How to fight multiple enemies: target-specific chemical defences in an aposematic moth

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Abstract

Animals have evolved different defensive strategies to survive predation, among which chemical defences are particularly widespread and diverse. Here we investigate the function of chemical defence diversity, hypothesising that such diversity has evolved as a response to multiple enemies. The aposematic wood tiger moth (*Arctia plantaginis*) displays conspicuous hindwing colouration and secretes distinct defensive fluids from their thoracic glands and abdomen. We presented the two defensive fluids from lab-reared moths to two biologically relevant predators, birds and ants, and measured their reaction in controlled bioassays (no information on colour was provided). We found that defensive fluids are target-specific: thoracic fluids, and particularly the 2-sec-butyl-3-methoxypyrazine (SBMP) which they contain, deterred birds, but caused no aversive response in ants. In contrast, abdominal fluids were particularly deterrent to ants, while birds did not find them repellent. Our study is the first to show evidence of a single species producing separate chemical defences targeted to different predator types, highlighting the importance of taking into account complex predator communities in studies on the evolution of prey defence diversity.

Keywords: predator-prey interactions, chemical defences, aposematism, pyrazines

1. Introduction

Predation is a key agent of natural selection in prey species[1]. In order to survive in a multi-predator world, animals have evolved different defensive strategies that vary in their nature and efficacy in relation to predator sensory abilities and attack tactics[2-4]. Which strategy, or set of strategies, is used as a defence depends on the benefits granted and the costs incurred. However, the strategy employed must ultimately aim to prevent the completion of a predation event as early as possible in the predation sequence (i.e. detection, identification, approach, subjugation and consumption *sensu* Endler 1986[2]).

Aposematic organisms gain protection from predators by displaying colourful warning signals, which are coupled with some form of unprofitability[5]. This unprofitability is frequently related to the possession of chemical defences that can be unpalatable or even toxic[1, 5-7]. Predators learn to associate the warning signal with a bad experience when tasting the prey, and remember it in subsequent encounters (e.g.[7-11]), leading to an aversive behaviour towards that particular prey.

Chemical defences in aposematic species can also vary in composition, quantity, and quality and, although this variation is relatively common[12-20], it has been addressed much less frequently than variation in warning signals[21]. Because these defences are usually effective during the subjugation and/or consumption stages of the predation sequence[2], chemical defences are often referred to as secondary defences. They can deter predators in a variety of ways, including volatile irritation, distastefulness or even toxicity[12]. Chemical defences can be costly[22-24], as they involve processes ranging from the sequestration of active compounds, either with or without subsequent modifications, through to their synthesis *de novo*[12, 24]. Therefore, these defences are expected to evolve only if needed, and to be effective against a wide array of predators[14].

The wood tiger moth (*Arctia (*formerly *Parasemia*) *plantaginis*[25]) is an aposematic arctiid species distributed across the Holarctic region[26]. Males display either white or yellow hind wings (except for the Caucasus, where males are mostly red), whereas females present a hindwing colouration that varies continuously from yellow through to red. This warning colouration is coupled with the possession of two types of seemingly distasteful chemical secretions[27, 28]. One type (hereafter 'neck fluids') is secreted from the prothoracic (cervical) glands, and the other one (hereafter 'abdominal fluids') is released from the abdominal tract. These fluids are released under different circumstances (i.e., seldom simultaneously). While abdominal fluids can be released in response to subtle disturbances, and mostly (if not only) during the early stages of adult life, neck fluids are most frequently secreted in response to the active 'squeezing' of the prothoracic glands (i.e., a bird attack; see video ESM1). The exact compounds in the defensive fluids of wood tiger moths have not yet been fully identified, but many other arctiids are well known for their chemical defences, which include pyrrolizine alkaloids, methoxypyrazines and iridoid glycosides, among others[17-20]. Given the possible costs associated with insect chemical defences[12, 24], it is intriguing that wood tiger moths are able to afford two different types of fluids.

Here, we test the hypothesis that these moths have two different types of chemical defences because they are targeted towards different predator types. We collected defensive fluids from lab-reared males, analysed their chemical composition and examined the reaction of two biologically relevant predators, birds and ants. We first show that the two defensive fluids are chemically distinct, and demonstrate that birds and invertebrate predators react to them differently. Following the results of these assays we identified a compound, 2-sec-butyl-3-methoxypyrazine (SBMP), which explains the target-specific nature of the thoracic defence fluid.

2. Material and methods

(a) Study species and collection of defensive fluids

The wood tiger moth, *Arctia plantaginis*, is an arctiid species distributed across the Holarctic region[26]. They are polyphagous and capital breeders[29], feeding only while larvae. Adults have a short lifespan (2-3 weeks for males, <1 week for females) and produce only one generation per year in the wild. Under laboratory conditions, wood tiger moths can be relatively easily bred and kept on a diet consisting mostly of dandelion (*Taraxacum sp*.) leaves, and can produce three generations per year. The individuals used in the present experiments were obtained from two laboratory stocks, established in 2010 and 2011, from wild moths collected from central and southern Finland, and reared at the University of Jyväskylä (Finland) under natural light conditions and a temperature ca. 23 C.

Fluids for the bird experiments were collected in 2012 from approximately 120 males, 60 white and 60 yellow, taken from the laboratory stock founded in 2011. Fluids for the ant experiments were collected in 2014 from 45 males from the same stock (see details about collection of defensive fluids in ESM2). There are no differences between wild and lab-reared moths in the volume of their defensive fluids, which appear to be produced *de novo* (Burdfield-Steel et al. *under review*).

(b) Chemical analyses

For the preliminary chemical analysis, neck and abdominal fluids from five individuals were pooled. 500 μl dichloromethane (DCM) was added to thoracic fluids and vortexed, and 20 μl of the abdominal fluid was pipetted to 500 μl DCM. The DCM was then evaporated under constant nitrogen flow and the dried samples re-dissolved with 250 μl pyridine and 250 μl

106 silylation reagent (BSTFA + 1% TMCS, Regisil). Extracted fluid samples were analysed with
107 an Agilent 6890 gas chromatograph - 5973 mass spectrometer (GC/MS) system. A sample
108 volume of 1 μl from both thoracic and abdominal fluid samples was injected into the injector
109 using a pulsed, splitless mode and the temperature was set to 290ºC. Compounds were
110 separated with a HP-5ms column (30 m x 0.25 mm I.D. with a film thickness of 0.25 μm;
111 J&W Scientific Inc.). Helium was used as a carrier gas at a constant flow (1 ml/min). The
112 oven temperature was programmed as follows: 2 min at 80ºC, then ramped to 180ºC at the
113 rate of 8ºC/min and from 180ºC to 290ºC at the rate of 7ºC/min, and kept at that temperature
114 for an additional 10 min. Electron ionization (70 eV) mass spectra were used for
115 identification. Chromatograms and mass spectra were evaluated using Agilent Chemstation
116 (version G1701CA) software, and the Wiley 7th edition mass spectral database.

117 A further chemical analysis was performed at TU Branschweig. The samples were collected
118 using Supelco Red (100 µm Polydimethylsiloxane, PDMS) and Black (75 µm
119 Carboxen™ /PDMS) solid phase micro extraction (SPME) fibers with neck fluids (1-10µL) of
120 freshly eclosed moths. Fibers were placed into the neck fluid and immediately transferred to
121 the injection port of the GC/MS system. GC/MS analyses were carried out on an Agilent GC
122 7890B system connected to a 5977A mass-selective detector (Agilent) fitted with a HP-5 MS
123 fused-silica capillary column (30 m×0.25 mm i.d., 0.22 µm film; Hewlett–Packard).
124 Conditions were as follows: carrier gas (He): 1.2 mL/min; injector: 250 °C; transfer line from
125 injector to column: 300 °C. The gas chromatograph was programmed as follows: 50 °C (5 min
126 isothermal), increased at 5 °C/min to 320 °C, and operated in splitless mode. The
127 identification of compounds was performed by comparison of mass spectra and retention
128 times with those of reference compounds (see ESM3).

129

(c) Bird response to moths' chemical defences

Birds (*Cyanistes caeruleus)* were observed through a mesh-covered window in one of the experimental cage's sides, and video-recorded with a digital camera (Sony DSC-HX1). The experimental cages were placed in a dark room, such that the observer was not noticeable for the birds (see details on bird housing and training in ESM2). Each bird was randomly assigned to one of five different groups, each with 13 birds. Groups were tested with either abdominal (A) fluids from yellow (Y) or white (W) moths; and neck (N) fluids from yellow or white moths. The fifth and final group was a control (C), tested with water only.

Each assay consisted of five trials, the first and last of which were done with water-soaked oats to ensure that the birds were feeding at the beginning of the experiment, and were not satiated at the end; in trials 2, 3 and 4 the birds were offered the treatment oats, which contained one type of the defence fluids. Therefore, only trials 2, 3 and 4 were included in the analysis. Each of these three trials was done with 25 μl of a specific blend of the fluids of three males of the same colour (see ESM2 for details on fluid collection) mixed with distilled water. Each blend was used twice (i.e. for two different birds). The 25 μl of fluids (or water, in case of the control group (C)), were distributed among three oat flakes, which were presented simultaneously to the birds, each of which had been food-deprived for a period no longer than two hours in order to ensure motivation to feed. During the experiment we recorded the 'latency to approach', defined as the time taken by the bird to approach and peck/eat the oats after seeing them, and recorded the number of oats eaten by the bird in a maximum trial duration of five minutes. The duration of the trial, taken as the time taken by the bird to finish the oats, was recorded in those cases where the birds ate all the oat flakes before the five-minute limit.

We ran two separate statistical analyses, one to test for differences in bird reaction towards

the abdominal (A) or neck (N) fluids in comparison to the controls (C), and a second one to compare bird reactions to the defence fluids of white (W) and yellow (Y) morphs. For the first analysis the differences in bird latency to approach the oats among treatments were analysed using a mixed-effects Cox model. The time before the bird started to eat the oats (i.e. time to event) was used as the response variable, and type of fluid (C, N or A), trial number and the interaction between the two were taken as explanatory variables, with bird ID as a random factor. Then, we ran a Generalised Linear Mixed Model (GLMM) with a Poisson distribution including the total number of oats eaten as response variable, using the same predictor variables as mentioned above. Trial duration was included as a covariate to account for the time it took for the birds to consume the oats, and bird ID was entered again as a random factor. Once we confirmed that bird reaction to the moths' chemical defences was different from that to controls, we ran the second analysis excluding the individuals from the control (C) group, using the same models described above, but with moth colour rather than fluid type as an explanatory variable. In order to see whether bird reaction changed over the course of the experiment, we compared trials 3 and 4 to trial 2, as birds were exposed for the first time to the moths' defences during trial 2. Model simplification (see ESM2) was done on the basis of differences in Akaike Information Criterion (AIC).

(d) Ant response to moths' chemical defences.

The assays with ants were done in September 2014 in a forest patch in the vicinity of Jyväskylä (62.193 N, 25.699 E), Finland. We identified 15 ant nests (*Formica sp.*) and their associated trails; two different trails per nest were chosen on the basis of their traffic (number of ants following the trail) in order to test ant response to the two different chemical defences of *A. plantaginis* following a protocol modified from previous studies[30, 31]. Once a trail

178 was chosen, an acetate disc of approximately 9 cm diameter was placed on the ground,
179 making sure that the ants would walk over it. Three drops of 10 μl each were added to the
180 disc at similar distances from each other, two containing a blend of chemical fluids coming
181 from three different males of the same colour, mixed with a 20% sugar solution, and one with
182 only the sugar solution, acting as a control. Using a sugar solution combined with a blend (in
183 a 10% concentration) of the chemical defences ensured that the ants would have the
184 motivation to drink despite the bad taste. We drew marks on the acetate disc with three
185 different randomly assigned colours to identify the fluid type in each droplet. Two discs were
186 used for each nest, one for each type of chemical defence. Both discs had fluids from both
187 colour morphs plus a control droplet (i.e. NY, NW and C were presented simultaneously in
188 one disc, and AY, AW and C were presented in the other one).

189 Ants were allowed to come to the disc and drink from the droplets for five minutes after
190 which the disc was removed. Each assay was filmed with a digital camera (SONY DSC-
191 HX1), and the videos were analysed in detail after the final experiment. For each disc we
192 counted the number of drinking events (an ant approaches the droplet and drinks from it) and
193 rejections (an ant approaches the droplet, tastes it and leaves immediately) in each droplet.
194 With this we calculated an 'acceptance score' as the number of drinking events divided by the
195 sum of drinking events and rejections, where values closer to 0.5 mean the ants have no
196 preference or repulsion, values closer to 1 mean the ants drank the fluid more than they
197 rejected it, and values close to 0 indicate that ants reject the fluid more than they drink it.
198 Additionally, we did scans every 30 seconds to count the number of ants drinking from each
199 droplet, and on the disc, and took the maximum number of ants over the five-minute period as
200 a proxy for ant traffic.

201 We ran a GLMM with binomial distribution where the acceptance score was the response
202 variable, and the interaction between morph and type of fluid was included as the explanatory

203 variable. We also included ant traffic as a covariate, and nest ID as a random factor. Main
204 effects were not included, as neck and abdominal fluid were not presented to the ants
205 simultaneously and, therefore, are not directly comparable. For this and all other analyses we
206 took a full-model approach. The variance explained by random effects was calculated
207 following[32]. This and all statistical analyses were done with the software R Studio[33],
208 using the packages *coxme*[34] and *lmer4*[35].

209

210 **(e) Bird and ant response to pure pyrazine**

211 Following the results of the second chemical analysis (see below) we performed a second
212 assay with ants (June 2016) and birds (November 2016) to determine whether the pyrazine
213 detected in the neck fluids was capable of eliciting aversive reactions on its own, and in the
214 concentrations found. The procedures followed the protocols described above for each
215 predator type. For details on the methods of these assays see ESM2.

216

217 **3. Results**

218 **(a) Preliminary chemical analysis**

219 We found that the two types of defensive fluids differ in their composition (ESM4). In
220 addition to containing a greater number of peaks, the peak areas obtained from the neck fluids
221 were essentially larger (ESM4a) compared to abdominal fluids (ESM4b). The main
222 compound groups in neck defensive fluids were amino and carboxylic acids (See Table I in
223 ESM2). The methods used in this first analysis did not allow for the identification of highly
224 volatile compounds because it aimed to identify as many compounds as possible using a

225 silylation derivatising step, in which the very volatile compounds are lost.

226

227 **(b) Bird response to moths' chemical defences**

228 Birds were overall significantly more deterred by the neck fluids than by the abdominal ones.
229 This was reflected in a higher latency to approach oats soaked with neck fluids compared to
230 control oats across trials (Table 1; Fig. 1a), whereas no differences were found between the
231 latency to approach oats soaked with abdominal fluids and controls (Table 1).

232 Likewise, birds ate oats soaked with neck fluids at a significantly lower rate than controls
233 (i.e., either took longer to finish the three oats presented, or ate less of them within the
234 maximum length (5 min) of each trial; estimate ± SE = -0.409 ± 0.152, z = -2.689, p = 0.007;
235 Fig. 2b), and than oats soaked with abdominal fluids (estimate ± SE = -0.317 ± 0.131, z = -
236 2.408, p = 0.016; Fig. 2b); however, there was no difference between the number of oats eaten
237 when soaked with abdominal fluids and water (estimate ± SE = -0.092 ± 0.124, z = -0.740, p
238 > 0.05; Fig. 2b). Oat eating rate did not differ either between trial 3 (estimate ± SE = -0.058 ±
239 0.124, z = -0.473, p > 0.05) or trial 4 (estimate ± SE =-0.031 ± 0.125, z = -0.247, p > 0.05)
240 and trial 2.

241 Having found that neck fluids repel birds whereas abdominal fluids do not, we checked with a
242 second analysis whether there were differences between the colour morphs in the efficiency
243 of their neck defensive fluids. This analysis revealed a significant interaction between moth
244 colour and trial, so that the latency to approach in the fourth trial was significantly higher in
245 response to the neck fluids of yellow males than to those of white males (Morph (Y) x Trial
246 (4): estimate ± SE = -2.057 ± 0.128, z = -3.16, p = 0.002; Fig. 2a); Table II in ESM2, Fig. 2a),
247 indicating that latency increases with time in response to fluids of yellow males (Fig. 2a), but

not in response to white males' fluids. The rate at which birds presented with neck fluids ate oats was not affected by moth colour (estimate ± SE = 0.057 ± 0.265, z = -0.215, $p > 0.05$; Fig. 2b).

(c) Ant response to moths' chemical defences

Ants reacted in a different way to the two types of moth fluids. Compared to the controls, neck fluids had a higher acceptance score, whereas abdominal fluids had a lower one (Fig. 3). As expected, there was no significant difference between the acceptance score of the controls in the discs containing abdominal fluids and those of discs containing neck fluids (Fluid (A) x Morph (C): estimate ± SE = 0.07 ± 0.24, z = 0.30, p = 0.77; Fig. 3). Nest ID accounted only for 5.3% of the variance in acceptance score. There was a significant interaction between the type of fluid and colour morph indicating that, compared to controls, abdominal fluids of both colour morphs are rejected more often than neck fluids (Fluid (A) x Morph (W): estimate ± SE = -1.09 ± 0.16, z = -6.77, $p < 0.001$; Fluid (A) x Morph (Y): estimate ± SE = -1.40 ± 0.17, z = -8.31, $p < 0.001$; Fig. 3). Taking a closer look at the disks of each fluid type, we found that the abdominal fluids of yellow males are rejected more often than those of white males (estimate ± SE = -0.459 ± 0.14, z = -3.26, p = 0.001; Fig. 3), whereas no significant differences in acceptance score were found between the neck fluids of white males and those of yellow males (estimate ± SE = -0.459 ± 0.14, z = -3.26, p = 0.001; Fig. 3). Neck fluids of white males, however, were accepted significantly more than the pure sugar solution contained in controls (estimate ± SE = 0.505 ± 0.22, z = 2.27, p = 0.023; Fig. 3).

(d) Further chemical analysis

Further chemical analysis of the neck fluids by SPME without derivatisation proved the presence of the volatile 2-sec-butyl-3-methoxypyrazine (SBMP; Fig. 4), which was not detected in abdominal fluids. The SBMP concentration in individual samples of neck fluids ranged from 0.1 to 1 ng/μl. As methoxypyrazines are known to be deterrent for birds[36], and they are commonly found in the defensive fluids of lepidopterans[37], we further tested bird reaction to oats coated with SBMP.

(e) Bird and ant response to pure pyrazine

Birds (n=10) showed a strong aversion to pure SBMP even at the lowest concentration (0.1 ng/μl), reflected in the significantly lower amount of oats eaten when soaked with the pyrazine than with water (estimate ± SE: -0.560 ± 0.177, t = -3.163, p = 0.005; ESM5a). Birds exposed to pyrazine-soaked oats also showed a tendency to hesitate for a longer time before approaching than did birds exposed to controls (estimate ± SE = -1.143, 0.604, z = -1.89, p = 0.059; ESM5b). In contrast, we did not find pure SBMP to have a deterrent effect on ants. There were no differences in acceptance score between a sugar solution containing 1ng/μl SBMP and the control solution (estimate ± SE = 0.139 ± 0.235; z = 0.589; $p > 0.056$; ESM5c).

4. Discussion

Many animals are prey to multiple species, spread across numerous taxa. This predator diversity poses a significant problem for the effectiveness of anti-predator defences, as

293 different taxa have different sensory capabilities, tolerances, and hunting strategies. Thus,
294 different predator types may produce differential selection pressures on the same prey[7, 38],
295 which may explain why defence chemicals vary so greatly between and within species[21].
296 This variation in selection pressures could even result in prey evolving defences targeted at
297 particular predators. Our experiments reveal a case of animal target-specific chemical
298 defences. Wood tiger moths produce two types of defensive fluids, which differ in function
299 and composition. While neck fluids successfully deter birds, abdominal fluids repel ants. In
300 both cases, the chemical defences of yellow individuals elicited a stronger aversion than those
301 of white males.

302 Previous studies on the chemical defences of several lepidopteran species have revealed that
303 their active compounds, mostly pyrrolizidine alkaloids, cardenolides and cardiac
304 glycosides[17, 18, 39-45], are unpalatable to a wide array of predators, including ants[30, 46],
305 spiders[47], bats[48], and birds[49-51]. Our findings suggest, however, that having only one
306 type of chemical defence would not be enough to deter all the different predator types that
307 wood tiger moths could encounter.

308 The two defence types found in *A. plantaginis* seem well suited for the different contexts in
309 which these moths may encounter avian and invertebrate predators. Because neck fluids are
310 secreted when the prothoracic glands are compressed, birds could be exposed to these
311 chemicals when attacking the moth, regardless of whether the moth is flying or resting on the
312 vegetation. Additionally, previous observations have revealed that birds tend to attack the
313 moths by their heads, which means an almost immediate exposure to the neck fluids (see
314 ESM1). Abdominal fluids, on the other hand, may be particularly useful for protection from
315 terrestrial predators (i.e. ants) at moments when the moths are resting on the vegetation
316 (especially females; Mappes, pers. obs.), or when fleeing is difficult, for example when the
317 moth is coming out of the pupa and its wings are not yet fully extended, or when the

318 temperature is too low to initiate flight. Indeed, the abdominal fluids may not be produced
319 solely for adult defence against predators, but might rather be the remains of the pupae liquid
320 (i.e. meconium), and hence available primarily at the very early stages of adult life, when
321 individuals are most vulnerable. Laboratory observations support this idea, as abdominal fluid
322 is typically (but not always) produced during the first few days of adulthood, and individuals
323 frequently release it if disturbed (Burdfield-Steel, pers. obs.).

324 Ants were, as expected, motivated to drink from the three droplet types, presumably because
325 of their content of sucrose, which they prefer over other sugar kinds[52]. However, the clear
326 differences in acceptance scores show that not only are abdominal fluids distasteful, but also
327 that neck fluids tend to be more accepted than the control solution. It is possible that neck
328 fluids have valuable nutrients for the ants in addition to sugar. For instance, some ant species
329 find a mixed solution of sugar and a blend of amino acids more appealing than a pure sugar
330 solution[52]. Indeed, our preliminary chemical analysis showed high levels of amino acids,
331 particularly in the neck fluids (Table II in ESM2; ESM4a), as is the case for some zygaenid
332 moths[15]. Future research into the wood tiger moth defences could therefore focus on
333 understanding why they invest in such costly products not related to the defence, or whether
334 those are instead just by-products of the haemolymph.

335 While the initial chemical analysis shows that the abdominal fluids contain fewer compounds
336 and are generally more dilute, it also shows that many of the major components of the two
337 fluids are the same. These included many acids, such as citric acid. However, the pH of the
338 fluids is close to neutral (Burdfield-Steel, pers. observ.), suggesting that acidity is unlikely to
339 be contributing to the predator response. Although there do appear to be some compounds
340 present in the abdominal fluids that are missing from the neck fluids, mostly notably glutamic
341 acid, it is still not clear what compound is responsible for the deterrent effect against ants.

342 Birds were significantly more deterred by neck fluids than by abdominal fluids. Furthermore,
343 their latency towards neck fluids from yellow individuals was the highest by the end of the
344 three trials (Fig. 2a). Because in our experiment bird predators did not have information on
345 prey colouration, their response was based purely on the odour and taste of the chemicals they
346 were exposed to. This might indicate that the odour of neck fluids from yellow males is more
347 of a deterrent than that of white males. While warning colours are always 'on', taste and smell
348 are hidden to predators until they come closer to the prey and/or attack them, in a similar
349 fashion to ultrasonic clicks emitted by tiger moths in response to echolocating bats[53].

350 As our initial chemical analysis did not detect any clear source of the strong odour and taste
351 associated with the neck fluids, we performed a second analysis to identify volatile candidate
352 compounds that may be contributing to the predator aversive response. This resulted in the
353 discovery of 2-sec-butyl-3-methoxypyrazine (SBMP). Pyrazines, most specifically
354 methoxypyrazines, have been previously found in the chemical defences of some arctiids[37,
355 54], and we believe SBMP is one of the major components explaining the anti-predator effect
356 of the neck fluids. It has been suggested that the odour of methoxypyrazines, which are
357 responsible for some of the strongest and most haunting odours known[55], could serve a
358 warning function towards predators which use smell to locate prey, in the same way that
359 certain colours or colour patterns would work as warning signals for visual predators[37].
360 Previous studies have indeed convincingly shown that odours from methoxypyrazines can
361 reinforce aversive responses of predators to certain colours[36, 56], or elicit taste-avoidance
362 learning on their own[57]. Domestic chicks have even been shown to be able to detect the
363 methoxypyrazine odour from a distance and to associate such smell with a bitter taste
364 provoking an aversive reaction[55]. However, there is little prior evidence that
365 methoxypyrazines are in itself strongly aversive to birds. Here we demonstrate that birds
366 exposed to pure SBMP indeed find it very repellent, even at the lower end of the

concentration range detected from the moths defences.

In contrast, much less is known about the role of pyrazines in invertebrate signalling (but see[58] for an illustration of the deterrent effect of SBMP against tropical invertebrate predators). We therefore also tested the effect of pure SBMP on ants and found, in keeping with the results from the neck fluid trials, that it did not deter them. Thus, SBMP seems the key behind the target-specific nature of the neck fluids, effective against bird predators, but not against insect predators such as ants.

Neck fluids of yellow males appear to be more effective than those of white males. Stronger defences in white males would have indicated a trade-off between warning signal efficacy and the strength of chemical defences that would help explaining why, against theoretical expectations, white and yellow males can co-exist in the same populations. With a more efficient warning signal[28] and somewhat better chemical defences (i.e. neck fluids that elicit bird increasing latency to approach with time (Fig. 2a), and abdominal fluids that are more often rejected than accepted by invertebrate predators (Fig. 3)), the reason(s) why the yellow morph has not reached fixation remains puzzling. These between-morphs differences in chemical defence quality are unlikely to be due to differences in larval diet between the two morphs, as larvae present no detectable differences in food choice (pers. observ.). Recent studies suggest that variation in the composition in predator communities[59], combined with differential mating success[60] and sufficient gene flow[60, 61], could contribute to the maintenance of this colour polymorphism. Further research should thus assess the relative importance of warning signals vs. chemical defences in wood tiger moths, and evaluate whether either defence overrides the other, or whether they have a synergistic effect and form a redundant multimodal display (*sensu* Partan & Marler[62]).

Chemical defences can vary in several ways, yet this has not been studied as thoroughly as

391 variation in colouration[21]. Here we demonstrate that the existence of two different,
392 seemingly costly ([28]; Suisto et al., unpublished; Burdfield-Steel et al., unpublished),
393 defensive fluids is justified by their predator specificity. Although the mechanisms by which
394 these chemicals are produced are not yet known, our findings will hopefully stimulate
395 research on the possible life-history trade-offs and fitness-related consequences faced by
396 species with one type of chemical defences vs. those faced by species with two (or more).
397 Comparative phylogenetic analyses could be a useful and interesting approach to track the
398 origin and evolution of general vs. specific chemical defences. We also show that there are
399 differences between yellow and white males in chemical defence quality. This aspect of
400 variation in chemical defences is not trivial for aposematic species[63]. Experiments are
401 needed where the probability of survival of individuals with different levels of chemical
402 defence is recorded, in order to gain a better understanding of the mechanisms underlying
403 intraspecific variation in chemical defences.

404 Our study not only highlights the largely overlooked importance of invertebrate predators as
405 selective agents on prey defences[64], despite their abundance in nature, but also stresses the
406 need to choose relevant predator species when studying the efficacy of chemical defences,
407 and drawing conclusions about the selective agent shaping prey defences. The presence of
408 enemy-specific chemical defences in a same prey animal hints at the importance of predator
409 community in shaping prey evolution, and suggests that selection on chemical defence may
410 be far more complex than we have previously assumed.

411

412 **Authors' contributions.** Study design: BR, EBS, KS, JM; implementation of bioassays: BR,
413 EBS, KS. Chemical analyses: HP, EBS, SS, MM, KS; video analyses: BR; statistical analyses
414 and first draft of the paper: BR, EBS; all co-authors contributed to final editing, and approved

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432 **References**

433 [1] Edmunds, M. 1974 *Defence in Animals: A Survey of Antipredator Defences*. New York,
434 Longman.
435 [2] Endler, J.A. 1986 Defense against predators. In *Predator Prey Relationships. Perspectives*
436 *and approaches from the study of lower vertebrates*. (ed. M.E.L.G.V. Feder), pp. 109-134.

437 [3] Hoverman, J.T. & Relyea, R.A. 2007 The rules of engagement: how to defend against
438 combinations of predators. *Oecologia* **154**, 551-560.
439 [4] Sih, A., Englund, G. & Wooster, D. 1998 Emergent impacts of multiple predators on prey.
440 *TREE* **13**, 350-355. (doi:http://dx.doi.org/10.1016/S0169-5347(98)01437-2).
441 [5] Poulton, E.B. 1890 *The Colours of Animals: Their Meaning and Use*. London, Kegan
442 Paul, Trench, Trubner; 558-612 p.
443 [6] Cott, H.B. 1940 *Adaptive Colouration in Animals*. London, Methuen.
444 [7] Ruxton, G.D., Sherratt, T.N. & Speed, M.P. 2004 *Avoiding Attack: the evolutionary*
445 *ecology of crypsis, warning signals and mimicry*. Oxford, Oxford University Press; i p.
446 [8] Alatalo, R.V. & Mappes, J. 1996 Tracking the evolution of warning signals. *Nature* **382**,
447 708-710. (doi:10.1038/382708a0).
448 [9] Guilford, T. 1990 The secrets of aposematism: unlearned responses to specific colors and
449 patterns. *TREE* **5**, 323-323. (doi:10.1016/0169-5347(90)90177-f).
450 [10] Mappes, J., Marples, N. & Endler, J.A. 2005 The complex business of survival by
451 aposematism. *TREE* **20**, 598-603. (doi:10.1016/j.tree.2005.07.011).
452 [11] Skelhorn, J., Halpin, C.G. & Rowe, C. 2016 Learning about aposematic prey. *Behav*.
453 *Ecol*. (doi:10.1093/beheco/arw009).
454 [12] Bowers, M.D. 1992 The evolution of unpalatability and the cost of chemical defense in
455 insects. In *Insect Chemical Ecology. An evolutionary approach* (eds. B.D. Roitberg & M.B.
456 Isman). London, Chapman & Hall.
457 [13] Maan, M.E. & Cummings, M.E. 2012 Poison frog colors are honest signals of toxicity,
458 particularly for bird predators. *Am. Nat.* **179**, E1-E14. (doi:10.1086/663197).
459 [14] Pasteels, J.M., Gregoire, J.C. & Rowellrahier, M. 1983 The chemical ecology of defense
460 in arthropods. *Annu. Rev. Entomol.* **28**, 263-289.
461 (doi:10.1146/annurev.en.28.010183.001403).
462 [15] Pentzold, S., Zagrobelny, M., Khakimov, B., Engelsen, S.B., Clausen, H., Petersen, B.L.,

463 Borch, J., Møller, B.L. & Bak, S. 2016 Lepidopteran defence droplets - a composite physical
464 and chemical weapon against potential predators. *Sci. Rep.* **6**, 22407.
465 (doi:10.1038/srep22407).
466 [16] Ritland, D.B. 1994 Variation in palatability of Queen Butterflies (*Danaus gilippus*) and
467 implications regarding mimicry. *Ecology* **75**, 732-746. (doi:10.2307/1941731).
468 [17] Rothschild, M., Aplin, R.T., Cockrum, P.A., Edgar, J.A., Fairweather, P. & Lees, R.
469 1979 Pyrrolizidine alkaloids in arctiid moths (Lep.) with a discussion on host plant
470 relationships and the role of these secondary plant substances in the Arctiidae. *Biol. J. Linn.*
471 *Soc.* **12**, 305-326. (doi:10.1111/j.1095-8312.1979.tb00062.x).
472 [18] Trigo, J.R. 2000 The chemistry of antipredator defense by secondary compounds in
473 neotropical lepidoptera: facts, perspectives and caveats. *J. Brazil. Chem. Soc.* **11**, 551-561.
474 [19] Triponez, Y., Naisbit, R.E., Jean-Denis, J.B., Rahier, M. & Alvarez, N. 2007 Genetic and
475 environmental sources of variation in the autogenous chemical defense of a leaf beetle. *J.*
476 *Chem. Ecol.* **33**, 2011-2024. (doi:10.1007/s10886-007-9351-9).
477 [20] Weller, S.J., Jacobson, N.L. & Conner, W.E. 1999 The evolution of chemical defences
478 and mating systems in tiger moths (Lepidoptera: Arctiidae). *Biol. J. Linn. Soc.* **68**, 557-578.
479 (doi:10.1111/j.1095-8312.1999.tb01188.x).
480 [21] Speed, M.P., Ruxton, G.D., Mappes, J. & Sherratt, T.N. 2012 Why are defensive toxins
481 so variable? An evolutionary perspective. *Biol. Rev.* **87**, 874-884. (doi:10.1111/j.1469-
482 185X.2012.00228.x).
483 [22] Reudler, J.H., Lindstedt, C., Pakkanen, H., Lehtinen, I. & Mappes, J. 2015 Costs and
484 benefits of plant allelochemicals in herbivore diet in a multi enemy world. *Oecologia* **179**,
485 1147-1158. (doi:10.1007/s00442-015-3425-0).
486 [23] Skelhorn, J. & Ruxton, G.D. 2008 Ecological factors influencing the evolution of insects'
487 chemical defenses. *Behav. Ecol.* **19**, 146-153. (doi:10.1093/beheco/arm115|ISSN 1045-2249).
488 [24] Zvereva, E.L. & Kozlov, M.V. 2016 The costs and effectiveness of chemical defenses in

herbivorous insects: a meta-analysis. *Ecol. Monogr.* **86**, 107-124. (doi:10.1890/15-0911.1).

[25] Rönkä, K., Mappes, J., Kaila, L. & Wahlberg, N. 2016 Putting *Parasemia* in its phylogenetic place: a molecular analysis of the subtribe Arctiina (Lepidoptera). *Syst. Entomol.***41,** 844-853. (doi:10.1111/syen.12194).

[26] Hegna, R.H., Galarza, J.A. & Mappes, J. 2015 Global phylogeography and geographical variation in warning coloration of the wood tiger moth (*Parasemia plantaginis*). *J. Biogeogr.* **42**, 1469–1481. (doi:10.1111/jbi.12513).

[27] Lindstedt, C., Eager, H., Ihalainen, E., Kahilainen, A., Stevens, M. & Mappes, J. 2011 Direction and strength of selection by predators for the color of the aposematic wood tiger moth. *Behav. Ecol.* **22**, 580-587. (doi:10.1093/beheco/arr017).

[28] Nokelainen, O., Hegna, R.H., Reudler, J.H., Lindstedt, C. & Mappes, J. 2012 Trade-off between warning signal efficacy and mating success in the wood tiger moth. *Proc. Roy. Soc. B* **279**, 257-265. (doi:10.1098/rspb.2011.0880).

[29] Tammaru, T. & Haukioja, E. 1996 Capital breeders and income breeders among Lepidoptera - Consequences to population dynamics. *Oikos* **77**, 561-564. (doi:10.2307/3545946).

[30] Molleman, F., Whitaker, M.R. & Carey, J.R. 2010 Rating palatability of butterflies by measuring ant feeding behaviour. *Entomol. Bericht.* **70**, 52-62.

[31] Müller, C., Boevé, J.-L. & Brakefield, P.M. 2002 Host plant derived feeding deterrence towards ants in the turnip sawfly Athalia rosae. *Entomol. Exper. Appl.* **104**, 153-157. (doi:10.1046/j.1570-7458.2002.01002.x).

[32] Nakagawa, S. & Schielzeth, H. 2010 Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biol. Rev.* **85**, 935-956. (doi:10.1111/j.1469-185X.2010.00141.x).

[33] RStudio. 2015 RStudio: Integrated development environment for R (Version Version 0.99.441) [Computer software]. Boston, MA.

515 [34] Therneau, T.M. 2015 coxme: Mixed Effects Cox Models. (2.2-5 ed).
516 [35] Bates, D., Maechler, M., Bolker, B. & Walker, S. 2015 Fitting Linear Mixed-Effects
517 Models Using lme4. *J. Statist. Softw.* **67**, 1-48. (doi:doi:10.18637/jss.v067.i01).
518 [36] Rowe, C. & Guilford, T. 1996 Hidden colour aversions in domestic clicks triggered by
519 pyrazine odours of insect warning displays. *Nature* **383**, 520-522. (doi:10.1038/383520a0).
520 [37] Rothschild, M., Moore, B.P. & Brown, W.V. 1984 Pyrazines as warning odour
521 components in the Monarch butterfly, *Danaus plexippus*, and in moths of the genera *Zygaena*
522 and *Amata* (Lepidoptera). *Biol. J. Linn. Soc.* **23**, 375-380. (doi:10.1111/j.1095-
523 8312.1984.tb00153.x).
524 [38] Vencl, F.V. & Srygley, R.B. 2013 Enemy targeting, trade-offs, and the evolutionary
525 assembly of a tortoise beetle defense arsenal. *Evol. Ecol.* **27**, 237-252. (doi:10.1007/s10682-
526 012-9603-1).
527 [39] Cogni, R., Trigo, J.R. & Futuyma, D.J. 2012 A free lunch? No cost for acquiring
528 defensive plant pyrrolizidine alkaloids in a specialist arctiid moth (*Utetheisa ornatrix*). *Mol.*
529 *Ecol.* **21**, 6152-6162.
530 [40] Moranz, R. & Brower, L.P. 1998 Geographic and temporal variation of cardenolide-
531 based chemical defenses of Queen Butterfly (*Danaus gilippus*) in Northern Florida. *J. Chem.*
532 *Ecol.* **24**, 905-932. (doi:10.1023/A:1022329702632).
533 [41] Rothschild, M., Euw, J.V. & Reichstein, T. 1973 Cardiac glycosides (heart poisons) in
534 the polka-dot moth *Syntomeida Epilais* Walk. (Ctenuchidae: Lep.) with some observations on
535 the toxic qualities of *Amata* (=Syntomis) phegea (L.). *Proc. Roy. Soc. Lond. B* **183**, 227-247.
536 [42] Hartmann, T., Theuring, C., Beuerle, T., Bernays, E.A. & Singer, M.S. 2005 Acquisition,
537 transformation and maintenance of plant pyrrolizidine alkaloids by the polyphagous arctiid
538 *Grammia geneura*. *Ins. Biochem. Molec. Biol.* **35**, 1083-1099.
539 [43] Hartmann, T., Theuring, C., Beuerle, T., Ernst, L., Singer, M.S. & Bernays, E.A. 2004
540 Acquired and partially *de novo* synthesized pyrrolizidine alkaloids in two polyphagous

arctiids and the alkaloid profiles of their larval food-plants. *J. Chem. Ecol.* **30**, 229-254. (doi:10.1023/B:JOEC.0000017975.16399.c3).

[44] von Nickisch-Rosenegk, E. & Wink, M. 1993 Sequestration of pyrrolizidine alkaloids in several arctiid moths (Lepidoptera, Arctiidae). *J. Chem. Ecol.* **19**, 1889-1903.

[45] Roque-Albelo, L., Schroeder, F.C., Conner, W.E., Bezzerides, A., Hoebeke, E.R., Meinwald, J. & Eisner, T. 2002 Chemical defense and aposematism: the case of *Utetheisa galapagensis*. *Chemoecology* **12**, 153-157.

[46] Molleman, F., Kaasik, A., Whitaker, M.R. & Carey, J.R. 2012 Partitioning variation in duration of ant feeding bouts can offer insights into the palatability of insects: experiments on African fruit-feeding butterflies *J. Res. Lepidopt.* **45**, 65-75.

[47] Carrell, J.E. 2001 Response of predaceous arthropods to chemically defended larvae of the pyralid moth *Uresiphita reversalis* (Guenée) (Lepidoptera: Pyralidae). *J. Kansas Entomol. Soc.* **74**, 128-135.

[48] Hristov, N. & Conner, W.E. 2005 Effectiveness of tiger moth (Lepidoptera, Arctiidae) chemical defenses against an insectivorous bat (*Eptesicus fuscus*). *Chemoecology* **15**, 105-113.

[49] Brower, L.P., Ryerson, W.N., Coppinger, L.L. & Glazier, S.C. 1968 Ecological chemistry and the palatability spectrum. *Science* **161**, 1349-1350.

[50] Cardoso, M.Z. 1997 Testing chemical defence based on pyrrolizidine alkaloids. *Anim. Behav.* **54**, 985-991. (doi:http://dx.doi.org/10.1006/anbe.1997.0505).

[51] Massuda, K. & Trigo, J. 2009 Chemical defence of the warningly coloured caterpillars of Methona themisto (Lepidoptera: Nymphalidae: Ithomiinae). *Europ. J. Entomol.* **106**, 253-259.

[52] Blüthgen, N. & Fiedler, K. 2004 Preferences for sugars and amino acids and their conditionality in a diverse nectar-feeding ant community. *J. Anim. Ecol.* **73**, 155-166.

[53] Ratcliffe, J.M. & Nydam, M.L. 2008 Multimodal warning signals for a multiple predator world. *Nature* **455**, 96-U59. (doi:10.1038/nature07087).

[54] Moore, B.P., Brown, W.V. & Rothschild, M. 1990 Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology* **1**, 43-51. (doi:10.1007/bf01325227).

[55] Guilford, T., Nicol, C., Rothschild, M. & Moore, B.P. 1987 The biological roles of pyrazines: evidence for a warning odour function. *Biol. J. Linn. Soc.* **31**, 113-128. (doi:10.1111/j.1095-8312.1987.tb01984.x).

[56] Lindström, L., Rowe, C. & Guilford, T. 2001 Pyrazine odour makes visually conspicuous prey aversive. *Proc. Roy. Soc. B* **268**, 159-162.

[57] Roper, T.J. & Marples, N.M. 1997 Odour and colour as cues for taste-avoidance learning in domestic chicks. *Anim. Behav.* **53**, 1241-1250. (doi:http://dx.doi.org/10.1006/anbe.1996.0384).

[58] Vencl, F.V., Ottens, K., Dixon, M.M., Candler, S., Bernal, X.E., Estrada, C. & Page, R.A. 2016 Pyrazine emission by a tropical firefly: an example of chemical aposematism? *Biotropica* **48,** 645-655. (doi:10.1111/btp.12336).

[59] Nokelainen, O., Valkonen, J., Lindstedt, C. & Mappes, J. 2014 Changes in predator community structure shifts the efficacy of two warning signals in Arctiid moths. *J. Anim. Ecol.* **83**, 598-605. (doi:10.1111/1365-2656.12169).

[60] Gordon, S.P., Kokko, H., Rojas, B., Nokelainen, O. & Mappes, J. 2015 Colour polymorphism torn apart by opposing positive frequency-dependent selection, yet maintained in space. *J. Anim. Ecol.* **84**, 1555–1564. (doi:DOI: 10.1111/1365-2656.12416).

[61] Galarza, J.A., Nokelainen, O., Ashrafi, R., Hegna, R.H. & Mappes, J. 2014 Temporal relationship between genetic and warning signal variation in the aposematic wood tiger moth (*Parasemia plantaginis*). *Mol. Ecol.* **23**, 4939-4957. (doi:10.1111/mec.12913).

[62] Partan, S. & Marler, P. 1999 Communication goes multimodal. *Science* **283**, 1272-1273.

[63] Rowland, H.M., Ihalainen, E., Lindström, L., Mappes, J. & Speed, M.P. 2007 Co-mimics have a mutualistic relationship despite unequal defences. *Nature* **448**, 64-67.

[64] Pekár, S., Petráková, L., Bulbert, M.W., Whiting, M.J. & Herberstein, M.E. 2017 The

golden mimicry complex uses a wide spectrum of defence to deter a community of predators. *eLife* **6**, e22089. (doi:10.7554/eLife.22089).

596 **Table 1.** GLMM showing the effect of fluid type on bird latency to approach during the three
597 trials with defensive fluids (fluid C and trial 2 are included in the intercept). A = abdominal,
598 N = neck, C = control (only water).

variable	estimate ± SE	*z*	*p*
fluid (A)	-0.577 ± 0.53	-1.08	0.280
fluid (N)	-0.511 ± 0.52	-0.98	0.330
trial 3	-0.328 ± 042	-0.77	0.440
trial 4	-0.524 ± 0.42	-1.25	0.210
fluid (A):trial 3	0.867 ± 0.52	1.66	0.098
fluid (N):trial 3	-1.182 ± 0.54	-2.20	**0.028***
fluid (A): trial 4	0.200 ± 0.52	0.38	0.700
fluid (N):trial 4	-1.051 ± 0.35	-1.97	**0.049***

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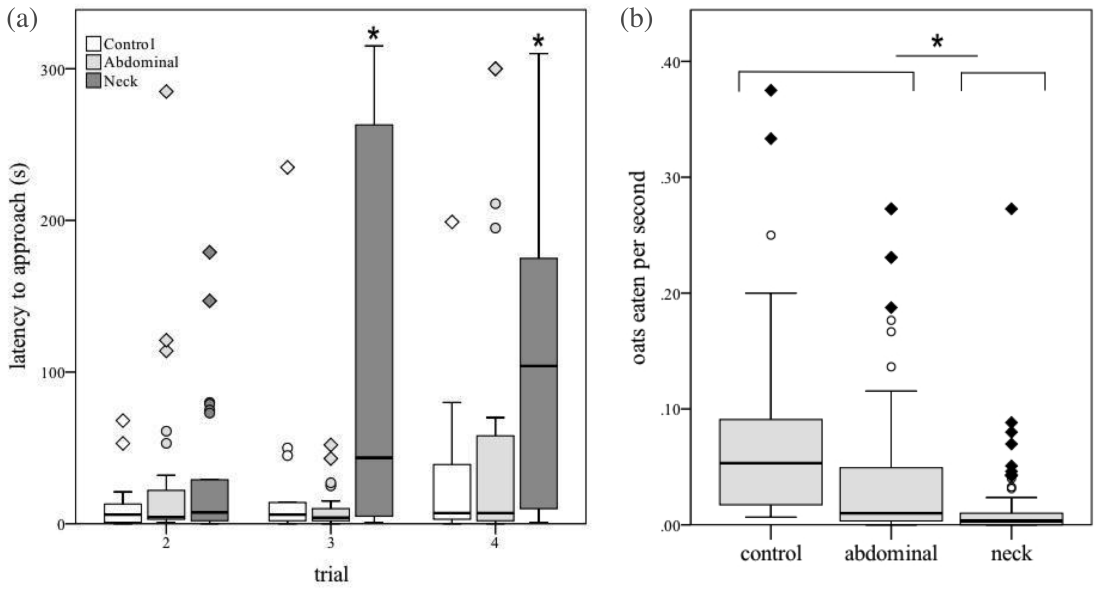

602

603 **Figure 1.** a) Latency to approach (time taken for blue tits to start eating the oat flakes) is
604 higher in response to neck fluids; and b) birds eat oats soaked in neck fluids at a significantly
605 lower rate. Asterisks indicate significant differences. Boxes show the median and the 25th and
606 75th percentiles of data distribution. Vertical lines indicate data range. Diamonds and circles
607 denote extremes and outliers in data distribution, respectively.

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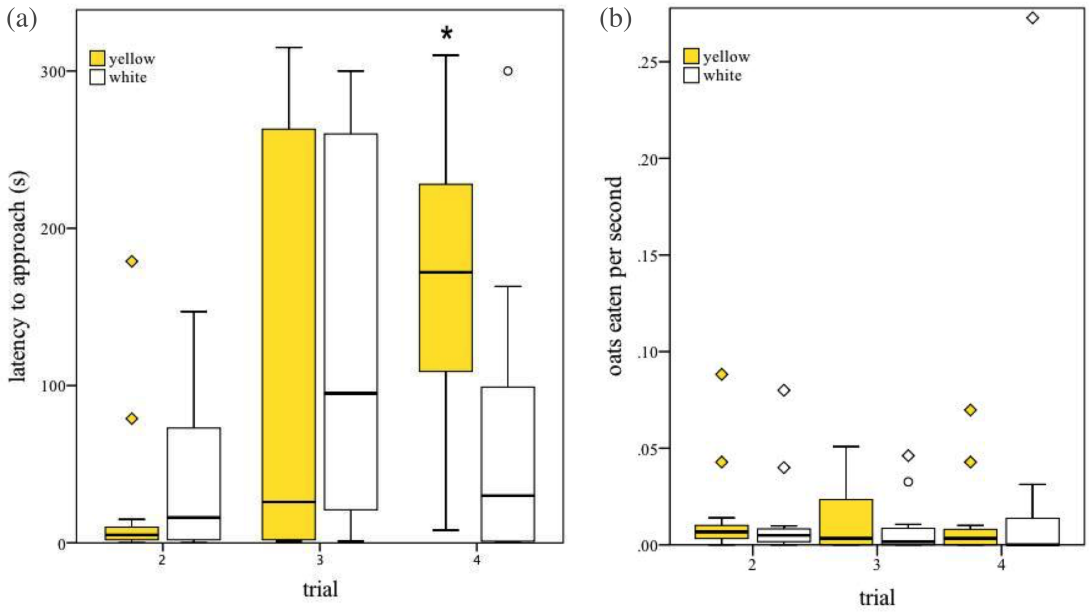

610

611 **Figure 2.** a) Latency to approach oats soaked in neck fluids (time taken for blue tits to start
612 eating the fluid-soaked oat flakes) increases with time for neck fluids coming from yellow
613 males; and b) oat flakes are eaten at similar rates when soaked with neck fluids of yellow or
614 white males. Asterisks indicate significant differences. Boxes show the median and the 25th
615 and 75th percentiles of data distribution. Vertical lines indicate data range. Diamonds and
616 circles denote extremes and outliers in data distribution, respectively.

617

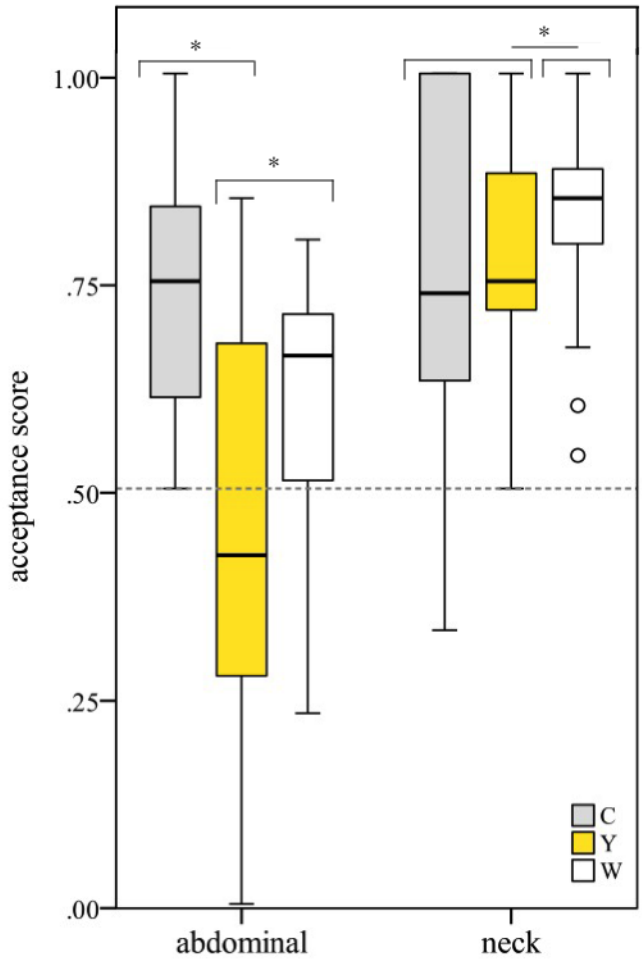

Figure 3. Acceptance score (see methods section for details on calculation) is lower for abdominal fluids, especially from yellow males, which tend to be more rejected than accepted. The variation in the acceptance score of abdominal fluids from yellow males, however, is the greatest. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines indicate data range, circles denote outliers and asterisks highlight

627 statistically significant differences.

628

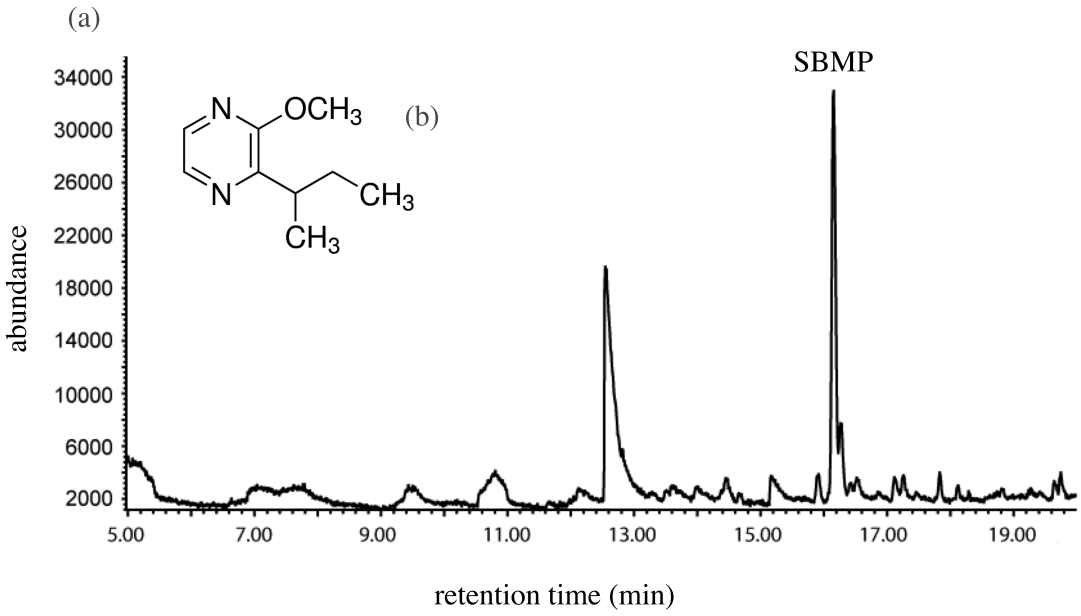

Figure 4. (a) Results of GC-MS analysis monitoring ions 124, 138 and 151 (Fig. S3); and (b) structure of 2-sec-butyl-3-methoxypyrazine (SBMP), the compound responsible for bird deterrence towards wood tiger moths' neck fluids.