysis. Both chasing (J. Fitzpatrick unpublished data) and netting for 10 min (Mileva et al. 2009) have been shown to elevate cortisol concentrations significantly in *N. pulcher*, and presumably elevate catecholamine levels also, as observed in other fish species (see Gamperl et al. 1994; Reid et al. 1998; Perry and Bernier 1999). Repeated exposure to acute stressors was used in the present study to elevate cortisol levels. Cortisol levels probably cycled, rising with each application of the acute stressor and then gradually falling again.

The day that females spawned for the second time marked the last day of the experiment. At this time the female and male breeders were caught and re-measured, and the eggs were removed (spawn 2) and processed as described above. Fulton's body condition factor [100 x (body mass/ standard length^3)] and specific growth rate [100 x (lnM2 - lnM1)(t2 - t1)$^{-1}$] were then calculated for each breeder (see Table 1). In the calculation of specific growth, M2 represented the mass of each fish at the second spawn (end of the experiment), M1 was the mass at first spawn (beginning of the experiment), and t2-t1 was the inter-spawn interval.

2.3. Fecundity and egg size analyses

Egg images were analysed using ImageJ v1.42 software (Wayne Rasband, NIH, USA, available at http://www.rsb.info.nih.gov/ij). Eggs were counted, and the length and width measured for 20 randomly chosen eggs per clutch. One female's second clutch included some visible egg-shells/scars (and some of these eggs were too soft to collect); these eggs and scars were counted in the fecundity analyses but were not included in egg size analyses. To compare laboratory fecundity with fecundity in wild fish, we analyzed the preserved ovaries of twenty dominant breeders from Lake Tanganyika. The 20 wild females were on average 5.16 ± 0.04 cm in standard length (ranging from 4.85 to 5.57 cm) and 3.49 ± 0.06 g in mass (range = 3.00 to 3.99 g). These dominant females were collected in Kaskalawa Bay between February and April 2005, and their ovaries were preserved in ethanol (see Stiver et al. 2006 or Fitzpatrick et al. 2006 for further details of the field site and collection methodology). In the laboratory, each ovary was gently teased apart using forceps and eggs were counted.

2.4. Egg cortisol analysis

Cortisol was extracted from egg samples and analyzed via enzyme-linked immunoassay (EIA) using protocols previously validated for fish eggs (de Jesus et al. 1991; Barry et al. 1995; Alsop and Vijayan, 2008). In brief, cortisol was extracted from homogenized egg samples three times using 5 ml of diethyl ether each time. Following evaporation of the ether, samples were reconstituted in either 200 µl (for all egg masses less than 150 mg) or 600 µL (for all egg masses above 150 mg) of enzyme immunoassay buffer (see below) and kept at 23 °C for 2-4 h, with occasional vortexing. Reconstituted samples were diluted further and used for cortisol analysis, which was carried out in duplicate with a colorimetric 96-well EIA kit (Cayman Chemicals, cat# 582121, Ann Arbor, MI, USA). All samples were analyzed on a single plate, with an intra-assay coefficient of variation of 3.3% - 15.3%. The detection limit of this kit is 12 pg ml^{-1}, and the kit detects cortisol, the primary corticosteroid in fish eggs (see Alsop and Vijayan, 2008), with high specificity, exhibiting 2% cross-reactivity to cortisone, 0.2% to DOC and <0.01% to progesterone. Measured cortisol concentrations were adjusted for dilution factors, and corrected for egg mass so as to be expressed in units of pg cortisol per mg of egg mass. Cortisol extraction efficiency was determined using the "cold spike" protocol described in the EIA kit to be 126 ± 9% (mean ± SE, $N = 3$), a value that was not significantly different from 100% (one sample Student's *t*-test, $p = 0.11$).

2.5. Statistical analyses

All statistical analyses were performed using JMP IN 5.0 (SAS Institute) and Microsoft Excel 12.0 2008 for Macintosh (Microsoft Corporation). Analysis of variance (ANOVA), Pearson's r tests, student's *t*-tests and paired *t*-tests were performed as appropriate where data were normally distributed and of equal variance (the latter applied only for ANOVA). When data did not meet these assumptions, equivalent non-parametric tests were employed. All student's *t*-tests assumed unequal variance. Non-significant interaction terms were removed from multi-factor models. No differences were found between wild caught and captive bred females and so the fish from both sources were analysed together. To account for differences in duration between successive spawning attempts (and related differences in body mass) among breeding females, specific growth rate was used as a co-variate in each model, when testing for differences between treatments in terms of fecundity, egg size, and egg cortisol concentration. One female (a control) was removed from egg cortisol analysis as a result of an outlier analysis; cortisol levels in the eggs of this female were over 2.5 standard deviations away from the mean in both her first and second spawns and greatly inflated variance for such a small sample size. Finally, *N. pulcher* eggs are elongated spheres, and hence the effective diameter of each egg was calculated by means of the widely-used formula, cube root of length x width^2 (Coleman and Galvani, 1998).

3. Results

3.1. Females

Prior to exposure to stress, control females and females in the stress treatment did not differ in body mass, standard length or body condition (body mass: Mann Whitney U test, $U = 0.61$, $p = 0.44$; standard length: *t*-test, $t_{15} = 1.41$, $p = 0.18$, body condition, $t_{15} = -1.18$, $p = 0.26$; Table 1). Over the course of the experiment (controlling for differences in spawning interval, see above), females in the stressed treatment gained less mass (some even lost mass) compared to control breeder females (ANOVA, overall model $F_{2,\ 14} = 3.342$, $p = 0.07$; effect of treatment $F_{1,\ 14} = 6.44$, $p = 0.02$). Accordingly, stressed females exhibited lower daily growth rates than control females (Table 1). As would be predicted, in control females, body mass and inter-spawn interval were strongly positively correlated (Spearman's rho = 0.89, $p = 0.0005$), i.e. females with longer inter-spawn intervals exhibited greater mass gain prior to spawning. Females in the stressed treatment did not show this relationship (Pearson's r, $r^2 = 0.08$, $p = 0.53$), consistent with their general loss of mass over the course of the experiment. To verify that the stressors (chasing and netting) targeted the focal breeder female and not the whole social group, we investigated the impact of the regular disturbance on breeder males from control versus stressed treatment tanks. No significant differences were observed over the course of the experiment in male breeders from the control versus the stressed treatment groups in terms of change in body mass ($t_{13} = 0.611$, $p = 0.55$), in body condition ($U = 2.6$, $p = 0.10$), or in specific growth rate ($t_{13} = 0.91$, $p = 0.38$). The finding that breeder males in the stressed treatment group did not lose weight ($t_6 = 0.63$, $p = 0.79$) supports the notion that the breeder females were effectively targeted.

3.2. Spawning Rates

In the wild, female *N. pulcher* can spawn every month and they will spawn even more frequently (every two weeks) in the laboratory,

where ample food is available (Balshine, unpublished data). In the present experiment, the mean inter-spawn interval was 42.5 days, ranging from 13 to 120 days. Females in the stressed treatment took significantly longer to spawn a second time than did control females (Mann-Whitney U test, $U = 6.70$, $p = 0.01$; Fig. 1).

3.3. Fecundity

Fecundity in laboratory females varied from 21 to 408 eggs per clutch with an overall mean of 133.9 ± 14.3 eggs per clutch. This fecundity for spawned clutches was much higher than the ovarian clutch fecundity observed for females captured in the field. The 20 ovarian samples from ripe wild female breeders revealed a mean (ovarian) clutch size of 42.7 ± 2.6 eggs, with a range of 23 to 61 eggs per female.

On Day 1 (i.e. the first spawn, before any stressors were applied), egg number did not differ between treatments (*t*-test, $t_{15} = -0.57$, $p = 0.58$; Fig. 2). However, when fecundity was compared at the second spawn (end of the experiment), stressed females laid significantly fewer eggs than did control females (*t*-test, $t_{15} = 2.21$, $p = 0.04$; Fig. 2). Interestingly, although both stressed and control females laid fewer eggs in their second spawn compared to their first spawn, the reduction in egg number between spawns was significantly greater for stressed females than for control females when controlling for specific growth rate (ANOVA, overall model $F_{2, 14} = 3.17$, $p = 0.07$, effect of treatment, $F_{1, 14} = 5.56$, $p = 0.03$, effect of specific growth rate $F_{1, 14} = 3.12$, $p = 0.10$).

3.4. Egg Size

Stressed females laid eggs of lower mass in their second spawn as compared to their first spawn (Paired *t*-test, $t_6 = -3.50$, $p = 0.01$; Fig. 3a), whereas unstressed control females laid eggs of similar mass across the two spawning events (Paired *t*-test, $t_9 = 1.13$, $p = 0.29$). When comparing the change in egg size from the first to the second spawn between the two treatment groups, we saw a significant treatment*specific growth rate interaction (Repeated measures ANOVA, overall model $F_{3, 13} = 7.59$, $p = 0.004$, effect of treatment $F_{1, 13} = 3.29$, $p = 0.09$, effect of specific growth rate $F_{1, 13} = 1.93$, $p = 0.19$, effect of treatment*specific growth rate $F_{1, 13} = 5.02$, $p = 0.04$).

Mean egg diameter was $1.24\ mm \pm 0.02$ across all laboratory females (first spawn only), and ranged between 1.02 and 1.40 mm. Egg diameter was smaller in the second spawn compared to the first

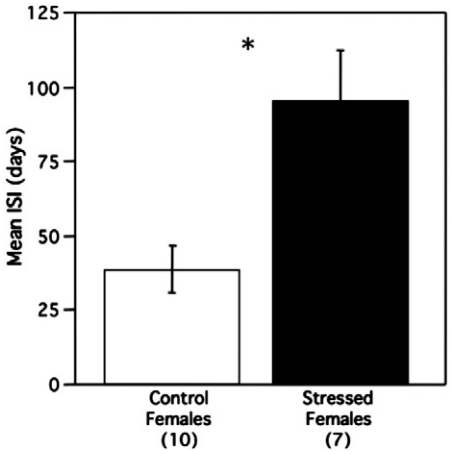

Fig. 1. The interval between the first and second reproductive events (inter-spawn interval, ISI) for *N. pulcher* breeder females in control and stressed treatment groups. Values are means ± se; *N* for each treatment group is indicated in parentheses. The asterisk indicates a significant difference between control and stressed females.

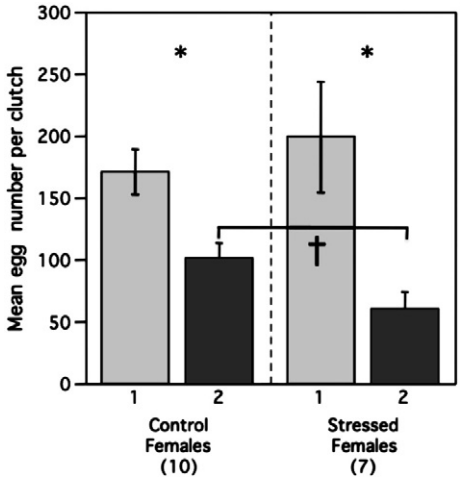

Fig. 2. Mean number of eggs per clutch ± se for the first and second spawn of *N. pulcher* breeder females assigned to control and stressed treatment groups. *N* for each treatment group is indicated in parentheses. Asterisks indicate a significant decrease in clutch size between spawns (control females: paired t-test, $t_9 = -4.02$, $p = 0.003$; stressed females: paired t-test, $t_6 = -3.30$, $p = 0.02$). Dagger symbol indicates a significant difference between treatments in the number of eggs at the second spawn.

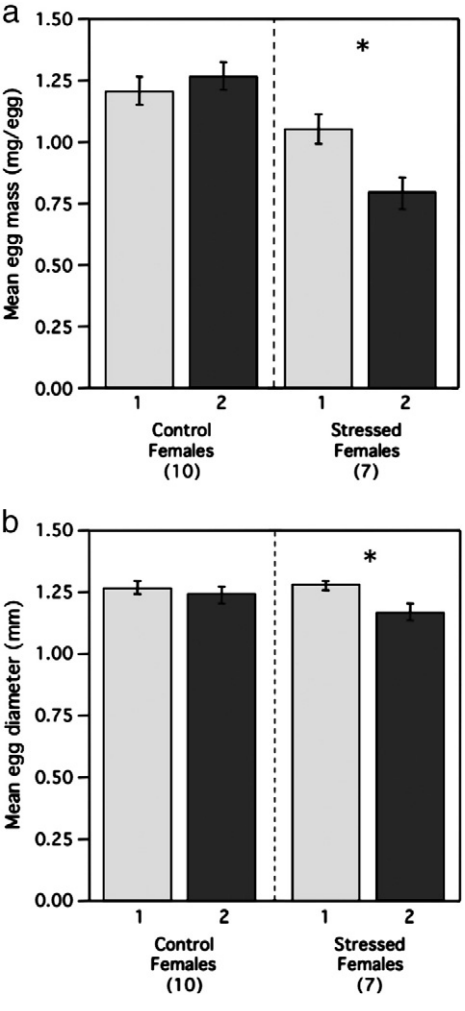

Fig. 3. Data presented are (a) egg mass (mg/egg) and (b) egg diameter (mm) for the first and second spawn of *N. pulcher* breeder females assigned to control and stressed treatment groups. Values are means ± se; *N* for each treatment group is indicated in parentheses. Asterisks indicate a significant change from spawn 1 to spawn 2 for females in the stressed condition.

spawn of stressed females (Wilcoxon signed-ranks test, U = -13.00, p = 0.03; Fig. 3b) but no such decrease was observed in control females (paired t-test, t_9 = -0.68, p = 0.51; Fig. 3b). Overall, however, the change in egg diameter from the first to second spawn did not vary between treatments (Repeated measures ANOVA, overall model: $F_{2, 14} = 1.05$, p = 0.38, effect of treatment $F_{1, 14} = 1.68$, p = 0.22, effect of specific growth rate $F_{1, 14} = 0.003$, p = 0.96).

3.5. Cortisol levels in eggs

At the first spawn, eggs from both control and stressed females had similar concentrations of cortisol (Mann-Whitney U test, U = 2.44, p = 0.12). In control females, cortisol levels in the second clutch were lower than those of the first clutch (Paired *t*-test, t_9 = -2.39, p = 0.04; Fig. 4), whereas stressed females exhibited no decline in egg cortisol concentration between spawning events (paired *t*-test, $t_6 = 1.43$, p = 0.20; Fig. 4). Control females tended to have decreased egg cortisol levels in the second compared to the first spawn while stressed females tended to have increased levels, however this pattern did not reach significance (Repeated measures ANOVA, Overall model $F_{2, 13} = 3.78$, p = 0.51, effect of treatment $F_{1, 13} = 3.82$, p = 0.07, effect of specific growth rate $F_{1, 13} = 0.69$, p = 0.42).

3.6. Helper Effects

Helper number had no effect on any egg characteristic (all p > 0.05). To increase the likelihood of detecting a helper effect, data for stressed and non-stressed females were pooled for this analysis and groups with helpers were compared to groups without helpers. Again, the presence of helpers had no effect on egg number, mass or diameter. Helper presence did affect egg cortisol levels. In groups with at least one helper, a smaller change in egg cortisol levels was observed between spawns compared to the change observed in groups without any helpers (Table 2). Finally, pooling both treatments together and examining eggs at first spawn only (prior to any stress treatment), there was no effect of helpers on the egg number (Mann-Whitney test, U = 0.01, p = 0.92), mass/egg (U = 0.00, p = 1.00), egg diameter (U = 0.09, p = 0.77), or cortisol concentration in eggs (U = 1.15, p = 0.28).

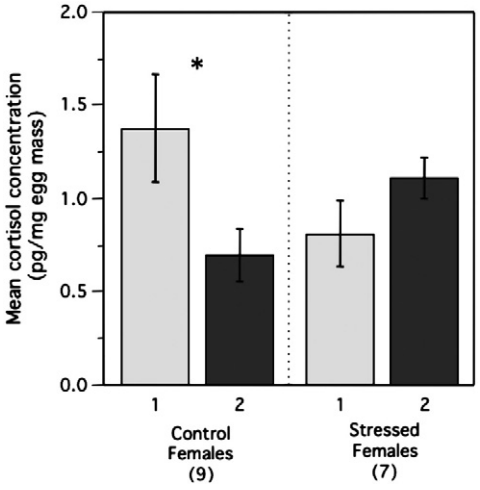

Fig. 4. Egg cortisol concentrations (pg/mg egg mass) are presented for the first and second spawn of *N. pulcher* breeder females assigned to control and stressed treatment groups. Values are means ± se; *N* for each treatment group is indicated in parentheses. The asterisk indicates a significant difference between spawn 1 and spawn 2 for females in the stressed condition.

Table 2
The effects of helper presence on changes in egg characteristics between spawns.

Variable	Groups without helpers (N = 7)	Groups with helpers (N = 10)	Critical statistic/ P value
Mean change in egg number ± se	-115.64 ± 47.11	-86.70 ± 16.48	t = -0.63, p = 0.55
Mean change in egg mass (mg/egg) ± se	-0.12 ± 0.11	-0.04 ± 0.07	t = -0.68, p = 0.51
Mean change in egg diameter (mm) ± se	-0.09 ± 0.04	-0.04 ± 0.03	t = -0.95, p = 0.36
Change in cortisol levels (pg/mg egg mass) ± se	0.43 ± 0.23	-0.31 ± 0.21	U = 5.95, p = 0.01

Data for control and stressed females were pooled for this analysis (17 groups: n = 7 groups without helpers and n = 10 groups with helpers). Helper presence/absence was tested irrespective of helper number (helper number was 0 in 7 groups; 1 helper in 2 groups; 2 helpers in 5 groups; 3 helpers in 1 groups; and 4 helpers in 2 groups).

4. Discussion

This study provides evidence that *N. pulcher* breeder females repeatedly exposed to acute stress experienced a decline in maternal fitness and is suggestive of possible offspring fitness costs. Stressed *N. pulcher* females experienced longer intervals between spawning events, and laid fewer eggs of smaller mass than control females. The results suggest that not only is maternal fitness influenced by repeated acute stress, but that maternal stress may have repercussions for offspring fitness owing to the production of smaller eggs. Both survival and reproductive success are impacted by offspring size, which in turn is strongly influenced by egg size (Marsh, 1986; Williams 1994). Finally, the data suggest that neither the presence nor the number of subordinate helpers played a strong role in determining maternal investment in gametes. However, when stressed and control mothers were considered together, mothers with helpers exhibited lowered egg cortisol concentrations between spawns whereas egg cortisol concentrations tended to increase for mothers without helpers. To our knowledge, this study is the first study to experimentally manipulate stress levels in a cooperative breeder, and to explore the effects of allocare on stress responses. The effect of helper presence on the change in egg cortisol concentration between spawns was contrary to our initial predictions. We had originally hypothesized that helpers would be a source of further stress for breeding females. However, the changes in egg cortisol concentration could be taken as support, albeit indirect, for the notion that having helpers or subordinates around decreases dominant breeder female stress. In a previous study (Mileva et al. 2009), group size (i.e. number of helpers) did not affect baseline cortisol levels in breeding females and males. However, the presence of helpers contributing to broodcare, territory defence, territory maintenance, (Taborsky 1984), may mitigate the effects of other stressors on breeder females, allowing breeder females with helpers to lay eggs with lower cortisol levels. Future studies are obviously needed to quantify the behavioural aspects of reproductive suppression and look at dominant policing behaviour in relation to cortisol released in eggs.

Repeated acute stress is likely to raise both catecholamine and glucocorticoid levels in blood plasma (Reid et al. 1994; Perry et al. 1996; Perry and Bernier 1999). Although the effects of acute stress on catecholamine concentrations in *N. pulcher* are not available owing to the small size of this species, each presentation of a stressor likely caused catecholamine levels to rise and then gradually return to baseline (reviewed by Gamperl et al. 1994). With time, the magnitude of the hormone elevation may have declined, as repeated stress often results in attenuation of the glucocorticoid response (see for example the work of De Boer et al. 1990 on rats, and that of Barton et al. 1987, Perry et al. 1996, Jentoft et al. 2005 on rainbow trout). We attempted to reduce behavioural habituation to the stressors by applying the

stressors at different times each day (always spacing out the two sessions by at least 4 hours).

In this study, we chose to employ two commonly used chronic stressors, in part because other approaches were not feasible. For example, crowding (Pickering and Stewart 1984; Montero et al. 1999; Ramsay et al. 2006) would have affected the entire social group and its dynamics. It would have been difficult to target a single individual with cortisol-treated feed, an approach often used to chronically elevate cortisol (see Barton et al. 1987). Similarly, elevation of cortisol using an intraperitoneal implant (e.g. McCormick 1998) is problematic in fish of such small size (approx total body mass = 6 g in field or 8 g in laboratory). Barton et al. (1987) found that repeated stress evoked physiological changes comparable, although smaller in magnitude, to those elicited by chronic elevation of cortisol. We chose to use repeated chasing and netting to selectively target the breeder female while keeping each *N. pulcher* social group intact. Breeder males in the stressed treatment group seemed unaffected by the selective stress exposure of their mates. The stressors occurred for a relatively short period of time and normal group dynamics were observed before and after the stressors were applied, ruling out the possibility that the failure of stressed breeder females to spawn was the result of disturbance of the whole social group.

In this study, experimentally stressed females exhibited reduced growth rates in comparison to control females. Decreased growth rates in response to repeated stress have been reported in other species (e.g. rainbow trout, Barton et al. 1987; Atlantic salmon, *Salmo salar*, McCormick et al. 1998; Japanese quail, Hayward and Wingfield 2004; Eurasian perch, *Perca fluviatilis*, Jentoft et al. 2005). When individuals are chronically or repeatedly stressed, tradeoffs occur between growth and survival (Wendelaar Bonga 1997; Schreck 2000). Mobilization of energy reserves by stress hormones is thought to contribute to this phenomenon (Pankhurst and Van Der Kraak 1997; reviewed by Gilmour et al. 2005). In addition, acute and chronic stress cause decreases in food intake (e.g. brown trout, *Salmo trutta*, Pickering et al. 1982; rainbow trout, Øverli et al. 2006; see reviews by Bernier and Peter 2001, Bernier 2006), and such appetite suppression may impact growth rates. All individuals in the present study were fed *ad libitum*, but we did not measure energetic status or specific food intake by the breeder females. Suppressed feeding and/or growth retardation may have contributed to the long inter-spawn interval of females in the stressed treatment group.

Our results indicated that repeated stress caused a delay or cessation in reproduction (3 of the 10 stressed females did not spawn a second time during the experimental period, 125 days). This result confirms previous findings in fish (Carragher et al. 1989; Campbell et al. 1992), ungulates (Dobson and Smith, 2000), and birds (Reynard and Savory 1999; Salvante and Williams 2003; Schoech et al. 2009). The mechanisms underlying the tendency for stress to impede reproduction have been particularly well studied in fish, where acute stress has been found to inhibit aspects of the fish endocrine system pertaining to reproduction (Pankhurst and Van der Kraak 1997; Pankhurst and Van der Kraak 2000). For example, Carragher et al. (1989) found that female brown and rainbow trout given cortisol implants had lower levels of circulating vitellogenin and estradiol, as well as smaller gonads. More recently, Pankhurst and Van der Kraak (2000) reported that sexually mature female rainbow trout showed a decrease in plasma testosterone and slight decreases in estrogen following exposure to an acute stressor.

In addition to the increased inter-spawn interval, exposure to repeated stress resulted in the production of fewer, smaller and lighter eggs. In both stressed and control treatment groups, clutch size decreased from the first to the second spawn. It is likely, however, that this effect was due to the provision of inadequate spawning substrate prior to the start of the experiment. Once adequate substrate was substituted (rough acetate sheets versus smooth ones), the fish began to spawn almost immediately, laying high numbers of eggs (9/17 fish laid more than 150 eggs, with two individuals laying 300+; laboratory observations show that 100-300 eggs per clutch is typical in a lab setting, Taborsky et al. 1984, Balshine, unpublished data), presumably because females had been sequestering eggs while no appropriate substrate was available for laying. Nevertheless, we still observed a greater decrease in the number of eggs laid by stressed females than was observed in control females. A significantly greater drop in egg mass occurred in the experimentally stressed females than in control females. Other studies have linked maternal stress to smaller gametes, smaller young, and higher juvenile mortality (Campbell et al. 1992; Coleman and Galvani, 1998; McCormick 1998, 2006; Contreras-Sánchez et al. 1998; Ostrand et al. 2004). The production of fewer, smaller gametes may have occurred in part because of the negative effects of cortisol on vitellogenin and estradiol production (see Carragher et al. 1989). In our experiment, eggs were used to quantify cortisol levels, and hence no young were produced. However, elevated egg cortisol levels have been associated with negative impacts on young in other fish species. For example, Gagliano and McCormick (2009) reported higher rates of mortality and otolith asymmetry in damselfish hatchlings that had been exposed to high cortisol levels as eggs. Similarly, McCormick (1999) found that damselfish eggs exposed to high levels of cortisol hatched into smaller juveniles and, Sloman (2010) found that juvenile brown trout hatched from eggs exposed to high, but ecologically relevant, levels of cortisol were more aggressive than their non-exposed siblings. Consequently, it would be valuable for future studies to use a split brood design for *N. pulcher* to explore the impacts of cortisol levels and decreased egg size on offspring survival rates as well as social, behavioural and reproductive repercussions in later life.

High maternal stress and/or cortisol levels result in elevated egg cortisol concentrations (Stratholt et al. 1997; McCormick 1998; Eriksen et al. 2006). In the present study, cortisol content of eggs produced by treated stressed females remained high or tended to increase whereas in control females egg cortisol levels decreased significantly from the first to the second spawn. It is possible that the sequestering of eggs, as noted above, resulted in elevated maternal, and hence egg cortisol concentrations. With this scenario, the subsequent fall in egg cortisol concentrations in the control group could reflect a re-establishment of normal spawning conditions, while continued high cortisol concentrations in eggs from stressed females reflected the impact of the stress exposure. Cortisol concentrations typically follow a U-shaped curve, starting with high cortisol in newly hatched eggs from maternal deposition, followed by a decrease until hatching, and then a post-hatch increase (de Jesus et al. 1991; Barry et al. 1995; Alsop and Vijayan 2008). In this study, eggs were collected in a fairly narrow time frame between 1 and 24 h post-spawn (the precise spawning time was not always known). However, as eggs typically take 3-4 days to hatch in *N. pulcher* (Balshine-Earn et al. 1998; Taborsky et al. 2007), collection during the first 24 hours probably controlled for a considerable proportion of the potential fluctuation caused by natural decreases in cortisol post laying.

In highly social species (e.g. wolves, *Canis lupus*, Creel 2005; meerkats, *Suricata suricatta*, Young et al. 2006; or Florida scrub jays, *Aphelocoma coerulescens*, Schoech et al. 2009), it is typically extremely challenging to manipulate maternal stress in an experimentally consistent manner as is the ability to investigate the effect of stress on maternal reproductive success and offspring characteristics. In contrast, in this study we used *N. pulcher*, a highly social fish species that performs naturalistic behaviour in the laboratory, facilitating investigation of stress responses in a group living species. Future studies that rigorously manipulate helper numbers are now needed to further explore the impacts of cooperative living on maternal stress levels and on offspring survival, growth rates, and social status.

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