Beyond species recognition: somatic state affects long-distance sex pheromone communication

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Long-range sex pheromones have been subjected to substantial research with a particular focus on their biosynthesis, peripheral perception, central processing and the resulting orientation behaviour of perceivers. Fundamental to the research on sex attractants was the assumption that they primarily coordinate species recognition. However, especially when they are produced by the less limiting sex (usually males), the evolution of heightened condition dependence might be expected and long-range sex pheromones might, therefore, also inform about a signaler’s quality. Here we provide, to our knowledge, the first comprehensive study of the role of a male’s long-range pheromone in mate choice that combines chemical analyses, video observations and field experiments with a multifactorial manipulation of males’ condition. We show that the emission of the long-distance sex pheromone of the burying beetle, *Nicrophorus vespilloides* is highly condition-dependent and reliably reflects nutritional state, age, body size and parasite load—key components of an individual’s somatic state. Both, the quantity and ratio of the pheromone components were affected but the time invested in pheromone emission was largely unaffected by a male’s condition. Moreover, the variation in pheromone emission caused by the variation in condition had a strong effect on the attractiveness of males in the field, with males in better nutritional condition, of older age, larger body size and bearing less parasites being more attractive. That a single pheromone is influenced by so many aspects of the somatic state and causes such variation in a male’s attractiveness under field conditions was hitherto unknown and highlights the need to integrate indicator models of sexual selection into pheromone research.

1. Introduction

Chemical communication is the most ancient and widespread form of communication in the animal kingdom and plays a fundamental role in mate choice [1,2]. Although chemical signalling remained understudied for a long time, it has been well established in recent years that sexual selection is a major force driving the evolution of chemical traits [2–5]. Both, the quantity as well as the composition of a pheromone have been shown to be used under sexual selection [6,7]. Although mate choice often exerts directional selection on signal traits [8], analyses of chemical traits, especially cuticular lipids, frequently revealed a complex pattern of linear and nonlinear selection (e.g. [7]). Recent empirical investigations suggest that animals base their choice of mate on chemical traits that are indicators of direct benefits, viability or attractiveness genes and/or genetic compatibility [1–3]. In the wasp, *Nasonia vitripennis*, for example, the quantity of the sex pheromone reliably reflects a male’s sperm number and females prefer males with higher pheromone titres to obtain a full sperm load [9,10]. In the cricket, *Teleogryllus oceanicus*, on the other hand, mate choice appears to be partly based on genetic compatibility, as individuals preferentially mate with partners who share more dissimilar cuticular hydrocarbon profiles and similarity in cuticular hydrocarbon patterns between mating pairs correlates with their genetic similarity [11].

Although there are numerous studies that found evidence for olfactory-mediated mate choice, the vast majority of them analysed contact, short range
or courtship pheromones and it remains unclear whether long-range sex pheromones, volatile pheromones released to attract conspecifics of the opposite sex from a distance, also can contain information about mate quality. Since 1959, when the first long-range sex pheromone was chemically characterized by Butenandt et al. [12], long-distance pheromone communication has been subjected to substantial research, capturing the attention of ecologists, evolutionary biologists, biochemists, neurophysiologists and molecular biologists (e.g. [13–15]). Long-range sex pheromones, which are usually composed of few components, have been assumed to serve solely as species recognition signals and therefore, to be subjected to a high degree of stabilizing selection (e.g. [16,17]). This early classical view had arisen because receivers often seem to be highly sensitive and narrowly tuned to the conspecific pheromone blend, and a slight change in the blend would thus result in a fitness loss of the signaler and therefore selected against (e.g. [18,19]). Nevertheless, various studies found considerable interindividual variation in the amount and ratio of pheromone components [20]. Although such variation can be caused by local environmental factors or due to heterozygote advantage [21], there is certainly the possibility that long-range sex pheromones covary positively with the condition of the sender and serve as reliable indicator of mate quality [22,23], especially if produced and emitted by the less limiting sex (usually the males). More importantly, we know from studies on acoustic signals used to attract conspecific mates that selection on species recognition does not necessarily preclude sexual selection (e.g. [24]).

Here, we provide, to our knowledge, the first comprehensive study on the role of an insect’s long-range sex pheromone in female mate choice that combines chemical analyses in the laboratory with a follow-up test under field conditions. Using males from the same population, we first looked for interindividual variation in the quantity and ratio of the pheromone components emitted as well as the time invested in calling. Secondly, by combining a dynamic headspace technique with video observations we analysed whether the quantity, ratio or the time invested in pheromone emission covaries with a male’s condition. Condition, which can be defined as the pool of resources an individual has available to allocate towards the production or maintenance of traits that enhance fitness, is determined by the somatic state, epigenetic state and genotype of an organism, and comprises several aspects including age, stored nutrients or number of parasites associated with the body [25–27]. Theory predicts that individuals of higher phenotypic condition have a larger resource pool from which to draw for allocation of different traits and are therefore better able to afford any costs associated with sexual trait expression [26,28]. To obtain a profound understanding of the possible condition dependence of a long-range sex pheromone, we manipulated several aspects of condition: (i) the actual nutritional state influenced by the quality and quantity of the diet; (ii) age; (iii) the nutritional state during development, which ultimately affects the adult body size of our study organism; (iv) parasite load; and (v) last but not least, and most importantly, we analysed whether females respond to the variation in pheromone quantity or quality under field conditions.

In this study, we used the burying beetle, *Nicrophorus vespilloides* as our model organism. Male burying beetles are well known for emitting a species-specific pheromone to attract females from a distance [29]. Their sex pheromone consists of two substances, ethyl 4-methyl heptanoate (EMH) and (E)-geranylacetone (GA) ([30]; W. Haberer, T. Schmitt and J. K. Müller 2014, personal communication). Although EMH can attract females on its own, a larger field study revealed that the second component significantly increases the number of attracted females. As burying beetles emit their long-range pheromone in a daily routine at a species-specific time and the time invested in pheromone emission is measurable because of a characteristic and observable ‘headstand’ posture the males adopt during calling [29,31,32], they are extraordinary well suited to quantify pheromone emission and to analyse the role of the volatiles in female mate choice.

2. Material and methods

(a) Insects

Burying beetles provide parental care in the form of parental defence and regurgitations to the young on small vertebrate carcases that they bury as a food resource for the developing young [29,33]. When a male has found and secured a carcass suitable for reproduction, he starts to attract females by emitting a long-range pheromone [29]. However, even if no carrion is available, males emit the sex pheromone [34]. *Nicrophorus vespilloides* males usually engage in pheromone emission every day in the late afternoon for approximately 2 h. Females are known to respond to the pheromone of non-resource owner, but there appears to be variation in the attractiveness between males based on the chemical signal [35]. Once attracted, however, a female will mate with the pheromone emitter before leaving in search of a carcass [35]. Although males primarily attract females in the field, some males also respond to the sex pheromone (approx. sex ratio 1:3.5), presumably because there is a certain chance that the pheromone emitter is in the possession of a carcass which can be taken over or because they seize the opportunity to mate with surplus females the pheromone emitter attracts [34]. Experimental *N. vespilloides* beetles used in this study were first to fourth generation offspring derived from wild-caught beetles trapped in a deciduous forest in Ulm, Germany (48°25’ N, 9°57’ E) in 2012 and 2013. Beetles were maintained in incubators at 20°C with a 16 L:8 D cycle and housed in clear plastic containers (10 × 10 × 6 cm) filled with moist peat. If not otherwise stated, experimental males were kept singly and fed decapitated mealworms (*Tenebrio*) twice a week.

(b) Quantifying pheromone emission

(i) Volatile collection and video analysis

For the volatile collection we used a standard dynamic headspace method (see, e.g. [36]). During headspace sampling, beetles were maintained in climate chambers at 20°C with a 16 L:8 D cycle. Five hours before lights were turned off (approx. 2 h before males start with pheromone emission), each experimental male was placed in a silanized glass jar (diameter: 3.2 cm; height: 13.0 cm) equipped with a ground glass stopper and two arms. Approximately 1.5 h later, we started to pump air through the glass at a flow rate of 200 ml min⁻¹ using a membrane pump (DC 12/16FK, Fürgtur, Aichstetten, Germany). Incoming air was cleaned by an activated charcoal filter (Supelco, Bellefonte, PA, USA); the effluent air stream passed an adsorbent tube filled with a 1:1 mixture of Tenax-TA (mesh 60–80; Supelco, Bellefonte, PA, USA) and Carbograph B (mesh 20–40; Supelco, Bellefonte, PA, USA). Collection took place until a male ended its calling behaviour (end of calling was determined by 30 min observation intervals; see the electronic supplementary material for chemical analysis). To get a precise measurement of how much time a male invested in pheromone emission, video observations were performed with infrared sensitive cameras (DCR-SR72 and DCR-SR35, Sony). Each male was recorded during the entire time of...
headspace sampling. After the onset of darkness, video recordings continued under infrared light. We quantified the duration of pheromone emission by determining the amount of time an individual spent in the characteristic calling posture.

(ii) Effect of nutritional state
The aim of this experiment was to analyse the effect of the quantity and quality of the diet on pheromone emission. We established three treatment groups: males were kept singly and were either food-deprived for 11.5 (+0.2 s.e.) days, fed with invertebrate carrion (decapitated mealworms) ad libitum for 15.0 (+0.3) days, or vertebrate carrion (small pieces of pinkie mice) ad libitum for 15.0 (+0.4) days before they were subjected to the combined headspace and video analysis. Prior to the experiment we tested the beetles’ starvation resistance (see the electronic supplementary material). Mature male beetles survived 18.6 (+1.2) days without food.

(iii) Effect of age
To test the effect of age on pheromone emission, males were either subjected to the combined headspace and video analysis at an age of approx. 20 days (21.3 ± 0.2 days; mean ± s.e.; treatment group ‘young’) or approx. 60 days (56.1 ± 0.3 days; treatment group ‘old’).

(iv) Effect of body size/nutritional condition during development
We manipulated the nutritional environment of the developing larvae which ultimately has an effect on adult body size [37]. Pairs of beetles were provided with a 20 g carcass (Mau-Ra Farm, Raddenseben, Germany) to initiate breeding. As soon as the developing larvae reached their third instar, the mass of each larva within a brood was measured three times a day. Of each brood, some larvae were removed once they had achieved a mass of 70–120 mg (treatment group ‘small’) and transferred to a new container for pupation [37]. The remaining larvae were left with their parents and the carcass until the carrion was entirely consumed or the larvae left the carcass for pupation on their own (treatment group ‘large’). After eclosion, we obtained males of two size classes: the ‘small’ experimental males had a mean pronotum width of 3.76 (+0.04) mm, the ‘large’ ones a mean pronotum width of 5.36 (+0.04) mm (Gaussian generalized linear model (GLM), F1,49 = 692.49, p < 0.0001).

(v) Effect of parasite load
Wild N. vespilloides beetles are usually infested by mites (mesostigmatic mites, especially Poecilochirus canthi) [38] and carrion-dwelling nematodes (Rhabditis stammeri) [39], which have negative effects on reproductive success and survival of their host ([40]; A.-K. Eggert and J. K. Müller 2010, personal communication). To test whether the parasites associated with a male’s body affect pheromone emission, we generated parasitized as well as parasite-free males. Nematodes as well as mites are transmitted a number of treatment groups affected the males’ attractiveness to females, we conducted four field experiments in the summers of 2013 and 2014. In a deciduous forest in Ulm, Germany (48°25’N, 9°57’E), we established 22 pitfall traps (two traps served as controls; see the electronic supplementary material for a schematic view of the set-up of a trap) that were at least 20 m apart. Males that served as bait were placed in small containers half-filled with peat and hanging above the opening of the traps. The lid and the upper part of each container were perforated to allow pheromone dispersal. In each field study, we compared males of two of the above-mentioned treatment groups: (i) food-deprived (for 10.8 ± 0.2 days) versus vertebrate carrion-fed males (for 11.0 ± 0.2 days), (ii) young (22.5 ± 0.4 days) versus old males (62.5 ± 0.3 days), (iii) small (pronotum width: 3.79 ± 0.05 mm) versus large males (pronotum width: 5.49 ± 0.05 mm), and (iv) parasitized versus parasite-free males. Every male was exposed in the field for 3 days, with the exception of the parasitized and parasite-free males, which were only exposed twice because of lasting bad weather conditions. In general, males were not brought into the field if the daytime temperature fell below 13°C and on days with precipitation. Within each study, the males of the two compared treatment groups were deployed in alternate traps and for each of the following exposition events males were moved clockwise to the next trap to minimize any possible position effects. After each trapping day, the trap catches were brought into the laboratory and the caught beetles were identified and sexed. In the case of the two control traps, the container was also filled with peat, but never baited with a male. During all four field experiments, control traps (n = 44) never caught any N. vespilloides individuals.

In general, the experimental design of our field experiments ensured that neither visual cues nor contact pheromones could have affected a female’s decision, but mate choice was based on a male’s long-range pheromone.

(d) Data analysis
Statistical tests were performed using R v. 2.15.3 (R Core Team 2013) and SPSS v. 20.0 (Chicago, IL, USA). We used GLMs with appropriate error structure to analyse the laboratory as well as the field data. Body size was either included as covariate (pronotum width as continuous variable) or as fixed effect (small and large as categorical variables). With regard to the laboratory data, we adopted two approaches: (i) within each experiment, we tested for an effect of treatment groups on pheromone emission (quantity, ratio, duration) using categorical variables; and (ii) across all treatment groups, we explored which factors explained most of the variation in pheromone emission based on partial eta squared (η²). In the latter case, age and body size were included as continuous variables, nutritional state and parasite load as categorical variables. If the video recordings revealed that a male did not show any calling behaviour, it was excluded from the analysis (approx. 10% of the males of each treatment group). With respect to the field data, we performed statistical tests on the sum of the beetles each male attracted during the days it was exposed in the field to avoid pseudoreplication and zero-inflated data.

3. Results
(a) Pheromone emission
(i) Variation across treatment groups
There was a large variation in the emitted quantity of the two pheromone components between the males (n = 224).

(b) Body size and mass effects
The developing larvae reached their third instar mass of 70–120 mg (treatment group ‘small’) and transferred to a new container for pupation [37]. The remaining larvae were brought into the laboratory and the caught beetles were identified and sexed. In the case of the two control traps, the container was also filled with peat, but never baited with a male. During all four field experiments, control traps (n = 44) never caught any N. vespilloides individuals.

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(c) Field experiments
To test whether the differences in the quantity or ratio of the pheromone components between the above-mentioned treatment groups affected the males’ attractiveness to females, we conducted four field experiments in the summers of 2013 and 2014. In a deciduous forest in Ulm, Germany (48°25’N, 9°57’E), we established 22 pitfall traps (two traps served as controls; see the electronic supplementary material for a schematic view of the set-up of a trap) that were at least 20 m apart. Males that served as bait were placed in small containers half-filled with peat and hanging above the opening of the traps. The lid and the upper part of each container were perforated to allow pheromone dispersal. In each field study, we compared males of two of the above-mentioned treatment groups: (i) food-deprived (for 10.8 ± 0.2 days) versus vertebrate carrion-fed males (for 11.0 ± 0.2 days), (ii) young (22.5 ± 0.4 days) versus old males (62.5 ± 0.3 days), (iii) small (pronotum width: 3.79 ± 0.05 mm) versus large males (pronotum width: 5.49 ± 0.05 mm), and (iv) parasitized versus parasite-free males. Every male was exposed in the field for 3 days, with the exception of the parasitized and parasite-free males, which were only exposed twice because of lasting bad weather conditions. In general, males were not brought into the field if the daytime temperature fell below 13°C and on days with precipitation. Within each study, the males of the two compared treatment groups were deployed in alternate traps and for each of the following exposition events males were moved clockwise to the next trap to minimize any possible position effects. After each trapping day, the trap catches were brought into the laboratory and the caught beetles were identified and sexed. In the case of the two control traps, the container was also filled with peat, but never baited with a male. During all four field experiments, control traps (n = 44) never caught any N. vespilloides individuals.

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3. Results
(a) Pheromone emission
(i) Variation across treatment groups
There was a large variation in the emitted quantity of the two pheromone components between the males (n = 224).
The amount of EMH ranged from less than 0.1 ng to 887.5 ng and the amount of GA from 0.3 ng to 816.3 ng. The average EMH: GA ratio was 1.4:1, but the ratio ranged between 14.9:1 and 1:65.6. Nevertheless, there was a positive correlation between the amounts of the two components (Pearson correlation, $n = 224$, $r = 0.39$, $p < 0.0001$). Males engaged in pheromone emission for 138 ± 4 min (mean ± s.e.). The time invested in calling affected positively the amount of EMH emitted (Gaussian GLM, $F_{1,222} = 8.15$, $p = 0.005$), but not the amount of GA (Gaussian GLM, $F_{1,222} = 1.86$, $p = 0.18$).

\[(ii)\] Effect of nutritional state

The nutritional state affected significantly the amount of both pheromone components emitted ($n = 79$, Gaussian GLM, EMH, $F_{1,75} = 5.21$, $p = 0.008$; GA, $F_{1,75} = 9.75$, $p = 0.0002$), but only the quantity and not the quality of the nutrition had an effect (figure 1a). Both, mealworm fed as well as vertebrate carrion-fed males emitted a higher quantity of the two substances than starved ones (figure 1a). The ratio of the two components was not affected by nutritional state ($F_{1,75} = 1.64$, $p = 0.20$; figure 1b). The different feeding regimes also had no significant influence on the duration males engaged in pheromone emission ($F_{1,75} = 2.7$, $p = 0.07$), but because there was a tendency that vertebrate fed males tended to spend less time than mealworm fed ones, we reran the analysis on pheromone quantity controlling for the time invested in calling. This was done to assess whether the difference in pheromone quantity is only driven by a difference in time invested or by a different ability/efficiency to produce and release the chemical substances. The feeding regime had a significant effect on the amount of both components emitted per minute (EMH, $F_{1,75} = 3.71$, $p = 0.03$; GA, $F_{1,75} = 10.45$, $p < 0.0001$). Differences could be detected between vertebrate carrion-fed beetles and food-deprived beetles (Bonferroni corrected pairwise comparison, EMH, $p = 0.03$, GA $p < 0.0001$) with carrion-fed ones having higher release rates.

\[(iii)\] Effect of age

Age significantly affected the amount of GA ($n = 51$, Gaussian GLM, $F_{1,48} = 13.97$, $p = 0.0005$), but not that of EMH released ($F_{1,48} = 2.09$, $p = 0.16$) (figure 1c). More importantly, the ratio between the two components changed significantly with age, with younger males emitting relatively more EMH than GA and older ones emitting more GA than EMH (figure 1d; $F_{1,48} = 8.12$, $p = 0.006$). Therefore, the overall pheromone quantity (sum of EMH and GA) did not change significantly with age ($F_{1,48} = 1.52$, $p = 0.22$). Age did not affect the time invested in pheromone emission ($F_{1,48} = 0.01$, $p = 0.98$).

\[(iv)\] Effect of body size

Body size significantly affected the overall amount of pheromone emitted (sum of EMH and GA, $n = 51$, Gaussian GLM, GA, $F_{1,49} = 13.97$, $p = 0.0005$), but this was primarily because of an effect on EMH ($F_{1,49} = 7.15$, $p = 0.01$; figure 1c). Nevertheless, there was a tendency for larger males also to emit more GA ($F_{1,49} = 3.82$, $p = 0.056$; figure 1c). The ratio between the two components was not affected by body size ($F_{1,49} = 0.33$, $p = 0.57$; figure 1f). Body size also did not influence the duration of pheromone emission ($F_{1,49} = 1.376$, $p = 0.19$). However, because a previous study found a significant, albeit small effect ($R^2 = 0.06$) of body size on the time invested in calling (larger males called less) [32], we performed a linear regression including the males of all treatment groups and body size as continuous variable. In this case, body size had a significant effect on calling duration ($n = 224$, $R^2 = 0.02$, $F_{1,222} = 5.36$, $p = 0.02$; see the electronic supplementary material, figure S3) with larger males calling less than small ones.

\[(v)\] Effect of parasite load

Parasite load had a significant effect on the amount of GA emitted, with parasitized males emitting less than unparasitized ones ($n = 43$, Gaussian GLM, $F_{1,42} = 5.71$, $p = 0.02$; figure 1g). Parasite load did not influence EMH quantity ($F_{1,42} = 0.15$, $p = 0.70$) and therefore, the overall pheromone quantity was not affected (sum of EMH and GA, $F_{1,42} = 1.06$, $p = 0.31$). Neither the ratio of the two components ($F_{1,42} = 1.12$, $p = 0.30$; figure 1h) nor calling duration ($F_{1,42} = 0.49$, $p = 0.49$) was affected by parasite load.

\[(vi)\] Explaining variation across treatment groups

Across treatment groups, the four tested factors (nutritional state, age, body size and parasite load) explained 14.3% of the variation in EMH quantity, 25.0% in GA quantity and 8.5% of the variation in the ratio between the two components. Nutritional state and body size explained most of the variation in EMH quantity, nutritional state and parasite load in GA quantity and age in the ratio of the two components (table 1).

\[(b)\] Attractiveness in the field

In the overall 440 exposition events, males attracted 161 males and 608 females (effect of sex, Quasi-Poisson GLM, Wald-$X^2_{3,63} = 66.78$, $p < 0.0001$; sex ratio 1:3.8). Daytime temperature affected the number of beetles a male was able to attract (Quasi-Poisson GLM, Wald-$X^2_{3,63} = 9.66$, $p < 0.002$). The phenomeral differences we found in the quantity and ratio between the tested treatment groups affected the attractiveness of the males in the field. With the exception of body size class, all tested fixed effects (nutritional state, age and parasite load) had a significant effect on the number of females attracted. Vertebrate carrion-fed males attracted more females than starved ones ($n = 40$, Poisson GLM, Wald-$X^2_{1,37} = 30.64$, $p < 0.0001$; figure 2a), older males were more attractive than younger ones ($n = 40$; Poisson GLM, Wald-$X^2_{1,37} = 6.35$, $p = 0.012$; figure 2b) and traps baited with parasitized males caught less females than those baited with unparasitized ones ($n = 40$; Poisson GLM, Wald-$X^2_{1,37} = 4.44$, $p = 0.035$; figure 2d; note: if size as covariate is removed from the model, the effect of parasite load is no more significant). With respect to body size, there was a tendency that larger males attracted more females than smaller ones ($n = 40$; Poisson GLM, Wald-$X^2_{1,37} = 3.63$, $p = 0.057$; figure 2j).

When combining all significant attractive groups and comparing them with the less attractive ones, it becomes apparent that attractive males were characterized by a pheromone ratio that is shifted in favour of GA ($n = 146$, Gaussian GLM, $F_{1,144} = 43.73$, $p = 0.002$).

4. Discussion

A central prediction underlying handicap models of sexual selection is that male sexual traits should covary positively
Figure 1. The amount (ng; mean ± s.e.) and ratio (mean ± s.e.) of the two male sex pheromone components depending on (a,b) nutritional state (starved males \( n = 26 \), mealworm-fed males \( n = 27 \), vertebrate carrion fed \( n = 26 \) ), (c,d) age (young males \( n = 27 \), old males \( n = 24 \) ), (e,f) body size (small \( n = 25 \), large \( n = 26 \) ), and (g,h) parasite load (unparasitized \( n = 22 \), parasitized \( n = 21 \)). Grey bars represent the amount of ethyl 4-methyl heptanoate (EMH), white bars the amount of \((E)\)-geranylacetone (GA). See text for statistical analyses.
with condition, allowing male sexual traits to serve as reliable indicators of male quality in female mate choice [28,42,43].

Our results confirm that also long-range sex pheromones can exhibit considerable variation in chemical composition and release rates, and covary with a male’s condition. In fact, our results reveal a hitherto unknown high sensitivity of long-distance pheromone communication to components of the somatic state, a key determinant of individual condition. The *N. vespilloides* long-range sex pheromone appears to reliably reflect the actual nutritional state, age, the nutritional condition during development, which in turn affects adult body size, and parasite load. Both the quantities as well as the ratio of the two pheromone components were affected by our treatments, whereas the time invested in chemical signalling was largely unaffected by a male’s condition. Dietary stress had an effect on the absolute amount of both pheromone components emitted, body size on EMH, parasite load on GA and age affected the ratio between the two components. But more importantly, our field experiments show that the condition-dependent variation in pheromone characteristics directly influences the attractiveness of a male. For example, males in a good nutritional condition released a higher pheromone quantity and were able to attract three times as many females as food stressed ones.

**Table 1.** p-values and effect sizes ($\eta^2_p$) of linear models with nutritional state (categorical variable), age (continuous variable), body size (continuous variable) and parasite load (categorical variable) as explanatory variables. (EMH, ethyl 4-methyl heptanoate; GA, (E)-geranylacetone. The ratio between EMH and GA is considered as the dependent variable (n = 224). Significant p-values are given in italics.)

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**Figure 2.** The number of attracted females per male in four different field studies (shown are box-and-whisker plots). Tested were the attractiveness of (a) starved versus vertebrate carrion-fed males, (b) young versus old males, (c) small versus large males, and (d) unparasitized versus parasitized males. Sample size for each treatment group, $n = 20$. Each male was exposed 3 days in the field, the unparasitized and parasitized males 2 days. Note: owing to differences in weather conditions and time of the year, the caught rates between experiments are not directly comparable, comparison can only be made within an experiment. See text for statistical analyses.
ornaments [44]. Many signals, be they acoustic, visual or olfactory ones, have been found to be affected by nutritional state (e.g. [45]). With regard to chemical signals, also the expression of cuticular hydrocarbons (e.g. [46]) or courtship pheromones (e.g. [9,47]) has been found to be dependent on the quality or quantity of diet. The only study besides ours investigating the effect of nutrition on long-range pheromone emission has received a similar result. In the nectar feeding moth, Heliothis virescens, sugar-stressed females released a lower pheromone quantity than sugar-replete ones and this affected their attractiveness, at least under laboratory conditions [22]. Foster [48] argued that the likely cause of the condition-dependent pheromone emission is due to an effect of sugar level on glycolysis and consequently levels of acetyl-CoA, the common precursor of fatty acid-derived pheromone components. This simple explanation might also account for our finding.

In this study, N. vespilloides males shifted their ratio towards GA when getting older and older males were more than twice as attractive as young ones. Age-graded changes in sexual advertisement and a corresponding alteration in mating success has also been reported by other studies. Theoretical considerations have suggested that because older males have demonstrated their survival ability they should be of higher genetic quality and consequently be preferred by females [49]. Although this so-called ‘age indicator mechanism’ hypothesis has been debated in the past, as for example the accumulation of spontaneous germ-line mutations throughout the lifetime of an individual could see older males more likely to carry unconditionally deleterious viability mutations, it might still hold under many circumstances (e.g. [50,51]). Interestingly, a recent study by Nieberding et al. [52] found, similar to our study, an age related change in the relative composition of a pheromone, the courtship pheromone of the butterfly, Bicyclus anynana. Female butterflies showed an olfactory-mediated preference for middle-aged over younger males and the authors argued in line with the age indicator hypothesis that older male butterflies might be of superior genetic quality as they have survived longer. However, in our study all experimental males survived until the age of 60 days and consequently, there was no selection for viability going on. That older male burying beetles are much more attractive to females than young ones was not known before. A recent experiment by Benowitz et al. [53] demonstrated that older N. vespilloides males invested more in parental care than younger ones and females adjusted their care in response to the age of their partner, providing less care when paired with older males than younger ones. This intuitively leads to the idea that the age-related female mate choice observed in our study might have evolved because of this direct benefit a female obtains, at least if the pheromone emitter is a resource holder. However, this automatically raises the question of why younger males do not cheat by mimicking the pheromone of older males. Rather than informing females specifically about a signaler’s age, the most likely explanation of the observed phenomenon is an age related resource allocation strategy that maximizes lifetime reproductive success [54,55]. Owing to the lower residual reproductive value [56], older male burying beetles might invest more resources into current mating effort, leading to the production of a pheromone of higher quality than that produced by younger males.

We found that males infested with mites and nematodes emitted less GA than unparasitized ones, however, the overall pheromone quantity or ratio was not influenced by parasite load. In the field this pheromonal difference had a less pronounced effect on the attractiveness of males than the variation in quantity or ratio of the emitted signal. Nevertheless, unparasitized males attracted more females than parasitized ones. Since 1982, when Hamilton & Zuk [57] suggested that females might use secondary sexual displays to assess a male’s parasite burden and resistance, the number of studies on the subject exploded, some finding support and others finding little evidence in favour. Parasite mediated sexual selection has been also investigated under the framework of chemical communication [4]. In accordance with our finding, female grain beetles, Tenebrio molitor, for example, were less attracted to the odour of males infected by a tape-worm than uninfected males [58]. However, the chemical signal itself was never measured and it remains unknown how it is altered by parasite load. To our knowledge there is, apart from our study, no other insect study which has found that parasitism affected the scent and consequently, a male’s attractiveness, and at the same time determined the chemical signal involved. In burying beetles, choosy females should especially gain the direct benefits of avoiding associatively transmittable parasites. Nevertheless, the effect on the attractiveness was not as drastic as in the case of age and nutritional state. One possible explanation may lie in the design of our study. In contrast to the parasitized males, the uninfested ones were raised without parents (to avoid the vertical transmission of nematodes and mites) and such a lack of parental care during development probably affects the condition. In fact, in the laboratory as well as the field study, the unparasitized males were of smaller body size than the parasitized ones (Gaussian GLM, laboratory, F1,41 = 11.48, p < 0.002; field F1,41 = 17.77, p < 0.0001). This might also explain why the parasitized males emitted on average less pheromone quantities than the males of some of the other treatment groups (figure 1).

Last, but not least, our study revealed that development plasticity affects pheromone emission. Males that had restricted food access during development and were therefore characterized by a smaller body size released a lower quantity of EMH and were tendentially less attractive to females than males with unrestricted food access during the larval stage and consequently of larger body size. The effect on attractiveness was not significant, a result which indicates that the more important part of the chemical signal relevant to female choice is GA. Apart from this, our results are in line with the results of an olfactometer choice test in the burying beetle N. orbicollis, where females were more likely to be attracted to the odour of larger males [59].

Our study is, to our knowledge, the first which combines a multifactorial test of the effect of nutritional condition, age, body size and parasite load on the emission of a chemical signal with an attractiveness test under natural conditions. Collectively our results demonstrate that the production and release rate of a male’s long-range sex pheromone can be highly condition-dependent and be linked to factors central to the somatic state. This in turn affects significantly the attractiveness of a male in the field. Consequently, our study provides good evidence that long-range sex pheromones contain information beyond species and are a reliable indicator of a male’s quality. Although it is possible that the N. vespilloides pheromone specifically informs about different aspects of an individual’s condition, it is more likely that it reliably reflects the current amount of allocatable
resources in a broad sense [26]. By manipulating food availability, body size or parasite load, we influenced the ability of males to acquire and process resources that can be allocated to pheromone signalling. By manipulating age, we created a variation in resource allocation between maintenance of body function and pheromone signalling. The fact that especially the GA component of the sex pheromone was closely linked to condition and attractive males were characterized by a higher GA : EMH ratio than unattractive ones indicates that particularly the production of GA is costly and consumes resources. Overall, our study underlines the importance of understanding long-range sex pheromones as a form of reproductive effort within the framework of sexual selection and life-history theory, and of measuring such chemical advertisement throughout a male’s lifetime.

Data accessibility. All data from this study are publicly available in the Dryad digital repository: http://dx.doi.org/10.5061/dryad.qp2g5.

Competing interests. We have no competing interests.

Funding. J.C. was financed by a PhD grant (1405 LGFG-E). The study was funded by a grant of the German National Foundation to S.S. (STE 1874/3-1).

Acknowledgements. We are grateful to Eva Keppler for helping to rear and maintain the beetles, Andreas Fischer and Dennis Jauch for establishing the pitfall traps and Wolf Haberer, Stefan Jarau, Josef K. Müller, Joachim Ruther and Johannes Stökl for stimulating discussions during the experiments and manuscript writing. We are also very thankful to Joachim Ruther for providing the synthetic pheromone component.

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