Learning enhances female control over reproductive investment in the Japanese quail

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The adaptive significance of learning is supported by studies showing its positive effects on mating behaviour, but they rarely go beyond fertilization success. Here we studied how learning contributes to qualitative reproductive investment, by testing the hypothesis that mating in the context that predicts male appearance has positive effects on female reproductive investment compared with unsignalled mating. Using Japanese quail (*Coturnix japonica*), we found that effects of mating in the context predicting mating opportunity depend on female body condition and receptivity, while the outcome of unexpected mating depends on male behaviour. In particular, among females mated with the familiar male in the context predicting that he will appear, female condition positively affected the number of fertilized eggs and egg mass and more receptive females tended to produce more sons. Additionally, conditioned females laid heavier eggs for daughters than for sons. In contrast, in females that were mated unexpectedly and with a novel male, the number of fertilized eggs was highly dependent on male behaviour and was negatively related to maternal body condition. Egg mass was not related to body condition, and there were no indications of sex allocation. This is, to our knowledge, the first study demonstrating how female body condition and behaviour interact with the context of mating in shaping maternal reproductive investment.

Keywords: Pavlovian conditioning; mate familiarity; body condition; maternal effects; sex allocation; fertilization success

1. INTRODUCTION

Maternal effects are non-genetic factors that are transmitted to the offspring and often affect its performance (Mousseau & Fox 1998). Characteristics of female state and body condition strongly predict maternal investment to the offspring and even their sex (Trivers & Willard 1973; Clutton-Brock *et al.* 1984). In birds, a taxon in which it is relatively easy to assess maternal investment, the significance of maternal effects transmitted via the eggs on future offspring performance is well known (e.g. Schwabl 1993). The relationship between female condition and quality of the offspring is not surprising, as egg content is strictly dependent on female physiology, including nutritional and hormonal status. However, also distal cues such as social environment (Mazuc *et al.* 2003), mate attractiveness (Cunningham & Russell 2000) and even the quality of the male mate's song (Gil *et al.* 2004) have been shown to affect maternal investment on the eggs. Furthermore, distal cues may also affect the sex ratio of the offspring (e.g. Komdeur *et al.* 1997). That evidence could suggest that there are some direct top-down (brain-regulated) pathways between what a female perceives and how her reproductive effort is managed (Pryke & Griffith 2009). Could learning and habituation contribute to that effect? The procedure of classical (Pavlovian) conditioning might shed some light here. Despite well-established and relevant

methodology, paradigms of learning are surprisingly rarely integrated in the studies of maternal effects.

Classical conditioning is a form of learning that involves behavioural adjustment to an impending biologically significant event. Pavlov's (1927) hypothesis that classical conditioning is adaptive has been supported by studies showing positive effects of conditioning on reproductive behaviour (reviewed in Pfaus *et al.* 2001; Woodson 2002). Signals enabling anticipation of copulation enhance female and male mating behaviour (Hollis *et al.* 1989) and male reproductive success in fishes (Hollis *et al.* 1997), fertilization success in female and male birds (Adkins-Regan & MacKillop 2003; Matthews *et al.* 2007), male mating behaviour (e.g. Silberbe & Adler 1974; Zamble *et al.* 1985) and female mate preferences in mammals (Coria-Avila *et al.* 2006). While there is evidence for positive effects of conditioning on quantitative reproductive success, i.e. number of fertilized eggs, in the Japanese quail (*Coturnix japonica*) (e.g. Adkins-Regan & MacKillop 2003; Mahometa & Domjan 2005; Matthews *et al.* 2007) and number of young 6 days after spawning in the blue gourami (*Trichogaster trichopterus*) (Hollis *et al.* 1997), it has not been determined how predictive signalling of mating opportunity affects qualitative investment on the offspring. It could be expected that conditioning prepares a female for reproduction (Hollis *et al.* 1997), and thus it should enhance the quality of the eggs. Given that the same factors often affect egg quality and offspring sex (reviewed in Alonso-Alvarez 2006; Rutkowska & Badyaev 2008), it could also be

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expected that conditioned females bias the sex ratio of their offspring.

Under natural circumstances, sexual conditioning is often interrelated to mate familiarity. For example, conditioning is suggested to be involved in the development of preference for the familiar partner in prairie voles (reviewed in Insel & Young 2001). In birds, highly repeatable fertilization success for a given female–male pair (Adkins-Regan 1995) might indicate that positive effects of conditioning are mediated by mate familiarity. Familiarity has also been hypothesized to explain improved breeding performance (Ens *et al.* 1996). Therefore, by incorporating mate familiarity, we enhanced the effect of Pavlovian conditioning and studied how they both affect maternal investment.

The Japanese quail is an important model organism in studies on maternal effects and sex allocation (Pilz *et al.* 2005; Pike & Petrie 2006) as well as on sexual conditioning (e.g. Gutierrez & Domjan 1997; Adkins-Regan & MacKillop 2003; Domjan *et al.* 2003). Here, we used this species to test the hypothesis that mating with a familiar male in the context that predicts that he will appear has positive effects on female reproductive investment compared with mating with a novel male in the context that predicts that the male will not appear. Specifically, we looked at fertilization success, egg mass and offspring sex, following signalled and unsignalled mating. Up to now, there were separate studies looking at behavioural effects of conditioning for mating opportunities (Gutierrez & Domjan 1997; Mahometa & Domjan 2005) and fertilization rates of conditioned and unconditioned individuals (Adkins-Regan & MacKillop 2003; Mahometa & Domjan 2005). Here we assess for the first time, to our knowledge, how the behaviour of both the sexes as well as their body conditions interact with Pavlovian conditioning to affect maternal investment beyond the fertilization rate.

2. METHODS

(a) Subjects

Birds used in the experiment were two- to three-month-old Japanese quail raised and housed in the animal facility at Cornell University. Fifty-two females involved in the study were laying eggs regularly, but were sexually naive. Twenty-eight males with previous mating experience confirmed by at least three successful copulations were used as stimulus birds. None of the males was used with more than two females and if so, those matings were at least a week apart. It has also been shown previously that fertilization rates when the same male mates with different females are not correlated (Adkins-Regan 1995). Prior to the experiment, all the birds were weighed using a Pesola balance to the nearest 1 g, and their tarsus length was measured with an electronic caliper to the nearest 0.1 mm. Birds were randomly assigned to the two experimental groups, and it was confirmed that groups differed neither in females' nor in males' mean mass, tarsus length or body condition (all $p > 0.4$).

(b) Conditioning and testing

Two cages differing in location, size and appearance were used for conditioning trials. One (A) was located in a room adjacent to the colony room, measured 28 cm high, 60 cm long and 45 cm wide, and was made of Plexiglas with a wire-mesh

floor over the Plexiglas floor. The other (B) was located in an empty room across the hall from the colony room, measured 35 cm high, 120 cm long and 40 cm wide, and was made of wire mesh with a white paper over the wire mesh floor. Both cages were divided in the middle with Plexiglas and wire mesh walls, respectively, so that birds could be separated during conditioning trials to prevent fertilization.

Each female was given two 7 min trials per day, one in each cage, for five consecutive days, always at the same times of the day. Trials were 90 min apart, and the order of the cages was counterbalanced. After a female was placed in a cage, a male was added to one cage after 2 min. For half the females, a male was added to cage A and for half the females, a male was added to cage B, again counterbalancing with cage order. Thus, by the end of 5 days, all females had spent an equal amount of time in both cages, all females had spent an equal amount of time with males; for half of the females, cage A had been the conditioned cage (CS+) that predicted a mating opportunity and for half of the females, cage B had been the CS+ cage. Each female always encountered the same male.

On the sixth day, each female was placed in a testing cage for 2 min, after which the male was introduced to the other half of the cage and the divider was removed, allowing birds to mate for 5 min at a time exactly between the times of the two conditioning trials. All mating trials took place between 11.00 and 13.00. Half of the females were tested in their CS+ cage with the same male they had encountered in that cage before and half in their unconditioned cage (CS−) with a male new to them, but which was used to encountering a different female in that cage. Thus, all the males had been mated in the cage for which they were conditioned, which aimed to enhance the fertilization success of mating (Mahometa & Domjan 2005). Similar procedures have been previously successfully used in Pavlovian conditioning in the Japanese quail (Adkins-Regan & MacKillop 2003). Here, males were not limited to one mating (insemination) but had 5 min to mate; some mated more than once.

(c) Behavioural observations

All mating trials were videotaped and subsequently analysed by a person blind to the group assignment of the birds. For females, it was noted whether they performed cricket calls before the male was introduced to the cage and, initial receptivity during the first few seconds after the male was introduced was assessed. The following scores were assigned: 0, female highly unreceptive, constantly moves away from male and refuses to squat; 1, female unreceptive but not markedly so; 2, female ambiguously receptive; 3, female clearly receptive, squats and does not move while mounted.

For male behaviour, latency to head grab the female and total duration of mating attempts were measured in seconds. The number of struts, feather fluffs and crows was counted.

(d) Measures of maternal investment

There were 52 females in the experiment, 19 of which were sacrificed after the trial as a part of another study (12 of those females were included in the analyses of behaviour during testing) and 33 of which continued laying eggs during the 10 days after the trial. At the day of laying, each egg was weighed to the nearest 0.1 g, marked with non-toxic marker and then incubated at 38°C for 5 days and examined for embryonic development. If present, a piece of embryonic tissue was preserved in ethanol for future sexing.

Figure 1. Variation in relative egg mass mean s.e. (standardized to the egg laid a day after the mating trial) with the egg laying sequence in (a) females mated in the context predicting mating opportunity (CS+, $n = 16$) and (b) females mated unexpectedly (CS-, $n = 17$).

Eggs were collected for 11 days starting 1 day after the mating trial. Laying gaps seldom occurred, and egg numbers were assigned according to the days elapsed after mating. The egg laid the day after the mating trial is considered not to be affected by the experimental treatment and was used as a reference egg.

DNA was extracted using Chelex resin, following the manufacturer's protocol. For PCR, we used primers 2718R and 2550F (Fridolfsson & Ellegren 1999), and reaction conditions were modified after Arnold *et al.* (2003) with an annealing temperature of 51°C. The products were scored on agarose gel stained with ethidium bromide for the presence of one band (male) or two bands (female). Accuracy of molecular sexing was confirmed using samples of six adult birds, three of each sex.

(e) Statistical analyses

In all models, experimental group was a fixed factor. Models that included multiple data points for a given female, i.e. analyses of offspring sex and egg mass variation, accounted for female identity as a random factor. Body condition was a residual value of regression of body mass on tarsus length and was introduced as a continuous variable. Egg laying order was a continuous variable. Some analyses involved behavioural measures as predictors of maternal investment, and in such cases, they were introduced as continuous variables. Behavioural variables were analysed using non-parametric tests, while egg mass, fertilization rates and offspring sex ratios were analysed using linear models. All tests were two-tailed. All statistical analyses were performed in SAS v. 4.1, and figures were prepared using STATISTICA.

3. RESULTS

(a) All females

Conditioning of behaviour was successful, as it significantly increased the initial receptivity of females (CS+: 2.36 ± 0.14 ; CS-: 1.56 ± 0.82 ; Mann-Whitney test: $U = 141$, $p = 0.011$, $n = 45$) and slightly reduced latency

to head grab by male (CS+: 3.0 ± 0.8 s; CS-: 10.3 ± 6.7 s; Mann-Whitney test: $U = 177.5$, $p = 0.086$, $n = 45$). Irrespective of the experimental group, heavier females were more likely to perform cricket calls during their initial time in the cage, before the appearance of the male (mass of females that performed cricket calls: 258.7 ± 10.0 g; those that did not: 234.5 ± 6.9 g; $F_{1,50} = 4.13$, $p = 0.047$).

Egg mass was positively related to female body condition (general linear mixed model (GLMM) controlling for female ID: $F_{1,287} = 9.24$, $p = 0.0026$), egg laying order ($F_{1,287} = 27.11$, $p < 0.0001$) and the interaction between experimental group and laying order ($F_{1,287} = 5.61$, $p = 0.018$). When analysed separately, in the CS+ group, the positive effect of female body condition was highly significant ($F_{1,139} = 9.78$, $p = 0.0021$) and egg laying order was not ($F_{1,139} = 3.23$, $p = 0.07$), whereas in the CS- group, egg mass increased with egg laying order ($F_{1,148} = 37.3$, $p < 0.0001$, figure 1) and was not related to female body condition ($F_{1,148} = 0.66$, $p = 0.42$).

Overall, females in the two groups did not differ in fertilization success (mean number of fertilized eggs in CS+ was 1.9 versus 2.35 in CS-). Yet, male behaviour during mating trials was a significant predictor of whether any eggs became fertilized, and the strength of that effect differed between experimental groups as indicated by the significant interaction between the group and number of feather fluffs performed by the male (logistic regression: 1, at least one egg was fertilized; 0, there were no fertilized eggs; group: Wald $\chi^2 = 2.02$, $p = 0.155$; number of feather fluffs: Wald $\chi^2 = 3.61$, $p = 0.057$; group $\times$ number of feather fluffs: Wald $\chi^2 = 4.81$, $p = 0.028$). Results for the number of struts were similar, but the interaction of that variable and experimental group was marginally non-significant at the level of $p = 0.060$. When analysed separately, in the CS+ group, there was no effect of male behaviour on the number of fertilized eggs (Spearman rank correlation; feather fluffs: $r_s = 0.11$,

Figure 2. Relationship between number of struts performed by males during the mating trial and number of fertilized eggs laid (a) by females mated in the context predicting mating opportunity (CS+, $n = 16$) and (b) females mated unexpectedly (CS-, $n = 17$). Size of the symbol reflects sample size for a given combination of male struts and number of fertilized eggs. Regression line fitted to the data is shown.

$p = 0.67$; struts: $r_s = 0.09$, $p = 0.73$; figure 2), but in the CS- group, both behaviours were highly predictive of fertilization success (feather fluffs: $r_s = 0.60$, $p = 0.01$; struts: $r_s = 0.70$, $p = 0.002$; figure 2). Duration of mating attempts did not predict fertilization success in either group ($r_s = -0.25$, $p = 0.16$).

(b) Females that laid at least one fertilized egg

Sixteen females laid at least one fertilized egg. Among those females, the experimental group had a significant effect on the number of fertilized eggs when female body condition was taken into account, and there was also a significant interaction between experimental group and female condition (ANOVA, group: $F_{1,15} = 5.90$, $p = 0.033$; condition: $F_{1,15} = 0.37$, $p = 0.55$; group $\times$ condition: $F_{1,15} = 19.15$, $p = 0.001$, figure 3). When analysed for the two groups separately, in the CS+ group, there was a positive relationship between female condition and number of fertilized eggs ($F_{1,7} = 6.41$, $p = 0.039$), while in the CS- group, that relationship was negative ($F_{1,5} = 27.37$, $p = 0.003$).

There were no differences in the offspring sex ratio between the groups (group: $F_{1,15} = 2.48$, $p = 0.141$). However, female receptivity (non-significant itself: $F_{1,15} = 1.88$, $p = 0.195$) tended to have a different relationship to offspring sex ratio in the two groups, which is indicated by the marginally non-significant interaction between experimental group and female receptivity ($F_{1,15} = 4.56$, $p = 0.054$). In the CS+ group, there was a trend for a male-biased offspring sex ratio in more receptive females (figure 4), but the sample sizes in the two groups are too small for further analyses. Interestingly, females in the two experimental groups differed in their qualitative investment to male and female offspring, as indicated by the significant interaction between offspring sex and group shaping egg mass variation (GLMM controlling for female ID, group: $F_{1,52} = 5.11$, $p = 0.028$,

offspring sex: $F_{1,52} = 0.11$, $p = 0.75$; group $\times$ offspring sex $F_{1,52} = 4.59$, $p = 0.037$). Within the CS+ group, female eggs (12.16 g) were slightly heavier than male eggs (11.93 g, GLMM controlling for female identity, $F_{1,22} = 4.56$, $p = 0.044$), but within the CS- group, there were no differences in the egg mass between the sexes ($F_{1,30} = 1.88$, $p = 0.18$).

4. DISCUSSION

Our study used Pavlovian conditioning to successfully enhance female receptivity in the context predicting mating opportunity in the Japanese quail. The experimental setup enabled us to show that in the context that predicts mating opportunity the egg outcome depends on female body condition (figure 3) and receptivity (figure 4), while the outcome of unexpected mating is more predicted by male behaviour (figure 2). Specifically, within females mated with the familiar male in the context predicting that he will appear, female condition positively affected the number of fertilized eggs and egg mass and more receptive females tended to have more sons in their clutches (figure 4). Additionally, conditioned females produced heavier eggs for female offspring than for male offspring. In contrast, in females that were mated unexpectedly and with a novel male, the number of fertilized eggs was highly dependent on male androgen-related behaviours during mating (Adkins & Adler 1972) and was negatively related to maternal body condition. In those females, egg mass was not related to body condition and they did not differentiate investment on male and female eggs.

Our experimental setup does not let us distinguish the effect of conditioning to the place where mating opportunities occur from the effect of mate familiarity (which could itself have similar influence); yet, any effect of mate familiarity is also likely to involve a conditioning

Figure 3. Relationship between female body condition and number of fertilized eggs in (a) females mated in the context predicting mating opportunity (CS+, $n = 7$) and (b) females mated unexpectedly (CS-, $n = 9$). Data for females with at least one fertilized egg. Regression line fitted to the data is shown.

Figure 4. Relationship between female receptivity and offspring primary sex ratio (proportion of males) in (a) females mated in the context predicting mating opportunity (CS+, $n = 7$) and (b) females mated unexpectedly (CS-, $n = 9$). Size of the symbol reflects sample size for a given combination of female receptivity and offspring sex ratio. Regression line fitted to the data is shown.

component and they both constitute the process of learning. Such a design strengthens the observed differences in maternal investment. More importantly, it resembles natural scenarios, where mating may repeatedly happen with the same partner in the same place and where within- and extra-pair copulations occur in distinct but different locations (Tryjanowski *et al.* 2007).

Strong effects of female body condition on maternal investment have well-established support (Trivers & Willard 1973; Nager *et al.* 1999; Velando 2002; Verboven *et al.* 2003), and therefore we hypothesize that it might affect the female response to mating in conditioned versus

unconditioned situations. Indeed, we found that female body condition allows her to adjust reproductive investment to the situation in which she is mated with a male. First, females in high body condition mated in the context that predicted mating opportunity laid heavier eggs, and also more of their eggs were fertilized, clearly indicating increased reproductive investment. In contrast, females in high body condition that were mated unexpectedly did not produce high-quality eggs, and they also laid fewer fertilized eggs than expected given their superior condition.

Adverse effects of mating with males unexpectedly are also visible in the variation in the egg mass with the

laying sequence. In CS– females, egg mass decreased for a couple of days (figure 1), which could be related to the fact that at the day of mating, those eggs were undergoing their most rapid growth (Hackl *et al.* 2003). The potentially stressful event of mating with a novel male in an unconditioned situation could have negative effects on growth of those follicles. The significant interaction between experimental group and laying sequence (figure 1) indicates that an egg mass decline after mating did not occur in the females mated with males in the conditioned situation. In CS+ females, there was little variation in egg mass with respect to the laying sequence.

In the Japanese quail, adult females are significantly larger than males (in our population, they are 30% heavier than males), and female mass and body condition are important predictors of reproductive success (this study, see also Correa *et al.* 2007). Therefore, the higher mass of eggs with female embryos in the CS+ group, i.e. under circumstances that give females more control over reproductive investment, could be interpreted as preferential maternal allocation aimed at producing higher quality daughters. A similar adaptive explanation for increased provisioning of the sex that might bring higher fitness returns has been evoked before (e.g. Mead *et al.* 1987; Cordero *et al.* 2000) to explain male-directed egg sexual size dimorphism in some passerine species. Given very little within-female variation in the egg mass (figure 1), it might be possible that differential allocation with respect to offspring sex is more pronounced in other maternal effects such as the amount of hormones or antioxidants in the eggs. Furthermore, given the lack of sibling competition in quail, selection might operate at a between-female/family level in this species. In such a case, overall differences between females in maternal investments should be expected.

In line with the above results on differential sex allocation, one would also predict a female-biased offspring sex ratio in the CS+ group. However, we found no differences between the groups in offspring sex ratios. The marginally non-significant interaction between experimental group and female receptivity shaping sex ratio (figure 4) could be explained by the fact that female receptivity depends on gonadal hormone levels (Adkins & Adler 1972; Adkins-Regan 2007), which might also be involved in chromosomal determination of offspring sex (reviewed in Rutkowska & Badyaev 2008). The causation pathway in CS+ females would be that less receptive females were more stressed during mating trials and therefore their corticosterone levels were elevated (Correa *et al.* 2007) to an extent leading to female-biased offspring sex ratios (Pike & Petrie 2006). In the CS– group, female receptivity does not seem to be related to sex allocation. Larger sample sizes and assessment of maternal hormonal levels resulting from mating in conditioned versus unconditioned situations would be required to further investigate the possibility of sex adjustment in the CS+ and CS– females.

The Japanese quail typically has very low average, yet also highly variable, fertilization success from short mating trials (e.g. Adkins-Regan 1995; Mahometa & Domjan 2005). Any factors that might increase fertilization rates should bring an evolutionary advantage. Our study contributes to the discussion of the extent to which a female has control over the fertilization of

her eggs. It has been shown previously that female receptivity by itself does not predict fertilization success (Adkins-Regan 1995), yet whether a female ran from the male's initial approach or not does determine if any eggs get fertilized (Adkins-Regan 1995). Here we demonstrate that male behaviour predicts whether any eggs become fertilized (figure 2), but in cases where at least one egg was fertilized, female body condition had a significant effect on their number. This might indicate fine-tuning of reproductive investment relative to the female's own physiological state. Receptivity differentiated by conditioning for mating opportunity (see also Gutierrez & Domjan 1997) significantly contributed to the direction of that effect. On the one hand, female body condition could underlay the proximate cause of variation in the number of fertilized eggs, but on the other hand, it can also be a trigger for male behaviour (see also Correa *et al.* 2007). For example, male behaviour could potentially be affected by cricket calls performed by heavier females before male appearance that signal their readiness to mate (Guyomarc'h & Guyomarc'h 1996). In our study, males could not hear females' calls before the trial and also that behaviour was not related to experimental treatment, but it could have adaptive significance in natural circumstances.

To our knowledge, this the first study that simultaneously looks at how learning affects mating behaviour in females and investigates maternal investment on the offspring beyond fertilization success. We demonstrate that classical conditioning, based on visual-only access to males without copulatory reinforcement, is sufficient to significantly affect maternal reproductive investment in birds. Our study gives rise to potential implications for the significance of learning for the evolution of female reproductive strategies. Would altricial species with between-sibling competition have more pronounced sex allocation patterns in relation to the context of maternal mating? Could learning contribute to post-copulatory mate choice and facilitate biased maternal investment in relation to paternity of the offspring (Johnson *et al.* 2009)? How does mate familiarity contribute to the process of conditioning? Finally, what neural mechanism underlies the effects of conditioning in females? So far, research has focused on those responsible for the increased success of conditioned males (Can *et al.* 2007; Taziaux *et al.* 2008). Integration of endocrine, behavioural and neural mechanisms should provide an important contribution to understanding the adaptive role of learning in maternal investment as well.

All procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

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