Master's Thesis

Influence of colour, smell and taste on the survival of the wood tiger moth (*Arctia plantaginis*) adults during predation event

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To ward off predators prey may use defences that stimulate multiple sensory modalities (i.e., multimodal signalling). For example aposematic organisms are defended against predators with a warning signal combined with a secondary defence. This study focused on how the wood tiger moth (*Arctia plantaginis*) colour (genotypes WW and Wy are white and yy is yellow), smell (from methoxypyrazines) and taste (from pyrrolizidine alkaloids) deter bird predators. Live moths of both colours, that were manipulated to have each chemical defence alone or in combination, were offered to birds to test how visual warning signals, smell and taste, interact through the predation event. White coloured moths with methoxypyrazine smell had the strongest effect on delaying the approach latency and increasing the number of times the bird dropped the moth. Taste alone did not deter birds and birds reduced the amount eaten only when smell was present in combination with taste. Overall, moths that had white hindwings, and had both smell and bad taste, had the best chance of survival. When defence efficacy of the white moths was explored in closer detail, heterozygous moths were found to have the most effective neck fluids (smell) in terms of delaying approach latency and reducing the drop latency of predators, which can help to explain the polymorphism in this species. These results indicate that multimodality improves prey anti-predator defence by providing protection throughout the attack.

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Saalistajien torjumiseksi saalis voi käyttää erilaisia puolustusmekanismeja, jotka stimuloivat useita eri aisteja (ts. multimodaalista signalointia). Esimerkiksi aposemaattiset eliöt puolustautuvat varoitussignaalin lisäksi sekundaarisella puolustuksella. Tässä tutkimuksessa keskityttiin siihen, kuinka täpläsiilikkään (*Arctia plantaginis*) väritys (genotyypit WW, Wy ovat valkoisia ja yy keltaisia), haju (metoksipyratsiinista) ja maku (pyrrolitsidiinialkaloidista) toimivat puolustuksena lintusaalistajien hyökkäyksiä vastaan. Linnuille tarjottiin kummankin värisiä eläviä siilikkäitä, joita oli manipuloitu niin, että niillä oli joko hajua, makua tai molempia näistä. Näin pystyttiin tutkimaan kuinka väritys, haju ja maku vuorovaikuttavat saalistustapahtumassa. Linnut lähestyivät hitaimmin valkoisia siilikkäitä, joilla oli pahaa hajua, ja myös pudottivat niitä useimmin. Maku yksinään ei torjunut lintuja, ja linnut söivät siilikkäästä vähemmän vain silloin, kun hajua esiintyi maun kanssa. Parhaimmat mahdollisuudet selvitä hengissä oli valkoisilla siilikkäillä, joilla oli sekä pahaa hajua että makua. Kun valkoisten siilikkäiden puolustustehokkuutta tutkittiin tarkemmin, heterotsygoottisten siilikkäiden hajun havaittiin olevan tehokkainta lähestymisajan pidentämiseen ja saaliin nopeampaan pudottamiseen, mikä voi auttaa selittämään tämän lajin polymorfismia. Nämä tulokset osoittavat, että multimodaalisuus parantaa saaliin selviytymistä saalistajaa vastaan suojaamalla saalista koko hyökkäyksen ajan.

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TERMS AND ABBREVIATIONS

TERMS

Multimodal signalling	The use of signal components from two or more sensory modalities to convey information between organisms (Partan and Marler 1999, Stevens 2013).
Aposematism	Aposematic organisms signal their unprofitability with a warning signal, for example being conspicuously coloured or releasing obnoxious sound or smell (Poulton 1890, Cott 1940).
Primary defence	A defence that acts at the detection and recognition stage of an attack (Ruxton et al. 2018).
Secondary defence	Defences that act to deter attack just before or during a contact between predator and prey (Ruxton et al. 2018).

ABBREVIATIONS

PA	Pyrrolizidine alkaloid
MP	Methoxypyrazine
UV	Ultraviolet
AIC	Akaike information criterion
SE	Standard error
Coxme	Mixed effects Cox proportional hazard model
GLMM	Generalized linear mixed effects model

LME	Linear mixed effects model

1 INTRODUCTION

Predation is one of the main threats to the survival of prey. This is why prey have evolved to have different defensive methods to keep predators away (Ruxton et al. 2018). For example, some species may avoid detection by predators by evolving to choose a particular background (Kang et al. 2012, Green et al. 2019, Stevens and Ruxton 2019) or body orientation (Kang et al. 2012, Stevens and Ruxton 2019, Rowland et al. 2020) or have colouration that disguises them through general resemblance to the background (Stevens and Ruxton 2019, Nokelainen et al. 2020). During a predation event there are different stages of attack, which can include for example detection of the prey, attacking the prey, handling and eating the prey (also called predation sequence) (Endler 1991, Bateman et al. 2014). Many animals have defence mechanisms that act sequentially in different stages of attack (Endler 1991, Bateman et al. 2014, Ruxton et al. 2018). These anti-predator defences can be separated into primary and secondary defences. Primary defences act at the detection and recognition stages of a potential interaction between the predator and prey, and they can be for example visual or behavioural. Secondary defences in turn act to deter predator once it has been contact with the prey. Secondary defences often make the prey unprofitable to predators and they can be chemical, mechanical or behavioural (Cott 1940). Most prey have more than one anti-predator defence as for example Longfin squid (*Loligo pealeii*), which uses either camouflage as primary defences or it flees and releases ink when predator has seen it despite its camouflage (secondary defence) (Staudinger et al. 2011). However, chemical defences may also be considered a primary defence if they are detected before contact with the prey (Guilford 1987, Rowe and Halpin 2013, Rojas et al. 2017, Rojas et al. 2019). Therefore, it is possible for a single defence mechanism to protect prey across multiple stages of a predator's attack.

1.1 Aposematism

Aposematic organisms signal their unprofitability with a warning signal, for example being conspicuously coloured or releasing obnoxious sound or smell (Poulton 1890, Cott 1940). For example, in olfactory aposematism compounds can give off an odour that signals for toxicity and makes recognition from a distance possible, so that prey can avoid being attacked (Cott 1940). For the aposematic signal to work, predators need to learn to associate certain signals with unprofitable prey and also remember that association (Cott 1940, Rowe 1999, Speed 2000). Some examples of predators that can learn their preys' signals include birds and bats (Siddall and Marples 2008, Conner and Corcoran 2010). For example, chemically defended moth's clicking sounds are effective at deterring bats only when bats had already experienced moths with a warning sound and chemical defence (Hristov and Conner 2005), meaning that bats learned to associate clicks to defended prey.

Aposematic signals are inherently multimodal, which means that signals are received through two or more sensory modalities by a receiver (Stevens 2013). Usually visual warning signals are used as a primary defence and chemicals, which can act to deter predators with a repellent taste, smell, or through toxicity, are used as a secondary defence (Ruxton et al. 2018). One of the common types of chemical primary defences are methoxypyrazines ("MPs"), because they have an odour that is repellent to many predators (Rojas et al. 2017). There are species from different insect orders that use MPs in their defences, such as *Cercopis vulnerata*, *Coccinella septempunctata* and *Dryas iulia* (Rowe and Halpin 2013). It has been shown that warning colours combined with pyrazines enhance the predator's avoidance learning (Rowe and Guilford 1999b). Taste can act as the prey's secondary defence, because it is noticed when the predator is already in contact with prey. Pyrrolizidine alkaloids ("PAs") can make prey distasteful (or even toxic) to predators (Trigo 2000, Ferro et al. 2006) and predators can learn to avoid preys that have PAs (Cardoso 1997). PAs are common defensive chemical

compounds and are used in different insect families and species, such as *Oreina cacaliae*, *Danaus plexippus* and *Utheisa ornatrix* (Klaas and Mirka 2002), and many insects can sequester PAs from food plants as for example from the family Asteraceae (Wink 2003). Multimodal effect of smell and taste is rarely differentiated in studies of aposematic displays (but see Marples et al. 1994). Thus, it remains unclear how interactions between smell and taste fit within the theoretical framework of multimodal aposematic signals (Rowe and Halpin 2013). Measuring the individual and combined effects of smell, taste, and warning colouration is essential for understanding the maintenance of both chemical diversity and multimodal warning signals and their evolution.

1.2 Advantages of multiple signals

Multiple signals can be advantageous in a few different ways. Having defences that incorporate multiple modalities can increase prey survival (Marples et al. 1994, Stevens 2013). Different warning signals are often more effective combined than alone, which means that the signals can be additive or amplified. For example, naive domestic chicks (*Gallus gallus domesticus*) took longer to taste novel-coloured than familiar food and when the novel-colour was paired with pyrazine odour, birds took even longer to taste the food. (Marples and Roper 1996). As defences can act through different sensory modalities, such as vision, smell and taste, predators can get warning signals through more than one sensory excitation. Multimodal defences can also protect the prey though multiple stages of an attack, because a predator can for example smell that prey is chemically defended before touching the moth or predator can taste the unpleasant compounds after grapping the prey (Rowe and Halpin 2013, Rojas et al. 2017, Rojas et al. 2019). In addition, multiple signals may be used by prey to deter predators in emergent ways (Rowe and Guilford 1996, Partan and Marler 1999, Rojas et al. 2018). For example, when warning coloured or pyrazine smelling crumbs were presented to *G. g. domesticus*, neither signal alone produced aversion.

However, when crumbs with both warning colour and pyrazine smell were presented, the combination produced aversion (Rowe and Guilford 1996).

There are also different advantages of having chemical diversity. If prey have multiple toxins, it may be more difficult for predators to evolve immunity to a suite of toxins compared to just one (Zhao et al. 2003), which increases the efficacy of secondary defences. Also, when prey has different chemicals, it can be better protected from multiple enemies if different compounds are targeted to different predators. For example, in *Arctia plantaginis* neck fluids defend against bird predators (but not invertebrates) and abdominal fluids defend against invertebrates (but not birds) (Rojas et al. 2017). Also different chemicals can deter predators at different stages of attack because different chemicals can be excreted at different ways and at different times. For example volatile (bad smelling) compounds, as methoxypyrazines, can be secreted before the predator has tasted the prey and bad tasting compounds, as pyrrolizidine alkaloids, can be stored in different parts of preys body and predators will be contact with these when they tastes the prey (Trigo 2000, Ferro et al. 2006, Rojas et al. 2017, A. Winters, unpublished).

When prey use multimodal signalling, predators are more likely to memorize their signal's meaning and therefore also learn to avoid them (Rowe 1999). Avoidance learning can also be affected by how strong the associative strength is between the warning signal and unpalatability. The stronger it is, the better a predator associates the warning signal to unpalatability (Skelhorn et al. 2016). However, a third signal can enhance (potentiate) the formation of association of the two signals (for example colouration and unpalatability) (Guilford and Dawkins 1991). An experiment using naive domestic chicks (*Gallus gallus domesticus*) showed that the presence of pyrazine odour not only increased the memorability of a colour signal, but also made the learning of unpalatable prey faster (Siddall and Marples 2008).

However, more studies are needed to see whether there is a difference if the prey has one or two defence mechanisms in addition to warning colours. Also, often assays to test multiple modalities are done using artificial stimuli, which often ignore prey behaviour and prey nutritional content and are focused only on predator behaviours and not survival of the real prey (Rowe and Guilford 1999a, Rojas et al. 2019). Because prey can defend themselves at different stages of an attack (Rowe and Halpin 2013, Rojas et al. 2017, Rojas et al. 2019), it is important to follow the predation sequence through to be able to evaluate the relative importance of multiple lines of defence and to see how the different defence mechanisms build up to a complete antipredator defence.

1.3 Study species

The wood tiger moth (*A. plantaginis*) uses visual warning signals accompanied with smell and taste to deter predators, making its status as an aposematic species clear (Nokelainen 2013). Each of these defence mechanisms can be manipulated in live moths, which is why *A. plantaginis* is a good species to study multimodal signalling. *A. plantaginis* belongs to the Arctiini tribe and has vast distribution across Holarctic realm (Hegna et al. 2015). They are capital breeders, which means that only larval stages feed (Tammaru and Haukioja 1996). The male's hindwings are colour polymorphic, in Europe the hindwings are typically either white or yellow whereas female hindwings vary more continuously from red to yellow (Hegna et al. 2015). Hindwing colour depends on the genotype. In males, genotypes WW and Wy produce the white morph and the genotype yy produces yellow (Suomalainen 1938, O. Nokelainen, unpublished).

A. plantaginis secrete defensive fluids from their prothoracic region (i.e., defensive neck fluids) to target avian predators and abdominal region to target invertebrate predators, but the latter fluid is often secreted only in the early stages of adulthood (Rojas et al. 2017). The neck fluids contain MPs that are produced de novo (Burdfield-Steel et al. 2018). *A. plantaginis* also have PAs (bad taste) that are

not synthesized by themselves, but sequestered from their food (such as *Senecio vulgaris*) and stored in all parts of their body (A. Winters, unpublished). PAs can also be toxic for example to rats and butterflies (Narberhaus et al. 2005, Ebmeyer et al. 2020). There is evidence that the neck fluids of yellow moths are more repellent because when Rojas et al. (2017) used blue tits and neck fluid –soaked oat flakes, bird hesitation to eat the oats increased across trials when the neck fluids came from yellow males. In contrast, Rojas et al. (2019) found no difference in latency to approach and attack between neck fluids of the two different colour morphs. However, in the same experiment yellow moths caused birds to wipe their beaks more while neck fluids of white moths caused birds to eat less (Rojas et al. 2019). Also, there seems to be variation in predator response to colouration; some studies show that white colouration seems to be more effective against predators from genus Paridae (Rojas et al. 2019) and others that yellow colouration would be better (Nokelainen et al. 2012, Nokelainen et al. 2014). Rojas et al. (2019) used artificial prey and Nokelainen et al. (2012) real (living or dead) moths. Nokelainen et al. (2014) also used artificial prey and they conducted the experiment in the field whereas Rojas et al. (2019) conducted research in the experimental enclosures. Because of these differences in earlier studies, it is important to do more studies to see which colour and which coloured moths neck fluids are really most effective or is there any difference. Also there are no studies investigating the anti-predator role of PAs in *A. plantaginis*, and it is important to investigate PAs together with the known defences of hindwing colour and methoxypyrazine smell to understand its role in the *A. plantaginis* multimodal defence.

1.4 Study questions

The aims of this study were to investigate 1) whether multimodality improves prey survival, 2) at which stage of attack each defence modality is effective and 3) in which way each defence modality influence predators' behaviour. Male *A. plantaginis* were used as prey and blue tits, *C. caeruleus*, were used as predators.

Male colour morphs of each genotype (WW, Wy and yy) were manipulated to either have both PAs (bad taste) and MPs (bad smell), have only PAs or MPs, or to have neither. In each treatment, moth survival was measured over three consecutive trials under controlled conditions in behavioural assays. As colour, smell and taste in *A.* plantaginis can be manipulated independently, the influence of each of these defence modalities can be tested using live moths. This means that the natural prey behaviours and nutritional content can be retained and also prey survival can be measured. Testing the three different sensory modalities helps to determine how each combination of these defence modes enhances learning and if they influence behaviours in additive or emergent ways. It also helps to determine which modes of defence are more important against bird predators, and also at which stage of the attack each defence is acting.

More specifically, there were three study questions and related hypotheses. 1) How do colour, smell and taste interact to influence the survival of prey during predation? As there is evidence that predators are more likely to leave prey with multimodal signals alone (Rojas et al. 2019, Rowe 1999), the hypothesis is that colour, smell and taste will interact so that moth survival chances are better the more defences the moth has. The prediction is that when introducing to the bird a moth that have only one chemical defence (either MPs or PAs), it will survive less often than moth with both chemical defences and when introducing moth with no chemical defence, it will survive the worst. 2) How does predators' ability to learn depend on multimodal signals? As multiple signals together enhance learning (Rowe 1999), the hypothesis is that bird will learn most efficiently when the moth has multiple signals. The prediction is that when introducing to the bird a moth that has less than two chemical defences, birds ability to learn will be weaker. 3) At which stage different defence modalities influence predators' behaviour and how? Colour is a primary defence so the hypothesis is that it will take effect before there is a contact with a prey, which is in at approach and attack stages. MPs create smell and it is shown to affect approach and attack latencies and also in the

proportion eaten (Rojas et al. 2019). Thus, MPs are hypothesized to take effect at approach and attack stage by delaying the attack or cancelling it and also in consumption stage to decrease the proportion eaten. As PAs influence the taste of a moth, the hypothesis is that it will influence predator behaviour at consumption stage. Thus, birds should drop the prey with PA's more often and consume them less.

2 METHODS

The experiment was run at Konnevesi Research Station in the winter and early spring of 2019 - 2020. The experiment took place in the winter because catching the birds from the winter feeding station makes obtaining the number of birds feasible. Also, as birds breed in the summer, conducting the experiments in winter was more ethical. Birds were captured and housed with permission of Central Finland Centre for Economic Development, Transport and Environment (VARELY/294/2015) and license from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014).

2.1 Lab stock and treatment manipulations

Moths that were used in the experiment were male offspring of 2019 lab stock (3rd generation). The moths were mated and raised in the greenhouse at the University of Jyväskylä. The 3rd generation matings were spread across time so that adults were emerging through November to early March. Clutches of *A. plantaginis* larvae from each genotype (WW, Wy or yy) were raised in climate cabinets set to growing conditions of *A. plantaginis* (approximately at 23 °C). There were 10 families per genotype and a total of 23 families; some families that were used to create WW or yy moths were also used to create Wy moths. Individuals of each family were split into two diet treatments: artificial porridge and artificial

porridge + freeze-dried *Senecio vulgaris*. Adult moths in the *S. vulgaris* treatment had the ability to sequester PAs, while those in the former treatment did not. Artificial porridge consists of Agar 4.6 g, yeast 8.58 g, semolina 32.1 g, wheat germ 8.3 g, boiling water 150 ml, Vanderzant vitamin mix 1.76 g, nipagen 1800 µl and acetic acid 180 µl. Artificial porridge + freeze-dried *S. vulgaris* consists of Agar 4.6 g, yeast 8.15 g, semolina 30.5 g, wheat germ 7.89 g, boiling water 150 ml, freeze-dried Groundsel 2.5 g, Vanderzant vitamin mix 1.76 g, nipagen 1800 µl and acetic acid 180 µl. Freeze-dried *S. vulgaris* was used because the fresh plants were no longer available in the winter. Liquid chromatography-mass spectrometry (LC/MS) was used to confirm the moths used in this experiment's PA treatments were in fact sequestering PAs, because they got freeze-dried and not fresh *S. vulgaris* and also because they might excrete rather than sequester PAs (Table 1).

Table 1. Liquid chromatography-mass spectrometry results for larvae, their food and faeces. Seneciphylline was about ten times more concentrated in the larvae compared to their food and only trace amounts were excreted in the faeces. Senecionine was about three times more concentrated in the larvae compared to their food and only trace amounts were excreted in the faeces. Seneciphylline and Senecionine content (µg/mg), standard error (SE) and sample size (n) are reported to every sample type.

PA	Sample	µg/mg	SE	n
Seneciphylline	Larvae	2.42	0.48	6
	Food	0.24	0.02	2
	Faeces	0.01	0	2
Senecionine	Larvae	0.14	0.03	6
	Food	0.05	0.04	2
	Faeces	0	0	2

From each diet treatment, the MP defence fluids were removed from half of the emerging adults by squeezing the thorax with fingers and collecting the fluid from the prothoracic glands using a microcapillary. Moths were squeezed a day before

the first trials and also on the day the experiment took place, 15 minutes before the experiment started. Through these manipulations there were 251 adult male moths, with a subset of at least n = 9 in each of the following 12 treatment combinations (Table 2). The data had fewer yellow moths because all the moths had lower mating success during wintertime than in autumn (when the first matings for this experiment were made) and especially moths that had genotype yy mated very poorly. Yellow moths have also been shown to have lower mating success (Nokelainen et al. 2012, Gordon et al. 2015). Also, the COVID-19 pandemic forced the experiment to end earlier than anticipated, so the remaining planned trials (partly consisting of yellow moths) could not be performed.

Table 2. Number of moths in each chemical treatment group for each hindwing phenotype (white and yellow) and genotype (WW, Wy or yy). If type of chemical defence is present in that treatment, it is marked as "+"and if not, it is marked as "-".

Phenotype	Genotype	+MP		-MP	
		+PA	-PA	+PA	-PA
White	WW	23	33	21	36
White	Wy	21	15	18	18
Yellow	yy	18	18	9	21

2.2 Birds used in this experiment

The bird species used in the experiment was wild *C. caeruleus* because they eat insects as their natural food source, overwinter in Finland, and are commonly used in similar behavioural experiments. *C. caeruleus* are natural predators of *A. plantaginis*, and that is why birds used in this experiment may have had previous experience with them. Birds were captured using bird feeding stations in

Konnevesi by placing food inside a feeder with a door that can be closed remotely. A total of 84 birds were used. The birds were housed at Konnevesi in separate plywood enclosures (65 x 50 x 80 cm) on a 11h : 13h (light : dark) cycle for at least one day before the experiment started so that they acclimatized. During this time, birds had free access to sunflower seeds, peanuts, a vitamin enriched food supplement and water. After experiments birds were sexed, aged, weighed and released back to the wild unharmed to the same location where they were caught.

2.3 Behavioural experiment

Each bird was presented with 3 moths (one moth per day for three consecutive days) from one of the 12 treatments (Table 1). This trial was repeated in order to test how modes of defence interact to influence avoidance learning. Before the experiment, birds were placed inside test enclosures (50 x 50 x 70 cm) for approximately 2h. Birds were deprived of food during that 2h time, which is enough time for them to be sufficiently hungry to search for prey. Only two sunflower seeds were fed to them from a small hatch on the side of the cage so that the birds could get used to finding food from the hatch and to ensure that birds are acclimated. The first seed was given when the bird was placed in the enclosure and second one after the bird ate its first seed. The experiment started one hour after the second seed had been eaten.

Enclosures were equipped with a water bowl, a perch, a visual barrier and an overhead digital camera (Sony DSC-HX1) for recording the experiment. There was also a small mesh window where the bird's behaviour was observed (Figure 1). The enclosure was lit from the inside and placed within a dark room to minimize the birds noticing the observer. Inside the enclosure, lighting was mimicking daylight (i.e., D65 standard) using one bulb also including UV-spectrum (Exo Terra Repti Glo 25 W 5.0 UVB).

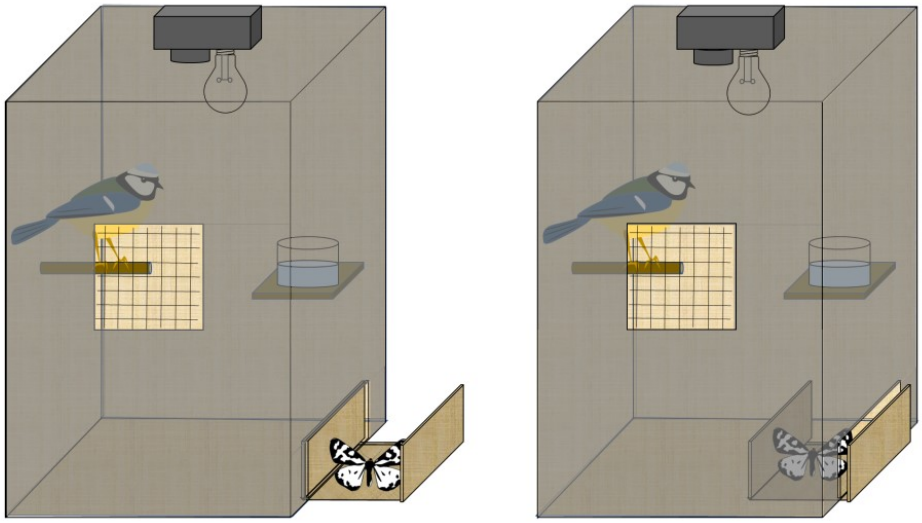

Figure 1. Experimental setup of the behavioural experiment demonstrating the placement of perch, water bowl, camera, light bulb, mesh opening for observation, and hatch for inserting moths into the enclosure.

The moth was placed in the enclosure, where the bird was, using the small hatch, and the bird's behaviour and interaction with the moth was recorded. The following bird behaviours were measured: approach latency (time from seeing the prey to approaching it), attack latency (time from seeing the prey to grabbing it), how many times the bird dropped the prey and drop latency, how long birds spent eating and handling (eating and holding) the moth, how many times the bird wiped its beak or drank water and latency to kill (time it took for the bird to kill the moth). Also, moth behaviour was recorded as follows: moth activity (the time the moth spent flying, crawling and flexing) and survival. The time at which each behaviour took place was recorded using a stopwatch (to nearest second) and marked on a paper sheet during the experiment, and the timing was also confirmed from video afterwards. After the bird had seen the moth, it had 15 minutes to attack, if it did not attack, the experiment was ended. If the bird touched the moth, the experiment was ended after the bird was not in contact with any part of the moth for one minute. After the experiment, survival of the moth was checked and proportion of different parts of the moth eaten (abdomen, thorax, head, antenna, legs and wings) were evaluated and later on used to

estimate the total proportion eaten. The proportions recorded were estimated based on what parts of the moth were found. Birds were kept in the enclosure for an additional 30 minutes before checking if the bird had vomited, as it can indicate that bird had eaten something bad (Brower et al. 1968). Finally, approximately 8 g of mealworms were given to check that birds were hungry enough to attack prey during the experiment. Birds had 10 minutes to eat as much mealworms as they wanted and after that the amount eaten was measured (in grams). Two birds did not touch the moth or the mealworms, so they were excluded. Mealworms are pleasant food for the birds, so if birds did not eat the mealworms, lack of hunger may explain why the bird did not touch the moth.

2.4 Statistical analyses

The response variables used were 1) survival = whether or not the moths survive the bird attack (binomial), 2) the proportion of the moth eaten (continuous proportion) , 3) bird approach latency = time between seeing the moth and approaching the moth (in seconds), 4) attack latency = the time it took for the bird to attack the moth after the bird had approached it (in seconds), 5) handling duration = how long bird handled (eating and holding) the moth (as seconds), 6) eating duration = how long bird ate the moth (as seconds), 7) drop latency = the time between when bird first grabbed the moth until the bird first dropped it (in seconds), 8) latency to kill = how long it took for moth to die from the time the bird saw the moth (in seconds), 9) the number of times the bird dropped the moth, 10) the number of times the bird wiped its beak, 11) the number of times the bird drank water.

Every model had four basic fixed effects explanatory variables; pyrrolizidine alkaloids (PAs, present-absent), neck fluids (MPs, present-absent), moth colour (either morph: white/yellow or genotype: WW, Wy = white or yy = yellow) (see below for model fitting information and AIC) and trial number (1–3). In addition, following covariates were tested by adding them one by one as fixed effects to the

basic model (i.e., model that had only basic fixed explanatory variables: PAs, MPs, moth colour/genotype and trial number) and they were included to the model if they improved the model fit. These covariates were 1) moth activity = in total how long moth crawled, flied or flexed (in seconds), 2) how long bird ate the moth (in seconds), 3) how long birds were in captivity (in days), 4) bird weight in (in grams) and 5) bird age (adult or juvenile). Bird ID was included as a random factor to account for multiple trials per bird to every model except for moth survival because the model would not converge if bird ID was as random factor due to the complexity of the model.

The following models were fitted to test the hypotheses of this study (Appendix 1, Table S1). To test the probability that blue tits would progress through the attack sequence (attacked, dropped, eaten) and if moth survived, generalized linear mixed model (GLMM) with binomial distributions was used (package lme4). For timed behaviours (approach and attack latencies, eating and handling durations, drop latency, latency to kill) Cox proportional hazards model (coxme) was used (package coxme). For dropping the moth and drinking water GLMM with Poisson distribution was used. For wiping the beak, a negative binomial distribution was used due to overdispersion. For proportion eaten, a linear mixed effects model (LME) with a Gaussian distribution was used. All analyses were conducted using the statistical program R, version 4.0.3.

Akaike's Information Criterion (AIC) was used for model comparison. It was used for every model to see if interactions between the basic fixed variables improved the model. It was done by dredge-function (package MuMIn) and the model that had lowest AIC score was selected. After seeing which interactions were improving the model, AIC was used to see if genotype improved the AIC score by more than 2 compared to moth morph, in which case genotype was used instead of morph (so some models contained moth morph and some moth genotype). Replacing moth morph with genotype improved the AIC score in models for approach latency and drop latency so genotype was used in those models

(Appendix 1, Table S1). AIC was also used to see which covariates improved the different models. It was done by comparing two models which one was simpler and contained only the fixed variables and the other contained the fixed variables and also one of the covariates. If the simpler model had AIC score lower than 2 compared to the other model, tested covariate was excluded from that model and otherwise it was selected as a covariate that improve the model. After that another covariate was tested. For the model of latency to kill, moth activity improved the AIC score, so it was included to that model. Bird age improved the AIC score for the model of dropping the moth, eating duration improved the model of water drinks and bird weight improved the of model total proportion eaten, so those covariates were included to their corresponding models (Appendix 1, Table S1).

3 RESULTS

3.1 Progression through attack sequence

Birds started to approach the moth quicker as the trials progressed (coxme estimate ± standard error (SE) = 1.316 ± 0.087, z = 3.15, p = 0.002; Appendix 2, Figure S1A). Birds approached moths of the WW genotype about three times slower compared to moths of the yy (coxme estimate ± SE = 0.517 ± 0.216, z = -3.05, p = 0.002; Figure 2). With moths of the genotype Wy, there were no significant differences in approach latency compared to either WW or yy genotypes. However, when neck fluids were included, birds approached WW and also Wy genotypes slower than yy without neck fluids (WW coxme estimate ± SE = 0.459 ± 0.305, z = -2.56, p = 0.011; Wy coxme estimate ± SE = 0.500 ± 0.339, z = -2.04, p = 0.041; Figure 2).

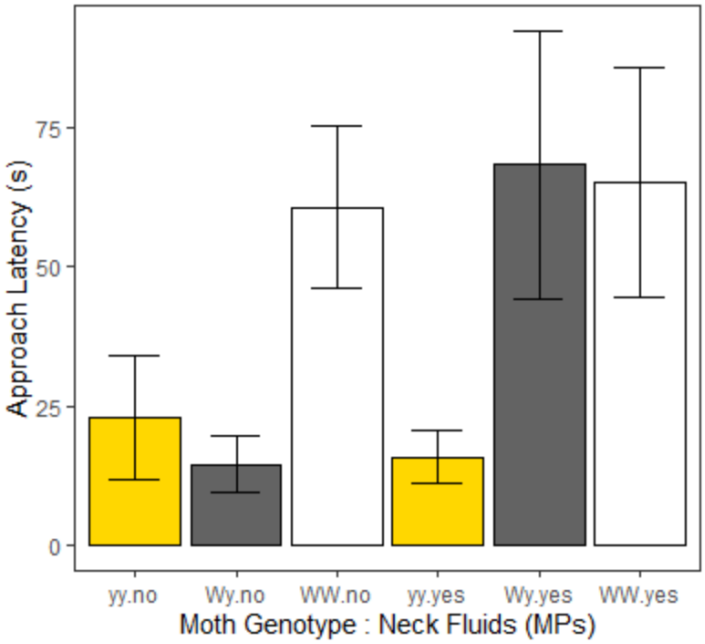

Figure 2. How long it took for bird to approach the moth (in seconds) after seeing it in response to the interaction between moth genotype (WW, Wy = white hindwings, yy = yellow hindwings) and neck fluids (bad smell) (yes = methoxypyrazines (MPs) were present, no = MPs were not present). Bar graph shows mean ± SE.

Only trial number affected the probability that birds would attack the moth. There was a trend for attack probability to increase as trials progressed (GLMM estimate ± standard error (SE) = 1.901 ± 1.031, $z = 1.85$, $p = 0.065$). Trial number influenced to how long it took for birds to attack; birds attacked the moth quicker as trials progressed (coxme estimate ± SE = 0.294 ± 0.084, $z = 3.51$, $p < 0.001$, Appendix 2, Figure S1B). There was also a trend for moths with only PAs (taste) to be attacked more quickly than moths with no chemical defences (coxme estimate ± SE = -0.823 ± 0.439, $z = -2.42$, $p = 0.015$; Figure 3).

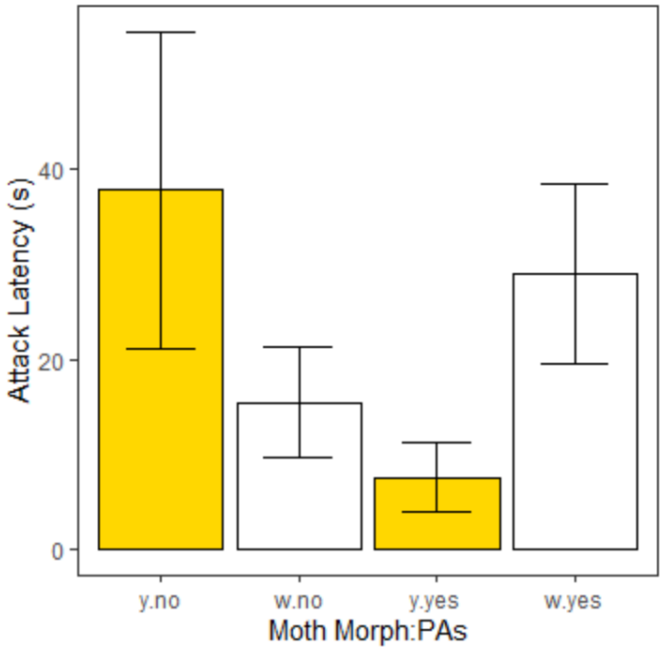

Figure 3. How long it took for bird to attack the moth (in seconds) after approaching it in response to interaction between moth morph (y = yellow, w = white) and bad taste, pyrrolizidine alkaloids (PAs) (yes = PAs was present, no = PAs was not present). Bar graph show mean ± SE.

The only thing to affect the likelihood for the bird to eat the moth was neck fluids (MPs). When the moth had neck fluids, the likelihood of being eaten was smaller than when it did not have them (GLMM estimate ± SE = -2.691 ± 1.031, z = -2.61, p = 0.009, Figure 4). The likelihood of being eaten was reduced by 18% when moth had neck fluids.

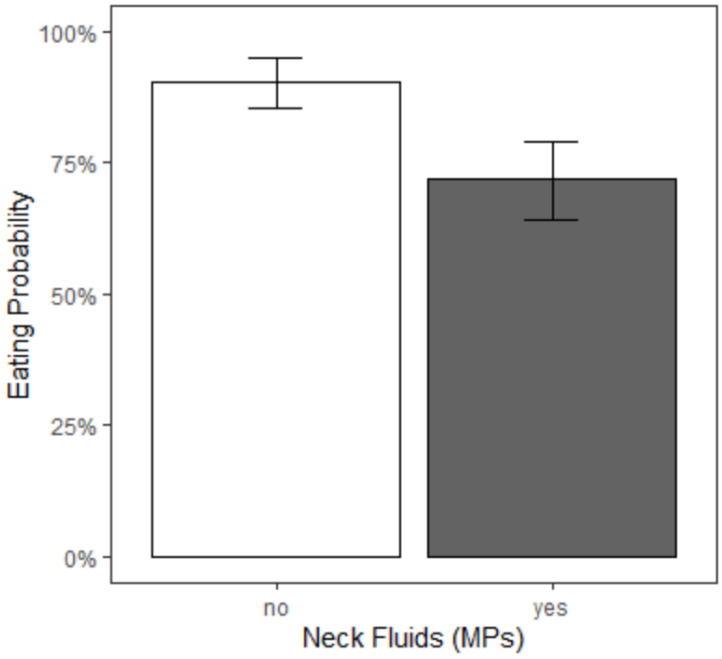

Figure 4. Probability for bird to eat the moth in response to neck fluids (bad smell) (yes = methoxypyrazines (MPs) were present, no = MPs were not present). Bar graphs show Bar graphs show the standard error from bootstrap.

3.2 Disgust behaviours

The probability for birds to drop the moth increased when the moth had neck fluids (MPs); 19% of the moths without neck fluids were dropped, while 36% of the moths with neck fluids were dropped (GLMM estimate ± SE = 1.407 ± 0.595, z = 2.37, p = 0.018; Figure 5A). However, the difference seemed to be from white moths that had neck fluids because yellow moths with neck fluids did not differ from either morph without neck fluids (GLMM estimate ± SE = 2.130 ± 0.980, z = 2.17, p = 0.030; Figure 5B) and so the relationship between colour and smell seems to be additive for white moths.

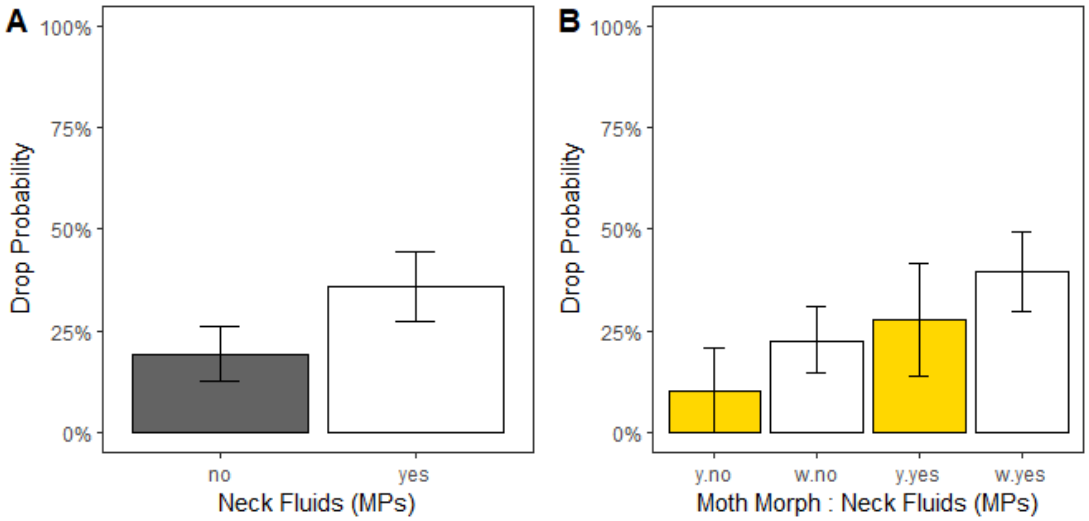

Figure 5. (A) Probability for bird to drop the moth in response to neck fluids (bad smell) (yes = methoxypyrazines (MPs) was present, no = MPs was not present). (B) Probability for bird to drop the moth in response to the interaction between moth morph (y = yellow hindwings, w = white hindwings) and neck fluids (yes = MPs was present, no = MPs was not present). Bar graphs show the standard error from bootstrap.

Birds dropped the moth before eating it almost twice as much when moth had neck fluids (MPs) than when it did not have them (GLMM estimate ± SE = 1.185, ± 0.428, z = 2.77, p = 0.006). When looking separately at both morphs with and without neck fluids, white moths with neck fluids were the ones to be dropped significantly more than yellow moths that did not have neck fluids (GLMM estimate ± SE = 2.041 ± 0.851, z = 2.40, p = 0.017; Figure 6A). There was also trend for the moth to be dropped more often when it had both chemical defences than when it had none (GLMM estimate ± SE = 1.048 ± 0.589, z = 1.78, p = 0.075) and combining all aspects, there was a trend for white moths with both chemical defences to be dropped more often than yellow moths with no chemical defences (GLMM estimate ± SE = 1.827 ± 0.956, z = 1.91, p = 0.056). Bird age also influenced the number of times the bird dropped the moth. Adults dropped the moth almost three times more often than juveniles (GLMM estimate ± SE = -0.919 ± 0.461, z = -1.99, p = 0.046; Figure 6B).

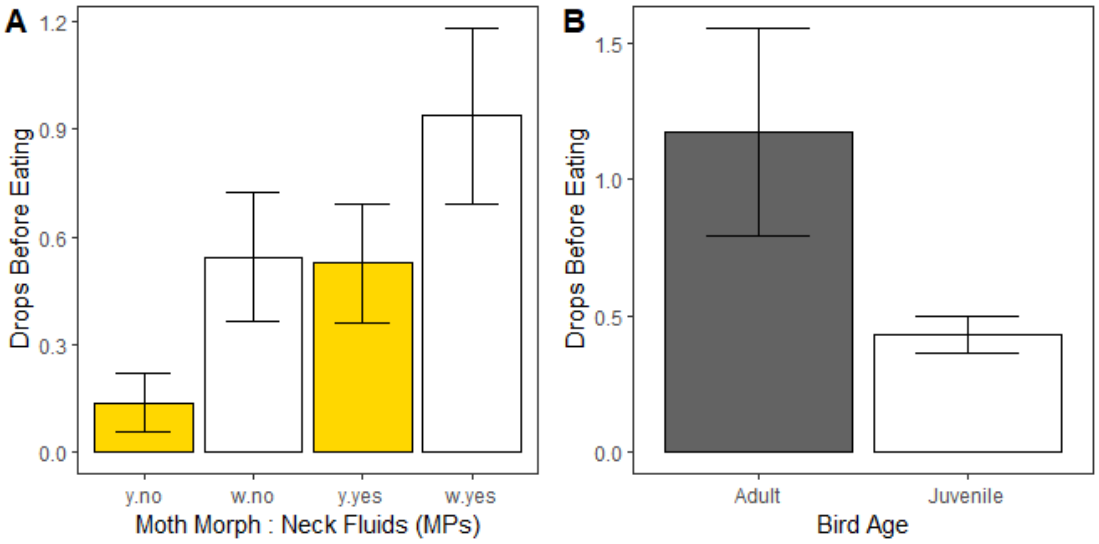

Figure 6. (A) The average number of drops birds did before eating the moths in response to the interaction between moth morph (y = yellow hindwings, w = white hindwings) and neck fluids (bad smell) (yes = methoxypyrazines (MPs) was present, no = MPs was not present). (B) The average number of drops for adult and juvenile birds before eating the moth. Bar graphs show mean ± SE.

Moth genotype influenced to the birds drop latency (Figure 7A). When the moth genotype was Wy, birds dropped the moth faster than when the genotype was yy (coxme estimate ± SE = 2.502 ± 0.394, z = 2.33, p = 0.020) or WW (coxme estimate ± SE = 2.712 ± 0.361, z = 2.76, p = 0.006; Figure 7A). There was a trend that moths with neck fluids were dropped quicker than those without (coxme estimate ± SE = 1.664 ± 0.302, z = 1.69, p = 0.091). However when looking separately at each genotype with and without neck fluids, the moths which had genotype Wy and neck fluids were dropped over five times quicker than those with genotype yy and no neck fluids (coxme estimate ± SE = 5.273, 0.533, z = 3.12, p = 0.002; Figure 7B).

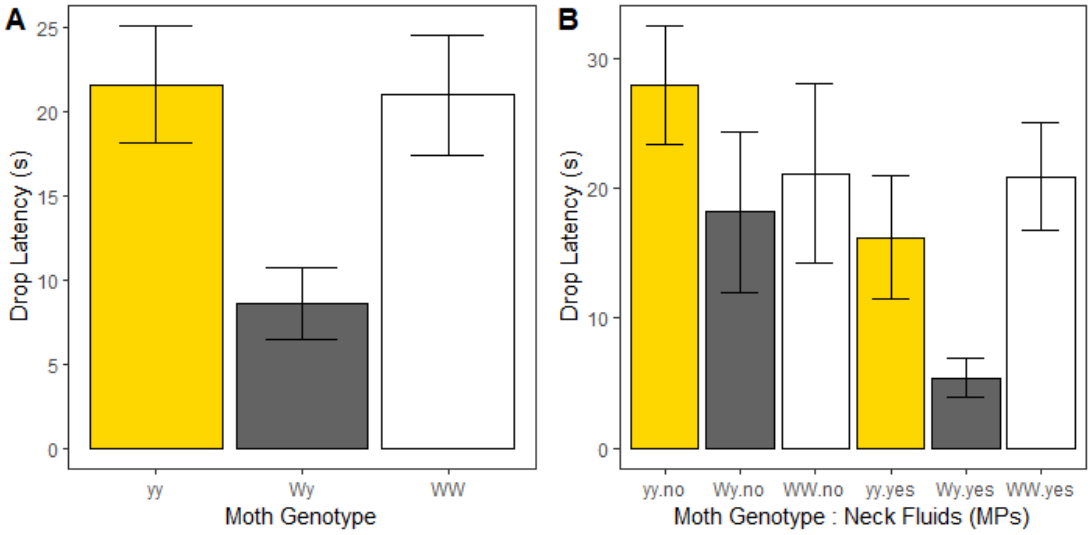

Figure 7. (A) Birds drop latency (in seconds) in response to each moth genotype (WW, Wy = white hindwings, yy = yellow hindwings). (B) Birds drop latency (in seconds) in response to the interaction between moth genotype and neck fluids (bad smell) (yes = methoxypyrazines (MPs) was present, no = MPs was not present). Bar graphs show mean ± SE.

Trial number influenced to handle duration; the more trials the bird had already done, the less time it spent handling the moth (coxme estimate ± SE = 2.342 ± 0.108, $z = 7.90$, $p < 0.001$; Appendix 2, Figure S1C).

In eating duration there was an interaction between trial number and neck fluids (coxme estimate ± SE = 0.685 ± 0.181, $z = -2.10$, $p = 0.036$; Figure 8). Birds ate the moths that did not have neck fluids quicker as trials progressed, but birds ate moths that had neck fluids for the same duration across trials.

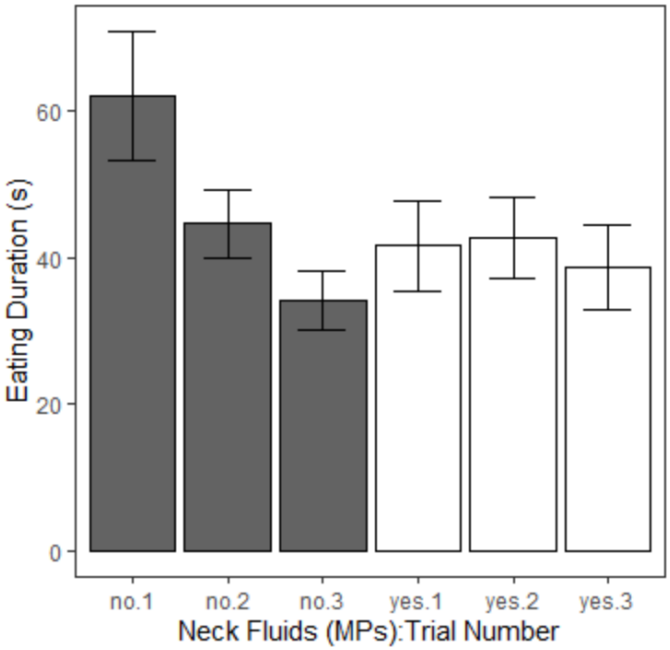

Figure 8. How long bird ate the moth in response to the interaction between neck fluids (bad smell) (yes = methoxypyrazines (MPs) was present, no = MPs was not present) and trial number (from 1 to 3). Bar graph show mean ± SE.

Only trial number affected to the number of times bird wiped its beak. Beak wiping decreased when trials progressed (negative binomial estimate ± SE = -0.796 ± 0.109, z = -7.31, $p < 0.001$; Appendix 2, Figure S1D).

The interaction between trial and moth morph significantly influenced bird drinking behaviour (GLMM estimate ± SE = -1.169 ± 0.386, z = -3.03, p = 0.002; Figure 9). Birds drank less water as trials progressed only when they were eating yellow moths but with white moths, birds did not significantly reduce water drinking. Bird eating duration also influenced the number of times it drank water. The longer the bird ate the moth, the more it drank water (GLMM estimate ± SE =0.014 ± 0.003, z = 4.77, $p < 0.001$; Appendix 2, Figure S2A). There was also a trend when looking at each morph with and without each chemical defence. When the moth was yellow and had only neck fluids (estimate ± SE = -2.362 ± 1.411, z = -1.67, p = 0.094) or when it was white and did not have any chemical defence (estimate ± SE = -1.513 ± 0.890, z = - 1.70, p = 0.089) birds drank less water than

when the moth was yellow and did not have chemical defence (Appendix, Figure S2B).

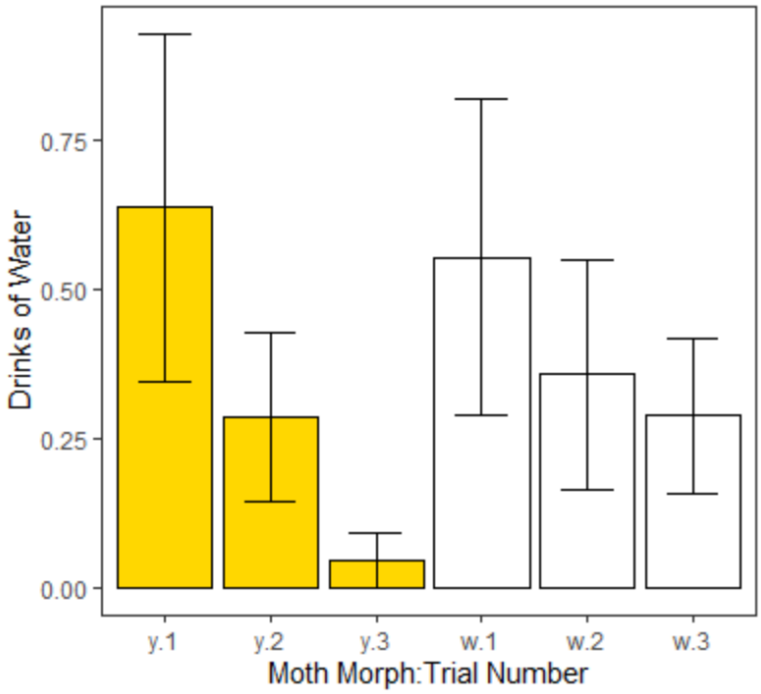

Figure 9. How many times bird drank water in response to the interaction between moth morph (y = yellow hindwings, w = white hindwings) and trial number (from 1 to 3). Bar graph show mean ± SE.

The total proportion of the moth that birds ate increased as the trials progressed (LME t-value = 2.053, DF = 151, p = 0.042; Appendix 2, Figure S1E). Total proportion eaten was smaller when moth had neck fluids (smell) (t-value = -2.406, DF = 79, p = 0.018). However, this result is probably driven by moths with both chemical defences because when looking separately at each chemical treatment, the proportion eaten of moths with both chemical defences was smaller than moths with no chemical defences (LME t-value = -2.265, DF = 79, p = 0.026; Figure 10A), but the proportion eaten did not differ between moths with only neck fluids and those with no defences (LME t-value = -1.282, DF = 79, p = 0.204; Figure 10A). Also, bird weight when they were captured had an effect, heavier birds ate a smaller amount of the moth (LME t-value = -3.772, DF = 79, p < 0.001; Figure 10B).

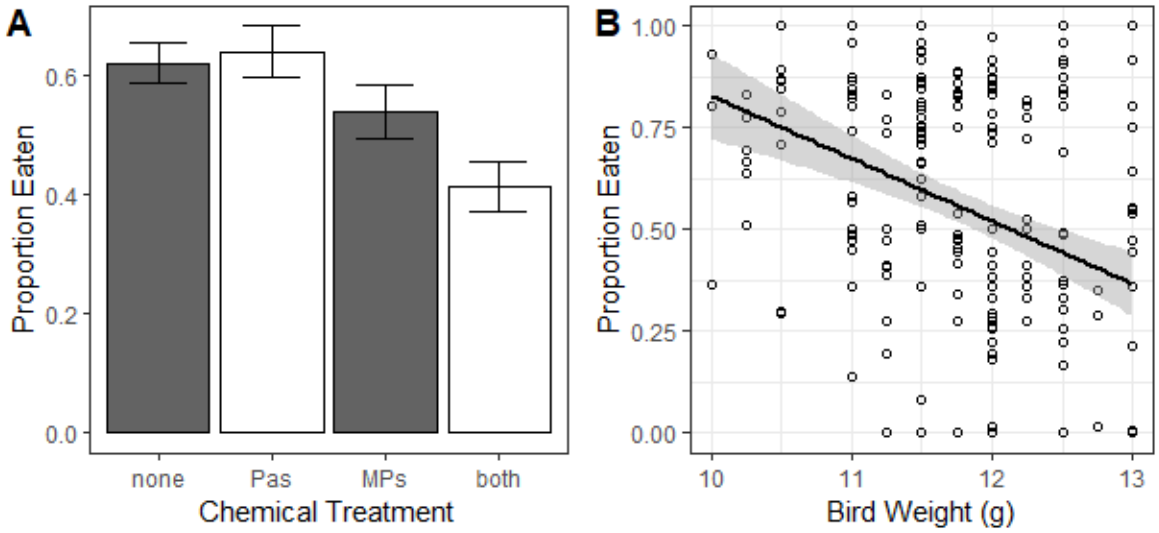

Figure 10. (A) Proportion of the moth eaten in response to moth chemical treatment (none = moth had no chemical defence, PAs = moth had only PAs (pyrrolizidine alkaloids as a bad taste), MPs = moth had only MPs (methoxypyrazines as a bad smell), both = moth had PAs and MPs). Bar graph shows mean ± SE. (B) Proportion of the moth eaten in response to bird weight (in grams). Shaded area in regression line represents the 95% confidence level interval.

3.3 Moth survival

Moths were three times more likely to survive when it had neck fluids compared to not having them (GLMM estimate ± SE = 1.257 ± 0.381, z = 3.30, p < 0.001, Figure 11A). There was also a trend that white moths survived more often than yellow ones (GLMM estimate ± SE = 0.801 ± 0.451, z = 1.78, p = 0.076). Moth survived twice more often when it had both chemical defences (MPs and PAs) compared to no chemical defence (GLMM estimate ± SE = 1.273 ± 0.473, z = 2.69, p = 0.007) and there was also a trend for moths to survive more often when they had only neck fluids compared to moths with no chemical defence (GLMM estimate ± 0.815 ± 0.485, z = 1.68, p = 0.093). When all of these defences are compared by looking interaction between colour, smell and taste, moths of the white morph with both chemical defences survived more often than moths of the yellow morph with none of the chemical defences (GLMM estimate ± SE = 1.610 ± 0.811, z = 1.99, p = 0.047; Figure 11B).

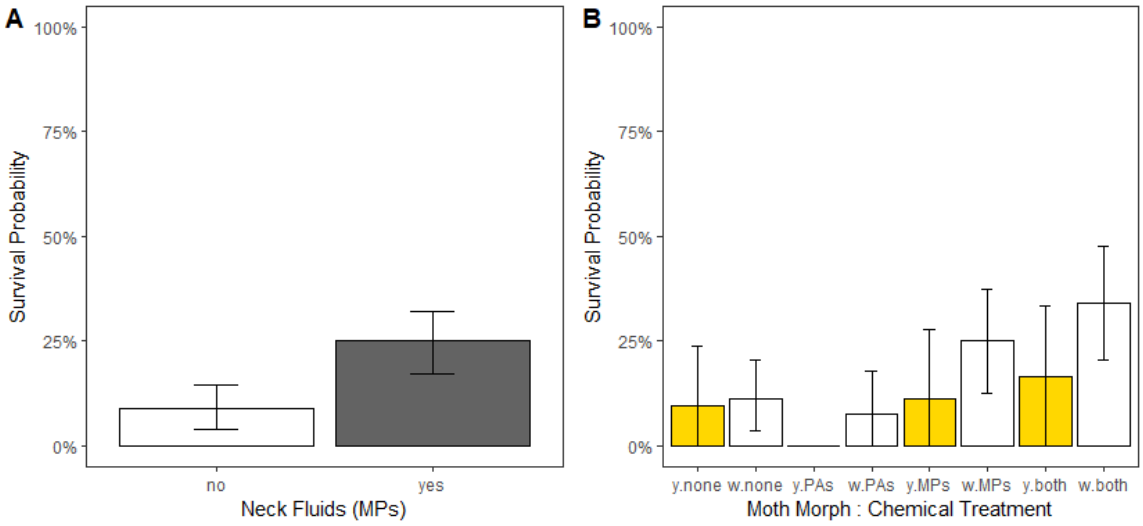

Figure 11. (A) Percentage of the moths survived after a trial in response to neck fluids (bad smell) (yes = methoxypyrazines (MPs) was present, no = MPs was not present). (B) Percentage of the moths survived after a trial in response to the interaction between moth morph (y = yellow hindwings, w = white hindwings) and chemical treatment (none = moth had no chemical defence, PAs = moth had only PAs (pyrrolizidine alkaloids as a bad taste), MPs = moth had only MPs, both = moth had PAs and MPs). Bar graphs show the standard error from bootstrap.

Bird latency to kill the moths was shorter as trials progressed (coxme estimate ± SE =0.718 ± 0.106, z = 86.77, p < 0.001; Appendix 2, Figure S1F) and also when moths had PAs (smell) than when they did not have them (coxme estimate ± SE = 1.350 ± 0.538, z = 2.51, p = 0.012; Figure 12). There was a trend that moth activity prolonged the death time so that birds killed more active moths later (coxme estimate ± SE = -0.018 ± 0.009, z = -1.95, p = 0.051; Appendix 2, Figure S3). When looking separately at each chemical treatment, there was a trend that moths with no chemical defences survived longer than moths with only PAs (coxme estimate ± SE = 0.529 ± 0.271, z = 1.95, p = 0.051).

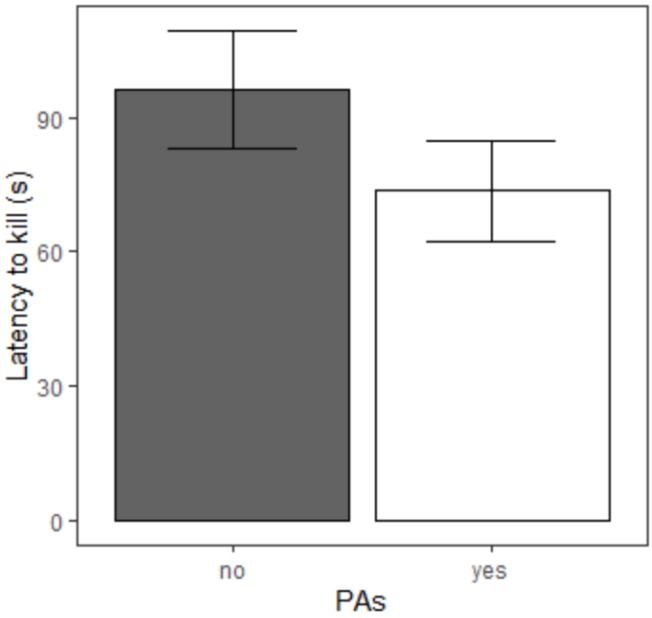

Figure 12. Latency to kill the moths (in seconds) in response to bad taste, pyrrolizidine alkaloids (PAs) (yes = PAs was present, no = PAs was not present). Bar graph show mean ± SE.

Moths that did not have PAs (taste) were more active (Appendix 2, Figure S3). Moths PA content relation to moth activity was investigated apart from the main analysis. It was done only by plotting PAs and activity, and did not include any statistical analysis. However, this cannot be proven statistically, because there were only so few moths that were active.

When plotting PAs and activity, moths that did not have PAs were more active (Appendix 2, Figure S4). However, because there were only so few moths that were active, this result was not part of the main analysis. The result was only based on the plotted picture, and thus it cannot be proven statistically.

When plotting PAs and moth weights, moths that did not have PAs were in average 0.7 g heavier (Appendix 2, Figure S5). However, because moth weight was not included in the model based on AIC, this result was not part of the main analysis.

4 DISCUSSION

This study shows how multimodal anti-predator defences work through the predation sequence and how multimodality improves moth survival. These results are in line with the first hypothesis; multimodality with three signals can indeed be better than only two signals. The second hypothesis, that birds will learn most efficiently when moth has multiple signals, was not correct because there was no evidence that birds learned to avoid prey with defences. The third hypothesis was that colour will take effect at approach and attack stage, smell (MPs) approach, attack and consumption stage and taste (PAs) at consumption stage. This was partly correct because colour influenced in approach and attack stage but also in consumption stage, MPs took effect at approach and consumption stage but not in attack stage and PAs influenced in consumption stage but also at the attack stage. Hesitation to approach and drop latency to moths of the Wy genotype (white morph) was dependent on the presence of neck fluids. White moths with neck fluids were also dropped greater number of times. Taste (PAs) alone did not deter bird predators, and birds responded to PAs only when they started to eat moths that also had neck fluids. Although neck fluids affected many predation events, the moth had the best chance to survive when it was white and had both secondary defences. By differentiating the effect of colour and smell and taste in a live prey, these results can be used to inform the theoretical framework of multimodal aposematic signals.

4.1 Progression through attack sequence

Neck fluids seem to be particularly important to moths of the Wy genotype. When birds are deciding whether or not to approach, neck fluids of Wy moths made the birds hesitate longer compared to Wy moths without neck fluids and compared to yy moths with or without neck fluids. For the WW genotype, neck fluids did not influence bird approach behaviour, they were approached later than yy moths

regardless of their neck fluid treatment. Rojas et al. (2019) also found that white hindwing colouration increases approach latency when there are neck fluids present, but the neck fluids of white and yellow moths did not differ. However, the authors only separated colour morphs, so the genotypes Wy and WW (and their neck fluids) were studied as one. Because in this study it is shown that Wy and WW genotypes may differ, Rojas et al. (2019) may have had different results if they had separated those two genotypes. There may be a difference between WW and Wy genotypes (both white hindwings), which blue tits can see that is not apparent to humans. Indeed, these two genotypes differ in their UV-reflectance (O. Nokelainen, unpublished). Because there was a difference in approach latency between moths of WW and Wy genotypes without neck fluids, it seems that to birds there indeed is some difference in visual appearance between these two genotypes. It has been shown that UV-reflective white colouration can act as a warning signal in some Lepidopteran species (Corral-Lopez 2020), while other studies suggest UV reflectance can attract birds (Lyytinen et al. 2001, 2004).

In contrast to this study and the results of Rojas et al. (2019), Nokelainen et al. (2012, 2014) found that yellow morph moths were better protected from predation by *C. caeruleus*. However, it must be noted that in this study the moths were presented against the plywood enclosure that had a yellow tinge and not against green background as in Rojas et al. (2019) and Nokelainen et al. (2012, 2014) studies. This may have muted the yellow warning signal and made it less effective. In addition, the difference between this study, Rojas et al. (2019) and Nokelainen et al. (2012, 2014) experiments may be because of different light environments (Rojas et al 2019, O. Nokelainen, unpublished). The natural light may differ from the artificial light used indoors and that may influence the way birds see the wing colour of *A. plantaginis*. Also, when using dead moths, as in Nokelainen et al (2012) study, the moths may not release methoxypyrazine (neck fluids – smell) as effectively as live ones because volatile methoxypyrazines may have evaporated or the moth may need to be alive to synthesize them as needed.

However, whether the prey was a model or natural prey does not seem to explain the differences in bird response observed between studies because Rojas et al (2019) (conducted in lab) and Nokelainen et al. (2014) (conducted in the field) used model prey and in this study and in Nokelainen et al. (2012) study (conducted in the lab with live prey and in the field with dead prey) the moths were natural.

Earlier studies found that moth neck fluids delayed attack latency (Rojas et al. 2019). In this study, contrary to the hypothesis, attack latency was not affected by neck fluids but only presence of bad taste (PAs). There could have been a possibility that moths that ate PA diet were larger and birds would not attack moths that had PAs quicker but moths that were larger and so were more attractive prey. However, the result does not indicate that because moth weight was not included in the model based on AIC and when comparing the moths' weights from diet with and without PAs, the moths without PAs were less than a gram heavier. There may also be a trade-off between sequestering PAs and synthesizing MPs. It could be that if moths have to invest energy in storing toxins they have less energy to invest in synthesizing MPs. Therefore, squeezed moths raised on a diet without PAs may have had neck fluid reserves in greater amounts compared to squeezed moths with PAs. Therefore, birds may not have noticed the MPs smell in the moths that had PAs and attacked them quicker than moths with no PAs. Although birds in this study and Rojas et al. (2019) study were both captured near the Konnevesi research station, there is a time difference between this study and the Rojas et al. (2019) study. This study was done in 2020 while the Rojas et al. (2019) study was conducted in 2012. By that time, the food preference of birds near the Konnevesi research station may have changed, which could also explain why neck fluids influenced to attack latency previously but not anymore. Such a shift in food preferences could dynamically happen through social information use among the predator community (Bennett 1996, Aplin et al. 2015, Thorogood et al. 2018, Hämäläinen et al. 2019, Hämäläinen et al. 2020).

4.2 Disgust behaviour

The probability for birds to drop the moth and the number of times the bird dropped the moth were greater when the moth had neck fluids. However, both of these results seem to have additive effects with white moths. Rojas et al. (2019) found that despite model colour, birds ate a smaller amount when the model had white moth neck fluids compared to yellow moth neck fluids and that the neck fluids of yellow moths made the birds wipe their beaks more. In this experiment, beak wipes were not affected by neck fluids but both of these results suggest that especially neck fluids from white moths are repellent for birds. Also, moths of genotype Wy (white) were dropped quicker than other genotypes (drop latency was shorter). This suggests that neck fluids from Wy moths are the most effective against birds at least at the grabbing and dropping stages of an attack. The number of drops and drop latency can also vary in response to the combination of visual and chemical signals as a context-setting signal. This means that neck fluids may set the context for colouration and possibly change the bird's response to it. Other studies have also shown this response (e.g., Jetz et al. 2001, Kelly and Marples 2004, Hebets and Papaj 2005). Bad taste (PAs) act at consumption stage to increase the times bird drop the moth as predicted. Although PAs alone did not increase the number of times the bird dropped the moth, but when paired with MPs, white moths with both chemical defences were dropped more than yellow moths with no chemical defence. It may be that MPs work as an attention-altering signal, that increases the bird's attention to PAs. So, when MPs are absent, birds do not recognise PAs as something bad or do not pay attention to the PA taste and reduce the amount eaten. Guilford (1994) suggested that visual warning signals may act as "go-slow" signals that alert predators to pay better attention in their assessment of prey palatability. This same may be true for methoxypyrazines, which may alert predators to "go-slow" in their taste assessment to avoid eating toxins such as pyrrolizidine alkaloids.

This study's results show that heavier birds ate less of the moth. Heavier birds may have been in better condition and could therefore choose to not eat so much. Similarly, Hämäläinen et al. (2020) found that male great tits attacked defended prey more when their body condition was low. Although birds did not drink water more when they ate defended prey, a longer time spent eating made the birds drink more water, which means the birds need to drink while eating. Also, neck fluids influenced bird eating behaviour. As predicted, neck fluids helped the moth to avoid getting eaten and also reduce the eating duration because the probability for the bird to start eating the moth and time spend eating was smaller when the moth had neck fluids. Similarly, Lindström et al (2001) showed that presence of pyrazines made birds avoid (did not start eating) conspicuously coloured food, which did not happen when pryrazines were absent. Although methoxypyrazines helped the moth to avoid getting eaten, pyrrolizidine alkaloids (that were expected to affect consumption) did not alone deter birds. Birds only reduced the amount consumed when PAs were combined with methoxypyrazines (neck fluids). Here, also as in number of times the bird dropped the moth, MPs may work as a "go-slow" signal and alert the bird to pay more attention to possible toxicity while eating their prey.

PAs are not just in moth tissues, but also found in neck fluids (A. Winters, unpublished), thus some of the PAs were removed in the PA treatment without neck fluids. This may lead to an underestimation of the PAs (bad taste) effectiveness. For example, birds in this study given moths from the treatment with only PAs did not experience PAs in neck fluids before eating the nutrient rich body. However, birds do not find moth abdominal fluids to be unpleasant (Rojas et al. 2017) even though these include PAs (A. Winters, unpublished) but not MPs. This suggests that PAs may be more effective against other predators than birds and indeed many studies show that PAs are effective against invertebrates (e.g., Dussourd et al. 1988, Eisner and Eisner 1991, Conner et al. 2000, Eisner et al. 2000). Rojas et al. (2017) already showed that wood tiger moths neck fluids were

deterrent to birds and abdominal fluids to ants. Because different defences may also asymmetrically target predators, PAs can target multiple types of predators but be more important to one predator than others. Thus, different predators may create different selection pressures and morph evolution of multimodal aposematic signals.

4.3 Moth survival

White moths with both chemical defences survived more often than yellow moths with no chemical defence. This indicates that all three defence mechanisms are important for survival against bird predators, as predicted in the hypothesis. Although Nokelainen et al. (2012) in contrast found that yellow moths would have higher probability to survive, they used already dead prey so the survival estimation may not be accurate in comparison to live prey. However, there are not many studies that assess prey survival, and more studies are needed.

As with attack latency, PAs seemed to increase predators' interest in killing the moth because birds killed moths more quickly if they had PAs than when they did not. There could be a trade-off so that moths with PAs are less active. When plotting the activity and PAs, moths with PAs are in average much less active. This however cannot be proven statistically because there were so few active moths. Also as stated earlier, if moths with PAs would have been larger, the moths would have been more attractive prey and also their ability to fly could have been worse. Moth activity prolonged bird latency to kill the moth, because it took more time for the bird to capture the moth. In the wild, such latency might allow moths to escape.

4.4 Predator learning

There was no evidence that birds learned to avoid moths that were chemically protected. Contrary to the prediction, approach latency, attack latency, eating

time, handling time and kill latency decreased as trials progressed regardless of the chemical treatment. So overall, birds became more efficient at attacking and handling the moths and also got more familiar with their defences because, independent of chemical treatment, disgust behaviours (beak wipes and water drinks) also decreased and total proportion eaten increased.

As there were adults and juveniles in this study, the birds may have had different prior experience with prey species that use MPs. If juveniles had not experienced warning signals before, they may not experience the aversive smell as a cue that prey should not be eaten. This may explain the result that adults dropped the moth a greater number of times compared to juveniles. Also, adults may sample the prey more cautiously because of prior experience with defended prey. Alternatively, adults might be expected to drop the prey less, because they should know that if the moth is dropped, it can escape.

Birds did not learn to avoid toxic moths. This may be because the bird's choices were either to eat the moth or to not eat it, and defended moths still have nutritional value. Barnett et al. (2007) showed that adult birds, that are familiar with the prey, make decisions depending on the prey's energetic value and toxins, and also its own energetic needs. If birds were offered an undefended option together with the defended moth, then birds might have learned to avoid defended moths. Indeed, when there are other, unprotected, prey available, predators tend to choose to eat them (Kokko et al 2003, Sherratt 2003). Also, the experiments were done in winter and birds were captured in winter when prey are sparse, so birds may have been more eager to eat live prey even with defences. For example, Chatelain et al. (2013) have shown that predators tend to eat even toxic prey when the temperature is cooler.

5 CONCLUSIONS

The aims of this study were to investigate whether and how multimodality (with colour smell and taste) improves prey survival, at which stage of attack each defence modality is effective and in which way they influence predators' behaviour. Colour showed a part in defence against bird predation because it delayed the approach to white moths more than yellow moths but it was not enough to stop the birds from approaching. PAs taste was not effective against blue tit predators; at least when acting without MPs smell. When taste was paired with smell, birds ate less and also bird dropped the moth more meaning that besides colour, a bad smell can also act as a "go-slow" signal. Smell on other hand had effect even when acting without taste because when moth had only a bad smell, birds approached the moths more slowly, were more likely to drop the moth, dropped the moth more and more quickly, were less likely to start eating the moth, ate for a longer period of time and also ate a smaller proportion of the moth. Birds seemed to pay attention to each defence mechanism, and each defence played a role in improving moth survival. Colour was used as a primary defence, smell was used partly as a primary defence and also as a secondary defence, while taste was only used as a secondary defence if the methoxypyrazine smell was also present. The fact that moths that are heterozygous for hindwing colouration had an advantage in approach latency and drop latency may provide an explanation to persistence of colour polymorphism in this species. These findings show that anti-predator defences should be studied together rather than separately because they can have different additive effects.

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APPENDIX 1. Result summary table

Table S1. Result summaries from the models used. Comparisions was done to see which treatments differ from the control (moths with genotype yy/yellow colour, without MPs and without PAs). MPs = methoxypyrazines (bad smell), PAs = pyrrolizidine alkaloids (bad taste), interaction = interaction between colour, MPs and PAs, distance = interaction between colour and MPs, distanceg = interaction between genotype and MPs, ChemTreatment = interaction between MPs and PAs.

Model type	Response variable	Model used				
GLMM (binomial distribution)	Probability to attack	glmer(AttackProb ~ MPs + PAs + MothMorph + TrialNumber + PAs : TrialNumber + (1 | BirdID), data=data, family=binomial)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	6.7610	2.9270	2.3100	0.0209
		MPsyes	-1.2460	1.4470	-0.8610	0.3891
		PAsyes	1.1910	2.1080	0.5650	0.5720
		MothMorphw	-1.6360	2.0170	-0.8110	0.4171
		TrialNumber	1.9010	1.0310	1.8450	0.0651
		PAsyes:TrialNumber	-1.6680	1.1830	-1.4100	0.1584
	Probability to drop the prey	glmer(DropProb ~ MPs + PAs + MothMorph + TrialNumber + (1 | BirdID), data= subsetattacked, family=binomial)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-2.7040	0.9026	-2.9960	0.0027
		MPsyes	1.4070	0.5947	2.3660	0.0180
		PAsyes	0.1615	0.5663	0.2850	0.7755
		MothMorphw	1.0553	0.6722	1.5700	0.1164

Model type	Response variable	Model used				
		TrialNumber	-0.1931	0.2268	-0.8510	0.3947
	Probability to start eating	glmer(AteProb ~ MPs + PAs + MothMorph + TrialNumber + MothMorph : TrialNumber + (1|BirdID), data=data, family=binomial)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	6.6967	2.2278	3.0060	0.0027
		MPsyes	-2.6912	1.0308	-2.6110	0.0090
		PAsyes	0.1417	0.9818	0.1440	0.8852
		MothMorphw	-3.0472	1.9215	-1.5860	0.1128
		TrialNumber	-0.3752	0.6208	-0.6040	0.5456
		MothMorphw:TrialNumber	1.0416	0.7279	1.4310	0.1524
	Survival	glm(SurvivalProb ~ MPs + PAs + MothMorph + TrialNumber, data=data, family=binomial)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-2.5281	0.6525	-3.8750	0.0001
		MPsyes	1.2575	0.3811	3.2990	0.0010
		PAsyes	0.1476	0.3481	0.4240	0.6715
		MothMorphw	0.8010	0.4511	1.7760	0.0758
		TrialNumber	-0.2610	0.2147	-1.2160	0.2241
		glm(SurvivalProb ~ interaction + TrialNumber, data=data, family=binomial)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-1.7339	0.8461	-2.0490	0.0404
		interactionw.none	0.1726	0.8621	0.2000	0.8413
		interactiony.PAs	-14.3101	796.1879	-0.0180	0.9857
		interactionw.PAs	-0.2345	0.9577	-0.2450	0.8066
		interactiony.MPs	0.1726	1.0583	0.1630	0.8704
		interactionw.MPs	1.1489	0.8167	1.4070	0.1595
		interactiony.both	0.6453	0.9786	0.6590	0.5096
		interactionw.both	1.6100	0.8110	1.9850	0.0471
		TrialNumber	-0.2684	0.2162	-1.242	0.2144

Model type	Response variable	Model used				
		glm(SurvivalProb ~MothMorph + ChemTreatment + TrialNumber, data=data, family=binomial)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-1.4452	0.6225	-2.3220	0.0203
		MothMorphw	0.8342	0.4525	1.8440	0.0652
		ChemTreatmentPAs	-1.4664	0.6726	-2.1800	0.0292
		ChemTreatmentnone	-0.8147	0.4848	-1.6810	0.0928
		ChemTreatmentboth	0.4583	0.4178	1.0970	0.2726
		TrialNumber	-0.2676	0.2158	-1.2400	0.2149
	Approach latency	coxme(Surv(ApproachLatency) ~ MothGenotype + MPs + PAs + TrialNumber + MPs : PAs + (1 | BirdID), data= approachlatdata)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothGenotypeWy	-0.2704	0.2350	-1.1500	0.2500
		MothGenotypeWW	-0.6598	0.2160	-3.0500	0.0023
		MPsyes	-0.0916	0.2326	-0.3900	0.6900
		PAsyes	0.4105	0.2561	1.6000	0.1100
		TrialNumber	0.2757	0.0869	3.1700	0.0015
		MPsyes:PAsyes	-0.5088	0.3563	-1.4300	0.1500
		coxme(Surv(ApproachLatency) ~ distance + PAs + TrialNumber + (1 | BirdID), data= approachlatdata)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		distancegWy.no	0.0191	0.3287	0.0600	0.9500
		distancegWW.no	-0.6527	0.3042	-2.1500	0.0320
		distancegyy.yes	-0.1774	0.3318	-0.5300	0.5900
		distancegWy.yes	-0.6931	0.3392	-2.0400	0.0410
		distancegWW.yes	-0.7793	0.3049	-2.5600	0.0110
		PAsyes	0.1461	0.1748	0.8400	0.4000
		TrialNumber	0.2730	0.0865	3.1600	0.0016
Coxme	Attack latency	coxme(Surv(AttackLatency) ~ MothMorph + MPs + PAs + TrialNumber + MothMorph : PAs + (1 | BirdID), data= attacklatdata)				

Model type	Response variable	Model used				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothMorphw	0.3726	0.2216	1.6800	0.0930
		MPsyes	-0.0958	0.1490	-0.6400	0.5200
		PAsyes	0.5348	0.2909	1.8400	0.0660
		TrialNumber	0.2944	0.0839	3.5100	0.0005
		MothMorphw:PAsyes	-0.8227	0.3396	-2.4200	0.0150
		coxme(Surv(AttackLatency) ~ MothMorph + ChemTreatment + TrialNumber + (1|BirdID), data= attacklatdata)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothMorphw	-0.3295	0.2091	-1.5800	0.1200
		ChemTreatmentPAs	0.5289	0.2710	1.9500	0.0510
		ChemTreatmentMPs	0.0337	0.2469	0.1400	0.8900
		ChemTreatmentboth	-0.1668	0.2534	-0.6600	0.5100
		TrialNumber	0.3966	0.0905	4.3800	<0.0001
	Eating duration	coxme(Surv(EatingTime) ~ MothMorph + MPs + PAs + TrialNumber + MPs:TrialNumber + (1|BirdID), data= eatdurdat, na.action="na.fail")				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothMorphw	-0.0973	0.3067	-0.3200	0.7500
		MPsyes	1.0359	0.4573	2.2700	0.0230
		PAsyes	0.0544	0.2766	0.2000	0.8400
		TrialNumber	0.4924	0.1336	3.6800	0.0002
		MPsyes:TrialNumber	-0.3791	0.1806	-2.1000	0.0360
	Handling duration	coxme(Surv(HandleTime) ~ MothMorph + MPs + PAs + TrialNumber + (1|BirdID), data= handledurdat)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothMorphw	-0.2013	0.3347	-0.6000	0.5500
		MPsyes	0.1371	0.3040	0.4500	0.6500
		PAsyes	-0.0241	0.3068	-0.0800	0.9400

Model type	Response variable	Model used				
		TrialNumber	0.6658	0.0981	6.7800	<0.0001
	Drop latency	coxme(Surv(ReactionTime) ~ MothGenotype + MPs + PAs + TrialNumber + (1|BirdID), data= reacttimedat)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothGenotypeWy	0.9170	0.3937	2.3300	0.0200
		MothGenotypeWW	-0.0805	0.3540	-0.2300	0.8200
		MPsyes	0.5091	0.3016	1.6900	0.0910
		PAsyes	-0.4156	0.2934	-1.4200	0.1600
		TrialNumber	0.0309	0.1488	0.2100	0.8400
		coxme(Surv(ReactionTime) ~ distance + PAs + TrialNumber + (1|BirdID), data= reacttimedat)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		distancegWy.no	0.3219	0.6499	0.5000	0.6200
		distancegWW.no	0.1597	0.5355	0.3000	0.7700
		distancegyy.yes	0.4466	0.5426	0.8200	0.4100
		distancegWy.yes	1.6627	0.5331	3.1200	0.0018
		distancegWW.yes	0.2337	0.4861	0.4800	0.6300
		PAsyes	-0.4103	0.2894	-1.4200	0.1600
		TrialNumber	0.0656	0.1487	0.4400	0.6600
	Latency to kill	coxme(Surv(DeathTime) ~ MothMorph + MPs + PAs + TrialNumber + MothActivity + MothMorph : MPs + MothMorph : PAs + MPs : PAs + (1|BirdID), data= killlatdat)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothMorphw	0.0383	0.3483	0.1100	0.9100
		MPsyes	0.1512	0.3220	0.4700	0.6400
		PAsyes	1.3505	0.5377	2.5100	0.0120
		TrialNumber	0.7182	0.1061	6.7700	0.0000
		Activity	-0.0179	0.0092	-1.9500	0.0510
		MothMorphw:PAsyes	-0.7554	0.5434	-1.3900	0.1600
		MPsyes:PAsyes	-1.0849	0.4916	-2.2100	0.0270

Model type	Response variable	Model used				
		coxme(Surv(DeathTime) ~ MothMorph + ChemTreatment + TrialNumber + MothActivity + (1|BirdID), data= killlatdat)				
		Fixed effects	Coef	Standard Error (coef)	z-value	p-value
		MothMorphw	-0.2643	0.2721	-0.9700	0.3300
		ChemTreatmentPAs	0.7680	0.3438	2.2300	0.0260
		ChemTreatmentMPs	0.1449	0.3266	0.4400	0.6600
		ChemTreatmentboth	-0.1135	0.3382	-0.3400	0.7400
		TrialNumber	0.7164	0.1064	6.7300	<0.0001
		Activity	-0.0185	0.0090	-2.0400	0.0410
GLMM (Poisson distribution)	Number of times bird dropped the moth	glmer(DropBeforeEat ~ MothMorph + MPs + PAs + TrialNumber + BirdAge + (1|BirdID), data=dropnumdat, family=poisson)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-1.8409	0.6643	-2.7710	0.0056
		MothMorphw	0.6779	0.4943	1.3710	0.1703
		MPsyes	1.1854	0.4279	2.7700	0.0056
		PAsyes	0.1332	0.4219	0.3160	0.7522
		TrialNumber	-0.1033	0.1069	-0.9660	0.3339
		BirdAgeJuvenile	-0.9192	0.4610	-1.9940	0.0462
		glmer(DropBeforeEat ~ interaction + TrialNumber + BirdAge + (1|BirdID), data=dropnumdat, family=poisson)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-1.4115	0.9358	-1.5080	0.1315
		interactionw.none	0.3273	0.9716	0.3370	0.7362
		interactiony.Pas	-19.9893	21295.0100	-0.0010	0.9993
		interactionw.Pas	0.4671	1.0111	0.4620	0.6441
		interactiony.MPs	1.1436	1.0992	1.0400	0.2982
		interactionw.MPs	1.2285	0.9494	1.2940	0.1957
		interactiony.both	0.7126	1.1639	0.6120	0.5404

Model type	Response variable	Model used				
		interactionw.both	1.8267	0.9555	1.9120	0.0559
		TrialNumber	-0.1033	0.1101	-0.9380	0.3481
		BirdAgeJuvenile	-0.9506	0.4615	-2.0600	0.0394
		glmer(DropBeforeEat ~ distance + PAs + TrialNumber + (1|BirdID), data= dropnumdat, family = poisson)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-2.7082	0.8393	-3.2270	0.0013
		distancew.no	1.0560	0.8607	1.2270	0.2198
		distancey.yes	1.4054	0.9585	1.4660	0.1426
		distancew.yes	2.0414	0.8510	2.3990	0.0165
		PAsyes	-0.0339	0.4347	-0.0780	0.9379
		TrialNumber	-0.1062	0.1069	-0.9930	0.3208
		glmer(DropBeforeEat ~ MothMorph + ChemTreatment + TrialNumber + (1|BirdID), data= dropnumdat, family = poisson)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-2.4196	0.6486	-3.7300	0.0002
		MothMorphw	0.8051	0.5145	1.5650	0.1176
		ChemTreatmentPas	-0.2561	0.6834	-0.3750	0.7078
		ChemTreatmentMPs	0.9090	0.5724	1.5880	0.1123
		ChemTreatmentboth	1.0479	0.5890	1.7790	0.0752
		TrialNumber	-0.1062	0.1069	-0.9930	0.3209
	Number of times bird drank water	glmer(Water ~ MPs + PAs + MothMorph + TrialNumber + MothMorph : TrialNumber + EatingTime + (1|BirdID), data=waterdat, family=poisson)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-1.2260	0.8076	-1.5180	0.1290
		MPsyes	0.3967	0.5148	0.7710	0.4409
		PAsyes	-0.1017	0.5171	-0.1970	0.8441
		MothMorphw	-2.3640	0.8362	-2.8270	0.0047

Model type	Response variable	Model used				
		TrialNumber	-1.0308	0.3358	-3.0700	0.0021
		EatingTimeM	0.0135	0.0028	4.7730	<0.0001
		MothMorphw:TrialNumber	1.1687	0.3858	3.0290	0.0025
		glmer(Water ~ interaction + TrialNumber + EatingTime + (1|BirdID), data=waterdat, family=poisson)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	-1.4796	0.7928	-1.8660	0.0620
		interactionw.none	-1.5133	0.8897	-1.7010	0.0890
		interactiony.Pas	-1.7493	1.5483	-1.1300	0.2586
		interactionw.Pas	-1.1341	0.9243	-1.2270	0.2198
		interactiony.MPs	-2.3617	1.4110	-1.6740	0.0942
		interactionw.MPs	-0.3500	0.8438	-0.4150	0.6783
		interactiony.both	-0.0418	1.0194	-0.0410	0.9673
		interactionw.both	-1.0801	0.9130	-1.1830	0.2368
		TrialNumber	-0.2055	0.1490	-1.3790	0.1679
		EatingTimeM	0.0118	0.0027	4.3050	<0.0001
GLMM (Negative binomial distribution)	Number of times bird wiped its beak	glmer.nb(BeakWipes ~ MPs + PAs + MothMorph + TrialNumber + (1|BirdID), data=bwdat)				
		Fixed effects	Estimate	Standard Error	z-value	p-value
		(Intercept)	1.8463	0.4432	4.1660	<0.0001
		MPsyes	0.2081	0.3379	0.6160	0.5380
		PAsyes	0.0726	0.3418	0.2120	0.8320
		MothMorphw	0.2717	0.3820	0.7110	0.4770
		TrialNumber	-0.7963	0.1089	-7.3110	<0.0001
LME	Total proportion eaten	lme(TotPropEaten ~ MPs + PAs + MothMorph + TrialNumber + BirdWeight, random = ~1|BirdID, data=propeatdat)				
		Fixed effects	Value	Standard Error	t-value (DF)	p-value
		(Intercept)	2.3482	0.4772	4.9205 (151)	<0.0001
		MPsyes	-0.1353	0.0562	-2.4056 (79)	0.0185

Model type	Response variable	Model used				
		PAsyes	-0.0346	0.0569	-0.6080 (79)	0.5449
		MothMorphw	0.0121	0.0634	0.1914 (79)	0.8487
		TrialNumber	0.0335	0.0163	2.0533 (151)	0.0418
		BirdWeightIn	-0.1526	0.0405	-3.7721 (79)	0.0003
		lme(TotPropEaten ~ MothMorph + ChemTreatment + TrialNumber + BirdWeight, random = ~1|BirdID, data=propeatdat)				
		Fixed effects	Value	Standard Error	t-value (DF)	p-value
		(Intercept)	2.2782	0.4862	4.6858 (151)	<0.0001
		MothMorphw	0.0079	0.0638	0.1241 (78)	0.9016
		ChemTreatmentPas	0.0135	0.0821	0.1643 (78)	0.8699
		ChemTreatmentMPs	-0.0956	0.0745	-1.2821 (78)	0.2036
		ChemTreatmentboth	-0.1759	0.0776	-2.2655 (78)	0.0263
		TrialNumber	0.0335	0.0163	2.0577 (151)	0.0413
		BirdWeightIn	-0.1480	0.0410	-3.6133 (78)	0.0005

APPENDIX 2. Figures

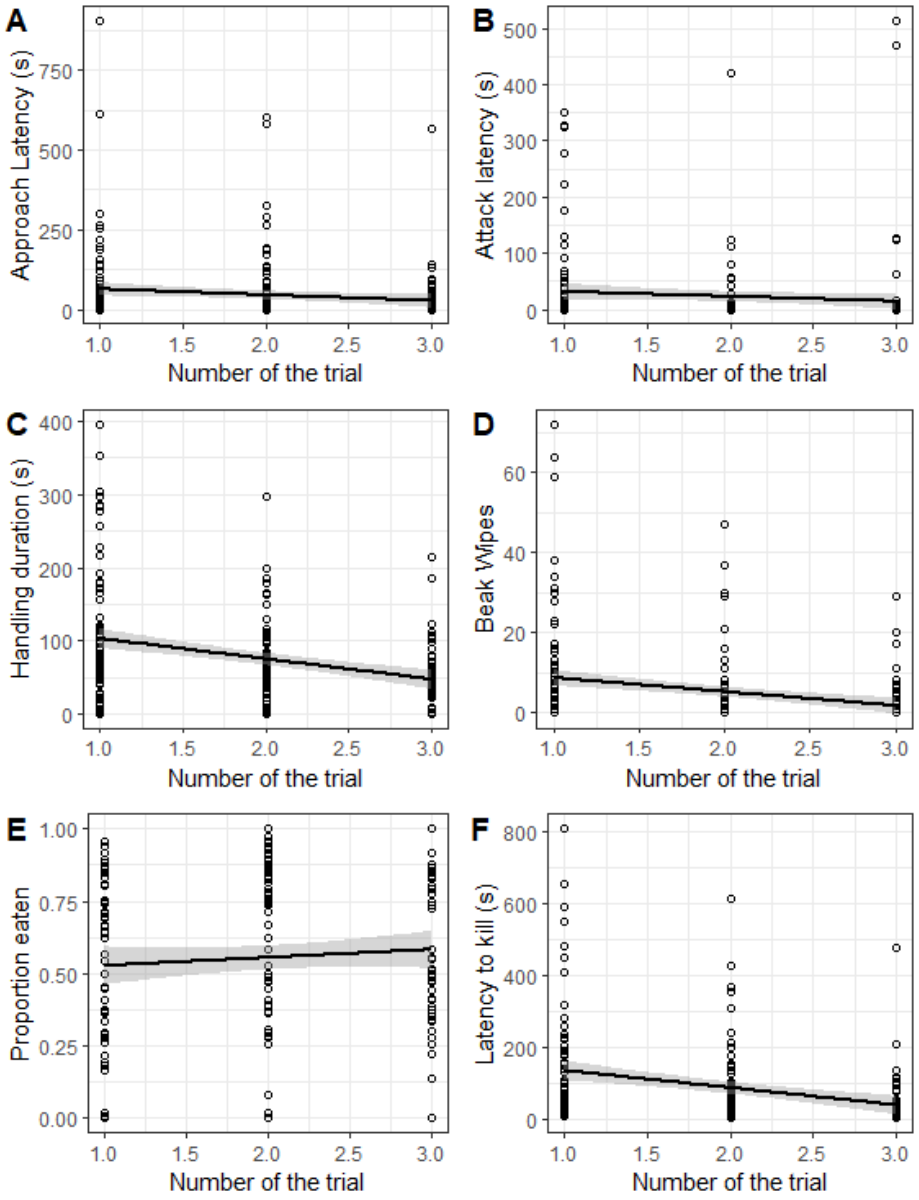

Figure S1 (A) Birds approach latency (in seconds) in response to trial number (from 1 to 3). (B) Birds attack latency (in seconds) in response to trial number (from 1 to 3). (C) Birds handling duration (in seconds) in response to trial number (from 1 to 3). (D) Number of times bird wiped its beak in response to trial number (from 1 to 3). (E) Total proportion of moth eaten in response to trial number (from 1 to 3). (F) Birds latency to kill the moth (in seconds) in response to trial number (from 1 to 3). Shaded area in regression lines represents the 95% confidence level interval.

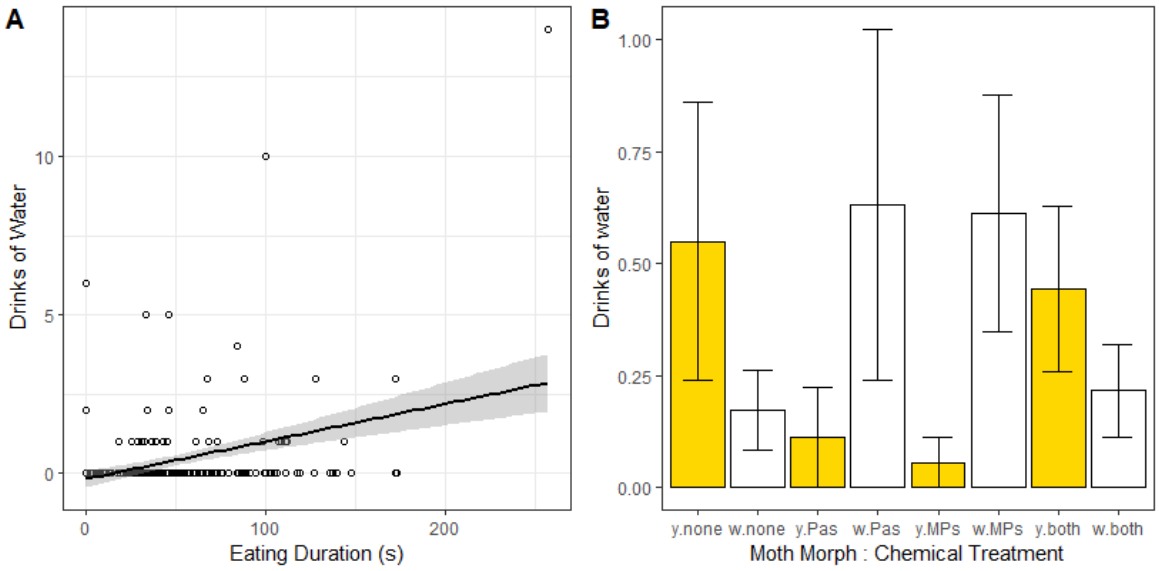

Figure S2. (A) How many times the bird drank water in response to how long bird spend eating the moth (in seconds). Shaded area in regression line represents the 95% confidence level interval. (B) How many times the bird drank water in response to the interaction between moth morph (y = yellow morph, w = white morph) and chemical treatment (none = moth had no chemical defence, PAs = moth had only PAs (pyrrolizidine alkaloids as a bad taste), MPs = moth had only MPs (methoxypyrazines as a bad smell), both = moth had PAs and MPs). Bar graph show mean ± SE.

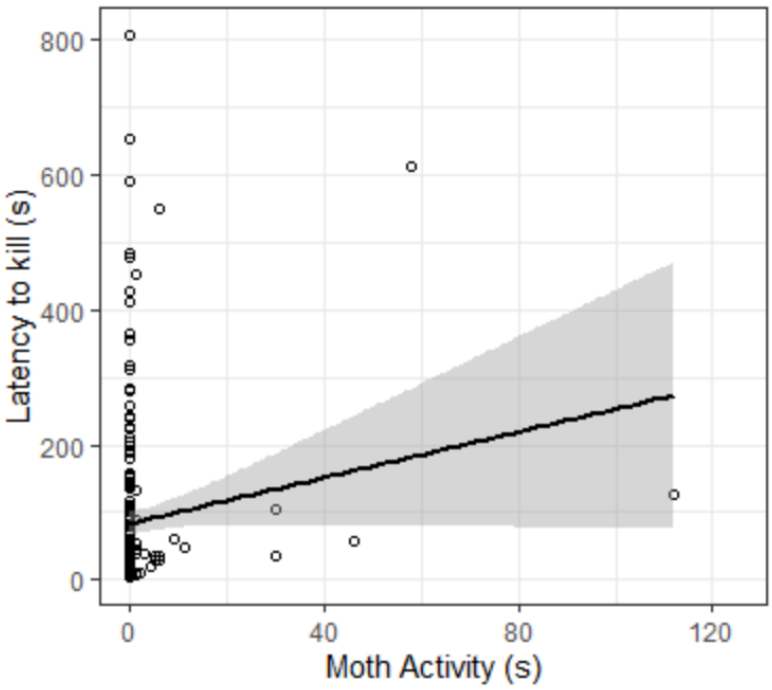

Figure S3. Latency to kill the moths (in seconds) in response to moth activity (in seconds). Shaded area in regression line represents the 95% confidence level interval.

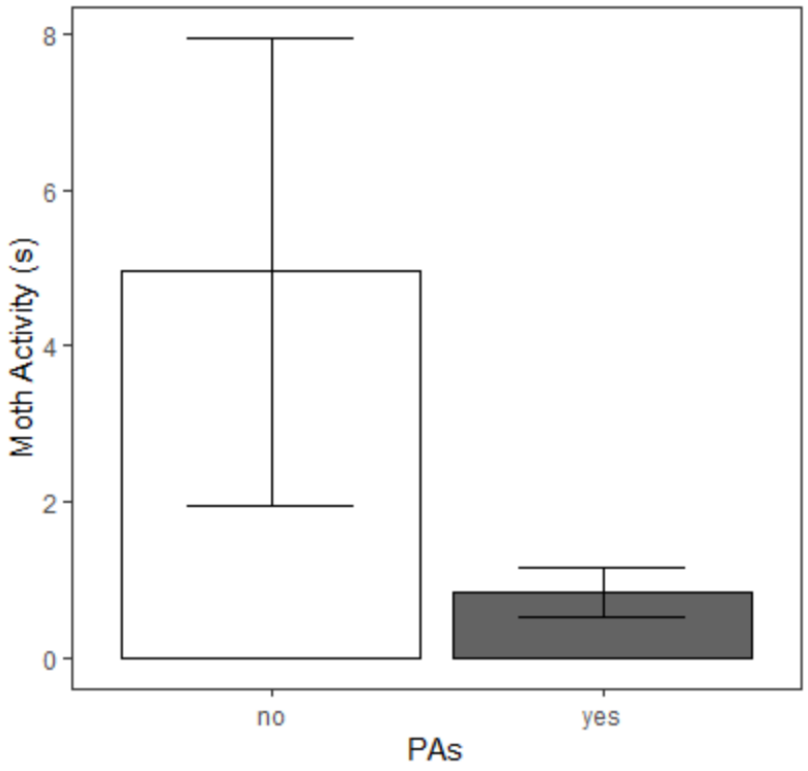

Figure S4. Average moth activity (in seconds) in response to whether or not moth had bad taste, pyrrolizidine alkaloids (PAs) (yes = PAs was present, no = PAs was not present). Bar graph show mean ± SE.

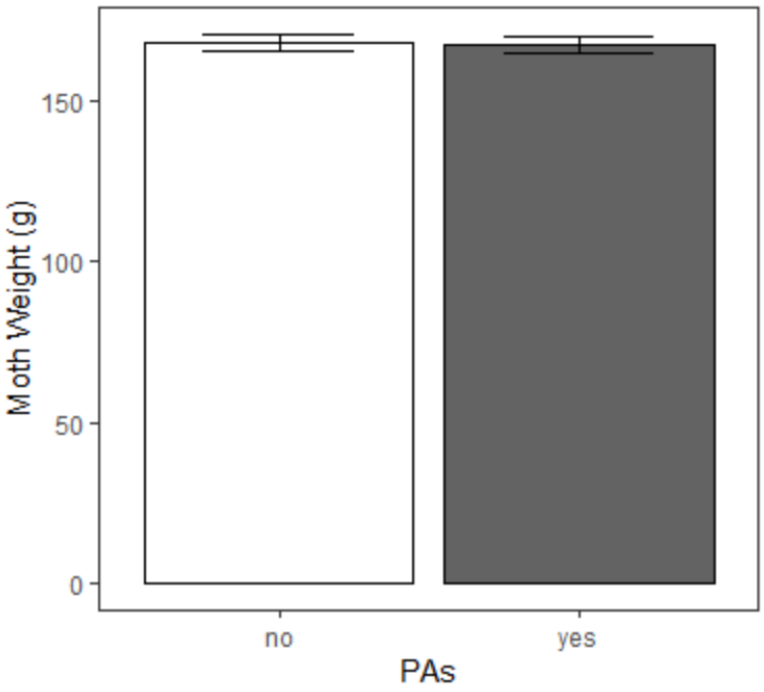

Figure S5. Average moth weight (in grams) in response to whether or not moth had bad taste, pyrrolizidine alkaloids (PAs) (yes = PAs was present, no = PAs was not present). Bar graph show mean ± SE.