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Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*

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ABSTRACT

Mygalomorph venom properties and active components, which have importance in medicine, agronomy, venomics, ecology and evolution, have been widely studied, but only a small fraction have been characterised. Several studies have shown inter-individual variation in the composition of venom peptides based on ontogeny, sexual dimorphism, season and diet. However, intra-individual variation in venom composition, which could play a key role in the evolution, diversification and function of toxins, is poorly understood. In this study, we demonstrate significant intra- and inter-individual variation in venom composition in the Australian funnel-web spider *Hadronyche valida*, highlighting that individuals show different venom profiles over time. Fourteen (four juvenile and ten adult females) funnel-web spiders, maintained under the same environmental conditions and diet, were milked a total of four times, one month apart. We then used reversed-phase high performance liquid chromatography/electrospray ionisation mass spectrometry to generate venom fingerprints containing the retention time and molecular weights of the different toxin components in the venom. Across all individuals, we documented a combined total of 83 individual venom components. Only 20% of these components were shared between individuals. Individuals showed variation in the composition of venom peptides, with some components consistently present over time, while others were only present at specific times. When individuals were grouped using the Jaccard clustering index and Kernel Principal Component Analysis, spiders formed two distinct clusters, most likely due to their origin or time of collection. This study contributes to the understanding of variation in venom composition at different levels (intra-individual, and intra- and inter-specific) and considers some of the mechanisms of selection that may contribute to venom diversification within arachnids. In addition, inter-specific variation in venom composition can be highly useful as a chemotaxonomic marker to identify funnel-web species.

1. Introduction

Spider venoms are a complex blend of peptides, proteins and small molecules (e.g. polyamines) that induce a variety of biological activities across a wide range of biological targets (Nentwig and Kuhn-Nentwig, 2013). Spider venom components commonly modulate ion channels, such as voltage-gated sodium (NaV) and calcium channels (CaV) (Gomes and Palma, 2016; Klint et al., 2012; Rash and Hodgson, 2002), affecting excitatory and inhibitory neurotransmission, neuronal and neuromuscular transduction in both vertebrates and invertebrates (Alewood et al., 2003; Nunes et al., 2008; Ushkaryov et al., 2004; Langenegger et al., 2019). For example, nucleosides block kainate receptors and L-type Ca2+ channels (Langenegger et al., 2019), while peptide toxins, such as hexatoxins from Australian funnel-web spiders, target NaV channels (Nicholson et al., 1996). Some protein toxins, acting as neurotoxins, affect Ca2+ channels and neurotransmitter release (Ushkaryov et al., 2004; Shatursky et al., 1995) or the extracellular matrix, causing necrotoxic effects in humans (Binford et al., 2009; Lopes et al., 2019). This molecular complexity and variety of potent activities across numerous targets has generated significant interest in the potential of spider venoms as an extensive source of natural, active molecules for use as therapeutic and bioinsecticide leads (Herzig et al., 2020a; Robinson

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et al., 2017; Saez et al., 2010; Wilson et al., 2017).

The variation in molecular complexity of spider venoms has also been studied. Venom composition of spiders varies between species, and provides sufficient consistency and resolution to be used as a chemotaxonomic marker down to the species variant level (Binford, 2001a, 2001b; Wilson and Alewood, 2006; Palagi et al., 2013). In addition, venom composition varies between individuals of the same species (Wilson and Alewood, 2006). However, while variation within the same individual over time has been reported to a limited degree in snakes (Ryabinin et al., 2019; Casewell et al., 2020; Tasoulis et al., 2020), scorpions (Pimenta et al., 2003), and cone snails (Jakubowski et al., 2005; Biass et al., 2009; Dutertre et al., 2010); this variation has not been reported in spiders. Identification and awareness of the level of variation in venom composition, both within and between individuals and species, is important for reproducibility in lead molecule discovery, and also medically. For example, variation in venom composition in the production of antivenoms, frequently still performed by inoculation of hosts with crude venom (e.g. funnel-web spider antivenom, https://www.seqirus.com.au/products), may impact the efficacy of these antivenoms (Isbister et al., 2014; Casewell et al., 2020).

Variation in the venom amount delivered and the composition observed within and between species (Atkinson and Wright, 1992; Arbuckle, 2017; Dutertre et al., 2014) can be affected by multiple factors. Some sources of this variation which can work synergistically and/or independently are: seasonality (variations in temperature and microhabitat conditions; Wong et al., 2016); sex (male and female lifestyles; Binford, 2001a, 2001b; Herzig, 2010; Wilson, 2016; Santana et al., 2017; Zobel-Thropp et al., 2018; Herzig et al., 2020b); type and size of prey (Kuhn-Nentwig et al., 2004; Barlow et al., 2009; Morgenstern and King, 2013; Nelsen et al., 2014); and age (selection pressures affect the availability of prey over development; Herzig, 2010; Cooper et al., 2015; Arbuckle, 2017). Depending on the stimulus spiders are experiencing, behaviour can trigger changes in spider responses, and the way they use venom (e.g. defense or predation; Schendel et al., 2019). Other sources of variation in venom components are related to geographic origin (Gomes and Palma, 2016), which can promote the divergence of species and communities, leading to changes at the genetic level (Escoubas et al., 2002). For example, different families of toxins in some scorpions differ based on their geographical locality; toxins belonging to the αNaScTx family that act on the Na_V receptor site 3, are a characteristic of Asian and Mediterranean scorpions belonging to the Buthidae family (Morgenstern, 2013).

The diversity and complexity of venoms can also vary depending on function, either for defense or predation (Casewell et al., 2013; Schendel et al., 2019). Predatory venoms are generally more complex and variable in composition, showing toxicity across a broad range of biological targets (Casewell et al., 2013; Arbuckle, 2017; Dutertre et al., 2014; Schendel et al., 2019). In contrast, defensive venoms are generally relatively simple in composition in some animals, such as bees and fish (Casewell et al., 2013), although in cone snails, defensive venoms can be more complex than predatory venoms (Dutertre et al., 2014). Divergence in predatory venom is linked to the ecological role of the venom, which may be driven by dietary differences and/or prey specialisation (Boevé et al., 1995; Wigger et al., 2002; Schendel et al., 2019). In general, variation in the complexity of defensive and predatory venoms, the modes of action, and biological targets, is present across all taxonomic levels, and can also occur in closely related species (Abdel-Rahman et al., 2009; Touchard et al., 2015; Zancolli et al., 2019).

Historically, Australian funnel-web spiders have been of research interest primarily because their venom components are responsible for an envenomation syndrome in humans that can lead to death, but also due to the extraordinary breadth and the potential of the toxin libraries they possess for commercial bioactive lead discovery (Gray and Sutherland, 1978; Nicholson and Graudins, 2002; Nicholson et al., 2004; Tedford et al., 2004; Chassagnon et al., 2017; Ikonomopoulou et al., 2018; Pineda et al., 2020). Funnel-web spiders show high complexity in venom composition (Palagi et al., 2013; Pineda et al., 2020), which is likely related to prey availability in different microhabitats, trophic adaptations, predator deterrance (Beavis et al., 2011; Pekár et al., 2018; Herzig et al., 2020b), genetics, molecular diversity (Pineda et al., 2020), ecological factors, and behaviour (Cooper et al., 2015).

While intra- and inter-specific variation in spider venoms has been previously reported (Escoubas et al., 1997; Palagi et al., 2013), the level of individual variation over time, and the conditions and factors that affect individual variation in venom properties, has not been established. Therefore, in this study, we analyzed venom fingerprints of female funnel-web spiders *H. valida* to assess the inter- and intra-individual differences in venom components of individual spiders over time. We then compared the venom components between *H. valida* and the closely related species *H. infensa*, which contributes to a broader understanding of the evolution of venom components, and the extent of the potential for the identification and characterisation of possible bioactive leads. Both species belong to a species complex group, the *infensa* group (Gray, 2010), where some of the species share similar morphological traits (Gray, 2010) and overlapping distributions, but differ in microhabitat, behaviour (Hernandez, unpub. obs.), and toxin composition (Palagi et al., 2013).

2. Materials and methods

2.1. Spider collection and husbandry

Fourteen *H. valida* (four juveniles and ten adult females; collected by manual excavation of burrows in the Currumbin Valley and Mount Tamborine) were purchased from Thargomindah Man Productions in 2019 (Varsity Lakes, QLD, Australia). Eighteen *H.* infensa (nine adult females and nine juveniles), were also collected manually in Toowoomba at Blue Meadow court and Ravensbourne (−27.5028782°S, 151.953638°E; −27.3665311°S, 151.1792198°E) by the authors in 2019. Sex differences in venom composition are known for these species (Wilson and Alewood, 2004), and we attempted to remove this variation by focusing on adult females. The spiders were transported alive in small plastic containers with damp cotton wool to the laboratory of the Australian Institute of Tropical Health and Medicine (AITHM), James Cook University Cairns campus, Queensland, Australia. Each spider was housed individually in a 3 L plastic container containing coconut coir peat as a substrate. The spiders were housed in a climate-controlled room (temperature: 20 ± 2 °C; relative humidity: 60%) on a reverse light:dark cycle (12L:12D; lights on at 6 p.m.). Each spider received one house cricket (*Acheta domestica*) once a week.

Adult females were identified by epigyne sclerotisation and the opening in the epigastric furrow (gonoslit, Zhan et al., 2019), which is very apparent in adult females, but is absent in juveniles (F. Perez-Miles, pers. comm.). Cephalothorax width was measured to assess spider size (Supplementary Material Table S1) after each repetition (see below). To obtain size, we photographed the dorsal aspect of each spider under Leica stereomicroscope, and processed the images using Image J 1.8.0 Software.

The research was conducted within the framework of the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013). Funnel web spiders are not a protected species in Australia. Consequently, the Department of Environment and Science of the Queensland Government advised that a scientific permit for collection and holding was not required.

2.2. Venom collection and analysis

The spiders were milked one week after they arrived at the laboratory to obtain a baseline venom profile. Venom expelled on the tips of the fangs of aggravated individuals was collected using a 200 μL Gilson P200 pipette with polypropylene micropitpette tips. To aggravate the spiders, we touched the first pair of legs using tweezers until the venom

was expressed on the fang tips (Wilson and Alewood, 2006). The process was repeated at short intervals for 10 min. The venom was then placed in a 1.5 mL microcentrifuge tube with 40 μL of Milli-Q water and stored at −20 °C. Thereafter, we milked the spiders three more times, one month apart, for a total of four venom samples per individual. Venom samples from each milking were kept separate. In each case, the spiders were not fed two weeks prior to venom extraction to minimise venom depletion and to reduce the potential effects of feeding on venom composition (Wigger et al., 2002).

Liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) analysis of the venom peptides of each individual from each species was performed to generate venom fingerprint profiles and observe differences in venom composition at the intra- and inter-individual levels, as well as the inter-specific level. To detect variation in venom composition, and to obtain venom profiles, the samples were injected via an autosampler (Shimadzu SIL-20AC HT) onto a reversed-phase high-performance liquid chromatography (RP-HPLC) column (Phenomenex Aeris 150 × 2.1 mm 3.6 μm PEPTIDE XB-C18 100 Å) at 30 °C. Solvent (buffer A: 0.1% formic acid/water; buffer B: 90% acetonitrile/0.09% formic acid/water) was delivered via Shimadzu LC-20AD pumps at a flow rate of 0.250 mL/min. The UV absorbance was observed at 214 nm and 280 nm on a Shimadzu SPD-20A detector. Mass spectra were collected in positive ion mode over a scan range of *m/z* 250–2000 with a detector voltage of 1.15 kV, nebulizing gas flow of 1.5 L/min, and drying gas flow of 3.0 L/min. Data were collected and analyzed using the Shimadzu LabSolutions v 5.96 software.

2.3. Statistical analyses

Statistical analyses were conducted using RStudio (version 1.0.153; https://www.rproject.org; R version 3.5.0, https://cran.rstudio.com). To compare the number of venom peptides within and between individuals of *H. valida* (Supplementary Material Table S2), we used the package UpSet plot (Lex et al., 2014). The package allowed us to quantify the number of sets (i.e. individuals) and intersections that are shared between elements (i.e. venom component masses shared between individuals).

To determine intra-individual variation in venom composition in *H. valida*, we plotted the venom fingerprint profile of each spider and its replicates (Supplementary Material Table S3, Fig. S1). Juveniles (A2, A4, A6, A10) and adults were separated (M, J, S, A1t, A5t, A7t, A8t, A9t, A11t, A12t) to quantify the number of venom components shared between each group. To obtain an UpSet plot with all the individuals, we binned the venom components found for each individual over all replicates. Some of the venom peptides were identified based on retention times from previous studies and mass and sequence information available in the Arachnoserver database (http://www.arachnoserver.org/). The other components currently remain unidentified.

A Jaccard matrix was constructed from the venom components obtained from each peak in the chromatograms of each individual. To measure the similarity of venom components within, and between, individuals of *H. valida*, we used the Jaccard similarity coefficient and the average linkage method to measure the distance between clusters (venom component masses of each individual). Dunn's index was used to determine the suitable average method to calculate the clusters. The correlation coefficient cophenetic distances were used to assess the best possible dendogram generated. Using the Jaccard matrix we carried out a Kernel Principal Component Analysis (KPCA) using the package mixKernel (Mariette and Villa-Vialaneix, 2017) to visualise in a better manner the relationships between how individuals were grouping, and the distances between them. The ten most important venom components that explained the majority of the variance of the first principal component of KPCA are shown in Supplementary Figs. S2a and S2b. We used the package factoextra (REF) to build a hierarchical cluster for all individuals (Altman and Krzywinski, 2017).

3. Results

3.1. LC/ESI-MS venom analysis

Venom fingerprints for each individual were generated from the LC/ESI-MS chromatograms of *H. valida* venom, which provided rentention time and mass data of the venom components present (Fig. 1). The venom components found ranged in mass from 295.030 Da to 8420.294 Da, with a predominant bimodal mass distribution in the ranges 3863.399–4854.311 Da and 6733.890–8420.294 Da.

3.2. Intra-individual variation

All individuals showed variation in venom composition over time (i.e. individual variation between replicates; Supplementary Material Fig. S1). In addition, the total number of venom components shared between each replicate varied depending on the individual (Fig. 2). Some components only appeared in a specific replicate and were not shared between replicates (Fig. 2; Supplementary materials Fig. S1). For example, in individual A1, the number of components shared between the baseline (A1) sample and replicates was 18 out of 42 (Fig. 2). Moreover, in the same individual, the baseline and replicates one (A1T1) and two (A1T2) shared only eight venom components overall, and seven venom components were exclusively present in the baseline sample.

3.3. Inter-individual variation

The total venom composition found in *H. valida* (when all four venom samples from each individual were considered) showed that, across all the spiders tested, a total of 83 discrete venom components were present, with up to 50 venom component masses evident in some individuals (see M, Fig. 3a) and only 37 in other individuals (See S, Fig. 3a). All spiders shared 17 venom components; however, some of the individuals also showed specific components. For example, the individuals M and J each showed four components that were unique to each of these individuals (Fig. 3a; Supplementary Material Fig. S1).

Comparing the venom fingerprints between just the juvenile specimens revealed 31 shared venom components (Fig. 3b), while a comparison between just the adult specimens showed only 18 shared venom components (Fig. 3c). A total of eighteen venom components were unique to the adult specimens. The Jaccard analysis of similarity (Supplementary materials Table S4) showed that three individuals (Cluster B, including individuals M, J and S) formed a separate cluster to the remaining individuals (Cluster A; Fig. 4). Interestingly, the component of mass 4079.420 Da was unique to individuals belonging to cluster A and was absent in individuals belonging to cluster B. Juveniles did not form a separate cluster to adults (Fig. 4a). In addition, the KPCA showed similar results to the ones returned by the hierarchical cluster, where Cluster B was completely separate from Cluster A (Fig. 4b). In addition, we found a sub-cluster grouping three individuals (A4, A6 and A5) inside cluster A, which are more separated from the rest of the cluster (Fig. 4b).

3.4. Variation in venom components between H. valida and H. infensa

Analysis of the venom fingerprints between specimens of the closely related *H. valida* and *H. infensa* species revealed that 26 venom components were shared between the species (Table 1). However, *H. valida* showed numerous species-specific compounds (50 venom components) that were not found in *H. infensa* and can be used as markers to characterise the species (e.g. 7069.219 Da at 15 min and 4175.818 Da at 36 min; Table 1). Similarly, *H. infensa* showed species-specific compounds (48 venom components) and specific markers characteristic of the species, such as 4795.449 Da (retention time 29.567–30.220 min) and 7120.841 Da (retention time 34.740–35.207 min). Similar to *H. valida*, the bimodal distribution in *H. infensa* showed that the majority of masses

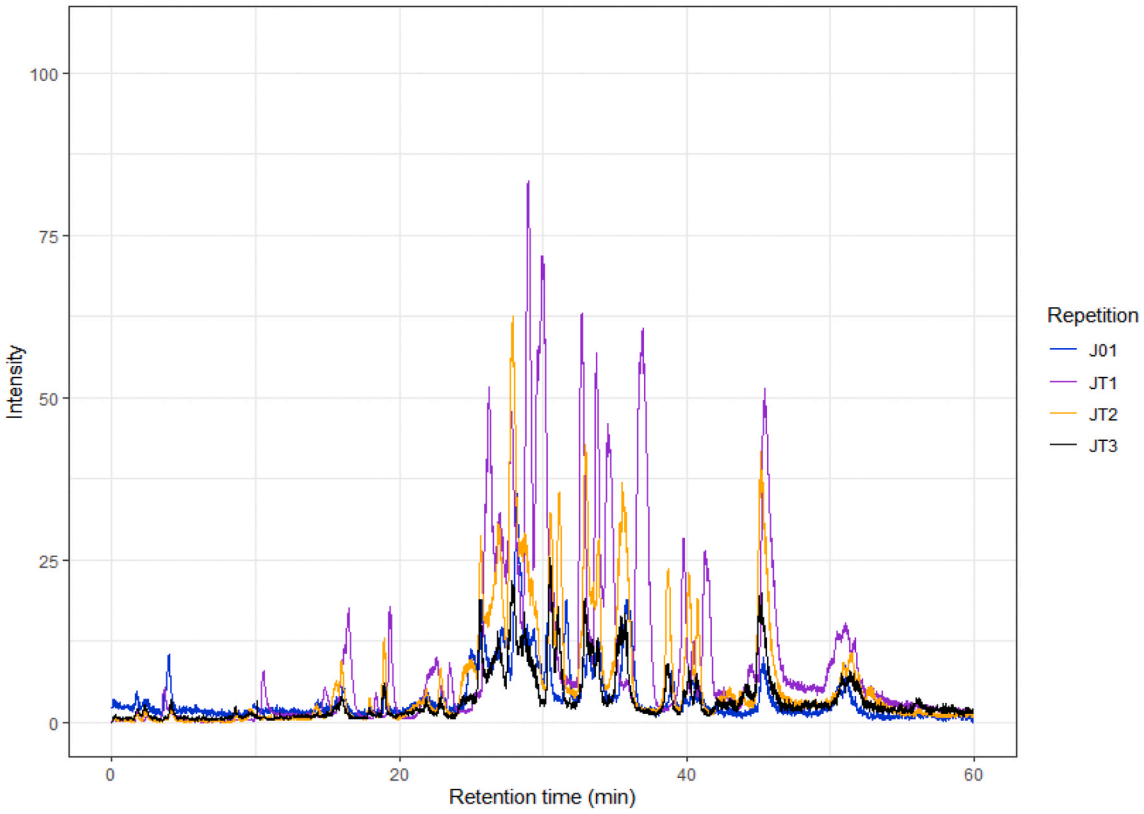


Fig. 1. Total ion chromatograms (TICs) of LC/ESI-MS analysis of venom sample repetitions over time from a female *H. valida* specimen (individual J). The chromatograms were visualised using the 'ggplot2' package (Wickham, 2009) in R version 4.0.1, using the normalised intensities and retention time from venom components obtained from the individual.

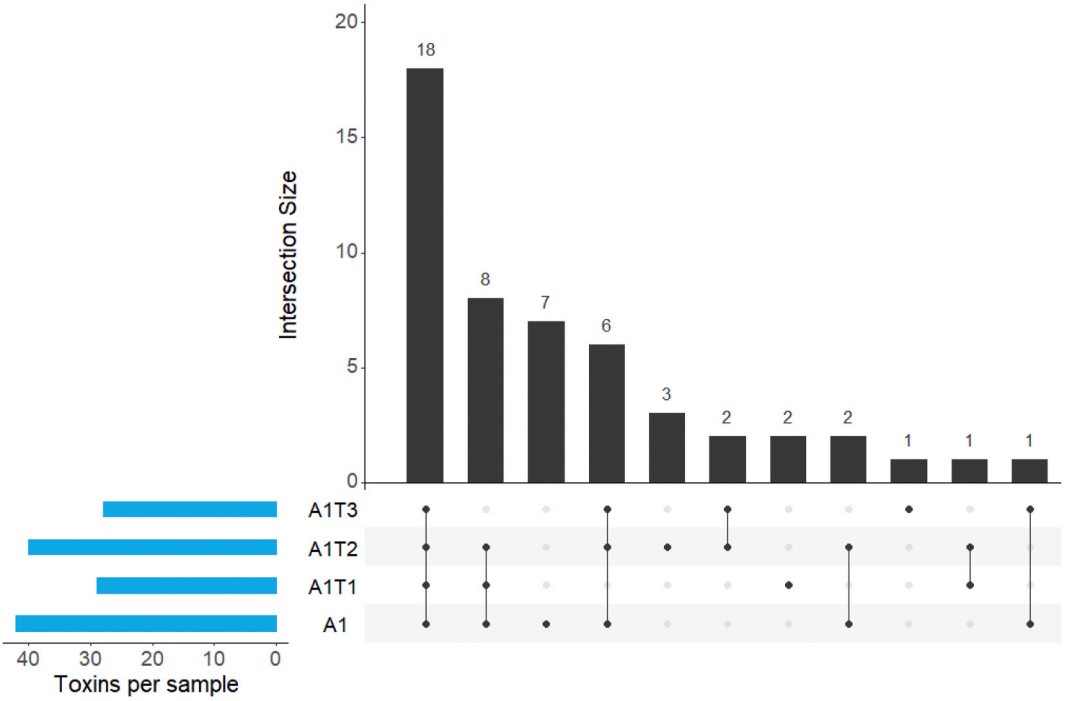
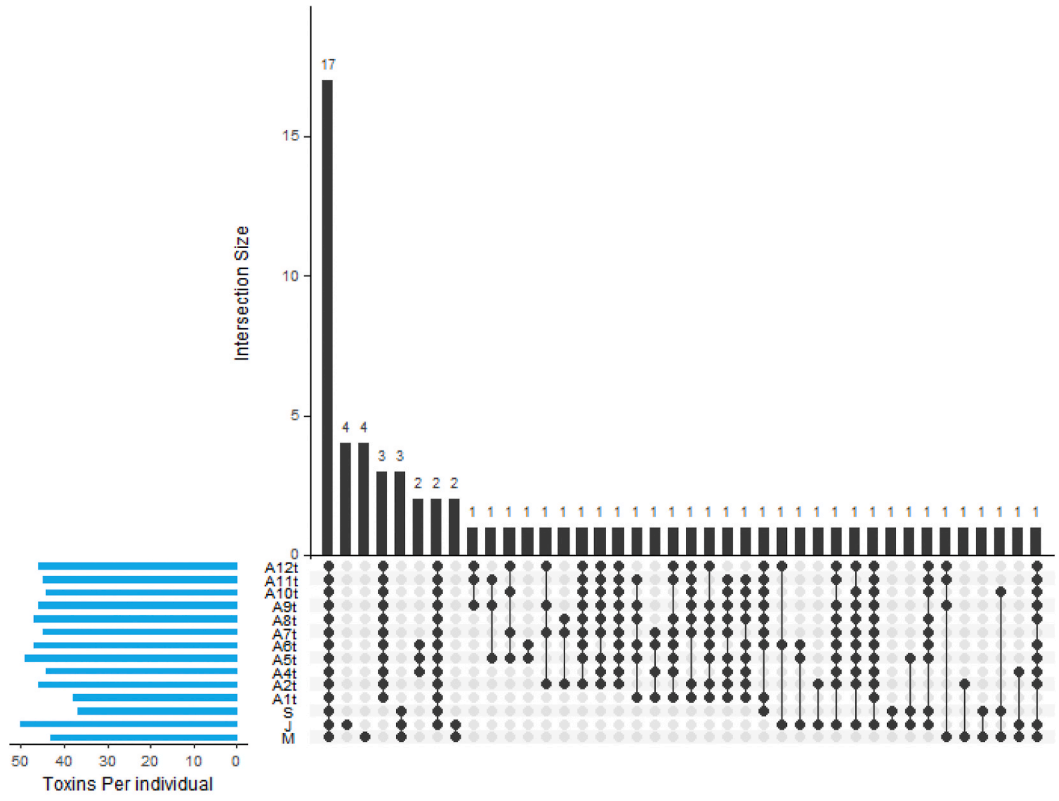


Fig. 2. Intra-individual variation in venom composition of a juvenile female *H. valida* (spider A1) over time. The Upsetplot shows the total number of uniquely individual venom components present in all replicates (bottom left: set size). A1 represents the baseline venom sample taken one week after the spider was collected. The order of the replicates is shown by the letter T (A1T1, A1T2, A1T3). The black dots show the venom components (intersections) shared between replicates (e.g. 18 peptides are present in all replicates).

a.



b.

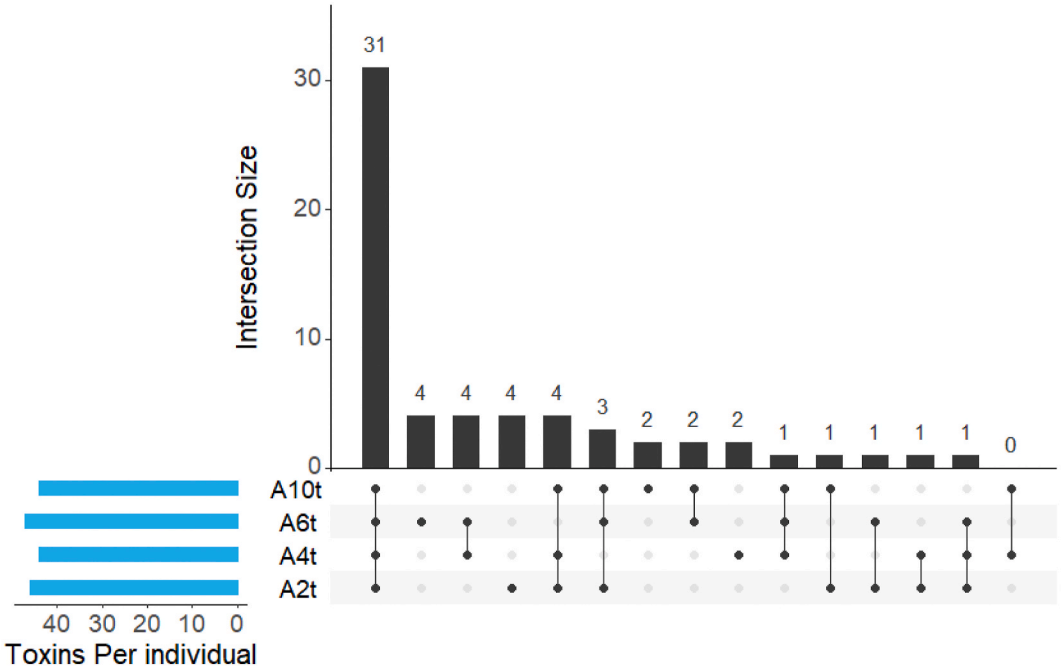


Fig. 3. (a) Upsetplot showing the number of toxins shared by all individual *H. valida*. The bars representing the intersection size show the number of venom components shared by the individuals highlighted by black dots in the matrix panel below. The bars in the panel to the left of the dot matrix panel show the total number of venom components per individual. (b) Upsetplots of juvenile and (c) adult specimen venom components.

c.

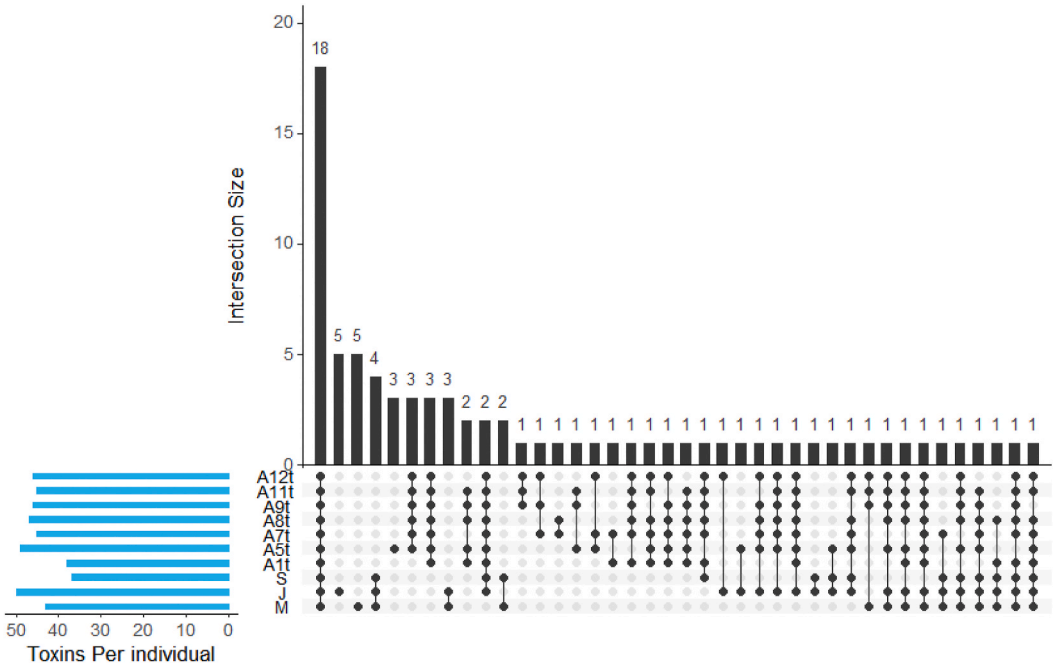


Fig. 3. (*continued*).

were found in the range of 3354.319–5216 Da, and a smaller group of masses in the range of 6764.1457–8420.2943 Da.

4. Discussion

4.1. Intra-individual variation

Through analysis of venom fingerprint profiles, we found considerable variation in venom components within *H. valida* individuals over time. Each individual spider showed the presence of unique components over time, some of which were present only once in one sample. Prey type and abiotic factors can affect variation in venom composition (Barlow et al., 2009; Casewell et al., 2013; Schendel et al., 2019). However, the individual *H. valida* specimens in this study still showed variation despite experiencing the same diet (house crickets) and housing under the same environmental conditions. To understand intra-individual variation, it is necessary to consider if multiple components in the venom are playing a particular role or have several functions in the individual (Casewell et al., 2013; Schendel et al., 2019). However, it is also necessary to consider the drivers of venom variation that cannot only be explained based on local diets (Schendel et al., 2019; Zancolli et al., 2019). For example, in the rattlesnake *Crotalus scutulatus*, neither diet nor genetic population structure explained intra-individual variation in venom composition, whereas both temperature and habitat conditions were the main drivers of variation in venom composition in this species (Zancolli et al., 2019).

Although the aggravation process used to milk spiders was identical for all individuals, changes in the behavioural responses associated with the aggravation process could trigger differences in the way that spiders respond to the threat stimulus, which in turn could lead to changes in venom composition (Nelsen et al., 2014). Variation in venom composition could be related to ecological function (Schendel et al., 2019). For example, Morgenstern (2013) reported unique peptide masses in different secretion series in *H. infensa*, suggesting that spiders can qualitatively and quantitatively modulate venom secretions for each stimulus they receive. In our study, spiders were most likely using defensive venom, which can have a higher complexity of components and greater variation in composition than offensive venom (Escoubas et al., 2006; Casewell et al., 2013; Schendel et al., 2019). In addition, the spiders could be showing a plastic response by varying venom properties after being exposed to a threatening stimulus (Nelsen et al., 2014), as occurs in the orb-web spider *Tetragnatha versicolor* (Zobel-Thropp et al., 2018) and Australian rainforest scorpion *Hormurus waigiensis* (previously *Liocheles waigiensis*) (Gangur et al., 2017). More studies testing intra-individual variation in venom composition including different factors (environment, predator/stimuli over time) are necessary to understand the underlying factors that lead to variation in venom composition.

4.2. Inter-individual variation

Juvenile and adult *H. valida* individuals had different venom compositions, and only shared a small number of venom components. This variation may be the result of the intra-individual variation observed but could also be complemented by other factors. For example, the variation in venom composition between developmental stages has been widely documented in spiders (Santana et al., 2017), scorpions (Fox, 2018), gastropods (Conoidea; Puillandre et al., 2017), and snakes (Andrade and Abe, 1999). Ontogenetic shifts in spider venoms can occur throughout a spider's development (e.g. tarantula venoms; Guette et al., 2006; Santana et al., 2017). Juveniles could have different predatory/prey interactions affected by different selection pressures that lead to variations in venom composition (Gibbs et al., 2011; Santana et al., 2017). The number of venom components in common between juveniles of *H. valida* were higher than in adults. However, juveniles did not form a specific cluster in the Jaccard similarity coefficient/matrix and KPCA. This lack of clustering may be a consequence of small sample size. However, it is also possible that venom could be continually changing in young individuals as they mature to adulthood, which would indicate that age is a factor affecting variation in venom composition, as has been observed in tarantulas of *Brachypelma* species (Escoubas et al., 2002) and *Phlogius crassipes* (Elias et al., 2006).

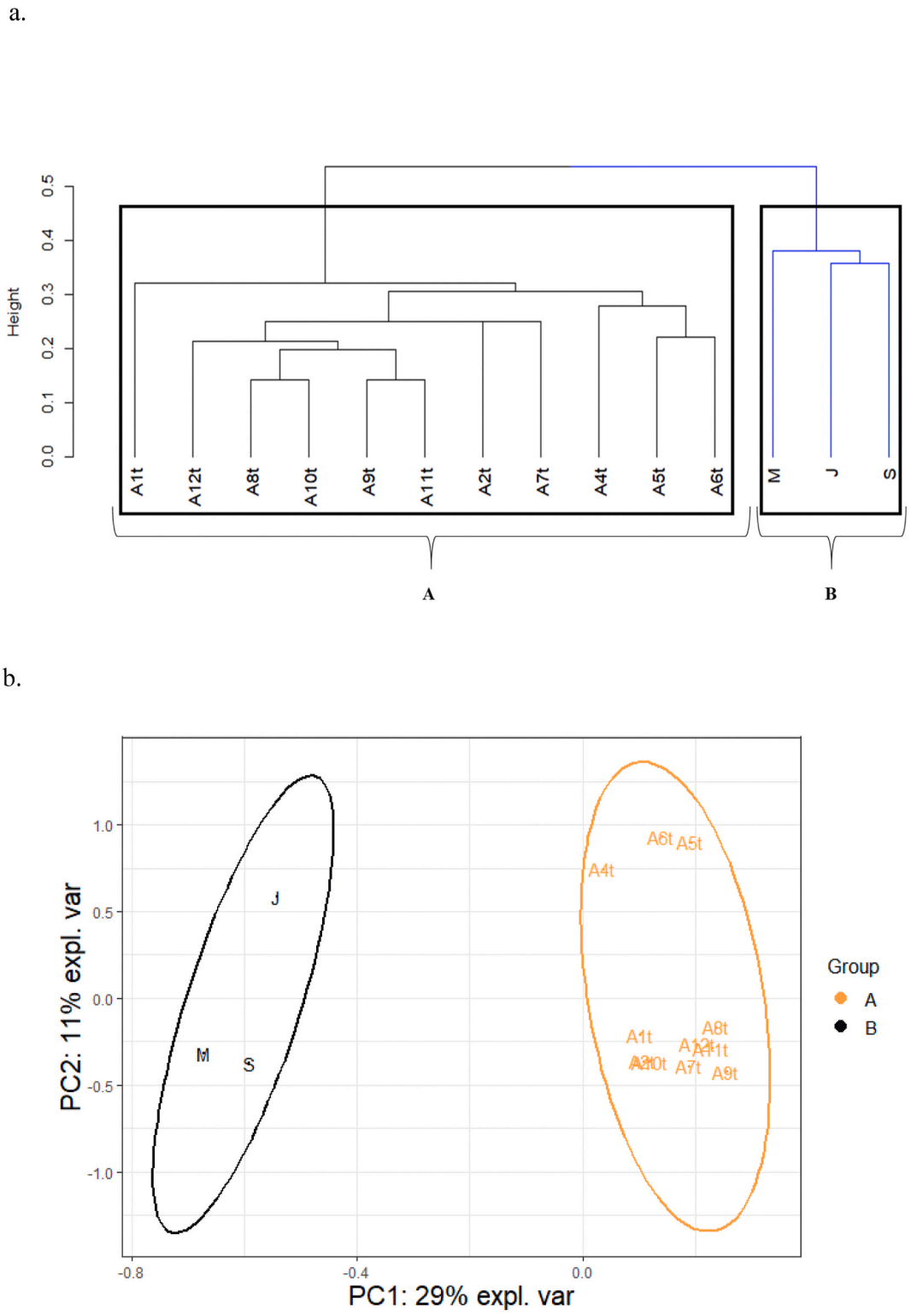


Fig. 4. (a) Hierarchical cluster of *H. valida* individuals based on the presence/absence of venom component masses (Da) using the Jaccard matrix and the average linkage method. Cluster A is clearly delineated from the Cluster B. (b) Projection of individuals of *H. valida* on the first two KPCA axes.

Although the juveniles and adults showed individual variation in venom components, most of the individuals were clustered together in the Jaccard similarity coefficient/matrix and KPCA, possibly due to geographic origin (Chippaux et al., 1991; Núñez et al., 2009; Touchard et al., 2015) or time of collection. Geographic origin could be a source of increased venom composition variation due to specific microhabitat differences and genetic diversity leading to intra-specific venom plasticity, as has been seen in the scorpion *Scorpio maurus palmatus* (Escoubas et al., 1997; Touchard et al., 2015). Unfortunately, as the *H. valida* spiders used here were collected by a commercial collector, we do not have specific locality information. The season of collection could also promote differences in venom components between individuals belonging to cluster A compared to the individuals belonging to cluster B, particularly as individuals from cluster A were collected in April while

Table 1
List of venom components of *H. valida* and the respective retention times, mass, sharing of toxins within individuals and with *H. infensa*, and the name of the component (if known). Masses indicated in red are the main peaks observed in the chromatogram.

Retention time (min)	*H. valida* (Mass Da)	Toxins shared between individuals (n = 17)	*H. infensa*	Toxins
2.273	294.0422	X	X	
3.713	437.8880	X	X	
3.907	346.9548	X	X	
3.907	307.4829	X	X	
5.267->5.600	362.9748			
8.613	266.9519	X	X	
8.613	277.9618			
13.713->14.633	3918.4483		X	
13.953->14.860	3863.3993			
14.773->15.127	4050.0898		X	
15.213->16.220	3992.5309		X	
15.367->16.068	4079.4200			
15.647->16.000	3921.1337	X	X	ω-hexatoxin-Hi1a (*H. infensa*)
16.027->16.173	4035.5376			
17.840->18.247	3950.1693			
18.967->19.280	4047.9459		X	ω-hexatoxin-Hi1b (*H. infensa*)
18.900->19.220	8095.2821		X	
20.400->22.133	4221.9554			
21.607->22.367	6968.5368			
21.920->22.132	7069.1648	X		
22.473->22.687	4249.3587			
22.753->23.153	6733.8906			
22.753->23.153	6764.1457	X	X	
24.327->25.033	4728.3849		X	
24.480->25.133	7066.5963			
24.840->25.387	4009.7675			
25.133	4702.8296			
25.347->25.747	4721.0204	X		
25.487->25.940	4809.7037		X	
25.507->25.720	7082.8107			
25.727->26.033	7101.7351			
25.893->26.747	4546.9051		X	
25.993->26.593	7049.4980			
26.167->26.447	8094.7175			
26.587->26.807	7575.5907	X		
26.587->26.808	8048.0931			
	8052.1272		X	

Table 1 (*continued*)

Retention time (min)	*H. valida* (Mass Da)	Toxins shared between individuals (n = 17)	*H. infensa*	Toxins
26.900->27.453				
27.393	4854.3118			δ-hexatoxin-Hva1a (*H. valida*)
27.400->27.653	4608.3235			
27.553->28.260	7545.1591			
27.707->28.713	4689.5631			
27.787	4699.2086			
28.013->28.207	4335.1019			
28.027->28.267	4840.0408			
28.107->29.520	8105.1082			
28.107->28.720	4659.8972	X		
28.62	1552.6191			
29.427->29.607	4011.0383			
29.467->30.527	4590.6332			
29.5	7478.6637			
29.953->30.233	7173.0626			
29.973->30.980	7461.1505			
30.3	7519.7387			
30.693->31.294	4792.7387			
30.693->31.295	7188.9628			
31.027->31.733	4107.9291			
31.667->31.840	4469.3004		X	
31.633->32.140	7516.3668			
32.240->32.893	7531.1108			
32.393	931.6511			
32.793->32.967	7238.0428			
32.800->32.020	4163.9178			
32.753->33.020	4090.1778			
33.233->33.407	7459.2294			
33.293->33.853	3992.1642		X	
33.133->33.460	7050.8973			
33.133->33.461	7512.8342			
33.307	7051.0478			
33.793->33.980	4391.6539			
34.027->34.287	7280.6407	X		
34.407->34.860	7339.1368			
36.02	4175.8185	X		
38.733->39.440	7183.3833			
38.733->39.340	8420.2943		X	
39.693->40.093	4397.0997			
	7197.532	X		

(*continued on next page*)

Table 1 (*continued*)

Retention time (min)	*H. valida* (Mass Da)	Toxins shared between individuals (n = 17)	*H. infensa*	Toxins
40.487->40.507				
40.500->41.153	7055.1345		X	
40.760->41.000	7165.2995		X	
40.853->41.307	7069.2197	X		
50.627	4009.2	X	X	ω-hexatoxin-Hi2a (*H. infensa*)
51.1	4023.4023	X	X	
50.367->50.793	4062.6339		X	

individuals from cluster B were collected in June. Differences in venom properties have been found in funnel-web spiders collected during different seasons. For example, in the funnel-web spider *Atrax sutherlandi*, specimens collected during winter showed a higher venom yield than those collected in autumn, although venom composition was not investigated (Keegan et al., 1960; Wong et al., 2016). However, venom yield and venom composition are not mutually exclusive and changes in both are possible (Morgenstern and King, 2013; Schendel et al., 2019).

4.3. Inter-specific variation in venom peptides

The complexity of venom in closely related species of funnel-webs such as *H. valida* and *H. infensa* has revealed a high degree of heterogeneity in venom components between species. Although similarities in the presence of different venom peptides exist between the two species (Table 1), both *H. valida* and *H. infensa* venom profiles can be easily distinguished by the variation of specific components.

Venoms from species belonging to the *infensa* species group, such as *H. valida* and *H. infensa*, may have venom components in common because of genetic and/or ecological factors (Palagi et al., 2013). However, each species also has unique venom components that vary in both composition and abundance, which could be related to venom adaptations specific to habitat and/or ecological function (Palagi et al., 2013; Cooper et al., 2015; Schendel et al., 2019). For example, Wilson and Alewood (2006) previously reported differences in venom components between similar species of the *infensa* species group collected from different geographical locations in South East Queensland. However, each species may have unique venom components simply due to genetic divergence over time. Without knowledge of the properties and function of each venom component, it is difficult to ascertain whether there is active selection driving differences between the species, or whether the differences are simply due to genetic drift. Nonetheless, our findings highlight the importance of venom fingerprint profiling for identification, which can be a useful tool for identifying and classifying closely related species. Similar findings have been observed in different species of *Brachypelma*, where common venom components are shared between closely related species, but each species retains venom components specific to that species (Escoubas et al., 1997).

While we observed substantial intra-individual, inter-individual and inter-specific variation in *H. valida* funnel-web venom composition, there is still sufficient consistency in the venom components present to identify specific character markers to use venom fingerprint profiles as chemotaxonomic tools. This level of variation, from the intra-individual to inter-specific levels, may also have medical implications in the production of antivenoms and efficacy in the treatment of envenomations. For example, the controversy surrounding the efficacy and use of antivenom to treat latrodectism caused by envenomation by widow spiders *Latrodectus* sp. (Isbister et al., 2014) may be a result of intra-individual or inter-specific variation in venom composition in the specimens sourced for antivenom production. More studies testing intra-individual variation in venom composition including different factors (environment, predator/stimuli over time) are necessary for understanding the underlying factors that could lead to variation in venom composition.

5. Conclusions

Different factors can trigger both intra- and inter-individual variation in venom composition in spiders, such as geographic origin, genetics, predator-prey interactions, behaviour and age. Our findings suggest that intra-individual variation in venom composition is likely a result of the way individuals respond to a particular stimulus over time, but more experiments including different predators and stimuli are necessary. Understanding inter- and intra-individual variation in venom composition in one species contributes to a broader understanding of the evolution and adaptation of venom in general. Venom fingerprint profiles can be used as chemotaxonomic markers to identify species, and possibly particular geographical populations, allowing the discrimination of species complexes such as in the genus *Hadronyche*.

Ethical statement

Spiders were observed daily and monitored weekly. Experimental procedures did not have any negative effects on the animals. Due to funnel web spiders are not a protected species in Australia, the Department of Environment and Science of Queensland Government advised that a scientific permit was not required. However, our research was conducted within the framework of the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Author contributions

Linda Hernandez Duran: Conceptualization, data collection, Methodology, visualization, writing-original draft preparation. Writing editing. Tasmin Rymer: Writing-Reviewing and editing, supervision. David Wilson: Methodology and data analysis, writing- Reviewing, editing, supervision. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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