

Exploring behavioral traits over different contexts in four species of Australian funnel-web spiders

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Handling editor: Claudio Carere

Abstract

Australian funnel-web spiders are arguably the most venomous spiders in the world, with much research focusing on this aspect of their biology. However, other aspects related to their life history, ecology and behaviour have been overlooked. For the first time, we assessed repeatability, namely risk-taking behaviour, aggressiveness and activity in the contexts of predation, conspecific tolerance and exploration of a new territory in four species of Australian funnel-web spiders: two are closely related, *Hadronyche valida* and *H. infensa*, and two have overlapping distributions but occupy different habitats, *H. cerberaea* and *Atrax robustus*. We also compared behaviors between species. At the species level, we found that *H. valida* showed consistency in risk-taking behavior when exposed to a predator stimulus, aggressiveness against conspecifics, and exploration of a new territory. In contrast, in the other species, only *A. robustus* showed repeatability in the context of exploration of a new territory. These results suggest that some behavioral traits are likely more flexible than others, and that the repeatability of behaviors may be species-specific in funnel-webs. When we compared species, we found differences in risk-taking behavior and defensiveness. This study provides novel insights to understanding variation in behavioral traits within and between species of funnel-web spiders, suggesting that some behavioral traits are likely context and/or species dependent, as a result of their evolutionary history. These findings provide key insights for understanding the ecological role of behavior and venom deployment in venomous animals, and a greater understanding of behavior in these medically significant and iconic spiders that are of conservation concern.

Key words: arachnids, behavior, ecological contexts, flexibility, mygalomorphae, repeatability.

Australian funnel-web spiders (Araneae: Mygalomorphae: Atracidae) are endemic species from eastern Australia (Gray 2010; Opatova et al. 2020). Funnel-webs are mostly forest ground-dwelling species, although some species do occur in other habitat types, such as montane herbland, open woodlands and closed forests (Gray 2010). Their microhabitat can vary from ground burrow retreats to logs, rocks, stumps, trees, and sand (Gray 2010). Funnel-webs are considered the most venomous spiders in the world because they can cause severe envenomation in humans, which is life-threatening (Isbister et al. 2005, 2015). More recently, funnel-web venom has also been a focus of research because individual components from different species possess therapeutic (Chassagnon et al. 2017; Ikonopoulou et al. 2018) and natural bioinsecticide properties (Windley et al. 2012; Herzig and King 2015).

While funnel-webs are iconic species, the majority of studies, including studies relating to their evolutionary history, have focused only on venom properties (Nicholson and Graudins 2002; Alewood et al. 2003; Herzig et al. 2020). Despite multiple studies on venom properties, the ecological function of venoms remains largely unexplored, and it is surprising that behavior and ecology have not been included in toxinology studies, particularly as both play a critical role in the evolution and diversification of venoms (Schendel et al. 2019; Hernández Duran et al. 2021). The study of behavior

is critical for understanding the complexity and variation of venom components because behavioral traits act synergistically with other intrinsic (genetics, physiology, neurohormones) and extrinsic factors (environment, experience, level of sociability) to affect the variation, abundance, and function of toxin molecules (Wullschleger and Nentwig 2002; Nelsen et al. 2014; Schendel et al. 2019; Hernández Duran et al. 2021). For example, in *Apis mellifera*, both ecological factors (temperature, plant flowering stage) and behavioral responses to a venom stimulating device affected the protein composition of bee venom in the species (Scaccabarozzi et al. 2021).

For funnel-web spider behavior in particular, only anecdotal or field collection observations have been registered previously (Levitt 1961; Gray 1981, 1987; 1992). This is surprising because behaviors associated with venom deployment, such as antipredator behavior (Dutertre et al. 2014; Nelsen et al. 2014; Arbuckle 2017; Hernández Duran et al. 2022), defensive and aggressive behaviors (Nekaris et al. 2020), courtship and mating (Arbuckle 2017), among others, affect fitness, as venomous animals depend on venom for the acquisition of resources and protection (Boevé et al. 1995; Nelsen et al. 2014; Cooper et al. 2015; Zobel-Thropp et al. 2018; Schendel et al. 2019; Herzig et al. 2020). Furthermore, funnel-webs are short-range endemic species, meaning they have a higher risk of extinction due to their restricted ability to disperse and

Received 19 June 2022; accepted 4 October 2022

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specific habitat conditions, which makes them vulnerable to habitat loss (i.e., fires, floodings, urbanisation; Mason et al. 2018). Therefore, given their potential conservation significance, it is surprising that ecological and behavioral studies have ignored these spiders in general.

Two particularly relevant avenues for behavioral studies in spiders are repeatability, or consistency, of behaviors and behavioral flexibility. Repeatability, or temporally consistent individual differences in behavior (Wolf and Weissing 2012), could explain how spiders use different resources, such as venom, to respond or adapt to different environmental conditions or threats (e.g., high predation risk or conspecific competition). Different spider behavioral types (defined as differences in behavioral traits among individuals), such as aggressive-docile (Gosling 2001), are likely the result of intrinsic (i.e., physiological traits such as venom, neurohormones, DiRienzo et al. 2015, metabolic rate, Shear and Pruitt 2014; silk production; Wullschlegel and Nentwig 2002) and extrinsic factors (i.e., environment and niche specialisation; Kralj-Fišer and Schneider, 2012; Kralj-Fišer et al. 2017) and) that work synergistically to affect spider survival (Chang et al. 2017; Hernández Duran et al. 2021). For example, in the grass spider *Agelenopsis aperta* (Agelenidae), females show different levels of aggression across different contexts (i.e., reproduction, prey capture, and conspecific interaction). More aggressive females are able to secure higher quality territories and capture more prey, but also suffer higher mortality (Riechert and Hedrick 1993). The balance of different behavioral types in a population could affect the ability of spiders to adapt to, and colonise, new environments.

Behavioral flexibility, which is compatible with repeatability, allows individuals to modulate their behavior across different contexts and conditions over time. While repeatability might be a consequence of strong selection pressure (i.e., changes in repeatability leading to higher fitness costs, Halpin and Johnson 2014), behavioral flexibility may occur when species are exposed to variable selection pressures (i.e., individuals can exhibit distinct flexibility levels for different behavioral traits; Sih, Bell, and Johnson 2004; Briffa et al. 2008; Kralj-Fišer and Schneider 2012) and). The constraints that limit behavioral flexibility include morphology, physiology, and environmental conditions (Briffa et al. 2008). For example, in the black widow spider *Latrodectus hesperus* (Theridiidae), plasticity and repeatability are both present when a specific context is assessed, and both are part of an individual's collective behaviors (Halpin and Johnson 2014). This suggests that the strength of plasticity and consistency may be the result of a trade-off between costs and benefits to cope with different conditions (Briffa et al. 2008). In spiders, both behavioral plasticity and consistency have implications for the use of resources, limiting or increasing the ability to colonise new environments (Kralj-Fišer and Schneider, 2012; Kralj-Fišer et al. 2017) and), as well as using or constraining the use of their biological weapons (Nelsen et al. 2014).

Given that different species of Australian funnel-web spiders can be found along a continuous gradient of habitats, we explored different behavioral characteristics in females of four different species (*Hadronyche infensa*, *H. valida*, *H. cerberaea*, and *Atrax robustus*—all species belong to the Atracidae family that includes the *Atrax* and *Hadronyche* genera; Hedin et al. 2018) across different ecological contexts (response to predation, conspecific tolerance, and exploration of a new territory) to assess the consistency

(i.e., repeatability) of spider behaviors over time. In addition, we compared behaviors across species, firstly because of the general public assumption that all funnel-webs behave the same (i.e., aggressively), and secondly because their ecologies suggest differing selection pressures that could affect their behaviors, venom composition and, ultimately, overall fitness. Each species occupies a different microhabitat, although some have overlapping distributions. *H. infensa* and *H. valida* belong to the *infensa* species group (Gray 2010). Both species have overlapping distributions (highlands ground-dwelling), show similar morphological characteristics, and share similar venom components, although each also has distinctive venom molecules (Hernández Duran et al. 2020). *H. cerberaea* belongs to the *cerberaea* group, and is a tree-dwelling species. In contrast to the other species of *Hadronyche*, *H. cerberaea* shows distinct morphological characteristics (Gray 2010) and venom profiles. Finally, *A. robustus*, most famously known for the lethal neurotoxin (δ -hexatoxin-Ar1a) found in the venom of mature males (Nicholson et al. 1996; Nicholson and Graudins 2002; Alewood et al. 2003; Klint et al. 2012), shares an overlapping distribution with *H. cerberaea* in the Sydney and Central Coast regions of New South Wales (Gray 2010). However, unlike *H. cerberaea*, *A. robustus* is a ground-dwelling spider, similar to *H. valida* and *H. infensa*.

We predicted that the closely related species, *H. infensa* and *H. valida*, would show similar and consistent behavioral responses across all different contexts due to both species sharing similar habitats and remaining confined in their ground burrows. For *H. cerberaea*, given the characteristics of its arboreal habitat and exposure to predators, such as birds, we expected more flexible risk-taking behaviors and aggressiveness towards predator stimuli and conspecifics. However, we predicted consistency in activity due to confinement in their tree burrows. Finally, because *A. robustus* are regularly found in urban and sub-urban environments (Gray 1992), we expected more flexible behavior in response to predator stimuli due to their habitat being more open and exposed to predators and other threats. However, we predicted consistency in aggressiveness against conspecifics and in activity because, as for the other species, *A. robustus* females are confined to a burrow. When comparing between species, we expected that if *A. robustus* is indeed more defensive than other species (based on anecdotal evidence), then *A. robustus* would show higher frequencies of fang movements (defensive behavior), higher frequencies of climbing, resume their movements faster and be more active compared to *Hadronyche* species.

Materials and Methods

Study species and locations

A total of 75 spiders were used in this study. Females are ground burrow-dwellers (*H. valida*, *H. infensa* and *A. robustus*) or tree-dwellers *H. cerberaea*. Burrow-dwellers can dig burrows of more than 1 m deep (pers. obs.). Therefore, it is difficult to know whether the spider being collected is an adult or juvenile prior to collection. Digging up an individual spider can take hours, so we collected any spiders we could find, regardless of size or stage, which led to unbalanced sample sizes. Twenty-three adult female *H. valida* (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). Sixteen adult female *H. infensa* were collected manually in Toowoomba and Ravensbourne by the authors in 2019 (collection permit SA 2016/08/55). Eighteen *H. cerbera* (nine adult females; nine juveniles) were collected across three *Eucalyptus regnans* in Gosford, New South Wales by the authors in 2019. Eighteen *A. robustus* (13 adult females; five juveniles) were collected by the authors from the Gosford/Central Coast region, New South Wales (collection carried out in cooperation with the Australian Reptile Park). The spiders were transported alive in plastic containers with damp cotton wool to the laboratory of the Australian Institute of Tropical Health and Medicine (AITHM), James Cook University Nguma-Bada (Cairns) campus, Queensland, Australia.

Spiders were kept individually in 5 L plastic containers with a 7-cm layer of coconut peat (L: 25 cm; W: 17.5 cm; H: 10 cm) 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.). Funnel-web spiders are solitary in nature, thus solitary housing is reflective of their natural social organization. The spiders were acclimated for 1 month before the start of behavioral assays (see below). Each spider received one house cricket *Acheta domestica* once a week. Compared to Araneomorph spiders, funnel-web spiders take a long time to mature and have a long life span. For example, males reach maturity between 2 and 4 years, while females can live for more than 10 years (Levitt 1961; Gray 1992). We did not include adult males in the study because once they reach maturity, they leave the burrow and wander in search of females (Bradley 1993). Furthermore, males have a significantly shorter life span than females, particularly in captivity, making it difficult to obtain repeated behavioral measurements over extended periods. Adult females were identified by epigyne sclerotization and the opening of the epigastric furrow (gonoslit, Zhan et al. 2019). Cephalothorax width was measured to assess spider size. It is impossible to tell males and females apart prior to the final molt to maturity.

Behavioral tests

Following the 1-month acclimation period, the spiders were starved for 2 weeks before behavioral assays commenced, and the same procedure was repeated before each bout of testing. We starved spiders to control for venom state and to ensure all individuals were at the same motivational state prior to behavioral tests. We assessed aggressiveness, risk-taking behavior and activity of each individual of each species in the context of predation (two separate tests), conspecific tolerance and exploration of a new territory (see below). Individuals were tested separately and tests were conducted on consecutive days (spiders were rested for 24 h between tests). The order of tests was randomized for each spider, with the exception of the prod test (see below), which always came first due to collection of venom samples (which we did not include here). Behavioral tests were repeated three times per individual, separated by 1 month between testing sessions, to measure repeatability. All assays were carried out between 6 a.m. and 12 p.m. during the dark phase, and were video recorded under red light. Behaviors were analyzed using the behavioral analysis software BORIS version 7.8.2. (Friard and Gamba 2016).

Predation context

The willingness of a spider to take risks was assessed using an aversive stimulus (a puff of air), which represents an air current that resembles the wing beat movements of a predator (e.g., a bird) or a parasite (e.g., a wasp; Riechert and Hedrick 1993; Keiser, Lichtenstein, Wright et al. 2018and). Following the methodology described in Hernández Duran et al. (2022), we gently moved the spider out of its retreat, and waited 60 s before commencing the test. Then, each spider received three rapid puffs of air to the anterior prosoma using a camera air blower. Thereafter, we recorded the spider's huddling behavior (a standard antipredator posture; Riechert and Hedrick 1993; DiRienzo et al. 2015; Parthasarathy et al. 2022) for 420 s, following the modified protocol of Riechert and Hedrick (1993) and Shearer and Pruitt (2014). Huddling was recorded as a binary response because the distribution of the data suggested a binomial distribution. Spiders that did not resume movement within 15 s of receiving the stimulus (i.e., that remained huddled) were assigned 0 (designated low risk-taking individual), while those that moved within 15 s were assigned 1 (designated high risk-taking individual).

Aggression against conspecifics and heterospecifics is common in spiders (Keiser et al. 2018). As for the puff test, we gently moved the spider out of its retreat, and waited 60 s before commencing the test. Following the protocols outlined by Hernández Duran et al. (2020) and Hernández Duran et al. (2022), we gently prodded the first pair of legs with blunt tweezers for 240 s, and then recorded each individual's behavior for a further 360 s (600 s in total). Australian funnel-webs respond to threatening stimuli by raising the forelegs, moving the fangs and expelling venom directly on the fangs (Wilson and Alewood 2004, and 2006). We collected expelled venom from aggravated individuals during the first 240 s using a 200- μ L Gilson P200 pipette with polypropylene micropipette tips (Hernández Duran et al. 2020). We did not collect venom after the second 360 s. As there was a strong positive correlation between lifting the first pair of legs and fang movements, and a negative correlation between huddling and fang movements in *A. robustus* (Hernández Duran et al. 2022), we used the number of fang movements as a measure of the aggressive response in all species. Spiders that showed a greater number of fang movements were considered aggressive, while spiders that showed fewer fang movements were considered submissive.

Conspecific tolerance

Aggressiveness and boldness can affect spider survival and fitness during foraging and mating (Riechert and Hedrick 1990). In funnel-webs, female retreats are often located close to each other (Gray 1992), indicating that burrow defence, mate choice, and food competition are likely occurring between females. To assess conspecific tolerance, following the methodology of Hernández Duran et al. (2022), we placed two individuals in a new container (L: 23 cm; W: 16.5 cm; H: 10.5), divided into two equal halves with a mesh barrier. This reduced the risk of fighting and death, but permitted detection of olfactory and mechanical cues. We randomly selected pairs of similar-sized spiders and video recorded the whole arena for 1800 s. We measured aggressiveness against conspecifics as the number of times the spider climbed the barrier. Aggressive behaviors (e.g., leg waving and lunging) were observed when spiders climbed the barrier, but not when

moving around the arena, indicating that climbing behavior occurs as a direct response to other individuals rather than an attempt to escape.

Exploration of a new territory

Spiders can disperse or relocate burrows to reduce predation risk and/or increase the chance of prey capture (Nakata and Ushimaru 2013; Bengston et al. 2014) and). To assess exploration of a new territory (fear of novel objects or environment, Réale et al. 2007; Bengston et al. 2014), we assessed the activity level of individuals in a new arena (L: 34.5 cm; W: 22 cm; H: 10.7 cm) by measuring the total time the spider spent moving around the novel arena for 900 s (Hernández Duran et al. 2022).

Statistical analyses

All statistical analyses were conducted using R (version 4.1.0, Core R Team 2021). We had both adult females and juveniles for only two species, *H. cerbera* and *A. robustus*. Following Hernández Duran et al. (2022), we tested for stage effects in these two species on the magnitude of individual behavioral measurements (risk-taking behavior and aggressiveness) between adults and juveniles in each context and over time using rank-based non-parametric analyses for longitudinal data (Supplementary Table S1). These analyses offer a robust framework for non-continuous variables, small sample sizes and skewed data (Noguchi et al. 2012). The design used was F1-LD-F1 in the nparLD package (Noguchi et al. 2012), where the first F1 refers to the number of factors in each group (juveniles and adults; whole-plot factor group) of each species. LD specifies the nature of the data (i.e., longitudinal), and the second F1 refers to the time level (sub-plot factor). We included the random effect of individual identity as a subject in this model. We did not observe differences between adults and juveniles for either *H. cerbera* or *A. robustus* for behavioral measurements and contexts (Supplementary Table S1). Therefore, we pooled juvenile and adult data for these two species to calculate repeatability within species and then to compare differences between species.

To assess repeatability in risk-taking behavior and aggressiveness across different contexts (Nakagawa and Schielzeth 2010; Stoffel et al. 2017; Dingemans and Wright 2020; and Schielzeth et al. 2020), we used separate generalised linear-mixed effect models (GLMM) for each species separately using the package glmmADMB (Stoffel et al. 2017). For risk-taking behavior (puff of air), given the distribution of the data, we used a binomial distribution with a logit-link function (1 = moved, 0 = huddled). For aggressiveness in the context of predation (frequency of fang movements) and conspecific tolerance (frequency of climbs), we used a negative binomial distribution with a log-link function. For activity in a novel field, we used a gamma distribution (log-link function) for *H. infensa*, *H. valida* and *A. robustus*. However, we could not calculate activity for *H. cerbera* because only three out of 18 individuals (17%) moved. There is individual variation in the size at which individuals molt to maturity, so we included size as a continuous predictor of stage, in preference to a categorical predictor of stage (Santana et al. 2017; Padilla et al. 2018). Therefore, in all models, the behavior (huddling, frequency of fang movements, frequency of climbs and activity) was the response variable, size was a continuous fixed effect, and spider

identity (ID) was included as a random effect. We report effect sizes for all tests (due to relatively small sample sizes).

For each species, we determined the proportion of variance explained by the fixed effects (marginal R^2), the proportion of variance explained by both the fixed and random effects (conditional R^2), and the intra-class correlation (ICC) without (see Supplementary material Table S2a; ICC: Null-model) and with fixed effects (see Supplementary material Table S2a; ICC: Full-model) following Nakagawa and Schielzeth (2013) and Stoffel et al. (2017). For models with negative binomial and gamma distributions, we used the trigamma function to calculate the marginal and conditional R^2 , as well as the ICC (Nakagawa et al. 2017). We tested the significant among-individual variation using likelihood ratio tests (LRT), comparing the model with the individual random effect to a model without it (Pinheiro and Bates 2000; Stoffel et al. 2019). To obtain the observation level variance in the negative binomial and gamma models, we used the trigamma function, whereas for the binomial model, we report the latent and link distribution specific variance (Nakagawa et al. 2017).

To compare changes in behavior between species, we used separate generalized linear-mixed effect models (GLMM) for each context. We used the same variable response (behavior), and random effect (ID) as for previous models, but we included species, size, and repetition, as fixed effects. In the activity context, we excluded *H. cerbera* from the species comparisons. To compare repeatability of behaviors between species, we calculated the mean standardization of each model to determine the magnitude of variation (VI; Doehrmann and Royauté 2019).

Results

Within species behavioral variation

Risk-taking behavior in a predatory context

Hadronyche valida showed consistent individual differences in risk taking behavior over time when individuals received a puff of air ($\%R_{\text{null,model}} = 25.360$; $P = 0.001$; Supplementary Table S2a). In contrast, we did not observe repeatability for *H. cerbera* ($\%R_{\text{null,model}} = 0.005$; $P = 0.987$; Supplementary Table S2a), *H. infensa* ($\%R_{\text{null,model}} = 0.000$; $P = 0.996$; Supplementary Table S2a), or *A. robustus* ($\%R_{\text{null,model}} = 1.000$; $P = 0.952$; Supplementary Table S2a). Size did not have a significant effect on risk taking behavior in any of the four species (*H. valida*: $Z = 1.27$, $SD = 1.11$, $P = 0.200$; *H. cerbera*: $Z = 1.26$, $SD = 0.01$, $P = 0.209$; *H. infensa*: $Z = 0.68$, $SD = 0.00$, $P = 0.500$; *A. robustus*: $Z = 0.27$, $SD = 0.30$, $P = 0.780$).

Aggressiveness in a predatory context

None of the four species (*H. valida*: $\%R_{\text{null,model}} = 0.000$, $P = 0.999$; *H. cerbera*: $\%R_{\text{null,model}} = 26.700$, $P = 0.068$; *H. infensa*: $\%R_{\text{null,model}} = 0.000$, $P = 0.985$; *A. robustus*: $\%R_{\text{null,model}} = 0.000$, $P = 0.987$, Supplementary Table S2a) showed consistent individual variation in aggression (frequency of fang movements) over time. Similarly, size did not have a significant effect on aggression in any of the three species (*H. valida*: $Z = 0.03$, $SD = 0.00$, $P = 0.980$; *H. cerbera*: $Z = -0.01$, $SD = 0.04$, $P = 0.990$; *H. infensa*: $Z = 0.13$, $SD = 0.00$, $P = 0.901$), with the exception of *A. robustus*, which showed a marginal significance ($Z = 2.00$, $SD = 0.04$, $P = 0.05$).

Aggressiveness in the context of conspecific tolerance

For aggressiveness measured in the context of conspecific tolerance, *H. valida* showed consistent individual variation in aggression ($\%R_{\text{null.model}} = 22.482$; $P \leq 0.001$; Supplementary Table S2a). However, neither *H. cerberaea* ($\%R_{\text{null.model}} = 7.101$; $P = 0.201$; Supplementary Table S2a), *H. infensa* ($\%R_{\text{null.model}} = 0.000$; $P = 0.992$; Supplementary Table S2a) nor *A. robustus* ($\%R_{\text{null.model}} = 3.280$; $P \geq 0.203$; Supplementary Table S2a) showed repeatability in aggression. In addition, size did not have a significant effect on aggressiveness in the context of conspecific tolerance in any species (*H. valida*: $Z = -1.22$, $SD = 1.09$, $P = 0.220$; *H. cerberaea*: $Z = -0.59$, $SD = 0.56$, $P = 0.560$; *H. infensa*: $Z = -0.04$, $SD = 0.00$, $P = 0.970$; *A. robustus*: $Z = -0.41$, $SD = 0.55$, $P = 0.160$).

Activity in the context of exploration of a new territory

Hadronyche valida ($\%R_{\text{null.model}} = 37.401$; $P \leq 0.001$; Table S2a) and *A. robustus* ($\%R_{\text{null.model}} = 40.489$; $P < 0.001$; Table S2a) showed repeatability for activity over time. Both species showed close average values in activity (*H. valida*: $\bar{x} = 131.507$; *A. robustus*: $\bar{x} = 91.857$), and similar repeatability values and magnitudes of among-individual variance relative to their means (*H. valida*: $I_1 = 31.649$; *A. robustus*: $I_1 = 34.129$). For *H. infensa*, we did not observe repeatability in activity ($\%R_{\text{null.model}} = 0.000$; $P = 0.068$; Supplementary Table S2a). There was no significant effect of size on activity in the context of exploration of a new territory in the three species (*H. valida*: $Z = 0.05$, $SD = 1.90$, $P = 0.960$; *H. infensa*: $Z = 0.31$, $SD = 0.01$, $P = 0.750$; *A. robustus*: $Z = 0.66$, $SD = 1.80$, $P = 0.510$).

Between species behavioral variation

Risk taking behavior in a predatory context

When we compared huddling behavior between species, we found significant differences between all species (Supplementary Table S2b, Figure 1). In general, *A. robustus* resumed their movements faster in response to a puff of air (antipredator stimulus), followed by *H. cerberaea*, *H. valida*, and *H. infensa* (Supplementary Table S2b, Figure 1). Repetition and size did not have a significant effect on huddling behavior (Supplementary Table S2b).

Defensive behavior in a predatory context

For frequency of fang movements in a predatory context, we found a significant difference between repetitions (Supplementary Table S2b), with an overall increase in the frequency of fang movements noted from T1 to T3, irrespective of spider species or size (Figure 2). No other factors had a significant effect on aggressiveness (Supplementary Table S2b).

Defensive behavior in the context of conspecific tolerance

For frequency of climbing in conspecific tolerance between species, we found no significant differences between species. Similarly, repetition and size did not have a significant effect on the frequency of climbing (Supplementary Table S2b).

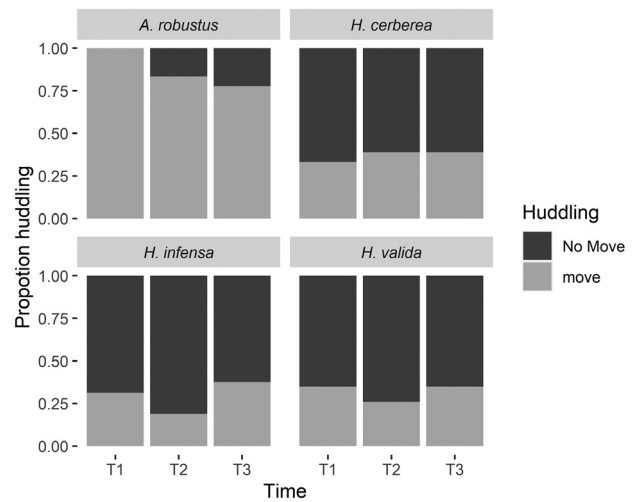


Figure 1. Proportion of individuals huddling when a puff of air was applied to the prosoma for each of four Australian funnel-web spider species over three time periods (T1: repetition one, T2: repetition two; T3: repetition three). The dark grey bars represent the proportion of spiders that moved, while the light grey bars represent the proportion of spiders that did not move.

Activity in the context of exploration of a new territory

For time spent moving around the arena, we found no significant differences between species, repetitions or size classes (Supplementary Table S2b).

Discussion

Different ecological contexts (i.e., foraging, mating, and exploration) affect the ways in which animals respond to their environments, either adjusting their behavior or maintaining consistent behavior over time (i.e., repeatability, Chang et al. 2019). Our study contributes to a broader understanding of intra- and inter-individual variation in Australian funnel-web spiders, which provides insights into the ability of individuals to respond to different environmental conditions, and potentially to use their biological weapons, namely silk and venom (Hernández Duran et al. 2021). Here, we assessed the repeatability of aggressiveness and risk-taking behavior in different contexts in four species of funnel-web spider over time.

We found that *H. valida* showed repeatability in risk-taking behavior (when individuals were exposed to an antipredator stimulus), aggressiveness (when individuals were exposed to conspecifics), and activity (when individuals were placed in a new territory). These findings suggest that development, and previous experience (Liedtke et al. 2015) in a particular environment (i.e., with predators, conspecifics) or in different contexts (Hernández Duran et al. 2021, 2022) may favor the maintenance of these behaviors over time in this species. In contrast, when we assessed temporal repeatability in *H. infensa*, *H. cerberaea*, and *A. robustus* across different contexts, only *A. robustus* showed repeatability in activity in the context of exploration of a new territory. The lack of repeatability in aggressiveness and risk-taking behavior suggests that these three species are not likely constrained in their behavioral responses, rather responding dynamically to different situations (Köhler and Vollrath 1995). Furthermore, these results also suggest species-specific responses, which is consistent

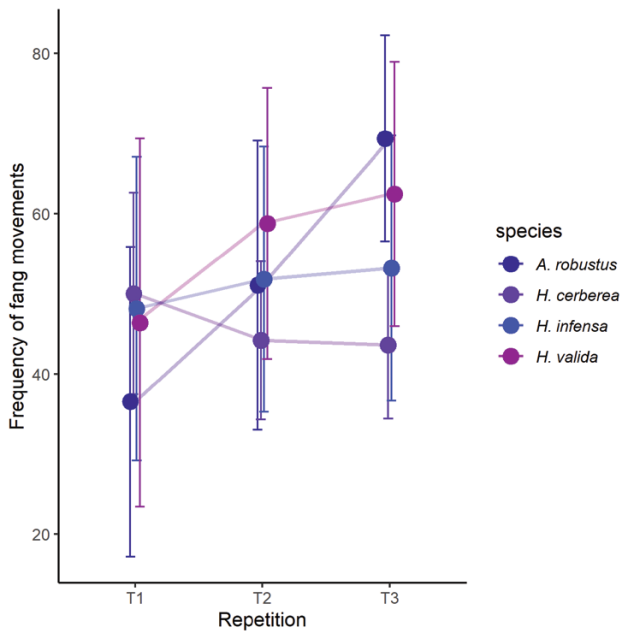


Figure 2. Frequency of fang movements (defensive behavior) for each of four Australian funnel-web spider species in response to a prod stimulus over three time periods (T1: repetition one, T2: repetition two; T3: repetition three).

with findings reported in other taxa (e.g., corvids, Vernouillet and Kelly 2020).

Species often show differences in behavioral responses (Bengston et al. 2014; Vernouillet and Kelly 2020), which could be a consequence of differences in the ecology and habitat complexity of each species (Bell 2007). For example, *H. cerberea* is arboreal, whereas the other three species are ground-dwelling (Gray 2010). The tree crevices where *H. cerberea* live may reduce or constrain the movement of individuals, which could explain why they barely moved when placed into an open environment. Interestingly, while we found repeatability for behavior in *H. valida*, we did not find the same response for *H. infensa*, even though these species belong to the same species group and experience similar broad ecological conditions. These results suggest that previous experience with the specific microhabitats in which these spiders occurred prior to collection may affect individual behavioral responses for extended periods, even when the spiders were kept under constant laboratory conditions for a period of time. However, the lack of repeatability in *H. infensa* might be affected by sample size (as suggested by the marginal value, see results).

Differences in behavior between life stages may be a direct consequence of changes in internal developmental processes (Bengston and Jandt, 2014). However, other extrinsic factors, such as experience and/or microhabitat conditions may also feedback to affect intrinsic physiological processes during development, which could explain why spiders of different ages respond behaviorally to different stimuli. We assessed behavioral differences between stages in *H. cerberea* and *A. robustus*, using size as a proxy of stage. We did not find differences in risk-taking behavior and aggressiveness in any context. The results are consistent with those observed for nymphs and adults of the cockroach, *Diploptera punctata*, where behaviors remain consistent across life stages (Stanley et al. 2017). The lack of difference in behavioral response

between stages may be a result of both stages sharing similar environments and foraging strategies (Stanley et al. 2017). However, these similarities in behavior between juveniles and adults in *H. cerberea* and *A. robustus* may only be temporarily stable during particular parts of the life stage (Parthasarathy et al. 2019). For example, juveniles (third and fourth instar) of the social spider, *Stegodyphus sarasinorum*, showed lower repeatability values in boldness and aggressiveness compared to subadults (Parthasarathy et al. 2019). Additionally, repeatability for both juveniles and adults tended to decline over longer periods of time (Parthasarathy et al. 2019). To determine whether or not funnel-webs show consistency during particular stages will require further research, which will take several years, given their long lifespans and relatively long periods required to reach maturity (Levitt, 1961; Gray, 1992).

In dangerous situations, such as under high predation risk, an animal that adjusts its behavior in response to the stimulus (Chang et al. 2019) may have a greater chance of survival. We did not find repeatability in aggressiveness in any of the funnel-web species in response to a direct predation threat (i.e., physical prodding, Jackson and Pollard 1990; Stankowich 2009), suggesting that spiders dynamically adjust or modulate behaviors in response to this type of stimulus. Funnel-web spiders showed increasing fang movements over time, indicating an increasing aggressive response over time. This flexibility in behavior is consistent with responses to the prod test in other species of spiders. For example, under laboratory conditions, the black widow spider *Lactrodectus hesperus* modulates its defensive behavior using venom and dry bites depending on the level of threat to which it is exposed (Nelsen et al. 2014). Different types of stimuli may also affect the ways in which spiders respond to a threatening situation. For example, in daddy long-legs spiders *Pholcus phalangioides* individuals reacted differently to a mechanical stimulus (i.e., whirled for longer periods) compared to a puff of air (Jackson and Pollard, 1990). In other arachnids, the behavioral response is also affected by the type of stimulus; in the southern unstriped scorpion, *Vaejovis carolinianus*, males and females perceived prods to the prosoma as the most threatening, and increased their stinging response as the threat persisted (Nelsen et al. 2020).

For assessment of variability in behavioral responses between species, we found species differences in huddling behavior. The proportion of time spent huddling by *A. robustus* was lower than in the other species of funnel-webs. Although funnel-web spiders are generally well known for their defensive behaviors, *A. robustus* typically performs obvious gaping displays, waving the first pair of legs, moving the fangs and expelling venom, even when the stimulus varies (Jackson and Pollard, 1990; Duran Hernández et al. 2022). These highly visual behaviors are possibly a consequence of the human-altered habitat in which *A. robustus* typically occurs (Gray 1992). Urban populations of different species often show differences in behavior to their rural counterparts. For example, individuals from urban populations of *Larinoidea sclopetarius*, show reduced fear of novel stimuli compared to individuals from rural populations (Kralj-Fišer et al. 2017). Faster resumption of movements could affect spider fitness, where higher risk-taking behavior could help spiders obtain more resources or deter predators, although this might also have increased costs associated with a higher risk of predation (Riechert and Hedrick 1993). *Hadronyche valida* and *H. infensa* showed a similar proportion of huddling,

most likely because both species inhabit similar environments and have overlapping distributions (Gray 2010), although risk-taking behavior was only repeatable in *H. valida*. For *H. cerberea*, the huddling response is likely affected by their arboreal life style (i.e., living in tree crevices). While birds may be common predators in trees, bark provides a strong fortress in which the spider can hide and defend itself. Conversely, the level of exposure to other predators and threats is likely to be higher for terrestrial species, particularly in fragmented patches (Gray 1992), where invasive predators may also pose a threat.

For frequency of fang movements in response to the puff of air across species, we observed differences over repetition, but not between species. The largest variation in aggression was observed in repetition three (T3), where *H. valida*, *H. infensa*, and *A. robustus* showed heightened defensive behavior, whereas the opposite was observed in *H. cerberea*. This could indicate that *H. cerberea* was capable of remembering the puff of air stimulus and had learned that the stimulus did not represent a threat. However, this requires additional testing. The levels of defensive behavior between species indicate that defensiveness (frequency of fang movements) is quite flexible, and can vary depending on the context and level of threat (Nelsen et al. 2014, 2020; Duran Hernández et al. 2022). While it has been suggested that solitary species, such as funnel-webs, may be more defensive than social species, this is not a general rule and other factors may constrain or trigger the level of aggression (Nelsen et al. 2014; Kralj-Fišer et al. 2017). While studying the behavior of these spiders in their natural habitats poses significant challenges (e.g., females are unlikely to leave their burrows if the burrows are built in favourable environments), it would be interesting to study funnel-webs under natural conditions. Factors such as microhabitat, predation pressure and population density may all play an important role in behavioral changes, providing information related to the ability to colonise, adapt to, and survive in different environments (particularly suburban and urban environments, Kralj-Fišer et al. 2017).

For climbing frequency and activity, we did not observe significant behavioral differences between funnel-web species. These findings suggest that these behavioral traits may be phylogenetically constrained (Riechert 1993). However, it is important to consider that other factors might be shaping behavioral responses across species, such as geographical variation (Van Dongen et al. 2010), microhabitat structure (Vernouillet and Kelly, 2020), and developmental experience, which can be related to interactions and exploratory behaviors (Vernouillet and Kelly 2020; Hernández Duran et al. 2021 and). If funnel-web spiders experience similar selection pressures that drive how they respond to conspecifics and how they interact directly with their environment to gain information, then this could explain why no differences in these behaviors were found.

In conclusion, the strength of selection pressures (i.e., predation, microhabitat, and location), as well as intrinsic factors, such as physiology, genetics, and neurohormones, may affect the expression and variability of repeatability of traits (Kralj-Fišer et al. 2017; Vernouillet and Kelly 2020; Hernández Duran et al. 2021) across different contexts and species. This is the first study to systematically explore behavioral flexibility and repeatability in four species of Australian funnel-web spiders. A comparison of the level of individual variability in behavioral responses within and

between species provides important information about how the dynamics of populations (i.e., dispersion, invasion processes; colonization, Fogarty et al. 2011) might be affected by behavior. Furthermore, our results provide information about behavioral traits of venomous arthropods that should be considered in ecology and toxinology studies to understand venom production, the costs and benefits of venom use, as well as the evolution and diversification of other traits (Cooper et al. 2015; Schendel et al. 2019). Understanding the link between particular behaviors (i.e., risk-taking behavior, activity) in different ecological contexts provides the basis for determining or unveiling how venomous animals use their defensive weapons (i.e., venom) and how, depending on the behavioral trait (e.g., aggressive/submissive), these traits are displayed during particular situations, as seen in vertebrates (e.g., bolder threespine sticklebacks *Gasterosteus aculeatus* have less armour; De Winter et al. 2016).

Australian funnel-web spiders are short-range endemic species, making them vulnerable to habitat loss due to urbanisation and fragmentation (Gray 1992; Harvey 2002). Understanding how different species of funnel-webs respond to different stimuli and contexts will provide greater insights into the ability of these species to cope with changing or challenging conditions, as well as valuable information for the conservation and management of funnel-webs. Conservation of these species is also critical given their medical importance.

Acknowledgments

The authors are grateful to Mr Rod Hobson, and the Sparshott and Hockly families for assistance with specimen collection. We acknowledge the help and assistance by the Toowoomba Regional Council, as well as the Australian Reptile Park. We thank Kellie Johns for helping with editing the manuscript, and Oscar Alvarado for statistical advice. This work was supported by the College of Science and Engineering Higher Degree by Research Student Support, James Cook University.

Funding

The funds obtained from the Higher Degree by Research Student Support from the College of Science and Engineering, James Cook University, were used for spider husbandry. This research received no external funding.

Conflict of Interest Statement

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.

Ethical Statement

Spiders were observed daily and monitored weekly. Experimental procedures did not have any negative effects on the animals. No spiders died during the experiment, and spiders readily resumed feeding following behavioral tests. As funnel web spiders are not 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).

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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