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Metabolic costs of sexual advertisement in the bank vole (*Clethrionomys glareolus*)

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ABSTRACT

Hypothesis: Sexual traits serve as honest signals of male quality because they are costly.

Question: Is olfactory signalling costly?

Organism: Bank voles (*Clethrionomys glareolus*) from generations 3–5 of a large laboratory colony reared from individuals trapped in the field.

Methods: We investigated the energetic and immune costs of male investment in olfactory signalling in the bank vole. The mass of the preputial gland, the main source of male sexual attractants, was a measure of investment in sexual signalling. We measured male basal metabolic rate both before and after pairing with females and exposure to conspecific males. After pairing, we also challenged males with a novel antigen (sheep red blood cells) and measured their antibody production.

Results: Preputial gland mass did not correlate with basal metabolic rate before pairing (both traits corrected for body mass). After pairing, basal metabolic rate increased significantly and the increase was significantly correlated with preputial gland size. Gland size was not significantly related to a humoral immune response following a challenge with sheep red blood cells.

Conclusion: Olfactory signalling in the bank vole is associated with energetic, but not immune, costs.

Keywords: chemical communication, mate choice, scent marking, sexual selection, social dominance.

INTRODUCTION

Honest signalling theory predicts that sexually selected traits may advertise the quality of their bearers provided that they are costly to produce and/or maintain (Zahavi, 1975; Grafen, 1990). The cost ensures that low-quality individuals cannot afford to produce elaborate traits and

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that the trait's elaboration is proportional to an individual's quality. Life-history costs of sexual advertisement are likely to be mediated through the energetic expenditure on the production or maintenance of sexually selected traits. The energetic costs of sexual advertisement have been studied in several major taxa (reviewed in Kotiaho, 2001; Ward *et al.*, 2003; Basolo and Alcaraz, 2003). Among mammals, mate choice is commonly based on olfactory signals (reviewed in Penn and Potts, 1998; Marchlewska-Koj *et al.*, 2001; Gosling and Roberts, 2001; Brennan and Keverne, 2004). However, we are aware of only a single study that has investigated the costs of olfactory signalling in mammals: Gosling *et al.* (2000) showed that male mice investing more in urine marking, a behaviour positively correlated with male sexual attractiveness (Rich and Hurst, 1998; Roberts and Gosling, 2003), paid a cost of reduced growth rate. Here, we investigate whether a male's investment in sexual signalling is associated with energetic and immune costs in the bank vole, *Clethrionomys glareolus*.

The preputial gland is located near the base of the penis and its products are released with urine. It plays an important role in rodent chemical communication (Brown and Williams, 1972). In bank voles, the preputial gland increases in size during the breeding season and then decreases afterwards (Christiansen, 1978). Preputial gland products, mixed with urine during scent-marking (Brown and Williams, 1972), were shown to be the source of sex-attractants (Kruczek, 1994). Males compete with each other for access to females and use urine marking to signal their dominance (Brinck and Hoffmeyer, 1984). Large preputial gland mass is associated with male social status, and females show preferences for dominant males (Horne and Ylönen, 1996; Kruczek, 1997). Thus, preputial gland size in bank voles is a good measure of male investment in sexual advertisement.

In this paper, we assess whether preputial gland size is correlated with metabolic costs in terms of an increased basal metabolic rate and the ability to mount an immune response to a novel antigen. Basal metabolic rate is a measure of the costs of somatic maintenance (McNab, 2002). If the maintenance of large preputial glands and the production of pheromones are energetically costly, gland size should be correlated with basal metabolic rate.

Preputial gland mass in mice increases in the presence of females (Koyama and Kamimura, 2001). As scent-marking functions both in mate choice and in intra-sexual competition (reviewed in Gosling and Roberts, 2001), we paired males with sexually active females and exposed focal males to conspecific males to stimulate male investment in signalling. We predicted that gland mass will be correlated with the basal metabolic rate of males after pairing with females and after exposure to conspecific males, but not necessarily before pairing. Basal metabolic rate, although measured in immobile animals, is correlated with field metabolic rates (e.g. Koteja, 1991; McNab, 2002; but see Speakman *et al.*, 2003), so basal metabolic rate may also reflect an animal's readiness to expend energy on activities other than somatic maintenance. Thus, a higher basal metabolic rate could indicate that a male is physiologically prepared for increased activity associated with reproduction.

Sexual advertisement has also been hypothesized to involve costs in terms of suppressed immunity (reviewed in Sheldon and Verhulst, 1996). Such costs may arise due to the immunosuppressive action of testosterone, an androgen necessary for the development of many sexual traits (Folstad and Karter, 1992), or because the production of sexual signals drains limited resources that could otherwise be invested in the immune system (Wedekind and Folstad, 1994; Cichon *et al.*, 2003). In the bank vole, a male's ability to respond to an immune challenge decreases during the breeding season (Saino *et al.*, 2000), while the preputial gland increases in size (Christiansen, 1978). Thus, we expected that preputial gland size should correlate negatively with the ability to mount an immune response.

MATERIALS AND METHODS

Animals

We used bank voles (*Clethrionomys glareolus*) from generations 3–5 of a large laboratory colony reared from individuals trapped in the forest area of Puszcza Niepołomnicka (Southern Poland) in autumn 2000 and 2001. The animals were maintained in standard mouse cages ($22 \times 16 \times 14$ cm) at 20 ± 1°C under a long photoperiod (16 h light/8 h dark), and fed Labofed H food pellets (Kcynia, Poland: 22% protein, 4% fat, 5% fibre). The young were weaned at 21 days. Before the experiments, males were kept individually and females were kept in groups of three to four. The experiments described here were performed in two blocks, in September and October 2003. The experimental protocols described below were approved by the State Ethical Committee for Experiments on Animals, Poland (DB/KKE/PL-111/2001), and the Local Ethical Committee in Kraków (62/OP/2003).

Schedule of measurements

For logistical reasons, we minimized the number of experimental animals and did not maintain unexposed control males in the laboratory. Instead, we measured the basal metabolic rate of each male both before and after pairing with females and exposure to conspecific males. In this way, we were able to examine the association between the magnitude of change in basal metabolic rate that was due to sexual stimulation, and male investment in signalling as measured by the size of the preputial gland post-stimulation (see below for statistical details). After measurement of basal metabolic rate, each male was challenged with a novel antigen and its humoral immune response and testosterone concentration were recorded.

A pre-pairing basal metabolic rate (hereafter referred to as BMR1) was measured in adult males (115–157 days old) after they had been weighed (± 0.1 g). Males were paired with females on the same day, 2–16 days after basal metabolic rate was measured. A week after pairing, males were also exposed to a 6-min contact with conspecific males in an open-field situation [$37 \times 21\frac{1}{2} \times 40$ cm plastic container (see Radwan *et al.*, 2004 for details)]. The male exposures were repeated five times, on 5 consecutive days, each time with a different conspecific male. After each exposure, the focal male was returned to his original cage where he remained paired with the female. The basal metabolic rate of paired males (BMR2) was measured 18–26 days after pairing with females and a second body mass measurement was taken.

The time between pairing, immunization, and preputial gland measurement was kept constant within each block. However, for logistical reasons, not all metabolic measurements could be carried out on a single day. Therefore, the time between metabolic measurements and other variables was controlled for statistically. Both BMR1 and BMR2 measurements were part of a 3-day series of multiple metabolic measurements, where aerobic capacity during exercise and thermoregulation were measured on the two remaining days as part of a separate project (not reported here; for details, see Sadowska *et al.*, 2005).

The males were separated from the females and placed in individual cages 2–11 days after the second metabolic measurements – that is, 26 (first block) or 29 (second block) days after the initial pairing – and 7 days later they were injected intraperitoneally with 20 μl $\cdot$ g body mass^{-1} of standard non-pathogenic T-cell-dependent antigen (sterile sheep red blood cells,

SRBC, Polish-American Institute of Pediatrics, Kraków, Poland) suspended in phosphate-buffered saline and adjusted to 6×10^6 cells $\cdot$ mm^{-3}. After 10 days, the voles were weighed and killed by decapitation, bled, and the preputial gland was dissected and weighed within an hour. Blood samples were centrifuged at 2500 rev $\cdot$ min^{-1} for 15 min, after which the plasma was extracted and heat-inactivated (56°C) for 30 min. Antibody production was assessed by a microhaemagglutination procedure, in which the number of titres showing positive haemagglutination represented antibody production (Hudson and Hay, 1989).

Measurement of basal metabolic rate

Basal metabolic rate was measured as the rate of oxygen consumption in an open-flow, positive pressure respirometric system (design 1b in Koteja, 1996) with an Applied Electrochemistry S-3A/II oxygen analyser (Ametek, Pittsburgh, PA). Basal metabolic rate was measured at +30°C [i.e. within the thermoneutral zone of voles (Petrusewicz, 1983)] after 5.5 h of food deprivation. The voles were weighed and placed in plastic chambers (800 ml) at 08.30 h. Dried air was passed through the chambers at approximately 22 litres $\cdot$ h^{-1} (measured with LO 63/33 rotameters; Rota, Germany). Samples of air were taken every 10 min sequentially from seven chambers (six containing animals and one empty reference chamber). Measurements were performed for 4 h (from 14.00 h to 18.00 h), so that 24 readings were obtained for each animal and the reference cell. Basal metabolic rate was defined as the average oxygen consumption calculated from the third, fourth, and fifth lowest readings (i.e. the two lowest readings were always rejected). Details of the protocol and rationale for this method of calculation are given in Labocha *et al.* (2004). Such a measure of basal metabolic rate is highly repeatable across a 2- to 4-week interval [$r = 0.69$ (Labocha *et al.*, 2004)] and heritable [narrow-sense heritability for mass-independent values = 0.4 (Sadowska *et al.*, 2005); all measurements done for unpaired males].

Testosterone assay

Serum testosterone was measured using a radioimmunoassay kit (TESTO-CTK, DiaSorin, Byk-Sangtec Diagonstica GmbH & Co., Germany) that had previously been validated for use in bank voles [parallelism demonstrated between standards and bank vole plasma concentrations (S.C. Mills, A. Grapputo, E. Koskela and T. Mappes, in preparation)]. In total, 50 μl of the seven testosterone standard concentrations or 20 μl of the blood serum samples were added with 500 μl of ^{125}I-labelled testosterone to tubes coated in a testosterone antiserum raised in rabbits. The assay was performed in duplicate for each sample and standard. The mixtures were equilibrated at 37°C for 3 h, during which time labelled testosterone and the testosterone contained in the samples or standards compete for a fixed and limited number of antibody binding sites. After 3 h, the incubation mixture was carefully aspirated so that no trace remained, and the radioactivity of the tubes was measured in a gamma counter. The amount of radioactivity measured is inversely related to the amount of unlabelled testosterone in the samples and standards. The amount of testosterone in the samples was therefore determined by interpolation from a calibration curve calculated from the standards.

Unfortunately, the plasma samples prepared for testosterone assays from the second block were lost due to a technical problem, so we could only analyse plasma testosterone concentrations for the first block.

Statistical analyses

To test for a mass-independent association between BMR1 and preputial gland mass we calculated coefficients of bipartial correlation, where BMR1 was adjusted for male body mass at the time of BMR1 measurement, and preputial gland mass was corrected for both male body mass at the end of the experiment (i.e. on the same day when the preputial gland was weighed) and the time between BMR2 measurement and preputial gland dissection. Both BMR1 and preputial gland mass were also adjusted for block. The mass-independent association between the preputial gland and BMR2 was tested with an equivalent model, but BMR2 was additionally adjusted for BMR1 values and for the time between pairing and BMR2 measurement. Statistically controlling for BMR1 allowed us to determine whether investment in the preputial gland after pairing was associated with an increase in basal metabolic rate above that of the pre-pairing level. Whether the increase in basal metabolic rate was mediated by an increased testosterone concentration was tested in a correlation analysis, where BMR2 was adjusted for male body mass and BMR1, and testosterone concentration was adjusted for preputial gland mass. The bipartial correlation analyses were performed with the SETCOR module of SYSTAT 10 for Windows (SPSS Inc.). Other analyses were performed with Statistica 6.1.

RESULTS

There was no significant change in male body mass between the first and the second basal metabolic rate measurement (repeated measures ANOVA, $F_{1,94} = 3.25$, $P = 0.074$; block effect, not significant) (Table 1). However, basal metabolic rate increased after pairing (repeated measures ANOVA, $F_{1,94} = 48.35$, $P < 0.001$; block effect, not significant) (Table 1).

The association between mass-independent BMR1 and gland mass (Fig. 1a) was not significant (coefficient of bipartial correlation, $r = 0.043$, $F_{1,91} = 0.168$, $P = 0.668$). BMR1 and BMR2 were highly correlated ($r = 0.499$, $F_{1,92} = 31.19$, $P < 0.001$). Nevertheless, the increase in basal metabolic rate after pairing with a female and exposure to conspecific males was positively associated with preputial gland mass ($r = 0.240$, $F_{1,90} = 5.525$, $P = 0.021$) (Fig. 1b).

Testosterone concentration was positively associated with preputial gland mass ($r = 0.31$, $n = 42$, $P = 0.049$), but did not correlate with the increase in basal metabolic rate after pairing ($r = 0.132$, $F_{1,38} = 0.740$, $P = 0.395$).

Preputial gland mass was not related to the amount of antibodies produced in response to sheep red blood cells (Table 2). In this analysis, the effect of the time interval between the second basal metabolic rate measurement and immunization was marginally non-significant

Table 1. Summary statistics of the variables measured (mean ± standard deviation)

Variable	*n*	Before pairing	After pairing	Final measure
Body mass (g)	96	22.2 ± 3.1	21.9 ± 2.1	24.9 ± 2.9
Basal metabolic rate (ml $O_2 \cdot h^{-1}$)	96	54.9 ± 6.8	59.5 ± 6.6	
Preputial gland mass (mg)	96			26.9 ± 12.0
Immune response	82			5.54 ± 2.47
Testosterone ($ng \cdot ml^{-1}$)	42			3.32 ± 2.74

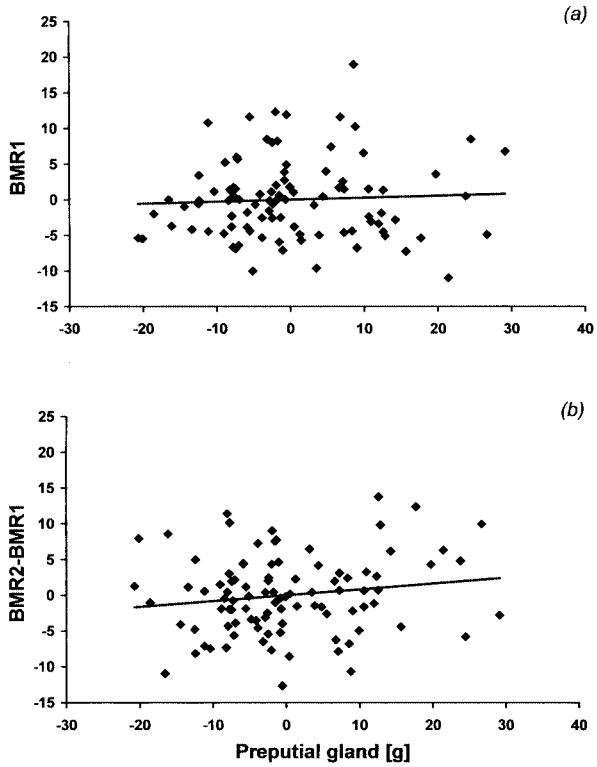

Fig. 1. The association between preputial gland mass and (a) BMR1 and (b) BMR2 corrected for BMR1 (after pairing with a female and exposure to conspecific males). Both basal metabolic rate and gland mass are expressed as residuals with respect to body mass (see statistical analyses in the Methods section for details).

Table 2. Partial correlation coefficients between the immune response following a challenge with a novel antigen and preputial gland mass, body mass, and the time between metabolic measurements and the injection of sheep red blood cells, adjusted for block

Source	*r*	d.f.	*F*	*P*
Preputial gland mass	0.11	1, 77	1.03	0.313
Body mass	– 0.07	1, 77	0.42	0.517
Time	0.21	1, 77	3.67	0.059

(Table 2). In a similar analysis, but only for the first block, we additionally tested the confounding effect of testosterone concentration, but it was not significant ($r = -0.12$, $F_{1,39} = 0.538$, $P = 0.46$).

DISCUSSION

The quality of olfactory signalling appears to be a major determinant of mammalian male sexual attractiveness (Penn and Potts, 1998; Gosling *et al.*, 2000). In mice, urinary odour may carry information about similar or dissimilar major histocompatability complex (MHC) genotypes (Penn and Potts, 1999), diet composition (Ferkin *et al.*, 1997), and parasite load (Kavaliers and Colwell, 1991; Penn and Potts, 1998), but it also appears to signal male quality (Gosling and Roberts, 2001; Fischer *et al.*, 2003). A recent analysis of the relative importance of MHC and male quality in mate choice of mice revealed that urine marking rate, thought to signal male quality, is a better predictor of female sexual preference than MHC type (Roberts and Gosling, 2003). To reliably reveal quality, the signal needs to be costly (Grafen, 1990). This study investigated the energetic and immune costs of investment in an important sexual signal of male bank voles, olfactory signalling, in which the preputial gland plays a significant role. Despite a positive correlation between preputial gland size and testosterone, we did not find significant costs in terms of suppressed immune response. However, we did detect significant metabolic costs. The costs were manifest by a significant correlation between preputial gland size and the increase in basal metabolic rate measured after pairing with females and exposure to conspecific males. This result implies that the pre-condition of honest signalling of male quality is fulfilled in the case of olfactory signalling in the bank vole. Costly signals are likely to be condition-dependent, and genetic variance in a multitude of traits affecting phenotypic condition is suggested to provide the heritable variance in fitness necessary for the 'good genes' mechanism of sexual selection to work (Andersson, 1986; Rowe and Houle, 1996). However, very little is known about quantitative genetics of condition in general (reviewed in Tomkins *et al.*, 2004), and without demonstrating the link between signal elaboration and genetic variation, it would be premature to conclude that mate choice based on olfactory cues brings female bank voles genetic benefits.

Although the observed correlation between basal metabolic rate and preputial gland size suggests that sexual advertisement is costly, it does not determine causation. We cannot separate whether the investment in signalling resulted in an increased basal metabolic rate, or whether a high metabolic rate enabled males to produce a larger gland [although in terms of costly advertisement, the latter still implies that to increase the investment in sexual signalling it is necessary to maintain (and pay the cost of) an increased metabolic rate]. However, the lack of correlation between gland size and pre-pairing basal metabolic rate suggests that high metabolic rates are unlikely to predispose individuals towards the development of large glands. Similarly, a previous study in the bank vole found no significant correlation between male dominance, a behavioural trait correlated with preputial gland size (Horne and Ylönen, 1998), and basal metabolic rate measured before males were paired with females or confronted with other males. Thus, it is more likely that the correlation between gland size and post-pairing basal metabolic rate (corrected for pre-pairing basal metabolic rate) reflects the costly investment in olfactory signalling. Such energetic costs may underlie the life-history cost reported by Gosling *et al.* (2000).

In house sparrows, the increase in basal metabolic rate of dominant males is mediated by changes in testosterone (Buchanan *et al.*, 2001). However, although preputial gland mass and testosterone were correlated in the present study, testosterone concentration did not predict the increase in basal metabolic rate after pairing. The association between testosterone and basal metabolic rate does not seem universal, even in birds: negative results have been obtained for jungle fowl (Chappell *et al.*, 1997) and the white-crowned sparrow (Wikelski *et al.*, 1999).

Buchanan *et al.* (2001) suggested that because the badge (chest bib), indicating social status in the house sparrow, is testosterone-dependent, the testosterone-mediated increase in basal metabolic rate represents a cost of status signalling. In contrast, our results indicate that olfactory signalling in the bank vole is costly irrespective of the action of testosterone. This may in fact render olfactory signalling a more reliable signal of male quality, as the mechanistic link between testosterone and metabolic rate may be uncoupled under selection. Our results indicate that the amplification of a sexual trait causes a concomitant increase in metabolic costs that is not mediated by testosterone.

The correlation between gland size and an increase in basal metabolic rate may reflect the cost of production and maintenance of the large gland, but it is more likely that investment in the preputial gland is just an indicator of the overall investment in olfactory signalling. Efficient signalling may require not only a large preputial gland, but also a simultaneous increase in the size of other organs that enable a higher turnover of proteins and other substances used for signalling, such as the major urinary proteins manufactured in the liver and filtered by the kidney. The major urinary proteins, involved in the controlled release of pheromones contained in scent-marks, are present in urine at very high concentrations (Beynon *et al.*, 2001). The rate of production of these proteins is likely to be correlated with the rate of production of the pheromones they bind, for example the sesquiterpenes released by the preputial gland (Novotny *et al.*, 1990). Additionally, an increase in activity associated with sexual signalling and/or intrasexual competition may also contribute to the metabolic costs we detected. In the field, such costs may thus be even higher, as signalling will also involve increased locomotion required to maintain adequate strength of signal across territories (reviewed in Gosling and Roberts, 2001). This may pose even more energetic demands than the 10% we detected in the laboratory, in turn invoking costs associated with the need for increased foraging. Indeed scent marking is reduced in the presence of predators (Roberts *et al.*, 2001).

We found that testosterone concentration was positively correlated with preputial gland size. Folstad and Karter (1992) predicted a dual effect of testosterone through the immunocompetence handicap hypothesis. It proposes that testosterone-dependent sexually selected traits advertise a male's quality/viability and serve as honest indicators of a male's ability to resist infections in spite of the immunosuppressive effect of testosterone. The immunosuppressive effect of testosterone, however, does not appear universal (reviewed by Roberts *et al.*, 2004). Here, we found no evidence for the negative association between testosterone concentration and the immune response to sheep red blood cells in the bank vole. Instead, we detected a marginally non-significant positive association between the time from metabolic measurements to immunization and the strength of the T-cell-dependent humoral response. This trend may be indicative of stress experienced by voles during metabolic measurements. Indeed, the suppression of immune function is often attributed to the stress hormones, corticosteroids (Kizaki *et al.*, 1997). Many studies have indicated that an elevated level of corticosterone induces immunosuppression due to the decreased proliferation and increased apoptosis of T-lymphocytes (Wyllie, 1980; Cohen and Duke, 1984).

In conclusion, our results suggest the presence of energetic costs associated with investment in olfactory signalling in male bank voles. This satisfies a necessary condition for olfactory signalling to be a reliable indicator of male quality (Grafen, 1990). However, the extent to which this allows females to accrue genetic benefits to their offspring remains to be determined.

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