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Preference of female rats for the odours of non-parasitised males: the smell of good genes?

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Abstract. Many animals obtain reliable information about potential mates, including whether they are parasitised or not, mostly from olfactory cues in urine. Previous experiments with rodents have shown that females can detect parasites in males that are potentially transmissible during copulation, so that females can directly avoid infection by discriminating against parasitised males. Here, using choice tests, we examine whether female rats can distinguish males infected with the tapeworm *Hymenolepis diminuta* Rudolphi, 1819, a parasite with a complex life cycle and thus not directly transmissible among rats. Female rats tended to spend more time investigating the urine of non-parasitised males than that of parasitised males. The magnitude of the parasite burden in the infected males had no effect on the females' preference for the non-parasitised males. We also found that parasitised males had lower testosterone levels in their blood than non-parasitised males. These results suggest that females use cues in male urine reflecting either the presence of the parasite and/or lower testosterone levels to avoid parasitised males and possibly secure resistance genes for their offspring.

Parasite-mediated sexual selection usually involves either direct benefits for choosy females in the form of extra resources (Howard and Minchella 1990) or avoidance of infection (Edwards and Barnard 1987, Kavaliers and Colwell 1995a,b), or indirect benefits obtained by securing genes for parasite resistance in offspring. Much work has focused on mate choice based on indicators of parasite resistance (Hamilton and Zuk 1982), usually visual cues such as bright body colouration. Hamilton and Zuk (1982) allude to the possibility that urine and faeces could contain information regarding a partner's health. Despite the fact that rodents use urine as a source of information (Drickamer 1992, Gheusi et al. 1997), studies focusing on urine as an indicator of male health and/or parasite resistance are relatively few (see Penn and Potts 1998, Klein et al. 1999).

Based on odours in urine, female mice can discriminate between males parasitised with the protozoan *Eimeria vermiformis* and non-parasitised males, and have a preference for non-parasitised males (Kavaliers and Colwell 1995a). *E. vermiformis* has a direct life cycle, therefore females directly avoid infection by not mating with parasitised males. No experiment has been conducted with mammals in which females can only obtain good genes for their offspring by choosing parasite-free males. The chemical detected by females in male urine could be some by-product of the parasite or of the male's immune reaction to infection. Females could also use testosterone levels in urine to detect the presence of parasites in potential partners. Testosterone mediates sexual characteristics and behaviours (Zuk 1996) and is required for regulation of spermatogenesis (Hillgarth et al. 1997), but it is also known to dampen the immune response of some animals (Zuk 1996, Hillgarth and Wingfield 1998). Testosterone might thus be a reliable indicator of male health.

Here we examine whether female rats discriminate between the urine of males parasitised with the cestode *Hymenolepis diminuta* Rudolphi, 1819 and the urine of non-parasitised males. Our experiments are social choice tests because we could not determine whether females were in oestrus and actual mating did not occur; however social choice tests correlate with mate choice tests, at least in mice (Egid and Brown 1989). Adult *H. diminuta* live in the small intestine of rats. Rats acquire the tapeworm when they prey on infected beetles, the parasite's intermediate host. Mettrick (1973) found a 20% decrease in growth of parasitised rats on an *ad libitum* feeding regime; however, the effect may be less drastic (Insler and Roberts 1976). Andreassen and Hopkins (1980) showed that immunity decreases with time following infection and concluded that the immune system probably prevents large accumulations of worms rather than conferring complete resistance. Therefore infection by *H. diminuta* bears a cost to the rat through activation of the immune system (Hindsbo et al. 1982) and by altering the intestinal environment and reducing digestive efficiency (Munger and Karasov 1989). Devoting resources to immunity also leaves less resources for other functions. *H. diminuta*'s complex life cycle ensures that discriminating females only receive indirect benefits (i.e. good genes) by not mating with infected males. We tested the hypotheses that (1) females prefer to spend time near the odour of non-parasitised males, and (2) parasitised males have less testosterone than non-parasitised males.

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MATERIALS AND METHODS

Animal care, infection procedure and worm recovery. All experimental procedures have been approved by the University of Otago Committee on Ethics in the Use of Laboratory Animals (permit no. 60-95). Male and female Sprague-Dawley rats aged between four to five weeks were obtained from the Department of Laboratory Animal Sciences, University of Otago. Rats were housed individually in rat breeding cages (RB3 rat cages, North Kent Plastic Cages Ltd, Kent, England; overall size 45 × 28 × 20 cm); all cages were kept in the same, well-ventilated room, with their position assigned at random within the room. Sawdust or wood shavings were used for bedding. Cages were cleaned twice a week. The rats were on a 10h light:14h dark photoperiod; the average room temperature was 23 ± 3°C. Water and food pellets were provided *ad libitum*.

A population of *H. diminuta* is maintained at the University of Otago by cyclical passage through Sprague-Dawley rats and the flour beetle *Tribolium confusum* (Jacqelin du Val). Between the age of five and six weeks, we infected 39 male rats with eight cysticercoids (the larval stage of *H. diminuta*). Rats were anaesthetised with ether and cysticercoids (contained in Hank's balanced salt solution) were introduced by stomach intubation. As a control, 30 male rats were 'sham' infected, i.e. they experienced the same infection procedure but received no cysts. All animals used in the experiments were 11-12 weeks old. At the end of the experiments animals were killed by CO_2 inhalation followed by cervical dislocation. Blood was then obtained from a subset of males by cardiac puncture. We dissected the infected animals, removed the small intestine and opened it in Hank's balanced salt solution. All tapeworms were carefully extracted with forceps. Scoleces were counted and the entire worm burden was placed in an oven at 65°C for at least 48 h before we obtained its dry weight. Worm biomass was used as a measure of parasite intensity.

Odour preference apparatus. We used a grey PVC Y-maze for all choice tests. The basal arm and both choice arms were 50 cm long. All arms were 10 cm wide; the walls of the maze were 10 cm high. The entire maze was fitted with a clear acrylic lid, and its floor was covered with clean sawdust. At the entrance to the two choice arms, removable barriers prevented the female rat from entering the arms during the 15 min acclimatisation period. These two barriers and the barrier at the base of the basal arm consisted of wire mesh set in plexiglass to allow air to move through the maze. Odour tanks, consisting of PVC inspection caps with screw-on lids, were connected by plastic tubing to the end of each choice arm. Plastic tubing also connected the odour tanks to a fan which provided a constant influx of air.

Urine collected from males was placed, in a watch glass, into the sealed odour tanks prior to each experiment. After each experiment we ventilated and cleaned all parts of the maze. An extractor fan was operating at all times to remove odours from the windowless experimental room. All experiments were conducted during the dark phase of the photoperiod. A red 25W bulb provided standardised low intensity lighting; red light is commonly used in experiments on rat behaviour (Blanchard et al. 1993). We video-taped each trial using a black and white, low-light intensity camera and a VHS recorder.

Standard procedures. The following protocol was standard in all experiments. Urine from males was collected on the day of a trial by placing a male into a 4-litre plastic container for 1-2 hours. This container had a gauze layer 1 cm off the bottom to limit contamination of urine by faeces. We then transferred the fresh urine to a watch glass. Each male, whether parasitised or not, provided a urine sample for a single test only.

A test female was placed into the basal arm and allowed to acclimatise for 15 min before the fan was turned on. This allowed the odour from the urine to permeate through the maze. After another 5 min we turned the video-recorder on and removed the barriers. The female's behaviour was recorded over the 10 min period after it first investigated male odours; this is similar to the 15min period used by Kavaliers and Colwell (1995b). Variables recorded were: (1) first choice arm entered; (2) time spent in each choice arm; (3) time spent investigating the odour (i.e., the time spent by the female with her nose in the last 2 cm of each choice arm, close to the air entry holes); and (4) time spent grooming in each arm. This last behaviour was only recorded to correct the other two recorded times. Grooming is very stylised and is something rats do for over a third of their waking hours (Bolles 1960). If the female was grooming while her nose was in the last 2 cm of the maze, we assumed that she was not investigating the odour and timing was stopped until grooming finished. In all experiments, preference for one arm over the other was assessed as the difference in relative time spent in the last 2 cm of both choice arms in the 10min observation period.

Experimental control. This experiment tested for any bias generated by the infection procedure. In these trials (N = 30) a female was presented with odours from two males, one sham-parasitised and one that had never undergone the infection procedure. The urine from the sham-parasitised male was randomly assigned to an odour box; urine from the other male went into the second box.

Odour preference experiment. This experiment (N = 39 trials) was designed to investigate whether females could discriminate between males that were parasitised with *H. diminuta* and non-parasitised males. The experimental procedure was the same as that for the experimental control except that instead of urine from a sham parasitised male, we used urine from a parasitised male.

Radioimmunoassay. Whole blood was used for RIA tests. A preliminary test showed that using whole blood gave results comparable to those obtained using plasma only. Blood was thawed and spun down for 8 min at 12000 g. The samples consisted of 1 ml of blood in an eppendorf tube. The top 50 µl of the sample was removed (in duplicate) and extracted with three washes of 1 ml of ether. Labelled testosterone (Amersham International PIC, Amersham, UK, TRK.402, Batch 82) (in PBS-BSA buffer) was added to the samples (about 3200 counts). Antibody was added (Endocrine Sciences RIA Reagents, California, USA; Lot 338A, Batch 1183) at a 1:100 dilution of NRS-EDTA-PBS. Samples were left between 16-20 h for incubation then dextran charcoal (5%) was added. Testosterone levels were counted in a liquid

scintillation analyser (Packard Instrument Company). The standard curve was spline. Standards used ranged from 3.75 pg/ml to 7.68 ng/ml. The correlation coefficient between standards and template curve was 0.9993. Non specific binding was around 6.3%. The minimum detectable concentration of testosterone was 3.75 pg/ml. When testing for testosterone recovery after extraction, the colour of the blood quenched the amount of light detected by the analyser. Therefore we spiked the buffer with the same concentration of testosterone as the extracted samples and compared the buffer sample to the spiked extracted sample for the percentage extraction recovery. Recovery of testosterone after extraction was 70%.

Statistical analysis. We used relative times (i.e. time spent investigating the odour in one arm divided by total time spent investigating the odours in both arms) for all analyses. Due to distributions that were not normal for data on relative time spent investigating odours in either choice arms, we used randomisation tests for this variable, computed with the RT 2.0 statistical package (Bryan Manly, Centre for the Application of Statistics and Mathematics, University of Otago). Randomisation tests and *t*-tests give similar *p*-values when data are normally distributed, but randomisation tests are robust to deviations from the assumptions of parametric tests (Manly 1991). In randomisation tests, the probability that an observed result is significant is computed as the probability of obtaining a more extreme result by rearranging the data at random between treatments; we performed 10,000 randomisations in all our tests. Other tests (two-tailed *t*-tests, Chi-square tests, regressions) were performed with the statistical package STATVIEW (Abacus Concepts, California).

RESULTS

Experimental control

None of the variables were significantly different between arms when the female chose between the urine from sham parasitised males and urine from non-parasitised males ($p > 0.60$ for all variables). We did not control for the weights of the two classes of males; however they were not significantly different (paired *t*-test: $t_{29} = 0.576$, $p = 0.569$).

Odour preference experiment

We did not control for the weights of males during this experiment. Pooled data suggest that parasitised males ($\bar{x}$ ± SD = 441 ± 28g) weighed slightly less than non-parasitised males (454 ± 33g) although the difference was not quite statistically significant (paired *t*-test: $t_{38} = 1.799$, $p = 0.08$). However, a regression showed that the relative preference of females for non-parasitised males, i.e. the difference between the times spent in the two choice arms, did not relate with the difference in male weight ($N = 39$, $r = 0.273$, $p = 0.389$).

The number of scoleces recovered ($\bar{x}$ ± SD = 5.5 ± 1.2 per male, $N = 39$) and dry weight biomass of worms ($\bar{x}$ ± SD = 401 ± 128mg) were not significantly correlated ($N = 39$, $r = 0.089$, $p = 0.573$). There was a weak, non-significant relationship between male weight and parasite biomass ($N = 39$, $r = 0.285$, $P = 0.08$). The relative preference of females for non-parasitised males did not relate with parasite intensity ($N = 39$, $r = -0.212$, $p = 0.193$).

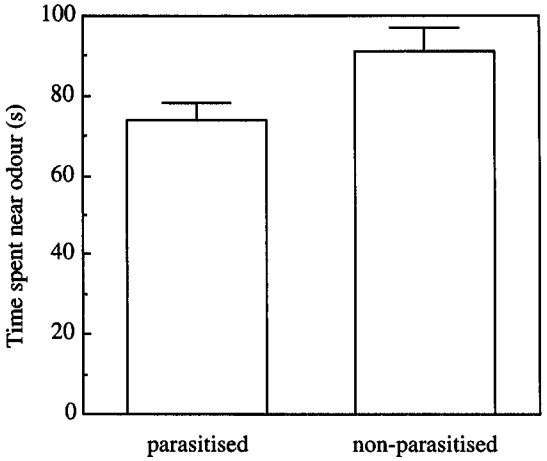

Fig. 1. Mean time (± SE) spent by female rats investigating male odours, i.e. spent in the last 2 cm of the choice arms containing either urine from a male parasitised by the cestode *Hymenolepis diminuta* or urine from a non-parasitised male.

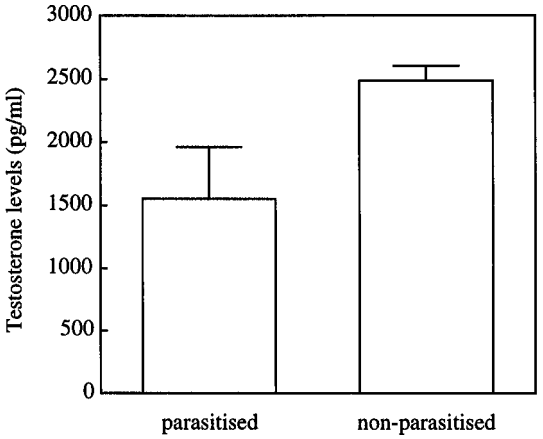

Fig. 2. Mean testosterone levels (± SE) in blood from male rats parasitised by the cestode *Hymenolepis diminuta* and in blood from non-parasitised males.

The arm first entered by females was no different than expected from random choice ($\chi^2_1 = 1.8$, $p > 0.10$). However, females spent more time in the last 2 cm of the choice arm that contained the odour of a non-parasitised male than that containing the odour of a parasitised male (Fig. 1), although this tendency does not quite attain significance at alpha = 0.05 (randomisation test: $p = 0.059$). Since non-parasitised males are slightly heavier than parasitised males, we also checked whether females spent more time with heavier males, and found no evidence of preferences based on male weight (randomisation test: $p = 0.342$).

Testosterone levels

In general parasitised males had lower levels of testosterone than non-parasitised males (Fig. 2); however the difference was not quite statistically significant (unpaired *t*-test: $t_8 = 2.124$, $p = 0.066$). There was no difference between sham-parasitised and non-parasitised males ($t_8 = 0.244$, $p = 0.25$). Testosterone levels in this RIA are consistent with levels found in the literature for serum or plasma (Gruenewald and Matsumoto 1993, Berdoy et al. 1995), therefore the results presented are valid even though whole blood was used.

DISCUSSION

In species where mate choice cues are gathered through olfaction (Gheusi et al. 1997), it is not surprising that information concerning the infection status of an individual is also contained in odour. Some of our results only approach statistical significance; nevertheless, our results suggest that female rats can use olfactory information to discriminate between cestode-infected males and non-parasitised males. Kavaliers and Colwell (1995a) used filter paper impregnated with urine and other substances (including faecal material) and therefore could not determine the source of the odour cue. Our experiment suggests that for rats, urine contains the necessary information for choices to be made. Olfactory cues may provide information about 'good genes' in species where secondary sexual characteristics have not evolved (Penn and Potts 1998, Klein et al. 1999). An alternative hypothesis, i.e. that non-parasitised males are better fathers that provide more or better resources to their offspring than parasitised males, cannot be dismissed but appears less likely as an explanation of our results.

The number of scoleces recovered and the worm biomass varied among rats, suggesting that susceptibility to infection may be at least partially under genetic control and varies within rat strains (see Andreassen and Hopkins 1980). The response of the female appears to be discrete rather than continuous, however. Females appeared to perceive the male as parasitised or non-parasitised rather than responding to the intensity of the male's infection. To understand fully how odour cues can influence mate choice, the signal in urine needs to be identified. By-products from *H. diminuta* may be excreted with urine, although this seems unlikely for two reasons. First, *H. diminuta* is situated in the intestinal tract, therefore its products should be excreted in faeces and not urine. Contamination of urine samples by faeces was extremely unlikely in our experiments. Second, if genes for resistance to *H. diminuta* are constantly being selected, the parasite would be under pressure in the evolutionary arms race not to give itself away. The cue may be linked to the major histocompatibility complex (MHC) which is responsible for encoding cell surface glycoproteins that bind peptides and present them to T cells (Hughes and Nei 1992). The MHC comprises highly variable genes that can confer resistance or susceptibility to various diseases in a variety of species (Apanius et al. 1997). The MHC has been found to influence mate choice in mice and rats (Potts et al. 1991). Information about a potential partner's MHC is conveyed in urine (Singh et al. 1987, Singer et al. 1997). Females may recognise either the specific binding of *H. diminuta* antigens or the more general presence of something not self.

Steroidic hormones may play a role in mate choice and may have co-evolved as a contagion indicator. According to the immunohandicap hypothesis (Folstad and Karter 1992), increased testosterone allows secondary sexual characteristics to become more elaborate, at the cost of a compromised immune system. Therefore, males that have elaborate secondary sexual characteristics and are also parasite free are likely to have genetic resistance. Males who are infected with *H. diminuta* may have less circulating testosterone as their immune system fights the tapeworm (see Addis 1946). Our results indicate that testosterone levels in rats parasitised with *H. diminuta* were reduced; the most likely reason why the difference was not quite significant would be a lack of statistical power. Morales et al. (1996) found that male mice infected with the tapeworm *Taenia crassiceps* had a 95% reduction in testosterone levels compared with their uninfected conspecifics. This reduction in testosterone completely inhibited sexual behaviour. Variation in levels of testosterone may thus be a valuable cue for females looking for resistance genes for their offspring.

Hamilton and Zuk (1982) stated that the type of parasites most likely to fit their hypothesis were those that would debilitate the host rather than either killing it or allowing total recovery after a brief sickness. The ideal disease would be acute and cause heavy juvenile mortality, but persist in chronic form in survivors. *H. diminuta* does not cause severe pathology in its host nor does it cause heavy juvenile mortality; nonetheless it appears that females have evolved to discriminate against it by choosing non-parasitised males. Finding whether the sensory cue in urine is specific to this parasite or more general would therefore be important. Parasites that are virulent and harm their host in obvious ways, i.e. loss of conditioning or of weight, lack of stamina, poor grooming, etc., may provide visual indications of their presence. The more general, more subtle odour cue may be useful in discriminating against males harbouring less harmful parasites.

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