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Article

Article

Females choose gentle, but not healthy or macho males in Campbell dwarf hamsters (*Phodopus campbelli* Thomas 1905)

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Abstract

Androgen-dependent male sexual traits (STs) as well as immunocompetence are theoretically assumed to be key indicators of a male's quality for the mate-choosing female. We studied mate choice by sexually motivated (SM) females of Campbell's dwarf hamsters. Females chose between 2 tethered male siblings that differed in expression of STs. Males were unrelated to the female and able to contact and copulate with her. In both males, we measured sex-related morphology of body mass, mid-ventral specific skin gland, ano-genital distance, and external testicular diameter. We also estimated levels of blood testosterone and cortisol, specific T- and B-cell immune responses to antigens, as well as aggressive and sexual dominance in sibling males through additional encounter experiments with another SM female (male sibs could freely compete for the female). We found that SM females chose a partner among 2 male sibs and spent over 80% of their time on average with the preferred male compared with the non-preferred one. Her choice was not associated with the first visit of the chosen male, with a higher expression of sex-related traits, higher levels of blood testosterone, or with aggressive dominance. The choice was not associated with the intensity of T-cell immune response to phitohemagglutinin (PHA). Instead there was a tendency for a negative relationship with the expression of STs and B-cell response to the antigen challenge. The only character that unambiguously influenced female choice was the non-aggressive male to female grooming during sexual contact. There was no difference in breeding success between preferred and non-preferred males paired with virgin females.

Key words: Campbell's dwarf hamsters, female mate choice, immunocompetence handicap, male–male aggression, sexual traits, testosterone.

According to sexual conflict theory females are the choosy sex (Trivers 1972; Parker 1979; Arnqvist and Rowe 2005) and should choose males of higher quality as potential mates (Zahavi 1975, 1977; Anderson 1994; Kokko et al. 2003; Neff and Pitcher 2005; Kotiaho and Puurtinen 2007; Jennions and Kokko 2010). By choosing a high quality partner the female provides offspring with resources (Hoelzer 1989) or with a better genetic background (Hamilton and Zuk 1982; Anderson 1986). Androgen-dependent male sexual traits (STs), including behavioral characteristics, as well as immunocompetence, are theoretically assumed to be key indicators for female mate choice. The expression of androgen-dependent traits and the maintenance of a male's physical health are costly processes causing a tradeoff between competing functional systems. According to the "Immunocompetence handicap" hypothesis, when the female chooses a male, she focuses on signs of his innate health, although high levels of testosterone may negatively affect immunity (Folstadt and Karter 1992). Theoretically, a female can base a choice on any optional trait indicating a male's quality, but the

interdependence between such traits and expression of secondary sexual characters, immunocompetence, androgens, or androgen-dependent behavior is assumed.

The Campbell dwarf hamster is a small, polyoestral, seasonally breeding rodent with highly pronounced sexual dimorphism, inhabiting the dry steppes and semi-deserts of Central Asia (Flint and Golovkin 1961; Sokolov and Orlov 1980; Feoktistova 2008). Ready to mate females (of different ages) weigh from 22 g to 45 g, and males weigh from 32 g to 60 g. Males have a large specific skin gland in the middle ventral part of the body. They use a secretion of the gland to mark extensive overlapping home ranges. Information about their mating system in nature is quite scarce, so it is safe to speak only about polygamy (Vasilieva et al. 1988; Wynne-Edwards et al. 1992; Telitsyna 1993; Surov 2006). Receptive females attract males from a distance of up to 1 km, and may mate with several males (Wynne-Edwards et al. 1992; Surov 2006). In captivity, adult males act aggressively toward each other from the age of 2 months, and their cohabitation in the same cage often leads to the death of one of them. Success in mating with the female can be related positively to aggressive dominance in male–male conflicts. However, this does not exclude the possibility of selective responses in the female. When a male and female share one cage, they demonstrate pronounced features of social monogamy including male participation in care of pups (Wynne-Edwards 1987, 1995; Vasilieva and Khrushcheva 2010). Fragmental observations in nature also signify to the close contact of a male with the female and juveniles (Wynne-Edwards et al. 1992; Sokolov and Vasilieva 1993; Wynne-Edwards 1995).

We studied experimentally the choice by receptive and sexually motivated (SM) female *Phodopus campbelli* between 2 male full-sibs that differed in the degree of expression of external STs. In addition to morphological characteristics in these males, we estimated levels of testosterone and cortisol in their blood, intensity of specific immune response to antigens, and their aggressive and sexual behavior when males competed for the female. We estimated cortisol concentrations to characterize stress level. Glucocorticoid stress hormones can play a role in sexual selection. In terms of mate choice, individuals can exhibit preferences for mates with either low baseline or peak glucocorticoid levels (Husak and Moore 2008). Siblings were used in order to minimize the genetic component of variance for the female choice since it is known that the female may choose a partner according to the difference in MHC genes (Yamazaki et al. 1976; Penn and Potts 1999; Milinski 2006).

We tested 3 hypotheses in this study: 1) The potential predictors of female mate choice (male STs, intermale aggressiveness and sexual dominance, endocrine and immune characteristics of males) will be correlated with each other. 2) A SM female will choose a partner between 2 male full-sibs divergent in the expression of ST. 3) Mate choice can be explained by a greater expression of ST, an increased level of testosterone, higher behavioral competitiveness, higher (indicator of health), or lower (reciprocal relationship with testosterone) specific immunocompetence, or by a combination of these characteristics.

Materials and Methods

Males

We obtained 18 litter with 3 or more juvenile males from 57 pairs of hamsters (aged from 6 months to a year) in 2014 and 30 litter from 120 such pairs in 2015. At 25 days we removed juvenile males from parental cages and kept them together by litter (males without females) in Ferplast plastic cages ($70 \times 40 \times 40$ cm) for up to 2 months. At 2 months the animals were photographed with a digital camera (Nikon 7000) in fixed positions with their ventrum up against a ruler for measurements of STs on the computer screen (Shekarova et al. 2010). We fixed animal by hand ventrum up, so that the testes became visible in scrotum. ST measurements included body mass and characteristics of male specific external morphology (both secondary and primary sexual characters): area (length-$\times$ width) of mid-ventral skin gland, distance between anal and genital openings, and the testes size (average length of testes) in their external outlines from a live animal.

In each litter we chose 2 males with maximal differences in body mass, area of mid-ventral gland, ano-genital distance, and testes size. From 8 to 9 AM we took a blood sample from the sublingual vein (0.3 mL) of each male. The whole procedure of sampling blood lasted no longer than 2 min, which is 2 times less than the time of glucocorticoid signal in the blood in response to handling. Samples were centrifuged at 3,000 rpm for 15 min, and the serum was separated from the hematocrite and frozen at -18°C. Then selected males were placed in individual cages $30 \times 22 \times 20$ cm, where they lived during the experiment (Figure 1).

Over 2 years 45 pairs of male sibs were selected for testing. One male died. Thus, for the statistical analysis we used the data set for 88 individuals (44 male pairs).

Females

Young females after removal from the parental cage at the age of 25 days were kept in groups of 7–8 individuals in plastic cages "Ferplast" ($70 \times 40 \times 40$ cm). At 3–4 months the majority of sexually mature females remained in diestrus. A week before the experiment we stimulated females by placing an adult male, confined in a small box of metal mesh, in the female's cage for 1 h, which was enough time to stimulate the female's estrous cycle. We tracked the females' cycle daily (1–2 h before testing) by viewing their vaginal smears under a microscope ($\times$20). In the tests we used virgin females 3–4 months old in the phase of transition from proestrus to estrus. Our preliminary observations showed that the female was SM only at this stage of the estrous cycle and actively looked for contact with a male. Although the female remains attractive to male during most of estrus, in the middle and at the end of estrus she opposes his attempts to mate and escapes into an area of the experimental enclosure inaccessible to the male. In Campbell's hamster a vaginal estrus, registered in smears, may not coincide with behavioral receptivity (Erb et al. 1993).

Thirty minutes before the test began the female was paired in a neutral arena with an adult male for 1–2 min to ensure that she was positively motivated. All females we used were not sisters of tested males. During the test, males were able to copulate with a female. In each test (with each pair of males) we used a new female.

Animals housing

The animals belonged to the laboratory population of hamsters at the A.N. Severtsov Institute of Ecology and Evolution, RAS. This population is descended from hamsters taken in the 1980s from Mongolia (MPR). All hamsters were kept in a room with a constant long-day regime (14 h). Food (formula feed for rats and mice, oat, vegetables, as additives, sunflower seeds, low fat cottage cheese, boiled chicken) and water were *ad libitum*. Thin wood shavings and sawdust were used as bedding, and pieces of cardboard served for

Litters birth	Litters removal from parental cages at 25 days age. Morphometry and weighing	Juvenile males housed in individual cages at 60 days age. Blood sampling, morphometry and weighing	DTH T-cell immunity test (cutaneous response to phytohemagglutinin)	Test 1. Mate choice by the female. Morphometry and weighing	Test 2. Encounter of males and free access to the female. Weighing	Immunization by SRBC and blood sampling 7 days after	Test 3. Reproductive success of males
10-25 Feb	5-25 Mar	10-25 Apr	1-10 May	10-25 May	4-10 June	21 June and 28 June	After 12 July

Figure 1. Time schedule of experimental study.

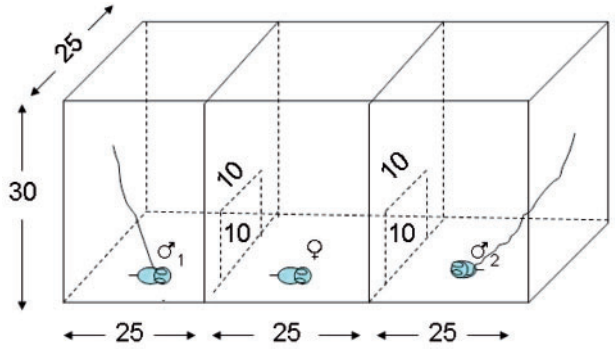

Figure 2. Experimental apparatus for Test 1. Dimensions are given in cm.

shelters. Animal care mandated that we change the bedding once every 2 weeks and check on the reproductive and health state every week.

Test 1: mate choice by the female

For each pair of male siblings at the age of 3 months (Figure 1) we selected a SM female (in transition from proestrus to estrus). Before testing we exposed this female to the odor of the litter from individual male cages for 5 min. This was done to minimize the likelihood that sexual intercourse would take place as a result of the female's accidental first entry into the compartment of 1 of the 2 males.

Testing was performed in a chamber made of organic glass (Plexiglas), consisting of 3 compartments—1 central and 2 lateral of $25 \times 25 \times 30$ cm each. Compartments were partially isolated by walls with gate-passes 10×10 cm on each side of the center compartment, but could be isolated completely by removable dividers—privacy screens (Figure 2).

We placed a SM female in the central compartment with gates, covered by screens. After a 5 min adaptation, 2 samples of the litter from male cages were exposed to the female in Petri plates (5 cm diameter) that were mounted at the mid-center line of the compartment at a distance of 5 cm one from another. The installation of plates took 2–3 s. During the next 5 min we recorded the time spent sniffing each odor sample using 2 stopwatches. After 5 min, the Petri plates were removed. The female was left for next 5 min in the central compartment. Meanwhile, flexible nylon collars were placed on each male. We used tightening nylon cable straps, usually used to tie up wiring (cable tie 3×100 white). The collars were attached to 25 cm of flexible tungsten fishing leads, the free end of which was fastened on the far wall of the lateral compartment. The fastening was designed so that three-fourth of the compartment floor was available for the male, while the female had free access to come into the compartment of each male, but remained inaccessible to the male in one-fourth of its area.

After a 5 min adaptation the screens that blocked passages to the side compartments were removed, allowing the female to enter freely the males' areas. Within the next 60 min one observer recorded female transitions from section to section using a computer program designed by A.V. Tchabovsky in Excel macros.

We gave animals only 5 min time for familiarization or adaptation to the experimental situation due to their very high sexual motivation. Our preliminary observations showed that longer periods of adaptation could impair the result of the test and, thus, would not provide any advantage.

For each female in each test, we estimated the number of transitions from compartment to compartment and the time the female spent in each compartment. The time intervals were used as a primary data for analysis (Williams et al. 1992). Simultaneously, during the entire time of the test a second observer recorded the following patterns of the males' behavior: 1) male grooming of the female, 2) series of mounts, and 3) intromissions (recognized as mounts associated with grooming of genitals; Sachs et al. 1988; Hicks et al.1999). In females we recorded: 1) approaches to the male, 2) defensive postures (fall on the back), 3) vocalizations (squeals associated with defense); and 4) aggression toward the male (rushes, bites). A male starts to groom the female in the shoulder area before mounting and holds her slightly biting on the shoulder while mounting, presumably stimulating the female for copulation. However, the actions of the male can be rough. Such "aggressive grooming" can be easily identified by the defensive postures of the female and squeals. In contrast, a "non-aggressive grooming" is not associated with any expression of discontent.

In parallel, we recorded the behavior of animals during the test with digital video camcorder Canon G30, and these records served as the main resource for further analysis. Afterwards the testing males were weighed and photographed in fixed positions with ventrum up against the ruler scale. If in one test the male with the higher expression of ST was tethered in the right compartment, in the next test this compartment was occupied by the male with the lower expression of ST.

The choice of a male by the female was based on the amount of time the female spent with each male. We assumed that a female did make a choice if she spent $\geq 2/3$ (66.7%) of the time with one male from the total time spent with males. In 75% of cases SM females spent less than 10% of the test time (Median = 8.4%, $n = 34$) in the central compartment. Although the social preference (estimated by time in the compartment) and the mating preference (sexual experience) may not be the same (for instance, in prairie voles, mating was not essential for partner preference formation; however, preferences developed more rapidly when mating occurred; Williams et al. 1992). The time the choosing individual spends with a potential

partner is a common measure in studies of mate preferences (Young at al. 2008).

Test 2: encounter of males and free access to the female

Ten days after the end of the mate choice experiment we estimated the competitiveness of male sibs (aggressive and sexual dominance) in a series of independent tests with free access of both males to another SM female (Figure 1). Males could freely contact with each other and mount the female. Males were paired in a round arena 50 cm in diameter. The arena was surrounded by an organic glass wall and was subdivided into 2 equal sections by a removable partition. A fresh sheet of filter paper was used as the substrate. After a 5 min adaptation of each male in a separate section we removed the partition wall, and during the next 5 min registered the patterns of aggressive dominance behavior (attacks, rushes, chases, fights), and defensive subordinate behavior (defensive postures on the back, escaping behavior and flights, submissive vocalization, positions under in fights). After 5 min of males' testing we placed a new non-relative SM female in the arena for 10 min and registered the aggressive and defensive behavior of males by the same scheme as before, as well as the frequency of mount series and intromissions. The behavior of the males was registered by video camcorder. We estimated the occurrence of each form of behavior within 5 s intervals of the time scale of the test. In the analyses we used number of intervals with a certain behavior during the test.

Test 3: reproductive success of males

Three-month old sexually mature virgin females in transition from proestrus to estrus (SM females) were introduced to males in their individual cages (1 per cage) 3 weeks after the males' immunization with sheep's red blood cells (SRBC) and 2 weeks after blood sampling (Figure 1). We recorded the date of birth of the first litter and the number of young per litter. If a female did not give birth within 50 days after pairing with a male she was considered non-breeding.

Male characteristics used as predictors for mate choice by the female

We used the following predictors, for female choice: 1) body weight (g), 2) mid-ventral gland area ($D_{max} \times d_{min}$; cm^2), 3) ano-genital distance (mm), 4) mean length of the testes (mm), 5) concentration of cortisol in the blood at 2 months old (ng/mL), 6) concentration of testosterone at the age of 2 months (ng/mL), 7) concentration of cortisol at the peak of the immune response to SRBC (ng/mL), 8) testosterone concentration at the peak of the immune response to SRBC (ng/mL), 9) intensity of the cutaneous DTH reaction to phytohemagglutinin (T-cell immunity test, mm), and 10) intensity of the immune response to SRBC (titre of antibodies in the blood). The characteristics of behavior recorded in Test 2 and used for evaluation of the males' competitiveness were as follows: 1) the aggressiveness of the male in encounter test with the sibling male (sum of 5 s intervals with aggression; first 5 min without the female), and 2) mounts on the female (sum of 5 s intervals with mounts; next 10 min with the female).

Immunity: T-cell immunocompetence

An estate of adaptive T-cell immunity of males was estimated using a cutaneous delayed type hypersensitivity response to phyto hemagglutinin (PHA-P, L8754-25mg, Sigma–Aldrich Co.), a mitogen of plant origin causing rapid T-cell recruitment and proliferation in the inflammation focus (Figure 1). This assay is widely used in ecological immunological research, and a study by Tella et al. (2008) confirmed its appropriateness for adaptive immunity studies.

We dissolved PHA-P in PBS (E404–200TABS, 100 mL Sigma) to 2.5 mg/mL (Sinclair and Lochmiller 2000), and 50 mL of this solution were injected intradermally in the heel of the right hind foot using an insulin syringe, while the left hind heel was injected for the control with the same amount of PBS. The paw thickness was measured before the injection and 24 h after the injection with a soft digital caliper, and the difference between the measurements ($\Delta = d_{after} - d_{before}$) was used to compare the reaction in the right and the left foot. Since the response of the control (left) foot to PBS was not pronounced and there was a highly significant difference in responses of the left and right (test) foot (T-test for matched pairs, $P < 0.001$), in our further analysis we used the difference in thickness of only the paw inoculated with PHA-P.

Immunity: B-cell immunocompetence

We injected experimental hamster males intraperitoneally with 2% suspension of SRBCs in saline and control animals ($n = 14$) with the same amount of saline (2 mL per gram of body weight). SRBCs were prewashed three times and then suspended in saline to the required concentration. The immune response was measured by hemagglutination assay (Wegmann and Smithies 1966; Roitt et al. 1998) with blood serum obtained from the sublingual vein 7 days post-immunization, at the immune reaction peak (observed between days 5 and 10; Figure 1). Reaction was performed with 0.5% SRBC suspension in saline. In the first well of a 96 well microbiological plate, 25 mL of experimental serum were thoroughly mixed with 75 mL of saline, and 50 mL of the resulting suspension were transferred into the next well; then the procedure was sequentially repeated. The incubation temperature was experimentally optimized as +38°C. The immune response to SRBC (serum antibody titer) was evaluated after 2 h of incubation as the number of the last well in this series of sequential saline dilutions that contained the quantity of antibodies sufficient for hemagglutination (Rogovin et al. 2015). As we expected, immunized animals showed a significantly stronger immune response than control ones, in whom the reaction was close to zero (Mann–Whitney U test: $Z = 4.6$; $P < 0.001$, $n_1 = 68$, $n_2 = 14$).

Hormones

We measured cortisol and testosterone concentrations in hamster blood serum specimens (Figure 1) by enzyme linked immunosorbent assay (ELISA) using IEA cortisol and IEA TS (testosterone) commercial test systems (NPO Immunotech, Russia) according to the protocol suggested by the manufacturer. The cortisol–testosterone cross reaction for the above kits was 0.08%. Optical densities were measured at 450 nm using a Uniplan plate spectrophotometer (Russia). In rare cases of high concentrations of testosterone we diluted samples with the "Buffer for serum dilution" produced by "NPO Immunotech".

Statistical analysis

We applied the principal component analysis (PCA) to all four variables describing male morphological traits in order to simplify the analysis and to reduce the dimensionality of the data. All variables were standardized, and the single principal component was extracted (Table 1). The component (ST expression) explained 60% of the total variance in the morphological variables and reflects the

increase of body mass, the size of ventral glands, the testes size, and the anogenital distance.

The testosterone and cortisol levels at 60 days and after SRBC immunization did not conform to the assumptions of normality (Shapiro–Wilk's W test, $P < 0.05$) and, therefore, were log-transformed to access the normal distribution.

To test the possibilities of future reduction of the dimensionality in the dataset and to reveal any high correlation among variables, the correlation analysis (Pearson's correlation) was performed among male individual characteristics (Table 2). Male–male aggressiveness and the intensity of the male's mounting behavior in TEST 2 (conducted with the same males that participated in TEST 1 and the other female) did not conform to the assumptions of normality (Shapiro–Wilk's W test, $P < 0.05$) and could not be transformed into a normal distribution. Thus, correlation statistics for these variables were assessed with Spearman's rank-order correlation.

Test 1. To determine the subset of variables that best discriminated between "preferred" and "non-preferred" males in tests with clear female choice ($\geq 66.7\%$ of time), we conducted General Discriminant Analysis (GDA; forward stepwise, $P_{\text{to enter/remove}} = 0.05$) (Hill and Lewicki 2006). All male individual characteristics were included as possible predictors in the GDA; additionally, the study year was fitted in the model.

To obtain a quantitative estimation of the female choice, we conducted forward stepwise general regression analysis (GRM, $P_{\text{to enter}} = 0.05$, $F_{\text{to enter}} = 1.0$) with the time that females spent with the preferred male in Test 1. Standardized differences between all individual characteristics of "preferred" males and "non-preferred" males (Table 3) were used as potential predictors in the model; in addition, the study year was included in the initial model as a categorical predictor. All tests were included in this analysis, $n = 44$.

We used McNemar's test for paired nominal data to estimate the effect of male behavior on the female choice in Test 1. The forward stepwise GDA was used in order to find variables that best discriminated between aggressive and nonaggressive males (with respect to male grooming).

Test 2. The Wilcoxon matched pairs test was used to estimate variation between the "preferred" and "non-preferred" males in the tests with clear female choice ($n = 34$). The differences between "preferred" and "non-preferred" males in aggressiveness and mounting behavior were normally distributed. Therefore, we performed the general regression model with differences in aggressiveness and mounting behavior as predictors and the time with the "preferred" male in TEST 1 as dependent variable (study year included as additional factor).

Test 3. To compare the litter sizes in the preferred and non-preferred males we used Student's T-test for paired samples. The birth dates of litters produced by preferred and non-preferred males did not fit demands for normality. To compare the birth dates the Wilcoxon matched pairs test was used.

Statistical analyses were performed using STATISTICA version 8.0 (StatSoft). All tests were 2-tailed, with a significance level of 0.05.

Table 1. Summary of principal components (PCs) analysis of 4 morphological variables describing male STs

Information	PC1
Eigenvalue	2.38
Percentage of total variance explained	60.0
Factor loadings:	
Body mass	0.68
Size of ventral glands	0.81
Testes size	0.85
Ano-genital distance	0.74

Ethical principles in treatment of animals

In our study we followed the requirements of the "Principles of Laboratory Animal Care" (NIH Publication Vol. 25, No. 28 revised 1996; http://grants.nih.gov/grants/guide/notice-files/not96-208.html), of "Guidelines for the treatment of animals in behavioural research and teaching" (ASAB/ABS 2012) and the Federal Law of the Russian Federation.

Results

Correlations between male traits

Contrary to expectation, we did not find any strong relationships among different male characteristics (Table 2). Namely, the correlations between ST expression and hormones, ST expression and immune status, and hormonal and immune status were low or absent. Most correlations were not significant, and the highest correlations did not exceed medium-sized effects (Cohen 1988; Hurlburt 2003). Thus, we did not reduce dimensionality of the dataset due to substantial information loss.

Male–male aggressiveness as well as the intensity of mounting in Test 2 (conducted with the same males that participated in Test 1 and the other female) also did not correlate with most individual characteristics of the males and between themselves (Table 3). The number of mount series per test the tethered male made in Test 1 did not correlate with the number of mount series the male made

Table 2. Correlation matrix (Pearson's r) among all male individual characteristics (boldface type indicates significant effects; $n = 88$)

	ST expression	Cortisol (60 days)	Cortisol (SRBC)	Testosteron (60 days)	Testosteron (SRBC)	Immune response to SRBC	Immune response to PHA
ST expression[a]							
Cortisol (60 days)[b,c]	**−0.25**						
Cortisol (SRBC)[c,d]	−0.06	0.17					
Testosterone (60 days)[b,c]	0.00	0.02	**0.21**				
Testosterone (SRBC)[c,d]	0.18	−0.01	**0.40**	**0.23**			
Immune response to SRBC	−0.04	−0.13	0.09	−0.10	**0.23**		
Immune response to PHA	0.15	−0.03	0.14	−0.12	**0.26**	0.16	

[a] The combined variable characterizing STs expression. Result of PCA of males' morphological characteristics.
[b] Hormone concentration in blood serum at the age of 60 days.
[c] Variables that were log-transformed.
[d] Hormone concentration in blood serum at the peak of immune response to SRBC.

Table 3. Spearman's correlation coefficients for male–male aggressiveness and male–female mounting activity in Test 2 (boldface type indicates significant effect, $p < 0.05$ $n = 88$)

	Aggressiveness	Mounting activity
ST expression	−0.13	0.10
Cortisol (60 days)	−0.10	0.04
Cortisol (SRBC)	−0.05	**−0.28**
Testosterone (60 days)	−0.07	−0.15
Testosterone (SRBC)	−0.10	−0.18
Immune response to SRBC	−0.03	−0.15
Immune response to PHA	−0.16	0.05
Mounting activity (Test 2)	−0.01	

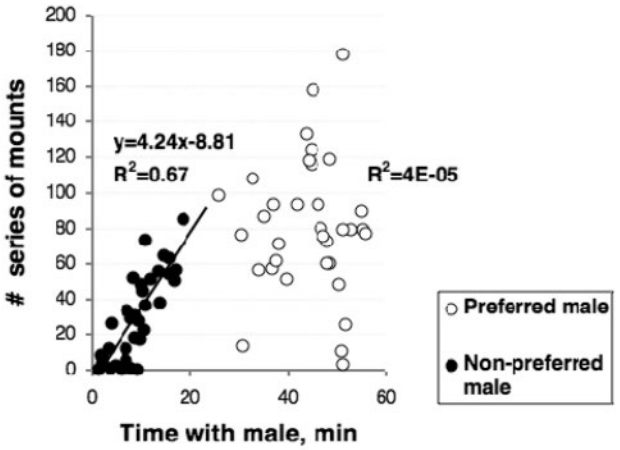

Figure 3. The relation between the number of series of mounts and the time that the female spent with preferred and non-preferred males.

when freely competing with a sibling for the female in Test 2 (Spearman's $r = 0.12$, $P = 0.26$, $n = 88$).

Female choice between 2 tethered males (Test 1)

In 77% of the tests ($n = 44$) females preferred 1 male and spent substantially more time with him (≥ two-third of the test duration) than with the other male. On average, females spent 82.4 ± 1.6% of the total time with the preferred male. Hence, in most tests the "preferred" and "non-preferred" males were evident. We defined tests in which females spent more than 67% of the total time with the "preferred" male as tests with a clear female choice.

In 41 of 44 tests (93%) females copulated with both males. Males preferred as social partners (justified by the time a female spent with the male) also were preferred as sexual partners. For non-preferred males the total number of series of mounts per test correlated positively with the total time that the female spent in the male's compartment ($r = 0.82$, $P = 0.0001$, $n = 34$). For preferred males the total number of mount series per test did not relate to total time the female spent with the male (Figure 3; $P = 0.97$, $n = 34$). The preferred males demonstrated significantly higher number of mount series per test than non-preferred males [median and limits: 79 (3–178) and 28 (0–85), respectively; Wilcoxon matched pairs test: $t = 160$, $P < 0.001$, $n_1 = n_2 = 34$]. The same was true for mounts with intromissions [median and limits for preferred male: 35 (0–95), for non-preferred males: 10 (0–67); Wilcoxon matched pairs test: $t = 86$, $P < 0.001$, $n_1 = n_2 = 34$]. The high sexual activity of the preferred male may lead to exhaustion; in many cases the male falls down on his back becoming rigid up to 1 min. This was the reason for the lower number of mounts per unit time in preferred males compared with non-preferred males [median and limits for preferred male: 1.62 (0.06–3.80), for non-preferred male: 3.29 (0–6.67); Wilcoxon matched pairs test: $t = 95$, $P < 0.001$, $n_1 = n_2 = 34$]. The same was true for mounts with intromissions [median and limits: 0.83 (0–1.92) and 0.95 (0–9.72), respectively; Wilcoxon matched pairs test: $t = 168$, $P = 0.073$, $n_1 = n_2 = 34$].

Table 4. Summary of forward stepwise general regression model (GRM) in which the dependent variable was the time that females spent with preferred males and independent variables (predictors) were represented by differences in trait values (Δ) between preferred and non-preferred males ($p_{\text{to enter}} = 0.05$, $F_{\text{to enter}} = 1.0$, $R^2 = 0.32$, $F = 5.9$, $P = 0.002$, all tests, boldface type indicates significant effect, $p < 0.05$ $n = 44$)

	Estimates	SE	*F*	*P*
Intercept	39.67	1.3		**<0.0001**
Δ ST expression	−2.27	0.9	5.9	**0.02**
Δ Testosterone (SRBC)	0.19	0.08	5.8	**0.02**
Δ Immune response to SRBC	−1.24	0.4	7.9	**0.008**
Δ Immune response to PHA	Out of the model		0.002	1.0
Δ Cortisol (60 days)	Out of the model		1.7	0.2
Δ Cortisol (SRBC)	Out of the model		0.06	0.8
Δ Testosterone (60 days)	Out of the model		0.2	0.6
Year effect	Out of the model		0.008	0.9

We did not find a relationship between the visiting order of the males and female choice: among 34 tests with clear female choice the first-visited male became the preferred male in 19 (55.9%) cases and in the other tests females preferred the second visited male ($\psi^2 = 0.47$, $P = 0.5$).

The stepwise GDA with the ST expression, hormonal characteristics and characteristics of the male immune status included as predictors, and the study year as an additional factor failed to discriminate between preferred and non-preferred males (in tests with clear female choice). No predictor contributed significantly in the discrimination and was selected for the model (Wilk's $\pi = 1.0$, $P > 0.1$).

From the initial set of predictors that included differences between the variables of preferred and non-preferred males, the stepwise GRM revealed 3 variables which significantly influenced the time that females spent with the "preferred" male. Namely, the lower values were the ST expression and the immune responses to SRBC, and the higher value was the testosterone level after SRBC immunization in the preferred males compared with the non-preferred males, the more time the females spent with the "preferred" males in the test (Table 4).

Effect of the male's behavior on the female's choice (Test 1)

The strongest factor that affected female choice in the tests was the male's behavior during copulations. Some males used aggressive grooming during sexual contact, and females obviously avoided such situations. From 34 tests with clear female choice, among the "preferred" males only 4 males groomed females aggressively and the other 30 did not; but among the non-preferred males there was a ratio of 20:14 aggressive to non-aggressive groomers (McNemar test for paired samples, $\chi^2 = 9.4$, $P = 0.002$). Females demonstrated significantly more defensive postures (fall on the back) per unit time

with non-preferred male [median and limits: 1.09 (0–6.96) compared with preferred males (median and limits: 0.65 (0–3.14); Wilcoxon matched pairs test: $t = 149$, $P < 0.011$, $n_1 = n_2 = 34$]. They also initiated more contacts per unit time with non-preferred males than with preferred males [median and limits respectively: 2.46 (0.28–23.32) and 1.15 (0.08–7.33); Wilcoxon matched pairs test: $t = 2$, $P < 0.001$, $n_1 = n_2 = 34$]. The reason is that the higher sexual activity of the preferred male may lead to his sexual exhaustion; from time to time the male falls down on his back becoming rigid up to 1 min.

The stepwise GDA model did not reveal any discrimination between males demonstrating aggressive and non-aggressive grooming (all 44 tests included). No predictor contributed significantly in the discrimination and was selected for the model (Wilk's $\pi = 1.0$, $P > 0.1$).

Encounter of males and free access to the female (Test 2)

Paired comparison of the "preferred" and "non-preferred" males from the tests with clear female choice (Test 1) did not reveal significant differences in their aggressiveness and mounting behavior in the encounter test (Wilcoxon matched pairs test: $t = 195.5$, $P = 0.6$, $n_1 = n_2 = 34$ and $t = 267$, $P = 0.8$, $n_1 = n_2 = 34$, respectively).

GRM with aggressiveness and mounting behavior as predictors and the time with "preferred" male in TEST 1 as dependent variable (study year included as additional factor) did not reveal any significant effects (multiple $r^2 = 0.16$, $F = 2.5$, $P = 0.07$).

Reproductive success in the "preferred" and "non-preferred" males (Test 3)

After the males that participated in Test 1 were paired with virgin females ready to mate in the male's cages, neither date of birth (Wilcoxon matched pairs test: $t = 99.5$, $P = 0.2$, $n_1 = n_2 = 28$) nor litter size differed for the first litters between the 2 categories of males (t-test for paired samples: $t = 0.98$, $P = 0.3$, $n_1 = n_2 = 22$).

Discussion

Among hypotheses we tested, only the second one received definite support. SM female hamsters chose their male partner from a pair of tethered male siblings, and the final mate choice was not a consequence of the first visit to the male. Other hypotheses did not receive support in results of our experiment. Correlations between male traits we studied and which are assumed to affect female choice were low or absent. Females did not make choice based on a higher expression of male STs, higher level of blood testosterone, with aggressiveness in the encounter with the sibling male or with sexual dominance in a situation when males could freely compete for the female. The choice was not associated with the intensity of T-cell immune response to phitohemagglutinin (PHA). The only characteristic that influenced female choice with high statistical support was non-aggressive grooming by males during sexual contact. Females also tended to choose males with a lower expression of ST and with a lower immune response to SRBC associated with higher testosterone after immunization.

We, therefore, are unable to explain our results in the framework of the widely discussed hypotheses of "good genes" (Hamilton and Zuk 1982) or immunocompetence handicap (Folstadt and Karter 1992). We expected females to prefer males with a higher expression of ST in combination with a lower specific immune response to antigens (in case of tradeoff between the expression of androgen-dependent traits and specific immunity). Inconsistency of our results with the theory can be explained by low correlations between dependent and predictor variables, but also by the vulnerability of any inferences based on the analysis of correlations with androgens. The result of recent cross-species meta-analyses of relationships between circulating sex hormone level and immune function was not statistically significant for either testosterone or estrogen. The meta-analysis of results of experiments, presupposing control of external and internal conditions, confirmed the effect (Foo et al. 2016).The testosterone concentration in blood is very unstable in *Ceteris paribus*. Thus, it would be more productive to focus on the concentration thresholds of sensitivity of the target tissue receptors rather than routine concentrations of the hormones, although it is difficult to realize this approach in practical work (Wingfield et al. 1990). Effects of sex hormones on immunity may depend on body conditions, and resources availability (McDade 2003; Ruiz et al. 2010). The leptin, which can prevent the immunosuppressive effect of testosterone, may be the reason (Alonso-Alvarez et al. 2007). The effect of testosterone on immune function may also depend on stress levels (Rantala et al. 2012, but see Roberts et al. 2007; Roberts and Peters 2009 for contradictory findings). Stress hormones can be the key mediators of many condition-dependent, sexually selected traits that serve as honest signals of mate quality. It is typically thought that 2-way interactions exist between glucocorticoid stress hormones, sex steroids, and body condition. In terms of mate choice, it appears that glucocorticoid stress hormones could mediate some of the condition-dependent traits used to assess mates (Husak and Moore 2008). It also needs to be taken into account that results obtained in the laboratory may not be the same as in natural conditions (Calisi and Bentley 2009). In a superb fairy-wrens *Malurus cyaneus* testosterone treatment depresses the immune response under laboratory conditions but not under natural conditions (Peters 2000).

The immunocompetence handicap hypothesis proposed by Folstad and Karter (1992) summarized what was known about the interactions between sexual signals, androgens, parasites, and the immune system. The main prediction was that there would be a trade-off between sexual displays on one hand and immune function on the other hand. At a later date most of the attention was directed to this dual effect of testosterone enhancing STs/displays, but suppressing immunity. Little attention was paid to the possible effect of testosterone on redistribution of immune cells rather than to its direct suppressive effect on the immune system (Braude et al. 1999). Roberts et al. (2004) revised studies that tested the suppressive role of testosterone in immunocompetence by means of meta-analysis and found this effect controversial; on average it was small and far from statistically significant. On the other hand, a meta-analysis of the effect of parasites on sexual signals revealed that experimental exposure to parasites significantly suppressed sexual signals (Møller et al. 1999). Finally, the meta-analysis of published data on the effects of experimental immune activation on testosterone showed a strong suppressive effect on testosterone. The trade-off between immunocompetence and STs/displays may primarily be generated by the effect of immune activation on testosterone, rather than the opposite effect that has received most attention (Boonekamp et al. 2008).

In addition to the above, a recent review of genetic benefits of extra-pair paternity in birds (Akçay and Roughgarden 2007) found no convincing evidence for genetic benefits of the phenomenon and rejects the good genes hypothesis. Taken all together these results show that Folstad and Karter's as well as Hamilton and Zuk's ideas may not be as solid as has been assumed by many people.

Although most empirical research is based on the assumption that females seek a male of the highest possible quality (in terms of the genes or resources the male can provide), manipulation of the female condition can lead to divergent female preferences (Riebel et al. 2010). For instance, it was shown on zebra finches (Holveck and Riebel 2010) and house sparrows (Griggio and Hoi 2010) that females of lower quality prefer low-quality males. High-quality female zebra finches preferred high-quality males and the high-quality female of house sparrows did not discriminate between the quality of a male (determined by the size of the black spot on the breast). Although we kept female hamsters under standardized conditions, we were limited in the possibility to control their internal state. Females that participated in the experiment were young (3–4 months), had no sexual experience, and were in the same stage of the estrous cycle. However, we were unable to standardize strictly their age and weight. These variables introduced into the model as predictors showed no statistically significant effects. However, this does not exclude the possibility of their limited impact, as well as of the impact of unaccounted factors, on the overall variation.

In a set of experimental studies the choosing females had greater reproductive success (in terms of offspring viability and survival to independence or reproductive age) when paired with preferred males (Drickamer et al. 2000; Sandvik et al. 2000; Bluhm and Gowaty 2004; Anderson et al. 2007). In house mice *Mus domesticus* per cent of juveniles at reproductive age was higher in females paired with the preferred male, but there was no significant difference in the number of pups born (Drickamer et al. 2000, 2003). Fecundity was even higher in parental pairs with non-preferred males ($P < 0.1$; Drickamer et al. 2000; Gowaty et al. 2007). In our study we found no difference in reproductive success between preferred and non-preferred males paired with a ready to breed new female. In test situation (Test 1) female choice was constrained by the experimental design. She was forced to choose between 2 sibling males which differed in ST expression. In nature she might not have preferred either male.

We also restricted our estimation of reproductive success to the first litters the females produced after pairing with experimental males. Usually the first litters born by young females of Campbell dwarf hamsters are more variable in size and viability, and we expected the impact of male traits on breeding success to be more pronounced at first reproduction event. At the same time, we cannot exclude completely the possibility of underestimation of reproductive differences between preferred and non-preferred males.

Interpreting our results we should take into consideration the species specific features of Campbell dwarf hamsters. In fact the spectrum of species for the experimental study of mate choice among rodents is still limited. Because many experiments were conducted on mice *Mus musculussensu lato*, the support of the "good genes" theory is largely associated with this species. Campbell hamsters differ from house mice in many aspects. Despite the high plasticity of the social organization of house mice (*M. musculus* s.l., Kotenkova and Munteanu 2006), at moderate densities of the population of synanthropic house mice (first and foremost, *M. m. domesticus*), despotic dominance of a male provides him with access to most of the group's females (Crowcroft 1966; Mackintosh 1970; Poole and Morgan 1973). It was the house mouse that provided good evidence for positive relationships between androgens, aggressive dominance, and expression of STs, or the negative impact of androgens on the system of acquired immunity (Schuurs and Verheul 1990; Olsen et al. 1991; Viselli et al.1995; Olsen and Kovacs 1996). The negative effect of activation of specific immunity function on testosterone level has also been confirmed in the house mouse (Boonekamp et al. 2008).

Despite the pronounced sexual dimorphism in Campbell hamsters, polygyny in the conventional sense (males desperately compete for females, and many females become available for the winner male) is uncommon for the species. The mating system of Campbell hamsters in nature, apparently, has some characteristics of promiscuity. Males mate with more than 1 female, and their female partners can mate with other males. Fragmentary observations made in nature support the opinion that males do not monopolize females (Vasilieva et al. 1988; Telitsyna 1993; Surov 2006; Wynne-Edwards et al. 1992). It is also possible that the female focuses on other quality markers of the male, unrelated to those, which determine the advantage in direct competition between males (body mass, aggressiveness, testosterone). On the other hand, the breeding system of Campbell dwarf hamsters has the features of social monogamy (Wynne-Edwards 1987, 1995; Vasilieva and Khrushcheva 2010). Among hamsters reared in captivity the male always cares for the young. Deprivation among pups of male paternal care, and in particular of the opportunity to receive a secretion of his specific glands, affects the growth and survival of offspring (Vasilieva and Khrushcheva 2010). Some observations in nature also point to the participation of a male in caring of juveniles. The male was observed carrying food into the burrow with pups (Sokolov and Vasilieva 1993; Wynne-Edwards 1995) and marking pups (Sokolov and Vasilieva 1993). In this respect it is possible that in choosing a non-aggressive male, the female focuses on the parental quality of a potential partner. This assumption suggests a separate study on parental behavior of preferred and non-preferred males; their actual reproductive success should be estimated by the number of juveniles surviving to reproductive age.

Also, results of experimental studies of mate choice, including ours, should be interpreted with accuracy due to their basic restriction—females in test situations are limited in making a totally free choice. Under other equal conditions the choice made by the female could be influenced by the activity (mating or courting behavior) of males. Direct tactile and subsequent sexual contacts with both males could enhance asymmetry in preference exhibited by the female. Our finding that females preferred gentle males supports this suggestion. This situation looks realistic in the natural context (in the wild), however, it leaves questions open. We did not find a correlation between the number of mount series of tethered males and number of mount series of freely competing males. This could indicate significant impact of female activity on the net result (ultimate choice), but a complete understanding of how the mechanism of female mate choice works seems feasible only through performing a separate experiment in which the participation of males in female mate choice is limited by a lattice, making sexual intercourse impossible. To what extent the results of this and earlier experiments will coincide is a question for future research.

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