Conspecific mortality cues mediate associative learning in crickets, *Acheta domesticus* (Orthoptera: Gryllidae)

ALEXANDER M. SHEPHARD[1], VADIM AKSENOV[1], C. DAVID ROLLO[1]

1 Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, L8S 4K1, Canada.

Corresponding author: *Alexander M. Shephard* (shephaam@mcmaster.ca)

Abstract

Many terrestrial and aquatic animals learn associations between environmental features and chemical cues of mortality risk (e.g. conspecific alarm pheromones or predator-derived cues), but the chemical nature of the cues that mediate this type of learning are rarely considered. Fatty acid necromones (particularly oleic and linoleic acids) are well established as cues associated with dead or injured conspecifics. Necromones elicit risk aversive behavior across diverse arthropod phylogenies, yet they have not been linked to associative learning. Here, we provide evidence that necromones can mediate associative olfactory learning in an insect by acting as an aversive reinforcement. When house crickets (*Acheta domesticus*) were forced to inhabit an environment containing an initially attractive odor along with a necromone cue, they subsequently avoided the previously attractive odor and displayed tolerance for an initially unattractive odor. This occurred when crickets were conditioned with linoleic acid but not when they were conditioned with oleic acid. Similar aversive learning occurred when crickets were conditioned with ethanol body extracts composed of male and female corpses combined, as well as extracts composed of female corpses alone. Conditioning with male body extract did not elicit learned aversion in either sex, even though we detected no notable differences in fatty acid composition between male and female body extracts. We suggest that necromone-mediated learning responses might vary depending on synergistic or antagonistic interactions with sex or species-specific recognition cues.

Key words

Introduction

In both terrestrial and aquatic environments, animals utilize chemical cues to detect and avoid prevailing mortality risks (Dicke and Grostal 2001, Ferrari et al. 2010). Predominant chemical cues include those derived from predators (e.g. kairomones) or from conspecifics (e.g. alarm cues). Organisms may avoid risky environments either by responding to risk cues directly or by avoiding environmental stimuli which the organism has learned to associate with a reliable cue of risk (Ferrari et al. 2010). Associative learning may greatly expand the environmental footprint of perceived danger, thus allowing animals to engage greater caution or avoid settings where cues of risk vary in space and time.

Learned risk avoidance via association with chemical cues has been experimentally demonstrated in a wide range of animal taxa, including insects (Wisenden et al. 1997, Dukas 1998, Nomikou et al. 2003, Yao et al. 2009), molluscs (Dalesman et al. 2006), crustaceans (Hazlett et al. 2002, Yao et al. 2009), fish (Magurran 1989, Chivers and Smith 1994), and amphibians (Polo-Cavia and Gomez-Mestre 2014). Protocols for assessing this type of learning usually consist of at least two phases. First, the animal is "conditioned" in an environment containing a novel stimulus (e.g. a food odor or a cue derived from a novel predator) paired with an innately recognized chemical risk cue (common examples include killed conspecifics or recognized predator cues) to serve as an aversive reinforcement. This is followed by a testing phase in which the animal re-experiences the stimulus without the reinforcement. Despite the applicability of this learning protocol across diverse taxa (Ferrari et al. 2010), the specific chemical nature of the cues that may mediate learned responses has rarely been considered.

For invertebrates, "necromone" cues might provide a chemical basis for learning about risks associated with conspecific mortality. Necromones are a class of chemicals released from dead or injured animals that elicit risk aversive behavior (e.g. alarm, avoidance, or hygienic behaviors) in living conspecifics (Wilson et al. 1958, Rollo et al. 1994, 1995, Yao et al. 2009). This concept traces back to E. O. Wilson et al.'s (1958) famous discovery on the chemical nature of "necrophoric" behavior in ants (i.e. the transport of dead individuals from the nest to the refuse pile). Wilson et al. (1958) showed that material treated with either acetone corpse extracts of *Pogonomyrmex badius* workers or oleic acid alone resulted in the same necrophoric behavioral response. Since Wilson et al. (1958), remarkable evidence for aversive behavioral responses to body extracts or chemical traces of dead conspecifics has accumulated across broad invertebrate taxa (Wilson et al. 1958, Rollo et al. 1994, 1995, Nilsson and Bengtsson 2004a,b, Yao et al. 2009, Green 2009, Chouvenc et al. 2012, Ulyshen and Shelton 2012, Sun and Zhou 2013, Aksenov and Rollo 2017). In eusocial insects, necromone recognition elicits undertaking or burial behavior (Wil-

son et al. 1958, Chouvenc et al. 2012, Ulyshen and Shelton 2012), whereas solitary and gregarious species respond by avoiding associated habitats (Rollo et al. 1994, 1995, Yao et al. 2009). Cockroaches, for instance, are strongly repelled from shelter habitats containing necromone cues (Rollo et al. 1994, 1995).

Chemical analyses have revealed that the major aversive components found in cockroach body extracts are (as discovered by Wilson et al. (1958) for ants) unsaturated fatty acids (Rollo et al. 1994). Oleic and linoleic acids were both highly repellent (Rollo et al. 1994). More recent studies have confirmed that unsaturated fatty acids alone (oleic and/or linoleic) elicited aversive behaviors in the same fashion as extracts of dead bodies. This has been documented in species ranging from primitive Collembola (Nilsson and Bengtsson 2004a) and Psocoptera (Green 2009) to the Blattodea (Rollo et al. 1994, Chouvenc et al. 2012), termites (Chouvenc et al. 2012), crickets (Aksenov and Rollo 2017), and even terrestrial isopods (Yao et al. 2009). Given the ancient divergence of insects and isopods, it appears that fatty acid necromone recognition is conserved across phyla that shared a common ancestor more than 400 million years ago. However, despite their broad taxonomic distribution and strength of responses, fatty acid necromones have not been considered as potential mediators of associative learning.

We recently extended necromone recognition to the house cricket, *Acheta domesticus* (L.) (Aksenov and Rollo 2017). Consistent with necrophobic behavior (Rollo et al. 1994, Yao et al. 2009), body extracts of cricket corpses as well as oleic and linoleic acids were all highly repellant to adult male and female crickets. Like many insects (Papaj and Prokopy 1989, Dukas 2008), crickets are capable of a remarkable degree of associative learning mediated by ecologically relevant cues (Matsumoto and Mizunami 2000, Wessnitzer et al. 2008), and *A. domesticus* has served as a useful model for olfactory learning and its underlying neurobiology (Cayre et al. 2007).

Here, we test whether conspecific body extracts as well as authentic unsaturated fatty acids can serve as aversive chemical reinforcements for associative learning in *A. domesticus*. Specifically, we tested the effectiveness of five different "necromone" cues in mediating olfactory learning: oleic acid, linoleic acid, and alcohol body extracts of male, female or combined male-female cricket corpses.

Methods

Experimental animals.—Adult *Acheta domesticus* were obtained from a large, genetically heterogeneous breeding population housed in an acrylic terrarium (78 cm × 56 cm × 39 cm) maintained at 30°C with a 12 h light/12 h dark photoperiod. Cardboard egg cartons and paper towels provided shelter. Chick feed pellets (Quick Feeds®) and water (soaked cellulose sponges) were provided *ad libitum*. All crickets used in learning experiments were less than 14 d past their imaginal molt.

Chemical preparation.—The "necromones" tested in the learning assay were oleic acid (Sigma-Aldrich cat# 364525), linoleic acid (Sigma-Aldrich cat# L1626), and ethanol extracts of adult cricket corpses. Cricket body extracts were obtained from mature crickets euthanized by freezing at -20°C and placed in vials containing 95% ethanol (1 mL of ethanol per cricket). Each batch of body extract was prepared using at least 10, but no more than 15, dead individuals. Vials were stored in a dark chamber at room tempera-

ture for five days to allow for extraction of body constituents into the alcohol, after which bodies were removed. Extracts were then stored at 4°C. Three types of cricket body extract were prepared: all-female extract (F Ex), all-male extract (M Ex), and a combined male-female extract consisting of equal numbers of crickets from each sex (MF Ex). This is in accordance with previous methods for obtaining repellant body extracts of crickets (Aksenov and Rollo 2017), cockroaches (Rollo et al. 1994, 1995), caterpillars and woodlice (Yao et al. 2009).

GC-MS analysis of body extracts.—Ethanol body extracts for the gas chromatography-mass spectrometry (GC-MS) analysis were prepared as described above. A male body extract and a female body extract were made, each using five individual corpses. For sample preparation, 50 μL of the ethanol extract was dried under a gentle stream of nitrogen gas and reconstituted in 1 mL methanol (2.5% H_2SO_4) and 10 μL 0.12 mg/mL stearic acid-d$_{35}$ (internal standard for GC-MS analysis). The samples were incubated at 80°C for 1 h and analyzed by GC-MS immediately.

GC-MS analyses were performed using an Agilent 6890N gas chromatograph (Santa Clara, CA, USA), equipped with a DB-17ht column (30m × 0.25mm i.d. × 0.15μm film, J & W Scientific) and a retention gap (deactivated fused silica, 5 m × 0.53 mm i.d.), coupled to an Agilent 5973 MSD single quadruple mass spectrometer. The derivatized cricket extract (1 μL) was injected using an Agilent 7683 autosampler in splitless mode. The injector temperature was 250°C and carrier gas (helium) flow was 0.7 mL/min. The transfer line was 280°C and the MS source temperature was 230°C. The column temperature was set at 50°C, raised to 300°C at 8°C/min, and held there for 15 min. After a five-minute solvent delay, mass spectra were acquired using electron ionization (EI) in full-scan mode. Fatty acid methyl esters were identified using NIST Mass Spectral Search Program version 2.0f (score > 800).

Learning assay.—Adult male or female crickets were individually tested under an olfactory learning paradigm consisting of three stages. First, in an initial olfactory preference test, crickets were monitored for their total amount of time spent attending to a favorable scent (vanilla) and an unfavorable scent (peppermint). Second, crickets underwent a conditioning period in which they were presented one of the five necromone cues (either oleic acid, linoleic acid, F Ex, M Ex, or MF Ex) in combination with the vanilla scent. For the control treatment, the conditioning period consisted of the vanilla scent in combination with evaporated 95% ethanol in place of a body extract or acid. Third, crickets underwent a post-conditioning olfactory preference test that was identical to the initial preference test. Throughout all experiments, each cricket was used only once. Therefore, all experimental crickets had no prior experience with necromone cues.

All olfactory preference tests were conducted in an enclosed circular arena (diameter = 29 cm; height = 29 cm), containing three vanilla-scented and three peppermint-scented filter papers (4 cm × 4 cm) equally spaced and oriented vertically along the inside base of the arena in an alternating fashion. Each filter paper was coated with 300 μL of either vanilla or peppermint solution (Clubhouse®, diluted 6-fold with water).

In each initial or post-training preference test, a single cricket was placed in the center of the circular arena and allowed to roam freely after the alcohol had completely evaporated. Each cricket was transferred to the arena in a plastic cylindrical vial (9.5 cm height, 2.5 cm width). Each test lasted for 10 min and was record-

ed with an overhead camera and digital video recorder (Diginet®). To control for spatial bias, the circular arena was manually rotated 180 degrees at 5 min after the initiation of each test to reverse the orientation of the filter papers relative to possible external cues visible outside the arena. Each ten-minute recording was subsequently scored for the total amount of time each cricket spent perching on vanilla- versus peppermint-scented papers.

During the conditioning period, each cricket was exposed to a piece of filter paper (4 cm × 12 cm) coated with 450 μL of vanilla extract solution and one of five necromone cues (oleic acid, linoleic acid, F Ex, M Ex, or MF Ex). Body extracts were applied at a dose of 0.45 body equivalents (b.e.) per filter paper, and oleic and linoleic acids were applied at 5 b.e. per filter paper. For body extracts, 1 b.e. was equivalent to 1 mL of ethanol extract (i.e. the estimated amount of material extracted from one cricket corpse). For oleic and linoleic acids, 1 b.e. was equivalent to approximately 4.65 mg and 7.9 mg respectively (estimated from fatty acid and lipid analyses of adult *A. domesticus*: see Grapes et al. 1989). Acids were dissolved in sufficient ethanol to ensure uniform application to the filter papers. In the control treatment, each cricket was conditioned with vanilla scent in combination with ethanol instead of body extract or acid. In all cases, ~30 min was allowed for the alcohol to entirely evaporate. Crickets were subjected to conditioning immediately after initial olfactory preference testing, and training lasted for 22 h. Post-conditioning olfactory preferences were tested immediately after the conditioning period.

Statistics.—An index of olfactory preference was created to reflect each cricket's time spent perching on a vanilla (T_v) or peppermint (T_p) scented cue in each preference test by applying the following calculation:

$$(T_v - T_p) \div (T_v + T_p)$$

where a positive index (0 to 1) indicated preference for vanilla, and a negative index (0 to -1) indicated preference for peppermint.

All data analyses were performed in R Studio version 1.1.456 (R Studio Team 2016). Results of the learning assay were analyzed using a linear mixed effects model in the "lme4" package (Bates et al. 2014). Olfactory preference index was used as the response variable. Predictor variables included sex, necromone treatment (Control, F Ex, M Ex, MF Ex, oleic acid, and linoleic acid), and preference test (initial and post-conditioning) as fixed effects. Also included were the two-way interactive effects between treatment and sex, treatment and test, and sex and test, as well as the three-way interactive effect between treatment, sex, and test. Individual was included in the model as a random effect. Statistical significance was recognized as p < 0.05.

Results

Learning assays.—During each preference test, crickets typically spent approximately one minute exploring the scented filter papers along the inside wall of the arena before selecting one to perch on. Crickets typically changed their perch several times over the duration of the test. Crickets displayed a strong inherent preference for vanilla over peppermint, inferred from perching durations in initial trials. Vanilla preference was 3.2-fold greater than peppermint in females (chi-square: p < 0.001) and 1.8-fold greater in males (chi-square: p < 0.001). Indeed, initial preference indices remained consistent among females and males

throughout experimental trials (see Fig. 1). Similar preferences were obtained for the cricket *Gryllus bimaculatus* by Matsumoto and Mizunami (2000).

Relative to the control treatment (n = 16), crickets conditioned in the MF Ex treatment (n = 25) showed a significant change in olfactory preference from vanilla to peppermint between initial and post-conditioning trials (Treatment × Preference Test interaction: t = -3.095, df = 97, p = 0.002; Fig. 1). Similarly, for crickets conditioned in the linoleic acid treatment (n = 19), there was a significant change in olfactory preference from vanilla to peppermint between initial and post-conditioning trials (Treatment × Preference Test interaction: t = -3.020, df = 97, p = 0.003; Fig. 1). Crickets conditioned in the F Ex treatment (n = 15) also showed a significant change in olfactory preference from vanilla to peppermint, relative to controls (Treatment × Preference Test interaction: t = -2.634, df = 97, p = 0.010; Fig. 1), and this preference change was greater in females than in males (Treatment × Preference Test × Sex interaction: t = 2.136 df = 97, p = 0.035). Significant changes in olfactory preference were not observed for crickets conditioned in the M Ex treatment (Treatment × Preference Test interaction: n = 15, t = -1.457, df = 97, p = 0.148) or oleic acid treatment (Treatment × Preference Test interaction: n = 20, t = -0.216, df = 97, p = 0.829) relative to the control treatment (Fig. 1). There were no significant interactions between treatment and sex or sex and preference test.

Chemical analysis.—GC-MS analysis showed that the fatty acid profiles of F Ex and M Ex cricket body extract samples were simi-
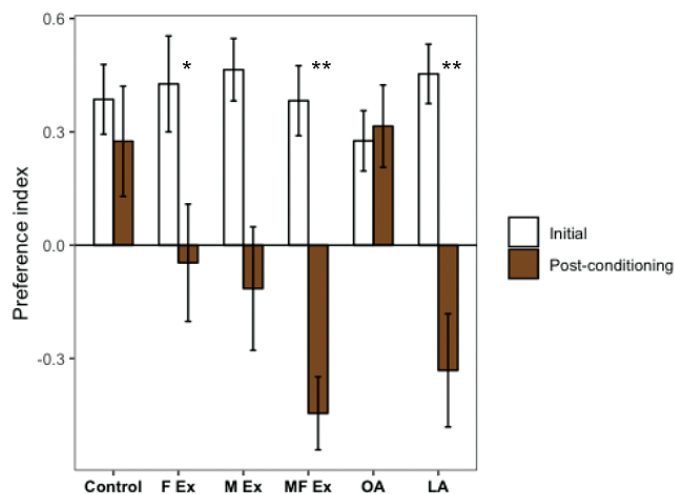

Fig. 1. Changes in olfactory preference index for adult crickets (*Acheta domesticus*) conditioned to associate a favorable vanilla olfactory scent with one of five necromone cues: all-female cricket body extract (F Ex), all-male extract (M Ex), combined male-female extract (MF Ex), oleic acid (OA), and linoleic acid (LA). In the control treatment, crickets were conditioned with ethanol instead of a necromone cue. Positive indices indicate vanilla preference and negative indices indicate peppermint preference. A significant change in preference index between initial and post-conditioning tests (relative to the control) indicates learning associated with a necromone cue (* p < 0.05, ** p < 0.01). All preference indices were derived from perching durations (i.e. total time spent attending to either the vanilla or peppermint cue during the preference test). Bars indicate standard error.

Table 1. Fatty acid methyl ester (FAME) analyses of adult female (F Ex) and male (M Ex) *Acheta domesticus* body extracts. Normalized peak areas of detected FAME. Four strongest peaks detected are in bold corresponding to: palmitic, stearic, oleic, and linoleic acids.

Compound	Male Extract (M Ex)	Female Extract (F Ex)
Myristic acid	0.43	0.53
Palmitic acid	**27.14**	**27.19**
Palmitoleic acid	1.10	1.96
Stearic acid	**14.15**	**11.33**
Oleic acid	**18.23**	**20.69**
Linoleic acid	**51.43**	**52.60**
γ-Linoleic acid	1.04	0.90
Paullinic acid	0.53	0.31

lar (Table 1). Samples consisted of bodies from five individuals to minimize bias resulting from potential variability among individual crickets. Strong peak areas were revealed corresponding to linoleic, oleic, stearic, and palmitic acids. In males, the acids were present in a ratio of linoleic:palmitic:oleic:stearic = 51.43:27.14:18.23:14.15. The female acid ratio was 52.60:27.19:20.69:11.33. The mean acid ratio for males and females combined was 52.01:27.16:19.46:12.74. It was not possible to distinguish between *cis* and *trans* isomers of the compounds.

Discussion

Learned responses to cues from dead or injured conspecifics are widespread (Dicke and Grostal 2001, Ferrari et al. 2010), but there has been little consideration of which chemicals may mediate these responses. Here, we examined a potential role for necromone cues as aversive chemical mediators of olfactory learning in an insect. Male and female *A. domesticus* showed learned aversion to an initially favorable odor after being conditioned to associate the favorable odor with a necromone cue. This was observed when crickets were conditioned with either body extracts of female crickets or body extracts of both sexes combined. In contrast, learning responses were not observed when crickets were conditioned with body extracts of male crickets (Fig. 1) even though male and female body extracts did not appear to differ in fatty acid composition (Table 1).

Next, we tested whether learning could be mediated by oleic and linoleic acids as necromone cues. Compelling reports suggest that unsaturated fatty acids (particularly oleic and linoleic) elicit aversive behavior associated with death recognition in a variety of invertebrate species (Wilson et al. 1958, Rollo et al. 1994, 1995, Nilsson and Bengtsson 2004a, Yao et al. 2009, Green 2009, Chouvenc et al. 2012) including *A. domesticus* (Aksenov and Rollo 2017). Chemical analyses of cockroach corpse extracts identified the active repellent fraction as oleic and linoleic acids. Chemical profiles obtained for *A. domesticus* here (Table 1) confirm strong prevalence of these fatty acids in our crickets. In this study, learning responses were observed when crickets were conditioned with linoleic acid but not when they were conditioned with oleic acid (Fig. 1). Our results suggest that cue strength, complexity or synergism (additive effects of multiple repellant or species-specific cues) or antagonism (masking of repellant cues by sex-specific attractants) might play important roles in mediating learned responses to conspecific mortality cues.

Our central finding that crickets modify their olfactory preferences following learned association with conspecific body extracts indicates that prior experience may impact behavioral decisions even when direct cues of risk are not apparent (i.e. the environment itself becomes perceived as risky). Similar examples of this form of associative learning mediated by conspecific mortality cues have been demonstrated in other insect species, suggesting that this could be a widespread phenomenon. Dukas (1998), for instance, demonstrated that *Drosophila* larvae learn to avoid odors associated with crushed conspecifics. Damselfly larvae reared in the absence of their natural predators responded to chemical cues from injured conspecifics, but not the predator-derived cues (Wisenden et al. 1997). However, after being exposed to conspecific and predator cues simultaneously, the larvae learned to respond directly to predator-derived cues. In a related study, damselfly larvae initially failed to respond to chemical stimuli from predators but did so after they had been exposed to chemical stimuli from predators that had been feeding on conspecifics or familiar heterospecifics (Chivers et al. 1996). Similarly, adult female whiteflies (*Bemisia tabaci*) learned to avoid ovipositing on plants harboring predatory mites that ate whitefly eggs and larvae (Nomikou et al. 2003). Although such learning responses were not explicitly linked to any type of alarm or avoidance chemistry, we suggest that necromones should be considered given their phylogenetic pervasiveness and strength of responses throughout the invertebrates (Yao et al. 2009, Sun and Zhou 2013).

Interestingly, the collembolan, *Sinella curviseta*ed no evidence of a conditioned response to predator cues from wolf spiders (*Pardosa milvina*) after pairing with crushed conspecifics (Sitvarin et al. 2015). While this negative result could be attributable to differences in experimental design (e.g. duration of the conditioning period), it is also possible that the wolf spider cues were not perceived as environmentally relevant to the Collembola, as they did not respond to the cues initially. In contrast, the conditioned stimulus utilized in our experiment (the vanilla olfactory cue) was initially attractive to both male and female crickets. An alternative possibility could be that *A. domesticus* has an evolved propensity for forming learned associations with olfactory cues. Indeed, olfactory learning has previously been reported in *A. domesticus* (Scotto-Lomassese et al. 2003) and other Gryllidae (Matsumoto and Mizunami 2000, 2002), and the neural mechanisms mediating this type of learning are well characterized in *Acheta* (Cayre et al. 2007).

Despite the strong presence of both oleic and linoleic acid in male and female cricket body extracts, learning responses were only observed for linoleic acid. This seems consistent with previous data demonstrating that both sexes showed weak initial aversion to habitats treated with oleic acid, but aversion to linoleic acid-treated habitats was stronger (Aksenov and Rollo 2017). Additionally, cricket body extracts tended to elicit stronger and more immediate aversion than either oleic or linoleic acid (Aksenov and Rollo 2017). It seems likely that the relative strength of responses

to body extracts arises via cue synergism (i.e. combinatorial effects of necromone cues or their synergism with species-specific recognition cues).

Further evidence for cue synergism was obtained when Yao et al. (2009) tested body extract repellency on a number of related species and found that extracts from one species also repelled others, but extracts of conspecifics were most repellant. Chouvenc et al. (2012) obtained similar evidence of cue synergism by showing that corpse burial by termites (*Pseudacanthotermes spiniger*) was elicited by material treated with conspecific body extract or a combination of chemicals found in the extract, but not when these chemicals were tested individually. Additionally, oleic acid elicited building behavior in the termite *Reticulitermes virginicus* but only when applied to imitation corpses of conspecifics, suggesting that synergism of chemical and tactile cues might also be important (Ulyshen and Shelton 2012).

Lack of learning responses when either male or female crickets were conditioned with body extracts of male crickets suggests that the F Ex and M Ex extracts differ in chemical composition. However, this is unlikely to reflect sex differences in necromone fractions given that the unsaturated fatty acid profiles of F Ex and M Ex did not notably differ (Table 1). One explanation could be that sex-specific extracts contain compounds relevant to sex such as pheromones or cuticular hydrocarbon signatures (Warthen and Uebel 1980, Hardy and Shaw 1983, McFarlane et al. 1983, Tregenza and Wedell 1997) that might have antagonistic (i.e. attractive) effects on aversive necromone cues. However, we must note that the quantity of body extract used in current experiments was low relative to the quantities shown to completely repel crickets in previous experiments (Aksenov and Rollo 2017). Therefore, another possible explanation could be that attractive components (e.g. sex-specific pheromones) may predominate in lower quantities of extract by overriding repellant fractions. Further research is required to understand complexities such as quantity of aversive fractions, duration of exposure, or antagonism by sex-specific cues.

The learning reported here occurred following a single conditioning event (i.e. crickets learned to avoid the vanilla olfactory cue after experiencing it together with the body extract reinforcement only once). Such "one-time learning" has been consistently reported for learned responses to chemical alarm cues in aquatic species (for review, see Ferrari et al. 2010), and in many cases, the learned association can persist long after it has been formed (Suboski 1990). Such robust learning seems particularly appropriate given the potential consequences of predation or disease. Albeit the reinforcing period employed in our study was extensive (22 hours), environments treated with alcohol body extracts have been repeatedly shown to remain highly repellant over similar durations (Rollo et al. 1994, Yao et al. 2009, Aksenov and Rollo 2017), suggesting that such an extended reinforcement period could be generally appropriate in this context.

The olfactory cues used to differentiate between environments in this experiment are somewhat artificial. It remains to be seen whether such learned aversion could have important impacts in natural settings. It seems possible that these conditions could somewhat resemble vegetation with characteristic scents (e.g. pine, cedar, mint, flowering herbs), which often occurs in patches. A foraging or dispersing animal could benefit by avoiding or increasing vigilance in environments previously identified as risky. In principle, this could limit the potential range of environments that organisms exploit (e.g. foraging, oviposition, shelter selection, etc.) and may even extend to foraging decision tradeoffs between environmental quality and mortality risk (see Lima and Dill 1990). Indeed, it has

been noted that risk aversion can lead to prey populations shifting to habitats of suboptimal quality (Werner et al. 1983).

Recognition of conspecific alarm or avoidance cues (including necromones) does not require the evolution or maintenance of multiple recognition systems for diverse risks, which might include predators, pathogens, or toxins. Associative learning broadens the range of risks and environments that can be avoided and may facilitate adaptive responses to risks varying in ecological space and time. For instance, necromone recognition could facilitate learned avoidance of generalist or even introduced predators (Nunes et al. 2013, Polo-Cavia and Gomez-Mestre 2014) that may not be innately recognized by prey. Perhaps even aversion to environmental toxins (e.g. Surinov 2007, Rollo et al. 2014) or poisonous foods (Bernays 1993) could be reinforced by necromones (or other cues) associated with ill, injured, or dead conspecifics.

In summary, we provide evidence that fatty acid necromones may serve as aversive chemical mediators of olfactory learning, thereby extending aversion to potentially risky environments. Given that both learning and necromone recognition are highly conserved, we suggest that such associative learning could impact foraging and habitat distribution across wide insect phylogenies, and such questions are amenable for study in a wide range of organisms. Particularly important outstanding questions pertain to how learning outcomes might be complexly mediated by synergistic or antagonistic interactions among various repellant or attractive cues as well as the broader ecological relevance of such learning.

Acknowledgements

References

Aksenov V, Rollo CD (2017) Necromone death cues and risk avoidance by the cricket *Acheta domesticus*: effects of sex and duration of exposure. Journal of Insect Behavior 30: 258–272. https://doi.org/10.1007/s10905-017-9612-6

Bates D, Maechler M, Bolker B, Walker S (2014) lme4: Linear mixed-effects models using Eigen and S4 (R package version 3.5.1). https://cran.r-project.org/web/packages/lme4/index.html

Bernays EA (1993) Aversion learning and feeding. In: Papaj DR, Lewis AC (Eds) Insect Learning: Ecological and Evolutionary Perspectives. Chapman and Hall, New York, 1–17. https://doi.org/10.1007/978-1-4615-2814-2_1

Cayre M, Scotto-Lomassese S, Malaterre J, Strambi C, Strambi A (2007) Understanding the regulation and function of adult neurogenesis: Contribution from an insect model, the house cricket. Chemical Senses 32: 385–395. https://doi.org/10.1093/chemse/bjm010

Chivers DP, Smith RJF (1994) Fathead minnows, *Pimephales promelas*, acquire predator recognition when alarm substance is associated with the sight of unfamiliar fish. Animal Behaviour 48: 597–605. https://doi.org/10.1006/anbe.1994.1279

Chivers DP, Wisenden BD, Smith RJF (1996) Damselfly larvae learn to recognize predators from chemical cues in the predator's diet. Animal Behaviour 52: 315–320. https://doi.org/10.1006/anbe.1996.0177

Chouvenc T, Robert A, Sémon E, Bordereau C (2012) Burial behaviour by dealates of the termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) induced by chemical signals from termite corpses. Insectes Sociaux 59: 119–125. https://doi.org/10.1007/s00040-011-0197-3

Dalesman S, Rundle SD, Coleman RA, Cotton PA (2006) Cue association and antipredator behaviour in a pulmonate snail, *Lymnaea stagnalis*. Animal Behaviour 71: 789–797. https://doi.org/10.1016/j.anbehav.2005.05.028

Dicke M, Grostal P (2001) Chemical detection of natural enemies by arthropods: an ecological perspective. Annual Review of Ecology and Systematics 32: 1–23. https://doi.org/10.1146/annurev.ecolsys.32.081501.113951

Dukas R (1998) Ecological relevance of associative learning in fruit fly larvae. Behavioral Ecology and Sociobiology 45: 195–200. https://doi.org/10.1007/s002650050553

Dukas R (2008) Evolutionary biology of insect learning. Annual Review of Entomology 53: 145–160. https://doi.org/10.1146/annurev.ento.53.103106.093343

Ferrari MCO, Wisenden BD, Chivers DP (2010) Chemical ecology of predator-prey interactions in aquatic ecosystems: a review and prospectus. Canadian Journal of Zoology 88: 698–724. https://doi.org/10.1139/Z10-029

Grapes M, Whiting P, Dinan L (1989) Fatty acid and lipid analysis of the house cricket, *Acheta domesticus*. Insect Biochemistry 19: 767–774. https://doi.org/10.1016/0020-1790(89)90058-9

Green PWC (2009) The effects of insect extracts and some insect-derived compounds on the settling behavior of *Liposcelis bostrychophila*. Journal of Chemical Ecology 35: 1096–1107. https://doi.org/10.1007/s10886-009-9688-3

Hardy TN, Shaw KC (1983) The role of chemoreception in sex recognition by male crickets: *Acheta domesticus* and *Teleogryllus oceanicus*. Physiological Entomology 8: 151–166. https://doi.org/10.1111/j.1365-3032.1983.tb00344.x

Hazlett BA, Acquistapace P, Gherardi F (2002) Differences in memory capabilities in invasive and native crayfish. Journal of Crustacean Biolology 22: 439–448. https://doi.org/10.1163/20021975-99990251

Lima SL, Dill LM (1990) Behavioral decisions made under the risk of predation: a review and prospectus. Canadian Journal of Zoology 68: 619–640. https://doi.org/10.1139/z90-092

Magurran AE (1989) Acquired recognition of predator odor in the European minnow (*Phoxinus phoxinus*). Ethology 82: 216–233. https://doi.org/10.1111/j.1439-0310.1989.tb00501.x

Matsumoto Y, Mizunami M (2000) Olfactory learning in the cricket *Gryllus bimaculatus*. Journal of Experimental Biology 203: 2581–2588.

Matsumoto Y, Mizunami M (2002) Lifetime olfactory memory in the cricket *Gryllus bimaculatus*. Journal of Comparative Physiology A 188: 295–299. https://doi.org/10.1007/s00359-002-0303-0

McFarlane IE, Steeves E, Alli I (1983) Aggregation of larvae of the house cricket, *Acheta domesticus* (L.), by propionic acid present in the excreta. Journal of Chemical Ecology 9: 1307–1315. https://doi.org/10.1007/BF00994799

Nilsson E, Bengtsson G (2004a) Endogenous free fatty acids repel and attract Collembola. Journal of Chemical Ecology 30: 1431–1443. https://doi.org/10.1023/B:JOEC.0000037749.75695.c5

Nilsson E, Bengtsson G (2004b) Death odour changes movement pattern of a Collembola. Oikos 104: 509–517. https://doi.org/10.1111/j.0030-1299.2004.12921.x

Nomikou M, Janssen A, Sabelis MW (2003) Herbivore host plant selection: Whitefly learns to avoid host plants that harbour predators of her offspring. Oecologia 136: 484–488. https://doi.org/10.1007/s00442-003-1289-1

Nunes AL, Richter-Boix A, Laurila A, Rebelo R (2013) Do anuran larvae respond behaviourally to chemical cues from an invasive crayfish predator? A community-wide study. Oecologia 171: 115–127. https://doi.org/10.1007/s00442-012-2389-6

Papaj DR, Prokopy RJ (1989) Ecological and evolutionary aspects of learning in phytophagous insects. Annual Review of Entomology 34: 315–320. https://doi.org/10.1146/annurev.en.34.010189.001531

Polo-Cavia N, Gomez-Mestre I (2014) Learned recognition of introduced predators determines survival of tadpole prey. Functional Ecology 28: 432–439. https://doi.org/10.1111/1365-2435.12175

R Studio Team (2016) RStudio: Integrated Development for R. RStudio, Inc. Boston, MA. http://www.rstudio.com

Rollo CD, Borden JH, Casey IB (1995) Endogenously produced repellent from American cockroach (Blattaria: Blattidae): function in death recognition. Environmental Entomology 24: 116–124. doi: http://dx.doi.org/10.1093/ee/24.1.116

Rollo CD, Czvzewska E, Borden JH (1994) Fatty acid necromones for cockroaches. Naturwissenschaften 81: 409–410. https://doi.org/10.1007/BF01132695

Rollo CD, Kumar A, Smith R, Wang J, Aksenov V, Han J, Kahnna P (2014) Trojan genes or transparent genomes? Sexual selection and potential impacts of genetically modified animals in natural ecosystems. Evolutionary Biology 41: 276–298. https://doi.org/10.1007/s11692-013-9268-x

Scotto-Lomassese S, Strambi C, Strambi A, Aouane A, Augier R, Rougon G, Cayre M (2003) Suppression of adult neurogenesis impairs olfactory learning and memory in an adult insect. Journal of Neuroscience 23: 9289–9296. https://doi.org/10.1523/JNEUROSCI.23-28-09289.2003

Sitvarin MI, Romanchek C, Rypstra AL (2015) Nonconsumptive predator-prey interactions: sensitivity of the detritivore *Sinella curviseta* (Collembola: Entomobryidae) to cues of predation risk from the spider *Pardosa milvina* (Araneae: Lycosidae). Environmental Entomology 44: 349–355. https://doi.org/10.1093/ee/nvv011

Sun Q, Zhou X (2013) Corpse management in social insects. International Journal of Biological Sciences 9: 313–321. https://doi.org/10.7150/ijbs.5781

Suboski MD (1990) Releaser-induced recognition learning. Psychological Review 97: 271–284. https://doi.org/10.1037/0033-295X.97.2.271

Surinov BP (2007) Mice with radiation or toxic damage or malignant tumors produce aversive chemosignals repelling intact animals. Doklady Biological Sciences 414: 199–201. https://doi.org/10.1134/S0012496607030088

Tregenza TOM, Wedell N (1997) Definitive evidence for cuticular pheromones in a cricket. Animal Behaviour 54: 979–984. https://doi.org/10.1006/anbe.1997.0500

Ulyshen MD, Shelton TG (2012) Evidence of cue synergism in termite corpse response behavior. Naturwissenschaften 99: 89–93. https://doi.org/10.1007/s00114-011-0871-3

Warthen JD, Uebel EC (1980) Comparison of the unsaturated cuticular hydrocarbons of male and female house crickets, *Acheta domesticus* (L.) (Orthoptera: Gryllidae). Insect Biochemistry 10: 435–439. https://doi.org/10.1016/0020-1790(80)90015-3

Wilson EO, Durlach NI, Roth LM (1958) Chemical releaser of necrophoric behavior in ants. Psyche 65: 108–114. https://doi.org/10.1155/1958/6939

Wisenden BD, Chivers DP, Smith RJF (1997) Learned recognition of predation risk by Enallagma damselfly larvae (Odonata, Zygoptera) on the basis of chemical cues. Journal of Chemical Ecology 23: 137–151. https://doi.org/10.1023/B:JOEC.0000006350.66424.3d

Werner EE, Gilliam JF, Hall DJ, Mittelbach GG (1983) An experimental test of the effects of predation risk on habitat use in fish. Ecology 64: 1540–1548. https://doi.org/10.2307/1937508

Wessnitzer J, Mangan M, Webb B (2008) Place memory in crickets. Proceedings of the Royal Society 275: 915–921. https://doi.org/10.1098/rspb.2007.1647

Yao M, Rosenfeld J, Attridge S, Sidhu S, Aksenov V, Rollo CD (2009) The ancient chemistry of avoiding risks of predation and disease. Evolutionary Biology 36: 267–281. https://doi.org/10.1007/s11692-009-9069-4