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

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SUPPLEMENTARY METHODS

1. Collection of defensive fluids

Adult male moths of both colour morphs were sampled between 0 and 10 days after eclosion (i.e. some were sampled on the same day they eclosed), however the majority were sampled between 0 and 3 days post-eclosion. Prior to sampling all moths were removed from the climate chamber in which they were stored at 7 °C and sprayed with water. They were then given one hour to drink and warm up (become active) at room temperature. Neck fluids were sampled by pinching the moth just below the prothoracic glands with a pair of tweezers. This stimulated the release of the fluid, which was then collected with 10 μl glass capillaries. The abdominal fluid was sampled by gently squeezing the moth's abdomen with tweezers and collected in the same manner. The fluid volume was recorded for each individual. The fluids were then pooled into groups of three male moths of the same hind wing colour to provide enough fluids for replication and diluted with distilled water to a volume of 50 μl. All samples were stored at -20°C until use to avoid transformation and prevent any breakdown of the components. Chemicals assays suggest that long term storage (up to 2 years) in this manner does not significantly influence the pyrazine detected in the samples (Burdfield-Steel et al. *unpublished data*).

Fluids for the initial chemical analysis were collected from the 2010 laboratory stock and stored in hexane at -80°C to prevent degradation. Fluids for the subsequent chemical analysis were collected in 2015 from adults from the 2011-2012 stock. Moths were transported to TU

Braunschweig as pupa and their fluids sampled upon eclosion following the protocol described above.

2. Bird housing and training prior to the assay

We used as predators 65 wild-caught blue tits (*Cyanistes caeruleus*), which were trapped at a feeding site at Konnevesi Research Station and kept in captivity for approximately five days. Once trapped, all birds were housed individually in plywood cages with a daily light period of 11h:13h (light:dark), fed on sunflower seeds, peanuts and a vitamin-enriched food supplement, and provided with fresh water *ad libitum*. After the experiment all birds were aged, sexed (when possible) and ringed for identification purposes before being released at the capture site. The experiment was conducted at Konnevesi Research Station (Central Finland) during winter 2012-2013 and 2013-2014.

Experimental trials were conducted in masonite cages (50 x 50 x 70cm, w x d x h) containing a perch and a water bowl, and lit with a daylight bulb (Exo Terra Repti Glo 10.0 UVB). Before the experiment birds were trained in their home cages to consume oat flakes, first mixed with their usual food, then on their own. Once birds ate the oat flakes regularly, each individual was placed in an experimental cage in order to habituate to the cage itself and with the feeding system. Food was offered through a hatch behind a visual barrier, which allowed us to record the exact moment at which food was detected by the bird. The birds were first familiarised with the experimental cage for 2h by letting them forage on sunflower seeds from a white dish.

Wild birds were used with permission from the Central Finland Centre for Economic Development, Transport and Environment and license from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014) and the Central Finland Regional Environment Centre (VARELY/294/2015), and used according to the ASAB guidelines for the treatment of animals in behavioural research and teaching.

3. Bird and ant response to pure pyrazine

To test whether the 2-sec-butyl-3-methoxypyrazine detected in the neck fluids was capable of eliciting aversive reactions on its own, we performed a second series of assays with both birds and ants. Birds were presented with oat flakes soaked in one of two concentrations of 2-sec-butyl-3-methoxypyrazine, 1ng/μl (N=5) and 0.1ng/μl (N=5), which fall within the range of concentrations found in the neck fluids. We measured latency to approach the oats and number of flakes eaten as our response variables, and the type of compound (water or pyrazine) as predictors. We analysed the differences in bird latency to approach the oats between treatments with a mixed-effects Cox model, and the total number of oats eaten with a GLMM with Poisson distribution.

Likewise, the procedure followed with the ants was essentially the same as with the previous assay, except that this time only one acetate disc was placed per nest (n=10 nests). Each disc had a droplet containing a solution 1ng/μl pyrazine in 20% sugar solution, and a control droplet containing only the sugar solution. This concentration was chosen as it fell on the high end of the range of concentrations detected in the neck fluids (Burdfield-Steel et al. *under review*). Differences in response to both droplets were analysed using a GLM with binomial distribution, with acceptance score (calculated as described above) as the response variable and type of chemical (pyrazine or control) as an explanatory variable.

4. Model selection procedures

FIRST ANALYSIS

(a) GLMM testing the effects of fluid type on bird latency to approach. Model chosen in bold letters.

Model 1.1 latency~fluid+trial+fluid:trial+birdID

Model 1.2 latency~fluid+trial+ birdID

	AIC	ΔAIC
model1.1	**1532.0**	**0.0**
model1.2	1551.8	19.8

(b) GLMM testing the effects of fluid type on oat eating rate. Model chosen in bold letters.

Model 2.1 oatflakes~trialduration +trial+fluid:trial+birdID

Model 2.2 oatflakes~trialduration +trial+ birdID

	AIC	ΔAIC
model2.1	586.1	4.3
model2.2	**581.8**	**0.0**

SECOND ANALYSIS

(a) GLMM testing the effects of colour morph on bird latency to approach neck fluids. Model chosen in bold letters.

Model 2.1 latency~morph+trial+morph:trial+birdID

Model 2.2 latency~fluid+morph+ birdID

	AIC	ΔAIC
model1.1	**461.4**	**0.0**
model1.2	479.5	18.1

(b) GLMM testing the effects of colour morph on bird amount of oats soaked in neck fluids eaten per unit of time. Model chosen in bold letters.

Model 2.1 oatflakes~trialduration+morph+trial+ morph:trial+birdID

Model 2.2 oatflakes~trialduration+morph +trial+ birdID

	AIC	ΔAIC
model2.1	220.3	3.7
model2.2	**216.6**	**0.0**

SUPPLEMENTARY RESULTS

Table I. Compounds identified in defensive fluids after derivatisation with BSTFA. Because of incomplete derivatisation some compounds occur at two different retention times.

Peak	RT	Compound
1	9.25	Serine
2	9.56	Threonine
3	10.48	Pyroglutamic acid
4	10.54	Parabanic acid
5	10.64	Malic acid
6	11.01	Pyroglutamic acid
7	12.04	Glutamic acid
8	12.15	Phenylalanine
9	13.81	Glutamine
10	14.36	Citric acid
11	15.16	Allantoin
11	15.21	Allantoin
12	15.5	Histidine

13	17.46	Lanthionine
14	17.53	Uric acid

Table II. GLMM showing the effects of colour morph on bird hesitation towards neck fluids (morph W (white) and trial 2 are included in the intercept)

Random effects	Variance	Std Dev			
(Bird ID)	1.692	1.301			
	Effect	exp(coef)	SE	z	p
MorphY	0.473	1.604	0.667	0.71	0.480
Trial3	-1.745	0.174	0.502	-3.48	**<0.001**
Trial4	-0.401	0.670	0.440	-0.91	0.360
MorphY:Trial3	0.359	1.432	0.659	0.54	0.590
MorphY:Trial4	-2.057	0.128	0.651	-3.16	**0.002**