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Japanese quail (*Coturnix japonica*) inseminations are more likely to fertilize eggs in a context predicting mating opportunities

Elizabeth Adkins-Regan[1,2*] and Emiko A. MacKillop[1]

[1]*Department of Psychology, and* [2]*Department of Neurobiology and Behavior, Uris Hall, Cornell University, Ithaca, NY 14853-7601, USA*

Theoretical developments in behavioural ecology have generated increased interest in the proximate mechanisms underlying fertilization, but little is known about how fertilization success is regulated by cues from the external or social environment in males and females. Here, we use a Pavlovian conditioning paradigm to show that inseminations resulting from mating male and female Japanese quail (*Coturnix japonica*) are more likely to fertilize eggs when they occur in a context predicting that an opposite-sex bird will appear than when they occur in a context predicting that an opposite-sex bird will not appear. This effect occurs when either the male or the female is the target of the conditioning. Thus, processes occurring during or after mating that contribute to fertilization success are subject to the influence of distal cues, confirming control by brain-level mechanisms. Conditioning is a widespread property of the nervous system and the demonstration that context conditioning can influence male and female reproductive success, and not simply mating success, has widespread implications for the fertilization successes of different types of copulation in natural mating systems.

Keywords: fertilization; Pavlovian conditioning; reproductive success; Japanese quail

1. INTRODUCTION

In internally fertilizing species, such as birds, reproductive success requires both that animals mate successfully and that sperm transferred during mating fertilize eggs. Insemination by no means guarantees fertilization (Adkins-Regan 1995). Some of the variability in fertilization success is likely to be caused by male factors such as the number of sperm ejaculated but there is much interest in the possibility that females contribute to it as well. Female birds have long convoluted oviducts containing sperm-storage tubules, the sites of insemination and fertilization are quite far apart and the female reproductive tract is thought to have a significant influence on fertilization success (Birkhead & Møller 1992). Female galliform birds have been observed to void ejaculates immediately after copulation, a possible form of female control over sperm usage (Pizzari & Birkhead 2000).

In recent years sexual-selection theory has been extended in important ways to the level of gametes and fertilization success (Simmons 2001; Birkhead & Pizzari 2002; Wedell *et al.* 2002). By building on important earlier work on sperm competition between males (Parker 1970, 1984), these developments have led to new hypotheses and insights about fertilization mechanisms in relation to social mating systems. The idea that fertilization can be regulated adaptively by males or females in response to other individuals means that some determinants of fertilization are influenced by external distal cues through top-down brain-level mechanisms. Yet male ejaculation is a set of largely spinal (mainly parasympathetic) reflexes triggered by tactile stimuli whose supraspinal control is poorly understood even in mammals (Froman 1995; McKenna 1999; Truitt & Coolen 2002), and little is known about possible pathways for adaptive regulation of the female oviduct other than the observation that the sperm-storage tubules are innervated (Freedman *et al.* 2001). Progress would be facilitated by bringing fertilization success under the control of external stimuli in an avian species in which neural pathways and physiological mechanisms linking distal cues to reproductive anatomy can be discovered. Here, we use a time-honoured method for showing control by an external stimulus, a Pavlovian (classical) conditioning paradigm, in Japanese quail (*Coturnix japonica*), a species already well established in neuroendocrinal research.

Domjan *et al.* (1998) reported that male Japanese quail ejaculating on a stuffed model released more sperm if tested in a cage where they had previously been given opportunities to copulate with live females (the conditioned stimulus cage or CS+ cage) than if tested in a control cage. This report is a significant step towards showing regulation of ejaculated sperm numbers by distal cues of the mating context and increased reproductive success through context conditioning. However, sperm release with a stuffed model is not a direct measure of reproductive success. Owing to the variability in the fertilization success of single inseminations in this species, it is not possible to conclude that males mating in the CS+ cage would actually fertilize more eggs. In addition, it is important to know whether a female's ability to predict mating opportunities affects the fertilizing success of inseminations. Although the sexual receptivity of female quail is subject to conditioning (Gutiérrez & Domjan 1997), which would clearly influence the male's mating success, it is not known whether external stimuli acting on the female would influence the fertilization consequences

* Author for correspondence (er12@cornell.edu).

of an insemination, especially in light of evidence that forced copulation with unreceptive females can fertilize eggs (Adkins-Regan 1995).

We used the context-conditioning experimental paradigm of Domjan *et al.* (1998) but tested males with live females, so that the fertilization success of confirmed inseminations could be determined, and used a within-male design to see whether the success of individual males varied with context. We also carried out a similar experiment with females as the subjects of the conditioning. Although the female anatomy suggests several possible mechanisms for a female influence over fertilization, it is important to determine whether external cues can actually influence fertilization success (and not simply mating success) in females by examining the consequences of confirmed inseminations.

2. MATERIAL AND METHODS

Successful insemination can be confirmed non-invasively by noting the presence of the male's white proctodeal gland foam in the female's cloaca (Adkins-Regan 1995). The fertilization success of single natural inseminations is quite variable within individual males and within individual females. About one-third of confirmed inseminations do not fertilize any eggs; the other two-thirds fertilize from one to 10 eggs (Adkins-Regan 1995).

(a) *Experiment 1: effects of male conditioning*

We used 26 male and 78 female Japanese quail raised in the laboratory; 26 females were used as mating partners for conditioning trials and 52 females were used as mating partners for test trials. Males were young (two months old) sexually naive adults at the beginning of the experiment. Conditioning-trial females were 6–12 months old and had sexual experience from participation in prior mating-behaviour experiments, to ensure an adequate level of mating success by the male subjects in the conditioning trials. Test females were the same age as the males and were sexually naive. All birds were housed on a 16 L : 8 D photoperiod and were physiologically reproductive: males had large foam glands and females were laying eggs regularly. The protocol was approved by the Cornell University Institutional Animal Care and Use Committee, and all applicable federal and institutional guidelines for animal care and use were followed.

Two cages differing in location, size and appearance were used for conditioning and testing. One (A) was located in an empty room across the hall from the animal colony room, measured 30 cm high, 30 cm wide and 46 cm long, and was made of Plexiglas with a wire-mesh floor over the Plexiglas floor. The other (B) was located in the colony room, measured 18 cm high, 38 cm wide and 24 cm long, and was made of wire mesh.

Each male was given two 7 min trials per day, one in each cage, for 5 consecutive days, always at the same times of day. Trials were 90 min apart and the order of the cages was counterbalanced. A female was added to one cage after 2 min. For half the males a female was added to cage A and for half the males a female was added to cage B, again counterbalancing with cage order. Thus, by the end of the 5 days: (i) all males had spent an equal amount of time in both cages; (ii) all males had spent an equal amount of time with females; and (iii) for half of the subjects cage A had been the CS+ cage that predicted a mating opportunity and for half of the subjects cage B had been the CS+ cage. Each male was given a different female each day, and no female was used more than once in a day.

Each male was then tested twice, once in his CS+ cage and once in his CS− cage, so that fertilization successes could be compared on a within-male basis. Fertilization success is not affected by copulations on prior days (E. Adkins-Regan, unpublished data). Tests were conducted on the 2 days following the conditioning trials, one test per male per day, with both tests at the same time of day. Tests were conducted at a time midway between the two prior daily conditioning-trial times. The order of the two tests was counterbalanced with respect to both cage status (CS+ versus CS−) and cage context (A versus B). For each test, the male was placed in the cage and a female was added 2 min later. Test females had never been in either cage, and each male was tested with a unique pair of females. The test was terminated when the male's behaviour indicated that he had ejaculated, or when 5 min had elapsed after the introduction of the female, whichever occurred first. The female's cloaca was examined for the presence of visible foam. Eggs laid by females with a confirmed insemination during the next 2–11 days (the possible fertilized-egg days; Adkins-Regan 1995) were collected daily, stored at 13 °C, incubated at 38 °C and 60% relative humidity, and broken open after 1 week of incubation to determine whether an embryo was present (Adkins-Regan 1995). The two measures of the test outcomes were: (i) insemination success (proportion of tests resulting in a confirmed insemination); and (ii) fertilization success (proportion of eggs fertilized by confirmed inseminations, as indicated by the presence of an embryo or, in two cases only, an early embryonic death). Data were analysed by G-tests with William's correction (Sokal & Rohlf 1995) or Wilcoxon tests. A two-tailed $p < 0.05$ was required for significance.

(b) *Experiment 2: effects of female conditioning*

The female experiment used the same design and procedures as experiment 1 with the following changes. First, the sexes were reversed. Subjects were 35 young adult (two months old) sexually naive females, partners for CS+ conditioning trials were 35 sexually experienced males 6–12 months old and partners for test trials were 35 young adult males with enough sexual experience to ensure successful mating. Second, because the fertilization success of test trials cannot be determined in females that have mated recently (owing to sperm storage), the female subject and male partner were separated by a wire-mesh or Plexiglas barrier in CS+ cage conditioning trials to prevent insemination. Third, for the same reason, the testing design was between-female rather than within-female. Each female was tested (allowed to mate) once with a male and all tests were conducted on the day after the fifth conditioning trial. Half of the females were tested in their CS+ cage and half in their CS− cage. Data were analysed by G-tests with William's correction or randomization tests. A two-tailed $p < 0.05$ was required for significance.

3. RESULTS

In the male experiment (experiment 1) most males successfully inseminated their test female regardless of the test cage (24/26 in their CS+ cage versus 22/26 in their CS− cage; a non-significant difference). Overall, 20 males succeeded in inseminating the females in both their tests, but in two cases females did not continue to lay eggs, so within-male comparison of fertilization success was possible for only 18 males. Their insemination fertilized more eggs when it occurred in their CS+ cage than in their CS− cage (figure 1). This effect was caused by a differ-

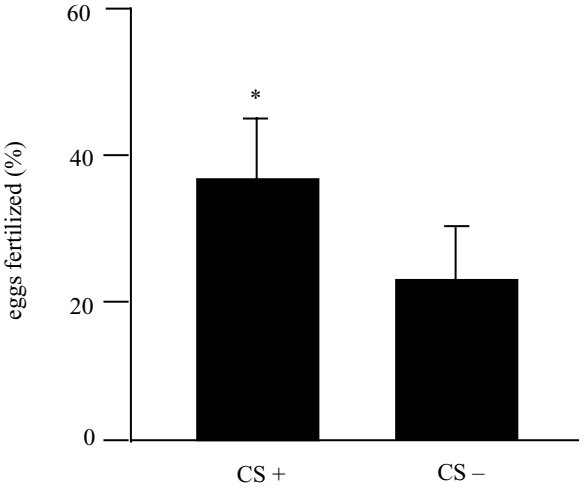

Figure 1. Mean (+ s.e.) percentages of eggs fertilized following a single insemination in a CS+ cage, where males had previously encountered and mated with females, and in a CS− cage, where males had never encountered females (experiment 1). The corresponding medians are 37% (CS+) and 0% (CS−). Eighteen males successfully inseminated the test female in both their CS+ cage and their CS− cage tests; $^{*}p < 0.03$, Wilcoxon test.

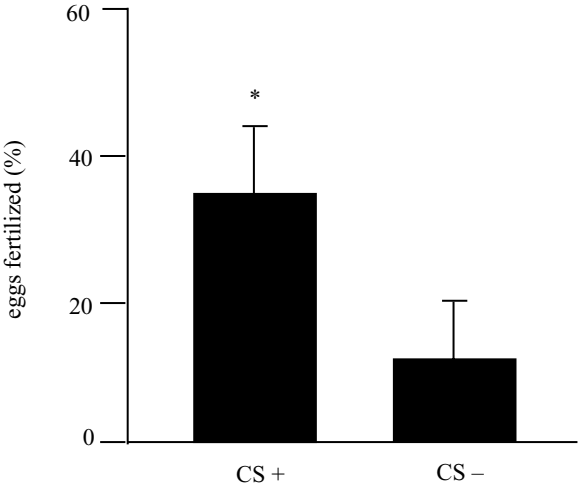

Figure 2. Mean (+ s.e.) percentages of eggs fertilized following a single insemination in a CS+ cage, where females had previously encountered males, and in a CS− cage, where females had never encountered males (experiment 2). The corresponding medians are 33% (CS+) and 0% (CS−). Fifteen females were successfully inseminated in CS+ cage tests and 10 females were successfully inseminated in CS− cage tests; $^{*}p < 0.05$, randomization test.

ence between CS+ and CS− tests in the proportion of inseminations that fertilized at least one egg (14/18 in their CS+ cage versus 7/18 in their CS− cage, $p < 0.02$) and not to a difference in the percentage of eggs fertilized in tests producing at least one fertilized egg (mean percentage fertilized excluding zero fertilizations of 55% in their CS+ cage versus 57% in their CS− cage).

In the female experiment (experiment 2), more females were successfully inseminated when tested in their CS+ cage, but this difference was not significant (16/19 in their CS+ cage versus 10/16 in their CS− cage, $p > 0.1$). One female inseminated in her CS+ cage failed to lay eggs following testing; therefore subsequent analyses are based on 15 rather than 16 inseminated females. Inseminations fertilized more eggs in females mated in their CS+ cage than in females mated in their CS− cage (figure 2). This effect appeared to be caused by a combination of an increased likelihood that an insemination would fertilize at least one egg (10/15 in their CS+ cage versus 3/10 in their CS− cage) and a greater percentage of eggs fertilized in tests producing at least one fertilized egg (mean percentage fertilized excluding zero fertilizations of 53% in their CS+ cage versus 40% in their CS− cage), because neither of these differences was significant by itself (both p-values were greater than 0.3). However, because the lower mean percentage fertilized in the CS− cage (40%) was the result of a very low percentage in only one female, it appears that, as in the male experiment, most of the difference in outcome is the result of differences in fertilization likelihood.

4. DISCUSSION

Inseminations by male subjects were more likely to fertilize eggs when they took place in the male's CS+ cage, and females' eggs were more likely to be fertilized when the female was inseminated by a male in the female's CS+ cage. Thus, both sexes achieved greater reproductive success when mating in the context that in the recent past had predicted the appearance of an opposite-sex bird and a potential reproductive opportunity. The territorial, courtship and mating behaviours of both vertebrates and invertebrates are subject to Pavlovian conditioning, as are sexual-partner preference and many other aspects of social communication (Hollis 1997; Owren & Rendall 1997; Domjan *et al.* 2000; Pfaus *et al.* 2001; Reif *et al.* 2002). The present results show that processes in addition to the mating act itself that influence fertilization success can also be conditioned. Pavlovian conditioning, which allows animal systems to predict and prepare for biologically significant events, has long been assumed to be adaptive. Our results add experimental support to the functional hypothesis that conditioning increases fitness (Hollis *et al.* 1997; Domjan *et al.* 1998; Woodson 2002), and show for the first time that context conditioning increases reproductive success in females as well as males.

One possible reason for the greater fertilization probability of males in their CS+ cage is that they transferred a larger or more effective ejaculate when mating there. This is supported by the Domjan *et al.* (1998) experiment, in which males ejaculated more sperm onto a stuffed model in the CS+ cage. Sperm are thought to leak passively out of the sperm-storage tubules of the oviducts of female birds such that the number of eggs fertilized reflects the number of sperm initially stored (Birkhead & Møller 1992). Another likely possibility is that males transferred more foam along with their sperm, or transferred both more sperm and more foam. Males show an increase in movements of the foam-gland musculature as soon as they see a female (Seiwert & Adkins-Regan 1998), a response that becomes conditioned to a CS+ cage (E. Adkins-Regan, E. A. MacKillop and C. H. Leung, unpublished data). These movements increase the amount of

foam produced, and the amount of foam has been shown to affect a male's fertilization probability if mating occurs when the female has a hard-shelled egg in the uterus (Adkins-Regan 1999).

What response could have become conditioned in the females that resulted in a greater fertilization probability in their CS+ cage? One possibility is that their behaviour was different in some way and affected the number of sperm or the amount of foam that the male could transfer. For example, they could have positioned their cloacal opening more advantageously. Although squatting, a component of female receptivity, is conditionable (Gutiérrez & Domjan 1997) and females are harder for males to inseminate if they are unreceptive, receptivity is not likely to be the conditioned response accounting for our results. Receptivity does not predict the fertilization success of confirmed inseminations (Adkins-Regan 1995). Could conditioning have enabled the oviduct to prepare better for sperm arrival and storage? Female subjects were not allowed to mate during conditioning trials, but, if females perform movements of the cloacal or oviductal musculature at the sight of a male (similar to males' foam-gland muscle movements at the sight of a female), such responses could have been conditioned in the absence of mating.

Regardless of what the specific conditioned responses were, the results clearly show that something influencing fertilization success, and not simply mating success, is subject to the influence of external distal cues, indicating regulation by brain-level processes. Such an effect has long been known to occur in male farm ungulates during semen collection. Semen volume and number of sperm ejaculated improve as the male becomes used to his surroundings, including the collection team (social cues from humans; Hafez 1968; see Mathevon *et al.* 1998 for a more recent example). In these species, however, the effect occurs because ejaculation consists of a series of semen expulsions, the entire series takes time and external cues affect the duration of ejaculation (the number of expulsions). It is far from obvious that context cues would affect the number of sperm ejaculated in birds such as quail, where insemination lasts one second or less (Adkins 1974), or that context cues would influence fertilization success in females.

One candidate brain-level process that could account for the present results is conditioned sexual arousal in the CS+ cage during the 2 min waiting interval before the arrival of the test partner (Pfaus *et al.* 2001), an interval during which male quail are known to show anticipatory locomotor activity (Akins 1998). Anticipation of sexual activity was shown many years ago to produce elevations in circulating luteinizing hormone and testosterone in mice in a classical conditioning paradigm (Graham & Desjardins 1980). Some of the neurotransmitter and neuropeptide mechanisms of sexual arousal in male mammals, such as dopamine, serotonin or oxytocin (Hull *et al.* 2002; Popova & Amstislavskaya 2002), might be involved in the regulation of fertilization success in birds and other animals. Morphine has been found to inhibit contractility of the vasa deferentia in the non-monogamous deer mouse (*Peromyscus maniculatus*) but not in the monogamous California mouse (*P. californicus*), suggesting the hypothesis that endogenous opiates released centrally in response to social cues are a mechanism for regulating the number of sperm ejaculated in response to perceived sperm competition (Pound 1999).

The mating system of wild Japanese quail is poorly understood because the birds are cryptic and hard to observe. In favourable habitats densities are high. Observations of a feral population housed in a semi-natural environment (Nichols 1991), together with a report of a wild population of the sibling species (*Coturnix coturnix*; Rodríguez-Teijeiro *et al.* 2003), suggest socially monogamous pairing combined with occasional mate switching and extra-pair copulations. Laboratory experiments with domestic quail suggest sensitivity to the social context during mate choice (White & Galef 2000). Clutch sizes in the wild range from five to 13 (Madge & McGowan 2002). A single insemination by domestic males seldom fertilizes this many eggs, and the fertilizing success of inseminations by feral-population birds is even lower (E. Adkins-Regan, unpublished data), suggesting that even a mated pair would have to mate several times to fertilize the clutch. In paired animals, there is the potential for the partner to be a conditioned stimulus along with other contexts predicting mating such as location relative to the nest or even (in this and other social species) the presence of any conspecific. Regardless of exactly how conditioning might promote fertilization success in the mating system of wild quail, Pavlovian conditioning is a universal property of nervous systems and therefore likely to be relevant to the fertilization success of wild animals in natural mating system contexts and to improvements in success with increased experience (Domjan *et al.* 2000). Conditioning could mean that fertilization success is achieved with fewer sperm when the male and female are already familiar with each other (e.g. paired) or that extra-pair matings are more successful when they occur in a location where they have occurred in the past.

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REFERENCES

Adkins, E. K. 1974 Electrical recording of copulation in quail. *Physiol. Behav.* **13**, 475–477.

Adkins-Regan, E. 1995 Predictors of fertilization in the Japanese quail, *Coturnix japonica*. *Anim. Behav.* **50**, 1405–1415.

Adkins-Regan, E. 1999 Foam produced by male *Coturnix* quail: what is its function? *Auk* **116**, 184–193.

Akins, C. K. 1998 Conditioned excitation and modulation of sexual conditioning in male Japanese quail. *Anim. Learn. Behav.* **26**, 416–426.

Birkhead, T. R. & Møller, A. P. 1992 *Sperm competition in birds*. London: Academic.

Birkhead, T. R. & Pizzari, T. 2002 Post-copulatory sexual selection. *Nature Rev. Genet.* **3**, 262–273.

Domjan, M., Blesbois, E. & Williams, J. 1998 The adaptive significance of sexual conditioning: Pavlovian control of sperm release. *Psychol. Sci.* **9**, 411–415.

Domjan, M., Cusato, B. & Villarreal, R. 2000 Pavlovian feed-forward mechanisms in the control of social behavior. *Behav. Brain Sci.* **23**, 235–249.

Freedman, S. L., Akuffo, V. G. & Bakst, M. R. 2001 Evidence for the innervation of sperm storage tubules in the oviduct of the turkey (*Meleagrus gallopavo*). *Reproduction* **121**, 809–814.

Froman, D. P. 1995 Biology of semen production and ejaculation. In *Proc. First Int. Symp. Artificial Insemin. Poultry* (ed. M. R. Bakst & G. J. Wishart), pp. 21–34. Savoy, IL: The Poultry Science Association.

Graham, J. M. & Desjardins, C. 1980 Classical conditioning: induction of luteinizing hormone and testosterone secretion in anticipation of sexual activity. *Science* **210**, 1039–1041.

Gutiérrez, G. & Domjan, M. 1997 Differences in the sexual conditioned behavior of male and female Japanese quail (*Coturnix japonica*). *J. Comp. Psychol.* **111**, 135–142.

Hafez, E. S. E. 1968 Sexual behavior. In *Reproduction in farm animals*, 2nd edn (ed. E. S. E. Hafez), pp. 155–172. Philadelphia, PA: Lea & Fibiger.

Hollis, K. L. 1997 Contemporary research on Pavlovian conditioning: a 'new' functional analysis. *Am. Psychol.* **52**, 956–965.

Hollis, K. L., Pharr, V. L., Dumas, M. J., Britton, G. B. & Field, J. 1997 Classical conditioning provides paternity advantage for territorial male blue gouramis (*Trichogaster trichopterus*). *J. Comp. Psychol.* **111**, 219–225.

Hull, E. M., Meisel, R. L. & Sachs, B. D. 2002 Male sexual behavior. In *Hormones, brain and behavior*, vol. 1 (ed. D. W. Pfaff, A. P. Arnold, A. M. Etgen, S. E. Fahrbach & R. T. Rubin), pp. 1–137. San Diego, CA: Academic.

McKenna, K. E. 1999 Ejaculation. In *Encyclopedia of reproduction*, vol. 1 (ed. E. Knobil & J. D. Neill), pp. 1002–1008. San Diego, CA: Academic.

Madge, S. & McGowan, P. 2002 *Pheasants, partridges, and grouse: a guide to the pheasants, partridges, quails, grouse, guineafowl, buttonquails, and sandgrouse of the World*. Princeton University Press.

Mathevon, M., Buhr, M. M. & Dekkers, J. C. M. 1998 Environmental, management, and genetic factors affecting semen production in Holstein bulls. *J. Dairy Sci.* **81**, 3321–3330.

Nichols, C. R. 1991 A comparison of the reproductive and behavioural differences in feral and domestic Japanese quail. Master's thesis, University of British Columbia.

Owren, M. J. & Rendall, D. 1997 An affect-conditioning model of nonhuman primate vocal signaling. In *Perspectives in ethology*, vol. 12 (ed. D. H. Owings, M. D. Beecher & N. S. Thompson), pp. 299–346. New York: Plenum Press.

Parker, G. A. 1970 Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**, 525–567.

Parker, G. A. 1984 Sperm competition and the evolution of animal mating strategies. In *Sperm competition and the evolution of animal mating systems* (ed. R. L. Smith), pp. 1–60. London: Academic.

Pfaus, J. G., Hippin, T. E. & Centeno, S. 2001 Conditioning and sexual behavior: a review. *Horm. Behav.* **40**, 291–321.

Pizzari, T. & Birkhead, T. R. 2000 Female feral fowl eject sperm of subdominant males. *Nature* **405**, 787–789.

Popova, N. K. & Amstislavskaya, T. G. 2002 Involvement of the 5-HT_{1A} and 5-HT_{1B} serotonergic receptor subtypes in sexual arousal in male mice. *Psychoneuroendocrinology* **27**, 609–618.

Pound, N. 1999 Effects of morphine on electrically evoked contractions of the vas deferens in two congeneric rodent species differing in sperm competition intensity. *Proc. R. Soc. Lond.* B **266**, 1755–1758. (DOI 10.1098/rspb.1999.0842.)

Reif, M., Linsenmair, K. E. & Heisenberg, M. 2002 Evolutionary significance of courtship conditioning in *Drosophila melanogaster*. *Anim. Behav.* **63**, 143–155.

Rodríguez-Teijeiro, J. D., Puigcerver, M., Gallego, S., Cordevo, P. J. & Parkins, D. T. 2003 Pair bonding and multiple paternity in the polygamous common quail *Coturnix coturnix*. *Ethology* **109**, 291–302.

Seiwert, C. M. & Adkins-Regan, E. 1998 The foam production system of the male Japanese quail: characterization of structure and function. *Brain Behav. Evol.* **52**, 61–80.

Simmons, L. W. 2001 *Sperm competition and its evolutionary consequences in the insects*. Princeton University Press.

Sokal, R. R. & Rohlf, F. J. 1995 *Biometry: the principles and practice of statistics in biological research*, 3rd edn. New York: Freeman.

Truitt, W. A. & Coolen, L. M. 2002 Identification of a potential ejaculation generator in the spinal cord. *Science* **297**, 1566–1569.

Wedell, N., Gage, M. J. G. & Parker, G. A. 2002 Sperm competition, male prudence and sperm-limited females. *Trends Ecol. Evol.* **17**, 313–320.

White, D. J. & Galef, B. G. 2000 Differences between the sexes in direction and duration of response to seeing a potential sex partner mate with another. *Anim. Behav.* **59**, 1235–1240.

Woodson, J. C. 2002 Including 'learned sexuality' in the organization of sexual behavior. *Neurosci. Biobehav. Rev.* **26**, 69–80.