Aposematism in the burying beetle? Dual function of anal fluid in parental care and chemical defence

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A short title: Aposematic display of burying beetle *N. vespilloides*?

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ABSTRACT

Burying beetles (*Nicrophorus vespilloides*) bear distinctive and variable orange-black patterning on their elytra and produce an anal exudate from their abdomen when threatened. During breeding, the anal exudates contribute to the antimicrobial defence of the breeding resource. We investigated whether the anal exudates also provide a responsive chemical defence, which is advertised to potential avian predators by the beetle's orange and black elytral markings. We found that that the orange-black elytral markings of the burying beetle are highly conspicuous for avian predators against range of backgrounds, by using computer simulations. Using bioassays with wood ants, we also showed that the burying beetle's anal exudates are aversive to potential predators. From these results, and other evidence in the literature, we conclude that the evidence for aposematism in the burying beetle is as strong as the evidence for many other classically aposematic species, such as defended Hymenopterans, ladybirds or poisonous frogs. Nevertheless, we also report unexpectedly high levels of individual variation in coloration and chemical defences, as well as sex differences. We suggest that this variation might be due partly to conflicting selection pressures, particularly on the dual function of the exudates, and partly to nutritional differences in the developmental environment. The ecology of the burying beetles (*Nicrophorus* spp.) differs markedly from better-studied aposematic insects. This genus thus offers new potential for understanding the evolution of aposematism in general.

INTRODUCTION

Prey individuals with toxic defences educate predators to avoid prey of similar appearance in future encounters (Speed et al. 2012). The avoidance learning rate of predators will be further enhanced if a defended prey bears a distinctive and memorable signal, such as bright coloration or a conspicuous display that predators can associate with the toxicity (i.e. aposematism) and so avoid attacking prey animals that carry that signal in future (Poulton 1890, Guilford 1990, Alatalo and Mappes 1996, Ruxton, Sherratt and Speed 2004). Predators have been shown to select for pronounced warning signals (Forsman and Merilaita 1999, Lindström et al. 1999, Lindstedt et al. 2008, Mappes et al. 2014) and signal uniformity (e.g., Mallet and Barton 1989, Joron and Mallet 1998, Kapan 2001, Beatty et al. 2004, Rowland et al. 2007) as well as high levels of chemical defence (Leimar et al.1986, Skelhorn and Rowe 2006, Ihalainen et al. 2007, Rowland et al. 2007) because all these characteristics enhance the efficiency of avoidance learning in the predator. Therefore, directional selection by predators is expected to decrease variation in the expression of these traits.

Nevertheless, it is widely acknowledged that both aposematic coloration (Ojala et al. 2007, Stevens and Ruxton 2011) and levels of chemical defence (Speed et al. 2012) can vary considerably among individuals. One explanation is that intrinsic constraints limit the response to directional selection from predators. For example, physiological costs of producing pigmentation (Grill and Moore 1998, Bezzerides et al. 2007, Ojala et al. 2007, Sandre et al. 2007, Lindstedt et al. 2010) or defensive chemicals (Higginson et al. 2011) can maintain variation in each of these traits. These costs can be further shaped by ecological (Grill and Moore 1998, Bezzerides et al. 2007, Ojala et al. 2007, Sandre et al. 2007, Lindstedt et al. 2010) and social (Daly et al. 2012) environments. In addition, the heritability of an aposematic trait and how it is genetically correlated with other traits can also influence the way in which it responds to directional selection from predators, and is a measure of the extent of variation in that trait (Lindstedt et al. 2016).

A different explanation for the persistence of variation is that aposematic coloration serves multiple functions, for example in thermoregulation (Brakefield 1985, Lindstedt et al. 2009, Hegna et al. 2013) or in mate choice (Summers et al. 1999, Maan and Cummings 2009). Thus, one of the key steps in understanding how this variation is maintained, has been to move the focus from the two-way interaction of the predator and prey towards considering the interactions of the prey species in greater complexity. This approach can identify additional selection pressures that may oppose directional selection imposed by predators, and thereby maintain variation in aposematic coloration (Friman et al. 2009, Nokelainen et al. 2011, Gordon et. al. 2015, Rojas et al. 2015, Crothers and Cummings 2013). Likewise, defensive compounds can also serve multiple functions and consequently be subjected to selection in different directions. For example, defensive toxins sequestered from the diet can sometimes be used to enhance immunological defence against parasites (Laurentz et al. 2012, Kollberg et al. 2014) or to produce pheromones at reproductive stage (Conner et al. 1981). Therefore to understand how variation in aposematic displays persists, despite directional selection from predators, it is important to establish new independent model species that differ ecologically and are therefore exposed to diverse selection pressures.

Here we consider whether the burying beetle (*Nicrophorus vespilloides*) exhibits aposematism and describe the extent of individual variation in its chemical defences and putative aposematic coloration. Burying beetles (*Nicrophorus* spp) are carnivorous Silphid beetles that are best known for their elaborate biparental care (Scott 1998, Eggert et al. 1998). They prepare carrion during reproduction, which they defend, maintain and feed to their offspring. Larvae of burying beetles feed on the carcass which parents smear with foul smelling dark brown anal exudate (Degenkolb et al. 2011), inhibiting microbial growth (Cotter et al. 2010, Cotter et al. 2013) and increasing larval survival (Arce et al. 2012).

The majority of *Nicrophorus* species also bear the distinctive orange-black coloration that is typical of other aposematic insects (Sillen-Tullberg 1985, Mappes and Alatalo 1997, Gamberale-Stille and Tullberg 1999, Exnerová et al. 2006; Sikes et al. 2002, Figure 1a.). Several reports in the literature suggest that the orange-black elytral markings of the burying beetle could function as part of a warning display (Morton Jones 1932, Lane and Rothschild 1965, Anderson and Beck 1985, Young 2014). Many Silphid beetles commonly feature in the diet of diverse vertebrates (Young 2014) and burying beetles specifically are potential prey for crows that scavenge upon carrion (Morton Jones 1932). Yet black Silphidae are more commonly described as prey than the orange and black *Nicrophorus* spp (Young 2014). Furthermore, Morton Jones (1932) reports that none of three different North American *Nicrophorus* spp were eaten by birds when presented alongside other Coleopteran species. The burying beetle species were unique among those species in being orange and black, whereas the species that were consumed were not. Further circumstantial evidence that the orange and black coloration of the burying beetle is aposematic comes from observations by Lane and Rothschild (1965), who describe a marked increase in agitation shown by captive blue tits (*Cyanistes caeruleus*) when orange-black *N. investigator* beetles were placed in their cages. These agitated behaviours are a characteristic avian response to several different species of aposematic insects (Rothschild and Lane 1960).

The orange-black colouration is just one component of a burying beetle's putative warning display. Upon handling, they also make a conspicuous 'buzzing' sound (Lane and Rothschild 1965, Hall et al 2013, C. Lindstedt pers obs). *N. investigator* even moves its abdomen in a style purported to resemble the stinging movements of bumble-bees (Lane and Rothschild 1965). These visual and auditory elements of the display accompany the responsive production of chemical defences. Upon handling, burying beetles produce the same anal exudate from their abdomen that is used by beetles to defend the carcass from rival microbes (Lane and Rothschild 1965, Cotter and Kilner 2010, Cotter et al. 2010, Degenkolb et al. 2011, Duarte et al. 2017). The odour of the exudate reportedly lingers for more than a year on unwashed 'inanimate objects' (Lane and Rothschild 1960), is very pungently putractive and has a very high pH (Degenkolb et al. 2011). In addition to compounds with antimicrobial properties, the anal exudate of *N. vespilloides* includes over 10 chemical compounds known to be repellent against invertebrates and vertebrates and some of these compounds can serve both antimicrobial and repellent functions (Degenkolb et al. 2011). Many of these repellent compounds have been found also in the defensive glands of other Coleopteran and Hymenopteran species (Degenkolb et al. 2011) suggesting that they could function in chemical

defence of the adult beetles as well as assist in defending the carcass from the rival microbes (Duarte et al. 2017). During the breeding chemical profile of the anal exudate changes as the number of antimicrobial compounds produced by *N. vespilloides* beetles increases. However, the repellent compounds are still present in the anal exudate during the breeding (Degenkolb et al. 2011, Haberer et al. 2014).

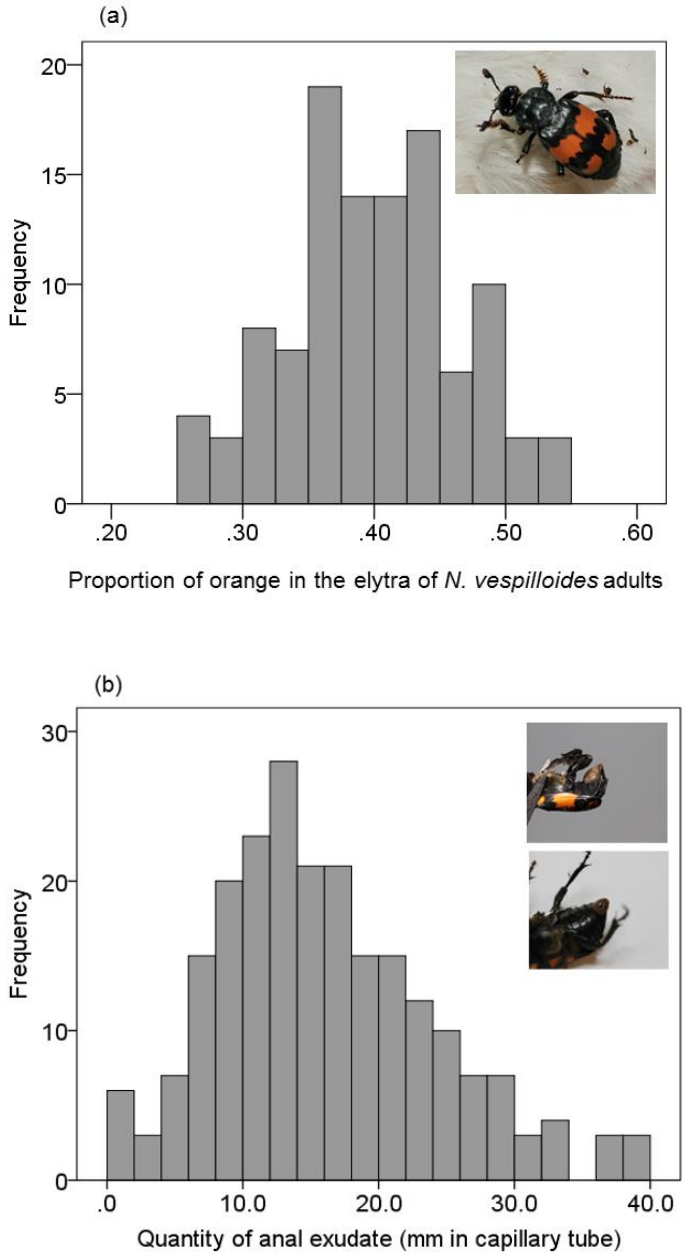

Figure 1. Individual variation in the aposematic signal for a) the size of the striking orange elytral pattern, and b) the quantity of anal exudate *N. vespilloides* produces when disturbed.

We have three aims in this paper: 1) to determine the salience of the burying beetle's orange and black coloration to avian predators, against a range of natural backgrounds (Stevens 2007); 2) to test whether the chemical defences in the burying beetle's anal exudates are aversive, using a standard bioassay with ants; 3) to quantify phenotypic variation and broad-sense heritability in each of these traits. Aims 1) and 2) are linked to understanding the nature of selection acting on the burying beetle's elytral markings and chemical defences, whereas aim 3) helps to understand how these traits might respond to selection.

METHODS

N. vespilloides colony

We used burying beetles from an outbred laboratory population established in 2005 at Cambridge University, and supplemented annually with wild-caught individuals from sites close to Cambridge, UK. Adults were housed alone in plastic boxes (12x8x2 cm) filled with moist soil, food (minced beef) was available *ad libitum* and boxes were kept at a constant temperature of 21 ˚C and 16h:8h light:dark cycle. Boxes were cleaned twice a week and at the same time old food was replaced. For breeding, unrelated pairs were placed in plastic boxes (17x12x6 cm) half filled with moist soil, provided with a freshly thawed mouse carcass (21.94 +- 0.33 SE g, range 15-35g) and kept in the dark. Larvae disperse from the carcass ca. 8 days after hatching and sexual maturity is reached ca. 5 weeks after dispersal.

Aim 1: Quantifying the salience of the orange-black coloration to avian predators

To test how insectivorous birds perceive the colour, luminance and contrast of colour patterns of beetles against various natural backgrounds, we used an avian vision model that assumes that receptor noise limits visual discrimination (Vorobyev and Osorio 1998, Vorobyev et al. 1998). This model is included in the Image Calibration and Analysis Toolbox (Troscianko and Stevens 2015). First, the regions of interest (ROIs) from the normalized and linearized images of beetles and different backgrounds (twigs from Scotch pine; stones; skin of museum samples of bank vole (*Myodes glareolus*); and birch leaf (*Betula pubescens*) were converted to predicted photoreceptor responses of single and double cone types of a blue tit (Hart, Partridge and Cuthill 2000, Hart 2001, Troscianko and Stevens 2015) by using a mapping function of the Image Calibration and Analysis Toolbox. This mapping is highly accurate compared to reflectance-based calculations of predicted cone responses (Stevens and Cuthill 2006, Pike 2011, Troscianko and Stevens 2015). Colour vision in birds stems from the four single cone types (Cuthill 2006), while the double cones are likely responsible for luminance-based tasks (Vorobyev et al. 1998, Osorio and Vorobyev 2005), such as detecting achromatic contrast differences. The vision model converts the ROIs to cone-catch data, i.e. to the relative photon catches of a blue tit's four single cones: longwave (LW), mediumwave (MW), shortwave (SW) and ultraviolet (UV) cones, as well as to luminance values based on the double cone sensitivity. To analyse the phenotypic and genetic variation in colour of the beetles, we calculated

saturation values (colour richness) similar to (Arenas et al. 2015) and brightness (double cone sensitivity) for the ROIs of the first and second orange stripes and black pattern.

To analyse the conspicuousness of burying beetles to avian predators, colour and luminance discrimination models (Vorobyev and Osorio 1998) were conducted on cone-catch data of backgrounds and colour patterns of beetles with ImageJ Toolbox (MICA) (Troscianko and Stevens 2015). We first tested how well blue tits can discriminate between the orange and black pattern elements of beetles against various natural backgrounds. Family mean values of cone catch data for the first and second orange stripe and black pattern of colour and luminance were compared against different backgrounds. To test the intrapattern contrast of orange and black pattern elements, we compared mean values of cone catch data of the first and second orange stripes and the black pattern within an individual. Finally, to test whether birds can detect the variation in conspicuousness of the colouration among *N. vespilloides* families, we compared the family mean values of cone catch data of different pattern elements among families. The discrimination model uses units called just noticeable differences (hereafter, JNDs) where values <1-3 indicate that the two colours are likely indistinguishable under optimal light conditions and values >3 indicate that two objects are likely discriminable and by increasing degrees: the greater the value the more distinguishable the colours should be even under less optimal light conditions (Siddiqi et al. 2004). Four single cones were used for the colour discrimination model, whereas the luminance discrimination model was based on the double cones (Siddiqi et al. 2004). In the colour discrimination model, a Weber fraction of 0.05 was used for the most abundant cone type, and the relative proportions of cone types in the blue tit retina (longwave = 0.96, mediumwave = 1 , shortwave = 0.85, and ultraviolet sensitive = 0.46). A Weber fraction 0.05 was also used for modelling luminance discrimination using the double cones (Siddiqi et al. 2004, Sandre et al. 2010).

Aim 2: Measuring noxiousness of the anal exudates using bioassays with ants

Ants are important predators of insects (Molleman et al. 2010, Pavis et al. 1992, Way and Khoo 1992) and one of the most important competitors with burying beetles for carcasses (Scott 1998). Ants can also reliably recognize the presence of repellent compounds, and thus are ideal for conducting bioassays of potentially noxious substances (Deroe and Pasteels 1977, Hare and Eisner 1993, Dyer and Floyd 1993). Often deterrence against ants correlates with the deterrence against avian predators (Lindstedt et al. 2006 and 2011, Lindstedt et al. 2008, Reudler et al. 2015).

We collected anal exudates from approximately 100 sexually matured beetles from the lab stock reared in standardized conditions. Anal exudates were collected by poking the abdomen of each beetle gently 1-2 times from the ventral side with a capillary tube, which caused the beetles to spray the fluid. Fluid was collected into the capillary tubes and pooled into 3 separate Eppendorf tubes and placed in a freezer (-20 C). Before presentation to the ants, samples were thawed and then diluted with a 20 % sugar solution (20% sugar, 80% water) to motivate the ants to feed on the solution. We conducted two separate bioassays with two concentrations to test how the variation in the concentration affected ants' willingness to feed on it. In the first assay, we tested the deterrence of anal exudate by offering 10% exudate solution (10% anal exudate / 90% sugar water) and palatable control solution (10% of plain

water / 90% sugar water) to ants simultaneously. In the second assay, we used 1 % exudate solution (1% anal exudate / 99% sugar water) and 1% control solution (1% plain water / 99% sugar water).

Bioassays were conducted similar to Reudler et al. 2015. We performed tests with the 10% exudate and control solutions on 10 different ant (*Formica sp.)* nests in the field in central Finland (62 °N, 26° E) in sunny and warm weather (15-20°C). To standardize the potential variation in activity and ant traffic among ant nests, we presented ants simultaneously with droplets of exudate and control solutions. In the vicinity of each nest we chose a spot on the trail where ant traffic was about 10 to 20 individuals/minute. We put 10 µl of both the control and exudate solutions close to each other (<2 cm) on a transparent, sterilized plastic circle (4 cm in diameter) and offered it to the ants. We repeated the assay three times per nest, each on a different ant trail, and order of control and exudate droplets was changed between repetitions. During the experiment we calculated the number of ants drinking from the different solutions in 1 minute intervals during the 10 minutes and counted the mean number of ants that drank each type of fluid to measure its aversiveness (Reudler et al. 2015). Recording was started after the first ant worker arrived at either of the droplets. We repeated exactly the same procedure one week later with the 1 % control and exudate solutions, using five of the same nests as those used in 10% solution assays. All of the experiments were run within a 2 week period in August 2010.

Aim 3: Variation in chemical defence, orange elytra pattern and colour

We set up 25 pairs for breeding with a carcass (mean ± S.E. carcass mass given above, in description of breeding conditions). Both parents remained with the offspring until larvae dispersed, at which point they were discarded and the larvae were transferred to separate individual boxes to pupate. After eclosion, when individuals had developed the typical black and orange coloration, they were sexed and the quantity of the defence fluid was measured by poking the abdomen of each beetle gently 1-2 times from the ventral side with a capillary tube, which caused the beetles to spray the fluid. Fluid was collected into the capillary tubes and the quantity produced was measured. Beetles were then weighed and killed by storing them in a freezer for -20˚C. Frozen individuals were photographed after the experiment using a calibrated Fuji IS digital camera, which records both ultraviolet and human visible signals. From the photographs, the size of the elytra and orange patterns were measured with the ImageJ - program and hue and brightness of the pattern components analysed with the Image Calibration and Analysis Toolbox (Troscianko and Stevens 2015) with the method described above. In total, we aimed to measure the anal exudate volume from 5 females and 5 males from each of 25 families. One individual was left out from the analyses as we failed to measure the defensive response and for some families the number of offspring was less than 10 individuals. We sampled 3-10 individuals per family (mean 8.96 ± 0.35 S.E.) yielding 224 samples in total. Signal size and colour measurements were taken from 98 individuals across 14 families.

Statistics

To take into account possible variation in ant behaviour and activity among the nests and trails, we used pairwise t-tests to compare the mean number of ants feeding on exudate solution and

control solution for the bioassays with 10% concentration and 1% exudate and control solutions. Data from the ant experiments were analysed using IBM SPSS Statistics 20 (IBM Corporation, NY, USA).

We used general linear mixed models to analyse the relationship between sex and elytra size on the volume of anal exudate produced, and on each of the other measures of the aposematic signal: the size, brightness and saturation of the two orange stripes and the brightness and saturation of the black portions of the elytra. The fit of each model was checked by examination of the residuals. The two measures of the black colour were log transformed as inspection of residuals suggested deviations from a normal distribution. We applied model selection by comparing nested models with ANOVA. In all models, family was included as a random effect to account for variation due to genetic or maternal effects. Variance components from the random model associated with family (V_G) and residual variance (V_R) were used to calculate broad sense heritability (H^2) for each of the traits, where $H^2 = V_G/(V_G + V_R)$. For mixed models, we used the "lme4" package in R (Bates et al. 2013); *t*-statistics, degrees of freedom and *p*-values were calculated using Satterthwaite's approximation, with the "lmerTest" package in R (Kuznetsova et al. 2013). The significance of the random effects was tested against a Chi-squared distribution. The coefficients of genetic (CV_G) and residual (CV_R) variation were calculated using untransformed data, as values for transformed data are meaningless (Houle 1992).

RESULTS

Aim 1: Quantifying the salience of the orange-black coloration to avian predators

The avian vision model for blue tits shows that avian predators should be able to discriminate orange and black patterns of burying beetles against various backgrounds (green leaves, grey stones, twigs, vole fur) both in terms of colour and luminance (Table 1). Within-pattern contrast of black and orange patterns was high and clearly visible for birds both in terms of colour and luminance (Table 1). Also, interestingly, the differences in the mean contrast values of the hue of pattern elements among families should be clearly visible for avian predators (Table 1). However, variation in the luminance contrasts of orange pattern elements among families are probably more difficult for birds to discriminate (Table 1).

Aim 2: Measuring noxiousness of the anal exudates using bioassays with ants

We found that significantly more ants took the sugar water than sugar water mixed with anal exudate of beetles (10% exudate: 90% sugar water) ($t = -6.678$, $n = 30$, $p < 0.001$). However, when the concentration was decreased (1% exudate: 99% sugarwater), we could not detect any difference between the treatments ($t = -0.400$, $n = 15$, $p = 0.695$) (Fig. 2). Thus, a higher concentration of anal exudates resulted in better defence against ants.

Table 1. Discrimination values (JND) for colour (hue) and brightness of different elytra pattern elements of *N. vespilloides* (values are average of 11 families) against various natural backgrounds according to model by Vorobyev et al. 1998. Variation in conspicuousness among families is based on two-way comparisons of average discrimination values of 11 families. Brackets show the range between minimum and maximum values). Values > 3 are easy to tell apart in most conditions.

Comparisons of elytra pattern elements	**Colour (hue)**	**Brightness**
	Mean discrimination value (min-max)	Mean discrimination value (min-max)
Intrapattern contrasts		
Black versus orange in the first stripe	90.09 (56.69-110.83)	34.20 (26.93-39-05)
Black versus orange in the second stripe	94.88 (77.39-111.17)	31.03 (25.59-35.86)
Orange in the first stripe versus orange in the second stripe	13.85 (53.31-3.00)	3.17 (0.20-7.06)
Elytra pattern contrasts against natural backgrounds		
Black against the pine twig	16.36 (3.66-23.26)	26.27 (19.53-30.61)
Black against the birch leaf	44.95 (34.07-53.40)	36.27 (26.09-43.20)
Black against the stone	12.17 (6.81-20.94)	39.63 (31.66-46.22)
Black against the bank vole fur	14.71 (3.12-24.07)	28.38 (13.94-38.77)
Orange in the 1st stripe against the pine twig	76.77 (49.94-94.63)	7.93 (3.47-12.70)
Orange in the 1st stripe against the birch leaf	58.55 (40.93-71.28)	3.22 (0.04-9.13)
Orange in the 1st stripe against the stone	93.93 (63.11-114.46)	5.47 (0.37-12.14)
Orange in the 1st stripe against the bank vole fur	79.61 (49.49-100.83)	6.18 (0.05-18.30)
Orange in the 2nd stripe against the pine twig	81.41 (72.12-101.33)	4.82 (0.44-8.27)
Orange in the 2nd stripe against the birch leaf	58.31 (46.57-82.53)	5.34 (0 - 13.04)
Orange in the 2nd stripe against the stone	98.97 (87.29-121.26)	8.60 (3.85-16.05)
Orange in the 2nd stripe against the bank vole fur	84.39 (72.03-107.53)	4.42 (0.08-13.87)
Variation in conspicuousness among families		
Black	8.60 (1.84 - 24.89)	3.78 (0.07-11.07)
Orange in the first stripe	53.72 (2.86-18.82)	2.33 (0.01-9.23)
Orange in the second stripe	11.67 (1.79-34.42)	2.66 (0.02-8.71)

Aim 3a): Variation in chemical defence

Body size was not associated with the amount of anal exudate beetles produced (Table 2). However, females produced significantly higher quantities of fluid than males (REML: Estimate= 2.89 $\pm$ 0.855; Table 2; Fig. 3). The amount of anal exudate produced upon disturbance showed a moderate broad-sense heritability of 0.38 (Table 3).

Table 2. ANOVA results for the fixed effects of sex, elytra size and their interaction on the amount of eclosion fluid produced and elements of the aposematic signal.

Trait	Sex	Elytra size	Sex:Elytra size
Eclosion fluid	**$F_{1,198}$ = 11.4** **P <0.001**	$F_{1,99}$ = 2.06 P = 0.15	$F_{1,94}$ = 2.88 P = 0.09
Orange total (mm)	$F_{1,98}$ = 0.17 P = 0.68	**$F_{1,109}$ = 92.68** **P <0.001**	$F_{1,96}$ = 0.61 P = 0.44
First stripe (mm)	$F_{1,98}$ = 0.01 P = 0.93	**$F_{1,110}$ = 78.03** **P <0.001**	$F_{1,96}$ = 0.01 P = 0.92
Second stripe (mm)	$F_{1,99}$ = 1.12 P = 0.29	**$F_{1,110}$ = 65.95** **P <0.001**	$F_{1,97}$ = 2.56 P = 0.11
Brightness stripe 1	$F_{1,87}$ = 1.05 P = 0.31	**$F_{1,35}$ = 11.13** **P = 0.002**	$F_{1,87}$ = 0.00 P = 0.96
Saturation stripe 1	$F_{1,85}$ = 0.49 P = 0.48	**$F_{1,41}$ = 4.70** **P = 0.036**	$F_{1,86}$ = 1.26 P = 0.26
Brightness stripe 2	$F_{1,88}$ = 1.36 P = 0.25	$F_{1,24}$ = 0.95 P = 0.34	$F_{1,86}$ = 0.00 P = 0.98
Saturation stripe 2	$F_{1,87}$ = 0.04 P = 0.84	**$F_{1,39}$ = 24.83** **P < 0.001**	$F_{1,87}$ = 0.23 P = 0.63
Brightness black	**$F_{1,87}$ = 4.27** **P = 0.04**	$F_{1,32}$ = 0.59 P = 0.45	$F_{1,85}$ = 1.01 P = 0.32
Saturation black	$F_{1,87}$ = 1.09 P = 0.30	$F_{1,35}$ = 0.10 P = 0.76	$F_{1,85}$ = 1.68 P = 0.20

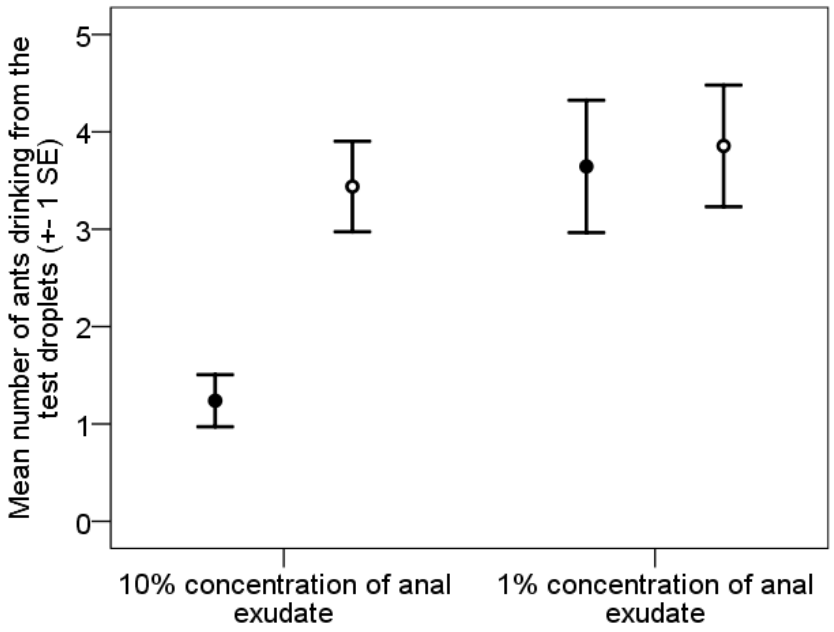

Figure 2. Mean number of ants (+- 1 SE) drinking the control solution (20% sugarwater) indicated by open circles and 10% (10% anal exudate: 90 % sugarwater) and 1 % experimental solution (1 % anal exudate: 99% sugarwater) indicated by closed circles.

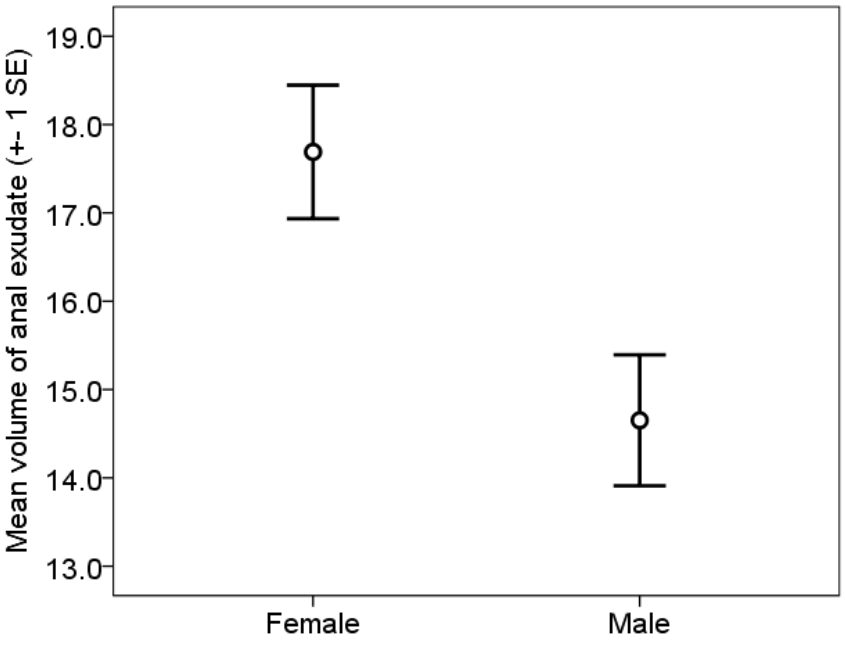

Figure 3. Mean volume of anal exudate produced under disturbance by *N. vespilloides* females and males.

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Aim 3b): Variation in orange elytra pattern and colour

The total size of the orange elytra pattern did not differ between males and females but did increase with the size of the elytra (REML: Estimate = 0.42 ± 0.043; Table 2). The same pattern was found if the two orange stripes were considered independently (Table 2).

The brightness of the first orange stripe was significantly higher than the second (Paired t-test, t_{184}=6.9, P<0.001), although the saturation of the stripes did not differ (Paired t-test, t_{187}=0.69, P=0.49). However, whilst the saturation of both stripes increased with elytra size (REML: Stripe 1 estimate = 0.0006 ± 0.00027, stripe 2 estimate = 0.0010 ± 0.00019; Table 2), the brightness of the first stripe decreased as beetles got bigger (REML: Estimate = 68.13 ± 20.42; Table 2) and elytra size had no effect on the brightness of the second stripe (Table 2). The brightness of the black sections of the elytra were lower in males (REML: Estimate = -195.12 ± 89.21; Table 2) but were not affected by the size of the beetles (Table 2).

The size of the orange pattern, both in total and in the first and second stripe separately, showed high broad sense heritabilities (range = 0.57-0.65, Table 3). None of the measures of saturation and brightness was significantly heritable, though the saturation of the first stripe and the brightness of the black were marginally non-significant (range – 0.03-0.12, Table 3).

Table 3. Genetic and residual variance in the amount of eclosion fluid produced and elements of the aposematic signal as estimated by REML using the lmer package in R. V_G represents additive, dominance and epistatic variation. H^2 is the broad sense heritability estimate V_G/ V_R., CV_G and CV_R are the coefficients of genetic and residual variance respectively. Significance was tested with chi squared. P>0.10 $^{n.s.}$, P<0.10^{+}, P<0.001 ***

Trait	No. families	$V_{G\ (SD)}$	$V_{R\ (SD)}$	H^2	chi	CV_G	CV_R
EF	25	24.53 (4.95)	40.02 (6.33)	0.38	54.6***	20.1	15.8
Orange total (mm)	14	12.09 (3.48)	6.51 (2.55)	0.65	81.8***	28.8	39.2
First stripe (mm)	14	5.57 (2.36)	3.15 (1.78)	0.64	75.3***	42.4	56.3
Second stripe (mm)	14	1.68 (1.30)	1.26 (1.12)	0.57	65.3***	77.2	89.1
Brightness stripe 1	11	158064 (398)	2244302 (1498)	0.06	1.25	0.25	0.07
Saturation stripe 1	11	4.84e-05 (0.007)	0.00019 (0.014)	0.12	2.93 $^{+}$	14374	5323
Brightness stripe 2	11	99341 (315)	1660766 (1289)	0.06	2.07 $^{n.s.}$	0.32	0.08
Saturation stripe 2	11	1.81e-05 (0.004)	1.92e-04 (0.014)	0.09	0.39 $^{n.s.}$	23525	7206
Brightness black	11	18432 (136)	191849 (438)	0.09	2.43 $^{+}$	0.74	0.23
Saturation black	11	1.73e-04 (0.013)	0.002 (0.045)	0.08	1.64 $^{n.s.}$	7604	2224

DISCUSSION

Our first aim was to determine the salience of the burying beetle's orange and black coloration to avian predators, against a range of natural backgrounds. We found that these elytral markings of the burying beetle are highly conspicuous for avian predators. Objectively, the burying beetle's orange-black elytral patterning does not differ much from the orange-black patterning of other insect species which are widely recognised to be aposematic, such as *Arctia plantaginis* larvae (Lindstedt et al. 2008) and adult females (Lindstedt et al. 2011), ladybirds (Linas et al. 2015) or *Heliconius* butterflies (Langham 2004). Furthermore, some *Nicrophorus* species have also been suggested to be Müllerian mimics of wasps and bumble-bees (Morton Jones 1932, Milne and Milne 1944, Lane and Rothschild 1965, Anderson and Beck 1985), each of which is known to deter avian predators. These observations, in conjunction with earlier reports that birds find burying beetles highly aversive (summarised in the Introduction), strongly suggest that many species of burying beetle use their orange and black elytral patterns as part of a warning display, and that these markings are under selection from avian predators. Collectively the evidence for aposematism (visual analyses about the conspicuousness of coloration combined with the bioassay for toxicity and presence of responsive defence) in the burying beetle is a strong as the evidence for a many other classical examples of an aposematism and Müllerian mimics such as defended Hymenopterans (e.g. Penney et al. 2012, Wilson et al. 2012), poison frogs (e.g. Maan & Cummings 2012), ladybirds (e.g. Linas et al. 2015) or marine opisthobranchs (e.g. Cortesi and Cheney 2010).

We fulfilled our second aim by demonstrating that the chemical defences in the burying beetle's anal exudates are aversive, using a standard bioassay with wood ants (Reudler et al. 2015). In our experiments, a greater concentration of anal exudate resulted in better defence against ants, suggesting that the production of more potent exudates should enhance the efficacy of the beetle's chemical defence. The most conservative interpretation of these results is that burying beetles can defend themselves, and their carrion breeding resource, specifically against ants (e.g Scott et al 1987). However, deterrence against ants often correlates with the deterrence against avian predators in chemically defended species (Deroe and Pasteels 1977, Hare and Eisner 1993, Dyer and Floyd 1993, Lindstedt et al. 2006 and 2011, Lindstedt et al. 2008, Reudler et al. 2015). Therefore a wider possible interpretation is that burying beetles possess a general chemical defence against their potential predators. If this is true, it means that the burying beetle's anal exudates serve a dual function by contributing to two public resources: the defence of the carrion breeding resource against microbes (Duarte et al. 2016, Duarte et al. 2017) as well as the collective education of potential predators via warning displays (Speed et al. 2012). The constituents within the exudates are therefore likely to be subjected to differing selection pressures from each of these two functions.

These contrasting selection pressures might explain why we found high levels of individual variation in the volume of anal exudate produced. We also found a sex difference in the volume of anal exudates produced by burying beetles, though this is harder to explain. One possibility is connected with a sex difference in the function of the anal exudates, namely the antimicrobial defence of the carcass during reproduction. When preparing carrion for reproduction, burying beetles strip the body of fur or feathers, mould the the flesh into a ball

and smear it with antimicrobial anal exudates (Scott 1998, Rozen et al 2008, Cotter and Kilner 2010). Females contribute exudates with greater lytic activity than males to this defence (Cotter and Kilner 2010), and likewise secrete a greater volume of fluid than males when handled (this study). In future work it would be interesting to test whether, and in what direction, the antimicrobial activity is correlated with the repellence of the anal exudate.

A second possibility is that females secrete a greater volume of exudates when threatened because they are more vulnerable to attacks by potential predators. The carcass is an attractive resource to scavengers and yet attended by parents during reproduction. Females spend much longer than males associated with the carcass, since males leave the brood before larval development is complete (Scott 1998, Boncoraglio and Kilner 2012, de Gasperin et al. 2015). Females might therefore be more likely than males to encounter a potential predator, and this could explain why they produce more exudate when threatened. However, it is important to remember that we only measured the quantity of the fluid here. Thus, it is possible that males can compensate the lower amount of exudate by making it more noxious. In addition, we measured the quantity of fluid only once per individual and therefore we do not know if males are not able to produce more fluid or if they were just not willing to do so.

Whatever the reason for this sex difference, it suggests that higher volumes produced by females are potentially contributing more to the education of naïve predators than are males. Understanding the evolutionary significance of this difference will again come down to understanding the cost of the chemical defence. If females can produce more anal exudates than males for the same cost, then they are simply contributing to a public good in relation to their ability to pay, as predicted by theory (Frank 2010, Duarte et al. 2016). But if females are paying a higher cost for educating predators with their greater noxiousness then they are vulnerable to exploitation by males, who can potentially gain the same protection from predation but for a lower price. If this is indeed the case then the puzzle for future work is to explain why such exploitation persists.

We have assumed throughout that an individual's chemical defences are fixed in their potency and producing higher volumes is favoured for both parental care and chemical defence. Yet burying beetles can flexibly adjust the antimicrobial function of their anal exudates, up-regulating it only when reproducing and varying its potency in relation to their partner's contributions, and the scale of microbial threat to the carcass (Cotter and Kilner 2010, Cotter et al. 2010, Haberer et al. 2014). Although a plastic response like this cannot account for our measurements, because they were taken when beetles were not breeding, it would be interesting to test whether burying beetles are similarly capable of adjusting the concentration of fluid they exude when threatened, increasing the potency when the threat of attack is greater during reproduction on the carcass.

We found high levels of individual variation in elytral markings as well as in the volume of the exudates produced. Each might be attributable to an environmental or genetic constraint upon the production of each trait (Lindstedt et al. 2009, Lindstedt et al. 2016). To understand how variation in colour patterning and chemical defences arise we need to know more about the costs associated with these traits and how they are affected by early developmental environment of the beetles. In addition, it is important to know the chemical structure of pigments (e.g. Lindstedt et al. 2010b) and defence chemicals. Burying beetles are

carnivorous insects and their diet is scarce in antioxidants in comparison to herbivores (Olson and Owens 1998, Bortolotti et al. 2000). If the orange pigmentation is protein based, it might be relatively cheaper for a carnivore to produce than if the orange colour was dependent on carotenoids or flavonoids, which are much rarer in a carnivorous diet. In the latter case, burying beetles would need to synthesize pigments and defensive chemicals *de novo* and this may require energy and resources that are scarce in their diet. It might even involve recruiting microbial symbionts for this purpose (Moran and Jarvik 2010, Tsuchida et al. 2010). For the repellent compounds in anal exudate it is already known that they are mainly based on amino-acids (Degenkolb et al. 2011) and therefore likely to be synthesized *de novo* and constrained by the quality and availability of proteins in the diet.

Since variation in both burying beetle elytral markings and their anal exudates are potentially connected to diet, it would be interesting in future work to determine the extent to which individual variation can be explained by variation in the level post-hatching care received during early life. Our calculations suggest that the broad-sense heritability of each trait is relatively high, but our measures cannot partition out the separate effects of the developmental environment from inherited genetic variation. Previous work on other burying beetle traits has found that once the developmental environment is accounted for, trait heritability is relatively low (e.g. Lock et al 2004). Nevertheless, this does not necessarily mean that traits cannot respond to selection by predators or other agents (Kilner et al 2015, Jarrett et al 2017) and exactly how this happens will need to be determined more explicitly in future work.

In conclusion, our experiments, together with evidence in the literature, strongly suggest that the orange-black colouring of the burying beetle's elytra serves an aposematic function and anal exudate of beetles can serve multiple functions in antipredator defence and parental care. The challenge for future work is to deduce the costs associated with producing both the colourful display and the chemical defence so as to better explain the intra-specific variation we have found. We also need more information about the selection pressures that visual predators, namely birds, impose on the colour and size of the pattern as well as toxicity of the anal exudate. We note that not all *Nicrophorus* species are orange and black, and that some entirely black species still produce a malodorous fluid when handled (e.g. *N. humator*, R. M. Kilner pers. obs.). Therefore, the genus *Nicrophorus* in general provides the opportunity to test: 1) why some chemically defended species have evolved conspicuous marking while others have not; 2) how aposematism is linked to different life-history strategies and social behaviour; and 3) how individuals can balance their contributions to two different sorts of public goods: chemical defence and antimicrobial defence of a carrion breeding resource.

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DATA ACCESSIBILITY STATEMENT

Analyses reported in this article can be reproduced using the data sets provided by Lindstedt et al. 2017. Data for the individual variation in the chemical defence, elytra colour and size of the markings in *Nicrophorus vespilloides*, will be released on 1st of August 2018. However, all reasonable requests for materials will be respected before that time on request.

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