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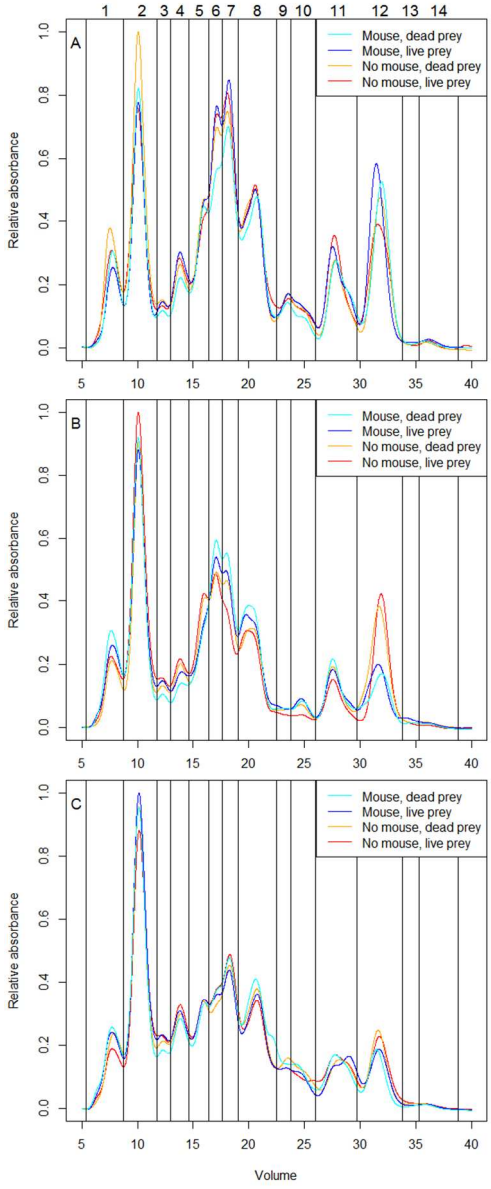

Figure 1. Averaged relative venom profiles for each of the four treatments taken: prior to treatments (A, t = 0 days), after the treatments ended (B, t = 49 days), and again after a recovery period (C, t = 70 days) divided into 14 fractions. Relative absorbance measures the absorbance units at 280 nm of any point along the venom profile relative to the point of maximum absorbance in the profile. Venom profiles obtained from scorpions subjected to the pressure for defensive venom (mouse exposure) treatment are given in dark blue (+ pressure for offensive venom, live cricket prey) and light blue (- pressure for offensive venom, dead cricket prey); profiles obtained from scorpions not subjected to this treatment are given in red (+ pressure for offensive venom) and orange (- pressure for offensive venom).

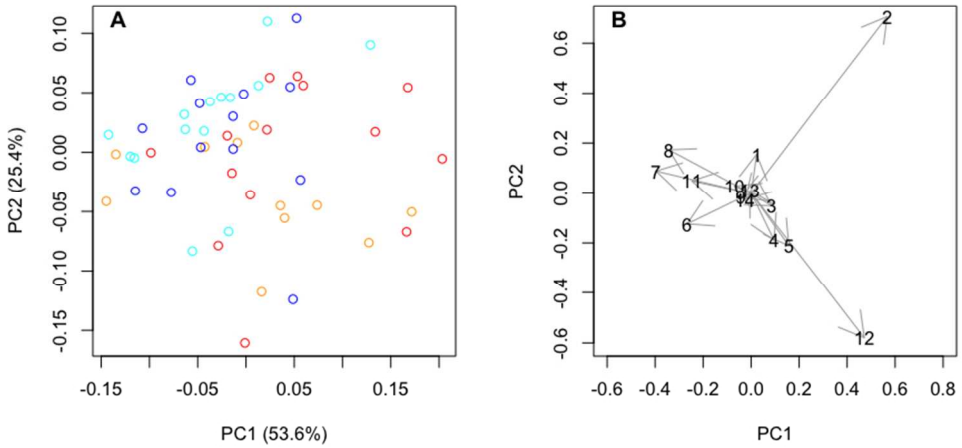

Figure 2. Loadings for principal components 1 and 2, separated into loadings for each scorpion (A) and loadings for each venom fraction (B, 14 total peaks) with fraction number indicated at the tips of each arrow. For clarity and consistency, colours are as per the curves presented in Figure 1; pressure for defensive venom treatment is indicated in dark (+ pressure for offensive venom) and light (- offensive pressure for offensive venom) blue, while data from scorpions not subjected to the pressure for defensive venom treatment are in red (+ pressure for offensive venom) and orange (- pressure for offensive venom). PC1 and PC2 described 53.6% and 25.4% of the overall variation, respectively.

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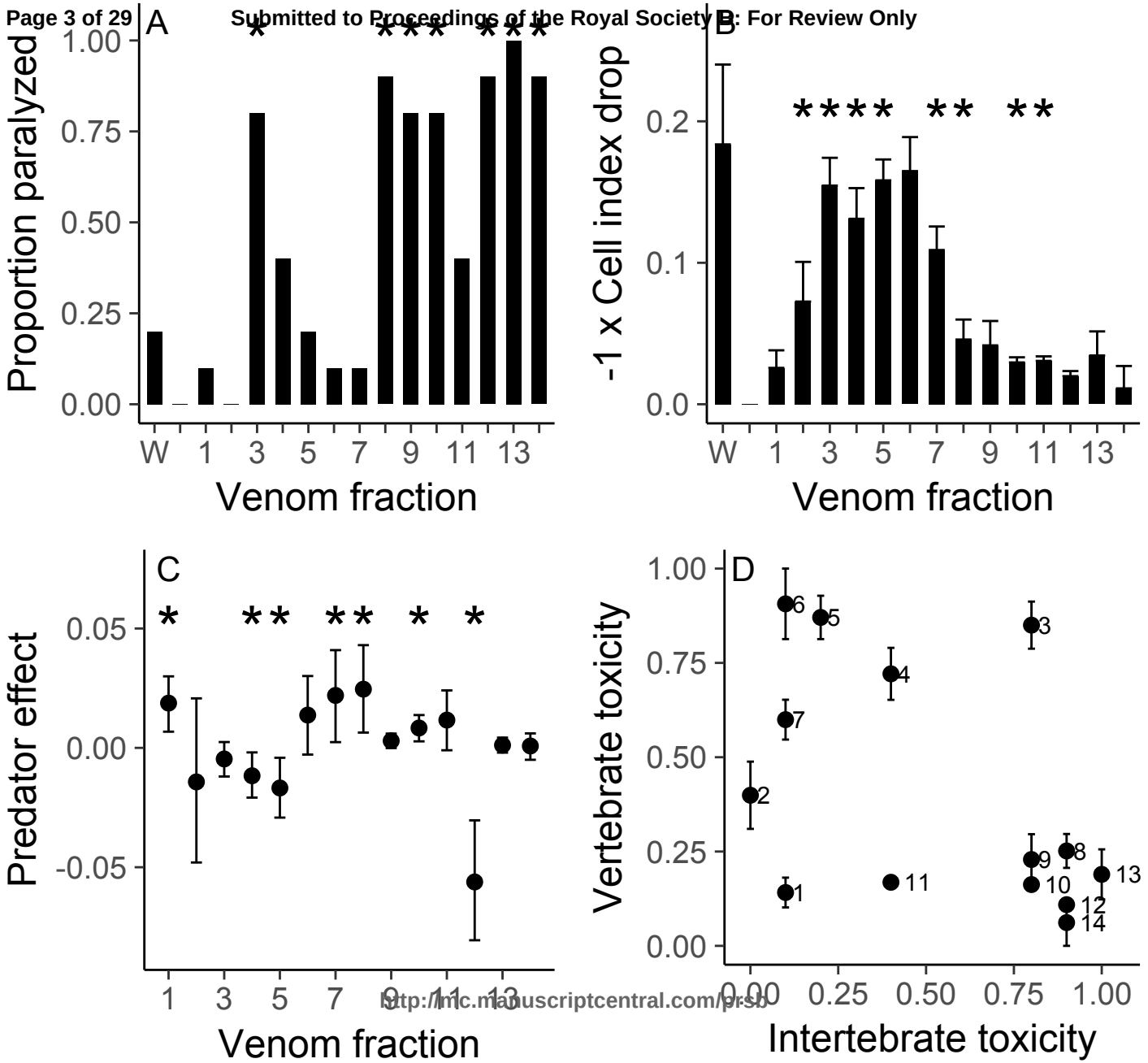

Changes in predator exposure, but not diet induce phenotypic plasticity in scorpion venom

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Abstract

Animals embedded between trophic levels must simultaneously balance pressures to deter predators and acquire resources. Venomous animals may use venom toxins to mediate both pressures, and thus changes in this balance may alter the composition of venoms. Basic theory suggests that greater exposure to a predator should induce a larger proportion of defensive venom components relative to offensive venom components, while increases in arms races with prey will elicit the reverse. Alternatively, reducing the need for venom expenditure for food acquisition, for example due to an increase in scavenging, may reduce the production of offensive venom components. Here, we investigated changes in scorpion venom composition using a mesocosm experiment where we manipulated scorpions' exposure to a surrogate vertebrate predator and live and dead prey. After six weeks, scorpions exposed to surrogate predators exhibited significantly different venom chemistry compared to naïve scorpions. This change included a relative increase in some compounds toxic to vertebrate cells, and a relative decrease in some compounds effective against their invertebrate prey. Our findings provide, to our knowledge, the first evidence for adaptive

plasticity in venom composition. These changes in venom composition may increase the stability of food webs involving venomous animals.

1 Introduction

Interspecific arms races are ubiquitous in ecological communities, and generally involve reciprocal selection pressures that drive the evolution of adaptations and responses between interacting organisms. Organisms investing in traits mediating these arms races need to balance the fitness benefits of winning the race against the cost of maintaining those traits [1]. Some organisms defend themselves in arms races using chemical toxins, and these toxins are often produced in low quantities unless induced by exposure to natural enemies to minimize the cost of maintaining unnecessary defences [2]. Similarly, when predators are exposed to prey with varying defensive adaptations, they may develop inducible chemical weapons [3, 4]. In venomous animals, the same delivery apparatus evolved for prey capture – such as fangs or a stinger – can also be used to inject chemicals to deter enemies [5], and this dual-purpose nature of the delivery apparatus also extends to the chemistry of the venom itself. Animals generally need to balance arms races involving both predators and prey, and these arms races drive the evolution of venom chemistry in both offensive and defensive contexts [5]. However, physiological differences between predators and prey may necessitate different toxins, and specificity of venom toxins to particular groups of animals has been identified in many venom-users [6-11]. For example, sodium channel blocking α-toxins in scorpions contain three separate subtypes of toxins that are effective against the voltage-gated sodium channels of mammals only, insects only, and both [6]. The whole venom mixture can be thought of as a cocktail of these different toxins, but whether the 'recipe' for this cocktail is fixed or can exhibit plasticity in response to different environments and predator/prey interactions remains unclear [12].

Broadly speaking, plasticity will be favourable when it enables an organism to have higher fitness across multiple environments, or within a variable environment [13]. Theoretical work has shown that plasticity can be selected for when: (a) populations are exposed to multiple environments or variability within an environment, (b) environments produce reliable cues, (c) different phenotypes are favoured in each environment, and (d) no single phenotype exhibits superior fitness across all environments [14-16]. Both plants and animals can in principle exhibit 'induced' plasticity to calibrate their defences in response to species interactions [17]. In this context, plasticity may provide a way of saving costs associated with defences, allowing resources to be allocated towards growth and reproduction instead [18]. For example, theory suggests that costly plant defensive compounds should be constitutive (i.e. permanent) where the probability of herbivory is high, while an induced defence in response to attack is optimal when the probability of herbivory is low but the threat of injury from an attack is high [19, 20] Though less well-understood, induced offensive traits enable a predator to capture certain prey more efficiently via plastic change in response to cues signalling the presence of that prey [21]. Induced offences are more favourable when a consumer can benefit from adapting to multiple resource (prey) species with a variety of defences, or resources that can exhibit variable levels of a defence [22, 23]. For example, plastic induced 'offensive traits' can be seen in feeding-morphologies, such as in snails from the genus *Lacuna*, which change the shape of their teeth to suit their prey [24], and *Nephila pilipes* spiders can plastically modify the composition of their silk chemistry in order to vary the architecture and physical properties of their webs to catch different prey [25].

In venom-users, the high cost of chemical warfare has selected for a range of behavioural 'venom-metering' strategies, and these plastic behaviours are used to minimize the quantity of venom delivered. Spiders, for instance, may evaluate venom resistance in prey

based on olfactory cues and use their venom accordingly [26]. A choice of whether or not to envenomate at all has been shown, based on the relative size and threat posed by the target [27, 28]. Once the decision to envenomate is made, a range of other cues can influence the delivery and volume of venom to minimize venom-use across venomous taxa [12]. By employing similar cues, a venom-user is able to modify the 'recipe' of its venom cocktail, thereby optimizing the fitness benefits of its costly venom in different environments exhibiting differences in densities and types of predators and prey [4]. Analogous to induced non-injected defences, a fixed, constitutive venom 'recipe' may be more favourable in environments with higher rates of predator attack and lower variability in predator type, while a plastic 'recipe' may be more favourable where rates of predator or prey encounters, or predator or prey types, are variable. However, to our knowledge neither induced defensive toxin production nor induced offensive toxin production have been demonstrated in venomous animals.

Here, we present an experimental exploration of induced plasticity in the composition of venom produced by scorpions in response to perceived predation risk, and reduced need for venom-use for prey capture. We test the hypotheses that, if induced plasticity of venom composition is exhibited by a model venom-user, higher predation risk will lead to higher relative production of predator-active toxins; and that relative production of prey-active toxins will increase in response to a prey-type that requires greater venom-expenditure to ensure a meal. We did this by manipulating exposure to a surrogate predator and access to live or dead prey, and evaluating changes in the relative concentrations of prey-specific toxins, predator-specific toxins, and general venom compounds. To investigate whether manipulated rates of predator-prey interactions would elicit the plastic changes in venom composition we used the Australian rainforest scorpion *Liocheles waigiensis* (Gervais) (Scorpionoidea: Hemisorpiidae). Our next goal was to evaluate the effects of the predator-

specific toxins present in *L. waigiensis* venom on mammalian cells and the prey-specific toxins on crickets that represent their invertebrate prey. Finally, we evaluated whether the relative proportions of vertebrate-toxic venom components would increase in response to a higher rate of vertebrate predator-interactions, and whether the relative proportions of invertebrate-toxic components would decrease in response to a lower need for venom in food consumption, through increased scavenging behaviour.

2 Methods

(a) Model organism

The Australian rainforest scorpion *Liocheles waigiensis* (Gervais) (Scorpionoidea: Hemisorpiidae) used in our experiments is a common species found in the wet tropics of Far North Queensland. *L. waigiensis* is a generalist predator of invertebrates, including crickets, and is in turn preyed upon by a range of invertebrate and vertebrate predators [29]. Scorpions sourced from rainforest areas around Cairns were individually held in 170 × 110 × 50 mm 650 ml plastic containers with one stone and moist organic soil (300 ml Searles Premium Potting Mix brand potting mix) to provide a suitable microclimate for the animal and to aid with moulting. These containers were randomly sorted and stacked two high, in two Wisecube WGC-450 temperature and humidity chambers at 28 ^{0}C on a 14/10 light/dark cycle. Relative humidity was maintained at 70%, and after 3 weeks of treatments all scorpions were moved to new containers containing freshly autoclaved soil to reduce fungal growth. All scorpions were maintained in the controlled environment for no more than 5 days prior to the first venom extraction.

(b) Experimental treatments

Given the general predation of invertebrates by *L. waigiensis* in the wild, we used the common house cricket *Acheta domesticus* L. (Insecta: Orthoptera) as a surrogate prey species. To simulate a vertebrate predator sometimes encountered by *L. waigiensis* in the wild, a frozen feeder mouse, *Mus musculus* L. (Rodentia: Muridae), was taxidermied by skinning, stuffing with cotton wool, and articulation with wire, then used as a simulated model vertebrate predator (as in Digweed and Rendall [30]). We used a 2 x 2 factorial design in which without and with pressure for induced offensive venom production (presence of live versus dead prey) was crossed with pressure for defensive venom production (simulated predator exposure). The number of replicates, accounting for scorpions that died during the treatments and were therefore excluded from the analysis, were as follows: 15 (pressure for offensive + defensive venoms), 14 (pressure for offense venom), 14 (pressure for defensive venom), 13 (control).

For the prey treatment, scorpions were each fed either a live (pressure for offensive venom) or dead cricket once per week. Live crickets were purchased 1-2 days prior to each feeding, and were killed by freezing for approximately 12 hours. In doing so, the quality of the diet was identical for both groups, but the pressure to use venom to obtain a meal was not. Our taxidermied mouse was used to provoke defensive stings from scorpions in the defensive venom pressure treatment three times a week, except for the first week to allow for acclimation. The mouse was used to continuously probe on the cephalothorax of defensive pressure treatment scorpions for 30 seconds. This stimulus readily stimulated anti-predator responses in the scorpions, including alert and threat postures (with chelae extended and open, and metasoma erect), grappling, pinching, stinging, squirming, and retreat [31, 32]. To ensure that scorpions excluded from the defensive pressure treatment were otherwise equally handled and exposed to laboratory conditions, the containers of these scorpions were opened and exposed to laboratory conditions for 30 seconds. Six weeks after commencement of the

149 experiment, scorpions were subjected to the control (no offensive, no defensive pressures)
150 treatment for one week before venom was extracted. Although this may have diminished the
151 measured effects of the with-pressure treatments due to relaxing of any induced response, a
152 brief recovery time was necessary to ensure sufficient volume of venom had recovered to
153 perform chemical analyses.
154
155 ***(c) Venom extraction***
156 We ran the treatments for 42 days so that the experiments lasted twice as long as the venom
157 regeneration time of 21 days, according to previous analyses [29]. Venom was first extracted
158 within 5 days of collection, and then again after a week of rest, following the end of the
159 experiment, 49 days later. Venom was then extracted from all scorpions a third time, 21 days
160 after the experimental treatments ceased, to assess how it had changed in the absence of
161 offensive and defensive pressures. By providing the scorpions with the full length of time
162 necessary to regenerate their venom, we ensured that there was ample time for the treatments
163 to elicit a response in the chemistry of the regenerated venom.
164
165 ***(d) Venom analysis***
166 Venom was collected using an Arthur H. Thomas Co. Z789 Square Wave Stimulator to
167 electrostimulate the telson at approximately 25 volts (5.5 pulses/sec, for 15 milliseconds per
168 pulse). Extracted venoms were diluted in 150 µL of degassed phosphate buffered solution
169 (PBS- Life Technologies), centrifuged for 10 minutes total at 32,000 RPM, and filtered
170 through a syringe-driven 4mm 0.22 µm filter (Millipore). Venom profiles were obtained
171 using size-exclusion fast protein liquid chromatography (FPLC) using a Superdex™ 75
172 10/300 (Tricorn) GL Column (13µm, 10mm×300mm – GE Healthcare) at 4 ^{o}C with 100%
173 PBS buffer at 0.50 ml/minute with 0.5 ml elutions for 45 mL on an ÄKTA™ FPLC (GE

Healthcare). Venom components were detected by absorbance measured at a wavelength of 280 nm. Finally, using venom collected from the same scorpions, but three weeks after the cession of the experimental treatments, we evaluated the toxicity of each venom fraction by performing toxicity assays on a human cardiac cell line to test for vertebrate toxicity (see section f), and by performing behavioural assays on crickets to test for effects on temporary or permanent invertebrate paralysis (see section g).

(e) Statistical analysis for profile changes

To compare between the venom profiles from each treatment, we split the FPLC venom profile into 14 different 'fractions', and differences in the amounts of each relative to the other treatments could then be evaluated statistically. To identify the different fractions, we first standardised each chromatogram to an area under the curve of 1, obtained the mean chromatogram for each of the four treatments by averaging all the curves within each treatment. We next fitted a spline curve to each of these mean chromatograms using the smooth.spline function in R [33], with the smoothing parameter, $\lambda = 0.5$ [33, 34]. The local minima in these splines were then designated as boundaries between two fractions. Local minima within 1 ml from each other were averaged to create a single break between fractions, with one exception: the local minima values of 38.64, 38.99, 39.78, 40.51, and 40.57 ml were divided into the two groups: 38.64, and 38.99; and 39.78, 40.51, and 40.57 ml, for which each was averaged to describe the combined fraction separation point. Principal component analysis (PCA) was then used to describe these 14 fractions across the data set [34]. MANOVA and two separate, follow-up ANOVA analyses were performed to evaluate treatment effects on the first two principal components. These analyses were conducted on venom samples collected at three time points: prior to the initiation of treatments (t = 0 days),

198 at the cessation of the treatments (t = 49 days), and 21 days after the cessation of treatments (t
199 = 70 days).

200 To evaluate the effects of predator and prey main effects on particular peaks, we
201 calculated the mean and 95% confidence intervals for each peak evaluating the difference
202 between the predator-no predator treatment means, or the live prey – dead prey treatment
203 means. The 95% confidence intervals were calculated using non-parametric bootstrapping
204 with 10,000 simulations. For each simulation, we resampled with replacement the
205 absorbances for a particular treatment (e.g., with or without simulated predator exposure) and
206 chemical fraction. Chemical fractions five through eight were not easily distinguishable and
207 likely represent a number of compounds, and therefore, we also calculated the mean
208 treatment effects and 95% confidence intervals for the sum of these fractions (summed
209 individually for each scorpion). Treatment effects were considered significant for $\alpha = 0.05$
210 when 95% confidence intervals did not overlap zero.
211
212
213 ***(f) Predator cell assays***
214 The biological consequences of observed changes to the venom profiles were evaluated using
215 toxicity assays. Fraction concentrations were determined using the A280 method [35]. A
216 human cardiac cell line (Sciencell) was used as a vertebrate assay, following Schneider [29].
217 Vertebrate cells were maintained and assays were performed as previously described by
218 Andreosso, Smout [36] and Chaousis, Smout [37]. An xCELLigence SP RTCA system
219 (ACEA Biosciences) with an E-plate seeded with 150 µL cardiac media (Sciencell) and 5000
220 human cardiac cells were incubated overnight at 37 ^{o}C and 5 % CO_2.

221 The cell response to each fraction (20 µL) and 100% PBS solution (control) was
222 measured by the xCELLigence system as changes to cell index. Cell response is a

combination measure of changes in media conductivity or cell contact/toxicity, which varies as the cells deform in response to exposure to a chemical sample. The cell index readouts were blanked against the PBS control, and the maximum drop value in 2 hours after venom addition was deemed the predator cell response. The relative response to whole venom as a percentage was then used to graph the activity level of the venom peaks. We used two-tailed t-tests to compare the response of each venom fraction to the PBS control to identify peaks that significantly altered media conductivity or cell contact/toxicity.

(g) Prey toxicity assays

Acheta domesticus cricket assays were performed by evaluating whether a given venom fraction was active towards immature crickets. To evaluate the effects of each fraction, 3 μL of one of the 14 chemical fractions was injected ventrally into the pronotum of an immature cricket varying in mass from 0.1 to 0.2 grams. Immediately after injection, the cricket was inserted into a clean, 9-dram clear styrene tube with snap-on lid and rolled onto its back every 10 seconds 18 times for a total of 3 minutes. A compromised righting response was recorded when a cricket was unable to right itself within 60 seconds of being rolled onto its dorsal side. Each fraction was replicated with 10 crickets. We used a 2 by 2 Fisher's exact test to compare the cricket response from each venom fraction to a PBS control.

3 Results

(a) Effects of predator-prey interactions on venom composition

The experimental results were used to evaluate our hypotheses on venom plasticity using a model animal the rainforest scorpion *Liocheles waigiensis*. The venom profiles obtained from venom extraction before the experimental treatments began were not significantly different from each other (Figure 1A, see Electronic Supplementary Material 1 for statistical analysis).

After treatment there was a difference between the venom profiles of the predator-treated and the predator-excluded scorpions.These profiles varied greatly in the relative concentration of multiple chemical fractions, with the greatest difference in treatments occurring in peak fraction 12 (Figure 1B). From the principal component analysis we obtained two major principal components, PC1 and PC2, which explained 53.6% and 25.4% of the overall variation, respectively. Venom profiles obtained from scorpions that were and were not subjected to the defensive pressure treatment were found to be significantly different using a MANOVA to evaluate the treatments on the principal component weightings (Table 1), with increased predator exposure leading to lower and higher values of PC1 and 2, respectively (Figure 2). This was most clearly associated with changes in fraction 12, which was reduced in the scorpions exposed to simulated predators (Figure 1B, 2), which significantly decreased with predator exposure (Figure 3). Fraction 2 also varied strongly in both principal components, but not in a way that was interpretable with the experimental treatments (Figure 2). There were no interaction effects, nor any significant effects from prey manipulation treatment (live versus dead prey) on the venom profile principal components 1 and 2 (Table 1). Profiles obtained after a 21-day recovery period following cession of treatments exhibited similar patterns of difference between treatments (Figure 1B, C, ESM 1), but the magnitude difference was reduced.

(b) Toxicity assays

Higher activity towards mammalian cells (>60%) was generally found in the toxin fractions containing larger proteins/peptides (fractions 2,3,4,5,7,and 8, Figure 3, ESM 1), which were likely 3-25 kDa due to the Superdex™ 75 resin that was used [37]. One section of the profile contained many fractions (5-8) that were not easily distinguishable, significantly increased in response to simulated predator exposure (95% bootstrap confidence limits: 0.004, 0.083), as

did fractions 7 and 8 when evaluated individually (Figure 3). In addition, fractions 10 and 11 had some activity against mammalian cells, although the magnitude of these effects were much lower than for other fractions (Figure 3, ESM 1). Toxicity towards crickets was generally found in a fraction containing larger proteins/peptides (fractions 3-4), and the fractions containing smaller compounds (fractions 8-14) (Figure 3A, ESM 1). Components of each of these sections were reduced in response to simulated predator exposure (Figure 3). It should be noted that many small molecules are not detectable at 280 nm and other detection methods may be required. Undetected compounds were the likely source of activity against crickets (>90%) exhibited by fractions 13 and 14, as the absorbance trace showed very minimal contents. Example cell responses are provided in ESM 2.

(c) Comparing treatment effects with toxicity assays

Simulated predator exposure had the strongest effect on reducing the relative production of fraction 12 that demonstrated activity on crickets, and to a lesser extent, reduced the relative production of a section (fractions 3-4) that exhibited effects on both crickets and mammalian cells (Figure 3). Fractions 5-8, which were not easily distinguishable (Figure 1), significantly increased in response to simulated predator exposure (predator treatment effect 95% limits: 0.003670351, 0.082905484) and portions of this section of the profile exhibited activity on mammalian cells (Figure 3B). Fractions 1 and 11 slightly increased in the presence of predators (Figure 3C), but neither of these had strong effects on invertebrates or mammals (Figure 3A,B). The presentation of live versus dead prey had little effect on the relative production of each chemical fraction, although it did slightly increase the production of fraction 3 (ESM 1) that effected both crickets and mammalian cells (Figure 3A,B).

4 Discussion

Given current theory relating selection pressures to plastic changes in defence and reproductive investment (e.g., Peacor, Peckarsky [38]), venomous mesopredators should shift the balance of venom composition towards the defensive components when predator exposure increases. In line with these predictions, we found evidence for a plastic change in venom composition in response to increased perceived predation risk, showing for the first time to our knowledge that organismal venom chemistry can change in response to a threat. These changes imply a rerouting of resource expenditure, which may be nutritional or energetic [12], to increase relative production of other venom fractions which are responsible for toxicity to vertebrates. Overall, simulated predator exposure appeared to decrease relative production of strong invertebrate toxins, while generally increasing the production of a section of the venom profile with activity towards mammalian cells. These results suggest for the first time to our knowledge that venoms can serve as inducible defences used against predators. Inducible defence theory suggests that plastic defences are more likely to evolve in highly variable or cyclic environments, where the fitness benefits of flexibility outweigh the costs of maintaining this capacity for variability [2, 13] Venomous animals evolve vast, complex armouries of peptides and proteins in their venoms [12], and it would appear that *L. waigiensis* is able to modify the production of a subset of their complex venom cocktail to suit a changing environment. The magnitude of the pressure to minimize venom cost and the predatory pressure may also relate to how closely venom production tracks the rate of ecological dynamics [17].

Resource type did not elicit a response in venom chemistry. This lack of effect, which was probed through removal of the need for venom expenditure, may have been due to a) insufficient variation in resource type, b) a time-lag in the scorpions' response which as a result was not detected, or c) an absence of inducible offence. It is well-documented that the

magnitude of environmental variability can influence both the magnitude and the speed of a plastic response [39]. For example, moderate levels of herbivore damage may only induce a 'primed' state in plants rather than the immediate chemical response to high damage, while low levels of damage may fail to provoke a plastic response at all [2]. In our experiment, we introduced two resource 'types' (live or dead) to represent variation in the need for venom during prey-capture. However, if the live resource type was not sufficiently different to the dead resource type (i.e. often not requiring venom-use to obtain a meal) than any inducible offence in the venom profile may not have been provoked. We tried to account for this by feeding larger prey (i.e. larger in size than a scorpion's chelae) to encourage the need for envenomation following van der Meijden, Coelho [32], but scorpions were still occasionally observed to be killing their prey without stinging. Secondly, there may have been a time-lag in any potential response to the treatment. In plants, induced chemical defences can be mounted in response to attack, followed by a substantially longer 'relaxation' period before returning to a ground state. For example, *Trifolium repens* mounts a systemic chemical defence within 51 hours of herbivory, but requires at least 28 days to relax (Gomez et al. 2010). Similarly, an induced offense in response to prey-type may exhibit a relaxation period. For example, in snails from the genus *Lacuna*, the longer an individual fed on previous diet, the slower its induced morphological offense switched to a new food source [24]. Finally, there may be no plastic response to variation in resource-type. This may be due to either insufficient variation in prey-type in the wild to drive the evolution of a plastic response capability, resembling the conditions under which constitutive defences are favoured by plants, or due to sufficiently high fluctuations in prey-type to favour a bet-hedging strategy rather than plasticity [40]. When traits respond to a selective pressure, evolution balances this response between optimising the trait for the maximum fitness benefit and over-investing in the trait to compensate for the effect of environmental stochasticity [15, 41]. Such bet

hedging strategies are ubiquitous in arms races [42], and may also be seen in venom-users [43]. In the absence of an alternate prey which doesn't require stinging to be subdued, it may be favourable to delay a plastic response (or exhibit none at all) and continue producing costly venom even in the absence of live prey to ensure success in future opportunities to catch a meal. Future work may be able to distinguish between these competing explanations by investigating the variability of food resources in the natural habitat of *L. waigiensis.*

Induced plastic defences can stabilize populations against fluctuating predatory pressures [17], and as such in ecological communities where venomous animals provide an important food resource (e.g. [44]) induced defences could act as an important stabilizing force for the community and diminish trophic cascades in food webs. Adaptive plasticity can mitigate the effects of sudden disturbances by allowing populations to evolve sufficiently quickly to survive abrupt change [45]. Phenotypic plasticity permits more time for evolutionary adaptation to occur, and may reduce the degree of evolutionary change necessary to track a moving optimal trade-off between the costs and benefits of venom production [45]. Indeed, populations which exhibit greater phenotypic plasticity are generally able to evolve more under global change and thereby adapt to changing environments [46]. However, plasticity may also slow the rate of evolutionary pressure by reducing the selection pressure for genetic change [16]; whether or not venom plasticity should facilitate or inhibit adaptation by venom-users to modified predator-prey interactions driven by environmental change remains an open question.

In our bioassays, we found some venom fractions (e.g., fraction 3) have activity against both the scorpion's cricket prey and mammalian heart cells, suggesting they may serve to improve both prey capture and defence against predators. This may lead to complicated tritrophic interactions where phenotypic changes in response to one arms race (e.g., with predators) can alter the investment in another arms race (e.g., with prey) (Gangur

et al. in review). Furthermore, we identified fractions of the venom profile (e.g, fraction 1) that increased in response to simulated predator exposure, but in isolation did not demonstrate activity against mammalian heart cells. These fractions may have effectiveness against another vertebrate biological pathway (e.g., pain activation), may interact with other fractions to improve potency, or may be increased incidentally due to physiological constraints in venom production. Thus, further research to clarify the role of these venom fractions in predator defence may shed light on adaptive advantage of the observed phenotypic changes in response to simulated predator exposure. Furthermore, we have only evaluated the effects of each venom fraction on two distantly related taxa (mammalian cells and arthropods). In some cases, organisms can target phenotypic changes in defence to the specific threats e.g. [47, 48]. Further research exposing these scorpions to a range of predator species and evaluating changes in venom composition may elucidate the specificity of this phenotypic plasticity.

Venom research has historically been intently focused on human toxicity, due to obvious reasons, the prevention of mortalities and a strong interest in medical advancements [49]. The ecological and evolutionary perspectives that have been increasingly explored, particularly in the last decade, offer critical insights into venomous animal ecology that has improved health outcomes as well as enriched our understanding of venom-use and production. Indeed, if plastic responses are widespread in venomous animals, antivenom production may be improved by accounting for this potential source of variation by ensuring live prey or simulated predation [50]. Furthermore, the potential role of venom in stabilising ecological dynamics needs to be further explored as in some cases this may be a substantial factor controlling community structure.

Data Accessibility: Venom extraction and assay data: Dryad doi:10.5061/dryad.sq2g4 [51]. Additional statistical analyses supporting this article have been uploaded as part of the supplementary material.

Ethics: There were no live vertebrate animals used as experimental subjects in this study.

Authors' Contributions: Experiments were designed by ANG, TDN, and MJL, JES, and conducted by ANG. Chemical analyses were conducted by ANG, MS, and DW. Mammalian and invertebrate bioassays were conducted by MS and TDN, respectively. ANG and TDN conducted statistical analyses with input from MJL and JES. The first draft was prepared by ANG and TDN, with all others contributing extensively to subsequent drafts.

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Competing Interests: We declare we have no competing interests.

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524 **TABLES**

525

526 **Table 1:** MANOVA results from venom collected one week after the cessation of the
527 experimental treatments, which demonstrated a significant overall difference between the
528 fraction loadings from scorpions that were and were not subjected to the defensive pressure
529 treatment. There was no significant interaction effect.

Source	d.f.	Pillai	*F*	Df den	*P*
Offence	1	0.040	0.978	2, 47	0.383
Defence	1	0.242	7.517	2, 47	0.001
Defence × Offence	1	0.036	0.887	2, 47	0.419
Residuals	48				

530

Table 2: ANOVA results from venom collected one week after cessation of experiments, demonstrating significantly different fraction loadings between the scorpions that were and were not subjected to the defensive pressure treatment along both PC1 and PC2. There were no significant interaction effects.

Source	**d.f.**	**M Sq**	***F***	***P***
PC 1				
Prey	1	0.012	1.933	0.171
Predator	1	0.048	7.643	0.008
Predator × *Prey*	1	0.001	0.170	0.682
Residuals	48	0.006		
PC 2				
Prey	1	0.001	0.173	0.679
Predator	1	0.018	5.928	0.019
Predator × *Prey*	1	0.005	1.744	0.193
Residuals	48	0.003		

538 **FIGURE LEGENDS**

539 Figure 1. Averaged relative venom profiles for each of the four treatments taken: prior to
540 treatments (A, t = 0 days), after the treatments ended (B, t = 49 days), and again after a
541 recovery period (C, t = 70 days) divided into 14 fractions. Relative absorbance measures the
542 absorbance units at 280 nm of any point along the venom profile relative to the point of
543 maximum absorbance in the profile. Venom profiles obtained from scorpions subjected to the
544 pressure for defensive venom (mouse exposure) treatment are given in dark blue (+ pressure
545 for offensive venom, live cricket prey) and light blue (- pressure for offensive venom, dead
546 cricket prey); profiles obtained from scorpions not subjected to this treatment are given in red
547 (+ pressure for offensive venom) and orange (- pressure for offensive venom).

548 Figure 2. Loadings for principal components 1 and 2, separated into loadings for each
549 scorpion (A) and loadings for each venom fraction (B, 14 total peaks) with fraction number
550 indicated at the tips of each arrow. For clarity and consistency, colours are as per the curves
551 presented in Figure 1; pressure for defensive venom treatment is indicated in dark (+ pressure
552 for offensive venom) and light (- offensive pressure for offensive venom) blue, while data
553 from scorpions not subjected to the pressure for defensive venom treatment are in red (+
554 pressure for offensive venom) and orange (- pressure for offensive venom). PC1 and PC2
555 described 53.6% and 25.4% of the overall variation, respectively.

556 Figure 3. Invertebrate (A) and vertebrate (B) toxicity assay results. Invertebrate toxicity was
557 measured by evaluating the proportion of crickets (10 crickets per treatment) that were
558 paralysed for longer than 60 seconds. Statistically significant difference from the control was
559 evaluated using a Fisher's Exact Test for each peak (ESM 1). Vertebrate toxicity was
560 evaluated by measuring vertebrate cell response to venom fractions relative to whole venom
561 response using the xCELLigence platform. Due to small sample volume, it was not possible

562 to completely separate fraction 5 from fraction 6. Statistically significant difference from the
563 PBS control (blanked at 0) was evaluated using a two-tailed t-test for each peak (ESM 1).
564 Panel C presents mean (and 95% non-parametric bootstrap confidence intervals) for the
565 difference between the treatments with and without simulated predator exposure. Confidence
566 intervals entirely above (or below) zero suggest significant effects of increased (or decreased)
567 production after simulated predator exposure. Differences were calculated after the
568 treatments ended (t = 49 days). Asterisks represent confidence intervals that do not overlap
569 zero. Chemical fractions five through eight were not easily distinguishable, and likely
570 represent multiple similarly sized compounds. Therefore, we have also calculated this
571 confidence interval separately (predator treatment effect 95% limits: 0.004, 0.083). Panel D
572 presents the relationship between invertebrate and vertebrate toxicity for each peak. The
573 feature scaling function $x' = \frac{x - \min(x)}{\max(x) - \min(x)}$ was used to convert max drop value into a
574 normalized vertebrate assay score in the range [0,1] for ease of comparison (invertebrate
575 assays were already scored in this range as a proportion of crickets out of 10 replicates
576 experiencing paralysis for >60 seconds after toxin injection). Error bars indicate standard
577 error. Due to low yield volume, the vertebrate assay score for fraction 5 includes both
578 fractions 5 and 6. Asterisks indicate significance for alpha = 0.05.