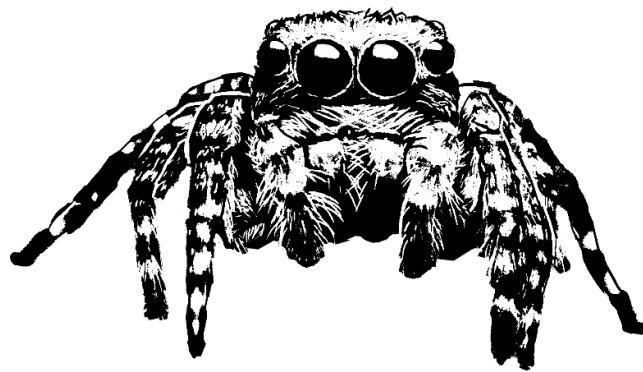


JUMPING SPIDERS AS A MODEL SYSTEM FOR COMPARATIVE VISUAL COGNITION



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September 2015

This thesis is presented for the degree of Doctor of Philosophy

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SUMMARY

Uncertainty is a common feature of the natural world. Associative learning, which enables animals to form predictive relationships between contingent events, reduces uncertainty and equips animals to respond adaptively. Associative learning is widespread across the animal kingdom, and has been particularly well characterized in a few key invertebrate model taxa. Invertebrates exhibit a broad repertoire of associative learning abilities, have significant molecular overlap with vertebrate systems, and are considerably more amenable to analyses at cellular and behavioural levels. Jumping spiders (Salticidae) - and arachnids as a whole - have been underrepresented in the comparative cognition literature but possess many traits that make them suitable and interesting models. The principle aim of this thesis was to begin to bridge the gap between spiders and traditional invertebrate model taxa. To do so, I developed methods that permitted rigorous and repeatable analysis of cognitive processes, and assessed how salticids integrate different sources of information during learning and memory. My research focuses on an Australian salticid, *Servaea incana*, but the methods and principles developed here should be relevant to other cursorial arachnids or invertebrates in which vision is well developed. I develop a novel method to produce electric shock platforms that is precise, easy to use, and highly repeatable, and isolate the changes in mobility and behaviour that are dependent on shock. Additionally, to overcome the constraints of commonly used playback methods, I develop an immersive, closed-loop virtual reality system tailored for studies of salticid cognition. Using passive and active avoidance conditioning assays coupled with the aversive electric shock stimulus, and a change detection paradigm in virtual reality, I characterize the critical determinants of learning and memory in salticids. These experiments reveal that the ecological relevance, or 'salience' of available visual cues, together with hunger level and the temporal nature of the training protocol, play a significant role in determining performance. At the same time, when spiders are provided with a number of cues predicting an aversive event, they learn an operant escape response while ignoring reliable visual information. These results are very surprising given the extent to which vision is known to mediate behaviour and decision making in jumping spiders.

DECLARATION

I certify that the work in this thesis entitled 'Jumping spiders as a model system for comparative visual cognition' has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help or assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

As this research was on invertebrates, the research presented was not subject to approval by the Macquarie University Ethics Review Committee.

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September 2015

ACKNOWLEDGEMENTS

To start at the start – I would like to thank Damian Elias for instilling in me a deep fascination with the spiders I would come to spend many years studying. His wisdom and curiosity propelled me down the path I find myself on today, and over a decade later, I still remember those years as amongst the most inspiring. I am also so grateful for his introduction to his wife and fellow arachnophile, Maddie Girard, who has been an incredible friend, and an endless well of warmth, wisdom and hilarity.

I am immensely grateful to my supervisor, Phil Taylor, for seeing the value in ‘arachnopsychology’ and initiating the project that grew to become my thesis. He has been a warm and patient mentor on this long journey and an incredibly astute editor.

I am grateful to Andy Barron, who, in the more stressful moments of my final year, welcomed me into his group and provided some critical perspectives on the cognitive themes of my research. I am also grateful to Beth Jakob and Fiona Cross, who also came on board in my final year to share their insights, and who became kind allies in the shared frustration of working with jumping spiders.

Developing a spider-sized virtual reality system was no small feat, and I am immensely thankful to all of those that contributed along the way. Bruno van Swinderen and his group from the Queensland Brain Institute contributed during the conceptual stages of development, and Chris Malloy, Keith Hassen, John Porte and Brian Su all contributed technical expertise to the project during different stages of execution. I am particularly thankful to Rob Lee, whose dexterous Unity coding skills saved the day on many occasions.

Across the field, office and lab, Rowan McGinley takes the cake for being the most reliable, informative and understatedly entertaining, colleague that one could wish for. This experience would not have been the same without him.

I am deeply grateful to my mother, who has always wholeheartedly supported my endeavours, no matter how strange they may have seemed or how far across the planet they have taken me. I am also thankful to my father, whose scientific curiosity surely catalysed my own interests, and who would have been so happy to discuss this work if he was here with us today.

Above all, I would like to thank Greg Hunsburger, for being a boundless well of love, good nature and encouragement. Greg contributed to so many aspects of this thesis that it would be difficult to name them, though bares mention that all artwork contained in this thesis is his doing. Most importantly, he helped create little mister Heilo Hunsburger, right in the middle of my thesis woes, whose mischievous ways kept me on my toes and ensured, even in the most challenging times, that I never strayed too far from a smile.

1 INTRODUCTION

Uncertainty is a common feature of the natural world. Even in seemingly static environments, resources ebb and flow, abiotic factors such as rainfall and sunshine fluctuate, and animals interact dynamically and transiently (Mangel, 1990). For an animal faced with change, associative learning provides an adaptive mechanism to reduce uncertainty by equipping individuals with a capacity to form predictive relationships about contingent events, which often possess positive or negative hedonic value for the animal (Carcaud, Roussel, Giurfa, & Sandoz, 2009). Two major forms of associative learning are usually recognized. In classical conditioning, animals learn to associate an originally neutral predictive stimulus with a subsequent biologically relevant stimulus, whereas in operant conditioning, animals learn to associate their own behaviour with its consequences (Carew & Sahley, 1986). Both allow for a predictive response to a changing environment, and are reflected internally by corresponding alterations in neuronal representations (Dukas & Duan, 2000). The essential prerequisites for learning to occur are thought to be quite simple: the capacity to sense some features of the environment, and to modulate cellular processes in response to these features (Dukas & Ratcliffe, 2009). Since all animals with nervous systems possess these two characteristics, it is not surprising that evidence of learning has been found in every major taxon where it has been sought (Heyes, 2012).

Understanding how ‘simple’ systems handle the complexities of daily life can inform us about the cognitive processing requirements that are necessary and sufficient to accomplish a given task. Invertebrates comprise over 95% of the earth’s animals (Perry, Barron, & Cheng, 2013) and have become increasingly popular as subjects for studies of associative learning. They possess relatively simple nervous systems that have substantial molecular overlap with vertebrate systems (Bailey, Bartsch, & Kandel, 1996; Chittka & Niven, 2009), while being

significantly more amenable to analyses at cellular and behavioural levels (Abramson & Feinman, 1988). Further, learning in invertebrates is diverse and is believed to enhance all major life activities including feeding, antipredatory behaviour, aggression, social interactions, courtship and mate choice (reviewed in: Dukas, 2008).

A principal goal of comparative cognition is to trace the evolutionary development of cognition by comparing the mechanisms employed by different taxa in solving analogous computational tasks (Soto and Wasserman, 2010). Taking a broad approach enables general processes to be separated from processes that are unique to an animal's particular ecology or neural circuitry (Lovell & Eisenstein, 1973), and clarifies the underlying mechanisms associated with conserved systems (Soto & Wasserman, 2010). However, despite the accepted value of a broad approach, the study of invertebrate cognition has focussed largely on a few key model systems and this narrow approach has precluded a truly comparative perspective on the generalities and evolutionary roots of learning. To bridge this gap, a much wider range of invertebrates must be considered and developed as points of comparison (Boogert, Fawcett, & Lefebvre, 2011; Perry et al., 2013).

The adequacy of an invertebrate model of cognition depends first on our concept of cognition (Vakarelov, 2011). Once focused solely on the human mind, cognition (literally, *cognitio*, 'to know') was seen as a process inherently interrelated with consciousness and awareness (Pickens & Holland, 2004). However, even in humans, complex processing of information in the absence of awareness has been well documented, and highlights the overly restrictive nature of this interpretation (Schacter, 1998; Weiskrantz, 1986). At the other extreme, this time focusing on bacteria, the term refers to the process of gathering sensory inputs which serve as guides for 'successful action' by regulating cellular functions (Shapiro, 2007). In this thesis, I adopt a more balanced interpretation that incorporates simple through to complex information processing, where cognition refers to 'the mechanisms by which animals acquire, process, store and act on information from the environment' (Shettleworth, 2010). These processes, which include perception, learning, memory and decision making, have been extensively characterized in model invertebrate taxa (reviewed in: Giurfa, 2007; Perry et al., 2013).

Spiders are a tremendously diverse group. With over 40 000 extant species (Platnick, 2016), spiders occupy nearly every terrestrial habitat (Foelix, 2011) and are considered to be the most important terrestrial predators (Wise, 1993). Despite having miniature nervous systems, they exhibit broad behavioural repertoires that clearly defy the historical view that

they, like insects, are ‘mindless machines’ driven by instinct alone (Gould, 1982). Rather, a growing literature demonstrates that many aspects of spider behaviour closely parallel much larger vertebrates, and are considered to be not only labile, but complex and cognitive in nature (Jakob, Skow, & Long, 2011).

Amongst spiders, jumping spiders (Salticidae) are considered to be particularly well suited for studies of visual cognition (reviewed in: Jackson & Cross, 2011; Jakob et al., 2011). With more than 5,400 species, ‘salticids’ are the largest and most diverse spider group (Platnick, 2016) and differ from other spiders in several key respects. While many spiders are considered to be ‘sit-and-wait’ predators, building webs to ensnare prey, most salticids are active, cursorial hunters that capture prey through visually mediated stalking routines (for an extreme example, see: Harland & Jackson, 2004). They possess a unique, modular, visual system with four pairs of functionally specialized ‘camera’ eyes (Land, 1985; Land & Nilsson, 2012) that collectively provide a wider field of view than any insect (Zurek & Nelson, 2012). Their large, forward-facing ‘principle’ eyes feature colour vision (Peaslee & Wilson, 1989), depth perception (Nagata et al., 2012) and a retina with greater spatial acuity than any animal of comparable size (Land & Nilsson, 2012), while their smaller secondary eyes act principally as motion detectors (Land, 1971). Having distinct functions in separate pairs of eyes enables the salticid visual system to perform complex functions while minimizing costly increases in eye size (Land & Nilsson, 2012).

Concomitant with their exceptional visual abilities, many aspects of salticid behaviour are mediated by vision. Using optical cues alone, salticids can identify prospective mates and rivals, classes of prey and predators, and features in their environment (Harland & Jackson, 2004; Jackson & Pollard, 1996; Tarsitano & Jackson, 1997). Vision also plays a central role in cognition, with salticids relying on vision for complex decision making (Jackson & Cross, 2013), learning (Liedtke & Schneider, 2014; Nakamura & Yamashita, 2000; VanderSal & Hebets, 2007), discrimination and categorization of prey (Dolev & Nelson, 2014), trial and error problem solving (Jackson & Nelson, 2011) and navigation (Hoefler & Jakob, 2006; Tarsitano & Jackson, 1997). Further, salticids respond to video images of prey, predators and conspecifics in a manner that closely resembles their responses to natural stimuli. Video or computer-generated stimuli are increasingly being incorporated into behavioural research, as they permit an otherwise unattainable level of control over visual stimuli (Clark & Uetz, 1992; Ord, Peters, Evans, & Taylor, 2002; Uetz & Roberts, 2002). The willingness of salticids to interact with 2D and 3D images, both abstract (e.g., Zurek, Taylor, Evans, & Nelson, 2010)

and lifelike (e.g., Clark & Uetz, 1990), greatly extends the breadth of experimental techniques and questions that can be addressed with this group

Experimental paradigms have been particularly well described for the few most frequently used invertebrate model taxa, but remain poorly developed for salticids. For example, in the honeybee, *Apis mellifera*, appetitive olfactory classical conditioning of the proboscis extension response has been developed over decades and is used by laboratories across the world (reviewed in: Giurfa & Sandoz, 2012). Using this paradigm alone, researchers have dissected the behavioural, genetic and neurobiological basis of olfactory learning in honeybees (e.g., Brandes, 1988; Giurfa & Sandoz, 2012; Takeda, 1961), as well as diverse aspects of honeybee cognition (e.g., de Brito Sanchez, Serre, Avargues-Weber, Dyer & Giurfa, 2015; Menzel, Manz, Menzel, & Greggers, 2001; Sandoz, Roger, & Pham-Delegue, 1995). In contrast, few studies have explicitly developed comparative methods or examined fundamental properties of learning in salticids (Jakob et al., 2011). Salticids offer great potential as an arachnid model for comparative cognition but in order to realize this potential there is a need for detailed analyses of processes mediating learning and memory that might allow for instructive comparisons with other taxa.

Prospectus

In this thesis, I develop novel methods that permit rigorous and repeatable analysis of cognition in salticids, with the principle goal of illuminating the characteristics of learning and memory in similar depth as current invertebrate model taxa.

This thesis is organized into five data chapters, each written as a separate manuscript that either has been submitted for publishing or is ready for submission. Chapters 2-4 detail the development and characterization of an aversive (electric shock) stimulus for use with salticids, and explore the role of temporal dynamics, cue salience, cue type, and contingency on learning and memory using passive and active avoidance paradigms. Chapter 5 describes a closed-loop, virtual reality (VR) system developed for studying salticid visual cognition alongside proof-of-concept tests, and Chapter 6 utilizes this VR system to explore the role that internal (e.g., hunger) and external (e.g., cue salience) factors play in mediating attention, working memory and change detection.

Chapter outline

Electric shock is used widely as an aversive stimulus in conditioning experiments, yet little attention is typically given to its behavioural and physiological effects. In **Chapter 2**, I discuss the need for a well-characterized aversive stimulus in conditioning experiments, and detail a novel method to produce electric shock platforms for use with cursorial invertebrates.

Through a series of behavioural, activity and conditioning tests, I characterize the effects of electric shock on jumping spider performance, behaviour and learning. This chapter has been published in *Behavioural Processes* (2015, Volume 113, Pages 99-104), and was co-authored by Phil Taylor who provided suggestions on experimental design and comments on the text.

Learning to avoid harmful events, stimuli, or places has clear adaptive value and is not surprisingly ubiquitous across invertebrates (Bernays, 1993). In **Chapter 3**, I develop a conditioned passive place avoidance paradigm to assess the relative importance of three parameters: the ecological relevance of the visual stimulus, the inter-trial interval, and the retention interval. This chapter has been prepared for submission to *Animal Behaviour*, and is co-authored by Phil Taylor who provided suggestions on experimental design and comments on the text.

In **Chapter 4**, I continue to explore the relative importance of the parameters involved in learning and memory in salticids. I develop an active avoidance conditioning task in which the relative weighting of visual stimuli, aversive stimuli, and stimulus contingencies can be explored. I find that spiders ignore predictive visual information in favour of learning about the aversive stimulus alone – a finding that contrasts the commonly held expectation that salticids consistently integrate visual information in decision making. This chapter has been prepared for submission to *Animal Behaviour*, and is co-authored by Phil Taylor who provided suggestions on experimental design and comments on the text.

Jumping spiders respond to artificial video and computer-generated stimuli as they would to natural stimuli, but so far all work with artificial stimuli has been conducted under open-loop conditions, where the presented stimuli are unresponsive to the behaviour of tested spiders. In **Chapter 5**, I detail a novel, immersive, VR system for jumping spiders that closes the loop for the first time. In this system, jumping spiders move freely through a 3D projected environment that updates in response to the spiders movements in real time. To investigate VR as a viable proxy for the real world, I conduct two experiments that examine the transference of behaviours and associations across real to virtual contexts. This work

benefited greatly from the thoughtful comments and technical expertise of a number of people: Bruno van Swinderen (insight), Greg Hunsburger (virtual environment & stimulus design), Rob Lee, Brian Su, Keith Hassen and John Porte (Unity coding). This chapter has been published in *Animal Behaviour* (2015, Volume 107, Pages 87-95), and was co-authored by Phil Taylor who provided suggestions on experimental design and comments on the text.

Jumping spiders live in topographically complex environments where they often lose sight of predators, prey and conspecifics. The ability to efficiently recognize a reappearing target would facilitate a quick and appropriate response. Recognition requires that spiders attend to the target when it was initially present and hold some form of ‘representation’ of this target in working memory until it reappears. Since both internal state (e.g., hunger) and external attributes (e.g., target salience) can modulate initial attention, they may additionally play a role in recognition by modulating the contents of working memory. In **Chapter 6**, I examine the interrelated role of attention, memory and target recognition using a modified expectancy violation paradigm in the virtual reality system described in Chapter 5. This chapter has been prepared for submission to *Animal Cognition*, and was co-authored by Fiona Cross and Phil Taylor, who both provided suggestions on experimental design and comments on the text.

Finally, in **Chapter 7**, I summarize and discuss my findings in the context of current frameworks of invertebrate cognition, and highlight promising directions for future work with salticids.

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2 ELECTRIC SHOCK FOR AVERSION TRAINING OF JUMPING SPIDERS: TOWARDS AN ARACHNID MODEL OF AVOIDANCE LEARNING

Abstract

Electric shock is used widely as an aversive stimulus in conditioning experiments, yet little attention has been given to its physiological effects and their consequences for bioassays. In the present study, we provide a detailed characterization of how electric shock affects the mobility and behaviour of *Servaea incana*, a jumping spider. We begin with four mobility assays and then narrow our focus to a single effective assay with which we assess performance and behaviour. Based on our findings we suggest a voltage range that may be employed as an aversive stimulus while minimizing decrements in physical performance and other aspects of behaviour. Additionally, we outline a novel method for constructing electric shock platforms that overcome some of the constraints of traditional methods while being highly effective and easily modifiable to suit the study animal and experimental context. Finally, as a demonstration of the viability of our aversive stimulus in a passive avoidance conditioning task, we successfully train spiders to associate a dark compartment with electric shock. Future research using electric shock as an aversive stimulus with terrestrial invertebrates such as spiders and insects may benefit from the flexible and reliable methods outlined in the present study.

Introduction

Electric shock is used extensively as an aversive stimulus in conditioning experiments, with species-specific avoidance responses documented in taxa as varied as insects (Vergoz et al, 2007), crustaceans (Abramson et al, 1988), fish (Wodinsky et al, 1960; Xu et al, 2007), rodents (Kimble, 1955; Iwata & Le Doux, 1988), and primates (Barrett, 1977), including humans (Glottbatch et al, 2012). As a conditioning stimulus, electric shock offers a number of advantages, including ease of use, immediacy of onset and offset, and a precisely controlled area of effect. There is a need for careful consideration of physiological effects induced by electric shock, and the consequences of such physiological effects for bioassays. It is difficult to isolate the behavioural changes that are contingent on the chosen learning or memory paradigm in the absence of an understanding of the changes to physical state that result from aversive stimuli (Pritchett, 1968). For example, a lack of mobility in a spatial maze owing to physical effects of an aversive stimulus could result in a poor performance score, as animals would be less likely to reach a target than their more mobile counterparts. Through awareness of such collateral effects of conditioning stimuli, it is possible to ameliorate risks through precautions or controls.

Invertebrates have long been used to study the behavioural, cellular and molecular basis of cognition, but in recent years, the focus has been on developing a few key model systems, such as honeybees and *Drosophila* in depth, rather than sampling widely across taxa (Sattelle & Buckingham, 2006; Wolf & Heberlein, 2003). While much can be learned from delving deep into the workings of select model animals, a broader perspective remains integral to the central tenet of comparative cognition, drawing on assessments of how taxonomically disparate groups perform in analogous physiological or behavioural tasks (Eisenstein, 1997, Shettleworth, 2010).

Spiders have been underrepresented in the comparative literature, yet they have much to offer. Spiders inhabit nearly all terrestrial environments and exhibit extraordinary diversity. For example, spiders vary from a solitary lifestyle to living in dense and cooperative social groups, with predatory behaviour ranging from sit-and-wait strategies in webs to active pursuit as cursorial hunters (Wise, 1993; Foelix, 2011). Jumping spiders (Araneae, Salticidae) are particularly well suited for studies of cognition, with exceptionally acute vision in their large forward-facing ‘primary’ eyes and complex, visually mediated behaviour (Jackson & Cross, 2011). Most jumping spiders are cursorial hunters that use their extraordinary visual abilities to mediate navigation, hunting and communication. It is known that they can learn in a variety of

contexts (Nakamura & Yamashita, 2000; Skow, 2005; Jakob et al, 2007; Leidtke et al, 2014), solve problems through trial and error (Jackson et al, 2001), perform challenging navigational detours (Tarsitano & Jackson, 1994) and behave flexibly in novel situations (Jackson & Wilcox, 2010). Despite a growing literature detailing impressive cognitive feats in spiders, much of this work has been conducted using methods that do not readily support comparisons with other taxa. For spiders in general and salticids in particular to enter the comparative framework, there is a need to adapt and apply the well-established standard tools of the comparative method.

There is a need for well-characterized aversive stimuli for use in conditioning experiments. In the present study, we provide a detailed characterization how electric shock, one of the most commonly used aversive stimuli, affects the mobility and behaviour of *Servaea incana*, a common Australian salticid species. We begin with four mobility assays and then narrow our focus to a single effective assay with which we assess performance after both short and long-term shock exposure. We also assess behaviour of spiders in the long-term exposure group. Based on these results, we suggest a voltage range that may be employed as an aversive stimulus while minimizing decrements in physical performance and other aspects of behaviour.

The passive-avoidance paradigm is based on the animal learning to avoid an environment in which an aversive stimulus was previously delivered. The response that is punished is typically one that the animal normally performs. This means that the task is for the animal to learn to suppress a normal response when in a given context, and thus behave contrary to their innate preferences (Bammer, 1982). The passive avoidance paradigm is well established in insects (bees: Abramson, 1986; Agarwal & Guzman, 2011; cockroaches: Disterhoft et al, 1971; Disterhoft, 1972; and ants: Abramson, 1981), and has been used successfully to assess colour discrimination in spiders through colour-heat pairings (Nakamura & Yamashita, 2000). Here, we conduct an initial preference test to determine if spiders prefer the dark or light compartment of a two-sided arena, then train spiders to avoid their preferred compartment by pairing it with electric shock. Following a 20-min break, we return spiders to the arena to see if avoidance behaviour persists in the absence of electric shock, and if spiders can retain at least short-term memory of this aversive event.

Methods and Results

General Method

Equal numbers of adult male (N=95) and female (N=95) *S.incana* were collected from *Eucalyptus* trees in Sydney, Australia. Spiders were maintained in a controlled environment laboratory (24-26°C; 65-75% relative humidity; 11:1:11:1 light:dusk:dark:dawn cycle) where they were individually housed in ventilated 1.125L plastic cages (11x11x12cm tall) containing a folded sheet of paper (2x3cm) that was shaped as a 'tent' under which spiders could shelter and build nests. Spiders were fed weekly on an alternating diet of two houseflies (*Musca domestica*) or two Queensland fruit flies (*Bactrocera tryoni*). All experiments were conducted 3-4 days following a feeding. Supplementary moisture was provided by lightly misting each cage with a spray bottle once each week. All experiments were conducted during daytime hours (8am to 4pm) under full light (50:50 metal halide and halogen ceiling lights).

Spiders were weighed to the nearest 0.1mg (Shimadzu Model N595, electronic balance, Shimadzu Corporation, Kyoto, Japan) within 3 hours following their final trial. In addition to weighing, each spider was photographed from above using a digital camera (ProgResC10) focused through a stereomicroscope (Olympus SZX12, Olympus Corporation, Tokyo, Japan). To minimize movement during photography, spiders were gently restrained on the flat surface of an inverted Petri dish under clear plastic film (Glad Products, Australia). Cephalothorax length and width were measured using the open source image-processing software Image J (v1.30, National Institutes of Health, Bethesda, MD, U.S.A.).

Shock chamber

Spiders were confined to a rectangular arena (75mm wide x 150mm long x 75mm tall) constructed from white corrugated plastic board (Corflute®). The top of the arena was open to permit video recording, while the walls were lightly dusted with non-scented talcum powder to make the walls slippery and prevent spiders from escaping. The shock platform - on the floor of each enclosure - was a rectangular board (2mm thick x 150mm long x 75mm wide) covered with a pattern of parallel copper bars alternately of positive and negative charge (Fig. 1). Previous studies have used adhesive copper tape (Skow, 2005), strips of aluminum (Bednarski et al, 2012), or wire (Agarwal et al, 2011) to achieve a similar design, but each has drawbacks owing to inconsistent voltage or susceptibility to damage. Here we adopt a novel technique that produces uniform voltage across the platform while eliminating risk of subjects

failing to contact bars or becoming injured. To create a shock platform, a grid-like pattern (5mm bars spaced 1mm apart) was chemically etched onto the copper side of a blank printed circuit board backed with epoxy fiberglass laminate (Jaycar Electronics, Australia). The pattern was designed using Adobe Photoshop CS5.5 (Adobe Systems, San Jose, CA, USA) and printed onto toner transfer film (Press-n-Peel, Techniks Inc., New Jersey) using a standard laser printer (Hewlett-Packard 4250 LaserJet). The pattern was then transferred to a blank copper board using a hot iron, followed by chemical etching in a hot ammonium persulphate bath. Boards produced using this method are durable and can be cleaned with circuit board cleaner, alcohol and water without affecting their conductivity.

Alternating bars of the copper shock platform were wired to the positive and negative terminals of a 60V (max 3amp) DC power supply (Sanke Electrical Co., Ltd., China) (see Fig.1). When the power supply was active, shock was delivered to spiders each time they bridged the gap between parallel copper bars, completing the circuit. Due to the narrow gap between opposite-charged bars, failure to complete the circuit was very rare.

Experiment 1: Finding an appropriate assay

In this experiment, we randomly assigned 40 spiders to one of four mobility assays (5 male, 5 female per assay) to determine the measure that best detected a voltage-related change in performance. Each spider was assigned as either a shocked (60V for 30s) or unshocked (0V) subject and was used only once. The sprint and ramp assays required the spider to run 60cm along a 5cm-wide textured (240 grit sandpaper) runway that was oriented horizontally (sprint) or at a 30-degree incline (ramp). For the climb assay, spiders were placed at the bottom a 2.5cm diameter wooden dowel coated with a textured spray paint (Spray Stone paint, White Knights Paint, Australia), and induced to climb its length without pause by gently brushing the spider's hind legs with a soft sable-hair paintbrush. The sprint, ramp and climb assays followed protocols from previous studies of spider locomotor performance (climb, Prenter et al, 2010; sprint & ramp, Prenter et al, 2012), and were all measured in terms of mean speed (distance travelled divided by the time taken to complete the task).

The general activity assay, in contrast, was a measure of spontaneous activity (total distance travelled) in a 5min period. Spiders were transferred from their home cages to a small holding vial, and then gently lowered into a rectangular arena identical to the shock arena but with the shock turned off. Spiders were allowed to move freely throughout the 5-min trial.

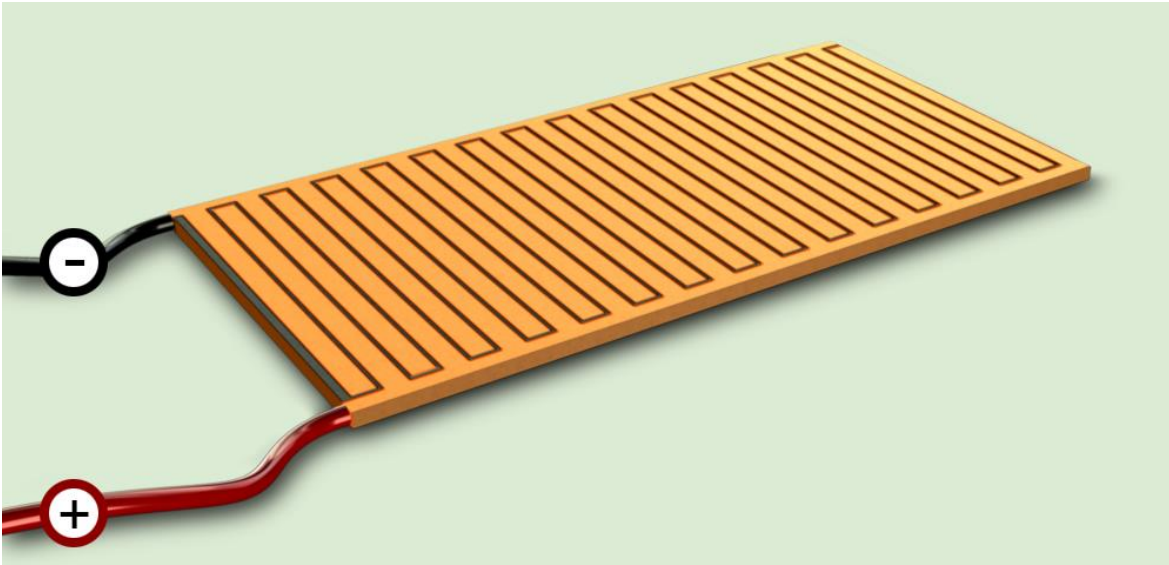


Figure 1. Schematic of custom-designed electric shock platform, constructed as a printed circuit. Spiders receive a shock when a power source is activated and they bridge parallel opposite-charged bars of the platform.

Trials were video-recorded from above (Panasonic HDC-SD900 video camera), and distance travelled was calculated by utilizing the motion tracking function in Adobe AfterEffects (CS5.5). Each video was first adjusted to isolate the spider as a high contrast image against the background onto which a tracker could be attached. Coordinate data were recorded throughout the trial by analysing the video frame-by-frame (30 frames per second), outputting locomotion as a series of changes in x and y coordinates. These values were then used to calculate the total distance travelled in Microsoft Excel.

Each spider provided three measures of performance in their respective assays. The first measure, T1, provided an individual baseline score. T2 was recorded 2h after T1, and immediately following 30s of 60V shock exposure or 30s in the shock apparatus without shock. Finally, T3 was recorded 2h after T2, to assess delayed or persistent effects of shock exposure. The differences in the performance of shocked and unshocked spiders was assessed using a repeated-measures MANOVA (group as predictor; speed at T1, T2 and T3 as responses), with a separate analysis run for each assay. To test whether shocked and unshocked spiders differed in performance across time points, we compared groups at each time pairing (T1-T2, T1-T3, T2-T3) using one-way ANOVAs. Huynh-Feldt corrections were applied to the degrees of freedom in all cases in which sphericity assumptions had been violated (Huynh & Feldt, 1970). Sex, weight and cephalothorax length were initially included in all analyses conducted in this study; however, as none exhibited a significant effect or interaction, these terms were omitted from the final model. The assay most sensitive to shock-related changes in performance was adopted for subsequent experiments designed to characterize effects of shock at a finer scale. A sensitive assay, in this case, was one that produced the greatest significance (P) value when comparing groups for performance changes between baseline and T2 and T3, and here we found this to be the ramp test. All experiments were analysed using JMP version 5.0.1.2 (SAS Institute Inc., Cary, NC).

The ramp assay was found to most clearly detect effects of shock on performance, with shocked, but not unshocked, spiders exhibiting a decrease in speed over time (group: $F_{1,8}=7.35$, $P=0.027$; time: $F_{2,7}=8.83$, $P=0.012$; time*group: $F_{2,7}=1.90$, $P=0.024$; Table 1). In particular, we found a significant difference between shocked and unshocked spiders between baseline and T2 ($F_{1,8}=7.72$, $P=0.024$, $\eta^2=0.49$) but not baseline and T3 ($F_{1,8}=3.50$, $P=0.098$) or T2 and T3 ($F_{1,8}=1.44$, $P=0.264$). Sprint, climb and general activity assays did not detect any significant changes in performance (sprint: $F_{2,7}=0.28$, $P=0.427$; climb: $F_{2,7}=0.84$, $P=0.469$; general activity: $F_{2,7}=0.82$, $P=0.478$) (Fig. 2). The ramp assay was subsequently adopted for experiments 2 and 3 as a sensitive test of electric shock-induced decrement in performance

Table 1. Mean speed in seconds (\pm SEM) on the ramp assay for experimental and control group spiders.

	T1	T2	T3
Experimental	6.48 ± 0.79	3.93 ± 0.40	4.32 ± 0.35
Control	7.35 ± 1.11	7.10 ± 0.58	7.10 ± 0.49

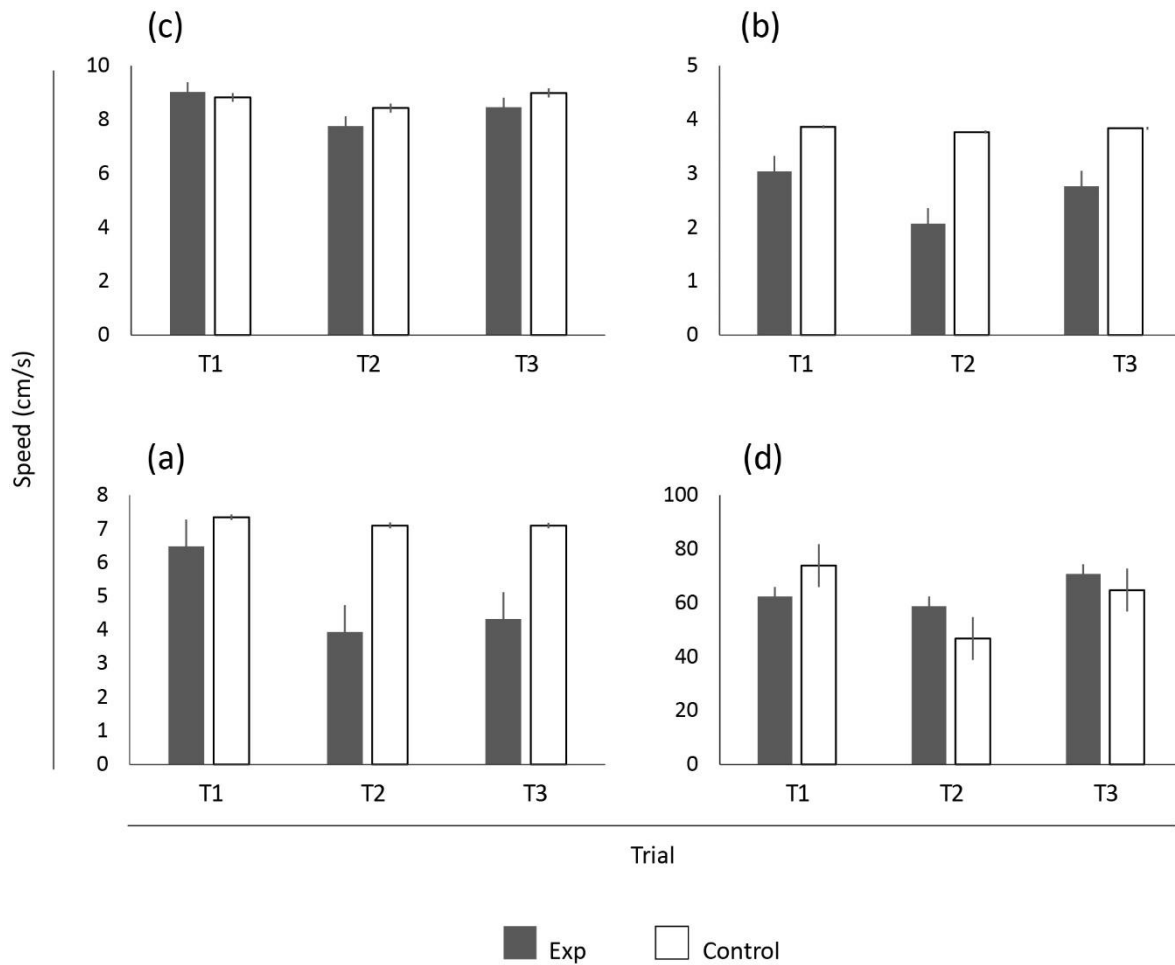


Figure 2. Mean speed on the (a) ramp, (b) sprint, (c) climb and (d) general activity (GA) assays in experiment 1. Spiders were tested 2h before shock (T1); immediately following 30s shock exposure (T2); and 2h after shock (T3). Blue bars represent experimental (EXP, shocked) spiders while grey bars represent control (unshocked) spiders. Bars represent \pm standard error of the mean.

Experiment 2: Short exposure to 0-60V

Spiders (5 male, 5 female) were assigned to each of five voltage groups (0, 15, 30, 45, 60V) and assessed for performance on the ramp mobility assay 2h before, immediately after and 2h after 30s of shock exposure. By comparing performance across 5 voltage levels, the goal of this experiment was to determine the maximum shock level for subsequent testing. Voltage levels associated with significant decrements in performance were excluded from testing in subsequent experiments that were designed to further refine recommended protocols. Results were analysed using an ANOVA with the change between baseline and T2 and T3 as the response variables and voltage group as a factor. Significant main effects were further analysed using a least squares means Student's t-test with Bonferonni corrections for multiple comparisons (adjusted alpha = 0.0125).

Voltage groups varied over time (voltage group by time interaction: Wilks' $\lambda=0.50$, $F_{8,88}=4.53$, $P<0.001$; Fig. 3), with significant differences found between baseline and T2 ($F_{4,45}=5.49$, $P=0.001$, $\eta^2=0.33$) as well as baseline and T3 ($F_{4,45}=5.46$, $P=0.001$, $\eta^2=0.33$). Using the baseline to T2 and baseline to T3 difference scores, we compared each voltage group to the 0V control group and found a significant difference at 45 (T1-T2: $F_{1,45}=17.72$, $P<0.001$; T1-T3: $F_{1,45}=7.79$, $P=0.008$) and 60V (T1-T2: $F_{1,45}=7.87$, $P=0.007$; T1-T3: $F_{1,45}=6.109$, $P=0.012$). In each case, speed decreased from baseline to both T2 and T3.

Experiment 3: Long exposure to 0-30V

Spiders (5 male, 5 female) were assigned to each of four long-exposure voltage groups (0, 20, 25 & 30V) and their subsequent performance was assessed using the ramp assay. The voltage levels used in this experiment were determined as safe working levels that were not associated with a significant decrement in performance in the previous experiments. Learning experiments typically involve observing a change in behaviour over a series of trials, with often-substantial cumulative exposure to the aversive stimulus in avoidance learning experiments. The goal of this experiment was to determine the effects of shock when delivered over longer periods. In each trial, spiders spent 15min in the shock apparatus, with intermittent (30s on/30s off) shock occurring throughout T2. Each spider provided three measures of speed (T1, T2 and T3), and trials were separated by a 15min inter-trial interval. Ramp test results were analysed using an ANOVA with repeated measures on one factor, time.

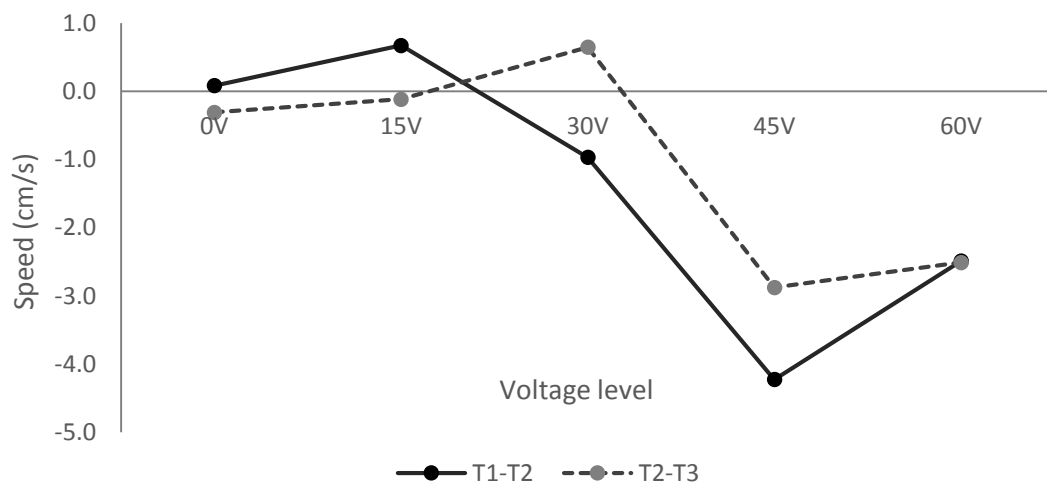


Figure 3. Mean performance difference between T1 (baseline, 2h before shock), T2 (immediately following shock) and T3 (2h after shock) on the ramp mobility assay across voltage groups. Negative numbers indicate a decrease in speed between time points. Bars represent +/- SEM.

Trials were video-recorded from above and analysed using event-recording software (JWatcher 1.0, Blumstein et al, 2000) for 5 behaviours: inactivity, walking, running, jumping and scrambling behaviour (the spider raised and moved its forelegs against a wall, as though attempting to climb). The proportion of each trial spent performing a behaviour and the number of instances that each behaviour occurred (count) were calculated for each individual at each time point. A behaviour was counted each time it was initiated, following either the start of a trial or a different behaviour. A separate two-way repeated measures MANOVA was run for each of the 5 behaviours, using each of the three measures (count, total time, proportion of time).

We found no significant time by voltage interaction in the running speed or behaviour (mean speed: Wilks' $\lambda=0.905$, $F_{6,70}=0.599$, $P=0.730$; behaviour: motionless: Wilks' $\lambda=0.92$, $F_{6,70}=0.33$, $P=0.922$; scramble: Wilks' $\lambda=0.94$, $F_{6,70}=0.37$, $P=0.895$). Similar results were obtained for analyses of count and total time. The lack of shock-induced change in behaviour or performance on a sensitive mobility assay suggests that intermittent exposure to 30V or below is a 'safe' level of shock to use as an aversive stimulus in conditioning experiments of this species.

Experiment 4: Passive avoidance conditioning

A separate group of adult female spiders ($n=38$) was used to test the efficacy of our electric shock stimulus as an aversive stimulus in a passive avoidance conditioning task. The testing environment consisted of a rectangular arena similar in design to that already described but twice the length (75mm wide x 300mm long x 75mm tall). The arena had a separate electric shock platforms at either end of the arena. In this way, we could electrify one half of the apparatus at a time while keeping the other half non-electrified and identical in appearance. A black piece of card stock was placed over one half of the apparatus creating a shaded 'dark' zone underneath.

Testing consisted of a shock-absent (S-) pre-training stage on day 1, a shock-present (S+) training stage 24h later, and an S- test stage 20 minutes following the completion of the training stage. Each stage was 20 minutes long and was conducted in the same arena, which was cleaned with warm water between trials to remove silk draglines and pheromone cues (see Jackson, 1987). The arena was rotated 90° between trials to avoid learning of visual extra-arena cues. For the shock-present group ($n=26$), the shock was deactivated during the pre-training and test stages, and activated on the dark side of the arena only during the training

stage. The task was thus for the spider to learn to avoid the electric shock by avoiding the dark side of the arena. Testing was similar for the control group (n=12), but with the shock switched off in all trials.

Trials were initiated by placing the spider in the centre of the illuminated compartment. All trials were video-recorded from above and subsequently scored from these recordings. We assessed the latency for spiders to first enter the dark side of the arena, as well as the proportion of each trial spent in the shocked side ($S+ - S- / S+ + S-$, where $S+$ is the time on the shocked side and $S-$ is the time on the unshocked side).

We performed a one-way ANOVA comparing shock-present and control spiders for each of the three time points, for both latency to enter the dark side of the arena and proportion of time spent on the dark side. The pre-training comparison ensured that, prior to treatment, experimental and control group spiders behaved similarly; the training comparison determined whether the aversive shock stimulus was indeed aversive to shock-present spiders; and the test stage comparison assessed whether this aversive experience was learnt and remembered after a 20-minute delay. We also ran repeated-measures ANOVA to compare group differences in both performance measures between pre-training and test sessions.

Overall, spiders spent more time in the dark side of the arena than the light side, spending on average 71% of the pre-training session in the dark. Since the task was for spiders to behave in opposition to their dark preference, we omitted spiders in the shock-present group that did not display this preference in the pre-training trial (n=4) from all subsequent analyses. We also omitted those that did not enter both compartments at least once in both the pre-training and training sessions (n=1). There were no significant differences ($P>0.1$) in shock-present and control groups in their latency to enter the dark side during pre-training and training, or the proportion of time spent in the dark side during pre-training. However, shock-present spiders spent significantly less time on the dark side of the arena during the training session than control spiders, indicating that the shock platform was indeed aversive (prop, shock-present mean: 0.52, control mean: 0.75; $t=2.25$, $df=33$, $P=0.031$, $\eta^2=0.13$). Additionally, shock-present spiders were slower to enter the dark side of the arena in the test trial (latency, shock-present mean=248s, control mean=82s; $t=-2.27$, $df=33$, $P=0.030$) and spent less time on the dark side (prop, shock-present mean=0.57, control mean=0.80, $t=2.51$, $df=33$, $P=0.017$, $\eta^2=0.16$) than control spiders, suggesting that spiders had learnt the dark-shock association and were able to retain this memory through the 20 minute interval preceding the test session (Fig. 4). Finally, shock-present and control groups differed

significantly in their latency to enter the dark side of the arena between pre-training and test (time*group, $F_{1,33}=6.97$, $P=0.013$), but not in the proportion of time the spent in this compartment (time*group, $F_{1,33}=2.41$, $P>0.1$).

Discussion

In the present study, we demonstrate the use of printed circuits as a novel, inexpensive and highly effective method for constructing electric shock platforms. Our approach overcomes many of the constraints associated with previous methods, such as inconsistent shock delivery, manufacturing complexity and cost (discussed in Long et al., in press). Since grid spacing and overall platform design are defined in computer software, measurements are precise, and most importantly, virtually identical across all platforms printed using a given design. The design itself is easily modifiable, allowing the experimenter to alter size, spacing or pattern to fit the study animal and experimental context. Future research using electric shock as an aversive stimulus with terrestrial invertebrates such as spiders and insects may benefit from the flexible and reliable methods outlined here.

Determining the appropriate intensity for aversive stimuli used in behavioural experimentation is an important step in technique validation. Previous work with rodents has demonstrated that the intensity of a shock stimulus can influence the outcome observed, with sub- or super- optimal levels leading to decrements in performance in learning tasks (Kimble, 1955; Moyer & Korn, 1966; Mcallister et al, 1971). Similarly, cockroaches perform worse in avoidance learning tasks as the intensity of shock exposure is increased (Pritchatt, 1970), and this is likely a product of shock-induced pathology. Since electric shock may produce changes at the level of primary sensory neurons, motor neurons, body fluids or hormones (Eisenstein, 1968), one should pay particularly close attention to the non-associative physical and behavioural changes that occur at various thresholds.

To this end, we conducted a series of experiments to assess the effects of shock on mobility, behaviour and learning in *S. incana*, determining 30V to be an optimal level. Here we use ‘optimal’ to refer to a voltage level that is sufficiently aversive so as to lead to avoidance, while at the same time producing no detectable detrimental effects on physical performance.

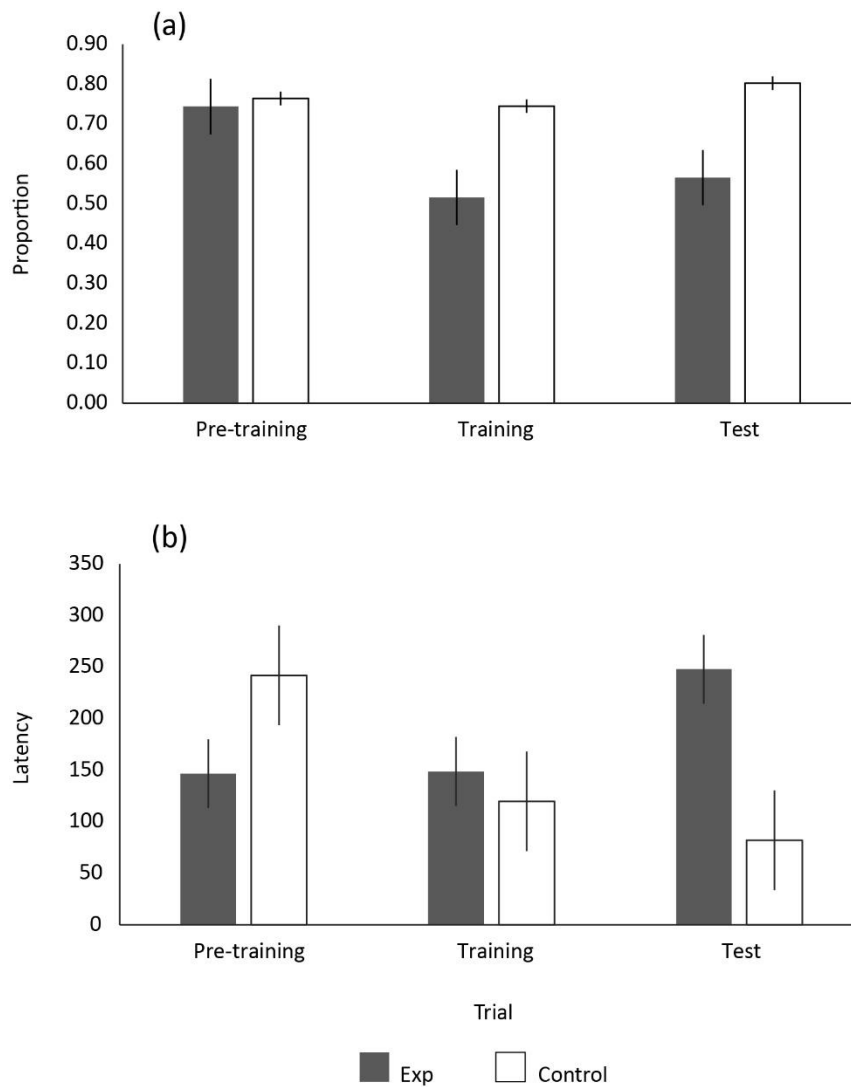


Figure 4. Spider performance on a passive avoidance conditioning test. Shock is activated during the training stage for the experimental (EXP) group only. **A.** The proportion of each trial spent in the dark compartment. **B.** The latency to enter the dark compartment. Stars represent trials in which shock-present and control group spiders are statistically different ($p < 0.05$), and bars represent \pm SEM.

Significant decrements in running speed on inclined ramps were noted in spiders exposed to 45V or greater, whereas even relatively long-term (15min) exposure did not yield significant effects on performance or behaviour when spiders were exposed to 30V or below. Future work using electric shock as an aversive stimulus with this species should consider intermittent exposure at around 30V as appropriate, and this provides a useful starting point for work with other salticids. Indeed, research with a salticid in the USA, *Phiddipus princeps*, indicated 33V as the maximum level of shock that could be delivered without impeding normal behaviour and movement (Skow, 2007).

The results from our passive avoidance conditioning experiment confirm that our shock stimulus was indeed aversive. Spiders spent significantly less time on the dark side of the arena when that side was paired with electric shock than when no shock was applied. Further, spiders exposed to this dark-shock pairing maintained this association in short-term memory over a 20-minute delay period and subsequently avoided the dark side of the arena in a shock-absent test trial. Previous work using aversive paradigms with salticids have demonstrated retention periods ranging from 3 minutes (Nakamura & Yamashita, 2000) to 24 hours (Jakob et al, 2007), with varying levels of acquisition. The paradigm outlined here could serve as a useful tool in methodically examining the speed of learning and the rate of memory decay in salticids, while being amenable to comparisons with other taxa. Additionally, since conditioning tends to be enhanced with increasing stimulus intensity in the passive avoidance paradigm (Seligman & Campbell, 1965), voltages between our known ‘safe’ level (30V) and the level demonstrated to produce physical decrements (45V) may be worth exploring in order to determine the parameters that produce maximal learning and memory.

Avoidance learning has been well characterized in invertebrates (Perry et al, 2013) but as with research on other aspects of cognition, investigations have focused heavily on a narrow range of model organisms. One of the primary goals of comparative cognition is to trace the evolutionary development of cognition by comparing the mechanisms employed by different taxa in solving analogous computational tasks (Soto & Wasserman, 2010). Salticids - and arachnids as a whole- have been underrepresented in the comparative cognition literature, yet possess many characteristics that make them suitable as models (Jakob et al, 2011). A small but growing body of research has demonstrated the capacity for spiders to learn in a variety of contexts (reviewed in Jakob et al., 2011), but drawing comparisons from these data to other taxa has been impeded by the disparate methodologies adopted in each of these studies. The passive avoidance paradigm is well described and may serve as a useful approach for further investigation of learning in spiders.

Acknowledgements

TP was supported by an Australian Government Endeavour Postgraduate Award. Research expenses were supported by Higher Degrees Research Funds of the Department of Biological Sciences at Macquarie University. We thank Rowan McGinley and Vivian Mendez for assistance with collection and maintenance of spiders; Ken Cheng for constructive advice during experimental design and two anonymous reviewers for insightful comments on the manuscript.

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3 FACTORS INFLUENCING PLACE AVOIDANCE LEARNING AND MEMORY IN A JUMPING SPIDER

Abstract

The ecological relevance of the stimuli used in conditioning tasks can play a significant role in learning, and has been well characterized in commonly used invertebrate model taxa. Jumping spiders are increasingly utilized in explorations of visually mediated behaviour and cognition, but a detailed characterization of the parameters influencing learning and memory is lacking. We used a conditioned passive place avoidance paradigm to assess the relative importance of three such parameters: the ecological relevance of the visual stimulus, the inter-trial interval, and the retention interval. Spiders exposed to a mild electric shock stimulus learned to associate the stimulus with a static visual background cue, but the extent to which they did so was dependent on the visual stimulus and the inter-trial interval (ITI). Spiders trained with a long ITI maintained performance throughout training, whereas spiders trained with a short ITI maintained performance only when the safe side was black. When the safe side was white, performance worsened steadily over time. There was no difference between spiders tested after a short (10 min) or long (24h) retention interval. These results suggest that the duration of the inter-trial interval, as well as the ecological relevance of the stimuli used, can influence learning and memory in jumping spiders, and should be taken into consideration when designing experiments with this taxon.

Introduction

Animals frequently encounter dangerous or noxious stimuli in their environments that can cause varying degrees of harm. The capacity to detect injurious stimuli is a basic sensory ability (Purves et al., 2012) that has been found across all major animal taxa and confers clear adaptive advantages (Sneddon, 2004). Protective behaviour varies from species to species, but normally includes an immediate and innate response to terminate the noxious stimulus, such as fighting, freezing or fleeing (Bolles, 1970; Sneddon, 2003). An innate reaction, or reflex, is a simple mechanism to remove the animal from harm's way without resorting to complex central processing (Elwood, 2011). While essential to reducing harm, reflexes do not improve over subsequent encounters. Learning and memory can play an important role in reducing further injury by contributing to sustained avoidance during re-exposure to the stimulus (Bateson, 1991). Arthropods have well characterized responses to noxious stimuli (Elwood, 2011), and despite possessing miniature nervous systems, arthropods exhibit a broad repertoire of learning abilities (Perry, Barron, & Cheng, 2013) which include robust avoidance responses to places, positions, visual stimuli and odors associated with aversive events (Bernays, 1993; Sneddon, Elwood, Adamo, & Leach, 2014).

Learning is thought to be particularly adaptive for animals living in more heterogeneous environments (Dukas, 2008), or those with more active foraging tactics (Punzo, 1980; Raine & Chittka, 2008). Jumping spiders (Salticidae) are an ecologically and behaviourally diverse group of cursorial arachnids that actively hunt diverse prey using flexible predatory behaviour (Bartos & Szczepko, 2012; Jackson & Pollard, 1996; Tarsitano & Jackson, 1994). Salticids are renowned for their exceptional visual acuity, and have attracted much interest in investigations of visually mediated behaviour and cognition. They possess a modular visual system with four pairs of functionally distinct 'camera eyes' that collectively enable the spider to view 360° of its surroundings (Land, 1971). In addition, salticids have been found to exhibit avoidance learning in varied contexts, including rapid avoidance learning about unpalatable prey (Edwards & Jackson, 1994), contextual learning (Skow & Jakob, 2006) and avoidance of visual stimuli associated with electric shock (Bednarski, Taylor, & Jakob, 2012) or vibration (Long, Leonard, Carey, & Jakob, 2015). However, compared to more traditional arthropod model systems, little is known about the factors affecting learning and memory. For example, in *Drosophila* olfactory learning alone, significant advances have been made in understanding the number of trials to acquisition (Beck, Schroeder, & Davis, 2000), the duration of memory (reviewed in Margulies, Tully, & Dubnau, 2005), the temporal determinants of learning (Tully, Preat, Boynton, & Del Vecchio, 1994), and the role of stress (Beck et al., 2000), age and

gender on associative learning (Neuser, Triphan, Mronz, Poeck, & Strauss, 2008). Salticids offer great potential as an arachnid model for comparative cognition, but in order to realize this potential there is a need for similarly detailed analyses of processes mediating learning and memory that might allow for instructive comparisons with other taxa.

In this study, our objective was to assess the relative importance of three experimental parameters on learning and memory in a salticid, *Servaea incana*. We used the conditioned passive place avoidance paradigm in which spiders encountered an aversive electric shock stimulus on one side of a two-sided arena. In this paradigm, conditioned place aversion is expressed when subjects withhold entry into an unsafe side of the arena. Our first experimental variable was the ecological relevance of the visual conditioned stimulus (CS). The ecological relevance of stimuli used in conditioning tasks can play a significant role in learning, to the extent that certain stimuli can be learned rapidly while others, of the same modality and in an identical experimental context, are not learnt at all (Hollis, 1997). *S. incana* build nests in dark spaces under loose pieces of bark and appear to prefer dark regions to light regions when given the choice (Peckmezian & Taylor, 2015). In this study, our visual cues were black and white backgrounds, which salticids are capable of discriminating (Nakamura & Yamashita, 2000), paired with safety or electric shock (e.g., black safe, white shocked, or the converse). We used black and white as visual cues rather than dark and light regions to facilitate video recording of behaviour. In pilot trials, we noted that spiders periodically climbed the walls and walked along the ceiling, which we would be unable to account for if the arena was obscured with a cover for darkness. Additionally, place preference studies have successfully used black backgrounds as a proxy for darkness (e.g., Serra, Medalha, & Mattioli, 1999). Given an underlying preference for dark places, and association of darkness with safety in nature, we predicted that spiders would learn to associate black backgrounds with safety more readily than white backgrounds.

Temporal characteristics surrounding an event can significantly influence the speed of learning and the strength of consequent memory (Balsam, Drew, & Gallistel, 2010; Gallistel & Gibbon, 2000). Generally, learning is positively related to the temporal spacing of learning trials (the inter-trial interval or ITI), such that learning occurs more quickly when successive trials are spaced further apart. This phenomenon is known as the trial-spacing effect, and has been observed across a wide range of taxa, including mammals (e.g., rats: Stout, Chang, & Miller, 2003; monkeys: Riopelle & Addison, 1962), birds (e.g., pigeons: Bizo & White, 1994), and insects (e.g., moths: Fan, Anderson, & Hansson, 1997; honeybees: Menzel, Manz, Menzel, & Greggers, 2001; and bumblebees: Toda, Song, & Nieh, 2009). Similarly, the duration of the

interval between the final training trial and the test session (referred to here as the retention interval, or RI) can determine whether and what type of memory will occur (Menzel et al., 2001; Sutton, Ide, Masters, & Carew, 2002). Providing the first insights to the importance of temporal spacing of training and test episodes on learning and memory in a salticid, we compared responses to short and long ITIs and RIs.

Methods

Spiders

Equal numbers of adult male (N=120) and female (N=120) *S. incana* were collected from the trunks of *Eucalyptus* trees in Sydney, Australia. We included both sexes in this study since previous studies with this species have revealed sex differences in visual perception (females are more responsive to moving stimuli: Zurek et al., 2010) and life history (females are longer-lived: McGinley, Prenter, & Taylor, 2013) that may influence visual learning performance. Spiders were maintained in a controlled environment laboratory (24–26°C; 65–75% relative humidity; 11:1:11:1 light:dusk:dark:dawn cycle) where they were individually housed in ventilated 1.125 L plastic cages (11 × 11 × 12 cm tall) containing a folded sheet of paper (2 × 3 cm) that was shaped as a ‘tent’ under which spiders could shelter and build nests. Spiders were offered one Queensland fruit fly, *Bactrocera tryoni*, every 3 days, and uneaten flies were removed every 4th day. Spiders were weighed to the nearest 0.1mg with an electronic balance (Shimadzu Model N595, Shimadzu Corporation, Kyoto, Japan) within 3 hours following their final trial.

Experimental arena

The experimental arena consisted of an inverted glass Petri dish (15cm diameter, 1.5cm tall) positioned symmetrically atop two rectangular (15cm long, 7.5cm wide, 0.2cm thick), independently controlled electric shock platforms (Fig. 1). The walls of the arena were covered with a thin film of petroleum jelly to impede spiders from climbing up the walls and/or onto the ceiling. The platforms were placed side by side lengthwise, forming a 15cm x 15cm square base. To visually distinguish one semi-circular half of the arena from the other, one wall was marked with black electrical tape, and the other half was marked with white electrical tape affixed to the outside of the Petri dish. The Petri dish was lined up with the shock platforms such that one background lined up with the first platform and the second background lined up

with the second platform. The shock platforms were constructed by chemically etching PC boards with a pattern of parallel copper bars, as described in Peckmezian & Taylor (2015). Alternating bars were wired to the positive and negative terminals of a 60V (max 3amp) DC power supply (Sanke Electrical Co., Ltd., China). When the power supply was active, 30V intermittent (3s on: 3s off) electric shock (the US) was delivered to spiders each time they bridged the gap between parallel copper bars, completing the circuit. Due to the narrow (1mm) gap between opposite-charged bars, failure to complete the circuit was rare.

Experimental procedure

To examine the effects of the CS (black or white safe background), ITI (short or long) and RI (short or long), equal numbers of male and female spiders were randomly assigned to a conditioned (shock present) or control (shock absent) treatment group based on these parameters (Table 1). For both ITI and RI, a short interval was 10 minutes and a long interval was 24 hours. While our RIs were within the range commonly used to assess retention in invertebrates (Gerber, Wüstenberg, Schütz, & Menzel, 1998), our ITIs were longer than the standard range (typically <20min; Fan et al., 1997; Menzel et al., 2001; see discussion). During training (T1-15), spiders in the conditioned treatment group received a shock whenever they entered the side of the arena corresponding to the shocked background assigned to them.

All spiders were initially run through a no-shock pre-training (PT) trial to test for any a priori background preferences and to establish baseline performance. Spiders then underwent 15 training sessions separated by either a short or a long ITI. Finally, spiders underwent a no-shock test session that followed either a short or a long RI. During the long ITI and RI, spiders were returned to their home cages, where they were fed one fly every 4 days, but were otherwise left undisturbed under normal laboratory conditions. During the short ITI and RI, spiders were collected into a transparent 15 mL plastic specimen jar that was placed into a larger open-topped opaque container, permitting light but not visual disturbance from movement in the room. We chose to hold spiders in a vial rather than return them to their home cages during the short intervals in order to minimize stress associated with recapture.

Spiders were randomly assigned to testing groups of four and run in these groups throughout all experimental stages. Each group comprised two female (one shock and one control) and two male (one shock and one control) spiders with matching ITI/RI assignments. Long-ITI groups were run at the same time each day. Shocked backgrounds were alternated by testing group, such that the first group of four experienced a black + shock

Table 1. Experimental parameters. Each spider was assigned to one of the two options within each parameter (safe background, ITI, RI and treatment) and tested with these conditions through all trials (*Total N=240*).

Background	ITI		RI
	10min	24h	
Black	Shock (n=30) Control (n=30)	Shock (n=30) Control (n=30)	10min
White	Shock (n=30) Control (n=30)	Shock (n=30) Control (n=30)	24h

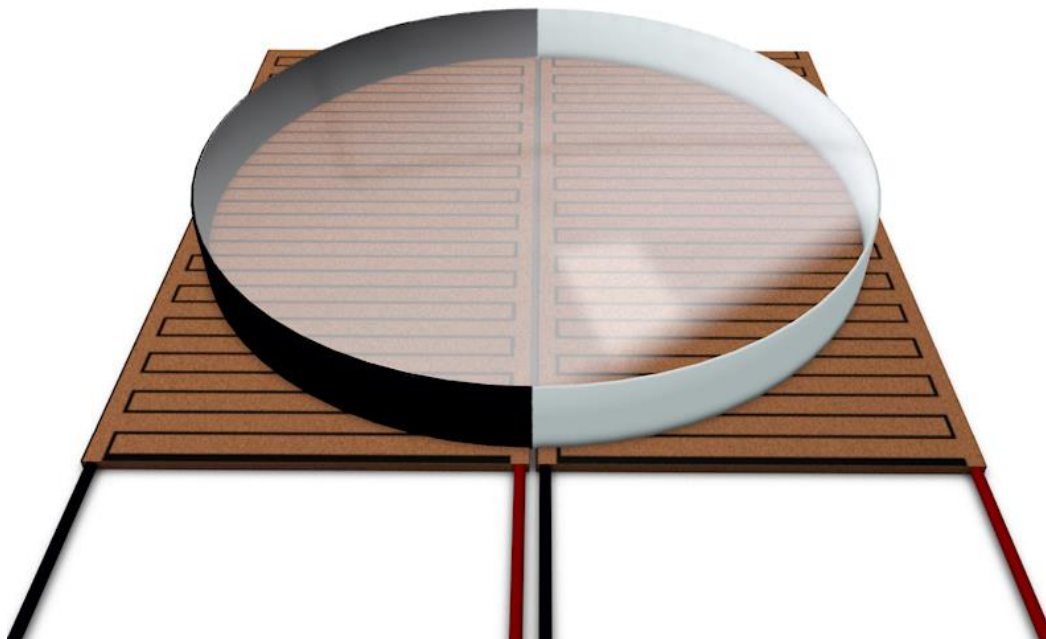


Figure 1. Schematic representation of the experimental arena, which comprised an inverted glass Petri dish upon two independently controlled electric shock platforms. The arena was divided into two semicircular halves, marked with black (one half) and white (other half) electrical tape on the Petri dish walls. Each half sat atop one of the two electric shock platforms. Spiders could move freely in the arena throughout each five-minute trial. For experimental spiders, one platform was electrically shocked while the other was safe (non-shocked); for controls, neither platform was shocked. Spiders could learn to avoid/escape the electric shock and/or to associate the paired background with the shock.

pairing, the second group white + shock, and so on. All trials were 5 minutes long. At the start of each trial, spiders were placed onto the midline of the two shock platforms, and the petri dish was lowered on top of the platforms, containing the spiders. For spiders in the conditioned treatment group, shock was turned on (on the side corresponding to the assigned background) after the petri dish was lowered and remained on throughout. The ‘incorrect’ side for control spiders was scored as the background assigned to the conditioned spiders within their testing group.

The arena was cleaned with warm soapy water and the shock platforms were wiped with a damp cloth between trials to remove silk draglines and pheromone cues (see Jackson, 1987). The platforms were rotated 90° clockwise or counter clockwise (direction randomized) each trial to impede positional cues from outside the arena from influencing behaviour. Trials were video-recorded from above (Panasonic HDC-SD900 video camera) and subsequently scored from video. We scored performance as the percentage of each trial that spiders spent in the ‘safe’ compartment compared to the non-safe compartment. In some instances (<6%), despite the petroleum jelly on the walls, spiders managed to climb the walls and onto the ceiling. When this occurred, we deducted this time from the overall trial length prior to calculating the performance measure.

Statistical analyses

Data were analysed using JMP version 5.0.1.2 (SAS Institute Inc., Cary, NC). We analysed baseline performance during PT by comparing the time spent on each background using a two-tailed least squares means Students t-test. For T1, we analysed the time spent on either background using two-way ANOVAs with treatment (conditioned or control) and background (black or white) as factors. To examine the time course of performance within this first shocked trial, we ran a separate analysis in which the 300s T1 trial was divided into 5, 60s time blocks and analysed using a two-way repeated measures ANOVA with the same factors (learning curve). For training trials (T1-15), we analysed the time spent on the safe side using factorial repeated-measures ANOVAs, with treatment, ITI (short or long) and background as factors. Both between and within (over time) subjects data are reported. For the final, no-shock test session, we analysed the time spent on the previously safe background using a factorial ANOVA with treatment, ITI, background and RI (short or long) as factors. For each of the above analyses we assessed the interaction of treatment with each factor, as well as the interaction between treatment, background and ITI during training.

Table 2. Statistical analysis of performance during the first training trial (T1). Performance was measured as the total amount of time spent on the safe side per trial. (a) Performance analysed using mean values from the entire 300s trial (two-way ANOVA). (b) Performance analysed as a learning curve, in 5, 60s time bins (two-way repeated measures within-subjects ANOVA).

Factors	(a) Whole trial			(b) Learning curve		
	df	F	P	df	F	P
Whole model	3, 122	30.97	<0.0001	9.9, 730.4	4.87	<0.0001
Treatment	1, 222	87.62	<0.0001	3.4, 731.4	5.53	<0.0001
Background			0.361		6.32	<0.0001
<i>Treatment * Background</i>			0.127		2.49	0.052

Fig. 2a

Fig. 2b

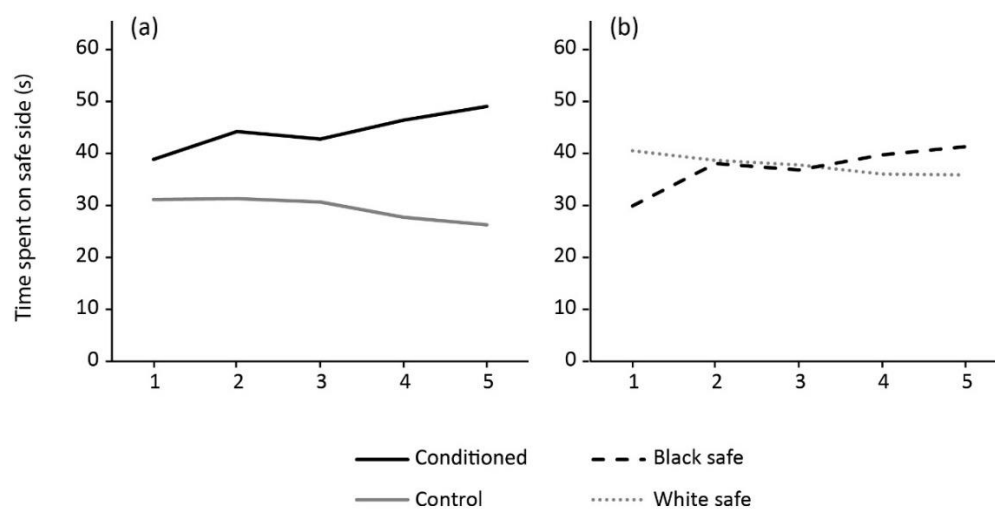


Figure 2. Avoidance conditioning learning curves, reflecting time spent on the safe side during T1. The learning curve was scored in 5, 60s time bins and analysed using a two-way repeated measures within subjects ANOVA. The interaction between treatment and time (2a: Shock or non-shock control) and background and time (2b: black or white) were significant ($P<0.001$).

Table 3. Statistical analysis of performance over the course of training (T1-T15). Both the between-subjects effects (a) and within-subjects effects (b) were assessed (factorial repeated measures ANOVA).

	(a) Between subjects			(b) Within-subjects		
	df	F	P	df	F	P
Whole model	5, 220	0.62	<0.0001	69.6, 3060.2	1.83	<0.001
Treatment	1, 220	125.11	<0.0001	14.1, 3052.4	3.47	<0.001
Background		12.16	<0.001		1.32	0.189
ITI		5.40	0.021		1.98	0.016
<i>Treatment * Background</i>		0.36	0.550		1.19	0.279
<i>Treatment * ITI</i>		10.16	0.002		1.07	0.380
<i>Treatment * Background * ITI</i>		5.35	0.024		1.15	0.309

Fig. 3

Sex and weight were initially included in all analyses; however, as neither exhibited a significant effect ($P > 0.1$ in all cases) at any time point, these terms were omitted from the final model. Only spiders that completed all 17 trials were included in the final analysis ($n=226$). Huynh and Feldt (1970) corrections were applied to the degrees of freedom in all cases in which sphericity assumptions had been violated.

Results

Spiders showed no evidence of background preference during the initial no-shock PT session ($t_{1, 224} = -0.27$, $P = 0.790$). During the first training trial (T1), conditioned spiders spent significantly more time on the side of the arena associated with the safe background than did control spiders (shock: 83.5%, control: 47.4%; Table 2). The learning curves showed that avoidance behaviour improved over the course of the trial in conditioned spiders but not controls (Fig. 2a), as well as in spiders that experienced black as the background associated with safety (and white as the background associated with shock) (Fig. 2b).

On average, over the course of training (T1-T15) spiders in the conditioned treatment group spent more time on the side of the arena associated with the safe background than did control spiders (shock: 70.8%, control: 51.1%) (Table 3). Performance was highest in T1 and gradually tapered off to its lowest point by T15 (shock: 63.9%, control: 52.9%). Background, independent of treatment group, played a role in performance, with spiders spending more time on the correct side when black was safe and white was shocked than the converse (black safe: 63.7%, white safe: 54.5%). Additionally, spiders trained with a long ITI outperformed spiders trained with a short ITI (long ITI: 63.5%, short ITI: 59.3%; Table 3). There was a significant interaction between treatment and ITI, which post-hoc contrasts revealed to be due to differing performance of shocked spiders in the short and long ITI groups. Specifically, performance in conditioned spiders trained with a long ITI was stable whereas performance in conditioned spiders trained with a short ITI worsened over time (LSMeans contrast $F_{1, 220} = 15.99$, $P < 0.0001$; Fig. 3). Further, there was a significant interaction between treatment, background and ITI. Pairwise post-hoc tests revealed that spiders trained with a black/shock, white/safe pairing worsened significantly over time, while all other pairings maintained performance. Finally, conditioned spiders outperformed controls during the non-shocked test session, spending less time on the side previously associated with shock (shock: 38.8%, control: 56.2%; Table 4, Fig. 4). Test scores were not significantly influenced by ITI, RI, background, or the interaction between these terms and treatment.

Table 4. Analysis of the effects of treatment, background, ITI and PTD on performance during the no-shock test session (factorial ANOVA).

	df	F	P
Whole model	7, 218	4.18	<0.0001
Treatment	1, 218	16.55	<0.0001
Background		1.36	0.244
ITI		1.44	0.232
RI		0.03	0.862
<i>Treatment * Background</i>		2.92	0.089
<i>Treatment * ITI</i>		1.45	0.230

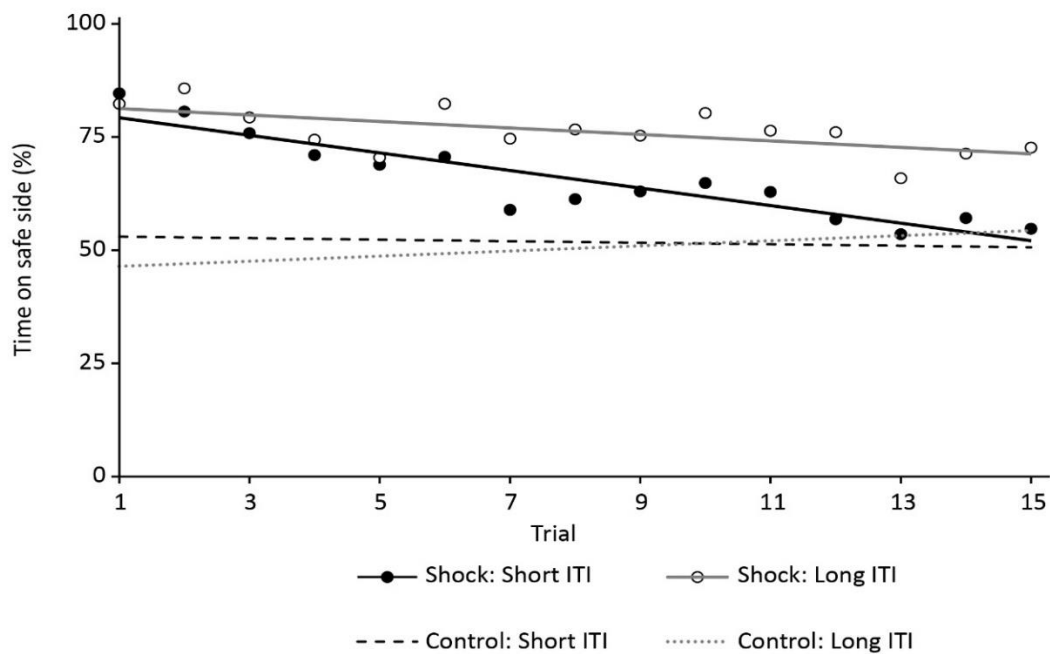


Figure 3. Avoidance during training by treatment (shock or no-shock control) and ITI (short or long). Performance is measured as the mean time (%) spent on the safe side per trial 300s trial. Higher values reflect better performance. Shocked spiders outperformed control spiders overall, and shocked spiders in the long-ITI group outperformed shocked spiders in the short ITI group ($P<0.0001$; see text & Table 2 for statistics).

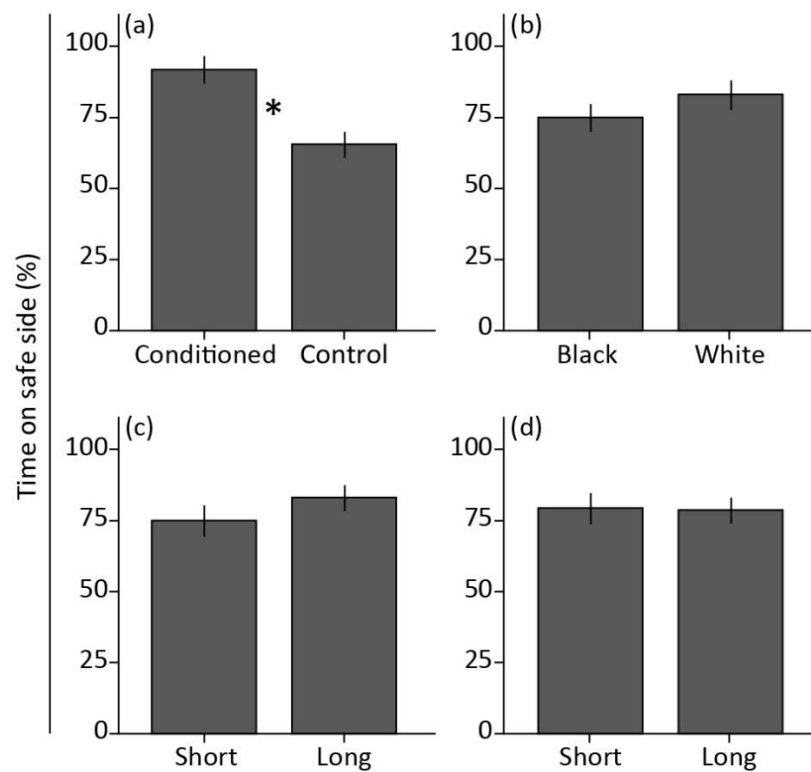


Figure 4. Avoidance during test by each factor. Performance is measured as the mean time (%) spent on the safe (safe) side per 300s trial. (a) Treatment (shock or no-shock control); (b) Background (black or white); (c) ITI (short or long) and (d), PTI (short or long). Asterisks denote significant differences between groups ($P < 0.001$). Graphs b-d reflect all spiders (including control spiders). Treatment * background and treatment * ITI were also insignificant (see Table 4).

Discussion

Exposure to noxious stimuli should lead to protective changes in behaviour that reduce future exposure (Magee & Elwood, 2013). In the present study, salticids exposed to a mild electric shock stimulus learned to associate the stimulus with a static visual background cue.

Performance in conditioned spiders progressively improved over the first 300s trial compared to controls, with conditioned spiders spending the majority (83.5%) of the trial on the safe side. While conditioned spiders outperformed controls throughout all stages of testing, the extent to which they did so varied with ITI and background. Spiders trained with a long ITI maintained a constant level of performance throughout training, whereas spiders trained with a short ITI maintained their performance only when the safe side was black. When the safe side was white, performance worsened steadily over time.

There are differences in the ease with which animals acquire ecologically relevant associations. In nature, *S. incana* shelter and build nests in shaded cavities under loose strips of bark on Eucalyptus trees, and may consequently be more prepared to associate black with safety than white. The idea that animals are adaptively prepared to learn some contingencies better than others is known as preparedness (Seligman, 1970), and is typified by a classic taste aversion experiment by Garcia & Koelling (1966) with rats. In this experiment, rats were simultaneously exposed to three CS: sweet water, light and sound. For some rats, these stimuli were paired with X-rays, which induced nausea, and for others they were paired with electric shock. When subsequently tested with the individual CS elements, the rats showed clear association between taste and nausea, and between the audio-visual cues and shock, but failed to learn the other elements in each case. Animals are frequently confronted with an abundance of sensory information, but have the capacity to process only a fraction of it (Dusenbery, 1992). A biological readiness to associate ecologically meaningful stimuli together while ignoring others would help filter pertinent information for processing, and could confer a survival advantage by directing attentional resources where they are most required (Mendl, Burman, Parker, & Paul, 2009).

While it is ecologically plausible that *S. incana* more readily learn that black predicts safety than white, this factor alone cannot explain our results, since spiders that were subjected to shock and a long ITI learned equally about both backgrounds. Our results also cannot be explained by a pre-existing bias towards the black background, as spiders spent comparable amounts of time on black and white sides during the non-shocked pre-training trial. Biases can change over time within an experiment as a result of handling or exposure to stimulus

alternatives (Cunningham, Ferree, & Howard, 2003), but here an acquired bias towards the dark background is unlikely as spiders in the control group spent a comparable amount of time on the black and white sides throughout the 15 training trials. Further, it is unlikely that performance was influenced by extra-arena visual cues, as the arena was rotated in a random direction each trial.

Temporal factors can play an important role in learning. For example, honeybees trained on a visual discrimination task successfully selected a simplified version of a trained pattern after a long training but not after a short one; that is, generalization was dependent on the duration of exposure (Stach and Giurfa 2005). Here we observed an effect of ITI during training that was consistent with the trial spacing effect reported previously for other taxa (e.g., Deisig, Sandoz, Giurfa, Lachnit, 2007; Sunsay, Stetson, Bouton, 2004, but see the introduction for a more detailed list). Spiders in the long ITI group outperformed spiders in the short ITI group over the course of 15 training trials. However, two important factors should temper interpretation of these findings as evidence for the trial spacing effect. First, the intervals used in this study were longer than those commonly used in the arthropod literature (Menzel et al., 2001). In memory spacing experiments with bees and moths, subjects are normally restrained for the duration of training and remain within the experimental context throughout all trials (and ITIs), permitting stimuli to be presented after very short intervals. However, since restraint can hamper mechanosensory feedback (e.g., flies: Fry, Sayaman, & Dickinson, 2003; mammals: Ravassard et al., 2013), can be stressful to subjects (Rodríguez Manzanares, Isoardi, Carrer, & Molina, 2005) and can impede acquisition (Locurto, Travers, Terrace, & Gibbon, 1980), we preferred here to utilize a free-moving paradigm that precluded very short ITIs due to the handling time associated with removal and return of subjects to the arena. Second, the difference between ITI groups varied strongly with background, such that spiders subject to a long ITI exhibited stable performance throughout training whereas spiders subject to a short ITI only exhibited stable performance when the safe side was black. The divergence in performance in the black and white background conditions observed in spiders subject to the short ITI but not the long ITI indicates that some other aspect of the training experience must contribute to the differences observed between ITI groups.

Stressful events can substantially modulate learning and memory (Martens et al., 2007). Honeybees exposed to simulated attack later display state-dependent modulation of stimulus categorization. Likewise, consistent application of a physical stressor leads to alterations in memory formation in the pond snail *Lymnaea stagnalis* (Martens et al., 2007). In the present study, both the electric shock US and handling associated with each trial could be considered

stressors. While the electric shock stimulus was identical between short and long ITI groups, handling differed. Spiders in the long ITI group were handled once every 24h and returned to their home cages between trials, whereas spiders in the short ITI group were collected into a vial between trials and handled once every 10 minutes for close to 3h. Even gentle handling can cause long-lasting behavioural and physiological changes. For example, in the vineyard snail *Helix pomatia*, gentle transport between laboratory rooms caused cardiac alterations that lasted up to 36h (Renwanz & Spielvogel, 2011), and similar results have been documented in diverse taxa including crustaceans (Listerman, Deskins, Bradacs, & Cooper, 2000), fish (Gomes, 2007), lizards (Cabanac & Cabanac, 2000) and mammals (Dantzer & Mormède, 1983). Considering that differences in an animal's internal state can alter how information is interpreted and acted upon (Pompilio, Kacelnik, & Behmer, 2006), the differing tendencies to associate black or white backgrounds with safety may relate to alterations in stress and behaviour induced by frequent handling of spiders in the short-ITI group.

While both background and ITI were significant determinants of performance during training, it is interesting to note that neither contributed significantly to performance in the final, no-shock test session. During this session, shocked spiders spent significantly less time on the side associated with the previously shocked background than controls, indicating that they had retained the CS-US association over the course of the RI. Further, this finding clarifies that the behaviour that we observed during training was indeed learning, and not simply an avoidance response to the aversive stimulus within each trial, or the result of nociceptive sensitization (Crook & Walters, 2011). It is particularly surprising that the duration of RI – either 10 min or 24h – did not contribute to performance in the test session. These results suggest that the 15 training sessions were sufficient to induce memory formation that lasted 24h, although it is unclear what stage of memory this is (medium or long-term) and whether fewer sessions would have produced a similar finding. In honeybees, multiple learning trials facilitate consolidation into long-lasting memory (Menzel et al., 2001) which develops after 24h (Menzel, 1999) and is interrelated with ITI, such that retention is not affected by ITI when tested after 24h but is when tested after 4 days (Gerber et al., 1998). The present study provides a platform for further detailed research into the time course of retention in spiders, testing memory after systematically varying the number of training trials and the duration of the inter-trial and retention intervals.

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4 JUMPING SPIDERS IGNORE PREDICTIVE VISUAL INFORMATION IN AN ACTIVE AVOIDANCE CONDITIONING TASK

Abstract

Associative learning is virtually universal across the animal kingdom, and has been well characterized in commonly used invertebrate model taxa. Jumping spiders are particularly well suited for studies of cognition, but the relative weighting of available cues in learning and memory has not been explored to the same extent as model taxa. In the present study, we use an active avoidance conditioning task to assess the relative contribution of visual environmental cues, a visual conditioned stimulus, an electric shock unconditioned stimulus, and the contingency between these stimuli to learning and memory in a jumping spider. We find that spiders learn to escape and avoid electric shock, and retain this task in short-term memory for at least 10 minutes. Spiders appear to do so by learning an operant avoidance and escape response while ignoring visual information provided by external positional cues and the associated visual stimulus. These results are very surprising given the extent to which vision is known to mediate behaviour and decision making in jumping spiders.

Introduction

Associative learning is virtually universal across the animal kingdom, and enables adaptive responses to predictive relationships or contingencies between events (Papini, 2008; Thorpe, 1956). Invertebrates have become increasingly popular as subjects for studies of associative learning, as they possess relatively simple nervous systems that have significant molecular overlap with vertebrate systems (Bailey et al., 1996), and are more amenable to analyses at cellular and behavioural levels (Abramson, Armstrong, Feinman, & Feinman, 1988). Invertebrates are the most abundant, speciose and diverse animal group on the planet and, despite possessing generally simple nervous systems, exhibit a broad repertoire of associative learning abilities (Dukas, 2008; Hollis & Guillette, 2011). Prominent amongst these abilities is the capacity to discriminate, learn about, avoid and escape aversive stimuli that are encountered in the environment (Sneddon, 2009).

Avoidance learning in invertebrates has primarily been studied using two paradigms; passive avoidance and active avoidance (Agarwal et al., 2011). In the passive avoidance paradigm, subjects learn to withhold behaviours that produce aversive stimuli, whereas in the active avoidance paradigm, subjects learn to express specific behaviours that reduce exposure to aversive stimuli. Unlike passive avoidance tasks, active avoidance tasks almost always utilize a discriminative stimulus (the conditioned stimulus; “CS”) which is paired with an aversive stimulus (unconditioned stimulus; “US”), providing additional predictive information to the subject (Olton, 1973). As with invertebrate cognition research in general, active avoidance studies have focused heavily on a narrow range of animal models (e.g., bees: Abramson, 1986; earthworms: Abramson & Buckbee, 1995; and sea slugs Davis et al, 1980) and this trade of breadth for depth has precluded a truly comparative perspective on the generalities and evolutionary roots of learning (Perry, Barron, & Cheng, 2013). One of the primary goals of comparative cognition is to trace the evolutionary development of cognition by comparing the mechanisms employed by different taxa in solving analogous computational tasks (Soto and Wasserman, 2010). Here, we broaden the scope of comparison by assessing active avoidance learning in an arthropod group that shows substantial promise, but has been historically underrepresented in the comparative cognition literature.

Spiders are highly diverse and speciose, with more than 40 000 described species (Platnick, 2010), and inhabit nearly all terrestrial environments (Wise, 1993). Among spiders, jumping spiders (Salticidae) are particularly well suited for studies of cognition (Jakob, Skow, & Long, 2011). ‘Salticids’ have exceptionally acute vision in their large forward-facing

‘primary’ eyes, and sensitive motion-detecting secondary eyes that provide almost 360° coverage (Homann, 1928). Consistent with their exceptional visual abilities, salticids are well known for complex, visually mediated behaviour (Jackson & Cross, 2011). Salticids rely largely on vision for navigation (e.g., Hoefler & Jakob, 2006), discrimination of environmental features (e.g., Bednarski, Taylor, & Jakob, 2012), hunting (e.g., Jackson & Pollard, 1996), and communication (e.g., Elias, Maddison, Peckmezian, Girard, & Mason, 2012). Further, salticids have been shown to exhibit avoidance learning in a variety of contexts, including rapid avoidance learning of unpalatable prey (Edwards & Jackson, 1994), avoidance of particular colours associated with heat (Nakamura & Yamashita, 2000), and avoidance of visual stimuli associated with electric shock (Bednarski et al., 2012).

Animals can attend to a vast diversity of cues during avoidance training, drawing on available associations to guide future adaptive responses (Olton, 1973). Providing a rare insight into the use of multiple cues during avoidance learning in a salticid, Skow & Jakob (2006) found that *Phidippus princeps* learned cues from background environment alongside cues from toxic prey, leading to increased avoidance when environmental cues matched those present during learning. However, the relative weighting of available visual and non-visual cues in salticid avoidance learning has not been explored. In the present study, we use an active avoidance conditioning task to assess the relative contribution of a visual CS, visual environmental cues, an electric shock US and the CS-US contingency to learning and retention in a salticid, *Servaea incana*.

Materials and methods

Collection and maintenance of spiders

Adult female *S. incana* were collected from the trunks of Eucalyptus trees in Sydney, Australia. Spiders were maintained in a controlled environment laboratory (24-26°C; 60-70% relative humidity; 11:1:11:1 light:dusk:dark:dawn starting at 07:00 AEST) where they were individually housed in 1.125L plastic cages (11x11x12cm tall, with a mesh-covered 80 mm diameter window for ventilation) containing a folded sheet of paper (2x3cm) that was shaped as a ‘tent’ under which spiders could shelter and build nests. Spiders were fed weekly on an alternating diet of two houseflies (*Musca domestica*) or two Queensland fruit flies (*Bactrocera tryoni*). Flies were removed 5 days prior to testing, since previous work with this species has demonstrated that hungry spiders are more responsive to moving stimuli than are sated spiders (Zurek,

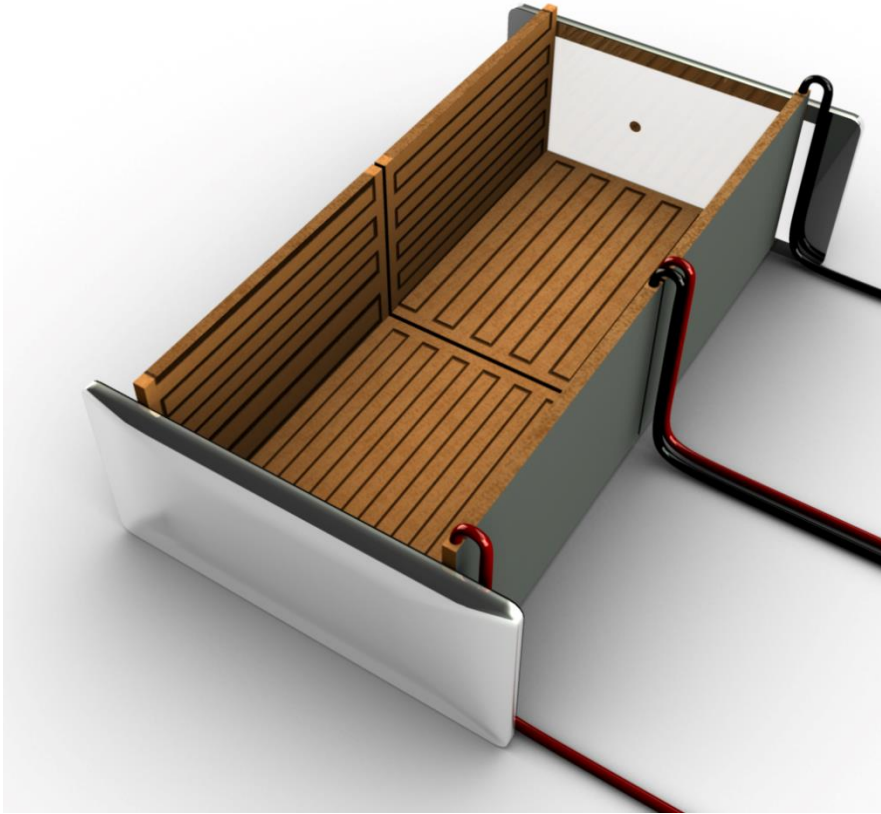


Figure 1. Schematic representation of the conditioning box apparatus. The floor and the two long walls of the box comprised two independently controlled electric shock platforms. Two iPod 4 Touch video screens formed the walls at either end of the box. Spiders in the experimental treatment received contingent CS/US pairings in one end of the box, alongside stable environmental cues. Spiders could move freely between the stimuli-present and stimuli-absent sides of the box throughout the five-minute trial. The experimental treatment was compared to each of four control treatments, which individually isolated the environmental cues, CS, US and CS-US contingency (see text).

Taylor, Evans, & Nelson, 2010). Supplementary moisture was provided by lightly misting each cage with a spray bottle once each week. No experiments were carried out during the first or last hour of the laboratory light phase.

Apparatus and visual stimulus

Spiders were trained and tested in a rectangular conditioning apparatus (50mm wide x 150mm long x 50mm tall, Fig. 1), in which a CS and an US could be controlled independently on each side. The top of the arena was a transparent Perspex lid through which all trials were video-recorded (Panasonic HDC-SD900 video camera). The floor and walls along the length of the arena were constructed from two 75mm x 150mm electric shock platforms that were ‘folded’ in thirds lengthwise and placed side by side. Platform design and construction were as described in Peckmezian & Taylor (2015), so are described here in abbreviated form. The platforms comprised a grid-like pattern of parallel copper bars chemically etched on blank printed circuit board backed with epoxy fiberglass laminate (Jaycar Electronics, Australia). Alternating bars of the copper shock platform were wired to the positive and negative terminals of a DC power supply (Sanke Electrical Co., Ltd., China). When the power supply was active, 30V intermittent (3s on: 3s off) electric shock (the US) was delivered to spiders each time they bridged the gap between parallel copper bars, completing the circuit. Due to the narrow gap between opposite-charged bars, failure to complete the circuit was very rare. Boards produced with this method are durable, easy to produce and clean, and highly replicable (Peckmezian & Taylor, 2015).

Previous studies have established that salticid responses to video and animated stimuli closely resemble their responses to natural stimuli (Clark & Uetz, 1990; Harland & Jackson, 2002; Bednarski et al. 2012). In recent psychophysics work with *S. incana*, video dot stimuli reliably elicited orientation turns (Zurek et al., 2010). We designed a video dot stimulus (CS) with attributes similar to the stimulus used by Zurek et al. (2010). The stimulus was a black dot with a diameter of 4° (20 pixels, from the midline of the arena), positioned 3cm above the substrate on a featureless white background. The dot moved continuously from one side of the screen to the other (7.5cm screen width) at a speed of 9°/s and was constructed in Adobe Aftereffects. The CS played on one of two iPod Touch 4 video screens (Apple, Inc) that were placed at either end of the arena.

Active avoidance paradigm

Individual spiders were randomly assigned to one of five treatment groups (N=15/group). The basic experimental procedure was the same for all groups. Spiders were collected from their home cages into a transparent 15mL plastic specimen jar, and then gently lowered into one side of the apparatus. During training for the experimental treatment ('Exp'), the CS was playing on this side from the start of each trial while the US was activated (same side) after a 15s delay. Stimuli were presented on the same side of the arena throughout all trials for a given spider (individual side assignment was randomized). Each trial ran for 5 minutes, during which spiders could move freely between the two sides. At the end of each trial, spiders were gently returned to their holding vials for a 10-minute inter-trial interval. Each spider underwent 5 training trials followed by a 10-minute pre-test interval and a single, no-US test session. The apparatus was cleaned between trials: the shock platforms were cleaned with damp paper towel dipped in warm soapy water to remove silk dragline and pheromone cues (Jackson, 1987), and the iPod video screens were wiped with a dry paper towel. The arena was placed on a table in the controlled environment laboratory and external visual cues (tall shelves on one side, wall on the other, and video camera directly overhead) were intentionally left unobscured.

The Exp group was compared to each of 4 control groups to isolate effects that were dependent on the contingent presentation of CS and US stimuli, in contrast to the effects of either stimulus alone, extra-arena cues, or non-contingent stimulus effects. Although the day, time and order of testing were randomized across the Exp and control groups, each Exp versus control group comparison was conducted separately.

To determine the degree to which performance was guided by attention to extra-arena contextual cues, the side of the arena containing the CS was randomized across trials for control group 1 (external), so that external cues were unreliable predictors of reinforcement. To determine the degree to which the CS contributed to learning, we omitted the CS for control group 2 (US-only). Similarly, to determine the degree to which the US contributed to learning, we omitted the US for control group 3 (CS-only). Finally, to determine whether spiders were learning predictive stimulus-response contingencies, control group 4 (yoked control) received non-contingent US presentation. Each yoked control spider was randomly assigned a 'master' spider from the Exp group. The yoked control spider received the US whenever its master did, irrespective of its location or responses in the arena, and was thus not given the opportunity to learn because the contingencies between the CS and US were absent

(Pritchatt, 1968). The US was activated and deactivated on both sides of the arena simultaneously to ensure that spiders experienced the US regardless of which side of the arena they were on. The CS played on one side of the arena only, as in the master Exp treatment.

In each case, we predicted a lower level of avoidance in the control group compared to the Exp group if the variable in question played a role in avoidance learning. Except for the specific variations outlined above, control trials were run identically to the Exp trials. All trials were video recorded from above and subsequently scored from these recordings. If spiders crossed the midline within the first 15s of the trial (before the US was initiated), we scored this as avoidance. We also recorded their latency to first switch sides (values greater than 15s reflect escape behaviour), as well as the total time spent on the US side.

Statistical analyses

Statistical analyses were performed using SPSS, version 16 (SPSS Institute). We performed 4 sets of identical analyses comparing the Exp group to each control group. Within each analysis, we ran an independent-samples t-test to detect differences between the experimental group and each control group on each response measure (avoidance, latency and time on shocked side) at two time points (mean training and test). Because our hypotheses were all directional, all tests were one-tailed, and the significance threshold for all analyses was $P < 0.05$.

RESULTS

On average, 41% of spiders in the Exp group avoided the onset of the US by switching sides within the first 15s during the training phase, and 60% during test. The mean latency to switch sides after US onset was 48.2s during training trials and 57.4s during the no-US test session. The mean time spent on the stimulus-present side (the side that had either the CS, US, or both, depending on group) over the course of a trial was 90.1s during training and 111.7s during test.

The external and US-only control groups were both statistically indistinguishable from the Exp group on each measure during training and test (external: $P > 0.15$ and US-only: $P > 0.35$ in all cases) (Fig. 2). In contrast, significant differences were found in nearly all measures for the CS-only group. During training, a higher proportion of spiders in the Exp group crossed the midline within the first 15s than did spiders in the CS-only group, in which a US did not ensue ($t = 2.99$, $df = 28$, $P = 0.003$). Spiders in the Exp group also had a shorter

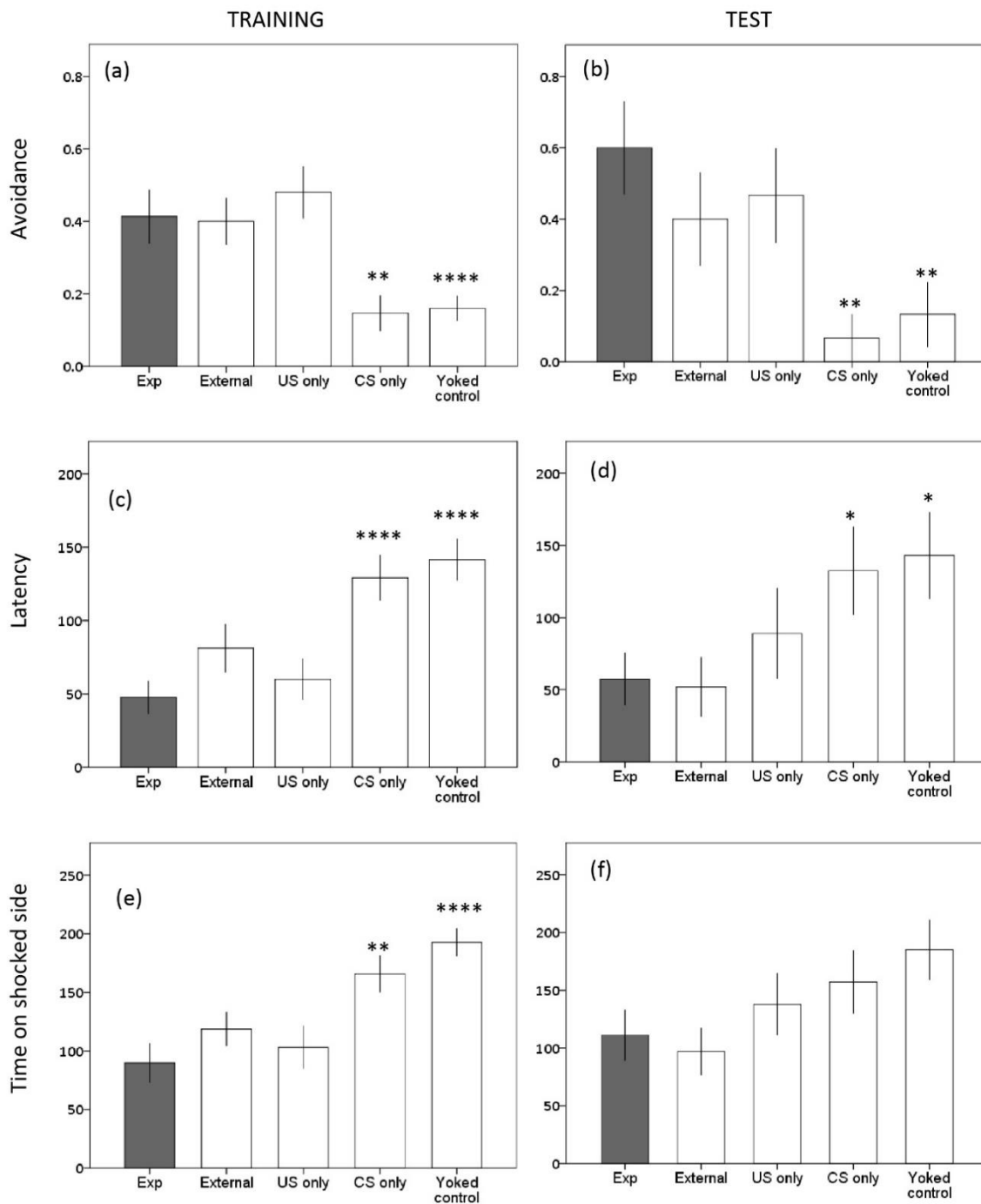


Figure 2. Summary of results from all analyses. All values reflect means. Avoidance was scored when spiders crossed the arena midline within the first 15s of the trial, prior to the onset of the US. Latency refers to the latency to cross the arena midline from the start of the trial; values >15s reflect escape after the US had been initiated. Time refers to the total amount of time spent on the starting (stimuli-present) side (s). Groups: Exp – the experimental group, received contingent CS-US pairing with stable environmental cues; External – received contingent CS-US pairing with unpredictable environmental cues; US-only – received the shock US only (no CS); CS-only – received the visual CS only (no US); and Yoked control – received *non-contingent* CS-US pairing governed by the behaviour of a yoked spider from the Exp group. Bars reflect group means (N=15/group), and asterisks denote significant differences between the Exp treatment and each control treatment: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

latency to leave the stimulus-present side ($t=-4.20$, $df=28$, $P<0.0001$) and spent less time on this side over the course of the trial ($t=-3.31$, $df=28$, $P=0.002$). During the no-US test session, a higher proportion of spiders in the Exp group crossed the midline within the first 15s ($t=2.68$, $df=28$, $P=0.005$) and had a shorter latency to leave the stimulus-present side ($t=-2.12$, $df=28$, $P=0.022$), though there was no significant difference in the total amount of time spent on the stimulus-present side ($t=-1.31$, $df=28$, $P=0.101$).

Spiders in the Exp group also differed significantly from yoked controls. During training, a higher proportion of spiders in the Exp group crossed the midline within the first 15s than did spiders in the yoked control group ($t=3.09$, $df=28$, $P<0.0001$). Spiders in the Exp group also had a shorter latency to leave the stimulus-present side ($t=-4.98$, $df=28$, $P<0.0001$) and spent less time on the stimulus-present side over the course of the trial ($t=-5.03$, $df=28$, $P<0.0001$). During the no-shock test session, a higher proportion of spiders in the Exp group crossed the midline within the first 15s ($t=2.93$, $df=28$, $P=0.007$), had a shorter latency to leave the stimulus-present side ($t=-2.44$, $df=28$, $P=0.021$) and spent less time on the side previously associated with the stimulus ($t=-2.01$, $df=28$, $P=0.033$).

DISCUSSION

Servaea incana jumping spiders were found to learn an active avoidance task in which an electric shock US is reliably associated with a region in space. Compared with a control group that received a conditioned stimulus (CS) alone, spiders in the Experimental group, for which the US was associated with a CS during training, avoided the onset of the US during training trials, had a shorter escape latency, and spent less time in the US-present side of the arena overall. Similar differences in avoidance and escape latency were found during the no-US test session, demonstrating that spiders in the Exp group held this task in short-term memory for at least ten minutes.

Studies of avoidance learning are often limited by lack of control for the potential effects of the aversive stimulus independently of the behavioural contingency. Differences in an animal's internal state can change how information is interpreted and acted upon (Pompilio, Kacelnik, & Behmer, 2006), and aversive stimuli (such as electric shock) can produce diverse physiological changes (Eisenstein, 1997). Here, our comparison of Exp and yoked control spiders, which were treated identically apart from the temporal relationship of the reinforcer to avoidance and escape behaviour during the training period (Eisenstein & Carlson, 1997), allowed us to demonstrate that the changes observed were due to the temporal

relationship between stimuli and response rather than effects associated with exposure to the stimuli themselves, such as sensitization. There were substantial differences in performance of Exp and yoked control groups, with yoked controls, having significantly lower tendency to move away from the stimulus-present side quickly when trials started during the no-US test session (see Fig 2). Overall, the performance of spiders in the yoked control group was very similar to that of the CS only group, which received no US during the training period.

Contrary to our expectations, performance was not influenced by the visual information of a moving dot (CS) presented on screens at the US-present end of the arena. Spiders in the control group that received the US alone performed as well as spiders in the Exp group that received a predictive pairing of the CS and the US, in terms of avoidance, escape latency, and the total amount of time spent on the shocked side during both training and test. Consequently, performance cannot be attributed to the transfer of the US's aversive properties to the CS. We found similar results in spiders that received contingent CS/US pairings with unpredictable contextual cues. During training and test, spiders in this group performed as well as spiders in the Exp group that had received predictable contextual cues. As with the results from the US-only control group comparison, this finding suggests that spiders are disregarding reliable and predictive visual information that could facilitate avoidance learning. Contextual cues that remain constant during learning often become associated with the reinforced stimuli (Tulving & Thomson, 1973) and can aid in decision making by guiding expectations within complex environments (Chun, 2000). There are numerous examples of invertebrates utilizing contextual cues in varied forms of decision making (Collett, Fauria, Dale, & Baron, 1997; Dale, Harland, Manning-Jones, & Collett, 2005; Liu, Wolf, Ernst, & Heisenberg, 1999; Weiss & Papaj, 2003), and they are generally thought to be a useful source of information. However, there may be costs and constraints associated with attending to contextual cues. The jumping spider *Phidippus princeps*, for example, learns contextual cues alongside cues about unpalatable prey, and subsequently shows avoidance of these prey items only when in the context where the association was learned (Skow & Jakob, 2006). When placed in a novel context, spiders attacked prey items they had earlier learned to avoid, reflecting a cost of attending to context. If processing constraints limited the capacity for spiders to learn the aversive properties of both the stimulus and the context, greater benefit would seem to accrue from learning about the stimulus (in this case, the prey item) alone rather than the context alone.

In the present experiment, spiders appear to acquire a tendency to avoid an electric shock US through learned avoidance of the aversive electric shock stimulus alone. What cues

they are attending to in order to do so are unclear. One possibility is that spiders are attending to the location itself, and not learning about the moving dot CS because location information is sufficiently predictive. Similar findings have been reported in rats that were trained on an active avoidance task and preferentially associated shock with a given location, rather than with concomitant visual or auditory stimuli (Olton & Isaacson, 1968). Alternatively, spiders may be acquiring a simple kinaesthetic rule such as, “walk forward” or “move from the place where you start”, upon placement in the start chamber. The vestibular and kinaesthetic feedback that occurs alongside movement away from harmful stimuli has itself been postulated to be rewarding, and can act as a form of reinforcement independent of other cues (Masterson & Crawford, 1982). For example, shore crabs discriminate between two shelters – one paired and the other unpaired with electric shock – on the basis of movement information alone, ignoring visual stimuli associated with the shocked shelter (Magee & Elwood, 2013). Learning to associate an action with an outcome, as appears to be the case here, fits the functional definition of operant conditioning, and is thought to be more complex than classical conditioning as behavioural responses are voluntary rather than reflexive (Perry et al., 2013).

Animals do not learn everything equally. When learning colours associated with food sources, bees can learn some colours after a single exposure, but require extended training to learn others (Giurfa & Menzel, 2001; Gould, 1986). At the same time, colours that are readily learned as indicators of food reward or the hive entrance are not learnt in the context of escape behaviour (Menzel & Greggers, 1985). Further, the presence of an aversive stimulus can play a significant modulatory role in directing attention, discrimination, and was it ultimately learnt (Avargues-Weber, de Brito Sanchez, Giurfa & Dyer, 2010). Psychophysics research with *S. incana* found that visual stimuli with characteristics similar to that used in the present study reliably elicited orientation (Zurek et al., 2010), and a recent neurophysiological study demonstrated that small, moving video stimuli elicit robust firing patterns in the salticid brain (Menda, Shamble, Nitzany, Golden, & Hoy, 2014). Further, salticids have been demonstrated to attend to visual environmental cues such as the extra-arena cues available here (Skow, 2007). As such, it is unlikely that visual stimuli as a whole are poor predictors of aversive events for salticids, but rather that some characteristic of the stimuli or their relationship may be un conducive to avoidance learning. Further investigation is required to determine the circumstances or cue parameters that promote attention to visual information in salticids, and those that preclude it.

Taken together, our results demonstrate that *S. incana* can learn to escape and avoid electric shock, and retain this task in short-term memory for at least 10 minutes. Spiders appear to do so by learning an operant avoidance and escape response while ignoring visual information provided by external positional cues and an associated visual CS. These results are very surprising given the extent to which vision is known to mediate behaviour and decision making in jumping spiders.

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5 A VIRTUAL REALITY PARADIGM FOR THE STUDY OF VISUALLY MEDIATED BEHAVIOUR AND COGNITION IN SPIDERS

Abstract

Jumping spiders (Salticidae) are well known for their unique, high-acuity visual system and complex, visually mediated behaviour. To overcome the limitations of video playback and other open loop systems that are currently available for the study of visually mediated behaviour in jumping spiders, we developed a closed-loop, virtual reality (VR) system in which a spider on a spherical treadmill walks through a projected 3D world that updates in real time in response to its movements. To investigate VR as an experimental technique for spiders as well as and validate it as a proxy of the real world, we conducted two experiments to assess whether individual behavioural tendencies and learning transferred from real to virtual environments. In the first experiment, we examined transference of individual behaviour tendencies (spontaneous locomotion and dark/light preference) between real and VR environments, and found that individual differences were conserved. In the second experiment, we investigated transference of beacon-learning tasks between real and VR environments. We found that spiders that had learned a beacon-nest site association in the real world tended to express similar associations in the virtual world. Virtual reality offers great promise as a new tool to explore the cognitive processes underlying vision-mediated learning, memory and navigation in jumping spiders.

Introduction

Virtual reality (VR) is increasingly used in the behavioural sciences to overcome the constraints of traditional open-loop systems. In the context of animal behaviour, VR refers to a simulated environment that is sensed by the animal and is updated by the animal's actions (Dombeck & Reiser, 2012), giving an experience of immersion within the simulation (Sherman & Craig, 2002). VR, by design, is a closed-loop system, in which responses to virtual stimuli are tracked and used to update the next 'view' of the virtual environment in real time. Since virtual environments are typically implemented as automated, computer-controlled systems, virtual stimuli and world features can be designed to be 'photo-real', abstract, or even selectively modified versions of real-world stimuli. In this way, VR can provide a valuable bridge between ecological validity and experimental control, supporting rich, multisensory environments alongside precise control of experimental variables (Bohil, Alicea, & Biocca, 2011).

Understanding how 'simple' invertebrate systems handle the complexities of daily life can inform us on the cognitive processing requirements that are necessary and sufficient to accomplish a given task. Virtual environments, in various forms, have been utilized to dissect diverse aspects of invertebrate behaviour and physiology. Perhaps the most common form has been the flight simulator for tethered flying insects, where the subject's intention to turn in response to visual motion is measured with a torque meter or wing beat analyser and used to update a virtual environment in real time. Using this approach, researchers have studied optomotor responses (Bender & Dickinson, 2006), flight kinematics (Gray, Pawlowski, & Willis, 2002), navigation (Gotz, 1987; Reiser & Dickinson, 2010), visual learning (Dill, Wolf, & Heisenberg, 1993; Wolf et al., 1998), decision making (Brembs, 2011) and operant and classical conditioning (Brembs, 2000). Systems have also been designed for tethered walking invertebrates (Dahmen, 1980; Strauss, Schuster, & Götz, 1997; Takalo et al., 2012), and recently, for subjects in free flight (Fry et al., 2004; Fry, Rohrseitz, Straw, & Dickinson, 2008).

Jumping spiders (Salticidae) are well known for their unique visual system and complex, visually mediated behaviour. In contrast to the compound eyes of insects, salticids possess four pairs of functionally specialized 'camera eyes', each of which use a single lens to project an image on to the retina (Land, 2005). Their large forward-facing principal eyes feature colour vision (Land, 1969; Peaslee & Wilson, 1989), depth perception (Nagata et al., 2012), and a retina with spatial acuity that greatly exceeds any animal with eyes of comparable size (Land & Nilsson, 2012). Smaller motion-detecting secondary eyes collectively enable the spider to view

nearly 360° of its surroundings (Land, 1971). By separating spatial acuity from motion detection, the modular design of the salticid visual system enables remarkable visual feats while minimizing costly increases in eye size (Land & Nilsson, 2012; Laughlin, de Ruyter van Steveninck, & Anderson, 1998). As a comparison, the high-resolution compound eyes of libellulid dragonflies combine these features in the same eye, but to achieve this their eyes are larger than the entire body of most salticids (Labhart & Nilsson, 1995).

Using optical cues alone, salticids can identify prospective mates and rivals, classes of prey and enemies, and features in their environment (Harland & Jackson, 2004; Jackson & Pollard, 1996; Tarsitano & Jackson, 1997). Vision is also central to intraspecific communication, such as during elaborate courtship displays (Elias, Land, Mason, & Hoy, 2006; Elias, Maddison, Peckmezian, Girard, & Mason, 2012), and during highly ritualized agonistic interactions between males (McGinley, Prenter, & Taylor, 2015; Taylor, Hasson, & Clark, 2001). Vision also plays a central role in cognition, with salticids relying on vision for complex decision-making (Jackson & Cross, 2013), learning (Jakob, Skow, Haberman, & Plourde, 2007; Nakamura & Yamashita, 2000; Skow & Jakob, 2006), discrimination and categorization of prey (Dolev & Nelson, 2014), trial and error problem solving (Jackson & Nelson, 2011) and navigation (Hoefler & Jakob, 2006; Tarsitano & Jackson, 1997).

Salticids respond to video images of prey, enemies and conspecifics in a manner that closely resembles their responses to natural stimuli. Clark and Uetz (1990) were the first to exploit this capability, pioneering the use of video playback for studies of spider communication. Video playback proved a significant advance over previous methods, such as mirrors, 2D static images and dummy lures, and has served as a valuable tool over the past 25 years (e.g., Bednarski, Taylor, & Jakob, 2012; Harland & Jackson, 2002; Menda, Shamble, Nitzany, Golden, & Hoy, 2014). The researcher can control the timing and features of a visual stimulus while ensuring that an identical stimulus is presented across trials and test subjects (D'Eath, 1998). However, while video playback offers many advantages over the available alternatives, it also shares some of the same constraints that have limited the utility of previous methods (and see Fleishman & Endler, 2000; Fleishman, McClintock, D'Eath, Brainard, & Endler, 1998 for general limitations). In particular, video playback is an open-loop system in which sets of stimuli are presented independently of the subject's responses. This lack of contingency between cue and response, as well as the absence of normal visual feedback to a mobile subject, interferes with the perception of depth and motion and ultimately hinders the realism of the simulation (Woo & Rieucou, 2011; Zeil, 2000). A method that closes the

feedback loop would offer significant advantages over traditional open-loop playback techniques.

The present study is the first to employ VR as an experimental approach to studying perception and cognition in salticids. In addition to detailing our VR methods, we validate VR as a representation of the real world (RW) through experiments demonstrating (1) consistency of individual differences across RW and VR contexts, and (2) transfer of associations learned in the RW to VR.

Methods

Source, Maintenance and Preparation of Spiders

Mature female *Servaea incana* jumping spiders (N=56) were collected from Eucalyptus trees in Sydney, Australia (Richardson & Gunter, 2012). Spiders were maintained in a controlled environment laboratory (24-26°C; 62-67% relative humidity; 11:1:11:1 light:dusk:dark:dawn starting at 07:00) where they were individually housed in cubic 1.125L plastic cages that had a 10cm diameter mesh-covered opening on one side for ventilation. Each cage contained a folded sheet of paper (2x3cm) that was shaped as a ‘tent’ under which spiders could shelter and build nests. Spiders were fed weekly on an alternating diet of two houseflies (*Musca domestica*) or two Queensland fruit flies (*Bactrocera tryoni*). Supplementary moisture was provided by lightly misting each cage with a spray bottle once each week. No experiments were carried out during the first or last hour of the laboratory light phase.

In preparation for virtual reality (VR) experiments, spiders were removed from their home cages and transferred in a 5ml plastic vial to a refrigerator (4°) where they were cooled until quiescent (2-3 minutes), then placed on a chilled granite block. A 2mm diameter neodymium magnet (0.4mm thickness, 8.5mg; approximately 13% of the average spider weight) was gently affixed to the dorsal carapace using a drop of dental cement (SynergyFlow A3.5/B3, Coltene Whaledent), taking care not to cover the eyes. The dental cement was cured with blue light from a LED dental curing light source (SDI radii plus, Henry Schein Dental). Spiders were then returned to their cages and allowed to recover for a minimum of 24 hours before being used in experiments. Spiders with magnets were maintained for 6 months following experimentation and we did not notice any changes in locomotion, behaviour or longevity in comparison to spiders without magnets.

At the start of each VR trial, spiders were gently lifted from their home cages using a magnetic pin and mounted so that they stood on a spherical treadmill (Fig. 1a). Movements of the treadmill were tracked and updated the virtual environment in real time (described below).

Following the completion of each VR trial, a strip of laminated paper was used to gently separate the neodymium magnet and magnetic pin, and each spider was returned to its home cage. Using pre-fixed magnets provided two significant advantages over direct tethering to a pin: (1) spiders could be tethered well in advance of a trial, and then have ample time to recover from stress associated with handling, and (2), each spider could be used repeatedly over days or weeks. The potential to use spiders repeatedly is valuable in this context since learning and memory trials typically occur over broader time scales than can be assessed within a single session.

VR System

A 3D-printed spherical treadmill was constructed using polylactide plastic. The treadmill comprised a semi-spherical chassis that held a 3.5cm diameter, 230mg expanded polystyrene ball, tracking sensors, and a clip above the ball to grip a magnetic pin that was attached to the magnet on the spider's carapace to hold the spider in place. The expanded polystyrene ball was supported by a constant, steady flow of air (Sparmax AT-250 mini-compressor; Comweld Medical air flow meter). The weight of the ball was unlikely to affect the spider's movement. The moment of inertia of the ball ($I_{\text{sphere}} = \frac{2}{5}m \cdot r^2$) was 112700 mg mm², which was 70 times greater than that of the spiders presumed moment of inertia (see Zurek & Nelson, 2012b for calculations); however, Land (1972) observed unimpeded movement in jumping spiders holding objects with moments of inertia 400 times greater than that of the spider. The spherical treadmill used here was similar to previously described optical mouse tracking systems (Harvey, Collman, Dombeck, & Tank, 2009; Takalo et al., 2012). Rotations of the ball were detected at a rate of 1000 frames/s using 4 PS/2 optical mouse (Logitech international) sensors (ADNS 2610 - Avago Technologies Ltd) positioned along the midline, with opposing pairs averaged to reduce measurement noise. The sensors connected to an Arduino Uno microcontroller board, in which motion (translation in X, Y and Z as well as rotation about the yaw, pitch and roll axes) was read by custom-written software. Commands were sent from the microcontroller board to a computer in ASCII format via a USB cable connecting to a virtual serial communication port at rate of 60 Hz (the closed-loop update rate).

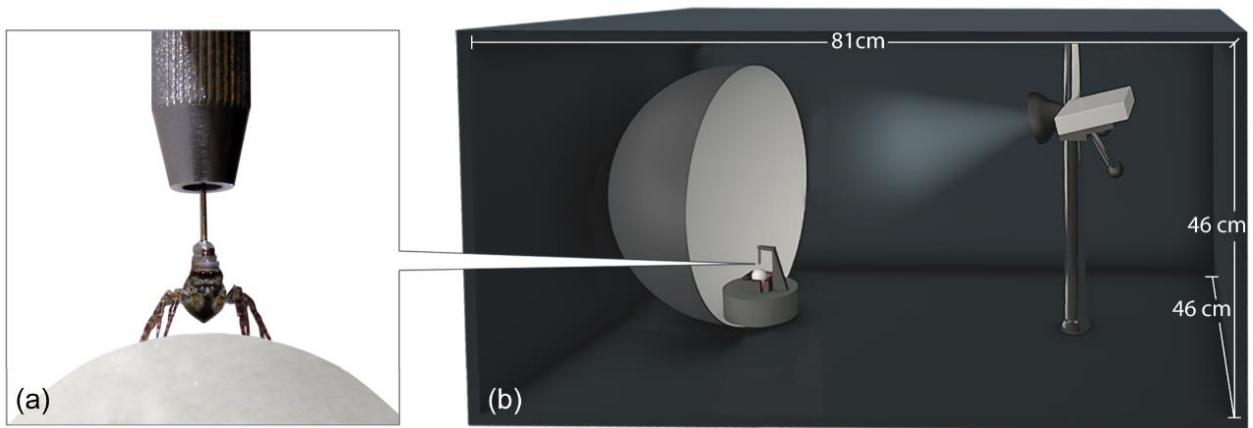


Figure 1. (a) A spider mounted above a spherical treadmill in preparation for a trial. A magnet is fixed to the spider's dorsal carapace and a magnetic pin holds the spider in position. (b) Schematic of VR system. Spiders are mounted above an air-supported spherical treadmill placed inside a hemispherical display screen. Dynamic computer generated 3D environments are front-projected onto the screen via a pocket projector with add-on fisheye lens, and spider movement updates the world in real-time (closed-loop).

The treadmill was positioned centrally in front of a hemispherical display screen (40cm diameter, painted with flat white epoxy enamel). The virtual environment was projected onto the inner surface of the hemispherical display screen using a LED pocket projector (Vivitek, Qumi Q2) with a supplementary fish eye lens (Zeikos 5.8cm, 0.43x wide angle). For spherical, projector based systems, a fish-eye design can produce higher image quality than traditional mirror-based systems (Takalo et al., 2012). This projector has a refresh rate of 60 Hz (at 1080p resolution) which is above the suggested flicker fusion frequency of salticid eyes (40 Hz, Forster, 1985). A plano-convex lens was used to focus the light from the projector into the fish eye lens (2.5cm diameter x 100mm focal length, Edmund Optics). The optics used here were chosen to maximize the ‘fill’ of the sphere whilst providing a focused image. A 3D-printed holder affixed the projector to its lenses, and this unit was mounted onto a movable arm approximately 25cm above the base of the screen and 50cm from the centre of the screen. The simulation projected 160° in both azimuth and elevation, covering the full field of view of both the forward-facing principal eyes and the anterior lateral secondary eyes, which have greater spatial acuity than the other laterally facing secondary eyes (Zurek & Nelson, 2012a). The entire system was enclosed in a light-tight black Plexiglas box to isolate spiders from distraction as well maximize projector brightness (maximum of 300 lumens in complete darkness) (Fig. 1b).

Care was taken in adjusting the spider’s position so that it could fully extend its legs and step normally, although jumping behaviour was inhibited by tethering. Most salticids, including *S. incana*, use stepping movements for normal locomotion in a manner similar to other spiders, and reserve jumping to span gaps, to escape enemies and for prey capture (Foelix, 2011). The inability to jump can be considered a limitation of tethered preparations in these circumstances, but is not expected to be a limitation under normal locomotion. Each spider was allowed 3 minutes to adjust to the setup with the screen dark before the simulation was started.

Visual Stimulus Design and Presentation

Three-dimensional simulated environments were created using the multi-platform game development software Unity3D (version 4.2.1; Unity Technologies) running on a Windows 7 operating system with an i7 processor and NVIDIA GTX 660 graphics card. On this system, Unity3D rendered at a rate of approximately 60 frames per second. We selected Unity3D as our software platform because it permits dynamic design of 3D stimuli and environments, allows full control of subject/object interaction and can be set up to communicate with

external devices. Simulated environments were created using a virtual 4-camera cube rig to reflect the position of the tested spider. The rig consisted of top (facing up) and bottom (facing down) cameras, as well as left and right cameras positioned at ± 45 degrees to the spiders forward-facing centre. The images output through the 4 cube faces resulted in a 90-degree perspective. In order to solve for a 180 degree fisheye projection, the images output from the 4 cube faces were applied as textures to 4 objects (known as 'meshes' in Unity), producing a single image pre-distorted to appear normal against the hemispherical projection geometry (Bourke, 2009).

Two closed-loop scenes were created for the experiments described below. Both environments contained a flat ground plane that was textured and coloured to resemble tree bark. In preliminary trials, we found that spiders displayed limited interaction with the simulation when the ground was uniform in appearance and lacking in texture. Lycosid spiders attend to the visual structure of the substratum while navigating home (Ortega-Escobar, 2011), and it is likely that substratum appearance is also relevant to salticid spiders, presumably by contributing to perceived ventral optic flow (Ortega-Escobar & Ruiz, 2014).

An interactive graphical user interface (GUI) was created to calibrate, run and play back our simulations. To calibrate distance in VR, we measured a single rotation of the treadmill ball and used this value to scale the virtual environment, so that 1 cm travelled on the treadmill in the RW equalled 1 cm travelled in VR. The system was recalibrated at the start of each test day. We recorded each spider's mean speed and total distance travelled for all trials. In addition, we observed spatial behaviour and responses to objects in a real-time replay function, which played back the trial from the spider's point of view, as well as a 'static replay' function, which displayed a line trace of the path taken throughout the trial. We also created a data collection tool that could be 'attached' to virtual objects to assist in our estimation of the level of interaction with these objects. The data collection tool allowed us to set a radius around a given object and subsequently record the latency to entering this radius (approach latency), number of entries and total amount of time spent in that region. Additionally, a 'dot product' function was included which, for each time point sampled, output a value ranging from -1 to 1, where -1 denotes that the spider is directly facing the object, 1 denotes that the spider is facing directly away from the object, and 0 denotes that spider is facing 90° away from the object. Multiple areas of interest could be set on a single object, and multiple objects could be set as objects of interest, collectively providing high-resolution output on the spider's orientation throughout the trial.

Experiment 1: Locomotor Activity & Light/Dark Preference Testing

To test whether inter-individual behaviour differences were conserved across contexts we measured spontaneous locomotor activity and light/dark preference of adult female spiders (N=32) in both RW and VR. Activity levels are often highly repeatable within species (Richardson 2001) and have been found to correlate with other behaviours in spiders (Pruitt, Riechert, & Jones, 2008). We started RW locomotor activity testing 24h after attachment of the magnet. Spiders were placed individually into glass Petri dishes (15cm diameter) and spontaneous locomotion was assessed for 10 minutes. Trials were video-recorded from above using a digital video camera (Panasonic HDC-SD900). The total distance travelled was measured from these recordings using LoliTrack 2.0 (Loligo Systems, Denmark). To test whether affixing or presence of the magnet affected mobility, locomotion of a randomly selected subset of 11 spiders was also recorded and analysed 24h before affixing the magnet. For these spiders, comparisons were made between pre- and post-magnet activity measures.

The light/dark preference test is commonly used to assess associative behaviours in invertebrates (Steenbergen, Richardson, & Champagne, 2011), including arachnids (Camp & Gaffin, 1999; Dos Santos, Hogan, & Willemart, 2013). RW dark preference testing began approximately 24h after locomotor activity testing with the same group of spiders. Spiders were placed in a rectangular arena (7.5cm wide x 15cm long x 7.5cm tall) constructed from white plastic board (Corflute®). The walls of the arena were lightly dusted with non-scented talcum powder to prevent spiders from escaping, and the top was open to permit video recording. A black piece of card stock was placed over one half of the arena creating a shaded 'dark' zone underneath. Trials lasted 20 minutes and were initiated by placing the spider in the centre of the 'light' zone. All trials were video recorded from above and subsequently scored from these recordings. We assessed the latency until spiders first entered the dark side of the arena, as well as the proportion of each trial spent on the dark side.

VR locomotor activity and dark preference were tested in a single, combined session approximately 24h following RW dark testing (see above for general procedures). The VR environment was a square arena (52cm wide, 22cm tall), designed to be either 'empty', containing no objects, or 'complex', containing 18 randomly placed pillars (3cm diameter, 8cm tall). The virtual arena was scaled to a larger size than the corresponding RW arena in order to reduce the probability of spiders reaching a virtual wall, which would not possess the properties of a physical wall and could create breaks in simulation 'reality' (Sherman & Craig, 2002). As in the RW, spiders began a trial in the centre of the 'light' zone. We measured the

relative ambient intensity contrast (watts per square meter per nanometer; $W/m^2/nm^{-1}$) of the light region compared to the dark region using a JAZ EL-200 portable spectrometer fitted with a spectralon diffused irradiance module (Ocean Optics Ltd., Dunedin, USA). The light region was approximately 5-6 times brighter than the dark region in both environments (real world 1:5.9; VR 1:4.9). Initially, spiders were assigned evenly to either VR condition, but we shifted this allocation in favour of the complex treatment (empty, $N=11$; complex, $N=21$) after observing abnormal behaviour in spiders in the empty condition (see Results).

Experiment 2: Beacon Learning Experiment

Salticids construct silken nests for shelter (Jackson, 1979) and commonly exhibit high nest site fidelity (Hoefer & Jakob, 2006; Jackson, 1988). An experiment was conducted with a new group of spiders to determine (a) whether spiders attended to beacons previously paired with a nest site, and (b) whether and how RW experiences influence behaviour in VR.

Following attachment of the magnet, spiders were placed in individual Petri dishes (1.5cm tall, 15cm diameter) and given 48 hours to build a nest under a folded sheet of paper (2x3cm) that was shaped as a 'tent'. On the second day in the Petri dish, and every second day following, spiders were provided one Queensland fruit fly as food. Spiders were then transferred in their nests to square individual 'home arenas' (52cm wide, 22cm tall) that they occupied for the duration of the experiment. Nest sites were placed 7.5cm from the middle of one wall. The arena walls were constructed from white plastic board (Corflute®) that was dusted with non-scented talcum powder to prevent spiders from escaping. Between trials, the arena was covered with a translucent polypropylene sheet fitted to the top of the walls that prevented spiders from seeing out of their enclosures while allowing diffuse illumination. Spiders were divided into two groups based on whether a beacon was present. 'Beacon spiders' ($N=12$) had a beacon (a red pillar or green cross) placed immediately behind their nest site, while 'No Beacon spiders' ($N=12$) had no beacon present. All spiders underwent an identical testing protocol of 5 RW trials and 2 VR trials: 2 RW trials on each of days 1 and 2, separated by 3 hours; 1 VR trial on each of days 3 and 4; and a single RW trial immediately following the final VR trial on day 4. The fifth RW trial was included to test whether two days of testing in VR had an effect on subsequent behaviour in the RW.

For RW trials, spiders were transferred into clean arenas that were identical to the arenas they had occupied previously with a beacon present but no nest (Fig. 2a). For Beacon spiders, the beacon was the same type that was present in their home arenas. No Beacon spiders,

which had no prior exposure to a beacon, were assigned one of the two beacon types and were subsequently tested with this beacon in all trials. The beacon was placed 7.5cm from the middle of one wall (as in home arenas), with a different wall randomly selected on each trial to prevent spiders from orienting to global cues beyond the arena. Spiders were given 3 minutes to acclimate while restrained in the centre of the arena by an upturned opaque vial before the vial was raised and the trial began. Each trial lasted 20 minutes and was video recorded from above.

The VR environment was a square arena designed to be similar in appearance and scale to the RW arena. The VR arena contained a single beacon in the same position relative to the spider's starting position as in the RW (Fig. 2b). We measured the relative radiance contrast (watts per square meter pre nanometer per steradian; $W/m^2/nm \cdot sr^{-1}$) of the beacon against the white wall in both environments. The background was approximately twice as 'bright' as the beacon in both worlds (beacon:background: real world 1:2; VR 1:1.5). Spiders began all trials in the centre of the arena facing the beacon, and all trials lasted 20 minutes. The treadmill was wiped with a damp cloth between trials to remove chemical cues left behind by the preceding spider (Jackson, 1987).

Spiders were scored for two measures in both contexts, recorded when the spider entered a 7.5cm radius circle surrounding the beacon: the proportion of the trial spent in proximity to the beacon and the number of beacon visits. In addition, total path length was recorded for spiders in the VR environment as a measure of general activity levels. We adopted an absolute proximity radius for scoring in order to have an objective measure of approach behaviours across trials. A radius of 7.5cm was selected based on data from pilot trials.

Analyses

Statistical analyses were performed using JMP version 11 (SAS Institute Inc., Cary, NC). We performed nonparametric statistics for all analyses, as our data did not meet the assumptions of normality. For experiment 1, descriptive data are presented for the empty VR arenas but due to abnormal behaviour this group was excluded from subsequent analyses. Spiders that did not participate in this task, defined here as path length of less than 30cm in the 20 min trial, were also excluded (N=2). A Wilcoxon matched pairs test was used to test for differences between RW activity and VR activity for spiders in complex VR arenas.

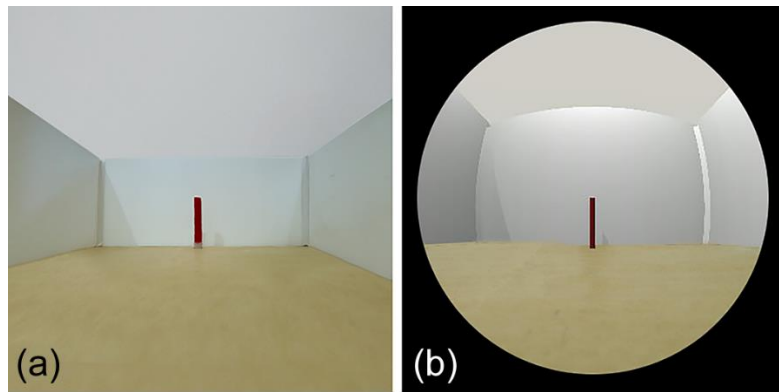


Figure 2. Spider's starting perspective in the beacon experiment in real (a) and virtual (b) environments. The virtual view reflects the image pre-transformed to fill the hemispherical display screen.

Spearman rank correlations were used to assess association between RW and VR in distance travelled, approach latency to enter the dark compartment and proportion of the trial spent in the dark compartment. A Wilcoxon matched pairs test was used to test for differences in RW activity before and after attaching the magnet.

For experiment 2, RW and VR conditions were analysed separately and then compared to identify consistency in performance across contexts. Wilcoxon matched pairs tests were used to compare the mean time spent in proximity to the beacon and the number of beacon visits in the RW and VR, and Wilcoxon signed ranks tests were used to detect group differences in performance on both measures within both RW and VR contexts. In the RW, Friedman's test was used to test for a trial effect in both measures across trials 1-4. A separate test was run comparing trials 4 and 5 to investigate whether VR testing had any effect on subsequent RW behaviour. Spearman rank correlations were used to assess association between RW and VR both each performance measures (outlined above). For the VR trials, we additionally compared the total distance travelled by Beacon and No Beacon spiders using a Wilcoxon signed-ranks test.

Ethical note

We followed the ABS/ASAB guidelines for the ethical treatment of animals. Attachment of the magnet in advance of experiments minimized stressful handling and allowed us to return spiders to their home cages after each trial. Feeding, behaviour and survival was not visibly affected by attachment of the magnet or participation in experiments. Spiders were subsequently used in pilot trials for other VR experiments.

Results

Experiment 1: Locomotor Activity & Light/Dark Preference Testing

In VR, spiders in empty virtual arenas, containing no objects, were significantly less active than spiders in complex arenas (mean distance travelled \pm SE: complex=160.3 \pm 18.4cm; empty=92.5 \pm 25.4cm; $Z=-2.40$, $P=0.016$), often stopping for prolonged periods during the trial. Due to their substantial reduction in general activity levels, spiders from the empty condition were excluded from further analyses.

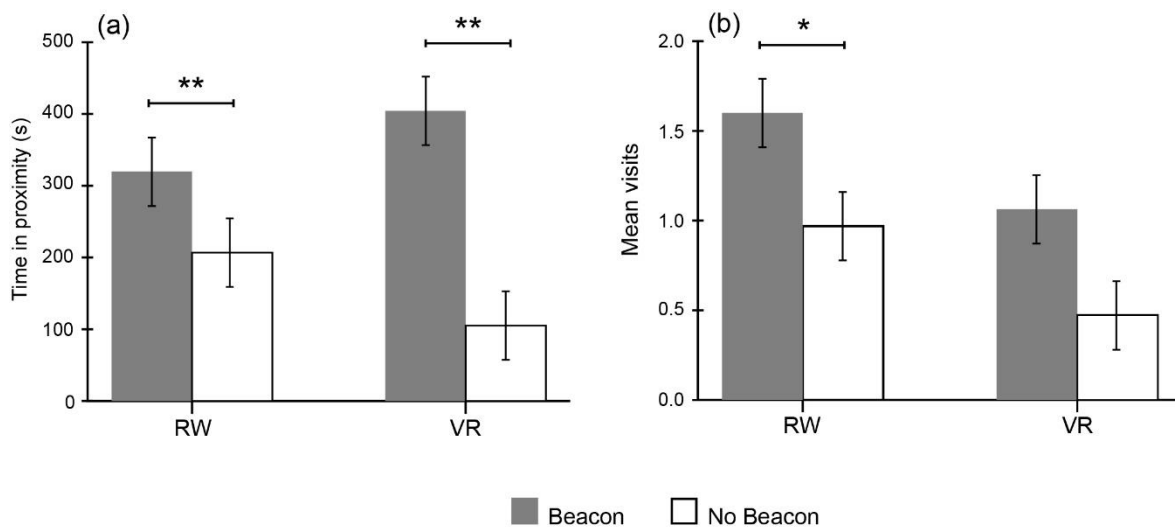


Figure 3. Spider performance in a beacon learning task in the RW and VR. (a) Time spent in proximity to the beacon. (b) Number of visits to the beacon. Beacon spiders were trained in the RW with a beacon/nest-site pairing while No Beacon spiders were provided no beacon. Asterisks denote significant differences between Beacon and No Beacon spiders. * $P < 0.05$; ** $P < 0.001$, Wilcoxon signed ranks test.

We found a significant positive correlation between each RW measure and its corresponding VR measure, although spiders tended to travel shorter distances in VR, were quicker to enter the dark compartment, and spent a greater proportion of the trial on the dark side of the arena than when in the RW (Table 1). Attachment of the magnet did not significantly influence activity levels in our RW activity trial (mean distance travelled in 10 min trials: pre-magnet = $407.9 \pm 46\text{cm}$, post-magnet = 390.5 ± 48.1 ; $Z = -0.46$, $P = 0.644$).

Experiment 2: Beacon Learning Experiment

In the RW, Beacon spiders, which had previously been exposed to a beacon beside their nest site in their home arena, made more visits to a beacon and spent significantly more time in its proximity than No Beacon spiders when tested in clean arenas (visits, $Z = 2.24$, $P = 0.025$; time in proximity, $Z = 3.30$, $P = 0.001$). There was no evidence of a difference across the four initial RW trials (visits: $\chi^2_3 = 6.79$, $df = 3$, $P = 0.080$; time in proximity: $\chi^2_3 = 1.80$, $df = 3$, $P = 0.615$) or between RW trial 4 (the trial preceding VR testing) and 5 (the RW trial immediately following VR testing) (visits: $\chi^2_3 = 2.57$, $df = 1$, $P = 0.109$; time in proximity: $\chi^2_3 = 1.19$, $df = 1$, $P = 0.275$). The similarity in performance between RW trials 4 and 5 indicates that the intervening VR trials did not disrupt subsequent performance in the RW.

The time spent in proximity to the beacon was positively correlated between the RW and VR for Beacon spiders but not for No Beacon spiders, suggesting that learning of the association between beacons and nest sites transferred across contexts (Table 2).

As was the case in the RW, when tested in VR Beacon spiders spent significantly more time in proximity to the virtual beacon than did the No beacon spiders ($Z = 2.81$, $P = 0.005$), although there was no significant difference in the number of visits ($Z = 1.50$, $P = 0.128$) (Fig. 3).

Beacon spiders had significantly longer path lengths in VR than No Beacon spiders (Beacon spiders = $118 \pm 17\text{cm}$, No Beacon spiders = $57.4 \pm 16\text{cm}$, $Z = 2.20$, $P = 0.028$). For both Beacon and No beacon spiders, activity peaked during the first 120 seconds and gradually declined over the course of the trial (Fig. 4).

Table 1. Wilcoxon matched pairs analyses signify differences between each measure in the RW and its corresponding measure in VR, while Spearman rank (*rho*) signify the correlation between each measure in the RW and its corresponding measure in VR. Distance refers to the total distance travelled; approach latency refers to the time to first enter the dark side of the arena and proportion refers to the proportion of the trial spent in the dark side (mean \pm SE, $N=21$).

Measure	<u>Means \pm SE</u>		<u>Wilcoxon matched pairs</u>		<u>Spearman Rank Correlation</u>	
	RW	VR	Z	P	Rho	P
Distance	383.8 \pm 38.8	160.3 \pm 18.6	4.02	0.001	0.579	0.006
Approach latency	466 \pm 99.1	188.6 \pm 76.1	2.94	0.003	0.637	0.002
Proportion	0.50 \pm 0.08	0.67 \pm 0.09	2.21	0.027	0.473	0.031

Table 2. Spearman rank (*rho*) signify the correlation between each measure in the RW and its corresponding measure in VR (averaged across all trials) while Wilcoxon matched pairs analyses signify differences between each measure in the RW and its corresponding measure in VR. Time in proximity refers to the amount of time (s) spent in proximity to the beacon and # Visits refers to the number of approaches to the beacon ($N=35$).

Measure	Group			<u>Spearman Rank Correlation</u>		<u>Wilcoxon matched pairs</u>	
				Rho	P	Z	P
Time in proximity	Beacon	320.2 \pm 43.2	404.4 \pm 104.6	0.625	0.009	0.78	0.437
	No Beacon	206.9 \pm 49.7	105.1 \pm 43.2	0.089	0.717		
# Visits	Beacon	1.6 \pm 0.2	1.1 \pm 0.3	0.409	0.116	-3.09	0.002
	No Beacon	1.0 \pm 0.1	0.5 \pm 0.1	0.193	0.429		

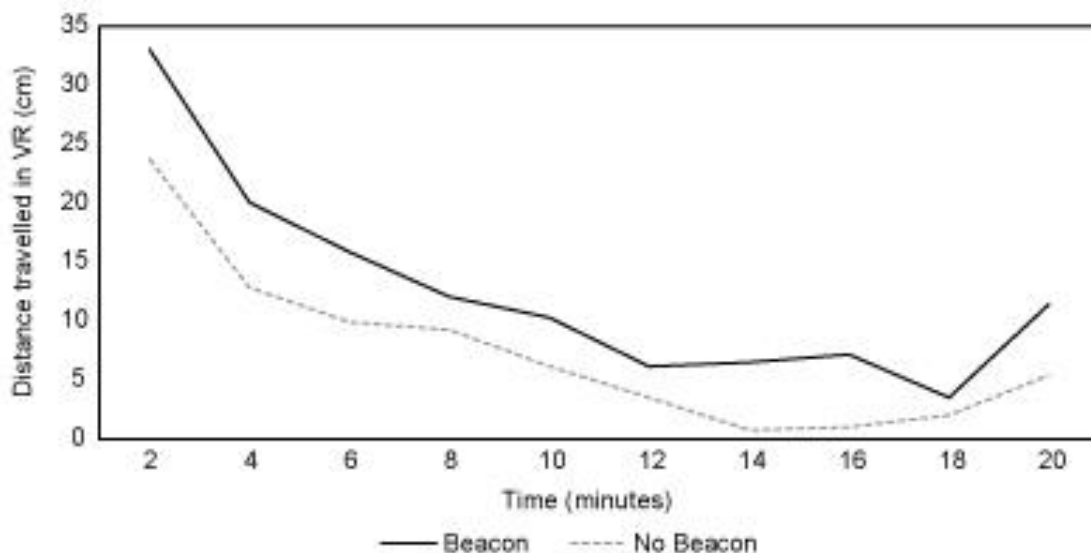


Figure 4. Mean distance travelled by Beacon and No Beacon spiders over the course of a 20 min trial in the VR system. Time is grouped into 120 s time bins and each time point reflects the distance moved since the previous time point.

Discussion

Video and computer animated stimuli have been presented to salticids in order to study psychophysics (Zurek, Taylor, Evans, & Nelson, 2010), cognition (Dolev & Nelson, 2014), courtship (Clark & Uetz, 1992), contests (Tedore & Johnsen, 2014) and predation (Bednarski et al., 2012), but these studies have always been conducted under open-loop conditions. The VR system described in the present study closes the loop for the first time, demonstrating that salticids detect and respond appropriately to the visual and motor feedback of a virtual environment. The present study comprises the first demonstration that spiders - here, female *S. incana* jumping spiders - display patterns of activity and behaviour in VR that closely parallel their RW counterparts. Individual tendencies were conserved across contexts; both individual locomotor activity levels and dark seeking behaviour in the RW were positively correlated with locomotor activity and dark seeking behaviour in VR. Further, associations learned in the RW transferred to VR. Spiders that were given the opportunity to learn about beacons associated with their nest site in the RW later tended towards these cues in both the RW and in VR.

Paying attention to local cues such as beacons is thought to be less computationally demanding than using distal cues (Shettleworth, 2010), and is part of the navigational strategy of many arthropods (Cheng, 2000; Collett, Graham, & Durier, 2003; Graham, 2003). A field study of a North American salticid, *Phidippus clarus*, found that spiders readily learned a beacon-nest site association and showed an increased tendency to approach beacons that were displaced from their original location (Hoefer & Jakob, 2006). Despite geographic and habitat differences we find a similar tendency to learn cues associated with nest sites. These results suggest that local cues may be an ecologically and taxonomically widespread element of the navigational strategy used by salticids, especially for homing.

VR may be a particularly useful tool in the study of navigation. Beacons, landmarks and environmental cues can easily be removed, manipulated or put into conflict with each other, and these manipulations can occur alongside neurophysiological recordings (Bohil et al., 2011). VR has been used extensively to study the neural underpinnings of behaviour in restrained primates, and more recently in rodents. Both rodents and primates display hippocampal place cell activity when navigating in VR (primates: Hori et al. 2005; rodents: Hölscher et al. 2005), but have critical differences in their activation patterns between real and virtual environments that can be attributed to lack of appropriate vestibular input in VR (Ravassard et al., 2013). Restraint can similarly lead to behavioural artefacts in invertebrate models that possess equilibrium sensors. Flies, for example, exhibit distortions in flight

behaviour when mechanosensory feedback from specialized stabilizing organs, the gyroscopic halteres, is impeded, as is the case during restraint (Fry, Sayaman, & Dickinson, 2003; Pringle, 1948). The limitations imposed by equilibrium sensors can be circumvented through VR studies of visually orienting walking invertebrates that lack equilibrium sensing organs, such as spiders.

Until recently, recording from salticid brains has been unfeasible due to their highly pressurized internal fluids, and the resulting fluid loss that accompanies incision. A recent study has developed methods that overcome this limitation, obtaining the first neurophysiological recordings from the brain of a salticid as it views traditional grating stimuli and naturalistic prey-like stimuli that were presented using open-loop methods (Menda et al., 2014). The effectiveness of a VR system for salticids in the present study raises the possibility of recording from brain cells in alert salticids interacting with an immersive, closed-loop environment.

The VR system described in the present study was designed to focus solely on vision, which is well known to mediate diverse aspects of salticid behaviour (Jackson & Harland, 2009; Land, 1969a). At the same time, many species routinely use other sensory modalities, such as chemoreception and vibration, both to assess the environment and to communicate (Elias et al., 2012; Girard, Kasumovic, & Elias, 2011; Jackson, 1987). A VR system could be designed to emphasize any modality, and indeed systems based on olfaction (Sakuma, 2002; Fry et al. 2008) and audition (Fry et al. 2004) have been used with other invertebrates. However, setting up a similar system for salticids would be challenging as salticids access these cues in part through the substrate (Barth 2002; but see Jackson, Clark, and Harland 2002; Jackson and Cross 2011 for examples of airborne chemoception in salticids), and the ‘substrate’ in our setup is an air-supported sphere atop which spiders are mounted. Although delivery of vibratory stimuli through this cushion of air would be particularly challenging, it would be straightforward to incorporate substrate-bound chemical cues applied to the ball prior to the start of each trial or to deploy puffs of airborne odours or airborne sound.

Irrespective of the modality emphasized in VR, it is important to ensure that the attributes present in the simulation are tuned to match the perceptual system of the subject. In a visual system, parameters such as size, shape, colour, texture, luminance and movement all require careful consideration (Woo & Rieucau, 2011; Zeil, 2000). The extensive body of research on visual perception of salticids provides an excellent framework for designing stimulus features that most effectively elicit a natural response. For instance, Zurek et al.

(2010) demonstrate that the orientation response in *S. incana* is dependent on stimulus size, contrast and speed; Bednarski et al. (2012) highlight the importance of motion characteristics in eliciting a predatory response; and Dolev and Nelson (2014) show that the relative angle between the body elements of abstract stimuli are sufficient and necessary for recognition. VR will provide an excellent resource to build from this foundation, as it broadens the scope of experimental possibility to encompass design features and contingencies that were previously inaccessible in salticids.

Acknowledgements

We thank Rowan McGinley for his assistance collecting spiders, Bruno Van Swinderen for his help conceptualising our VR system, Thomas White for providing spectral measurements and three anonymous reviewers for their helpful suggestions on the manuscript. We are grateful to Chris Malloy, Rob Lee, Brian Su, Keith Hassan and John Porte for their invaluable technical assistance. We are especially thankful to Greg Hunsburger for his assistance in designing and implementing the 3D environment, and his continual support throughout this project. This research was supported by an Australian Government Endeavour Postgraduate Award. Research expenses were supported by Higher Degrees Research Funds of the Department of Biological Sciences at Macquarie University.

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6 VISUAL WORKING MEMORY IN JUMPING SPIDERS: AN EXPECTANCY VIOLATION PARADIGM IN VIRTUAL REALITY

Abstract

Animals often live in topographically complex environments where predators, prey and conspecifics can disappear from view and reappear some time later. An ability to anticipate the likely identity of a reappearing target can facilitate a rapid and appropriate response that could spell the difference between life and death, or securing a mate or meal. Target recognition requires that animals attend to the target when it initially appears, hold a representation of the target in working memory during the target's absence, and subsequently recognize the target as matching or mismatching to its initial presentation when it reappears. In the present study, we examine the role of hunger and target salience on visual working memory and mismatch detection in a jumping spider, *Servaea incana*. We developed an automated expectancy violation paradigm in a virtual reality system that enables precise control of stimulus features and delay timing, and high reproducibility between subjects and trials. In the first stage of the two-stage trial, spiders viewed a moving virtual target (high salience fly or low salience sphere) that disappeared into the substrate. Following a short delay (3s or 10s), a matching or mismatching target appeared and the spider's looking time towards the target was recorded. We found that, consistent with predictions of expectancy violation, spiders looked for longer towards the mismatching target, but only when the object initially viewed was the more salient type. Hunger increased looking time, while the duration of the delay had no effect. Taken together, these results suggest that hunger and the salience of the target play a role in guiding attention, working memory and target recognition in the jumping spider *S. incana*, and that working memory persists for at least 10 s.

Introduction

Although features in an animal's environment may change in content from moment to moment, 'working memory' (see Baddeley, 2012) can assist the animal with making decisions in its day to day life (Brembs, Menzel, & Giurfa, 2007; Brown & Demas, 1994) by combining priority information with other cognitive processes such as selective attention (Kamil & Bond, 2006). The capacity to hold key information 'online' has been demonstrated across diverse vertebrate and invertebrate taxa (e.g., Carruthers, 2013; Matzel & Kolata, 2010; Brembs et al., 2007; Menzel, 2009; Zhang, Bock, Si, Tautz, & Srinivasan, 2005). Temporally, working memory lies between short-term memory (in the scale of seconds) and long-term memory (in the scale of hours; but see Ericsson & Kintsch, 1995), and can be distinguished from both by its dependency on sustained attention and sensitivity to attentional interference (Carruthers, 2013).

Attention is thought to be important for binding features into a perceptual representation of an object (Treisman & Gelade, 1980), maintaining that representation in working memory (Brown & Brockmole, 2010), and for detecting changes in object features (Makovski, Shim, & Jiang, 2006). Representation, here, refers to the functional correspondence between a salient aspect of the environment and processes in the animal's brain that target the animal's behaviour to that particular aspect (Gallistel, 1990; Gallistel, 1989). The classical view is that attention gates what information is stored as a representation in working memory (Downing, 2000). However, recent studies suggest that this process may work in the opposite direction as well, such that the contents of working memory may influence where attention is subsequently directed (Desimone & Duncan, 1995; Downing, 2000). This process has been proposed to work in one of two ways. On the one hand, representations held in working memory could bias and involuntarily guide attention via top-down control in favour of matching items (Duncan & Humphreys, 1989), such that expected stimuli are more easily perceived (Jacoby & Dallas, 1981). On the other hand, attention may be biased in favour of novel, unexpected stimuli that catch the animal by surprise (Yantis & Jonides, 1990). Attention towards unexpected stimuli is thought to confer survival value by increasing the ease with which novel objects are detected and identified in familiar environments (Johnston, Hawley, Plewe, Elliott, & DeWitt, 1990).

One of the most commonly used methods to study change detection is the "expectancy violation" paradigm (Baillargeon, 2000), previously also known as the "preferential looking" paradigm (Spelke, 1998), which was originally developed for pre-verbal infants (Spelke, 1985),

and has since been adapted to diverse vertebrate species (primates: Hauser & Carey, 2003; horses: Proops, McComb, & Reby, 2009; dogs: Müller, Mayer, Dörrenberg, Huber, & Range, 2011; and crows: Kondo, Izawa, & Watanabe, 2012). In this paradigm, a subject views a scene in which a target item disappears behind an occluder. Following a short delay, the scene is restored with a target that either matches or mismatches the initial presentation, and the subject's gaze duration ('looking time') towards the reappearing target is recorded. Instances in which the subject looks longer towards the mismatched target are taken as evidence that the subject detected a change between the current target and the representation of the target that had previously been loaded into working memory. Since the expectancy violation paradigm can be conducted within a single test session, it avoids many of the motivational confounds associated with repeated exposure to reinforcers (Blaser & Heyser, 2015). As such, it has been considered to be a 'pure' working memory test that is unbiased by either reference memory (such as rule learning) or associated effects of positive or negative reinforcement (Ennaceur & Meliani, 1992).

The expectancy violation paradigm tests the observer's sensitivity to perceptual changes and reflects the capacity to build and hold salient visual representations in working memory for short periods of time (Hauser & Carey, 2003). There have been few attempts to adapt this paradigm (or other change detection paradigms) to invertebrates, despite a growing literature on their diverse cognitive capacities (Perry et al., 2013), which include robust demonstrations of working memory (Brown & Demas, 1994; Brown, Moore, Brown, & Langheld, 1997; Zhang, Si, & Pahl, 2012). Amongst invertebrates, jumping spiders (Salticidae) are well known for their high acuity vision and complex, visually mediated behaviour. Recent studies have demonstrated that working memory plays a likely role in vision-based and olfaction-based selective attention (Cross & Jackson, 2009, 2010), as well as during detour routes when they temporarily lose sight of their target (Tarsitano & Jackson, 1997; for a review of spider cognition see Jackson & Cross, 2011; Jakob, Skow, & Long, 2011). Further, the only study of expectancy violation in arthropods to date was conducted with the araneophagic ('spider-eating') salticid *Portia africana*, in which spiders were demonstrated to detect a change in prey type, as evidenced by fewer leaps towards mismatched prey (Cross & Jackson, 2014b).

Virtual reality (VR) is increasingly used in the behavioural sciences to overcome the constraints of traditional, open-loop experiments in which stimulus conditions are presented independently of the subject's responses to the stimuli (Dombeck & Reiser, 2012). In closed-loop systems such as VR, motor behaviour feeds back into and updates the stimuli that the subject interacts with in real time (Fry et al., 2004). Since virtual environments are typically

implemented as automated, computer-controlled systems, virtual stimuli and world features can be designed along a spectrum from abstract to photo-real, and can include parameters and contingencies that would be difficult or impossible to create in the real world. Further, a recent study has demonstrated that salticids interact readily with virtual environments, and that behavioural tendencies and associations in VR paralleled their real world counterparts (Peckmezian & Taylor, 2015).

In the present study, we developed an automated expectancy violation paradigm in a virtual reality system in which object characteristics and delay could readily be modified. Our principle aim was to assess spontaneous (single-trial) working memory and visual detection of mismatch using objects that varied in salience, and under increasing hunger conditions, in a salticid, *Servaea incana*. Internal factors, such as hunger (Lang, Bradley, & Cuthbert, 1997), and external factors, such as the salience of an object (Giurfa & Menzel, 1997; van Swinderen & Greenspan, 2003), are known to modulate attention and consequently, the representations that are stored in working memory (Menzel, 2009). We compared looking time (the proportion of the trial oriented) towards a moving object that was either matched or mismatched to an earlier presentation. Although salticids have movable eye tubes that extend their field of view, they commonly turn to face objects detected in the periphery by their secondary eyes (Land, 1972), and looking time has previously been shown to be a good predictor of subsequent choice (Tarsitano & Andrew, 1999). In line with expectancy violation research with vertebrates (Hauser & Carey, 2003), we interpreted longer looking time towards the mismatched object as indication that there had been a violation of expectancy (Shettleworth, 2010; Spelke, 1985). If spiders form a spontaneous representation of the object during their initial encounter, then we predicted that they would spend more time looking when the object changed from their memorized representation, thus violating their expectation (Kondo et al., 2012). Specifically, we predicted that spiders would spend more time looking at a reappearing object (1) with increasing hunger levels, (2) following shorter delay and (3) when viewing more salient object.

Methods

General

Experiments were conducted using mature, field-collected *Servaea incana* females ($N=71$), maintained under standard controlled laboratory conditions of $25\pm 1^\circ\text{C}$ and $65\pm 5\%$ RH (for

details, see Peckmezian & Taylor, 2015). All testing was conducted between 0800 and 1500 (laboratory photoperiod 11:1:11:1 light:dusk:dark:dawn, lights on at 0700). Spiders were fed two Queensland fruit flies (*Bactrocera tryoni*) 5-9 days prior to testing (see *experimental protocol* for details of hunger treatment). Each spider was randomly assigned to an initial treatment group (described below), and participated in three different trials separated by a minimum of 7 days.

VR System

The virtual reality (VR) system and basic experimental procedures of this study were largely as described in Peckmezian and Taylor (2015), and only essential details are provided here. In preparation for VR experiments, spiders were first restrained on a foam-backed plunger inside a 30 ml syringe (tip of syringe cut off). A sheet of clear plastic film (Glad® ClingWrap) stretched over the opening of the syringe and the plunger was depressed until the spider's dorsal surface was pressed firmly against the film. A fine needle was used to make a hole in the film, exposing the cephalothorax, while leaving the abdomen and legs immobilized. A neodymium magnet (diameter, 0.2 cm) was gently affixed to the dorsal carapace using a drop of dental cement (SynergyFlow A3.5/B3, Coltene Whaledent), with care taken to avoid covering the spider's eyes. The dental cement was then cured with blue light from a LED dental curing light source (SDI radii plus, Henry Schein Dental). Each spider was then returned to its cage and allowed to recover for a minimum of 24 hours before being used in experiments.

At the start of each trial, spiders were lifted from their home cages using a magnetic pin and positioned on to a 3D-printed spherical treadmill that was located centrally in front of a hemispherical display screen (40 cm diameter, painted with flat white epoxy enamel) (Fig. 1a). Care was taken to ensure that the spider could fully extend its legs and step normally. The treadmill held a 3.5 cm diameter, 230 mg expanded polystyrene ball that was supported by a constant flow of air (Sparmax AT-250 mini-compressor; Comweld Medical air flow meter). The moment of inertia of the ball was low enough to ensure that the spider's movement was unlikely to be impaired (Land, 1972; Peckmezian & Taylor, 2015). The ball was marked with an irregular pattern of black spots using a permanent marker for motion tracking. Three-degrees of freedom (roll, pitch and yaw) rotations of the ball were tracked using images captured by a webcam (Logitech Quickcam Pro 9000) and analysed by the motion tracking software FicTrac (Moore, 2012; Moore et al., 2014).

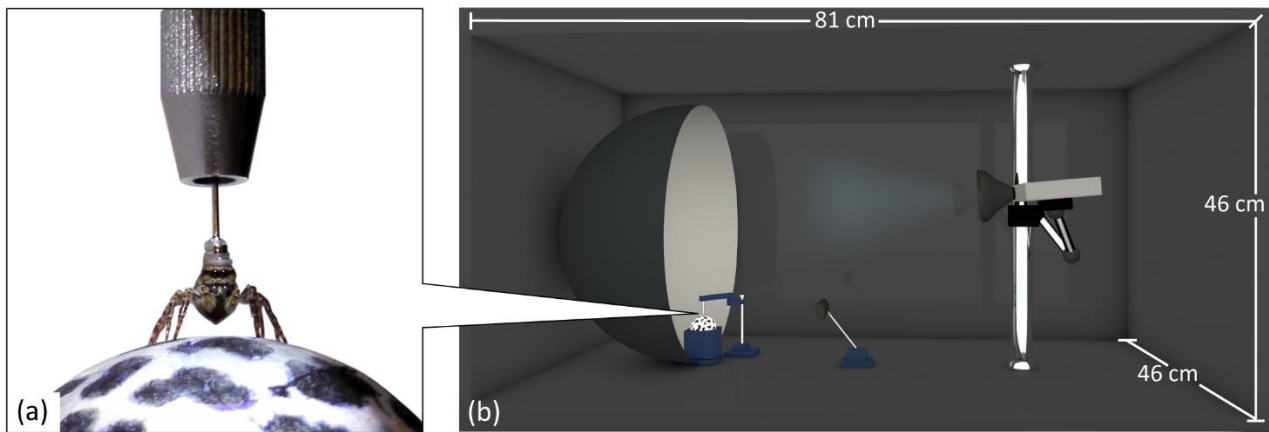


Figure 1. (a) Spider mounted above a spherical treadmill in preparation for a trial. A magnet is fixed to the spider's dorsal carapace and a magnetic pin holds the spider in position. (b) Schematic of VR system. Spiders are mounted above an air-supported spherical treadmill placed inside a hemispherical display screen. Dynamic computer generated 3D environments are front-projected onto the screen via a pocket projector with add-on fisheye lens, and spider movements are detected by a camera based tracking system (FicTrac) and update the world in real-time (closed-loop).

Using visual input, FicTrac computes the absolute orientation of a patterned ball by localizing the currently visible region of the ball with a learned map of its entire surface. This procedure has been shown to outperform standard optical mouse-based systems for fictive path tracking.

A virtual environment was projected onto the inner surface of the hemispherical display screen using a Vivitek Qumi Q2 LED pocket projector (300 lumens; 60 Hz at 1080p resolution) with a supplementary fish eye lens (Zeikos, 5.8 cm, $0.43\times$ wide angle). This projector has a refresh rate of 60 Hz (at 1080p resolution) which is above the estimated flicker fusion frequency of salticid eyes (40 Hz; Forster, 1985). A plano-convex lens (Edmund Optics 2.5 cm \times 10 cm focal length) was used to focus the light from the projector into the fish eye lens. A 3D-printed holder affixed the projector to the two lenses as a single unit, and this unit was mounted onto a movable arm approximately 25 cm above the base of the screen and 50 cm from the centre of the screen. The simulation projected 160° in both azimuth and elevation, covering the full field of view of both the forward-facing principal eyes and the anterior lateral secondary eyes. The entire system was enclosed in a light-tight black Plexiglas box to maximize projector brightness and isolate spiders from visual distractions (Fig. 1b).

A three-dimensional simulated environment was created using the multi-platform game development software Unity3D (version 4.2.1; Unity Technologies). Due to the requirements of the motion-tracking software FicTrac, Unity3D ran on an Ubuntu (Linux) virtual machine (VMware Inc) operating on a Windows 7 operating system. This system ran on an i7 processor and used a NVIDIA GTX 660 graphics card. Using these specifications, Unity3D rendered at a rate of approximately 60 frames per second. Simulated environments were pre-distorted in Unity3D to map normally onto the hemispherical projection geometry (Bourke, 2009). An interactive graphical user interface was created to calibrate, run and play back our simulations. To calibrate distance in VR, we measured a single rotation of the treadmill ball and used this value to scale the virtual environment, so that 1 cm travelled on the treadmill in the real world equalled 1 cm travelled in VR.

The VR environment was a square arena (52 cm wide, 22 cm tall) with white walls and a flat ground plane that was textured and coloured to resemble tree bark. Depending on the treatment group, spiders were presented with one or both of the two moving virtual object (VO) types (a black sphere, constructed in Unity, and an animated house-fly prey object, purchased from Biometric Games). The wings of the prey VO fluttered, but otherwise moved along the same trajectory as the sphere VO. Both objects (excluding the wings on the prey VO) had a diameter of approximately 4° and moved at a speed of 9deg s^{-1} . These values had

elicited the strongest orientation propensity in an earlier study of *S. incana* (Zurek et al., 2010). Prior to this experiment, we conducted a ‘preference’ test to determine the more salient VO. We exposed a separate group of spiders (N= 14) to each of the two VO types (random order, 30s exposure), separated by two days, and compared looking time to both types. Based on greater looking time, the prey VO was deemed the more salient object type. While we refer to the fly object as ‘prey’, we did not measure predatory behaviour (e.g., stalking, leaping and attacking) which was constrained in our tethered system, and we make no assumptions regarding how spiders classify either object.

Experimental protocol

The experimental protocol was designed to assess the roles of salience and hunger on working memory and expectancy violation in a single (two-stage) trial. We began each trial with a 3min dark period to allow the spider to acclimatize to the setup. Then, to begin stage 1 of the trial, we presented the spider with one of two VOs that differed in salience (fly: more salient; sphere: less salient). This VO appeared directly in front of the spider before moving in a straight line for 7s to the right side of the arena (i.e., to the spider’s visual periphery). Once it had reached the right of the arena, the VO then moved in a straight line for 14s to reach the left side of the arena. It then moved back towards the right side for 7s until it reached the centre of the arena. After reaching this point, the VO receded through the substrate for 2s before disappearing.

We elected to have the VO disappear and reappear through the substrate because *S. incana* often interacts with prey and conspecifics that temporarily move out of sight in nature by moving above or below pieces of bark, or around trunks of the *Eucalyptus* trees that they inhabit (pers. observation). Furthermore, having the VO disappear into the substrate at the end of stage 1 allowed the VO to logically re-emerge from the substrate at the beginning of stage 2. The point of re-emergence was directly in front of the spider, irrespective of where the spider happened to be in virtual space at the start of stage 2. In this way, all spiders viewed at least the start of the stage 1 and stage 2 VOs in the same way, creating a reliable metric for comparison between trials.

Stages 1 and 2 were separated by a retention interval (RI) of 3s or 10s, with these times being chosen because they have been used in previous working-memory experiments with invertebrates. Bees, for instance, hold sample patterns in visual working memory for as long as

5s, after which their performance decays until reaching levels of chance by 8.9s (Chittka, Gumbert, & Kunze, 1997; Chittka, Thomson, & Waser, 1999; Zhang et al., 2005). Spiders moved freely through the virtual arena during the RI.

After the RI, we then began stage 2 of the trial and, during this stage, the spider was presented with a VO (fly or sphere) that was either the same or different to the VO presented in stage 1. This VO emerged from the substrate directly in front of the spider and then moved in a straight line for 7s to either the left or right side of the arena (side determined at random) before disappearing and ending the trial. At the end of each trial, we used a strip of laminated paper to gently separate the neodymium magnet and magnetic pin, and each spider was returned to its home cage.

Spiders were randomly assigned to the experimental groups: (1) stage 1 fly VO (more salient) or sphere VO (less salient), (2) short RI (3 s) or long RI (10 s) and (3) stage 2/stage 1 VO same or stage 2/stage 1 VO different. Moreover, before spiders began a trial, they were deprived of food for between 5 and 9 days (period determined at random).

Each trial ran for a total of 40s (3s RI) or 47s (10s RI) and was closed-loop throughout. ‘Closed-loop’ meant that the movements of the spider updated the simulation and that the spider could orient towards or away from the VO. We created a data collection tool that was ‘attached’ to the VOs, allowing us to estimate the level to which the spider interacted with these objects. The tool contained a ‘dot product’ function that, for each time point sampled, gave us a value that ranged between -1 and 1, with -1 denoting that the spider was directly facing the VO, 0 denoting that the spider was facing 90° away from the VO, and 1 denoting that the spider was facing directly away from the VO (Fig. 2). We set an orientation ‘threshold’ of 10°, meaning that spiders were considered ‘oriented’ for each time point sampled only when facing the VO within a 10° window (corresponding to a dot product of <-0.94).

Finally, we ran two baseline procedures that determined, and controlled for, orientation values that could not be accounted for by a spider’s directional behaviour. In the first baseline test, we used a separate group of spiders (N=28) in a two-stage (paired design) motion tracking test to consider the likelihood of the VO passing in front of an active spider that had not oriented towards it intentionally. This ensured that our observed values were not simply due to the virtual object crossing the spider’s central field of view during non-directed movements. In the “real VO” stage, spiders viewed the prey VO as it moved twice from the

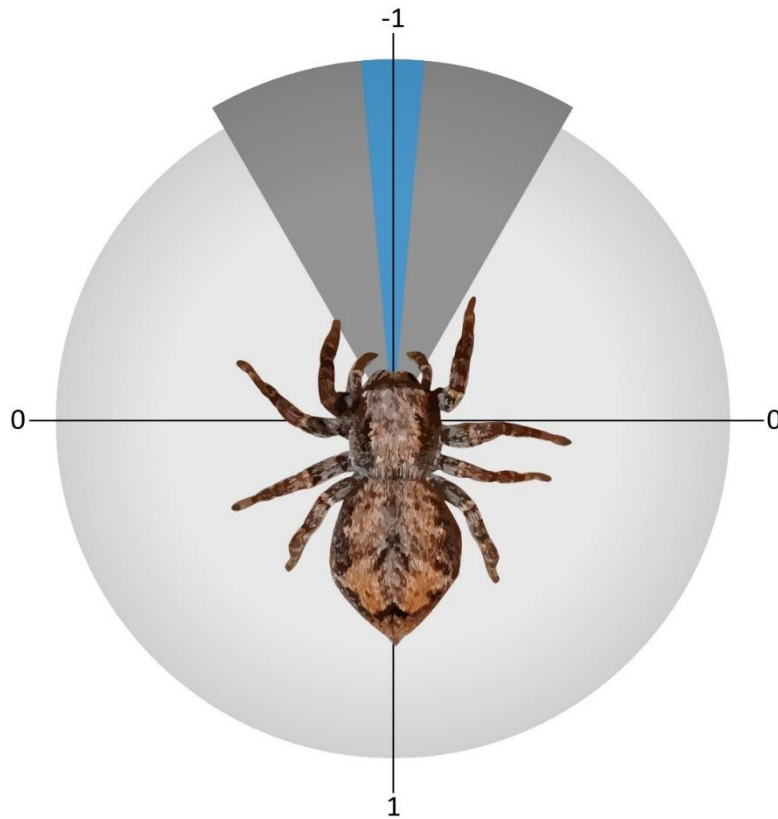


Figure 2. Looking time towards virtual objects was measured with a 'dot product' function. For each time point sampled, the function produced a value that ranged between -1 and 1, with -1 denoting that the spider was directly facing the VO, 0 denoting that the spider was facing 90° away from the VO, and 1 denoting that the spider was facing directly away from the VO. Spiders were considered 'oriented' when facing the VO within 10° (blue section; corresponding to a dot product of <-0.94). The high acuity region of salticid principle eye retinas is approximately 5° per eye, but complex eye tube movements increase this field of view to approximately 28° per eye corresponding to dark grey region (Land, 1969b).

left to the right side of the screen. In the “invisible VO” stage, the prey VO was replaced by an ‘invisible’ VO that moved along the same trajectory as the prey object and had the same data collection tool attached. Each stage was 28s long (order determined at random), separated by a 180s interval. We compared orientation values between these two stages to determine if orientation towards the prey VO exceeded chance orientation values.

The second baseline test was run to account for the fact that the VO crosses the spider’s field of view even when the spider is completely motionless. We ran 20 trials without the spider to determine the average system (computer-generated) orientation values. This system ‘chance’ level is included as a reference line in all figures.

Analyses

Statistical analyses were performed using IBM SPSS version 20. For the first baseline test, we used a paired t-test to compare orientation values towards the prey VO with orientation values towards the ‘invisible’ VO. For experimental Stage 1, we ran two identical statistical tests, with the first test assessing performance over the full 30s trial, and the second test assessing performance during only the first 7s of that trial, in order to allow direct comparison with Stage 2 (which was a 7s trial). Our initial model was a factorial ANOVA with orientation as our response variable, hunger and Stage 1 VO type as factors, and resampling trial order as a covariate. For stage 2, our initial model was a factorial ANOVA with Stage 1 VO type, Stage 2 VO type, RI group and hunger treatment as factors, and with trial order and object direction (R/L) as covariates, along with the interaction between Stage 1 and Stage 2 VO types.

Results

Resampling trial order, hunger, RI, and object direction were all found to be non-significant ($P>0.1$) and were omitted from the final model. During the first baseline test, spiders spent significantly more time oriented towards the prey VO than towards the invisible VO (means \pm SE, prey VO: 0.24 ± 0.01 , invisible VO: 0.13 ± 0.01 ; $t=4.05$, $DF=27$, $P<0.001$), confirming that orientation responses could not be explained by chance movements. The average system-generated orientation was 0.11 for Stage 1 and 0.20 for Stage 2. These values are reflected as a reference line in Figures 3 and 4.

In the first 7s of Stage 1, the length of time that the spider oriented towards the prey VO was not significantly different than the length of time it oriented towards the sphere VO

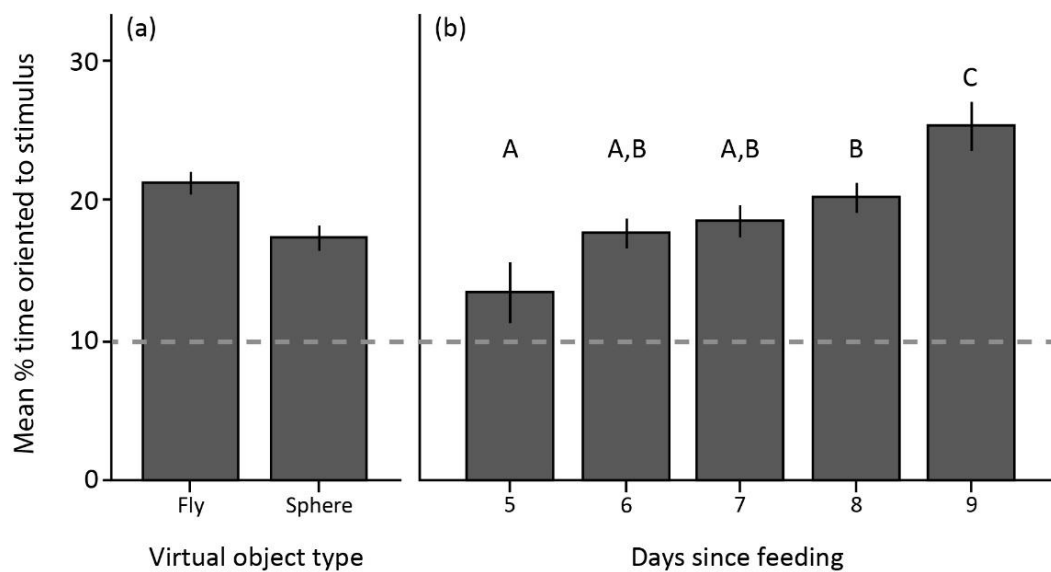


Figure 3. Mean percent of time spent oriented toward a moving virtual object during Stage 1 (28s). (a) Orientation by virtual object type ($P=0.007$). (b) Orientation by hunger level (days since last feeding). Levels not connected by the same letter are significantly different (Tukey HSD test, $P<0.05$). Bars reflect standard error; the dotted line indicates the 'chance' level of orientation if the spider did not move from its starting position.

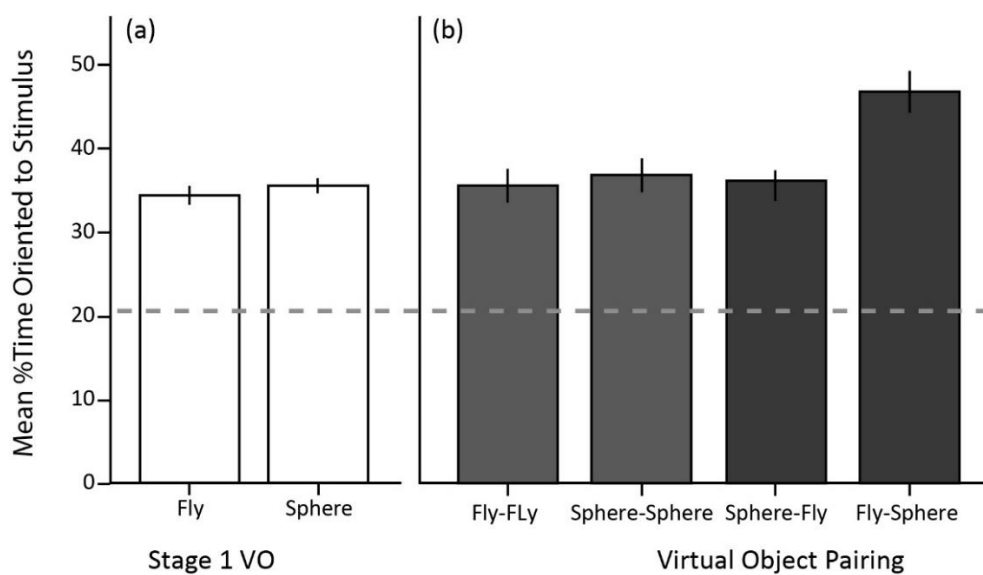


Figure 4. Mean percent of time spent oriented towards a moving virtual object during Stages 1 and 2. (a) Orientation to either VO in the first 7s of Stage 1. (b) Orientation to the Stage 2 VO based on both Stage 1 and Stage 2 VO types. Light grey bars indicate matching virtual objects; dark grey bars indicate mismatching virtual objects. Orientation was measured as the percent of the trial spent oriented towards the virtual object. Levels not connected by the same letter are significantly different (Tukey HSD test, $P<0.05$). Bars reflect standard error; the dotted line indicates the 'chance' level of orientation if the spider did not move from its starting position.

($p>0.5$). However, orientation differed significantly during the full 30s trial, with spiders orienting towards the prey VO significantly longer than the sphere VO ($F_{1,212}=7.48$, $P=0.007$) (Fig. 3a). Hunger treatment was marginally significant in the first 7s of Stage 1 ($F_{4,212}=2.54$, $P=0.039$), but very significant over the full 30s trial ($F_{4,212}=7.97$, $P<0.001$; Fig. 3b). Mean orientation values increased in a stepwise manner with each additional day of hunger. Five, six and seven days of starvation were not statistically different ($P>0.05$), but eight days differed from five days ($P=0.015$) and nine days differed from all other days (five days: $P<0.0001$; six days: $P=0.001$; seven days: $P=0.004$; eight days: $P=0.040$). The interaction between VO type and hunger was not significant ($P>0.5$), indicating that hunger increased orientation irrespective of object type.

During Stage 2, orientation was affected by both the Stage 1 and Stage 2 VO types (Stage 1: $F_{1,212}=5.25$, $P=0.023$; Stage 2: $F_{1,212}=10.15$, $P=0.002$) and their interaction ($F_{1,212}=6.97$, $P=0.009$). Specifically, spiders that had viewed a prey VO in Stage 1 and a Sphere in Stage 2 oriented for longer in stage 2 than all other combinations (Fig. 4).

Discussion

In this study, we examined working memory and mismatch detection in the jumping spider *Servaea incana*, using a modified expectancy violation paradigm in a virtual reality system. When initially exposed to the virtual object (VO), spiders oriented for longer as they became hungrier and also oriented for longer towards the prey VO than towards the sphere VO. When the VO reappeared in stage 2, spiders oriented for significantly longer towards the VO when the more salient prey VO (in stage 1) reappeared as the less salient sphere VO (in stage 2). By contrast, when the sphere reappeared as the prey, or when the reappearing object matched the object in stage 1, no such effect was found, suggesting that our results are not simply due to a general preference for novelty (as is the case with rodents; e.g., Bello-Medina et al., 2013).

Duration of the retention interval (RI: 3s or 10s) had no effect on orientation duration in the present study. Previous research with honey bees and bumblebees has demonstrated a role for working memory in mediating arthropod behaviour (Giurfa, Zhang, Jenett, Menzel, & Srinivasan, 2001). After reinforced trials, bees learn to select an object that matches (delayed match-to-sample task) or mismatches (delayed non-match-to-sample task) an object that they had viewed earlier. Using these tasks, working memory for sample patterns is high after a 5s delay and reduced to chance levels after a 8.9s delay (Zhang et al., 2005). In our study,

however, performance was similar after a 3s and a 10s RI. This suggests that salticid working memory persists for longer than bee working memory.

Jumping spiders often live in heterogeneous, topographically complex environments where predators, prey and conspecifics frequently disappear and reappear some time later. The capacity to recognize reappearing targets would allow spiders to respond quickly and appropriately, which could spell the difference between life or death, or securing a mate or meal. However, to do so, spiders must be able to distinguish it from other objects that they encounter and detect changes when they occur, a process that is influenced by the salience of both the initial and reappearing objects (Wright, 2005). Further, salience can modulate attention and consequently the formation and maintenance of intact representations of visual objects in working memory (Rensink, 2002). Salient stimuli draw attention, often through a change from a previous state, and working memory provides a mechanism for an animal to detect that change (Swinderen, 2005). For example, *Drosophila* react selectively to the stimuli that are the most behaviourally relevant or ‘salient’ in their environment, a process that is modulated by memory (Waddell & Quinn, 2001). Moreover, since salience and memory are subserved by the same brain region (the mushroom bodies) in *Drosophila*, and memory mutants show a diminished ability to perform selective discrimination (Wu, Gong, Feng, & Guo, 2000), this shared neuroanatomical substrate has been postulated to reflect a functional interconnectivity in insects (van Swinderen & Greenspan, 2003).

In the present study, spiders attended to both high and low salience virtual objects during their initial encounter, but looked for longer towards the more salient prey-like stimulus. In their subsequent re-encounter, spiders looked for longer only when the prey object had changed to the non-prey object, but not vice-versa, and not when objects were the same in both stages. We propose that, in stage 1, the spiders held in working memory a more robust or persistent representation of the more salient object, increasing the likelihood of detecting a mismatch when a different object appeared in stage 2.

In addition to varying with external factors, such as object salience, experimental expression of working memory can also be modulated by internal physiological and motivational factors such as hunger. Spiders frequently face extended periods of starvation, and possess physiological adaptations to not only survive but also to prosper on a limited and unpredictable food supply (Anderson, 1974; Wise, 1993). Despite being well equipped to go for extended periods without a meal, hunger still plays an important modulatory role in many aspects of spider behaviour. For example, hunger can influence risk-taking while foraging

(Gillespie & Caraco, 1987), locomotor activity (Walker, Marshall, Rypstra, & Taylor, 1999), web morphology and reproductive investment (Sherman, 1994). Hunger also increases the propensity for salticids to orient towards moving objects (Gardner, 1966; Zurek et al., 2010) and increases the likelihood that salticids will attack non-preferred prey (Jackson, Li, Barrion, & Edwards, 1998; Jackson, 2000). Previous studies investigating the role of hunger in spider behaviour typically include two conditions – hungry or sated. In our study, we included five hunger levels (5 to 9 days without food), and we found that orientation increased with increasing hunger. How orientation and performance is affected beyond 9 days of hunger remains to be investigated, but would be a worthwhile step in clarifying the experimental parameters best suited for studies of salticid visual cognition.

An objective of this study was to test for differences in looking time towards objects that differed in saliency, rather than to determine the particular object attributes that facilitated discrimination and preference. However, since both VOs were matched for size, contrast and global motion (speed and pattern of movement), the discriminable features must be related to the few elements that differed – namely object shape and features of local motion. Even after aversive conditioning, individuals of a North American salticid, *Phidippus audax*, were unable to discriminate between a moving snapshot of a cricket and a moving rectangle, suggesting that shape was not a critical feature in object discrimination. By contrast, differences in local motion facilitated both discrimination and preference (Bednarski, Taylor, & Jakob, 2012). It would be straightforward to assess the relative role of local and global motion, shape, size, speed, colour, and a myriad of other visual features that could influence discrimination using the VR-based expectancy violation paradigm described in the present paper. Having determined that spiders attend to moving virtual prey, store such encounters in working memory for at least 10s, and subsequently detect a visual mismatch when this object changes to a sphere, the next logical steps include a detailed examination of the specific visual features that contribute to such discrimination.

Acknowledgements

We thank Rowan McGinley for his assistance collecting spiders and Andrew Barron for his help conceptualizing these experiments. We are grateful to Richard Moore for developing and sharing FicTrac, and Bruno Van Swinderen, Gavin Taylor and Matthew Van de Poll at the Queensland Brain Institute for their kind technical assistance integrating FicTrac with our system. We are especially thankful to Rob Lee, for his invaluable technical assistance, and Greg Hunsburger, for his assistance in designing and implementing the 3D environment as

well as his continual support throughout this project. This research was supported by an Australian Government Endeavour Postgraduate Award. Research expenses were supported by Higher Degrees Research Funds of the Department of Biological Sciences at Macquarie University. FRC was supported by a Royal Society of New Zealand Marsden Grant (UOC1301).

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7 GENERAL DISCUSSION

All animals face the challenge of surviving in a changeable environment. Learning about contingencies in the world (classical or Pavlovian conditioning) and about the consequences of one's own actions (operant conditioning) allows an animal to adaptively modify its behaviour in accordance with current environmental conditions. In establishing predictive relationships between contingent events, associative learning reduces environmental uncertainty and contributes to adaptive behaviour (Giurfa, 2007), and has been demonstrated in diverse animal taxa (Heyes, 2012).

Associative processes have been characterized in great detail in model invertebrate taxa, and these studies have contributed insights to the mechanisms and evolution of learning (Giurfa, 2013), and how differently constructed brains solve similar problems (Bitterman, 1975). However, it is now generally agreed that these studies have not been sufficiently comparative (reviewed in: Perry, Barron, & Cheng, 2013). Salticids – and arachnids as a whole – have been underrepresented in the cognition literature, but possess many characteristics that make them suitable as models (reviewed in: Jakob, Skow, & Long, 2011).

The principle aim of my thesis was to begin to bridge the great gulf between spiders and conventional invertebrate model taxa. To do so, I developed new methods that permitted rigorous and repeatable analysis of cognitive processes, and assessed how salticids integrate different sources of information during learning and memory. While the experiments conducted for this thesis were with a single species, these methods and principles should be relevant to other salticids as well as to other visually orienting invertebrates.

Spiders in virtual space

Animal behaviour researchers commonly seek a middle ground between providing realistic, ecologically relevant experimental conditions, and providing conditions that can be precisely controlled and reproduced across trials. Typically, this involves a trade-off between realism and tractability. Conducting experiments in an animal's home environment would clearly be the most 'natural' approach, but control over environmental variables would be highly constrained. Conversely, precise 'synthetic' stimuli can be generated in the laboratory and presented using automated systems, but these do not provide important feedback information to the interacting subject. Virtual reality, which couples naturalistic simulated environments with precise control of experimental variables and real-time feedback, provides a valuable bridge between ecological validity and experimental control (Bohil, Alicea, & Biocca, 2011).

Salticids possess a unique visual system with higher acuity than any other terrestrial arthropod, and respond to video images of prey, enemies and conspecifics in a manner that closely resembles their responses to natural stimuli (e.g., Bednarski, Taylor, & Jakob, 2012; Clark & Uetz, 1990; Menda, Shamble, Nitzany, Golden, & Hoy, 2014). However, up until now, studies using computer-generated or automated stimuli with salticids have been under open-loop conditions that fail to update stimuli in response to the test subject's behaviour. In Chapter 5, I outline the development and proof-of concept testing of a novel virtual reality system for salticids that closes the loop for the first time. I show that salticids detect and respond appropriately to the visual and motor feedback of a virtual environment, and that individual tendencies and associations learnt in the real world transfer to VR. These methods and findings broaden the scope of experimental possibility to encompass design features and contingencies that were previously inaccessible in salticids.

Parameters of learning

Early learning theorists believed that there was a 'general process' to learning in all species that allowed any two associated events to be learned with equal ease. This 'law of equipotentiality' was brought into question in a famous series taste-aversion experiments in the 1960s (Garcia & Koelling, 1966). Rats were exposed to simultaneous pairings of three conditioned stimuli (sweet water, light and sound) with one of two aversive stimuli (X-rays, which induced nausea, or electric shock). When subsequently tested with the individual conditioned stimulus elements, the rats showed clear association between taste and nausea, and between the audio-visual cues and shock, but failed to learn the other elements in each case. These findings

contributed to a shift in perspective and the development of a new theory known as preparedness (Seligman, 1971) that proposed that animals are adaptively prepared to learn certain, biologically meaningful, contingencies.

The ecological relevance, or ‘salience’ of available cues turns out to be an important factor mediating salticid learning and memory. In Chapter 3, I found that spiders more readily learnt to associate a black background with safety than a white background, although this effect was interrelated with the temporal characteristics of the training regime. In Chapter 4, when aversive reinforcement was associated with multiple cues, I found that spiders attended to the aversive cue in exclusion of predictable visual cues, a surprising finding given the otherwise visual nature of spider cognition. Finally, in Chapter 6, I demonstrated that change detection was critically dependent on the salience of the initially viewed target. Taken together, these results suggest that the ecological relevance of the stimuli used can play an important modulatory role in salticid learning and memory.

The temporal characteristics surrounding an event can significantly influence the speed of learning and the strength of consequent memory (Balsam, Drew, & Gallistel, 2010; Gallistel & Gibbon, 2000), yet their influence has not been explicitly examined with spiders. Consistent with literature addressing the ‘trial spacing effect’, spiders trained over 15 trials with a long inter-trial interval outperformed spiders trained with a short inter-trial interval (Chapter 3). Contrary to our expectations, however, spiders tested after a long retention interval (24h) performed as well as spiders tested after a short retention interval (10min). These results suggest that 15 training sessions were sufficient to induce memory formation that lasted 24h, although it is unclear what stage of memory this is (medium or long-term) or whether fewer sessions would have produced a similar finding.

In humans and non-human animals alike, the duration and capacity of working memory is assumed to be an essential component of intelligence (Baddeley, 1986; Matzel & Kolata, 2010). Since intelligence is approximately correlated with brain size (Roth & Dicke, 2005), it is particularly interesting to assess the extent of working memory in animals with miniature central nervous systems (Menzel, 2012). Amongst arthropods, working memory has been well characterized for bees trained to select an object that matches (delayed match-to-sample task) or mismatches (delayed non-match-to-sample task) an object that they had viewed earlier. Using these tasks, working memory for sample patterns is high after a 5s delay and reduced to chance levels after a 9s delay (Zhang et al., 2005). In contrast, I found no significant difference

in performance in spiders tested after a 3s and 10s retention interval, indicating that salticid working memory persists for longer than bee working memory (Chapter 3).

Aversively motivated behaviour

Food reward is the most common form of positive reinforcement, but for many animals, changes in palatability over repeated feedings may confound performance (Bello-Medina et al., 2013). For example, satiation can reduce motivation towards unconditioned stimuli in bees (Abramson, 1994b; Menzel et al., 2001), and can reduce performance on appetitive memory tests in flies (Krashes & Waddell, 2008). In spiders, appetitive reinforcement with food is further confounded by the fact that spiders frequently face extended periods of starvation, and possess physiological adaptations to not only survive but prosper on a limited and unpredictable food supply (Anderson, 1974; Wise, 1993).

In contrast to appetitive stimuli, the effects of aversive stimuli on learning are relatively free of satiation effects, and have unconditioned effects that are less influenced by the animals state (Beninger, 1989). Further, a number of studies with bees have illustrated the modulatory role that aversive stimuli may play in attention and consequent learning. For example, Rodriguez-Girones and colleagues (2013) demonstrated that early contact with an aversive stimulus was sufficient to induce several hours of increased attention, while Avargues-Weber and colleagues (2010) demonstrated that aversive reinforcement can modulate visual discrimination through alterations in attentional processes.

Electric shock is a consistent and fast-acting conditioning stimulus that has been used extensively with diverse vertebrate and invertebrate taxa (e.g., Glotzbach, Ewald, Andreatta, Pauli, & Mu, 2012; Kimble, 1955; Vergoz, Roussel, Sandoz, & Giurfa, 2007). Like any conditioning stimulus, it is important to consider its physiological and behavioural effects, in order to isolate these from the behavioural changes that are a product of the learning or memory test (Pritchatt, 1968). In Chapter 2, I develop a novel method for constructing electric shock platforms and characterize the effects of shock on spider mobility and behaviour. Based on my findings, I suggest a voltage range for learning experiments with spiders that minimizes decrements in physical performance. The method for shock platform construction outlined in this study uses customizable printed circuits, which overcomes many of the constraints of previous methods such as inconsistent shock delivery, manufacturing complexity and cost.

Spiders, like all animals, face risks in their environments. Injury or even death can follow encounters with conspecific rivals, predators, or toxic prey. The ability to learn about and subsequently avoid aversive events has clear adaptive value, and has been demonstrated in insects (e.g., Agarwal et al., 2011; Bernays, 1993; Vergoz et al., 2007) as well as spiders (e.g., Bednarski et al., 2012; Skow, 2007). Avoidance conditioning involves learning a response that prevents the reoccurrence of an aversive event, and is generally divided into two types. In active avoidance, the animal must emit a certain response to postpone or prevent the aversive event, while in passive avoidance, the animal must withhold some response (Olton, 1973). Because these two types test fundamentally different behaviours (action versus inhibition), they can highlight different learning attributes. For example, since active avoidance tasks are normally paired with a discriminative stimulus, they have been used to uncover species differences in cue utilization, such as the preferential use of location information over audio-visual information in rats (Denny, Koons, & Mason, 1959) but not cats (McAdams, 1964) or monkeys (Krieckhaus, 1967).

In this thesis, I examined the relative importance of seven experimental parameters on salticid aversive learning using both passive and active conditioning assays, and electric shock as the aversive stimulus. I used a passive avoidance paradigm in Chapter 2 to train spiders to avoid a previously preferred dark compartment, and in Chapter 3 to avoid a white or black background cue. In contrast to the static cues presented in Chapters 2 & 3, I trained spiders to avoid a moving dot video stimulus in Chapter 4 using an active avoidance paradigm.

Rapid avoidance learning coupled with prolonged memory, as was found in each of the aforementioned tests, indicates central processing and is consistent with current definitions of pain (e.g., Elwood, Barr, & Patterson, 2009). Pain has two main components. First, nociception refers to the ability to detect noxious stimuli and coordinate a reflexive response to get out of harm's way. Second, what we typically refer to as 'pain' is the negative internal interpretation of that experience (Sneddon, Elwood, Adamo, & Leach, 2014), that involves awareness, interpretation and long-term behavioural change (Broom, 1998). Most pertinent to the findings in this thesis is the idea that animals in pain should quickly learn to avoid the noxious stimulus and demonstrate sustained changes in behaviour that have a protective function to reduce further injury and pain, prevent the injury from recurring, and promote healing and recovery (Sneddon, 2003). While nociception coordinates the initial move away from harm, the associative behavioural changes that occur with pain have significantly longer-term protective effects (Bateson, 1991), and thus, greater implications for fitness. As a result,

pain is widespread in the animal kingdom (Elwood, 2012), and is believed to play a role in mediating aversively motivated behaviour in invertebrates (reviewed in: Elwood, 2011).

Inferring mental states in animals is fraught with difficulty (Dawkins, 2006). This is particularly the case with invertebrates, that, even when displaying identical behaviours to vertebrates in response to an aversive stimulus (e.g., writhing in response to electric shock) are dismissed as irrelevant (Sherwin, 2001). While attributing pain states to invertebrates might be ‘inconvenient’ (Kellert, 1993), the detection and assessment of pain in any animal is a crucial step in developing humane experimental procedures and policies (Dawkins, 2006). Further, a detailed behavioural and physiological characterization of nociception and pain in invertebrates would contribute to our understanding of the generalities and divergences between vertebrate and invertebrate systems.

Outlook

This thesis has only begun to scrape the surface of understanding how spiders learn. In addition to the variables examined here, invertebrate learning can be influenced by the amount, probability, frequency, and delay of reinforcement; the types of response being measured; and the duration, intensity, and temporal order of stimuli used during training, alongside a multitude of other factors (Abramson, 1994a). Moreover, my research focused strictly on associative learning processes that encompass predictive and unambiguous relationships between events, but there are other forms of associative learning that occur when the relationships between events are ambiguous. These *nonelemental* forms of associative learning are considered to be more complex, as they cannot be solved by learning simple associations between two elements (Rudy & Sutherland, 1992). For example, in ‘negative patterning’, the animal must learn to respond to two single stimuli when presented alone, but not their compound. Since the compound is necessarily different than the sum of each stimulus alone, the animal cannot use ‘simple’ associative processes to learn the task. Amongst invertebrates, the traditional framework of nonelemental learning protocols has primarily been applied to bees (Deisig, Sandoz, Giurfa, & Lachnit, 2007; Giurfa, 2003; Giurfa et al., 1999), but has not yet been explicitly tested with spiders. However, nonelemental learning also underlies problem solving in which animals must respond to stimuli that they have never encountered by generating a novel response that is adaptive within that given circumstance (Giurfa, 2007). Such behavioural flexibility has been demonstrated in the araneophagic (spider-eating) salticid *Portia*, in diverse contexts (e.g., Cross & Jackson, 2014; Jackson, Carter,

& Tarsitano, 2001). Further research on nonelemental processes in spiders is required to understand the degree to which such complex processes mediate learning and memory in this group.

Purely behavioural studies of cognition have the limitation that motor output (such as orientation or approach) can be dissociated from attention: it is possible to attend to an object without moving, or to move towards an object without attending to it (Swinderen, 2005). This is particularly the case in salticids, since the retinae of their principle eyes are located on movable eye tubes that significantly extend the visual field without requiring additional movement (Land, 1969a, 1969b). The findings from this thesis could be greatly extended by incorporating neurophysiological recordings or retinal eye tracking, both of which have recently been developed for salticids (Menda et al., 2014; Schiesser, Canavesi, Long, Jakob, & Rolland, 2014). The virtual reality methods outlined in Chapter 5 offer a particularly compelling experimental paradigm for coupling behavioural with neuroanatomical techniques. Indeed, the possibility of conducting neurophysiological testing while an animal interacts with a realistic virtual environment is thought to be one of the greatest benefits and most important advances of VR compared with other methods (Bohil et al., 2011; Dombeck & Reiser, 2012; Tarr & Warren, 2002).

Finally, for spiders as a whole to be of real utility within the comparative framework, a greater taxonomic range needs to be considered. The spider cognition literature is dominated by only a few families, but there is evidence for behavioural modification across the Araneid order (reviewed in: Jakob et al., 2011). An excellent case study for the utility of examining within-order cognitive differences is that of parasitoid wasps, that show significant inter-species ecological variation. This variation is accompanied by species-specific differences in learning and memory formation (Bleeker et al., 2006; Geervliet, Vreugdenhil, Dicke, & Vet, 1998; Smid et al., 2007) that appear to reflect adaptations to each species' unique ecological constraints (Hoedjes et al., 2011). Considering the extraordinary diversity of spiders, it is very likely that detailed comparisons across families, and even species, will reveal similar ecologically dependent cognitive differences.

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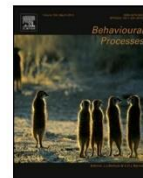
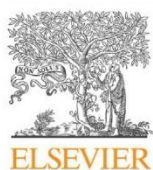
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8 APPENDIX A

This section contains the published version of chapter 2

Peckmezian, T., & Taylor, P. W. (2015). **Electric shock for aversion training of jumping spiders : Towards an arachnid model of avoidance learning.** *Behavioural Processes*, 113, 99–104.



Electric shock for aversion training of jumping spiders: Towards an arachnid model of avoidance learning

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ARTICLE INFO

Article history:

Received 26 August 2014

Received in revised form 19 January 2015

Accepted 25 January 2015

Available online 28 January 2015

Keywords:

Aversive stimulus

Electric shock

Jumping spider

Learning

Mobility

ABSTRACT

Electric shock is used widely as an aversive stimulus in conditioning experiments, yet little attention has been given to its physiological effects and their consequences for bioassays. In the present study, we provide a detailed characterization of how electric shock affects the mobility and behaviour of *Servaea incana*, a jumping spider. We begin with four mobility assays and then narrow our focus to a single effective assay with which we assess performance and behaviour. Based on our findings, we suggest a voltage range that may be employed as an aversive stimulus while minimizing decrements in physical performance and other aspects of behaviour. Additionally, we outline a novel method for constructing electric shock platforms that overcome some of the constraints of traditional methods while being highly effective and easily modifiable to suit the study animal and experimental context. Finally, as a demonstration of the viability of our aversive stimulus in a passive avoidance conditioning task, we successfully train spiders to associate a dark compartment with electric shock. Future research using electric shock as an aversive stimulus with terrestrial invertebrates such as spiders and insects may benefit from the flexible and reliable methods outlined in the present study.

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1. Introduction

Electric shock is used extensively as an aversive stimulus in conditioning experiments, with species-specific avoidance responses documented in taxa as varied as insects (Vergoz et al., 2007), crustaceans (Abramson et al., 1988), fish (Wodinsky et al., 1960; Xu et al., 2007), rodents (Kimble, 1955; Iwata and LeDoux, 1988), and primates (Barrett, 1977), including humans (Glötzbach et al., 2012). As a conditioning stimulus, electric shock offers a number of advantages, including ease of use, immediacy of onset and offset, and a precisely controlled area of effect. There is a need for careful consideration of physiological effects induced by electric shock, and the consequences of such physiological effects for bioassays. It is difficult to isolate the behavioural changes that are contingent on the chosen learning or memory paradigm in the absence of an understanding of the changes to physical state that result from aversive stimuli (Pritchett, 1968). For example, a lack of mobility in a spatial maze owing to physical effects of an aversive stimulus could result in a poor performance score, as animals would be less likely to reach a target than their more mobile counterparts. Through awareness

of such collateral effects of conditioning stimuli it is possible to ameliorate risks through precautions or controls.

Invertebrates have long been used to study the behavioural, cellular and molecular basis of cognition, but in recent years the focus has been on developing a few key model systems, such as honeybees and *Drosophila* in depth, rather than sampling widely across taxa (Sattelle and Buckingham, 2006; Wolf and Heberlein, 2003). While much can be learned from delving deep into the workings of select model animals, a broader perspective remains integral to the central tenet of comparative cognition, drawing on assessments of how taxonomically disparate groups perform in analogous physiological or behavioural tasks (Eisenstein, 1997; Shettleworth, 2010).

Spiders have been underrepresented in the comparative literature, yet they have much to offer. Spiders inhabit nearly all terrestrial environments and exhibit extraordinary diversity. For example, spiders vary from a solitary lifestyle to living in dense and cooperative social groups, with predatory behaviour ranging from sit-and-wait strategies in webs to active pursuit as cursorial hunters (Wise, 1993; Foelix, 2011). Jumping spiders (Araneae, Salticidae) are particularly well suited for studies of cognition, with exceptionally acute vision in their large forward-facing 'primary' eyes and complex, visually mediated behaviour (Jackson and Cross, 2011). Most jumping spiders are cursorial hunters that use their

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extraordinary visual abilities to mediate navigation, hunting and communication. It is known that they can learn in a variety of contexts (Nakamura and Yamashita, 2000; Skow, 2005; Jakob et al., 2007; Leidtke et al., 2014), solve problems through trial and error (Jackson et al., 2001), perform challenging navigational detours (Tarsitano and Jackson, 1994) and behave flexibly in novel situations (Jackson and Wilcox, 2010). Despite a growing literature detailing impressive cognitive feats in spiders, much of this work has been conducted using methods that do not readily support comparisons with other taxa. For spiders in general and salticids in particular to enter the comparative framework, there is a need to adapt and apply the well-established standard tools of the comparative method.

There is a need for well-characterized aversive stimuli for use in conditioning experiments. In the present study, we provide a detailed characterization how electric shock, one of the most commonly used aversive stimuli, affects the mobility and behaviour of *Servaeia incana*, a common Australian salticid species. We begin with four mobility assays and then narrow our focus to a single effective assay with which we assess performance after both short and long-term shock exposure. We also assess behaviour of spiders in the long-term exposure group. Based on these results, we suggest a voltage range that may be employed as an aversive stimulus while minimizing decrements in physical performance and other aspects of behaviour.

The passive-avoidance paradigm is based on the animal learning to avoid an environment in which an aversive stimulus was previously delivered. The response that is punished is typically one that the animal normally performs. This means that the task is for the animal to learn to suppress a normal response when in a given context, and thus behave contrary to their innate preferences (Bammer, 1982). The passive avoidance paradigm is well established in insects (bees: Abramson, 1986; Agarwal and Guzman, 2011; cockroaches: Disterhoft et al., 1971; Disterhoft, 1972 and ants: Abramson, 1981), and has been used successfully to assess colour discrimination in spiders through colour-heat pairings (Nakamura and Yamashita, 2000). Here, we conduct an initial preference test to determine if spiders prefer the dark or light compartment of a two-sided arena, then train spiders to avoid their preferred compartment by pairing it with electric shock. Following a 20-min break we return spiders to the arena to see if avoidance behaviour persists in the absence of electric shock, and if spiders can retain at least short-term memory of this aversive event.

2. Methods and results

2.1. General Method

2.1.1. Animals

Equal numbers of adult male ($n = 95$) and female ($n = 95$) *S. incana* were collected from *Eucalyptus* trees in Sydney, Australia. Spiders were maintained in a controlled environment laboratory (24–26°C; 65–75% relative humidity; 11:11:11 light:dusk:dark:dawn cycle) where they were individually housed in ventilated 1.125 L plastic cages (11 × 11 × 12 cm tall) containing a folded sheet of paper (2 × 3 cm) that was shaped as a 'tent' under which spiders could shelter and build nests. Spiders were fed weekly on an alternating diet of two house flies (*Musca domestica*) or two Queensland fruit flies (*Bactrocera tryoni*). All experiments were conducted 3–4 days following a feeding. Supplementary moisture was provided by lightly misting each cage with a spray bottle once each week. All experiments were conducted during daytime hours (8 am to 4 pm) under full-light (50:50 metal halide and halogen ceiling lights).

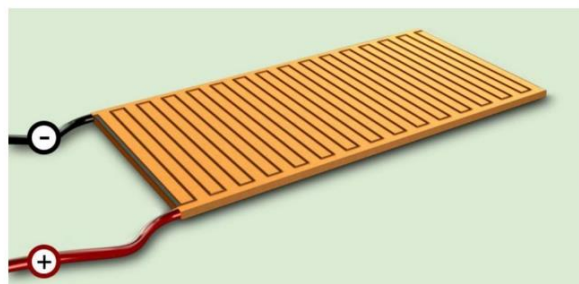


Fig. 1. Schematic of custom-designed electric shock platform, constructed as a printed circuit. Spiders receive a shock when a power source is activated and they bridge parallel opposite-charged bars of the platform.

Spiders were weighed to the nearest 0.1 mg (Shimadzu Model N595, electronic balance, Shimadzu Corporation, Kyoto, Japan) within 3 h following their final trial. In addition to weighing, each spider was photographed from above using a digital camera (ProgResC10) focused through a stereomicroscope (Olympus SZX12, Olympus Corporation, Tokyo, Japan). To minimize movement during photography, spiders were gently restrained on the flat surface of an inverted Petri dish under clear plastic film (Glad Products, Australia). Cephalothorax length and width were measured using the open source image-processing software ImageJ (v1.30, National Institutes of Health, Bethesda, MD, U.S.A.).

2.1.2. Shock chamber

Spiders were confined to a rectangular arena (75 mm wide × 150 mm long × 75 mm tall) constructed from white corrugated plastic board (Corflute®). The top of the arena was open to permit video recording, while the walls were lightly dusted with non-scented talcum powder to make the walls slippery and prevent spiders from escaping. The shock platform – on the floor of each enclosure – was a rectangular board (2 mm thick × 150 mm long × 75 mm wide) covered with a pattern of parallel copper bars alternately of positive and negative charge (Fig. 1). Previous studies have used adhesive copper tape (Skow, 2005), strips of aluminium (Bednarski et al., 2012), or wire (Agarwal et al., 2011) to achieve a similar design, but each has drawbacks owing to inconsistent voltage or susceptibility to damage. Here we adopt a novel technique that produces highly uniform voltage across the platform while eliminating risk of subjects failing to contact bars or becoming injured. To create a shock platform, a grid-like pattern (5 mm bars spaced 1 mm apart) was chemically etched onto the copper side of a blank printed circuit board backed with epoxy fibreglass laminate (Jaycar Electronics, Australia). The pattern was designed using Adobe Photoshop CS5.5 (Adobe Systems, San Jose, CA, U.S.A.) and printed onto toner transfer film (Press-n-Peel, Techniks Inc., New Jersey) using a standard laser printer (Hewlett-Packard 4250 LaserJet). The pattern was then transferred to a blank copper board using a hot iron, followed by chemical etching in a hot ammonium persulphate bath. Boards produced using this method are durable and can be cleaned with circuit board cleaner, alcohol and water without affecting their conductivity.

Alternating bars of the copper shock platform were wired to the positive and negative terminals of a 60 V (max 3 amp) DC power supply (Sanke Electrical Co., Ltd., China) (see Fig. 1). When the power supply was active, shock was delivered to spiders each time they bridged the gap between parallel copper bars, completing the circuit. Due to the narrow gap between opposite-charged bars, failure to complete the circuit was very rare.

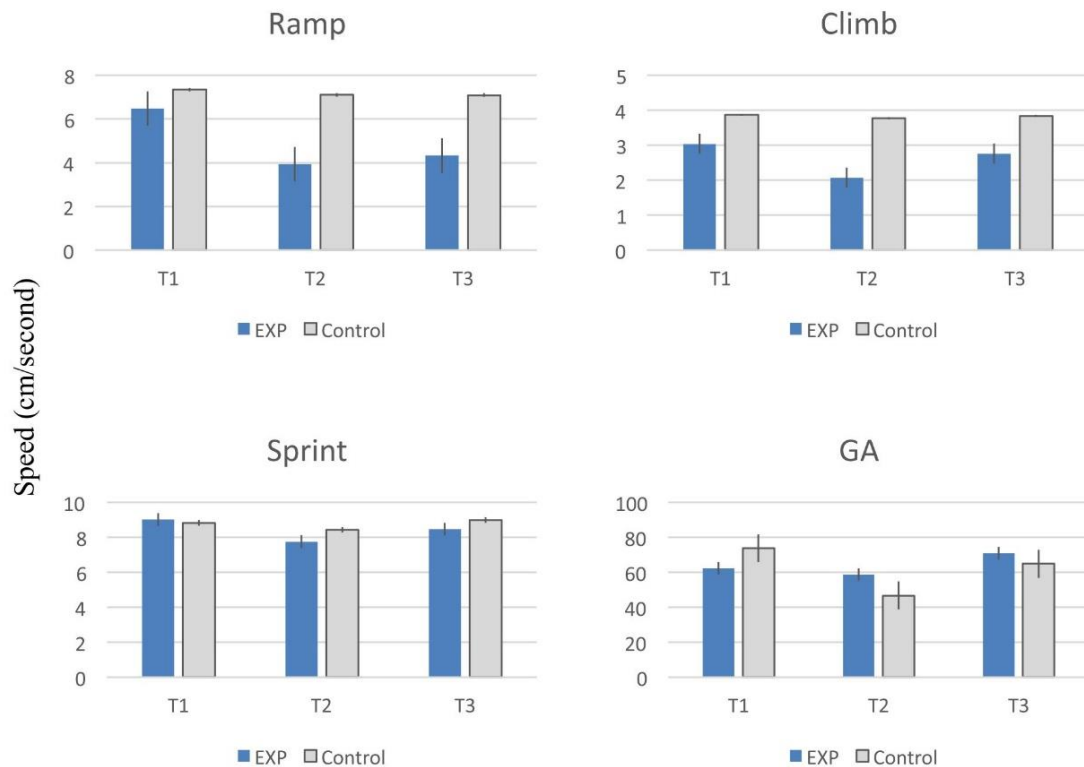


Fig. 2. Mean speed on the ramp, sprint, climb and general activity (GA) assays in experiment 1. Spiders were tested 2 h before shock (T1); immediately following 30 s shock exposure (T2); and 2 h after shock (T3). Blue bars represent experimental (EXP, shocked) spiders while grey bars represent control (unshocked) spiders. Bars represent \pm standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Experiment 1: finding an appropriate assay

In this experiment, we randomly assigned 40 spiders to one of four mobility assays (5 male, 5 female per assay) to determine the measure that best detected a voltage-related change in performance. Each spider was assigned as either a shocked (60 V for 30 s) or unshocked (0 V) subject and was used only once. The sprint and ramp assays required the spider to run 60 cm along a 5 cm-wide textured (240 grit sandpaper) runway that was oriented horizontally (sprint) or at a 30-degree incline (ramp). For the climb assay, spiders were placed at the bottom a 2.5 cm diameter wooden dowel coated with a textured spray paint (Spray Stone paint, White Knights Paint, Australia), and induced to climb its length without pause by gently brushing the spider's hind legs with a soft sable-hair paintbrush. The sprint, ramp and climb assays followed protocols from previous studies of spider locomotor performance (climb, Prenter et al., 2010 sprint & ramp, Prenter et al., 2012), and were all measured in terms of mean speed (distance travelled divided by the time taken to complete the task).

The general activity assay, in contrast, was a measure of spontaneous activity (total distance travelled) in a 5 min period. Spiders were transferred from their home cages to a small holding vial, and then gently lowered into a rectangular arena identical to the shock arena but with the shock turned off. Spiders were allowed to move freely throughout the 5-min trial. Trials were video-recorded from above (Panasonic HDC-SD900 video camera), and distance travelled was calculated by utilizing the motion tracking function in Adobe After Effects (CS5.5). Each video was first adjusted to isolate the spider as a high contrast image against the background onto which a tracker could be attached. Coordinate data were recorded throughout the trial by analysing the video frame-by-frame

(30 frames per second), outputting locomotion as a series of changes in x and y coordinates. These values were then used to calculate the total distance travelled in Microsoft Excel.

Each spider provided three measures of performance in their respective assays. The first measure, T1, provided an individual baseline score. T2 was recorded 2 h after T1, and immediately following 30 s of 60 V shock exposure or 30 s in the shock apparatus without shock. Finally, T3 was recorded 2 h after T2, to assess delayed or persistent effects of shock exposure. The differences in the performance of shocked and unshocked spiders was assessed using a repeated-measures MANOVA (group as predictor; speed at T1–T3 as responses), with a separate analysis run for each assay. To test whether shocked and unshocked spiders differed in performance across time points, we compared groups at each time pairing (T1–T2, T1–T3, T2–T3) using one-way ANOVAs. Huynh and Feldt, 1970 corrections were applied to the degrees of freedom in all cases in which sphericity assumptions had been violated. Sex weight and cephalothorax length were initially included in all analyses conducted in this study; however, as none exhibited a significant effect or interaction, these terms were omitted from the final model. The assay most sensitive to shock-related changes in performance was adopted for subsequent experiments designed to characterize effects of shock at a finer scale. A sensitive assay, in this case, was one that produced the greatest significance (p) value when comparing groups for performance changes between baseline and T2 and T3, and here we found this to be the ramp test. All experiments were analysed using JMP version 5.0.1.2 (SAS Institute Inc., Cary, NC).

The ramp assay was found to most clearly detect effects of shock on performance, with shocked, but not unshocked, spiders exhibiting a decrease in speed over time (group: $F_{1,8} = 7.35$, $p = 0.027$; time:

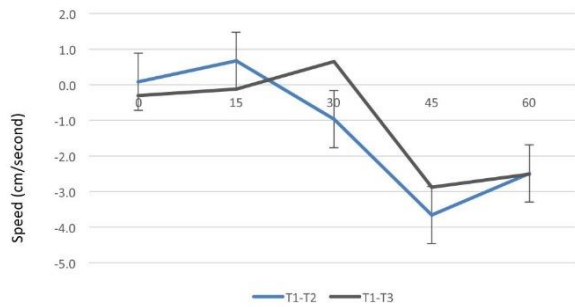


Fig. 3. Mean performance difference between T1 (baseline, 2 h before shock), T2 (immediately following shock) and T3 (2 h after shock) on the ramp mobility assay across voltage groups. Negative numbers indicate a decrease in speed between time points. Bars represent \pm SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$F_{2,7} = 8.83$, $p = 0.012$; time \times group: $F_{2,7} = 1.90$, $p = 0.024$; Table 1). In particular, we found a significant difference between shocked and unshocked spiders between baseline and T2 ($F_{1,8} = 7.72$, $p = 0.024$, $\eta^2 = 0.49$) but not baseline and T3 ($F_{1,8} = 3.50$, $p = 0.098$) or T2 and T3 ($F_{1,8} = 1.44$, $p = 0.264$). Sprint, climb and general activity assays did not detect any significant changes in performance (sprint: $F_{2,7} = 0.28$, $p = 0.427$; climb: $F_{2,7} = 0.84$, $p = 0.469$; general activity: $F_{2,7} = 0.82$, $p = 0.478$; Fig. 2). The ramp assay was subsequently adopted for experiments 2 and 3 as a sensitive test of electric shock-induced decrement in performance.

2.3. Experiment 2: short exposure to 0–60 V

Spiders (5 male, 5 female) were assigned to each of five voltage groups (0, 15, 30, 45, 60 V) and assessed for performance on the ramp mobility assay 2 h before, immediately after and 2 h after 30 s of shock exposure. By comparing performance across 5 voltage levels, the goal of this experiment was to determine the maximum shock level for subsequent testing. Voltage levels associated with significant decrements in performance were excluded from testing in subsequent experiments that were designed to further refine recommended protocols. Results were analysed using an ANOVA with the change between baseline and T2 and T3 as the response variables and voltage group as a factor. Significant main effects were further analysed using a least squares means Student's t -test with Bonferroni corrections for multiple comparisons (adjusted $\alpha = 0.0125$).

Voltage groups varied over time (voltage group by time interaction: Wilks' $\lambda = 0.50$, $F_{8,88} = 4.53$, $p < 0.001$; Fig. 3), with significant differences found between baseline and T2 ($F_{4,45} = 5.49$, $p = 0.001$, $\eta^2 = 0.33$) as well as baseline and T3 ($F_{4,45} = 5.46$, $p = 0.001$, $\eta^2 = 0.33$). Using the baseline to T2 and baseline to T3 difference scores, we compared each voltage group to the 0 V control group and found a significant difference at 45 (T1-T2: $F_{1,45} = 17.72$, $p < 0.001$; T1-T3: $F_{1,45} = 7.79$, $p = 0.008$) and 60 V (T1-T2: $F_{1,45} = 7.87$, $p = 0.007$; T1-T3: $F_{1,45} = 6.109$, $p = 0.012$). In each case, speed decreased from baseline to both T2 and T3.

Table 1

Mean speed in seconds (\pm SEM) on the ramp assay for experimental and control group spiders.

	T1	T2	T3
Experimental	6.48 \pm 0.79	3.93 \pm 0.40	4.32 \pm 0.35
Control	7.35 \pm 1.11	7.10 \pm 0.58	7.10 \pm 0.49

2.4. Experiment 3: long exposure to 0–30 V

Spiders (5 male, 5 female) were assigned to each of four long-exposure voltage groups (0, 20, 25 and 30 V) and their subsequent performance was assessed using the ramp assay. The voltage levels used in this experiment were determined as safe working levels that were not associated with a significant decrement in performance in the previous experiments. Learning experiments typically involve observing a change in behaviour over a series of trials, with often substantial cumulative exposure to the aversive stimulus in avoidance learning experiments. The goal of this experiment was to determine the effects of shock when delivered over longer periods of time. In each trial, spiders spent 15 min in the shock apparatus, with intermittent (30 s on/30 s off) shock occurring throughout T2. Each spider provided 3 measures of speed (T1, T2 and T3), and trials were separated by a 15 min inter-trial interval. Ramp test results were analysed using an ANOVA with repeated measures on one factor, time.

Trials were video-recorded from above and analysed using event-recording software (JWatcher 1.0, Blumstein et al., 2000) for 5 behaviours: inactivity, walking, running, jumping and scrambling behaviour (the spider raised and moved its forelegs against a wall, as though attempting to climb). The proportion of each trial spent performing a behaviour and the number of instances that each behaviour occurred (count) were calculated for each individual at each time point. A behaviour was counted each time it was initiated, following either the start of a trial or a different behaviour. A separate two-way repeated measures MANOVA was run for each of the 5 behaviours, using each of the three measures (count, total time, proportion of time).

We found no significant time by voltage interaction in the running speed or behaviour (mean speed: Wilks' $\lambda = 0.905$, $F_{6,70} = 0.599$, $p = 0.730$; behaviour: motionless: Wilks' $\lambda = 0.92$, $F_{6,70} = 0.33$, $p = 0.922$; scramble: Wilks' $\lambda = 0.94$, $F_{6,70} = 0.37$, $p = 0.895$). Similar results were obtained for analyses of count and total time. The lack of shock-induced change in behaviour or performance on a sensitive mobility assay suggests that intermittent exposure to 30 V or below is a 'safe' level of shock to use as an aversive stimulus in conditioning experiments of this species.

2.5. Experiment 4: passive avoidance conditioning

A separate group of adult female spiders ($n = 38$) was used to test the efficacy of our electric shock stimulus as an aversive stimulus in a passive avoidance conditioning task. The testing environment consisted of a rectangular arena similar in design to that already described but twice the length (75 mm wide \times 300 mm long \times 75 mm tall). The arena had a separate electric shock platforms at either end of the arena. In this way, we could electrify one half of the apparatus at a time while keeping the other half non-electrified and identical in appearance. A black piece of card stock was placed over one half of the apparatus creating a shaded 'dark' zone underneath.

Testing consisted of a shock-absent (S^-) pre-training stage on day 1, a shock-present (S^+) training stage 24 h later, and an S^- test stage 20 min following the completion of the training stage. Each stage was 20 min long and was conducted in the same arena, which was cleaned with warm water between trials to remove silk draglines and pheromone cues (see Jackson, 1987). The arena was rotated 90° between trials to avoid learning of visual extra-arena cues. For the shock-present group ($n = 26$), the shock was deactivated during the pre-training and test stages, and activated on the dark side of the arena only during the training stage. The task was thus for the spider to learn to avoid the electric shock by avoiding the dark side of the arena. Testing was similar for the control group ($n = 12$), but with the shock switched off in all trials.

Trials were initiated by placing the spider in the centre of the illuminated compartment. All trials were video-recorded from above and subsequently scored from these recordings. We assessed the latency for spiders to first enter the dark side of the arena, as well as the proportion of each trial spent in the shocked side ($S^+ - S^- / S^+ + S^-$, where S^+ is the time on the shocked side and S^- is the time on the unshocked side).

We performed a one-way ANOVA comparing shock-present and control spiders for each of the three time points, for both latency to enter the dark side of the arena and proportion of time spent on the dark side. The pre-training comparison ensured that, prior to treatment, experimental and control group spiders behaved similarly; the training comparison determined whether the aversive shock stimulus was indeed aversive to shock-present spiders; and the test stage comparison assessed whether this aversive experience was learnt and remembered after a 20-minute delay. We also ran a repeated-measures ANOVA to compare group differences in both performance measures between pre-training and test sessions.

Overall, spiders spent more time in the dark side of the arena than the light side, spending on average 71% of the pre-training session in the dark. Since the task was for spiders to behave in opposition to their dark preference, we omitted spiders in the shock-present group that did not display this preference in the pre-training trial ($n=4$) from all subsequent analyses, as well as those that did not enter both compartments at least once in both the pre-training and training sessions ($n=1$). There were no significant differences in shock-present and control groups in their latency to enter the dark side during pre-training and training (pre-training: $t=1.58$, $df=33$, $p>0.1$; training: $t=-0.42$, $df=33$, $p>0.1$), or the proportion of time spent in the dark side during pre-training ($t=0.28$, $df=33$, $p>0.1$). However, shock-present spiders spent significantly less time on the dark side of the arena during the training session than control spiders, indicating that the shock platform was indeed aversive (prop, shock-present mean: 0.52, control mean: 0.75; $t=2.25$, $df=33$, $p=0.031$, $\eta^2=0.13$). Additionally, shock-present spiders were slower to enter the dark side of the arena in the test trial (latency, shock-present mean = 248 s, control mean = 82 s; $t=-2.27$, $df=33$, $p=0.030$) and spent less time on the dark side (prop, shock-present mean = 0.57, control mean = 0.80, $t=2.51$, $df=33$, $p=0.017$, $\eta^2=0.16$) than control spiders, suggesting that spiders had learnt the dark-shock association and were able to retain this memory through the 20 min interval preceding the test session (Fig. 4). Finally, shock-present and control groups differed significantly in their latency to enter the dark side of the arena between pre-training and test (time \times group, $F_{1,33}=6.97$, $p=0.013$), but not in the proportion of time spent in this compartment (time \times group, $F_{1,33}=2.41$, $p>0.1$).

3. Discussion

In the present study we demonstrate the use of printed circuits as a novel, inexpensive and highly effective method for constructing electric shock platforms. Our approach overcomes many of the constraints associated with previous methods, such as inconsistent shock delivery, manufacturing complexity and cost (discussed in Long et al., 2015). Since grid spacing and overall platform design are defined in computer software, measurements are precise, and most importantly, virtually identical across all platforms printed using a given design. The design itself is easily modifiable, allowing the experimenter to alter size, spacing or pattern to fit the study animal and experimental context. Future research using electric shock as an aversive stimulus with terrestrial invertebrates such as spiders and insects may benefit from the flexible and reliable methods outlined here.

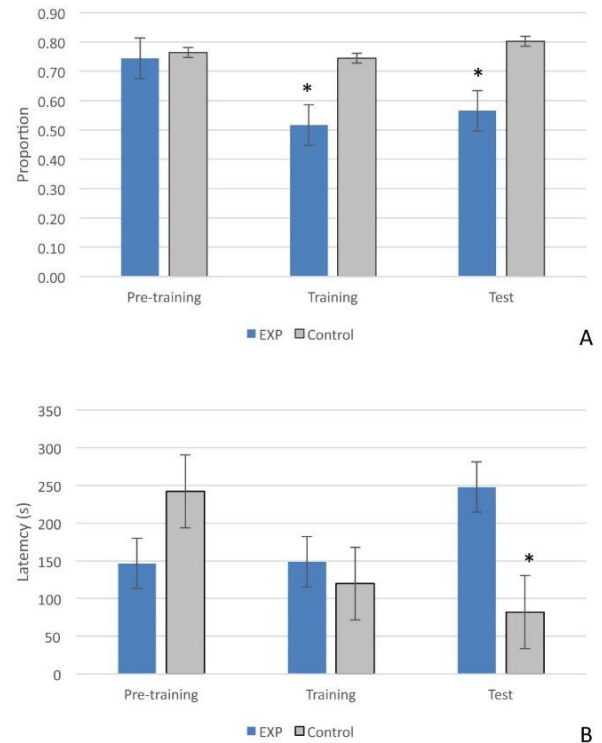


Fig. 4. Spider performance on a passive avoidance conditioning test. Shock is activated during the Training stage for the experimental (EXP) group only. A. The proportion of each trial spent in the dark compartment. B. The latency to enter the dark compartment. Stars represent trials in which shock-present and control group spiders are statistically different ($p < 0.05$), and bars represent \pm SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Determining the appropriate intensity for aversive stimuli used in behavioural experimentation is an important step in technique validation. Previous work with rodents has demonstrated that the intensity of a shock stimulus can influence the outcome observed, with sub- or super-optimal levels leading to decrements in performance in learning tasks (Kimble, 1955; Moyer and Korn, 1966; Mcallister et al., 1971). Similarly, cockroaches perform worse in avoidance learning tasks as the intensity of shock exposure is increased (Pritchatt, 1970), and this is likely a product of shock-induced pathology. Since electric shock may produce changes at the level of primary sensory neurons, motor neurons, body fluids or hormones (Eisenstein, 1997), one should pay particularly close attention to the non-associative physical and behavioural changes that occur at various thresholds.

To this end, we conducted a series of experiments to assess the effects of shock on mobility, behaviour and learning in *S. incana*, determining 30 V to be an optimal level. Here we use 'optimal' to refer to a voltage level that is sufficiently aversive so as to lead to avoidance, while at the same time producing no detectable detrimental effects on physical performance. Significant decrements in running speed on inclined ramps were noted in spiders exposed to 45 V or greater, whereas even relatively long-term (15 min) exposure did not yield significant effects on performance or behaviour when spiders were exposed to 30 V or below. Future work using electric shock as an aversive stimulus with this species should consider intermittent exposure at around 30 V as appropriate, and this also provides a useful starting point for work with other salticids. Indeed, research with a salticid in the U.S.A, *Phidippus princeps*,

indicated 33 V as the maximum level of shock that could be delivered without impeding normal behaviour and movement (Skow, 2007).

The results from our passive avoidance conditioning experiment confirm that our shock stimulus was indeed aversive. Spiders spent significantly less time on the dark side of the arena when that side was paired with electric shock than when no shock was applied. Further, spiders exposed to this dark-shock pairing maintained this association in short-term memory over a 20 min delay period and subsequently avoided the dark side of the arena in a shock-absent test trial. Previous work using aversive paradigms with salticids have demonstrated retention periods ranging from 3 min (Nakamura and Yamashita, 2000) to 24 h (Jakob et al., 2007), with varying levels of acquisition. The paradigm outlined here could serve as a useful tool in methodically examining the speed of learning and the rate of memory decay in salticids, while being amenable to comparisons with other taxa. Additionally, since conditioning tends to be enhanced with increasing stimulus intensity in the passive avoidance paradigm (Seligman and Campbell, 1965), voltages between our known 'safe' level (30 V) and the level demonstrated to produce physical decrements (45 V) may be worth exploring in order to determine the parameters that produce maximal learning and memory.

Avoidance learning has been well characterized in invertebrates (Perry et al., 2013) but as with research on other aspects of cognition, investigations have focused heavily on a narrow range of model organisms. One of the primary goals of comparative cognition is to trace the evolutionary development of cognition by comparing the mechanisms employed by different taxa in solving analogous computational tasks (Soto and Wasserman, 2010). Salticids – and arachnids as a whole – have been underrepresented in the comparative cognition literature, yet possess many characteristics which make them suitable as models (Jakob et al., 2011). A small but growing body of research has demonstrated the capacity for spiders to learn in a variety of contexts (reviewed in Jakob et al., 2011), but drawing comparisons from these data to other taxa has been impeded by the disparate methodologies adopted in each of these studies. The passive avoidance paradigm is well described and may serve as a useful approach for further investigation of learning in spiders.

Acknowledgements

TP was supported by an Australian Government Endeavour Postgraduate Award. Research expenses were supported by Higher Degrees Research Funds of the Department of Biological Sciences at Macquarie University. We thank Rowan McGinley and Vivian Mendez for assistance with collection and maintenance of spiders; Ken Cheng for constructive advice during experimental design and two anonymous reviewers for insightful comments on the manuscript.

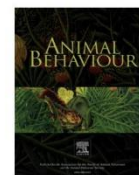
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9 APPENDIX B

This section contains the published version of chapter 5

Peckmezian, T., & Taylor, P. W. (2015). **A virtual reality paradigm for the study of visually mediated behaviour and cognition in spiders.** *Animal Behaviour*, 107, 87–95.



A virtual reality paradigm for the study of visually mediated behaviour and cognition in spiders



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ARTICLE INFO

Article history:
Received 23 March 2015
Initial acceptance 21 April 2015
Final acceptance 3 June 2015
Published online

MS. number: 15-00234

Keywords:
jumping spider
learning
Salticidae
virtual reality
vision

Jumping spiders (Salticidae) are well known for their unique, high-acuity visual system and complex, visually mediated behaviour. To overcome the limitations of video playback and other open loop systems that are currently available for the study of visually mediated behaviour in jumping spiders, we developed a closed-loop, virtual reality (VR) system in which a spider on a spherical treadmill walks through a projected 3D world that updates in real time in response to its movements. To investigate VR as an experimental technique for spiders as well as validate it as a proxy of the real world, we conducted two experiments to assess whether individual behavioural tendencies and learning transferred from real to virtual environments. In the first experiment, we examined transference of individual behaviour tendencies (spontaneous locomotion and dark/light preference) between real and VR environments, and found that individual differences were conserved. In the second experiment, we investigated transference of beacon-learning tasks between real and VR environments. We found that spiders that had learned a beacon–nest site association in the real world tended to express similar associations in the virtual world. Virtual reality offers great promise as a new tool to explore the cognitive processes underlying vision-mediated learning, memory and navigation in jumping spiders.

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Virtual reality (VR) is increasingly used in the behavioural sciences to overcome the constraints of traditional open-loop systems. In the context of animal behaviour, VR refers to a simulated environment that is sensed by the animal and is updated by the animal's actions (Dombeck & Reiser, 2012), giving an experience of immersion within the simulation (Sherman & Craig, 2002). VR, by design, is a closed-loop system, in which responses to virtual stimuli are tracked and used to update the next 'view' of the virtual environment in real time. Since virtual environments are typically implemented as automated, computer-controlled systems, virtual stimuli and world features can be designed to be 'photo-real', abstract, or even selectively modified versions of real-world stimuli. In this way, VR can provide a valuable bridge between ecological validity and experimental control, supporting rich, multisensory environments alongside precise control of experimental variables (Bohil, Alicea, & Biocca, 2011).

Understanding how 'simple' invertebrate systems handle the complexities of daily life can inform us about the cognitive

processing requirements that are necessary and sufficient to accomplish a given task. Virtual environments, in various forms, have been utilized to dissect diverse aspects of invertebrate behaviour and physiology. Perhaps the most common form has been the flight simulator for tethered flying insects, where the subject's intention to turn in response to visual motion is measured with a torque meter or wing beat analyser and used to update a virtual environment in real time. Using this approach, researchers have studied optomotor responses (Bender & Dickinson, 2006), flight kinematics (Gray, Pawlowski, & Willis, 2002), navigation (Gotz, 1987; Reiser & Dickinson, 2010), visual learning (Dill, Wolf, & Heisenberg, 1993; Wolf et al., 1998), decision making (Brembs, 2011) and operant and classical conditioning (Brembs, 2000). Systems have also been designed for tethered walking invertebrates (Dahmen, 1980; Strauss, Schuster, & Götz, 1997; Takalo et al., 2012), and, recently, for subjects in free flight (Fry et al., 2004; Fry, Rohrseitz, Straw, & Dickinson, 2008).

Jumping spiders (Salticidae) are well known for their unique visual system and complex, visually mediated behaviour. In contrast to the compound eyes of insects, salticids possess four pairs of functionally specialized 'camera eyes', each of which uses a single lens to project an image on to the retina (Land, 2005). Their large forward-facing principal eyes feature colour vision (Land,

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1969b; Peaslee & Wilson, 1989), depth perception (Nagata et al., 2012), and a retina with spatial acuity that greatly exceeds that of any other animal with eyes of comparable size (Land & Nilsson, 2012). Smaller motion-detecting secondary eyes collectively enable the spider to view nearly 360° of its surroundings (Land, 1971). By separating spatial acuity from motion detection, the modular design of the salticid visual system enables remarkable visual feats while minimizing costly increases in eye size (Land & Nilsson, 2012; Laughlin, de Ruyter van Steveninck, & Anderson, 1998). As a comparison, the high-resolution compound eyes of libellulid dragonflies combine these features in the same eye, but to achieve this their eyes are larger than the entire body of most salticids (Labhart & Nilsson, 1995).

Using optical cues alone, salticids can identify prospective mates and rivals, classes of prey and enemies, and features in their environment (Harland & Jackson, 2004; Jackson & Pollard, 1996; Tarsitano & Jackson, 1997). Vision is also central to intraspecific communication, such as during elaborate courtship displays (Elias, Land, Mason, & Hoy, 2006; Elias, Maddison, Peckmezian, Girard, & Mason, 2012) and during highly ritualized agonistic interactions between males (McGinley, Prenter, & Taylor, 2015; Taylor, Hasson, & Clark, 2001). Vision also plays a central role in cognition, with salticids relying on vision for complex decision making (Jackson & Cross, 2013), learning (Jakob, Skow, Haberman, & Plourde, 2007; Nakamura & Yamashita, 2000; Skow & Jakob, 2006), discrimination and categorization of prey (Dolev & Nelson, 2014), trial and error problem solving (Jackson & Nelson, 2011) and navigation (Hoeffler & Jakob, 2006; Tarsitano & Jackson, 1997).

Salticids respond to video images of prey, enemies and conspecifics in a manner that closely resembles their responses to natural stimuli. Clark and Uetz (1990) were the first to exploit this capability, pioneering the use of video playback for studies of spider communication. Video playback proved a significant advance over previous methods, such as mirrors, 2D static images and dummy lures, and has served as a valuable tool over the past 25 years (e.g. Bednarski, Taylor, & Jakob, 2012; Harland & Jackson, 2002; Menda, Shamble, Nitzany, Golden, & Hoy, 2014). The researcher can control the timing and features of a visual stimulus while ensuring that an identical stimulus is presented across trials and test subjects (D'Eath, 1998). However, while video playback offers many advantages over the available alternatives, it also shares some of the same constraints that have limited the utility of previous methods (and see Fleishman & Endler, 2000; Fleishman, McClintock, D'Eath, Brainard, & Endler, 1998 for general limitations). In particular, video playback is an open-loop system in which sets of stimuli are presented independently of the subject's responses. This lack of contingency between cue and response, as well as the absence of normal visual feedback to a mobile subject, interferes with the perception of depth and motion and ultimately hinders the realism of the simulation (Woo & Rieucau, 2011; Zeil, 2000). A method that closes the feedback loop would offer significant advantages over traditional open-loop playback techniques.

The present study is the first to employ VR as an experimental approach to studying perception and cognition in salticids. In addition to detailing our VR methods, we validate VR as a representation of the real world (RW) through experiments demonstrating (1) consistency of individual differences across RW and VR contexts, and (2) transfer of associations learned in the RW to VR.

METHODS

Source, Maintenance and Preparation of Spiders

Mature female *Servaea incana* jumping spiders ($N = 56$) were collected from *Eucalyptus* trees in Sydney, Australia (Richardson &

Gunter, 2012). Spiders were maintained in a controlled-environment laboratory (24–26 °C; 62–67% relative humidity; 11:11:11 light:dusk:dark:dawn starting at 0700 hours) where they were individually housed in cubic 1.125-litre plastic cages that had a 10 cm diameter mesh-covered opening on one side for ventilation. Each cage contained a folded sheet of paper (2 × 3 cm) that was shaped as a 'tent' under which spiders could shelter and build nests. Spiders were fed weekly on an alternating diet of two house flies, *Musca domestica*, or two Queensland fruit flies, *Bactrocera tryoni*. Supplementary moisture was provided by lightly misting each cage with a spray bottle once each week. No experiments were carried out during the first or last hour of the laboratory light phase.

In preparation for VR experiments, spiders were removed from their home cages and transferred in a 5 ml plastic vial to a refrigerator (4 °C) where they were cooled until quiescent (2–3 min), then placed on a chilled granite block. A 2 mm diameter neodymium magnet (0.4 mm thickness, 8.5 mg; approximately 13% of the average spider weight) was gently affixed to the dorsal carapace using a drop of dental cement (SynergyFlow A3.5/B3, Coltene Whaledent, Burgess Hill, U.K.), taking care not to cover the eyes. The dental cement was cured with blue light from an LED dental curing light source (SDI radii plus, Henry Schein Dental, www.henryschein.co.uk). Spiders were then returned to their cages and allowed to recover for a minimum of 24 h before being used in experiments. Spiders with magnets were maintained for 6 months following experimentation and we did not notice any changes in locomotion, behaviour or longevity in comparison to spiders without magnets.

At the start of each VR trial, spiders were gently lifted from their home cages using a magnetic pin and mounted so that they stood on a spherical treadmill (Fig. 1a). Movements of the treadmill were tracked and updated the virtual environment in real time (described below).

Following the completion of each VR trial, a strip of laminated paper was used to gently separate the neodymium magnet and magnetic pin, and each spider was returned to its home cage. Using prefixed magnets provided two significant advantages over direct tethering to a pin: (1) spiders could be tethered well in advance of a trial, and then have ample time to recover from stress associated with handling, and (2) each spider could be used repeatedly over days or weeks. The potential to use spiders repeatedly is valuable in this context since learning and memory trials typically occur over broader timescales than can be assessed within a single session.

VR System

A 3D-printed spherical treadmill was constructed using polylactide plastic. The treadmill comprised a semispherical chassis that held a 3.5 cm diameter, 230 mg expanded polystyrene ball, tracking sensors, and a clip above the ball to grip a magnetic pin that was attached to the magnet on the spider's carapace to hold the spider in place. The expanded polystyrene ball was supported by a constant flow of air (Sparmax AT-250 mini-compressor; air flow meter, Comweld Medical, Preston, VA, Australia). The weight of the ball was unlikely to affect the spider's movement. The moment of inertia of the ball ($I_{\text{sphere}} = 2/5 \times mr^2$) was 112 700 mg mm², which was 70 times greater than that of the spiders' presumed moment of inertia (see Zurek & Nelson, 2012b for calculations); however, Land (1972) observed unimpeded movement in jumping spiders holding objects with moments of inertia 400 times greater than that of the spider. The spherical treadmill used here was similar to previously described optical mouse tracking systems (Harvey, Collman, Dombeck, & Tank, 2009; Takalo et al., 2012). Rotations of the ball were detected at a rate of 1000 frames/s using four PS/2 optical mouse (Logitech

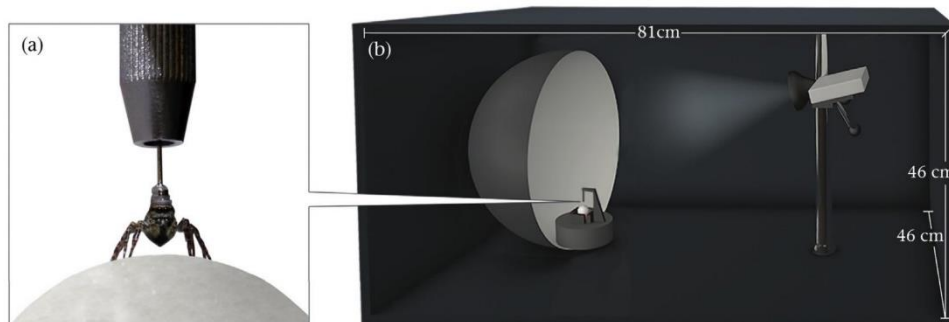


Figure 1. (a) A spider mounted above a spherical treadmill in preparation for a trial. A magnet is fixed to the spider's dorsal carapace and a magnetic pin holds the spider in position. (b) Schematic of VR system. Spiders are mounted above an air-supported spherical treadmill placed inside a hemispherical display screen. Dynamic computer-generated 3D environments are front-projected onto the screen via a pocket projector with add-on fish-eye lens, and spider movement updates the world in real-time (closed-loop).

International, Newark, CA, U.S.A.) sensors (ADNS 2610, Avago Technologies Ltd, Singapore) positioned along the midline, with opposing pairs averaged to reduce measurement noise. The sensors connected to an Arduino Uno microcontroller board, in which motion (translation in X, Y and Z as well as rotation about the yaw, pitch and roll axes) was read by custom-written software. Commands were sent from the microcontroller board to a computer in ASCII format via a USB cable connecting to a virtual serial communication port at a rate of 60 Hz (the closed-loop update rate).

The treadmill was positioned centrally in front of a hemispherical display screen (40 cm diameter, painted with flat white epoxy enamel). The virtual environment was projected onto the inner surface of the hemispherical display screen using an LED pocket projector (Qumi Q2, Vivitek, Hoofddorp, The Netherlands) with a supplementary fish-eye lens (Zeikos, Edison, NJ, U.S.A., 5.8 cm, $0.43\times$ wide angle). For spherical, projector-based systems, a fish-eye design can produce higher image quality than traditional mirror-based systems (Takalo et al., 2012). This projector has a refresh rate of 60 Hz (at 1080p resolution) which is above the suggested flicker fusion frequency of salticid eyes (40 Hz, Forster, 1985). A planoconvex lens (2.5 cm diameter \times 100 mm focal length, Edmund Optics, Barrington, NJ, U.S.A.) was used to focus the light from the projector into the fish-eye lens. The optics used here were chosen to maximize the 'fill' of the sphere while providing a focused image. A 3D-printed holder affixed the projector to its lenses, and this unit was mounted onto a movable arm approximately 25 cm above the base of the screen and 50 cm from the centre of the screen. The simulation projected 160° in both azimuth and elevation, covering the full field of view of both the forward-facing principal eyes and the anterior lateral secondary eyes, which have greater spatial acuity than the other laterally facing secondary eyes (Zurek & Nelson, 2012a). The entire system was enclosed in a light-tight black Plexiglas box to isolate spiders from distraction as well maximize projector brightness (maximum of 300 lumens in complete darkness; Fig. 1b).

Care was taken in adjusting the spider's position so that it could fully extend its legs and step normally, although jumping behaviour was inhibited by tethering. Most salticids, including *S. incana*, use stepping movements for normal locomotion in a manner similar to other spiders, and reserve jumping to span gaps, to escape enemies and for prey capture (Foelix, 2011). The inability to jump can be considered a limitation of tethered preparations in these circumstances, but is not expected to be a limitation under normal locomotion. Each spider was allowed 3 min to adjust to the set-up with the screen dark before the simulation was started.

Visual Stimulus Design and Presentation

Three-dimensional simulated environments were created using the multiplatform game development software Unity3D (version 4.2.1; Unity Technologies, San Francisco, CA, U.S.A.) running on a Windows 7 operating system with an i7 processor and NVIDIA GTX 660 graphics card. On this system, Unity3D rendered at a rate of approximately 60 frames/s. We selected Unity3D as our software platform because it permits dynamic design of 3D stimuli and environments, allows full control of subject/object interaction and can be set up to communicate with external devices. Simulated environments were created using a virtual four-camera cube rig to reflect the position of the tested spider. The rig consisted of top (facing up) and bottom (facing down) cameras, as well as left and right cameras positioned at $\pm 45^\circ$ to the spider's forward-facing centre. The images output through the four cube faces resulted in a 90-degree perspective. To solve for a 180-degree fish-eye projection, the images output from the four cube faces were applied as textures to four objects (known as 'meshes' in Unity), producing a single image predistorted to appear normal against the hemispherical projection geometry (Bourke, 2009).

Two closed-loop scenes were created for the experiments described below. Both environments contained a flat ground plane that was textured and coloured to resemble tree bark. In preliminary trials we found that spiders displayed limited interaction with the simulation when the ground was entirely uniform in appearance and lacking in texture. Lycosid spiders attend to the visual structure of the substratum while navigating home (Ortega-Escobar, 2011), and it is likely that substratum appearance is also relevant to salticid spiders, presumably by contributing to perceived ventral optic flow (Ortega-Escobar & Ruiz, 2014).

An interactive graphical user interface was created to calibrate, run and play back our simulations. To calibrate distance in VR, we measured a single rotation of the treadmill ball and used this value to scale the virtual environment, so that 1 cm travelled on the treadmill in the RW equalled 1 cm travelled in VR. The system was recalibrated at the start of each test day. We recorded each spider's mean speed and total distance travelled for all trials. In addition, we observed spatial behaviour and responses to objects in a real-time replay function, which played back the trial from the spider's point of view, as well as a 'static replay' function, which displayed a line trace of the path taken throughout the trial. We also created a data collection tool that could be 'attached' to virtual objects to assist in our estimation of the level of interaction with these objects. The data collection tool allowed us to set a radius around a given object and subsequently record the latency to enter this radius (approach

latency), number of entries and total amount of time spent in that region. Additionally, a 'dot product' function was included which, for each time point sampled, output a value ranging from -1 to 1 , where -1 denotes that the spider is directly facing the object, 1 denotes that the spider is facing directly away from the object, and 0 denotes that the spider is facing 90° away from the object. Multiple areas of interest could be set on a single object, and multiple objects could be set as objects of interest, collectively providing high-resolution output on the spider's orientation throughout the trial.

Experiment 1: Behaviour in RW and VR

To test whether interindividual behaviour differences were conserved across contexts we measured spontaneous locomotor activity and light/dark preference of adult female spiders ($N = 32$) in both RW and VR. Activity levels are often highly repeatable within species (Richardson, 2001) and have been found to correlate with other behaviours in spiders (Pruitt, Riechert, & Jones, 2008). We started RW locomotor activity testing 24 h after attachment of the magnet. Spiders were placed individually into glass petri dishes (15 cm diameter) and spontaneous locomotion was assessed for 10 min. Trials were videorecorded from above using a digital video camera (Panasonic HDC-SD900). The total distance travelled was measured from these recordings using LoliTrack 2.0 (Loligo Systems, Tjele, Denmark). To test whether affixing or presence of the magnet affected mobility, locomotion of a randomly selected subset of 11 spiders was also recorded and analysed 24 h before affixing the magnet. For these spiders, comparisons were made between pre- and postmagnet activity measures.

The light/dark preference test is commonly used to assess associative behaviours in invertebrates (Steenbergen, Richardson, & Champagne, 2011), including arachnids (Camp & Gaffin, 1999; Dos Santos, Hogan, & Willemart, 2013). RW dark preference testing began approximately 24 h after locomotor activity testing with the same group of spiders. Spiders were placed in a rectangular arena (7.5×15 cm and 7.5 cm high) constructed from white plastic board (Corflute). The walls of the arena were lightly dusted with nonscented talcum powder to prevent spiders from escaping, and the top was open to permit video recording. A black piece of card was placed over one half of the arena creating a shaded 'dark' zone underneath. Trials lasted 20 min and were initiated by placing the spider in the centre of the 'light' zone. All trials were video-recorded from above and subsequently scored from these recordings. We assessed the latency until spiders first entered the dark side of the arena, as well as the proportion of each trial spent on the dark side.

VR locomotor activity and dark preference were tested in a single, combined session approximately 24 h after RW dark testing (see above for general procedures). The VR environment was a square arena (52 cm wide, 22 cm high), designed to be either 'empty', containing no objects, or 'complex', containing 18 randomly placed pillars (3 cm diameter, 8 cm high). The virtual arena was scaled to a larger size than the corresponding RW arena in order to reduce the probability of spiders reaching a virtual wall, which would not possess the properties of a physical wall and could create breaks in simulation 'reality' (Sherman & Craig, 2002). As in the RW, spiders began a trial in the centre of the 'light' zone. We measured the relative ambient intensity contrast (W/m^2 per nm) of the light region compared to the dark region using a JAZ EL-200 portable spectrometer fitted with a spectralon diffused irradiance module (Ocean Optics Ltd., Dunedin, FL, U.S.A.). The light region was approximately five to six times brighter than the dark region in both environments (real world

1:5.9; VR 1:4.9). Initially, spiders were assigned evenly to either VR condition, but we shifted this allocation in favour of the complex treatment (empty, $N = 11$; complex, $N = 21$) after observing abnormal behaviour in spiders in the empty condition (see Results).

Experiment 2: Beacon Learning

Salticids construct silken nests for shelter (Jackson, 1979) and commonly exhibit high nest site fidelity (Hoefer & Jakob, 2006; Jackson, 1988). An experiment was conducted with a new group of spiders to determine (1) whether spiders attended to beacons previously paired with a nest site, and (2) whether and how RW experiences influence behaviour in VR.

Following attachment of the magnet, spiders were placed in individual petri dishes (1.5 cm high, 15 cm diameter) and given 48 h to build a nest under a folded sheet of paper (2×3 cm) that was shaped as a 'tent'. On the second day in the petri dish, and every second day following, spiders received one Queensland fruit fly as food. Spiders were then transferred in their nests to square individual 'home arenas' (52 cm wide, 22 cm high) that they occupied for the duration of the experiment. Nest sites were placed 7.5 cm from the middle of one wall. The arena walls were constructed from white plastic board (Corflute) that was dusted with nonscented talcum powder to prevent spiders from escaping. Between trials, the arena was covered with a translucent polypropylene sheet fitted to the top of the walls that prevented spiders from seeing out of their enclosures while allowing diffuse illumination. Spiders were divided into two groups based on whether a beacon was present. 'Beacon spiders' ($N = 12$) had a beacon (a red pillar or green cross) placed immediately behind their nest site, while 'No Beacon spiders' ($N = 12$) had no beacon present. All spiders underwent an identical test protocol of five RW trials and two VR trials: two RW trials on each of days 1 and 2, separated by 3 h, one VR trial on each of days 3 and 4 and a single RW trial immediately following the final VR trial on day 4. The fifth RW trial was included to test whether 2 days of testing in VR had an effect on subsequent behaviour in the RW.

For RW trials, spiders were transferred into clean arenas that were identical to the arenas they had occupied previously with a beacon present but no nest (Fig. 2a). For Beacon spiders, the beacon was the same type that was present in their home arenas. No Beacon spiders, which had no prior exposure to a beacon, were assigned one of the two beacon types and were subsequently tested with this beacon in all trials. The beacon was placed 7.5 cm from the middle of one wall (as in home arenas), with a different wall randomly selected on each trial to prevent spiders from orienting to global cues beyond the arena. Spiders were given 3 min to acclimate while restrained in the centre of the arena by an upturned opaque vial before the vial was raised and the trial began. Each trial lasted 20 min and was video-recorded from above.

The VR environment was a square arena designed to be similar in appearance and scale to the RW arena. The VR arena contained a single beacon in the same position relative to the spider's starting position as in the RW (Fig. 2b). We measured the relative radiance contrast (W/m^2 per nm per sr) of the beacon against the white wall in both environments. The background was approximately twice as 'bright' as the beacon in both worlds (beacon:background: real world 1:2; VR 1:1.5). Spiders began all trials in the centre of the arena facing the beacon, and all trials lasted 20 min. The treadmill was wiped with a damp cloth between trials to remove chemical cues left behind by the preceding spider (Jackson, 1987).

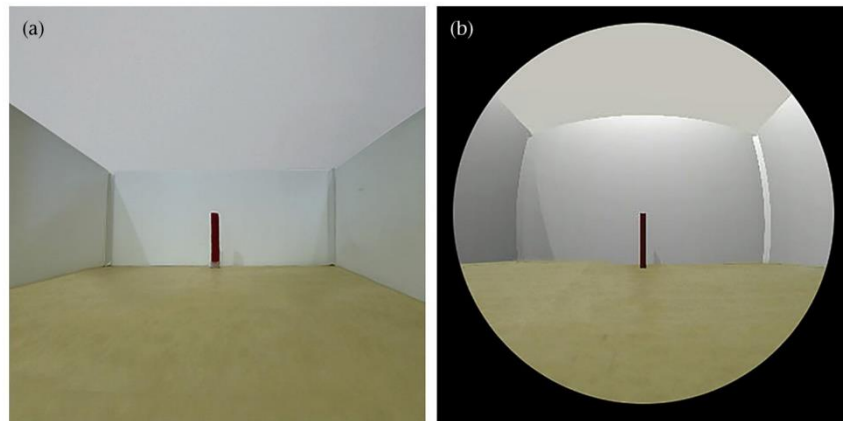


Figure 2. Spider's starting perspective in the beacon experiment in (a) real and (b) virtual environments. The virtual view reflects the image pretransformed to fill the hemispherical display screen.

Spiders were scored for two measures in both contexts, recorded when the spider entered a 7.5 cm radius circle surrounding the beacon: the proportion of the trial spent in proximity to the beacon and the number of beacon visits. In addition, total path length was recorded for spiders in the VR environment as a measure of general activity levels. We adopted an absolute proximity radius for scoring in order to have an objective measure of approach behaviours across trials. A radius of 7.5 cm was selected on the basis of data from pilot trials.

Analyses

Statistical analyses were performed using JMP version 11 (SAS Institute Inc., Cary, NC, U.S.A.). We performed nonparametric statistics for all analyses as our data did not meet the assumptions of normality. For experiment 1, descriptive data are presented for the empty VR arenas but due to abnormal behaviour this group was excluded from subsequent analyses. Spiders that did not participate in this task, defined here as path length of less than 30 cm in the 20 min trial, were also excluded ($N = 2$). A Wilcoxon matched-pairs test was used to test for differences between RW activity and VR activity for spiders in complex VR arenas. Spearman rank correlations were used to assess association between RW and VR in distance travelled, approach latency to enter the dark compartment and proportion of the trial spent in the dark compartment. A Wilcoxon matched-pairs test was used to test for differences in RW activity before and after attaching the magnet.

For experiment 2, RW and VR conditions were analysed separately and then compared to identify consistency in performance across contexts. Wilcoxon matched-pairs tests were used to compare the mean time spent in proximity to the beacon and the number of beacon visits in the RW and VR, and Wilcoxon signed-ranks tests were used to detect group differences in performance on both measures within both RW and VR contexts. In the RW, a Friedman's test was used to test for a trial effect in both measures across trials 1–4. A separate test was run comparing trials 4 and 5 to investigate whether VR testing had any effect on subsequent RW behaviour. Spearman rank correlations were used to assess association between RW and VR for each performance measure (outlined above). For the VR trials we additionally compared the total distance travelled by Beacon and No Beacon spiders using a Wilcoxon signed-ranks test.

Ethical Note

We followed the ABS/ASAB guidelines for the ethical treatment of animals. Attachment of the magnet in advance of experiments minimized stressful handling and allowed us to return spiders to their home cages after each trial. Feeding, behaviour and survival were not visibly affected by attachment of the magnet or participation in experiments. Spiders were subsequently used in pilot trials for other VR experiments.

RESULTS

Experiment 1: Behaviour in RW and VR

In VR, spiders in empty virtual arenas, containing no objects, were significantly less active than spiders in complex arenas (mean distance travelled \pm SE: complex = 160.3 ± 18.4 cm; empty = 92.5 ± 25.4 cm; $Z = -2.40$, $P = 0.016$), often stopping for prolonged periods during the trial. Owing to their substantial reduction in general activity levels, spiders from the empty condition were excluded from further analyses.

We found a significant positive correlation between each RW measure and its corresponding VR measure, although spiders tended to travel shorter distances in VR, were quicker to enter the dark compartment, and spent a greater proportion of the trial on the dark side of the arena than when in the RW (Table 1). Attachment of the magnet did not significantly influence activity levels in our RW activity trial (mean distance travelled in 10 min trials:

Table 1
Statistical comparison of measures in the RW and VR in experiment 1

Measure	Means \pm SE		Wilcoxon matched-pairs		Spearman rank correlation	
	RW	VR	Z	P	r_s	P
Distance	383.8 \pm 38.8	160.3 \pm 18.6	-4.02	0.001	0.579	0.006
Approach latency	466 \pm 99.1	188.6 \pm 76.1	-2.94	0.003	0.637	0.002
Proportion	0.50 \pm 0.08	0.67 \pm 0.09	-2.21	0.027	0.473	0.031

Differences between each measure in the RW and its corresponding measure in VR were analysed with Wilcoxon matched-pairs tests. Correlations between each measure in the RW and its corresponding measure in VR were analysed with Spearman rank correlations. Distance refers to the total distance travelled; approach latency refers to the time to first enter the dark side of the arena and proportion refers to the proportion of the trial spent in the dark side (mean \pm SE, $N = 21$).

premagnet = 407.9 ± 46 cm; postmagnet = 390.5 ± 48.1 cm; $Z = -0.46$, $P = 0.644$).

Experiment 2: Beacon Learning

In the RW, Beacon spiders, which had previously been exposed to a beacon beside their nest site in their home arena, made more visits to a beacon and spent significantly more time in its proximity than No Beacon spiders when tested in clean arenas (visits: $Z = 2.24$, $P = 0.025$; time in proximity: $Z = 3.30$, $P = 0.001$). There was no evidence of a difference across the four initial RW trials (visits: $\chi^2_3 = 6.79$, $P = 0.080$; time in proximity: $\chi^2_3 = 1.80$, $P = 0.615$) or between RW trial 4 (the trial preceding VR testing) and 5 (the RW trial immediately following VR testing; visits: $\chi^2_1 = 2.57$, $P = 0.109$; time in proximity: $\chi^2_1 = 1.19$, $P = 0.275$). The similarity in performance between RW trials 4 and 5 indicates that the intervening VR trials did not disrupt subsequent performance in the RW.

The time spent in proximity to the beacon was positively correlated between the RW and VR for Beacon spiders but not for No Beacon spiders, suggesting that learning of the association between beacons and nest sites transferred across contexts (Table 2).

As was the case in the RW, when tested in VR Beacon spiders spent significantly more time in proximity to the virtual beacon than did the No beacon spiders ($Z = 2.81$, $P = 0.005$), although there was no significant difference in the number of visits ($Z = 1.50$, $P = 0.128$; Fig. 3).

Beacon spiders had significantly longer path lengths in VR than No Beacon spiders (Beacon spiders = 118 ± 17 cm; No Beacon spiders = 57.4 ± 16 cm; $Z = 2.20$, $P = 0.028$). For both Beacon and No Beacon spiders, activity peaked during the first 120 s and gradually declined over the course of the trial (Fig. 4).

DISCUSSION

Video and computer-animated stimuli have been presented to salticids in order to study psychophysics (Zurek, Taylor, Evans, & Nelson, 2010), cognition (Dolev & Nelson, 2014), courtship (Clark & Uetz, 1992), contests (Tedore & Johnsen, 2015) and predation (Bednarski et al., 2012), but these studies have always been conducted under open-loop conditions. The VR system described in the present study closes the loop for the first time, demonstrating that salticids detect and respond appropriately to the visual and motor feedback of a virtual environment. The present study comprises the first demonstration that spiders, here, female *S. incana* jumping spiders, display patterns of activity and behaviour in VR that closely parallel their RW counterparts. Individual tendencies were conserved across contexts: both individual locomotor activity levels and dark-seeking behaviour in the RW were positively correlated with locomotor activity and dark-seeking behaviour in VR. Further, associations learned in the RW transferred to VR.

Table 2

Statistical comparison of measures in the RW and VR in experiment 2

Measure	Group	RW	VR	Spearman rank correlation		Wilcoxon matched-pairs	
				r_s	P	Z	P
Time in proximity	Beacon	320.2 ± 43.2	404.4 ± 104.6	0.625	0.009	-0.78	0.437
	No Beacon	206.9 ± 49.7	105.1 ± 43.2	0.089	0.717		
No. of visits	Beacon	1.6 ± 0.2	1.1 ± 0.3	0.409	0.116	-3.09	0.002
	No Beacon	1.0 ± 0.1	0.5 ± 0.1	0.193	0.429		

Correlations between each measure in the RW and its corresponding measure in VR were analysed with Spearman rank correlations. Differences between each measure in the RW and its corresponding measure in VR were analysed with Wilcoxon matched-pairs tests. Time in proximity refers to the amount of time (s) spent in proximity to the beacon and no. of visits refers to the number of approaches to the beacon ($N = 35$).

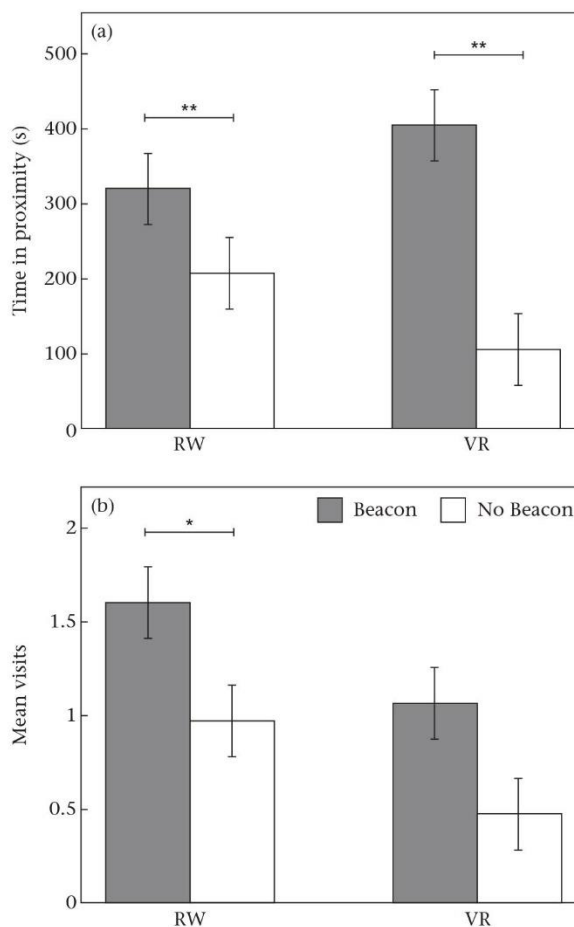


Figure 3. Spider performance in a beacon learning task in the RW and VR. (a) Mean time spent in proximity to the beacon \pm SE. (b) Mean number of visits to the beacon \pm SE. Beacon spiders were trained in the RW with a beacon/nest site pairing while No Beacon spiders were not provided with a beacon. Asterisks denote significant differences between Beacon and No Beacon spiders. * $P < 0.05$; ** $P < 0.001$; Wilcoxon signed-ranks test.

Spiders that were given the opportunity to learn about beacons associated with their nest site in the RW later tended towards these cues in both the RW and in VR.

Paying attention to local cues such as beacons is thought to be less computationally demanding than using distal cues (Shettleworth, 2010), and is part of the navigational strategy of many arthropods (Cheng, 2000; Collett, Graham, & Durier, 2003; Graham, 2003). A field study of a North American salticid,

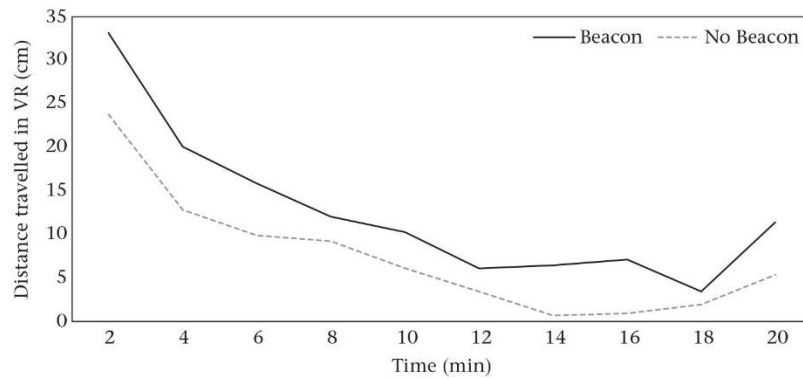


Figure 4. Mean distance travelled by Beacon and No Beacon spiders over the course of a 20 min trial in the VR system. Time is grouped into 120 s time bins and each time point reflects the distance moved since the previous time point.

Phidippus clarus, found that spiders readily learned a beacon–nest site association and showed an increased tendency to approach beacons that were displaced from their original location (Hoeffler & Jakob, 2006). Despite geographical and habitat differences we found a similar tendency to learn cues associated with nest sites. These results suggest that local cues may be an ecologically and taxonomically widespread element of the navigational strategy used by salticids, especially for homing.

VR may be a particularly useful tool in the study of navigation. Beacons, landmarks and environmental cues can easily be removed, manipulated or put into conflict with each other, and these manipulations can occur alongside neurophysiological recordings (Bohil et al., 2011). VR has been used extensively to study the neural underpinnings of behaviour in restrained primates, and more recently in rodents. Both rodents and primates display hippocampal place cell activity when navigating in VR (primates: Hori et al. 2005; rodents: Hölcher, Schnee, Dahmen, Setia, & Mallot, 2005), but have critical differences in their activation patterns between real and virtual environments that can be attributed to lack of appropriate vestibular input in VR (Ravassard et al., 2013). Restraint can similarly lead to behavioural artefacts in invertebrate models that possess equilibrium sensors. Flies, for example, exhibit distortions in flight behaviour when mechanosensory feedback from specialized stabilizing organs, the gyroscopic halteres, is impeded, as is the case during restraint (Fry, Sayaman, & Dickinson, 2003; Pringle, 1948). The limitations imposed by equilibrium sensors can be circumvented through VR studies of visually orienting walking invertebrates that lack equilibrium-sensing organs, such as spiders.

Until recently, recording from salticid brains has been unfeasible owing to their highly pressurized internal fluids, and the resulting fluid loss that accompanies incision. A recent study has developed methods that overcome this limitation, obtaining the first neurophysiological recordings from the brain of a salticid as it views traditional grating stimuli and naturalistic prey-like stimuli that were presented using open-loop methods (Menda et al., 2014). The effectiveness of a VR system for salticids in the present study raises the possibility of recording from brain cells in alert salticids interacting with an immersive, closed-loop environment.

The VR system described in the present study was designed to focus solely on vision, which is well known to mediate diverse aspects of salticid behaviour (Jackson & Harland, 2009; Land, 1969a). At the same time, many species routinely use other sensory modalities, such as chemoreception and vibration, both to assess the environment and to communicate (Elias et al., 2012;

Girard, Kasumovic, & Elias, 2011; Jackson, 1987). A VR system could be designed to emphasize any modality, and indeed systems based on olfaction (Fry et al. 2008; Sakuma, 2002) and audition (Fry et al. 2004) have been used with other invertebrates. However, setting up a similar system for salticids would be challenging as salticids access these cues in part through the substrate (Barth, 2002; but see Jackson, Clark, & Harland, 2002; Jackson & Cross, 2011 for examples of airborne chemoception in salticids), and the 'substrate' in our set-up was an air-supported sphere atop which spiders were mounted. Although delivery of vibratory stimuli through this cushion of air would be particularly challenging, it would be straightforward to incorporate substrate-bound chemical cues applied to the ball prior to the start of each trial or to deploy puffs of airborne odours or airborne sound.

Irrespective of the modality emphasized in VR, it is important to ensure that the attributes present in the simulation are tuned to match the perceptual system of the subject. In a visual system, parameters such as size, shape, colour, texture, luminance and movement all require careful consideration (Woo & Rieucan, 2011; Zeil, 2000). The extensive body of research on visual perception of salticids provides an excellent framework for designing stimulus features that most effectively elicit a natural response. For instance, Zurek et al. (2010) demonstrated that the orientation response in *S. incana* is dependent on stimulus size, contrast and speed; Bednarski et al. (2012) highlighted the importance of motion characteristics in eliciting a predatory response; and Dolev and Nelson (2014) showed that the relative angle between the body elements of abstract stimuli are sufficient and necessary for recognition. VR will provide an excellent resource to build from this foundation, as it broadens the scope of experimental possibility to encompass design features and contingencies that were previously inaccessible in salticids.

Acknowledgments

We thank Rowan McGinley for his assistance collecting spiders, Bruno Van Swinderen for his help conceptualizing our VR system, Thomas White for providing spectral measurements and three anonymous referees for their helpful suggestions on the manuscript. We are grateful to Chris Malloy, Rob Lee, Brian Su, Keith Hassan and John Porte for their invaluable technical assistance. We are especially thankful to Greg Hunsburger for his assistance in designing and implementing the 3D environment, and his continual support throughout this project. This research was supported by an Australian Government Endeavour Postgraduate

Award. Research expenses were supported by Higher Degrees Research Funds of the Department of Biological Sciences at Macquarie University.

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10 APPENDIX C

This section contains the F1000 recommendation for the published version of chapter 5.

Peckmezian, T., & Taylor, P. W. (2015). **A virtual reality paradigm for the study of visually mediated behaviour and cognition in spiders.** *Animal Behaviour*, 107, 87–95.

1 A virtual reality paradigm for the study of visually mediated behaviour and cognition in spiders.

Peckmezian T, Taylor PW
Anim Behav. 2015 Sep; 107:87-95

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RECOMMENDATIONS 1 | ABSTRACT | COMMENTS

Recommendations:

Good

28 Jan 2016



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TECHNICAL ADVANCE

DOI: 10.3410/f.726099645.793513805

Spiders in Virtual Reality

This paper studies visually-mediated behavior in spiders placed in a virtual reality situation. Virtual reality refers to an artificial environment that simulates part of the real world and that is sensed and updated by an animal's actions (closed-loop situation) {1}. It gives the possibility to study behavior allying ecological context and very controlled conditions. Here, the authors achieved a remarkable technical advancement, which is the establishment of a virtual-reality visual environment for the jumping spider (*Salticidae*) *Servaea incana*, which has remarkable visually-mediated prey-capture behaviors. They tested the validity of their set-up by comparing visually-guided behaviors in the real world and in virtual reality conditions.

Jumping spiders were individually tethered on a tracking ball (a "treadmill") whose displacements were monitored by four PS2 optical mice to reconstruct trajectories of the spider walking on the ball. A 3D environment was programmed and projected onto a semi-circular screen by an elevated pocket projector with a fish-eye lens. The screen was placed in front of the spider. The movements of the spiders were tracked and used to update the visual surroundings in the virtual reality, thus providing a closed-loop situation.

Two kinds of behavioral experiments were performed. In each experiment, spiders were tested both in the real world and in virtual reality to compare performances. In the first experiment, the authors measured the spontaneous locomotor activity and the light/dark preference of female spiders (spiders prefer to spend more time in dark areas than in illuminated ones) confronted with a real and a virtual box presenting dark and clear halves. In the second experiment, they placed a real beacon in the proximity of a nest built by spiders within a folded paper in order to determine if spiders learn to associate the beacon with the nest site and thus orient towards the beacon (e.g. a real cylinder) both in a real arena and in a virtual arena when transferred to it.

The authors showed that, in the first experiment, locomotor activity and light/dark preference (distance traveled, time before first entrance in the dark side, and proportion of trials spent in the dark side) were comparable and correlated in the real and the virtual world. In the second experiment, spiders that had previously associated a beacon with their nest made more visits to the beacon and spent more time in its proximity than spiders not exposed to a beacon. This result was obtained both in a real arena with a real cylinder and in a virtual arena with a virtual cylinder, thus showing behavioral transfer between both experimental conditions and the validity of the virtual-reality set-up.

The publication has the merit of introducing a remarkable methodological achievement. The authors developed their own system and provide many details about their experiments and procedures, thus making them adaptable to other situations and studies. The demonstration that there was no big difference between spider behavior in the real world and in virtual reality is a major technical breakthrough in the use of virtual reality for the study of invertebrate behavior. Some points remain to be clarified, like, for instance, the occurrence of "abnormal behaviors" in virtual reality: what exactly do these behaviors reflect and what are their underlying mechanisms? And besides this point, does the nervous system of a spider encode the real word using the same neural mechanisms as for the virtual world? With the set-up established, this latter question will be tractable in an immediate future.

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PMID: 22048061 DOI: 10.1038/nrn3122

Disclosures

None declared

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