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ON THE LATERALISED MOTOR BEHAVIOUR OF INSECTS

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This thesis is submitted to the University of Sussex in application for the degree of

Doctor of Philosophy

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DECLARATION

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration with others except where specifically indicated in the text. No part of this dissertation has been submitted to any other university in application for a higher degree.

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26th September, 2016

SUMMARY

The late 20th century brought with it substantial evidence showing that many vertebrate species are capable of exhibiting brain and behavioural lateralisation (*i.e.* functional and/or structural specialisations of the left and right sides of the brain/behaviour), undermining the traditional view that this attribute is uniquely human. Motor lateralisation, a specific form of behavioural lateralisation, in which an organism displays a directional preference whilst making movements, has previously been identified in many vertebrates but has received little attention in invertebrate species, particularly insects. Considering this, I investigated motor lateralisation in the desert locust (*Schistocerca gregaria*) and the red wood ant (*Formica rufa*) using a combination of a gap-crossing paradigm and a 'Y'-maze choice experiment. Using these paradigms, I show that the relatively small nervous systems of insects are capable of producing lateralised motor behaviour, providing evidence that being strongly lateralised can be advantageous and, more generally, adding support to the hypothesis that social organisms are more likely to align their lateralisation with others in their group. The gap-crossing paradigm demonstrates that both desert locusts and red wood ants display a forelimb preference, the first direct evidence that an arthropod possesses a preference in the use of otherwise symmetrical limbs. Studying locusts in this paradigm also produced the first evidence that strong lateralisation confers an advantage to the individual because more strongly biased locusts display fewer reaching errors. In addition, wood ants display inter-colony variation in their preference, the first demonstration of a previously undescribed form of lateralisation, colony-level lateralisation. By testing wood ants over both the gap-crossing and 'Y'-maze paradigms I show that within the same insect species, lateralisation can be expressed in different forms of motor behaviour.

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PUBLICATIONS

To date, the following publications have arisen in whole or part from this thesis:

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Bell, A.T.A. and Niven, J.E. (2016). Strength of forelimb lateralisation reduces motor errors in an insect. *Biology Letters*, **12**, 20160547.

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“I learned to recognise the thorough and primitive duality of man; I saw that, of the two natures that contended in the field of my consciousness, even if I could rightly be said to be either, it was only because I was radically both.”

Robert Louis Stevenson, *Strange Case of Dr. Jekyll and Mr. Hyde*

Chapter 1

General Introduction

1.1 What is lateralisation?

Bilaterally symmetrical organisms can be characterised by a figurative cut into two mirror-imaged halves along the sagittal plane. Lateralisation is a broad term which refers to different structural and/or functional specialisations between these halves. Although the term lateralisation may be used interchangeably with the term 'asymmetry' in this thesis I will, when appropriate, use the term lateralisation for simplicity.

Lateralisation may occur in otherwise bilaterally symmetrical organisms in several ways. Firstly, all organisms are lateralised at the molecular and macromolecular levels (Fig. 1.1A). At the molecular level living organisms utilise only L-amino acids and D-sugars and at the macromolecular level proteins containing α -helical regions will have them arranged in their most stable form of right-handed helices. Despite all organisms being lateralised at this lower level of biological complexity, symmetry may prevail at higher levels, such as body shape in humans.

Secondly, external structure as viewed from outside the organism may be lateralised (Fig. 1.1B). Despite being exceptionally rare, lateralisation has evolved independently many times (Palmer, 2009). Obvious examples include crossbills, sole and male fiddler crabs that all display obvious morphological lateralisation in their external anatomy.

Thirdly, internal structure may be lateralised despite bilateral symmetry being retained externally, for example the heart and arterial organisation in humans (Fig. 1.1C). This is the default condition (Palmer, 2004) is thought to have evolved due to the requirements to balance forces on bodies or body parts exposed to gravity or to the stresses of a moving medium (Ludwig, 1932; McManus, 2002).

Fourthly, behaviour may be lateralised, which is the type of lateralisation this thesis will focus on, for example escape response in some teleost fish (Fig. 1.1D). Perhaps the most

relatable example is that humans show clear lateralisation in hand use, with approximately 90% of us showing a preferential use of our right hand (McManus, 2002). I will now move on to describe lateralisation in a framework of Tinbergen's four questions (Tinbergen, 1963). Please note that each section is aimed to serve as a brief introduction to the relevant aspect of lateralisation and not intended to be a comprehensive review.

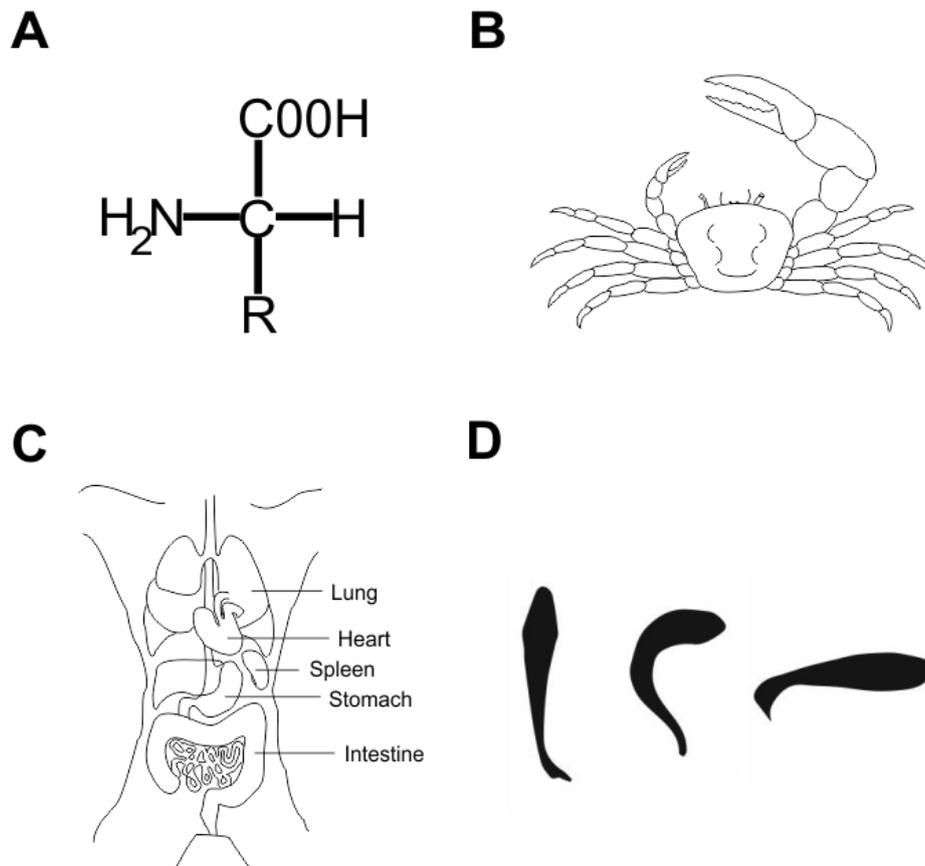


Figure 1.1. Types of lateralisation. **A.** Molecular lateralisation - an amino acid containing a lateralised carbon atom. **B.** Structural lateralisation - male fiddler crabs display structural lateralisation in its chelae. **C.** Internal lateralisation - the internal arrangement of organs in humans is lateralised whilst external morphological symmetry is maintained. **D.** Behavioural lateralisation - lateralised escape behaviour of fish. (Adapted from Neville, 1976).

1.2 Evolution of lateralisation

Lateralisation is a fundamental property of the universe and can be even observed in the forces which form its structure (Wu *et al.*, 1957). This phenomenon has led to the speculation that the forces of nature may have given rise to life's asymmetry (Gibney, 2014). Evidence of lateralisation can be seen at the cellular level in their left-right axis (Xu *et al.*, 2007) and indeed, we can observe lateralisation in all levels of biological complexity from atomic, molecular, macromolecular, microfibril, fibril, fibres, cells, organs and organismal levels (Neville, 1976). At the organismal level lateralisation has evolved in all complex, bilaterally symmetrical animals (Annett, 1987), however, pinpointing its phylogenetic origins and unravelling the evolutionary dynamics that shaped it in extant species is difficult. The fossil record is one useful source that allows us to investigate when lateralisation may have evolved. Perhaps evidence of the earliest form of behavioural lateralisation is inferred from asymmetric distribution of sub-lethal scars on trilobites. Scarring on one side of these extinct animals bodies suggests that either their predators were lateralised in their attack strategy or trilobites were themselves lateralised in their escape response (Babcock and Robison, 1989a,b). Furthermore, this evidence suggests that lateralised nervous systems were in existence at least 500 million years ago in the Early Cambrian.

Evidence from early hominids suggests that handedness in the form of limb control has been in existence pre-Holocene epoch and biologists now agree that right-handedness evolved in our hominid ancestors as they learned to build and use tools, about 2.5 million years ago (Faurie *et al.*, 2016). Damage or wear and tear to integuments such as tools and teeth belonging to early hominids also provide evidence of early behavioural lateralisation. A clockwise rotation of Pleistocene stone cores used during flaking suggest that a right hand preference in tool-makers existed 1.9 – 1.4 million years ago (Toth, 1985). It has been demonstrated that striations on the surfaces of permanent teeth found in the late Pleistocene were probably caused by tool use. These striations were inferred to have been cut with tools used in the right hand (Bermúdez de Castro, 1988). Behavioural asymmetry has also been correlated with morphological asymmetry in cranial endocasts in various hominid species for

example *Homo erectus*, *H. sapiens neanderthalensis* and *Australopithecus* (Holloway, 1981a, b; Holloway and de la Coste-Lareymondie, 1982). It is argued that specialization of each hemisphere in the human brain was already present in its basic form when vertebrates emerged about 500 million years ago (MacNeilage *et al.*, 2009).

Fossils from non-hominid species too provide evidence that not only hominids were capable of producing lateralisation in behavioural and morphology. Mastodon, large animals resembling modern elephants, show lateralisation in the length of their tusks, thought to be required for manipulating food (Kürten and Anderson, 1980). A group of fossils considered an ancestral form of chordate or echinoderm families shows signs of structural lateralisation in the shape of the head (Jefferies and Lewis, 1978). Shells from fossils of gastropod molluscs show both sinistral (leftward) and dextral (rightward) coiling (Vermeij, 1975). Finally, the fossil record provides many examples of both structural and behavioural lateralisation members of Arthropoda, Annelida, Bryozoa, Echinodermata, Cnidaria, Mollusca, Chordata and Conodonta (Babcock, 1993).

Extant species too can give us insights into the evolution of lateralisation. Lateralisation is present in the line of evolution that gave rise to vertebrates. Lancelets, considered a common ancestor of vertebrates, possess structural lateralisation. As larva they have an asymmetrically placed mouth which they use to feed by swimming down, always to the left with the mouth open (Webb, 1975). Lateralisation in structure and behaviour is documented widely across all vertebrate classes (Rogers and Andrew, 2002; Vallortigara and Rogers, 2005; MacNeilage *et al.*, 2009; Vallortigara *et al.*, 2011). Given that structural and behavioural lateralisation is documented early on in evolutionary history, and that it is present across a wide range of extant species we have some insight into the evolution of lateralisation however it is difficult to discern whether it is a persistent trait i.e. homologous (Campbell, 1988) or it has evolved independently many times i.e. is homoplastic (Hodos, 1988). Regardless of however it has evolved, that most organisms show some form of lateralisation in structure and/or function (Babcock, 1996) shows support for the idea that it provides significant advantages.

1.3 Development of lateralisation

Whilst it may be difficult to accurately identify when in evolutionary time lateralisation of structure and function emerged it might be possible to outline when and how lateralisation occurs during an organism's development. The ontogeny of lateralisation can, in some species, be traced back to cell cleavage. Most species of gastropods have dextrally coiled shells and the direction of coiling can be traced back to when the cells are undergoing cleavage from the two to four cell stage (Huxley and De Beer, 1963). Extensive work investigating the inheritance of sinistral coiling in the shell of the pond snail *Lymnaea* (Boycott *et al.*, 1931) found the direction of twisting is determined in the unfertilised egg and not in the sperm that fertilises it.

In insects, sex is determined in every cell by chromosomes. Males have XX chromosomes and the loss of one of these chromosomes results in a female cell. If, during the first nuclear division, a cell loses one of the X chromosomes the left axis of the butterfly will develop into a female and the right axis will develop into a male. The result is a bilateral gynandromorph, a remarkable display of lateralisation in the pattern of the adult butterfly. Gynandromorphs are not restricted to lateralisation of pattern. The male cockroach *Byrostria fumigata* is winged therefore the gynandromorph has wings on the male half which are absent on the female half. *Drosophila* gynandromorphs offer an opportunity to study behavioural lateralisation of motor output. Ikeda and Kaplan (1970) observed leg shaking during anaesthetization in hemizygote male and homozygote females. Creating gynandromorphs of these they found only leg shaking in the male side of the thorax. These mutants allow an opportunity to investigate the development of lateralisation. In amphibians the gut roof determines the lateralisation of the placement of internal organs. If the gut roof of the frog *Bominator* is manipulated so that it is rotated 180° the internal organs follow suit which results in *situs inversus*, the gut developing on the opposite side of the body (Meyer, 1913).

Life experience too can affect the development of lateralisation. Maguire *et al.* (2000) demonstrated that London taxi drivers have a larger than average region at the back of the right hippocampus, associated with spatial navigation. Homologous examples are from

animals that *cache* food. Squirrels, kangaroo rats, marsh tits and Clarke's nutcrackers all have larger hippocampus than closely related *non-caching* species (Johnson *et al.*, 2010; Jacobs and Spencer, 1994; Shettleworth, 2003). Also, homing pigeons allowed to navigate as fledglings develop cerebral asymmetries which were not found in those that were neglected the chance to navigate at this early stage (Mehlhorn *et al.*, 2010). These studies suggest that behavioural and cerebral lateralisation develops naturally. Early life experience seems to play a crucial role in the natural development of lateralisation. There are many studies on the zebrafish (e.g. Budaev and Andrew, 2009a,b; Andrew *et al.*, 2009) and the domestic chick (e.g. Rogers, 1982, 1990, 1991, 2000) demonstrating the effect of light exposure on visual and behavioural lateralisation during development.

It is well known that the brain is not as 'hard-wired' as originally thought and is modified throughout an animal's life through interaction with the environment. The research on the development of lateralisation appears support this, suggesting that an animal's genetics lay the foundation of lateralisation and during development, especially during early life experience, the lateralisation can be enhanced, modified or suppressed (Rogers *et al.*, 2013).

1.4 Mechanism of lateralisation

Lateralisation of brain and behaviour is considered to be generated through two types of neural asymmetry (Concha *et al.*, 2012). In one, bilateral circuits contain similar assemblages of cell types and connectivity patterns but differ in frequency between left and right (Fig 1.1A). In this type of neural lateralisation, the asymmetry could be generated through a difference in absolute number, scaling or ratio of neurons between left and right. The difference may not be restricted to the level of the cell but could also be brought about through the differences in somas, dendritic arbours, axon terminals and subcellular components, such as vesicles release, between the left and right sides. This type of neural lateralisation is hypothesised to be the 'simplest' to manifest because it is a fluctuation from an initially bilateral circuit (Concha *et al.*, 2012). An example is the difference in the size of the arcuate fasciculus between the left

and right hemispheres of the human brain, known to be associated with language production (Takao *et al.*, 2011a,b; de Schotten *et al.*, 2011).

The other type of neural lateralisation contains unique, unilateral circuit components which are found exclusively on one side (Fig. 1.1B). The unilateral circuit components can either develop independently on one side and not the other or they both can both develop but one regresses or is incorporated into other structures (Concha *et al.*, 2012). An example can be found in wild-type fruit flies (*Drosophila melanogaster*) in which a neural structure called the asymmetrical body is present in the right but not the left hemisphere of the brain (Pascual *et al.*, 2004). Although this classification does help to categorise the mechanisms that form the basis of lateralisation it must be emphasised that the production of lateralisation behaviour is often too complex to fit this dichotomy because interaction between left and right hemispheres of the brain is required to produce lateralised behaviour.

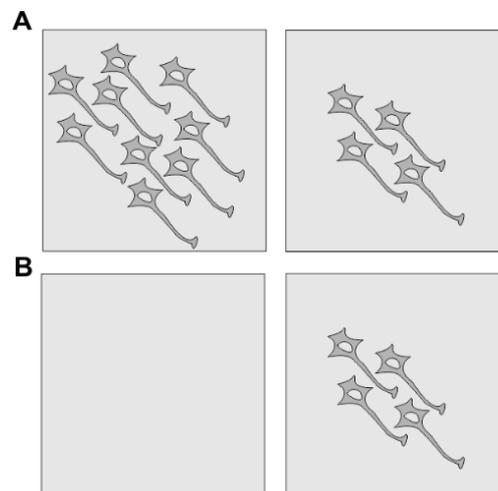


Figure 1.2. Types of neural lateralisation. **A.** The same types and patterns of neurons found on both sides but differ in frequency from left to right. **B.** Unique circuit components are found on only one side of the brain. (Reproduced from Concha *et al.*, 2012).

Attempt has been made to identify structural and/or physiological characteristics underpinning cerebral lateralisation. Perhaps the first and best known example is Paul Broca

(1861) who identified an area in the left hemisphere of the human brain that was involved in the production of speech. Since then, studies have demonstrated which lateralised structures are responsible for the control of certain behaviours in non-human animals. For example, hemispheric lateralisation in the control of song production in songbirds (Nottebohm, 1971; 1977), habenular asymmetries in the most primitive living vertebrates, the jawless fish (Braitenberg and Kemali, 1970) and the cellular and molecular mechanisms of lateralised neural development in the nematode worm *C. elegans* and zebrafish (Taylor *et al.*, 2010). Also, in *Drosophila* Pascual *et al.*, (2004) identified an asymmetric structure called the asymmetric body in some individuals that is responsible for the storage of long-term memories.

Studies also document asymmetry in cerebral structures responsible for lateralised control, for example in discrimination ability in chicks (Rogers and Anson, 1979; Rogers, 1982, 1990, 1991, 2000) and spatial preference in rats (Glick, 1977). In male fiddler crabs Young and Govind (1983) revealed that the motor neurons controlling the larger claw had larger somata and dendritic arbors and there are more sensory axons in the nerve root of the minor claw. Locusts (*Schistocerca gregaria*) flying in a dark wind tunnel show asymmetrical motor nerve output to flight muscles. The locusts spontaneously roll either clockwise and some roll anticlockwise which is coordinated by asymmetric motor nerve output (Wilson, 1968). Lateralisation of the motor output to milkweed bugs (*Oncopeltus*) hind legs is thought to produce a turning bias in the absence of visual feedback (Chapple, 1966a).

1.5 Function of lateralisation

Lateralisation of brain and behaviour, has been shown in many vertebrates (Rogers & Andrew, 2002; Vallortigara & Rogers, 2005) and in invertebrates (Frasnelli, 2013; Frasnelli *et al.*, 2012b), suggesting that it contributes significantly to biological fitness (Ghirlanda *et al.*, 2009). Evidence from chimpanzees (McGrew & Marchant, 1999), cats (Fabre-Thorpe *et al.* 1993), pigeons (Güntürkün *et al.*, 2000) and chicks (Rogers *et al.*, 2004), shows that lateralised animals outperform non-lateralised ones in many circumstances and researchers agree that a lateralised brain may confer several advantages (Ghirlanda *et al.*, 2009).

One advantage of lateralisation is that it may serve to increase neural capacity. If one side of the brain is specialising in one function it leaves the other hemisphere free to carry out other functions (Levy, 1977). Evidence from *Drosophila* supports this. Compared to individuals that don't possess an asymmetrical body, Pascual (2004) demonstrates that individuals which do possess this lateralised brain structure have better ability forming long-term memories. Another advantage of lateralisation is that it may confer an advantage by permitting separate and parallel processing to take place. Rogers showed that strongly lateralised chicks were able to respond faster to overhead predators (Rogers, 2000) and discriminate better between food and pebbles (Rogers *et al.*, 2004) than their weakly lateralised conspecifics. Being lateralised can also be advantageous because dominance in one side of the brain is likely to prevent the simultaneous initiation and incompatible response on the other. This is especially pertinent for animals with laterally placed eyes (Andrew, 1991; Vallortigara, 2000). Some fish species show lateralisation in their 'C-start' reaction to aversive stimulus. Heuts (1999) showed that the Mauthner cells and their axons, which are responsible for this behaviour, are themselves lateralised.

The function of lateralisation can be discussed with reference to the distribution of lateralisation individuals within a group. The distributions of lateralised individuals fall into one of three categories (van Valen, 1962; Leary and Allendorf, 1989) (Fig. 1.3) which are produced by differing genetic and/or environmental factors controlling the development of lateralisation. 'Fluctuating asymmetry' (Ludwig, 1932) is produced when variation in the development of otherwise symmetrical traits cause disturbances in the left-right axis during developing (Fig. 1.3A). The result is that the majority of the individuals within the group are lateralised with a small proportion of left and right biased individuals. Adaptive asymmetry (Palmer, 1996) is formed from genetically or epigenetically influenced traits, selected for a specific function. This type of lateralisation can produce two distributions which constitutes the second two categories. Population-level asymmetry (or species-level/directional asymmetry) occurs when the majority of individuals are lateralised in the same direction, causing a population bias to either the right or the left (Fig. 1.3B). Individual-level asymmetry is observed when the majority

of individuals are lateralised but there are approximately equal numbers of left- and right-biased individuals in the population (Fig. 1.3C).

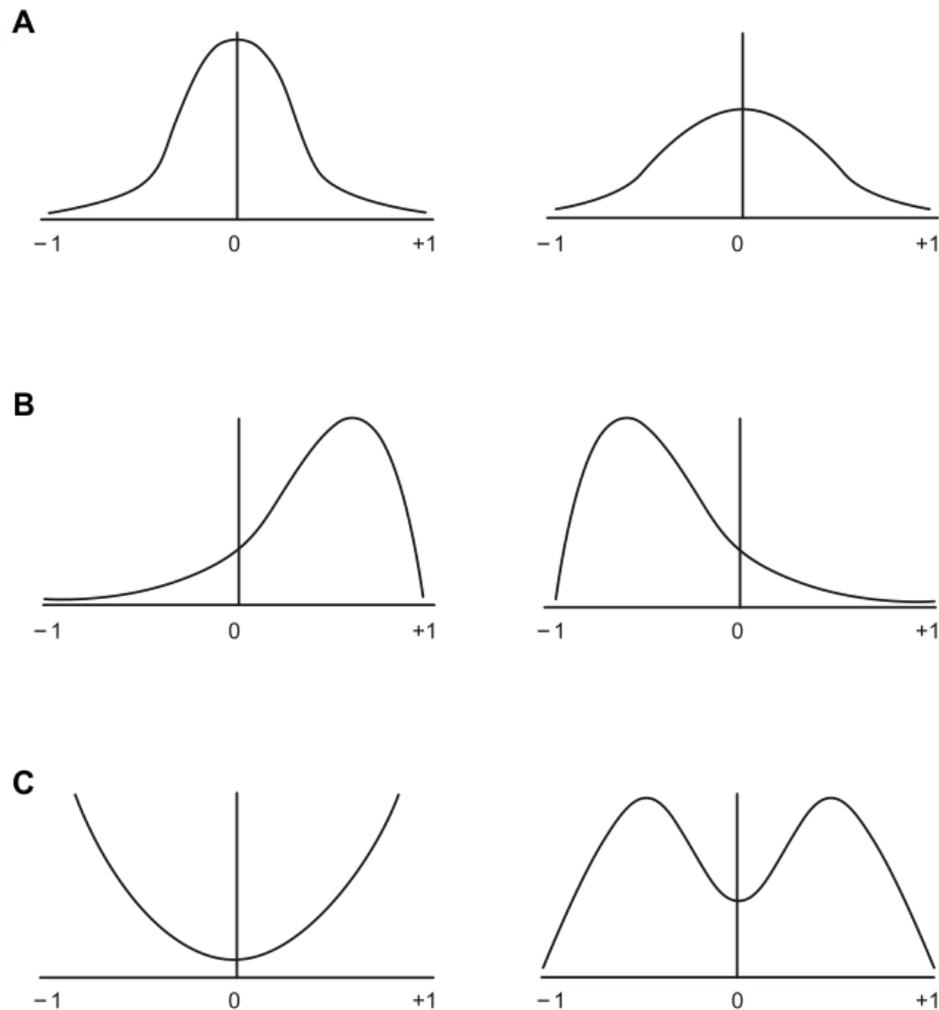


Figure 1.3. Frequency distributions of lateralised individuals. The x axis values represent the lateralisation index (right - left/right + left) **A.** Fluctuating asymmetry - the majority of the individuals within the group are lateralised with a few left and right biased individuals. **B.** Population-level asymmetry - the majority of individuals are lateralised in the same direction, causing a population bias to either the right or the left **C.** Individual-level asymmetry - the majority of individuals are lateralised but there are approximately equal numbers of left- and right-biased individuals in the population. (Reproduced from Vallortigara and Rogers, 2005).

Although these advantages illustrate the function of lateralisation to an individual (Fig. 1.3C) they fail to resolve the question of why a group of individuals may be lateralised in the same direction (Fig. 1.3B), because an individual's computational efficiency is unrelated to the direction of other lateralised individuals. In addition, it may be surprising that population-level lateralisation emerged at all because it may create predictable behaviour, such as turning in a particular direction during escape, which could be exploited by predators. Moreover, it is unclear why the proportion of left *versus* right individuals in lateralised populations varies from ~90%, as is the case in humans (McManus, 2002) and species of parrots (Magat and Brown, 2009) to 35 – 40%, as is the case in toads (Bisazza *et al.*, 1996).

Two explanations using mathematical models have thus been proposed for population-level lateralisation. The first is that population-level lateralisation reflects a balance between symmetry and asymmetry. The models (McManus, 1999; Annett, 2002) suggests that alleles exist for the dominant direction (D) and 'chance' alleles (C) that cause the other direction or the dominant direction at random. A population displaying population-level lateralisation can be maintained in these models if DC genotypes have higher fitness than CC and DD genotypes (heterozygotic advantage). For instance, if intermediate levels of brain asymmetry are superior to both extreme asymmetry and symmetry (Corballis, 2006).

The second, and possibly simpler, model suggests that the alignment of lateralisation at the population-level may arise as an "evolutionarily stable strategy" (Maynard Smith, 1982), when it is advantageous for lateralised individuals to coordinate their behaviour with that of other lateralised individuals within the group (Ghirlanda and Vallortigara, 2004). This hypothesis requires that brain lateralisation produces lateralised behaviour and has costs and benefits associated with interactions with other animals. Benefits may include synergistic interactions with conspecifics and costs may involve predictability of behaviour to predators such as anticipation of escape direction. The competition between the costs and benefits then determines the left/right proportion of lateralised individuals in the group (Ghirlanda and

Vallortigara, 2004; Fig. 1.4). Population-level lateralisation therefore is more likely to be observed in social rather than solitary species.

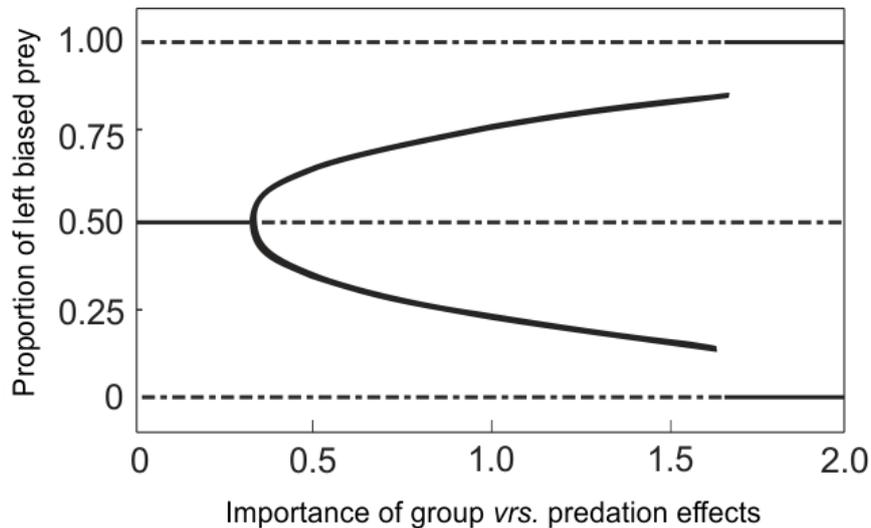


Figure 1.4. Game theoretical model illustrating of group versus predation effects in group. When social factors are low individual-level lateralisation is likely to exist however when sociality increases the probability of population-level lateralisation increases. (Reproduced from Ghirlanda and Vallortigara, 2004).

1.6 Lateralisation in insects

Traditionally, it was considered that only humans were capable of producing brain and behavioural lateralisation. Research now documents extensive examples of lateralisation throughout all vertebrate classes (Rogers and Andrew, 2002; Vallortigara and Rogers, 2005; MacNeilage *et al.*, 2009; Vallortigara *et al.*, 2011). Recently, a wealth of evidence on brain and behavioural asymmetries in invertebrates has begun to emerge (Frasnelli *et al.*, 2012b; Frasnelli, 2013). Although there are many examples of structural lateralisation in the general morphology in invertebrates (Palmer, 2009), here I will focus specifically on lateralisation in insect nervous systems and behaviour. The following studies are highlighted because they

are the most relevant to this thesis (see Frasnelli (2012b) and Frasnelli *et al.*, (2013) for more thorough reviews on the lateralisation of invertebrate brain and behaviour).

Research has shown that insects are cable of lateralisation in a variety of modalities. The fruit fly (*Drosophila melanogaster*) has been shown to possess olfactory lateralisation. Duistermars *et al.* (2009) demonstrated experimentally that when flying, the flies used their left antenna more than their right to track an odour plume. Honeybees also display lateralisation in olfaction when learning to associate an odour with a food reward (Letzkus *et al.*, 2006) although it is difficult to discern whether this lateralisation is due to sensory asymmetry in the antenna or lateralisation in learning and memory of olfactory pathways (Frasnelli *et al.*, 2012b). In another study on honeybees Letzkus *et al.* (2007) present evidence for lateralised eye use in honeybees, showing they use their right eye when learning to associate a visual stimulus with a food reward. Three species of Australian stingless bee have all been shown to (*Trigona carbonaria*, *T. hockingsi* and *Austroplebeia australis*) display an olfactory bias during an associative learning task (Frasnelli *et al.*, 2011).

Lateralisation in the form of motor preference has also been documented in insects. Kells and Goulson (2011) report that three species of bumble bee (*Bombus lapidarius*, *B. lucorum* and *B. pascuorum*) show a lateral bias when circling florets. When looking at the side of which ants walk along paths, Heuts *et al.* (2013) showed that twelve species walked on the right hand side compared to only one which preferred to walk along the left. Cockroaches (*Periplaneta americana*; Cooper *et al.*, 2010) and giant water bugs (*Belostoma flumineum*; Kight *et al.*, 2008) have an innate turning bias when presented with a choice to turn left or right in a 'Y'-maze. In a similar turning paradigm *Drosophila melanogaster* show a strong turning preference when presented with a left or right choice, although there is strong individual variation (Buchanan *et al.*, 2015). Lateralisation in motor behaviour is observed in blowflies (Romano *et al.*, 2015), tephritid flies (Benelli *et al.*, 2015a) and mosquitos (Benelli *et al.*, 2015b) when responding aggressively to conspecifics. Rowell (1963) also documents a form of limb preference in response to stimulation in the locust (*Schistocerca gregaria*), though the

numbers used in the study are small and there is little control regarding the stimulus site, leaving the conclusions from substantiated.

Although sparse, there is some evidence that the nervous systems of insects can be lateralised and that lateralised behaviour is correlated with lateralisation of the nervous system (Chapple, 1977). As mentioned earlier, some *D. melanogaster* individuals have a structure called in the right side of the brain called the asymmetric body, which is thought to be associated with the processing of long-term memories (Pascual *et al.*, 2004). The milkweed bug, *Oncopeltus fasciatus*, shows lateralisation in motor neuron output to the metathoracic legs (Chapple, 1966a). When vision was excluded the result was a bias in turning, however, the turning bias was corrected by when the visual feedback was permitted (Wilson and Hoy, 1968). In the mason bee (*Osmia cornuta*), Anfora *et al.* (2010) demonstrated a higher electrophysiological response of olfactory receptor neurons in the right antenna in comparison to the left. Aidley and White (1968) showed that Brazilian cicadas (*Fidicina rana*) produce asymmetry in their motor output to muscles controlling their song, with the left and right muscles are out of phase with one another.

1.7 Insects as models for lateralisation research

Insects provide powerful models for research into behavioural lateralisation. They are the most numerous and successful group of invertebrates in terms of numbers and are generally morphologically symmetrical (Chapple, 1977). Possessing large, identifiable motor neurons is also advantageous because it allows homologies to be identified, not only between right and left sides of nervous system or from organism to organism, but also from species to species (Harnad and Doty, 1977). Knowledge of lateralisation in insect nervous system may be crucial to understanding the evolution of brain and behavioural asymmetry. Due to the relative simplicity of their brains compared to vertebrate species they offer an excellent opportunity to investigate molecular, genetic and developmental aspects of lateralisation (Frasnelli *et al.*, 2013). The studies listed prove that insects provide an excellent model for research into lateralisation but much still remains to be discovered.

1.8 Context-dependency of forelimb lateralisation

Animals may exhibit lateralised motor behaviour of their forelimbs in one context i.e. targeted reaching behaviour, but fail to show comparable lateralised motor behaviour in another context i.e. non-targeted stepping to initiate walking. This is what is referred to as 'context' within this thesis.

When comparisons are drawn between species' limb preference in this thesis it should be noted that it is only the frequency distribution of lateralised individuals within their groups that is being compared, not that insect forelimb reaching and human reaching behaviours are analogous, and can be discussed in similar context. Comparison of human handedness with that of non-human animals should be taken with caution because human handedness is linked with fine motor abilities associated with object manipulation and tool use. The findings are primarily derived mainly from self-report and surveys of exclusively tool use (e.g. Edinburgh Handedness Inventory). The methodology for collecting data is not transferrable to non-linguistic animal species. Additionally, observational studies of both ape and human handedness have reported that the strength of lateral bias varies between contexts (Forrester *et al.* 2013) and may even be reversed in some contexts for individuals who are right-hand biased for object manipulation e.g. self-directed behaviour (Forrester *et al.* 2014). Unimanual reaching in apes is also suspected to be a less sensitive indicator of hand dominance compared with bimanual hand dominance because simple reaching may be as effective with either limb, not requiring specific neural resources (e.g. Tabiowo and Forrester 2013).

1.9 Summary of contributions

This thesis makes a number of original contributions to the field of lateralisation research. We show that desert locusts (*Schistocerca gregaria*) are biased in the forelimb they use to reach across a gap in the substrate upon which they are walking. This preference is context-dependent and is expressed by individuals rather than at the population-level. To our knowledge this is the first direct evidence from an insect, or indeed any arthropod, possessing individual-level limb preference in the use of otherwise symmetrical limbs.

Building upon this initial finding, we show that strongly biased locusts perform better during gap-crossing, making fewer errors with their preferred forelimb. The number of targeting errors locusts make negatively correlates with the strength of forelimb lateralisation. Lateralised behaviours are widespread in both vertebrates and invertebrates, suggesting that lateralisation is advantageous. Yet evidence demonstrating proximate or ultimate advantages remains scarce, particularly in invertebrates or in species with individual-level lateralisation. This provides evidence that stronger lateralisation confers an advantage in terms of improved motor control in an invertebrate with individual-level lateralisation.

We also demonstrate forelimb preference in another arthropod species, the red wood ant (*Formica rufa*) in the same gap-crossing paradigm. As was the case for desert locusts, we found some individuals preferred to use their right forelimb, others their left and the remainder showing no preference – the hallmark of individual-level lateralisation. Interestingly, the majority of individual ants within a colony show a preference for using the same forelimb to cross the gap. This directional preference differs between colonies, thus, wood ants exhibit two forms of lateralisation at the individual-level and at the colony-level the latter being an entirely novel, previously undescribed form of lateralisation.

Furthermore, we show that red wood ants are also lateralised in the movement of their entire body, *i.e.* they display a turning preference, within a 'Y'-maze paradigm. In this context, however, the lateralisation is displayed at the individual-level only, with neither the colony nor the population showing any directional bias. We find no correlation between the lateralisation of limb preference and the lateralised turning of the whole body, suggesting the independence of lateralised neural components.

The 'Y'-maze paradigm is used often when testing animals for lateralisation in turning preference because it provides a distinct 'choice' that is easy to score. Through close inspection of the ants' behaviour in these mazes, we reveal that their 'choices' are dominated by wall-following behaviour. Therefore, we question the validity of using the 'Y'-maze paradigm when assessing turning preferences, and suggest it is more likely to be assessing the persistence and switching of wall-following.

Chapter 2

Individual-level, context-dependent forelimb preference in the desert locust, *Schistocerca gregaria*.

2.1 Abstract

Despite evidence of asymmetries in insect sensory perception and motor control, there is no direct evidence for functional left-right asymmetry in their limb control, equivalent to that of vertebrates such as humans. The desert locust (*Schistocerca gregaria*) can be used as a powerful tool to investigate limb preference because morphologically they are bilaterally symmetrical and they have been shown to use their forelimbs whilst crossing gaps using visually-targeted behaviour. Here, we show that locusts are biased in the forelimb they use to reach across a gap in the substrate upon which they are walking. The strength of this bias differed among individuals, as did the forelimb with some locusts favouring their right forelimb more often and others their left. In contrast, the locusts' forelimb movements immediately prior to reaching, or whilst walking, were unbiased. This pattern was repeated when the gap was replaced with a glass platform; forelimb use was unbiased when stepping onto the glass surface but biased when stepping onto the other side. Thus, locusts show limb preference during targeted forelimb placement, but not whilst walking, the switch initiated by visual inputs. This preference is context-dependent and is expressed by individuals rather than at the population level. We also show that strongly biased locusts perform better during gap-crossing, making fewer errors with the targeted forelimb. This provides evidence that being biased is advantageous for individuals. To our knowledge this is the first direct evidence from an insect, or indeed any arthropod, possessing individual-level limb preference in the use of otherwise symmetrical limbs.

2.2 Introduction

Lateralisation is a functional and/or structural specialisation of the left and/or right side of the brain/body. Humans exhibit a form of lateralisation – handedness, whereby 90% of the population favour their right hand over their left (McManus, 2002). Traditionally, functional lateralisation was thought to occur only in humans (Annett, 1995), however, it has now been documented in all vertebrate classes (Vallorigara and Rogers, 2005; Vallortigara *et al.*, 2011). Lateralised animals have been shown to out-compete non-lateralised animals in various situations suggesting lateralised behaviour contributes to biological fitness (Güntürkün *et al.*, 2009; McGrew and Marchant, 1999; Rogers *et al.*, 2004). However, these studies have focused on vertebrates, many of which show population-level lateralisation in which unequal proportions of right- and left-biased individuals coexist (Rogers *et al.*, 2013).

Investigating limb preference in over 100 non-vertebrate species, Ströckens *et al.* (2013) found that around 70% of them exhibited a preference, indicating that limb preferences are clearly not restricted to humans (Versace and Vallortigara, 2015). It is unknown, however, whether non-human animals generally display a similar ratio of left to right-handed individuals within their respective populations, *i.e.* do they show population-level lateralisation in their limb preference? It is also unclear whether the limb preferences seen in non-human animals are consistent over time and context, *i.e.* does the animal show the same preference as a juvenile as that of an adult and does the preference appear stronger in different tasks?

Such population-level lateralisation is often proposed to have evolved from individual-level lateralisation, in which there is no obvious mode in the strength and direction of the bias (Rogers *et al.*, 2013). In turn, individual-level lateralisation is supposed to confer an advantage in terms of proximate performance that improves ultimate success, only coalescing into population-level lateralisation when the benefits of cooperativity outweigh the costs of predictability (Levy, 1977; Ghirlanda and Vallortigara, 2004).

Emerging research has produced evidence that invertebrates too are capable of lateralisation ranging from sensory perception to motor control (Frasnelli *et al.*, 2012b; Frasnelli, 2013). The left antenna of fruit flies (*Drosophila melanogaster*), for example,

contributes more to odour tracking than the right (Duistermars *et al.*, 2009), whilst honeybees mainly use their right eye when learning associations with food rewards (Letzkus *et al.*, 2007). Twelve species of ant have been shown to travel along the right side of their foraging paths (Heuts *et al.*, 2003) and giant water bugs (*Belostoma flumineum*) have an innate preference to turn left in a T-maze paradigm (Kight *et al.*, 2008). Although some research suggests invertebrates are capable of producing behavioural asymmetry in motor control there is relatively little specific evidence relating to limb preference.

Although most invertebrates are symmetrical (Palmer, 2009), gastropod molluscs and decapod crustaceans are exceptions (Chapple, 1977), both exhibiting morphological lateralisation. During development gastropod molluscs go through a 180-degree torsion of the viscera, producing bilateral asymmetry of the body. Decapod crustaceans such as the European fiddler crab (*Uca tangeri*), show heterochely (asymmetric claw size) in which each claw is specialized for determined motor actions and functions such as fight, attracting females (Govind and Blundon, 1985; Oliveira and Custodio, 1998), grip, hold, grasp, pull, cut during feeding (Hartman *et al.*, 1997). Therefore, although there is evidence that crustacean species show a limb preference, the preference is related to obvious morphological asymmetry.

Within Arthropoda there is also some circumstantial evidence of lateralisation at the behavioural level in the form of limb control (Ruppert *et al.*, 2004; Frasnelli *et al.*, 2012b). Spitting spiders (*Scytodes globula*) retrieved from the field displayed a higher frequency of left-leg loss, which researchers indicate is likely due to prey handling during capture (Ades and Ramires, 2002). Indeed, many arachnid species display lesions to the left leg (Heuts and Lambrechts, 1999) suggesting the asymmetry may be widespread. Locusts show motor asymmetry in their limbs in response to stimulation, however, this was recorded only when the legs were isolated from the rest of the central nervous system (Rowell, 1964). These promising findings suggest that relatively small invertebrate nervous systems are capable of lateralised behaviours, though there exists no direct evidence for functional left-right asymmetry in insect limb control, equivalent to that of vertebrates.

Desert locusts (*Schistocerca gregaria*, Forskål 1775) present a powerful model system in which to investigate forelimb preference in an invertebrate species. Their bilateral symmetry provides an opportunity to test for behavioural asymmetry in forelimb use in an insect. Easily maintained, they can be tested over time and context. Upon encountering a gap in the substrate upon which they are walking locusts use vision to target their forelimbs (Niven *et al.*, 2010), demonstrating that they use their forelimbs in controlled functional ways that may be analogous to vertebrates, including humans.

Here, we test the desert locust for limb preference over several contexts in which they can use either one of their two forelimbs. Our experiments determine whether individual locusts display a limb preference, manifested as a bias in the forelimb they use for particular tasks. By testing limb preference over various contexts we were able to assess the context-dependency of the locusts' preference and compare it with handedness in vertebrates, including humans, which may be absent in some movements (for example, walking) but pronounced in others (for example, reaching) (Rogers *et al.* 2013). We also determined whether the strength of an individual's lateralisation confers an advantage in performance by assessing the accuracy of targeted forelimb placement during gap-crossing.

2.3 Materials and Methods

2.3.1 Animals

Fifth-instar desert locusts were selected at random from a crowded colony maintained by a commercial supplier (Blades Biological Ltd, UK). These locusts were then kept throughout their final moult at the School of Life Sciences, University of Sussex, UK in heated holding tanks (160 x 100 x 120 mm, 24°C) and given a diet of wheat germ, grass and water from which they fed *ad libitum*. Except during testing, the locusts remained in the tanks to maintain crowding conditions. Only healthy animals with intact eyes, limbs and antennae were selected for experiments. Individual locusts were identified throughout the experiment by making a small unique cut in their wings.

2.3.2 Experimental arena and platform

Individual locusts were tested at 23°C in a rectangular white Perspex arena measuring 800 x 600 x 600 mm (Fig. 2.1A). The arena was lined with cardboard to conceal the corners, which can distract the locusts. A 'peephole' 60 mm in diameter was cut in the arena wall at a height of 6 cm from the base to admit a 50 mm diameter camera lens with which the locusts were filmed. An identical hole was cut into the opposite wall of the arena into which a black disk was inserted to maintain symmetry.

Two horizontal platforms 150 x 50 x 20 mm constructed from 5 mm thick Perspex were placed in the centre of the arena (Fig. 2.1A). The two platforms were placed opposite separated by 25 mm creating a gap consisting of one straight face and one 'diving board' face (Figure 2.1B) (Triphan *et al.*, 2010). The size and shape of the gap was determined from pilot studies that investigated the propensity of locusts to cross gaps (unpublished data). The platform to which each locust crossed was elevated by 5 mm from the platform on which they were initially placed. The horizontal surface of each platform was covered with white paper to allow it to be cleaned easily if locusts deposited faeces and/or spit. This paper was marked with fine horizontal lines. The edge of the elevated platform was marked with black acrylic paint. A rectangle of black card (60 x 250 mm) was placed at the end of this platform to attract locusts (Wallace, 1958).

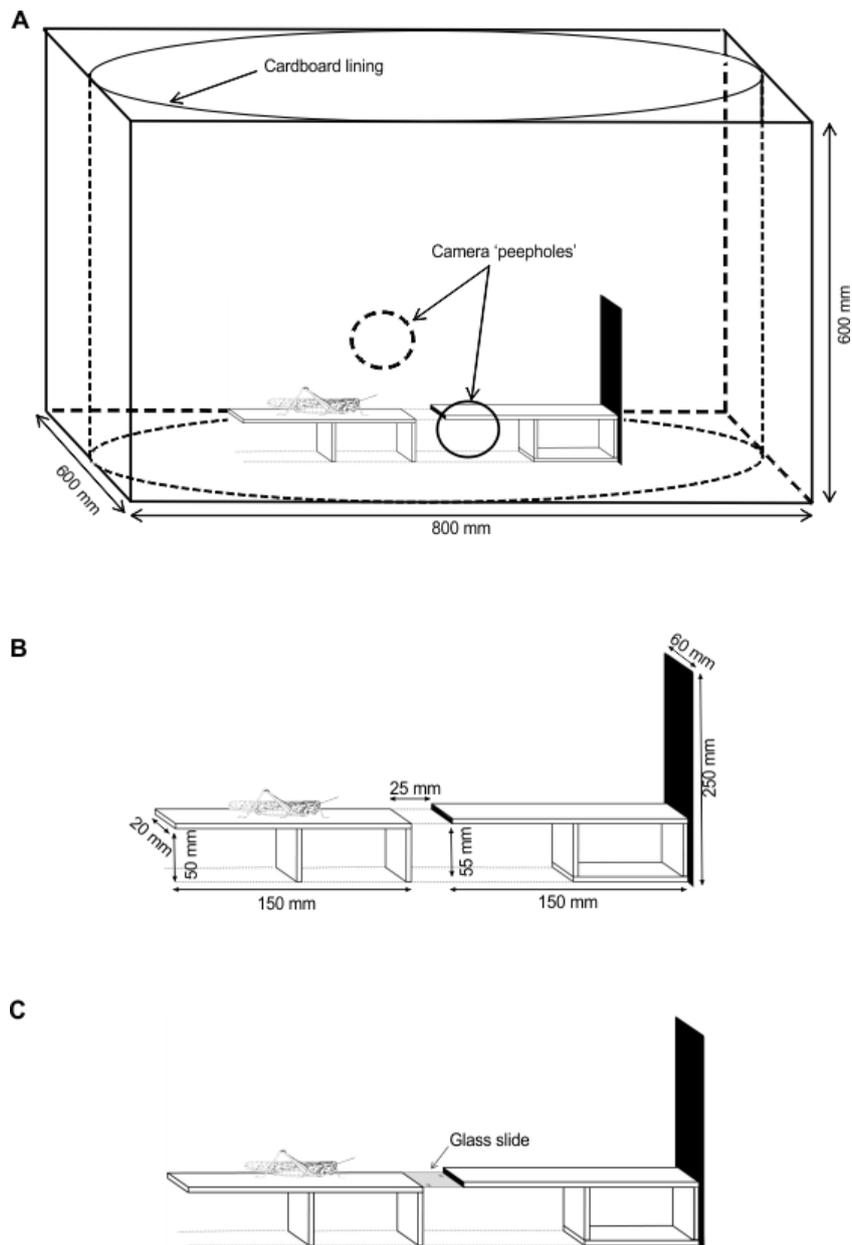


Figure 2.1. The experimental arena for testing locust limb preference and the modification of the platform with a glass slide. A. The testing arena. The arena contains two platforms upon which the locusts walk, two camera 'peepholes' to record the gap cross and a cardboard lining to cover corner edges, which can distract the locusts. **B.** Positioning of the two platforms inside the arena. The arrangement and dimensions of the two platforms that created the gap for the locusts to cross. **C.** Positioning of the glass slide between the platforms.

2.3.3 Testing

All locusts were the same size and age when tested for limb preference. Individual locusts were tested multiple times during the experiment, which lasted between 21 and 30 days. Each individual locust had only one gap crossing trial before being returned to a holding tank and another individual being chosen at random. This ensured there was an appreciable time interval between replicates, minimising any possible effect of task familiarity on the locusts' behaviour. The time of day was also randomised for each trail.

2.3.4 Gap-crossing

A cohort of 29 locusts was used to assess gap crossing. A locust was placed 80 mm from the gap, on the platform opposite the 60 x 250 mm black rectangle. Filming began after the locust started to walk and continued until it crossed to the opposite platform. Occasionally, locusts made searching or flailing movements with both forelimbs to grasp the opposite platform. These trials were excluded because they did not involve deliberate forelimb targeting. Trials in which locusts did not initiate any gap-crossing behaviour were counted as refusals and were omitted from analysis. Each locust crossed the gap a total of 20 times. On half the trials the locusts crossed from left to right, whilst on the other half they crossed right to left to exclude any possible asymmetrical bias in the arena. In addition to these 29 locusts, we added a further cohort of 11 to investigate whether the occlusion influenced limb preference (section 2.3.8).

2.3.5 Steps prior to gap-crossing

We used a second cohort of 29 locusts over 30 trials to record the forelimb used to initiate walking on the platform (Fig. 2.2A). We modified the arena by blocking the side viewing hole used in the previous experiments and positioning the camera directly above the platforms. This arrangement maintained the arena's symmetry, whilst allowing us to film the locusts walking on the platforms from overhead. The original cohort of 29 locusts was used to assess the forelimb used to enter the gap, immediately prior to gap-crossing (Fig. 2.2B).

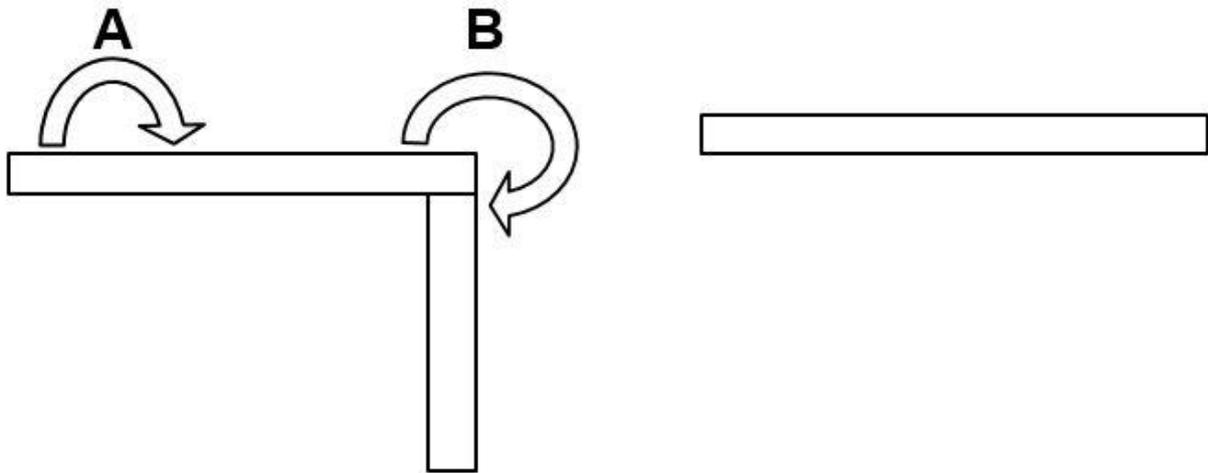


Figure 2.2. Schematic representation of steps recorded prior to gap crossing. A. The step used to initiate walking along platform. **B.** The step into gap immediately prior to gap crossing.

2.3.6 Bridging of the gap with a glass slide

We modified the arena a second time to insert a transparent glass surface was between the platforms, bridging the gap between them (Fig. 2.1C). This glass bridge allowed a locust to cross to the other platform by walking whilst maintaining the visual impression of the gap. We recorded a third cohort of locusts walking on the platform, assessing the forelimb first placed onto the glass slide (Fig. 2.3A) and the forelimb that stepped from the glass onto the opposite platform (Fig. 2.3B). In this paradigm we recorded 30 locusts performing 30 trials each.

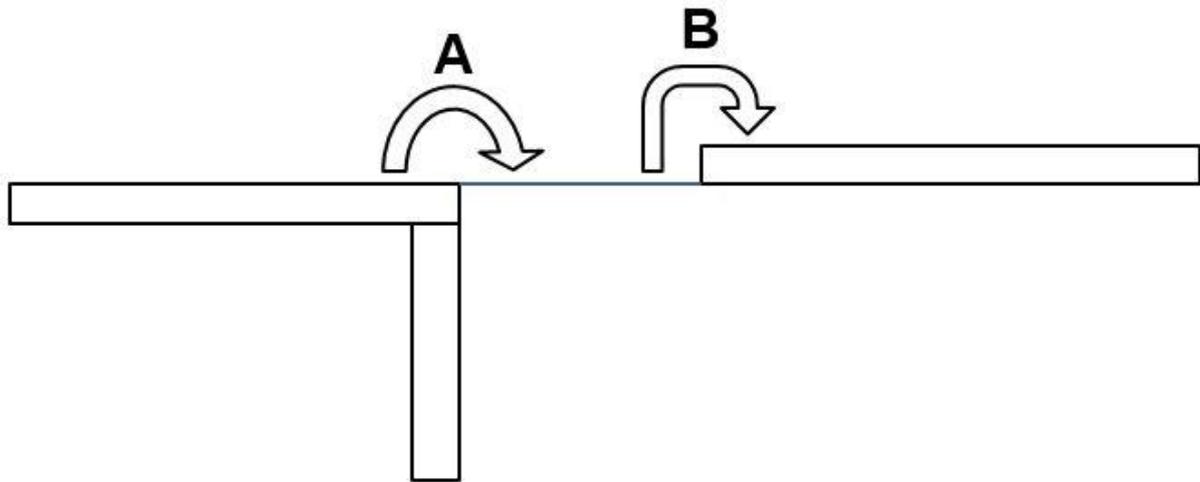


Figure 2.3. Schematic representation of steps recorded onto and off the glass slide. A. The step used to initiate walking on the glass slide. **B.** The step used to mount the opposing platform.

2.3.7 Influencing limb preference

Limb preference in this context may be produced through learning, emerging via repetition during development. To investigate this possibility, we occluded the left eye of locusts immediately after their second moult by painting the eye with opaque, acrylic paint preventing them from seeing through this eye. Locusts were checked on a daily basis to ensure they hadn't scraped off their occlusion and if they did the paint was immediately reapplied. After approximately 7 days into adulthood the occlusion was removed and the locusts were tested for limb preference. We tested 40 locusts over 20 trials and compared these with the original cohort of locusts, in addition to 11 more, to investigate whether the occlusion influenced limb preference. We refer to locusts that experienced the visual occlusion as the treated cohort and the normal cohort of locusts that did not experience the occlusion as the untreated cohort.

2.3.8 Assigning strength of preference

The strength of forelimb bias was based on preferred forelimb usage; zero was assigned to locusts that crossed with their right forelimb in 10 of 20 trials, while 10 was assigned to those that crossed with their right forelimb in either 20 or 0 of 20 trials.

2.3.9 Video analysis

The videos for the gap crossing experiments were recorded with a video camera (SONY Handycam HDR-CX105E) fitted with a wide angle lens positioned parallel to the horizontal plane of the gap (Fig. 2.1A). The videos for the control experiments were recorded with a Logitech HD Webcam C615). Videos were saved and analysed offline.

2.3.10 Statistical analysis

The distribution of the observed number of forelimb reaches, preferred forelimb reaches and steps performed by each locust was tested for deviation from the expected distribution, which was calculated from binomial distribution assuming no forelimb preference ($p = 0.5$). Any classes in which the expected numbers were <3 were amalgamated with the adjacent classes to produce expected numbers of the appropriate size. The degrees of freedom were adjusted to account for amalgamation and for the use of an intrinsic hypothesis based on the binomial distribution. A single-classification G-test for goodness of fit to the intrinsic hypothesis was used with William's adjustment (G_{adj}) applied (Sokal and Rohlf, 1997). This statistical analysis was used to test for the deviation from the binomial distribution for each distribution. A G-test for heterogeneity (G_H) was calculated (Sokal and Rohlf, 1997) to test for heterogeneity among the locusts, G pooled (G_P) to compare the pooled data across all locusts and G total (G_T) the sum of G_H and G_P . In the text I give G_T , however, in all cases where G_T is significant this was due to heterogeneity among locusts (G_H). The deviation of individual locusts from the expected binomial distribution ($p = 0.5$) was calculated using a 2-tailed Exact Binomial Test using the method of small p -values. All statistical tests were carried out using custom programs written in Excel (Microsoft) (Tables 2.1-2.7).

Laterality Index (LI) scores were calculated for each individual to establish the degree of lateral asymmetry, using the formula $[LI = (R - L) / (R + L)]$, with R and L being the frequency counts for right- and left limb actions. LI values vary on a continuum between -1.0 and +1.0, where the sign indicates the direction of hand preferences. Positive values reflect a right limb

preference while negative values reflect a left limb preference. When $R = L$, the LI is taken to be zero. Mean Laterality Index scores (MLI) were also be calculated for cohort analyses where appropriate.

An independent samples *t*-test (Baldi and Moore, 2014) was conducted to compare the mean number of missed reaches between strongly (significant) and weakly (non-significant) biased individuals. A Spearman's rank-order correlation (Baldi and Moore, 2014) was computed to test for a significant relationship between the strength of individual preference and the number of reaching errors. A dependent samples *t*-test (Baldi and Moore, 2014) was conducted to compare the mean number of reaching errors between the preferred and non-preferred forelimb. To test whether reaching errors were independent between trials, we compared the observed distribution of reaching errors against a Poisson distribution with the same mean.

2.4 Results

2.4.1 Gap-crossing behaviour

Desert locusts (*Schistocerca gregaria*) placed at one end of a platform in a white arena walked along the platform until they encountered a gap (Fig. 2.4). They then crossed the gap to the horizontal platform on the far side in 82% (580/707) of trials, the remaining trials being refusals. Typically, during gap crossing locusts placed one or both forelimbs into the gap before replacing them on the platform edge. They then reached with one forelimb towards the opposite platform (Fig. 2.4). Once they made contact with one forelimb, the locusts stepped across the gap with the opposite forelimb and crossed.

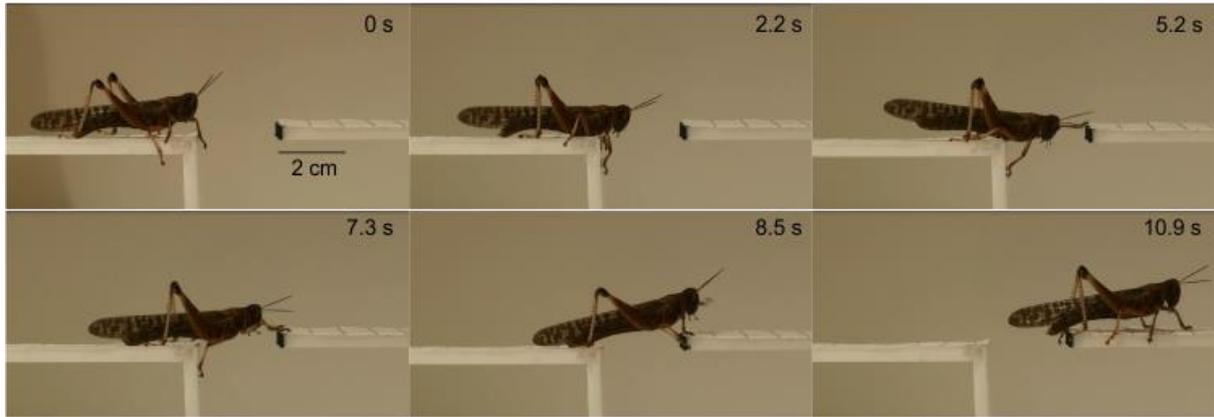


Figure 2.4. A video sequence showing a gregarious locust crossing a gap. Locusts cross gaps by reaching across with one forelimb to contact the opposite platform.

2.4.2 Locust forelimb preference

Each locust ($N = 29$, where N refers to the number of locusts and n refers to the number of trials throughout this chapter) performed 20 gap-crossings during which they could reach across the gap using either forelimb. We found that individuals varied in the strength of their bias; some showed a strong bias towards a particular forelimb (e.g. 90% of trials), whilst others showed a weak bias (e.g. 55% of trials). Were locusts unbiased, their forelimb use would be expected to approximate a binomial distribution. The distribution we observed deviated significantly from the binomial expectation (G -test, $G_{adj} = 19.73$, $3df$, $p < 0.005$, $N = 29$) (Fig. 2.3A) with many individuals showing a strong bias for one forelimb. However, individual locusts differed from one another significantly in the strength and direction of their forelimb bias (G -test, $G_{adj} = 17.75$, $5df$, $p < 0.005$, $N = 29$) suggesting there was no consistent bias towards a particular limb among the population. Indeed, the population contained individuals that were significantly biased towards either their left or right forelimb (Exact Binomial tests, Table 2.1). This cohort of locusts had a MLI of -0.08 .

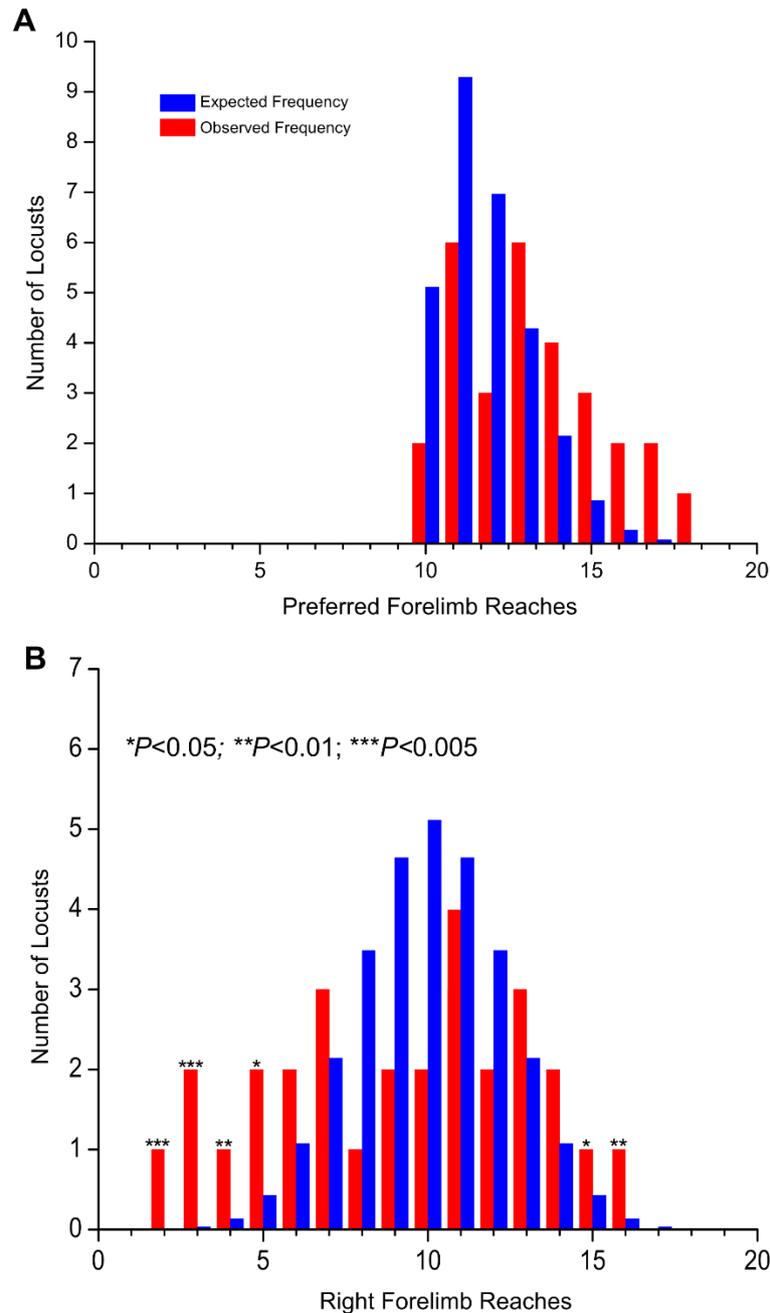


Figure 2.5. Limb preference in targeted forelimb movements during gap-crossing in 29 locusts. **A.** The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 29$, $n = 20$) (red). The binomial expectation of no preferred forelimb ($p = 0.5$) (blue) obtained by mirroring the binomial distribution to incorporate both left and right forelimb use. **B.** Frequency distributions of movements initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue). Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests (Table 2.1).

Table 2.1. Binomial Exact tests of goodness-of-fit for reaches across gap. Data from Figure 2.5; $N = 29$, $n = 20$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Locust	Right Forelimb Reaches	2-tailed probability	Laterality Index
1	11	0.664	0.10
2	4	0.0072**	- 0.60
3	16	0.0072**	0.60
4	2	0.000221***	- 0.80
5	14	0.0784	0.40
6	12	0.383	0.20
7	3	0.00149***	- 0.70
8	15	0.0266*	0.50
9	14	0.0784	0.40
10	11	0.664	0.10
11	9	0.664	- 0.10
12	13	0.189	0.30
13	11	0.664	0.10
14	7	0.189	- 0.30
15	13	0.189	0.30
16	6	0.0784	- 0.40
17	3	0.00149***	- 0.70
18	12	0.383	0.20
19	5	0.0266*	- 0.50
20	7	0.189	- 0.30
21	11	0.664	0.10
22	13	0.189	0.30
23	7	0.189	- 0.30
24	5	0.0266*	- 0.50
25	6	0.0784	- 0.40
26	10	1	0
27	9	0.664	- 0.10
28	10	1	0
29	8	0.383	- 0.20

2.4.3 Steps prior to gap-crossing

To determine whether biased forelimb use was restricted to gap-crossing, we assessed the forelimb placed into the gap immediately prior to reaching in the same cohort of locusts. On this step the distribution of forelimb use did not differ from the binomial expectation (G -test, $G_{adj} = 2.54$, $3df$, $p > 0.05$, $N = 29$) showing it was unbiased (Fig. 2.6A). This cohort of locusts had a MLI of -0.10 .

We also assessed the first step that initiated walking on the platform in a second cohort of locusts. In each trial the locust started from a stationary position. We recorded the forelimb first placed forward towards the gap. We found the distribution of forelimb use on this step did not differ from the binomial expectation (G -test, $G_{adj} = 4.14$, $3df$, $p > 0.05$, $N = 29$) showing it too was unbiased (Fig. 2.6B). Thus, the bias in locusts' forelimb use was restricted to reaching during gap-crossing. This cohort of locusts had a MLI of 0.01 .

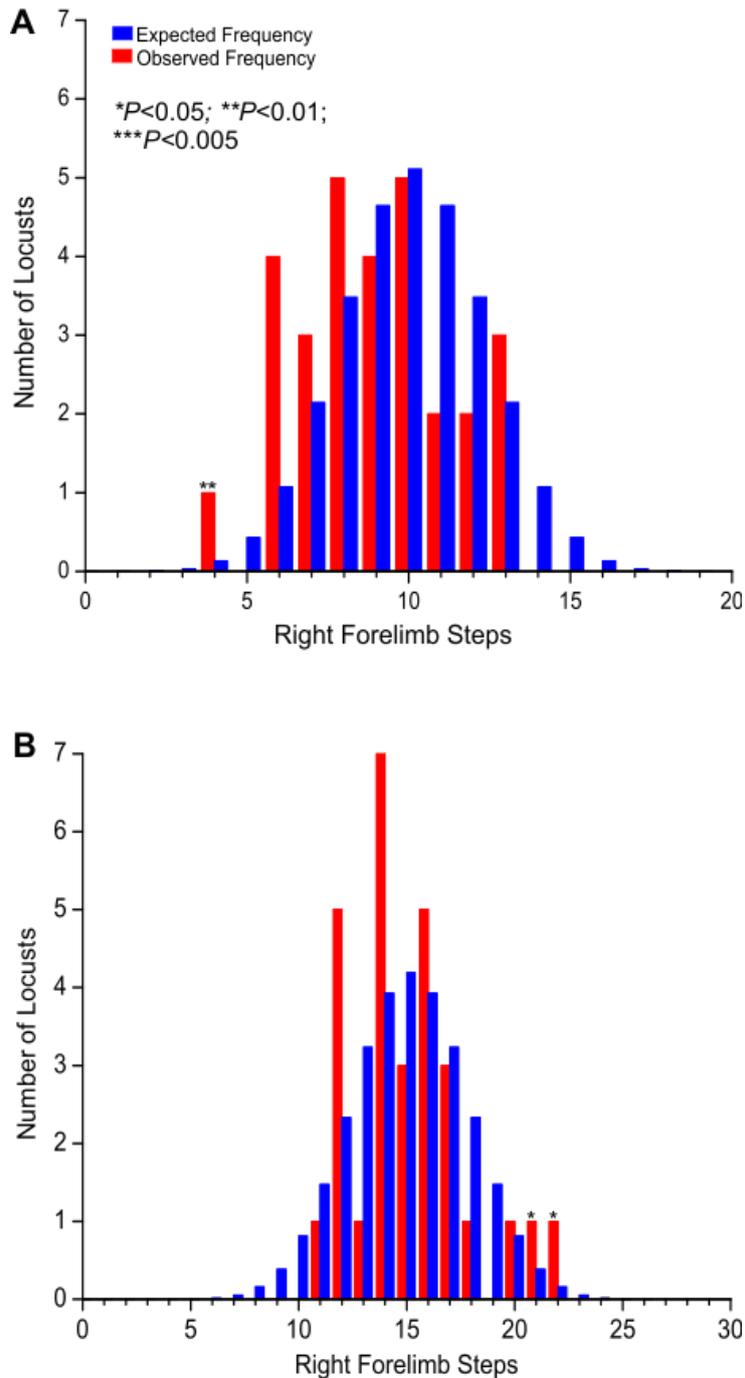


Figure 2.6. Forelimb movements of locusts prior to gap crossing. A. Steps into the gap ($N = 29$, $n = 20$) Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests (Table 2.2). **B.** Steps initiating walking on the platform ($N = 29$, $n = 30$). Frequency distribution of movements initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue). Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests (Table 2.3).

Table 2.2. Binomial Exact tests of goodness-of-fit for steps into gap. Data from Figure 2.6A; $N = 29$, $n = 20$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Locust	Right Forelimb Steps	2-tailed probability	Laterality Index
1	9	0.664	- 0.10
2	7	0.189	- 0.30
3	10	1	0
4	11	0.664	0.10
5	8	0.383	- 0.20
6	10	1	0
7	9	0.664	- 0.10
8	12	0.383	0.20
9	8	0.383	- 0.20
10	7	0.189	- 0.30
11	10	1	0
12	10	1	0
13	13	0.189	0.30
14	4	0.0072**	- 0.60
15	12	0.383	0.20
16	7	0.189	- 0.30
17	8	0.383	- 0.20
18	13	0.189	0.30
19	6	0.0784	- 0.40
20	9	0.664	- 0.10
21	9	0.664	- 0.10
22	8	0.383	- 0.20
23	6	0.0784	- 0.40
24	8	0.383	- 0.20
25	11	0.664	0.10
26	6	0.0784	- 0.40
27	13	0.189	0.30
28	10	1	0
29	6	0.0784	- 0.40

Table 2.3. Binomial Exact tests of goodness-of-fit for walking initiation. Data from Figure 2.6B; $N = 29$, $n = 30$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Locust	Right Forelimb Steps	2-tailed probability	Laterality Index
1	16	0.72	0.07
2	15	1	0
3	14	0.72	- 0.07
4	12	0.281	- 0.20
5	12	0.281	- 0.20
6	17	0.473	0.13
7	16	0.72	0.07
8	14	0.72	- 0.07
9	12	0.281	- 0.20
10	14	0.72	- 0.07
11	16	0.72	0.07
12	18	0.281	0.20
13	20	0.0708	0.33
14	22	0.0107*	0.47
15	14	0.72	- 0.07
16	14	0.72	- 0.07
17	17	0.473	0.13
18	15	1	0
19	17	0.473	0.13
20	16	0.72	0.07
21	13	0.473	- 0.13
22	12	0.281	- 0.20
23	14	0.72	- 0.07
24	15	1	0
25	21	0.0294*	0.40
26	16	0.72	0.07
27	14	0.281	- 0.20
28	12	0.72	- 0.07
29	11	0.15	- 0.27

2.4.4 Steps on the glass bridge

The bias in forelimb use may be triggered by detecting the gap visually (Niven *et al.* 2010; Niven *et al.* 2012) or by placing a forelimb into it. We used the third cohort of locusts to distinguish these possibilities. We bridged the gap with a transparent glass slide, retaining the visual impression without the actual gap itself (Fig. 2.1C). Typically, locusts paused upon encountering the gap before walking across the bridge, slowing or pausing again before stepping onto the far platform. We found the distribution of the forelimb placed first onto the glass slide did not differ from the binomial expectation (G-test, $G_{adj} = 4.69$, 3df, $p > 0.05$, $N = 30$), suggesting the locusts were unbiased on this step (Fig. 2.7A). This cohort of locusts had a MLI of 0.01. However, the distribution of the forelimb used to step from the glass slide onto the opposite platform differed significantly from the binomial expectation (G-test, $G_{adj} = 8.90$, 2df, $p < 0.05$, $N = 30$; Fig. 2.7B). This cohort of locusts had a MLI of 0.04. Consistent with this, individual locusts differed significantly in the strength and direction of their bias on this step (G-test, $G_T = 79.29$, 29df, $p < 0.005$, $N = 30$; Fig. 2.7B). Thus, forelimb movements are unbiased when the locusts step onto the bridge but are biased when stepping off it suggesting that visual perception of the gap and/or step influences the switch from unbiased to biased.

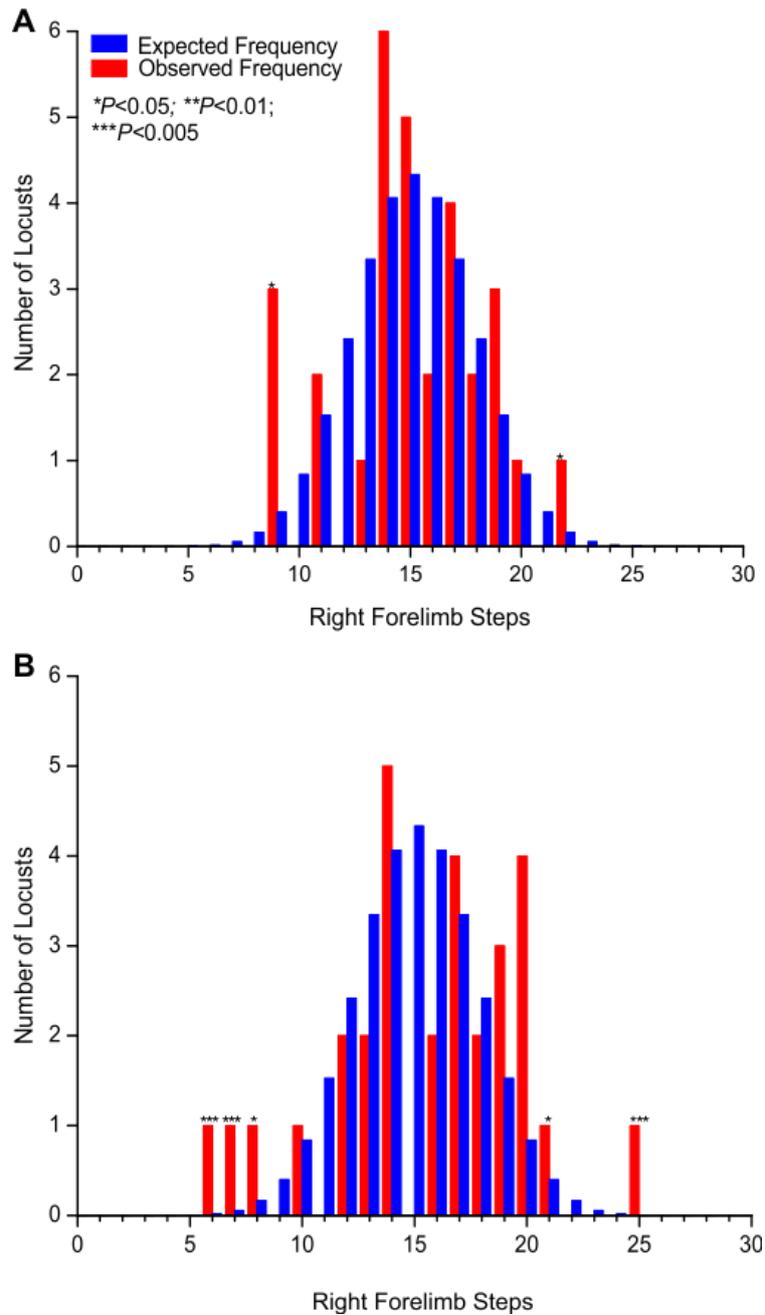


Figure 2.7. Forelimb movements of locusts walking on a glass bridge. **A.** The frequency distribution of right forelimb steps onto the glass slide compared with the expected binomial distribution ($N = 30$, $n = 30$). Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests (Table 2.4). **B.** The frequency distribution of right forelimb steps up to opposite platform compared with the expected binomial distribution ($N = 30$, $n = 30$). Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests (Table 2.5).

Table 2.4. Binomial Exact tests of goodness-of-fit for steps onto glass slide. Data from Figure 2.7A; $N = 30$, $n = 30$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Locust	Right Forelimb Steps	2-tailed probability	Laterality Index
1	11	0.15	- 0.27
2	15	1	0
3	15	1	0
4	22	0.0107*	0.47
5	15	1	0
6	16	0.72	0.07
7	14	0.72	- 0.07
8	15	1	0
9	14	0.72	- 0.07
10	17	0.473	0.13
11	9	0.0294*	- 0.04
12	9	0.0294*	- 0.04
13	15	1	0
14	14	0.72	- 0.07
15	17	0.473	0.13
16	19	0.15	0.27
17	18	0.281	0.20
18	11	0.15	- 0.27
19	19	0.15	0.27
20	13	0.473	- 0.13
21	14	0.72	- 0.07
22	14	0.72	- 0.07
23	17	0.473	0.13
24	20	0.0708	0.33
25	19	0.15	0.27
26	9	0.0294*	- 0.04
27	17	0.473	0.13
28	14	0.72	- 0.07
29	16	0.72	0.07
30	18	0.281	0.20

Table 2.5. Binomial Exact tests of goodness-of-fit for steps up to opposite platform.

Data from Figure 2.7B; $N = 30$, $n = 30$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Locust	Right Forelimb Steps	2-tailed probability	Laterality Index
1	19	0.15	0.27
2	21	0.0294*	0.40
3	20	0.0708	0.33
4	20	0.0708	0.33
5	7	0.00333***	-0.53
6	14	0.72	-0.07
7	14	0.72	-0.07
8	19	0.15	0.27
9	20	0.0708	0.33
10	17	0.473	0.13
11	16	0.72	0.07
12	8	0.0107*	-0.47
13	25	0.000192***	0.67
14	14	0.72	-0.07
15	12	0.281	-0.20
16	13	0.473	-0.13
17	19	0.15	0.27
18	12	0.281	-0.20
19	14	0.72	-0.07
20	18	0.281	0.20
21	18	0.281	0.20
22	17	0.473	0.13
23	16	0.72	0.07
24	20	0.0708	0.33
25	17	0.473	0.13
26	6	0.000878***	-0.60
27	17	0.473	0.13
28	13	0.473	-0.13
29	14	0.72	-0.07
30	10	0.0708	-0.33

2.4.5 Influencing limb preference

We wanted to investigate whether visually occluding one eye during development could drive a preference for the contralateral forelimb. As locusts use their eyes for targeted limb movements (Niven et al., 2010) we predicted the occlusion would force greater usage of contralateral forelimb and, consequently, influence the preference of this forelimb. We occluded the left eye of third instar locusts during development, then removed the occlusion and tested for limb preference in adulthood, comparing the distribution with an untreated cohort of locusts.

The addition of 11 more untreated locusts to the original cohort of 29 may have had an effect on the result that showed a significant deviation from the binomial distribution. To exclude this possibility, we re-tested the entire cohort of 40 locusts against the expected binomial distribution (Fig. 2.8). The inclusion of extra locusts did not affect the distribution of preferred forelimb use, which still deviated significantly from the folded binomial distribution (G -test, $G_{adj} = 20.9$, $2df$, $p < 0.005$, $N = 40$; Fig. 2.8A). The distribution of right handed reaches also still deviated significantly from the binomial expectation (G -test, $G_{adj} = 21.46$, $5df$, $p < 0.005$, $N = 40$; Fig. 2.8B).

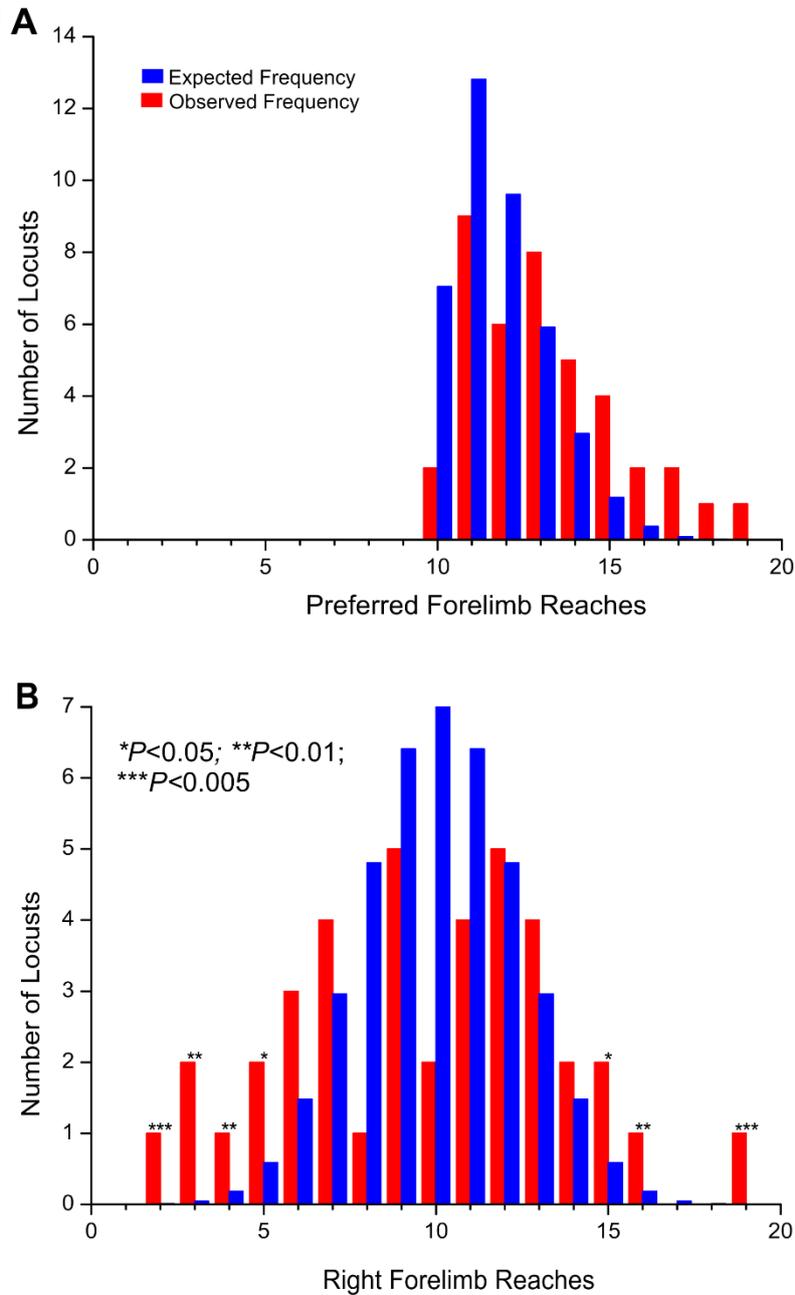


Figure 2.8. Limb preference in targeted forelimb movements during gap-crossing in 40 locusts. **A.** The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 40$, $n = 20$) (red). The binomial expectation of no preferred forelimb ($p = 0.5$) (blue) obtained by mirroring the binomial distribution to incorporate both left and right forelimb use. **B.** Frequency distributions of movements initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue). Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests.

We then compared the untreated cohort of locusts with the treated cohort (Fig. 2.9). We found the distributions of preferred forelimb reaches did not differ from one another (G -test, $G_{adj} = 4.04$, $5df$, $p > 0.05$, $N = 40$, $n = 20$) (Fig. 2.9A) suggesting the occlusion had no effect on the strength of limb preference. The distribution of right forelimb reaches in occluded locusts did not differ from original cohort of locusts (G -test, $G_{adj} = 13.88$, $10df$, $p > 0.05$, $N = 40$, $n = 20$) suggesting the visual occlusion did not induce a preference for the right forelimb (Fig. 2.9B).

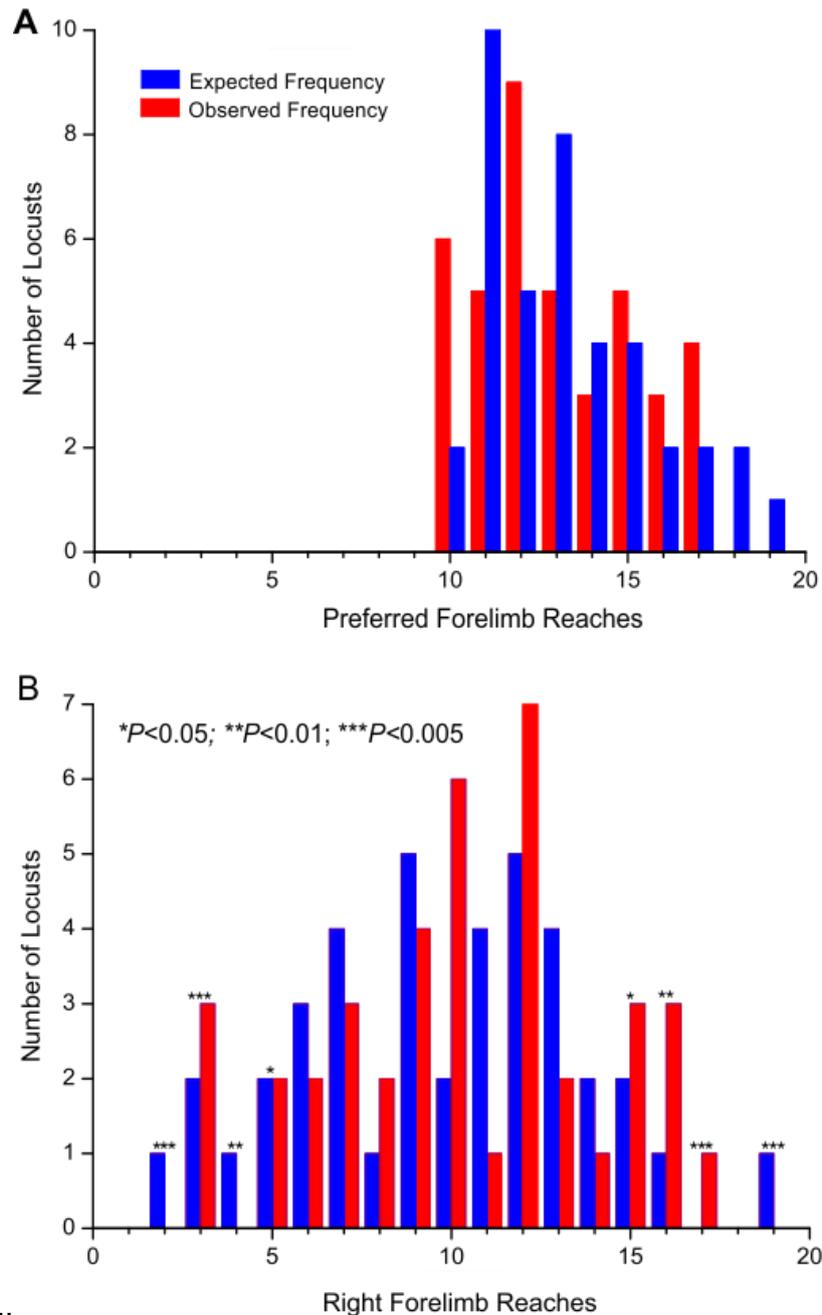


Figure 2.9. Limb preference in targeted forelimb movements during gap-crossing in locusts that were visual occluded during development. Histogram of original cohort of locusts (blue; no visual occlusion) against locusts with left eye visual from 3rd instar (red). **A.** The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 40$, $n = 20$). **B.** Frequency distributions of movements initiated by the right forelimb (red) compared with the expected distribution (blue). Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests (Table 2.6 and 2.7).

Table 2.6. Binomial Exact tests of goodness-of-fit for steps up to opposite platform by untreated locusts. Data from Figure 2.11; $N = 40$, $n = 20$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Locust	Right Forelimb Steps	2-tailed probability	Laterality Index
1	11	0.664	0.10
2	4	0.0072**	-0.60
3	16	0.0072**	0.60
4	2	0.000221***	-0.80
5	14	0.0784	0.40
6	12	0.383	0.20
7	3	0.00149***	-0.70
8	15	0.0266*	0.50
9	14	0.0784	0.40
10	11	0.664	0.10
11	9	0.664	-0.10
12	13	0.189	0.30
13	11	0.664	0.10
14	7	0.189	-0.30
15	13	0.189	0.30
16	6	0.0784	-0.40
17	3	0.00149***	-0.70
18	12	0.383	0.20
19	5	0.0266*	-0.50
20	7	0.189	-0.30
21	11	0.664	0.10
22	13	0.189	0.30
23	7	0.189	-0.30
24	5	0.0266*	-0.50
25	6	0.0784	-0.40
26	10	1	0
27	9	0.664	-0.10
28	10	1	0
29	8	0.383	-0.20
30	19	0.000021***	0.90
31	11	0.664	0.10
32	13	0.189	0.30
33	12	0.383	0.20
34	6	0.0784	-0.40
35	9	0.664	-0.10
36	7	0.189	-0.30
37	9	0.664	-0.10
38	15	0.0266*	0.50
39	12	0.383	0.20
40	9	0.664	-0.10

Table 2.7. Binomial Exact tests of goodness-of-fit for steps up to opposite platform for treated locusts. Data from Figure 2.11; $N = 40$, $n = 20$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Locust	Right Forelimb Steps	2-tailed probability	Laterality Index
1	5	0.0266*	-0.5
2	5	0.0266*	-0.5
3	4	0.0072*	-0.6
4	8	0.383	-0.2
5	17	0.00149***	0.7
6	12	0.383	0.2
7	4	0.0072**	-0.6
8	10	1	0
9	10	1	0
10	13	0.189	0.3
11	7	0.189	-0.3
12	11	0.664	0.1
13	13	0.189	0.3
14	15	0.0266*	0.5
15	10	1	0
16	10	1	0
17	8	0.383	-0.2
18	10	1	0
19	17	0.00149***	0.7
20	12	0.383	0.2
21	13	0.189	0.3
22	4	0.0072**	-0.6
23	8	0.383	-0.2
24	17	0.00149***	0.7
25	11	0.664	0.1
26	8	0.383	-0.2
27	5	0.0266*	-0.5
28	7	0.189	-0.3
29	8	0.383	-0.2
30	3	0.00149***	-0.7
31	14	0.0784	0.4
32	11	0.664	0.1
33	11	0.664	0.1
34	9	0.664	-0.1
35	8	0.383	-0.2
36	15	0.0266*	0.5
37	10	1	0
38	14	0.0784	0.4
39	8	0.383	-0.2
40	6	0.0784	-0.4

Given that the cohort of treated locusts did not deviate significantly with the untreated cohort, we combined these data to produce larger cohort of 80 locusts and re-tested them to determine whether they deviated from a binomial distribution (Fig. 2.10). As before, the distribution deviated significantly from the binomial expectation (G -test, $G_{adj} = 56.24$, $3df$, $p < 0.005$, $N = 80$) (Fig. 2.10A) with many individuals showing a strong bias for one forelimb. Individual locusts differed significantly in the strength and direction of their bias (G -test, $G_{adj} = 42.38$, $7df$, $p < 0.005$, $N = 40$) (Fig. 2.10B) suggesting there was no consistent bias towards either direction within the population.

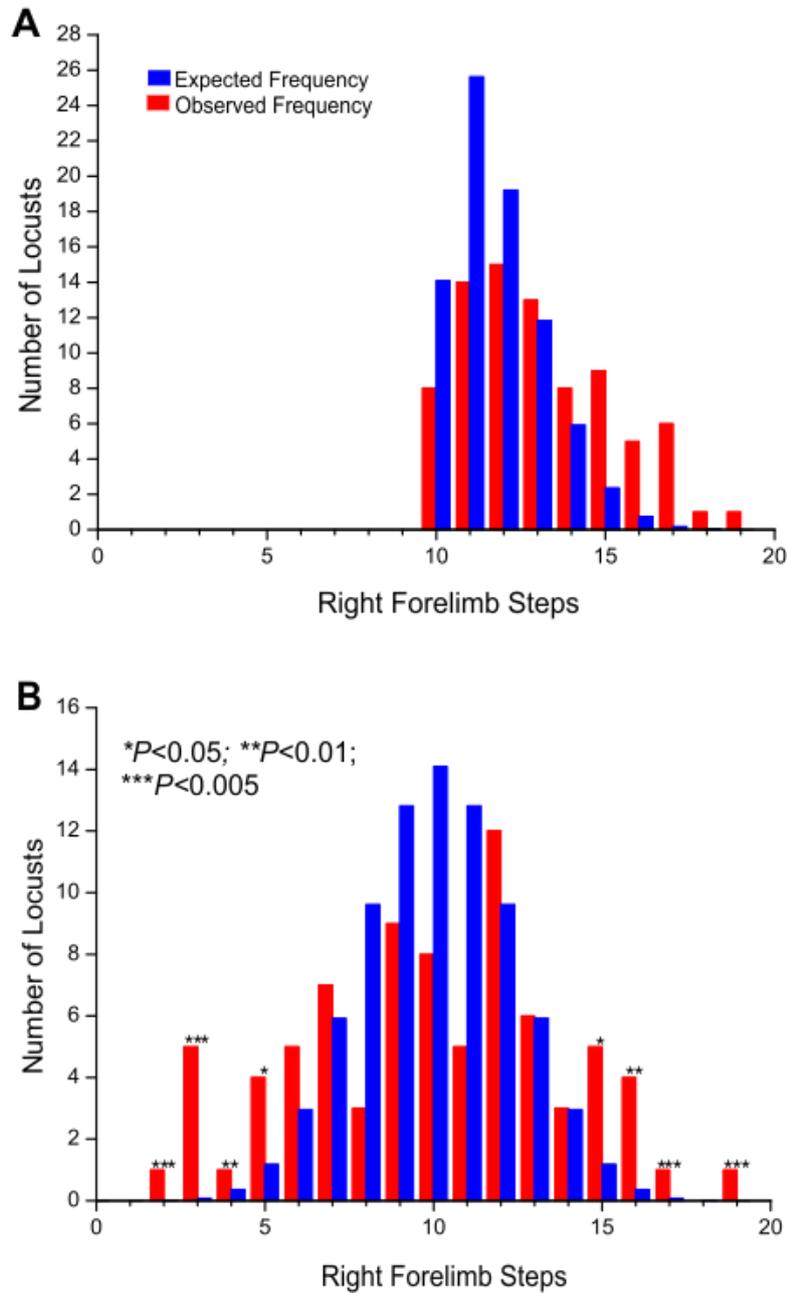


Figure 2.10. Limb preference in targeted forelimb movements during gap-crossing in 80 locusts. A. The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 80$, $n = 20$) (red). The binomial expectation of no preferred forelimb ($p = 0.5$) (blue) obtained by mirroring the binomial distribution to incorporate both left and right forelimb use. **B.** Frequency distributions of movements initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue). Asterisks indicate significant deviations from the binomial distribution determined by exact binomial tests.

2.4.6 Strong forelimb preference confers a direct advantage

Behavioural asymmetry may offer an advantage to an individual. To assess a possible advantage, we compared the performance of the strongly biased locusts (individuals that displayed a significant bias in the 2-tailed Exact Binomial test) with weakly biased locusts (individuals that did not display a significant preference in the 2-tailed Exact Binomial test) over the 20 gap-crossing trials. We amalgamated the data from the untreated (Table 2.6) and treated (Table 2.7) conditions to compare the reaching errors between 22 strongly bias locusts and 58 weakly biased locusts.

The performance of an individual was based on the accuracy of forelimb placement during gap-crossing. An accurate forelimb placement was observed when a locust reached for the opposite platform with its leading leg and made contact during the first attempt at placement without having to readjust the trajectory of the forelimb (Fig. 2.11). A reaching error was observed when a locust used its leading leg to make contact with the opposite platform edge, but undershot and therefore had to readjust before contact was made (Fig. 2.12). Individuals could accumulate multiple reaching errors per trial.

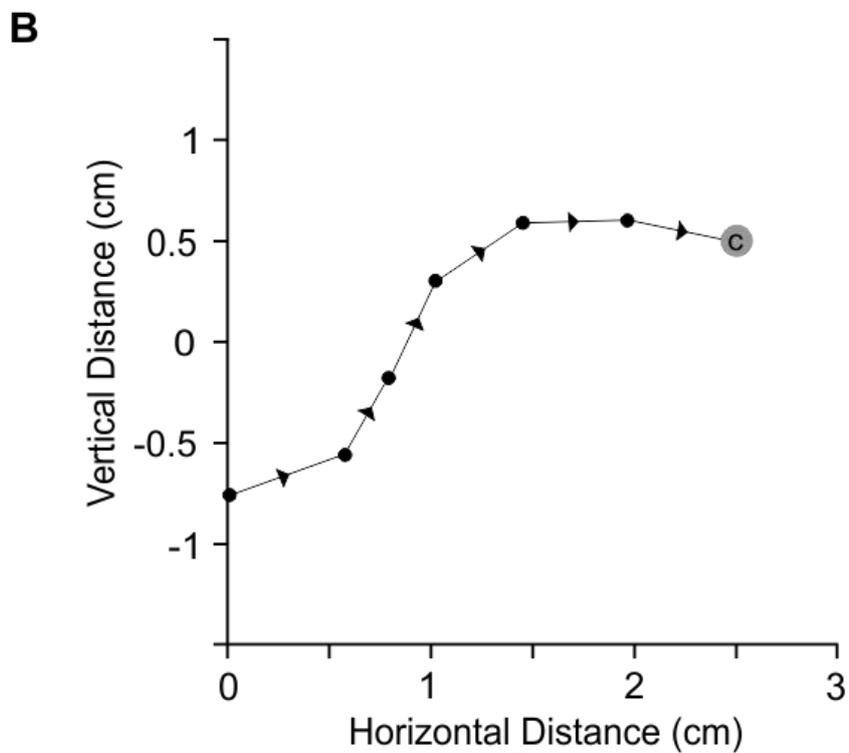
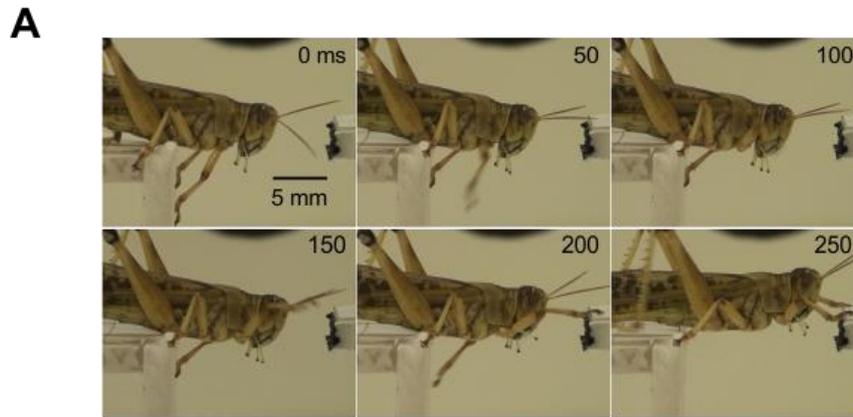


Figure 2.11. An accurate forelimb placement. **A.** A typical video sequence showing a gregarious locust making an accurate reaching attempt. **B.** The trajectory of the tibiotarsal joint during accurate forelimb placement. The forelimb is accurately aimed at the opposite platform edge during initial attempt without need for retargeting. Triangles represent the direction in which the forelimb is moving. The 'C' represents when contact is made with the opposite platform.

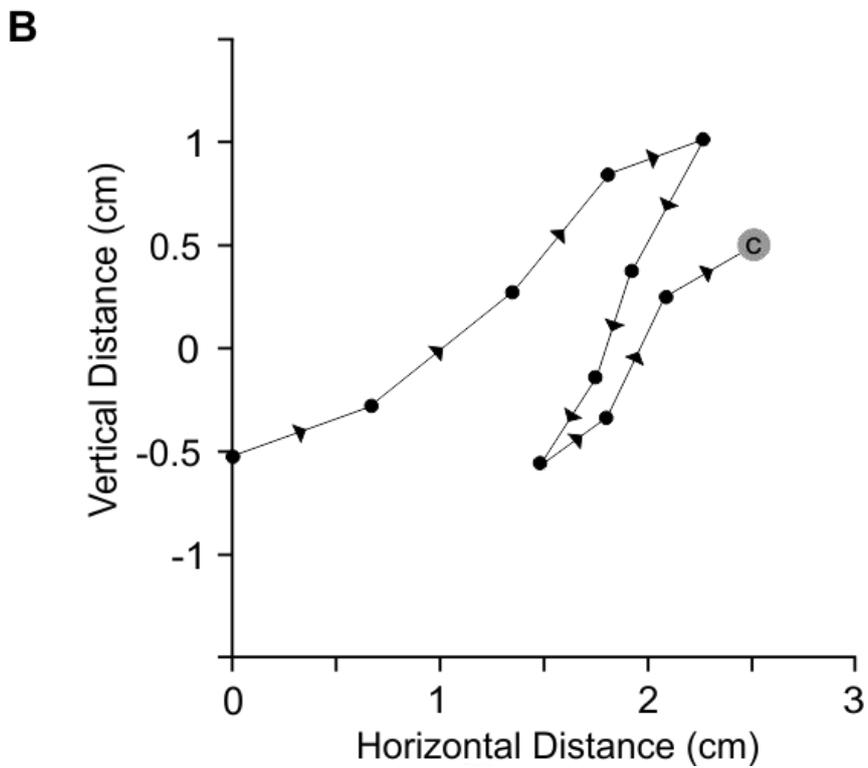
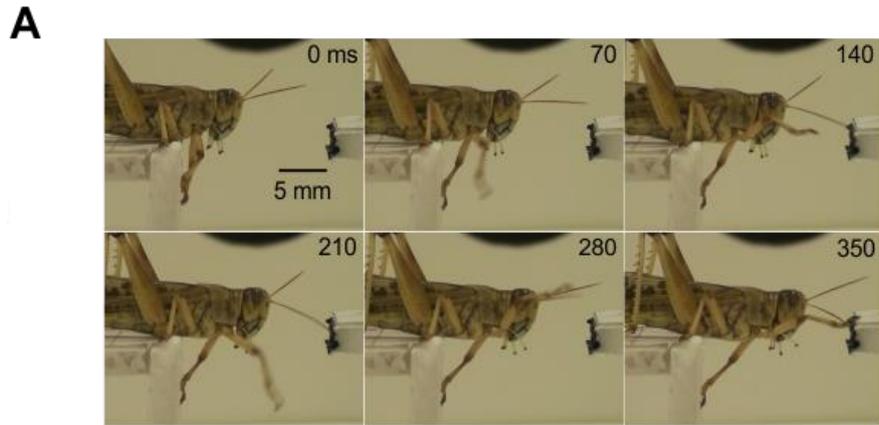


Figure 2.12. A reaching error. **A.** A typical video sequence showing a gregarious locust making a reaching error. **B.** The trajectory of the tibiotalar joint during the reaching error. The forelimb undershoots the opposite platform edge, producing an error, but is retargeted. Triangles represent the direction in which the forelimb is moving. The 'C' represents the contact made with the opposite platform.

To determine whether a strong bias conferred an advantage to an individual whilst gap-crossing, we compared the number of reaching errors between strongly and weakly biased locusts. We found a significantly larger number of reaching errors in weakly biased locusts (mean = 13.34, s.d. = 4.22, $N = 22$) compared to strongly biased locusts (mean = 8.77, s.d. = 3.84, $N = 58$; Independent samples t -test; $t = 4.43$, $78df$, $p < 0.005$; Fig. 2.13A). This suggests that being strongly biased improves the accuracy of forelimb placement and, consequently, provides an advantage during gap crossing.

We then determined whether the strength of the locusts' forelimb bias (see Methods) was correlated with the number of reaching errors they made. We assessed the relationship between the strength of bias and the number of reaching errors and found a significant negative correlation between the two (Spearman's rank-order correlation; $\rho = -0.404$, $N = 80$, $p < 0.005$, $78df$; Fig. 2.13B), which suggests that the strength of the bias is related to the accuracy of forelimb placement. This supports the previous result that a strong bias confers an advantage whilst gap-crossing.

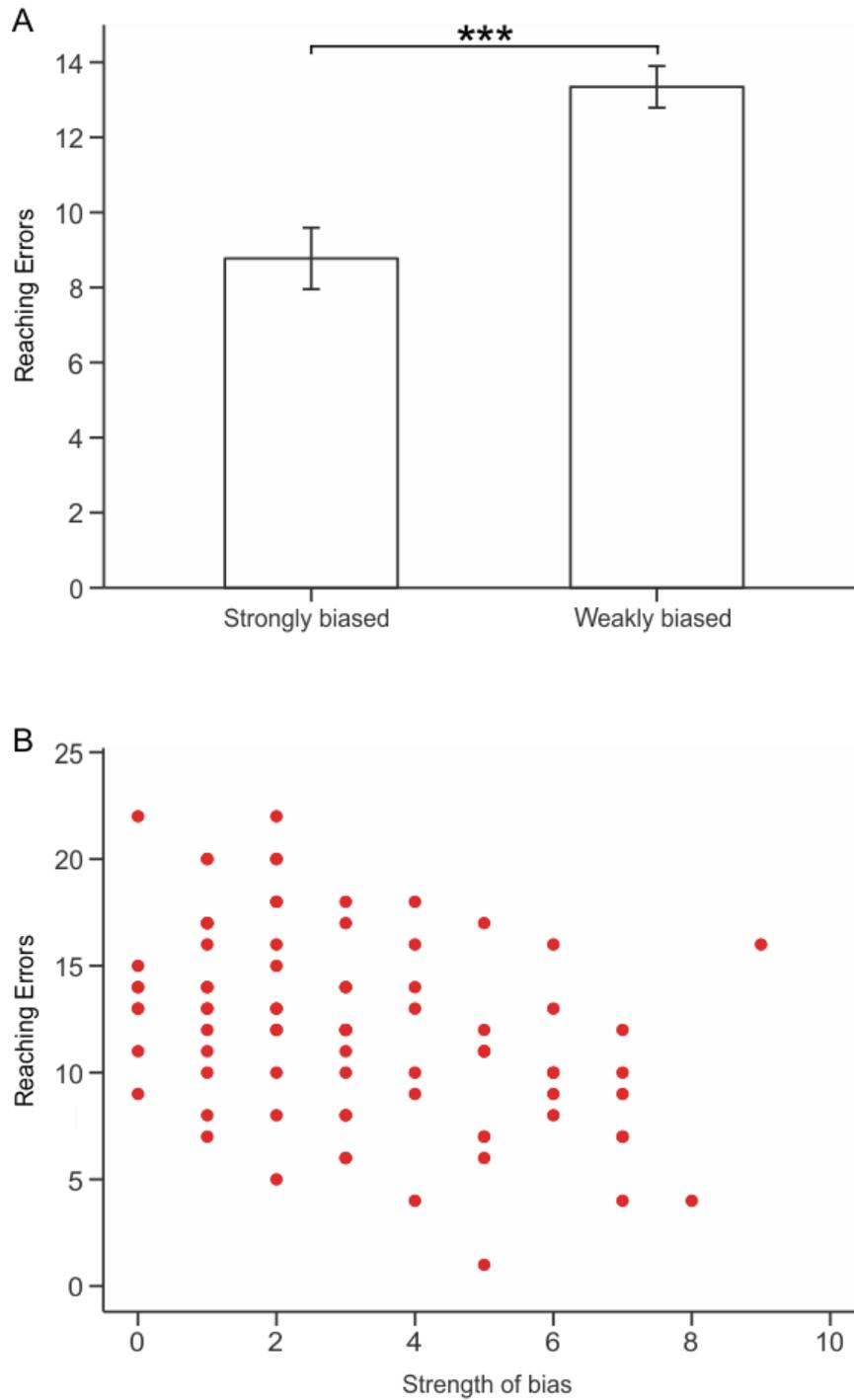


Figure 2.13. Strong forelimb preference is advantageous whilst gap-crossing. A. Strongly biased individuals make fewer reaching errors than weakly biased individuals whilst gap-crossing. Error bars indicate the standard error. ($N = 80$, $n = 20$) **B.** The strength of an individual's preference is inversely related to the number of reaching errors ($N = 80$, $n = 20$).

Over 20 trials locusts made on average 12.5 reaching errors. We plotted the distribution of reaching errors and compared it with the expected Poisson distribution, which assumes errors are randomly distributed between trials (Fig. 2.14). The distribution we observed did not deviate significantly from the Poisson expectation (G -test, $G_{adj} = 15.01$, 11 df , $p > 0.05$, $N = 80$, $n = 20$) (Fig. 2.14). This suggests that each trial was independent and that a reaching error in one trial is not dependent upon one in the previous or subsequent trials.

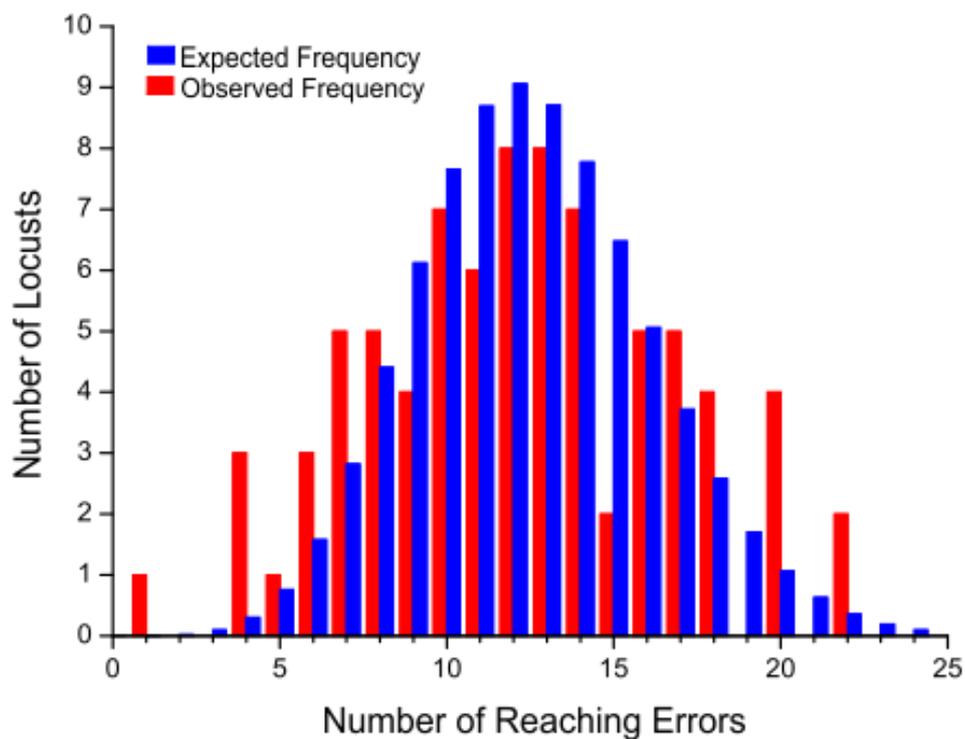


Figure 2.14. Frequency of locusts' reaching errors does not deviate from the expected Poisson distribution. Frequency distribution of reaching errors made by locusts ($N = 80$, $n = 20$) (red) compared with the expected Poisson distribution (blue).

We wanted to investigate whether the preferred forelimb made fewer reaching errors than the non-preferred forelimb during gap-crossing. Locusts that made 10 right forelimb gap-crosses (*i.e.* were unbiased) were removed from this analysis because they displayed no preference. We found no significant difference in the error rate between the non-preferred forelimb (mean 0.48, s.d. = 0.23, $N = 72$), and the preferred forelimb (mean = 0.48, s.d. = 0.19, $N = 72$); (Paired sample *t*-test; $t = 0.09$, 71 *df*, $p > 0.05$, Fig. 2.15).

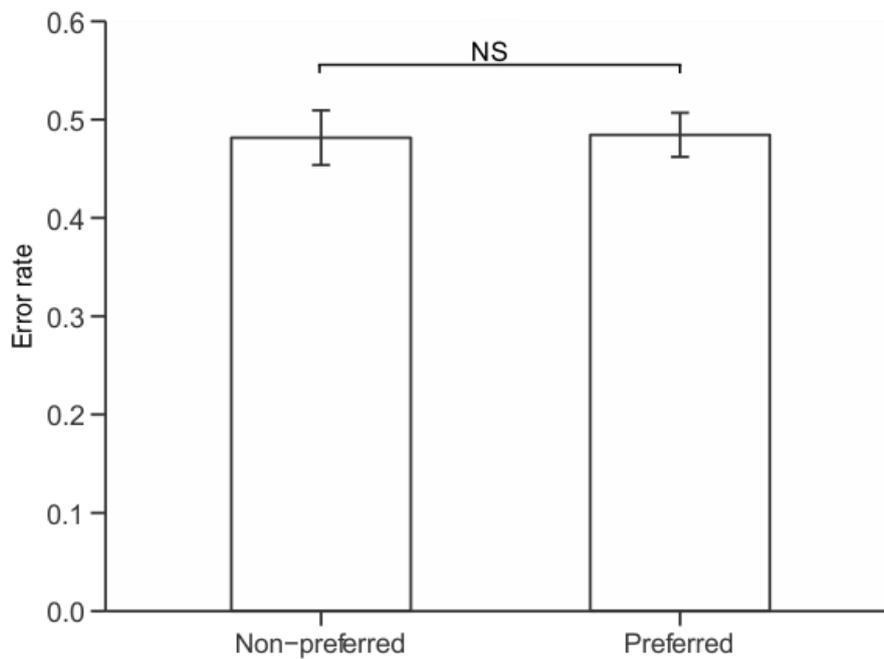


Figure 2.15. Locusts do not make more reaching errors with preferred forelimb. Bar chart to no difference in the error rate between preferred and non-preferred forelimb. Error bars indicate standard error ($N = 72$, $n = 20$).

We wanted to determine whether there was a relationship between the strength of preference and the error rate for both the preferred and non-preferred forelimb. We found a significant negative correlation between strength of bias and number of reaching errors in the preferred forelimb (Spearman's rank-order correlation; $\rho = -0.339$, $N = 72$, $p = 0.004$, $70df$; Fig. 2.16A). However, we found no correlation between strength of bias and number of reaching errors in the non-preferred forelimb (Spearman's rank-order correlation; $\rho = -0.153$, $N = 72$, $p > 0.05$, $70df$; Fig. 2.16B). This suggests that the strength of limb preference only affects the error rate of the preferred limb but not the non-preferred limb.

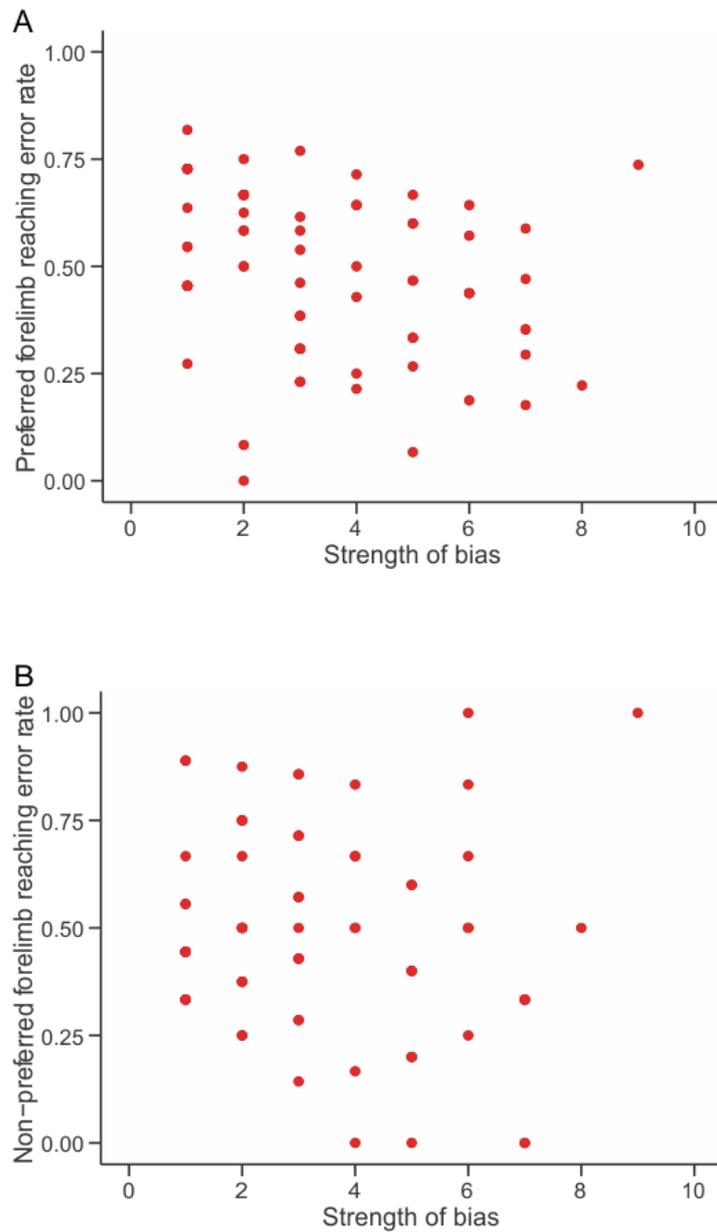


Figure 2.16. Strong forelimb preference is advantageous while gap-crossing. A. Scatterplot showing the relationship between strength of bias and error rate in preferred forelimb ($N = 72$, $n = 20$). **B.** Scatterplot showing the relationship between strength of bias and error rate in non-preferred forelimb ($N = 72$, $n = 20$).

2.5 Discussion

We used a gap-crossing paradigm to demonstrate that freely-moving desert locusts (*Schistocerca gregaria*) show a preferential use for one forelimb over the other whilst crossing a gap in the substrate they are walking. We find the forelimb preference varies within the population, with some individuals preferring to cross the gap with their right forelimb and some preferring to cross with their left. Furthermore, the strength of this bias differed among individuals. Moreover, we show that preferential leg usage confers a direct advantage to the individual whilst gap-crossing.

Arthropods have been shown to possess perceptual and motor asymmetries (Frasnelli *et al.*, 2012) but previous studies have either inferred asymmetrical limb use from circumstantial evidence (Heuts and Lambrechts, 1999; Rowell, 1964), focussed on asymmetrical limb use determined by morphological asymmetry (Govind, 1989), or reported biased forelimb use from single behavioural observations (Ades and Ramires, 2002). In this study, we used direct observation of a bilaterally symmetrical insect over repeated trials. Thus, to our knowledge this is the first direct evidence an insect, or indeed any arthropod, possessing individual-level limb preference in the use of otherwise symmetrical limbs.

In this study, locusts were reused to track the frequency of forelimb use enabling us to test the strength of an individual's preference. This produced the possibility that locusts were simply conditioned to cross with a particular limb because it previously proved successful, instead of a spontaneous decision making process. If a successful crossing was reinforced by multiple trials a motor memory could result. To counter this, each locust was tested once and then all other locusts were tested in the interval before the second trial. This strategy allowed a significant amount of time to elapse between trials reducing a possibility of a memory being formed from frequent reinforcement. Displaying the strength of individual limb preference is important because it supports the idea that preference should be reported on a continuous scale unlike many studies in which preference is generally reported as a discrete left or right.

It is conceivable that the asymmetric use of antennae or eyes of locusts could contribute to the asymmetric forelimb preference. Insects are capable of possessing

lateralisation of different sensory modalities (Frasnelli, 2012; Frasnelli *et al.*, 2013) but we are unaware of these existing within one species and, therefore, it is unknown how they might influence one another. Indeed, an important unknown is how many circuit asymmetries are present within an individual animal's nervous system. Even in *C. elegans*, in which the entire wiring diagram of the nervous system is known, the extent of asymmetry is unresolved. More work needs to be done to establish if locusts are capable of other forms of lateralisation and how they might influence forelimb preference. Nevertheless, it is insightful that these small nervous systems can exhibit a preference in their forelimbs, not dissimilar to that seen in other classes such as vertebrates.

We demonstrate that locusts possess a limb preference, however, much remains to be done if we are to understand how the circuit asymmetry arises and how it contributes to the behaviour. Although there is evidence that insect brains can contain structural asymmetries (Pascual *et al.*, 2004) within the literature there is often little attempt to relate underlying circuit asymmetry with cognitive or behavioural outputs indeed. Now we can move beyond correlative studies to look for casual links between the two by using the desert locust as a model for understanding the mechanistic basis of limb preference. With modern techniques, we can not only record from large cohorts of neurons but silence circuit components and study the consequence on associated left-right asymmetry in behaviour.

It has been suggested that handedness *per se* can only be inferred if the limb preference is shown to persist over time and context (Versace and Vallortigara, 2015). In this study locusts' limb preference fluctuated between tasks and, therefore, the behaviour does not fit with the Versace and Vallortigara (2015) definition of handedness. Further work needs to be done to establish that individual locusts' preferences persist over context. For example, is the same limb used to cross gaps as that used to grasp a food reward? We would also need to establish that the same limb is used preferentially over the locusts' lifetime. This could be done by recording the preference at juvenile stages, between moults and into adulthood. Nevertheless, the context-dependency of the locusts' limb preference is reminiscent of

aspects handedness in vertebrates, including humans, which is absent in some movements (e.g. walking) but pronounced in others (e.g. reaching) (Rogers *et al.*, 2013).

Theory predicts that animals living in groups should possess population-level lateralisation of some behaviours (Vallortigara and Rogers, 2005; Ghirlanda and Vallortigara, 2004). With no particular right or left forelimb bias, and variation in the strength of the bias, there was no evidence for population-level lateralisation in locusts. Instead, our results suggest the locusts' forelimb preference is equipotential, with an equal probability of an individual being either right or left biased. However, there is no conflict between the theory and our results because not all behaviours need be lateralised, and it is unlikely that forelimbs are used in locust social interactions. Consequently, it is unsurprising that the preference is not aligned with the majority of the population. In addition, although locusts congregate in large numbers in their gregarious phase they are not strictly considered social species. In fact, it has been demonstrated experimentally that when the locusts do form these large, co-ordinated congregations they are driven by cannibalistic interactions between one another (Bazazi *et al.*, 2008).

Locusts benefit from lateralised behaviour during gap-crossing through the accuracy of their forelimb placement. Although lateralised animals have been shown to outcompete non-lateralised animals in previous studies (Güntürkün *et al.*, 2009; McGrew and Marchant, 1999; Rogers *et al.*, 2004) these studies have all focussed upon vertebrates. Locusts' natural habitat consists of discontinuous food sources, which they must reach by traversing across foliage and uneven terrain. Our results may reflect the benefit of accurate forelimb placement in this context. This fits with the theory that being lateralised at the individual level does have advantages by reducing the computations involved in forelimb selection for targeted movements (Levy, 1977) and avoiding duplication of functions by saving neural tissue required for certain behaviours, though this does not explain the weak limb preference of some locusts.

Previous studies demonstrate that life experience can affect the development of lateralisation in vertebrates (Budaev and Andrew, 2009a,b; Maguire *et al.*, 2000; Mehlhorn *et al.*, 2010). To test whether a locusts' limb preference could be manipulated we occluded the

left eye of young 3rd instar locusts until adulthood then tested their preference to see if the occlusion induced the preferential use of the right forelimb. Our results suggest that the visual occlusion failed to produce an effect. It is possible that the forelimb preference cannot be modified through any environmental means, however, it is also possible it did not work for several other reasons: The occlusion may have been insufficient due to the ocular overlap, with locusts still being able to see the contralateral leg to aid placement; the antennae may play a larger role in accurate forelimb placement; the visual occlusion may also not have been implemented at a young enough age to have an effect; or the limb preference of juvenile locusts may be reset during moulting. Further work needs to be done to investigate the possible environmental effects on lateralisation at the individual level.

Despite the fact the insects evolved locomotion independent of vertebrates and share striking differences in the structure of their limbs our results show that they have converged on at least some functional solutions to the physical problems that face animals with versatile forelimbs.

Chapter 3

Colony-level, context-dependent forelimb preference in the red wood ant, *Formica rufa*

3.1 Abstract

Lateralisation in limb control has been well-documented across a wide range of vertebrate taxa, including humans who possess population-level lateralisation thought to have evolved in response to social living. In contrast to vertebrates, there has been relatively little research on lateralisation in invertebrates. Individual-level lateralisation, in which individuals within the population vary in their preference, has been documented in the desert locust (*Schistocerca gregaria*), which swarms but is not considered social. The red wood ant (*Formica rufa*) lives in social groups providing an opportunity to study the possible effects of social factors on the evolution of lateralisation in insects. Whilst crossing a gap in the substrate upon which they are walking ants, like locusts, use their forelimbs to reach across and contact the opposite side. We investigated whether they displayed a preference for using a particular forelimb. In this context, some individual ants preferred to use their right forelimb, others their left and the remainder showing no preference – the hallmark of individual-level lateralisation. Remarkably, the preference differed between colonies, the majority of individuals within a colony showing a preference for using the same forelimb to cross the gap. Thus, wood ants exhibit two forms of lateralisation at the individual and colony-levels the latter being an entirely novel, previously undescribed form of lateralisation.

3.2 Introduction

Lateralisation of brain and behaviour is ubiquitous among vertebrate classes (Vallortigara and Rogers, 2005; Vallortigara *et al.*, 2011), with more recent research showing that it may also be pervasive among invertebrates (Frasnelli *et al.*, 2012; Frasnelli, 2013), suggesting that it

contributes significantly to biological fitness. We previously demonstrate that strongly lateralised locusts make fewer motor errors than their weakly lateralised counterparts (Bell and Niven, 2014). Indeed, lateralised animals have been shown to outperform non-lateralised animals in various scenarios (Güntürkün *et al.*, 2009; McGrew and Marchant, 1999; Rogers *et al.*, 2004). Although the actual mechanisms are unknown, there is consensus on several advantages of having a lateralised brain: the production and maintenance of neural tissue is spared by avoiding duplication of functions in the two hemispheres (Levy, 1977); information can be processed in parallel (Rogers 2002; Rogers *et al.*, 2004); and prevention of simultaneous initiation of incompatible responses by allowing one hemisphere to have control over actions (especially in animals with laterally placed sensory organs; Andrew, 1991; Vallortigara, 2000).

Despite these compelling arguments for the advantages of lateralised brain, a caveat excludes them from explaining one intriguing aspect of lateralisation. The population structure of lateralised behaviour, i.e. the frequency distribution of individuals with lateral biases within the population, is traditionally split into one of two categories; population-level asymmetry wherein more than half of the population display the same direction of asymmetry (see General Introduction, Fig. 1.3B) or alternatively, individual-level asymmetry in which the frequency of left/right biased individuals within the population approximates an equal proportion (see General Introduction, Fig. 1.3C). Because individual efficiency does not require alignment of lateralisation at the population-level the above arguments do not help to explain the existence of population-level lateralisation.

Computational modelling based on a game theoretical approach has been used to explain the alignment of an individual's lateralisation with that of others in the population (Ghirlanda and Vallortigara, 2004; Vallortigara and Rogers, 2005; Ghirlanda *et al.*, 2009). It suggests population-level lateralisation may have arisen as an evolutionarily stable strategy (ESS; Maynard-Smith, 1982) in which individually lateralised organisms must coordinate their behaviour with that of other lateralised organisms in the population. This arrangement assumes that there are associated frequency-dependent costs and benefits to the individual

(see General introduction). If an individual is lateralised in the same direction as the rest of its group an advantage may be gained. For example, if an individual can predict the behaviour of a conspecific, say whilst escaping from a predator, staying with the group may be advantageous. However, the predictability of such behaviour may be disadvantageous because it can be exploited by predators (see General Introduction, Fig. 1.4). Furthermore, the minority of individuals lateralised in the other direction may incur an advantage by having less predictable behaviour during confrontations with conspecifics.

The suggestion that population-level lateralisation evolved as an ESS led to the hypothesis that it is found in social, cooperative species due to interactions with conspecifics (Ghirlanda and Vallortigara, 2004; Anfora *et al.*, 2010). According to this hypothesis, the evolution of population-level lateralisation is a two-step process. It begins with individuals evolving lateralisation because of the associated advantages to the individual. This is followed by the alignment of lateralisation with the population when the behaviour is relevant to others. Research supports the idea that population-level lateralisation may have arisen in social animals. Humans and chimps, considered social animals, preferentially use their right hand in particular tasks (McManus and Bryden, 1992; Hopkins *et al.* 2011), seven marsupials have demonstrated left handedness at the population-level (Giljov, 2015), and 20 species of shoaling fish show a turning bias for predator evasion (Bisazza *et al.*, 2000). The proportions of individuals that display the same direction of bias can vary between 90%, as is the case in humans (McManus, 2002), to 35-40% in, for example, toads (Bisazza *et al.*, 1996).

Experimental evidence suggests that insects too are capable of displaying different modalities of lateralisation (Frasnelli *et al.*, 2012; Frasnelli, 2013). Eusociality is the most advanced form of social organisation in the animal kingdom and is found in many insect species (Hölldobler and Wilson, 1990). Eusocial animals have three defining features: the cooperative care of the young from more individuals than just the mother, specialised sterile castes for a range of behaviours, including reproduction, and overlapping generations of young to provide an opportunity for the young to help with their sibling rearing (Wilson, 1971; Hölldobler and Wilson, 1990). It has been hypothesised that population-level lateralisation is

more likely to exist in social species (Ghirlanda and Vallortigara, 2004; Anfora, 2010) therefore eusocial insects such as bees, wasps and ants, exhibiting an extreme form of sociality provide an opportunity to test this hypothesis. We have demonstrated that locusts exhibit individual-level lateralisation in the form of limb preference (Bell and Niven 2014, 2016) however locusts are not strictly considered social and therefore can't be used to directly test the ESS hypothesis alone.

Red wood ants (*Formica rufa*; Linnaeus, 1761) are a species of ant native to the Northern Europe, extending into southern parts of the U.K. (Hölldobler and Wilson, 1990). Being eusocial, red wood ants provide a great opportunity to test the ESS theory of population-level lateralisation (Ghirlanda and Vallortigara, 2004; Anfora, 2010) and investigate the evolution of population-level lateralisation. In addition, with bilateral symmetry and a relatively simple neural system, compared to the numbers of neurons that exist in vertebrate neural systems (Chitkka and Niven, 2009), they are a suitable model for research into lateralisation of behaviour. In the present study, we use red wood ants to test for a behavioural lateralisation in the form of limb preference. We use the same gap-crossing paradigm as in the previous chapter to permit direct comparison with the results of the desert locusts. Ants must cross the gap in the substrate upon which they are walking by using a particular forelimb. We test for behavioural lateralisation at the individual level and determine whether it relates to the alignment of other colony members, and the colony as a whole. We then compare these preferences between colonies.

3.3 Materials and Methods

3.3.1 Animals

Four red wood ant (*Formica rufa*) (Linnaeus, 1761) nests were removed from Broadstone Warren Scout Camp, Ashdown Forest between July and August 2014. Prior to removal, each colony was located within 30 m² of open meadow in which wood ants forage. Each nest was maintained in separate holding tanks (140 x 80 x 60 cm, 24°C) at the School of Life Sciences, University of Sussex, U.K. They were fed *ad libitum* on a diet of sugar water (3:1), crickets and water. When not being trained or tested the wood ants remained in these holding tanks.

Ants were selected at random from foragers present on the surface of the nest. Foragers with damage to their eyes, limbs or antennae were discarded. Ants were also not used if they showed any signs of lethargy, which is indicative senescence. All the ants selected were relatively large and of similar size. Ants were marked using various colours and combinations of paint (Testors Pactra®) allowing them to be individually identified throughout training and testing. A dot of paint was placed on each side of the thorax to assist in determining which forelimb was used to cross the gap. After individually marking each ant they were placed in isolation for several minutes to allow the paint to dry and avoid attack from conspecifics (due to masking of cuticular hydrocarbons). Ants were then released back in the nest for 24 hours before training commenced.

3.3.2 Experimental arena

Ants were tested at 23°C in a rectangular, white Perspex® arena measuring 800 x 600 x 600 mm (Fig. 3.1A). A direct, overhead light source was used to illuminate the arena whilst avoiding any directional asymmetry. The light source also had a large surface area to reduce shadow sharpness within the arena. A 'peephole' 60 mm in diameter was cut in the arena wall at a height of 60 mm from the base to admit a 50 mm diameter camera lens with which the ants were filmed. An identical hole was cut into the opposite wall of the arena into which a black disk was inserted to maintain symmetry. This was the same arena set-up as used in the previous chapter.

Two horizontal platforms (150 x 50 x 15 mm) constructed from 5 mm thick Perspex® were placed in the centre of the arena (Fig. 3.1B). These platforms were modified from the previous chapter, being narrower and with a smaller gap width. The platforms were placed opposite one another separated by 8 mm creating a gap consisting of two 'diving board' faces. The size and shape of the gap was determined from pilot studies that investigated the propensity of ants to cross gaps (section 3.3.3). The platform to which each ant crossed was elevated by 5 mm from the platform on which they were initially placed. At one end of the lower platform was a plastic releasing chamber. The chamber functioned as a standardised starting point for each ant. From here they could pass through a rectangular opening at the bottom of the chamber, allowing them to exit and walk along the platform until they encountered the 1 mm gap. The horizontal surface of each platform was covered with white paper to enhance the grip for walking and to block any reflected light off the Perspex surface. The paper was marked with fine horizontal lines. The edge of the elevated platform was marked with black acrylic paint. A rectangle of black card (60 x 250 mm) was placed vertically at the end of this platform to act as a landmark, which helped the ants navigate towards the food reward (Fig. 3.1B).

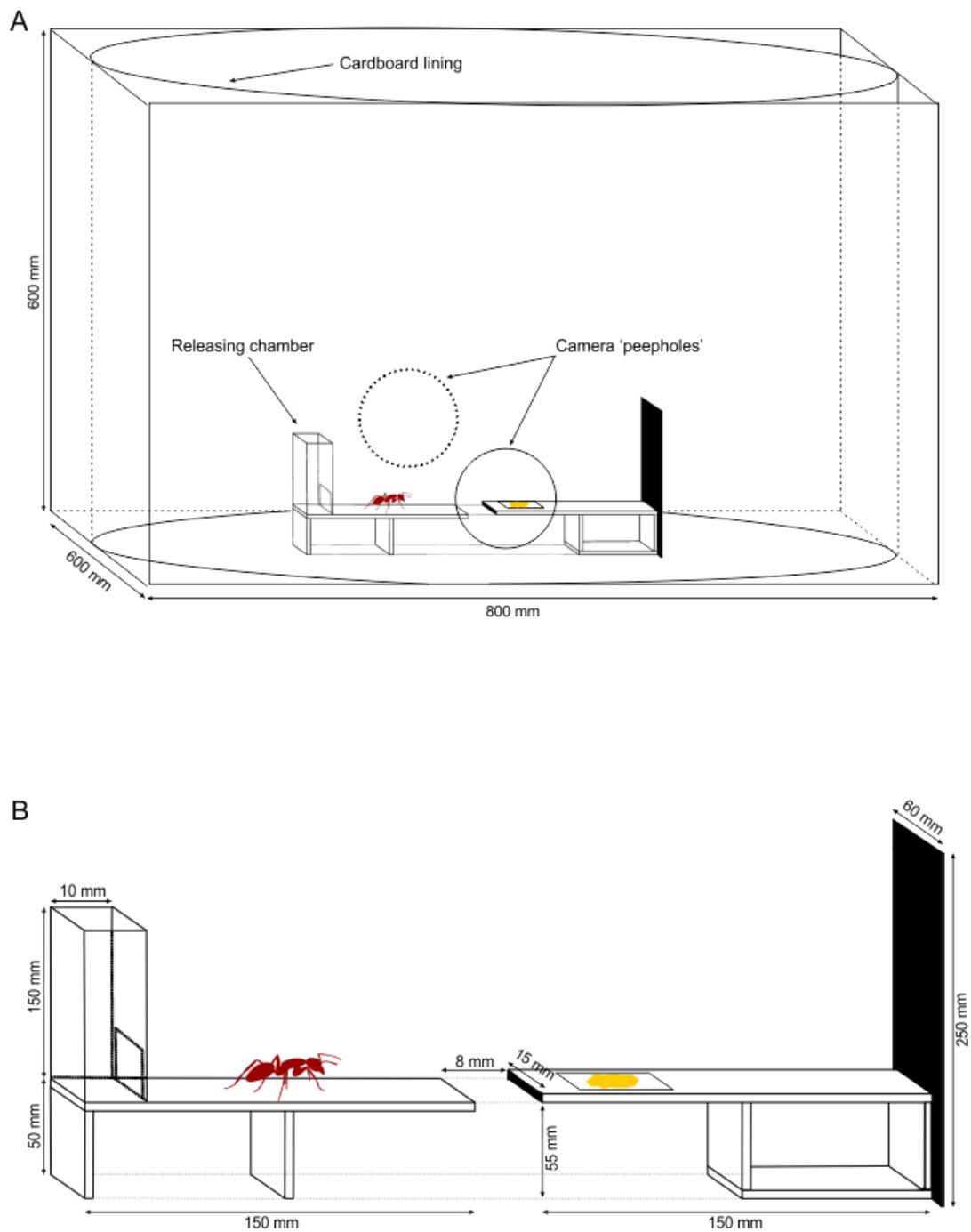


Figure 3.1. Arena and platforms used for training and testing ant limb preference. A. Ants were tested in a white, symmetrical arena. **B.** Individual ants crossed from one platform to the other to achieve a food reward.

3.3.3 Gap width determination

It was important to ascertain a gap width that was sufficiently large enough to simulate a demanding task, whilst remaining small enough to supply an efficient number of crosses in a limited time. We used a cohort of 10 ants in order to establish such gap widths. Six gap widths were investigated ranging from 5 to 10 mm in increments of 1 mm. Each day the ants crossed each of the six gap widths 10 times in a randomised manner. Individual ants were tracked over 10 days during which each ant crossed each gap a total of 10 times.

3.3.4 Determining the preferred crossing strategy

Ants can successfully cross a gap by contacting the opposite platform with one forelimb whilst using their other limbs remain in contact with the platform from which they are crossing, acting as a support or an anchor. Alternatively, they place both forelimbs into the gap, waving them until contact is made with the opposite platform, whilst supporting the body with the remaining four legs. The same as the previous chapter, this chapter uses N to refer to the number of ants used in each experiment and n to refer to the number of trials per ant. To assess the preferred crossing strategy, we tested a second cohort of 10 ants over 10 trials ($N = 10$, $n = 10$) in which they crossed over a gap width of 8 mm and another gap width of 10 mm, and then compared the strategy implemented over each.

3.3.5 Training

Although wood ants walk over uneven ground in their natural habitat, the arena-based, gap-crossing paradigm is out of context for them. Consequently, when presented with this novel apparatus the frequency with which foraging wood ants crossed the gap was initially low. To increase the number of successful crossings, the ants were trained using a simple reinforcement paradigm.

Ants were fed less than their usual allocation of sugar water to motivate them to search for food. Upon crossing the gap, they obtained a reward of sugar water, encouraging them to cross the gap in future trials. Once training had started ants were kept in 'starved conditions'

to maintain motivation. Some ants never explored the entire platform, and a small number of ants fell off the side of the platform in many trials, preventing them from completing a gap-cross during training. These ants were discarded.

During each training event an ant was always alone within the arena. To avoid handling the ants, a straw with a small, square piece of paper attached at one end was used to remove the ants from a container and transfer them to the plastic releasing chamber at one end of the starting platform.

Ants from each colony were trained and tested consecutively. Batches of 30 ants were trained before being tested. Training continued until sufficient numbers of ants crossed to the food source in less than approximately 2 minutes.

3.3.6 Testing

Testing took place between January 2014 and March 2015 using the same apparatus and procedure as training, and was carried out in the same room at the same temperature. A cohort of trained ants from each colony were tested. For the limb preference experiment 50 ants completed 10 gap-crosses from colonies # 1 - 3 ($N = 50$, $n = 10$) and 46 ants completed 8 gap crosses from colony 4 ($N = 46$, $n = 8$). When referring to these colonies I will use the following: colony 1 = C#1, colony 2 = C#2, colony 3 = C#3 and colony 4 = C#4. The trained ants were removed from their nest and placed in a starting enclosure. For each trial, one ant was removed and placed in the releasing chamber. Filming began when the ant emerged from the releasing chamber was approaching the gap and continued until it crossed to the opposite platform. After ten seconds of drinking from the sugar water solution, the ant was removed and placed back in the starting enclosure. Trials in which ants did not initiate any gap-crossing behaviour were counted as refusals and were omitted from analysis. Individual ants were tested multiple times during the experiment. Once 10 gap-crossings had been filmed for 50 ants from each colony testing stopped (excluding ants from colony 4 in which 46 ants did 8 such crossings). Ants were tested over multiple days. Two final year undergraduate students

assisted with data collection for this chapter: Jamie Sneddon collected data from 30 ants in colony #1 in 2014 and Johnathan Stone collected data from 46 ants for colony #4 in 2015.

If during testing ants were unresponsive or showed a lack of motivation to cross the gap, they were placed in a separate container that held a randomly selected naïve group of hungry ants from the same colony. The ant being tested could then off-load any ingested sugar water to the hungry ants through trophallaxis (Frasnelli *et al.*, 2012a). This procedure ensured the trained ants remained motivated to cross the gap towards the food reward throughout testing.

The platforms in the arena were rotated 180° when the ants had completed half of their gap-crosses, therefore, half of their trials were left to right and the other half right to left, reversing the polarity of any directional environmental cues or remaining asymmetries within the arena.

3.3.7 Anatomical correlates of behavioural asymmetry

Behavioural biases may arise from morphological asymmetry such as the difference in leg or antennal length. To determine whether this was the case in wood ants we measured and compared forelimb femur length (as a proxy for leg length) and scape length (as a proxy of antennal length) of 20 ants from each colony ($N = 80$) using digital callipers (Mitutoyo Absolute IP67). Legs were removed at the coxa, and the ant's head was severed from the body. Legs, head and body were placed on a glass slide and fixed in position with the aid of petroleum jelly (Vaseline®).



Figure 3.2. Preparation for the measurement of the forelimbs and antennae of the red wood ant. Eighty ants were tested for morphological asymmetries. The legs, head and antennae were removed from the body. The lengths of the left and right femurs from the forelimbs and the left and right antennal scape were measured and compared. A representative preparation is shown.

3.3.8 Assigning strength of preference

The strength of forelimb bias was based on preferred forelimb usage; 0 was assigned to ants that crossed with their right forelimb in 5 out of 10 trials. A score of 1 was assigned to ants that crossed with their right forelimb in 4 or 6 trials, a score of 2 for right forelimb crossings in 3 or 7 trials, a score of 3 for right forelimb crossings in 2 or 8 trials, a score of 4 for right forelimb crossings in 1 or 9 trials and a score of 5 for right forelimb crossings in 0 or 10 trials.

3.3.9 Video analysis

The videos of the gap crossing trials were recorded at 200 Hz using a high-speed video camera (JVC, GC-PX100) positioned parallel to the horizontal plane of the gap (Fig. 3.1A). Videos of images with a resolution of 640 x 360 pixels were saved directly to a computer for later analysis offline.

3.3.10 Statistical analysis

A Spearman's rank-order correlation was used to measure the strength of association between antennal contacts and gap-crossing behaviour. It was also used to measure the association between the strength of forelimb preference and crossing latency. To test for differences in number of single limbed crossing between the 8 mm and 10 mm gap widths, a Wilcoxon signed-rank test was used for the two related ant cohorts. A dependent samples *t*-test was used to compare differences between left and right antennae and femur lengths within individuals. To compare the effect of colony on the number of right forelimb crossings we used a Kruskal-Wallis test. Mann-Whitney U tests on each pair of groups and adjusted *p*-values using the Bonferroni method for multiple comparisons were used for *post-hoc* analysis. The following statistical tests were parametric because latency, scape and femur length were Normally distributed and had equal variances between the groups. A one-way analysis of variance (ANOVA) was used to determine whether there were any significant differences between the means of the four colonies for crossing latency, scape and femur length. The software R (version 3.0.2) was used for these statistical analyses (R Development Core Team, www.cran.r-project.org). An LI score was also calculated for each individual and where appropriate, a MLI score was calculated to assess colony-level preference (see Materials and methods, Chapter 2).

When testing for intra-colony variation in forelimb preference, the deviation of each ant from the expected binomial distribution ($p = 0.5$) was calculated using an Exact Binomial Test using the method of small *p*-values. The distribution of the observed number of forelimb reaches performed by each ant was tested for deviation from the expected distribution, which

was calculated from the binomial distribution assuming no forelimb preference ($p = 0.5$). Any classes in which the expected numbers were < 3 were amalgamated with the adjacent classes to produce expected numbers of the appropriate size. The degrees of freedom were adjusted to account for the amalgamation of classes, and for the use of an intrinsic hypothesis. A single-classification G-test for goodness of fit to the intrinsic hypothesis was used with William's adjustment (G_{adj}) applied (Sokal and Rohlf, 1997). This statistical analysis was used to test for the deviation of each distribution from the binomial expectation. These statistical tests were carried out using custom written programs in Excel (Microsoft). All tests were two-tailed.

3.4 Results

3.4.1 Gap width affects behaviour

Upon encountering the gap at the end of an approach, ants perform one of three behaviours depending on the size of the gap. Firstly, upon encountering the gap they may make no initiation to cross (*no initiation*). In this case, their forelimbs enter the gap before they probe the gap with their antennae, which often contact the opposite platform. No further action is initiated, however, and they make no attempt to cross (Fig. 3.3A). Secondly, upon encountering the gap they attempt to cross but abandon gap-crossing (*attempt*). In this case, their forelimbs enter the gap before they probe the gap with their antennae, which often contact the opposite platform. One or both forelimb/s is/are then targeted towards the opposite platform by raising it towards the opposite platform edge but failing to contact. The ant then terminates the attempt and abandons the crossing, remaining on the starting platform (Fig. 3.3B). Thirdly, upon encountering the gap they make a successful gap-crossing (*success*). In this case, their forelimbs enter the gap before they probe the gap with their antennae, which often make contact with the opposite platform. A forelimb is then targeted towards the opposite platform by raising it towards the opposite platform edge. Contact is made and the forelimb/s is/are used to hoist the rest of their body up onto the opposite platform (Fig. 3.3C).

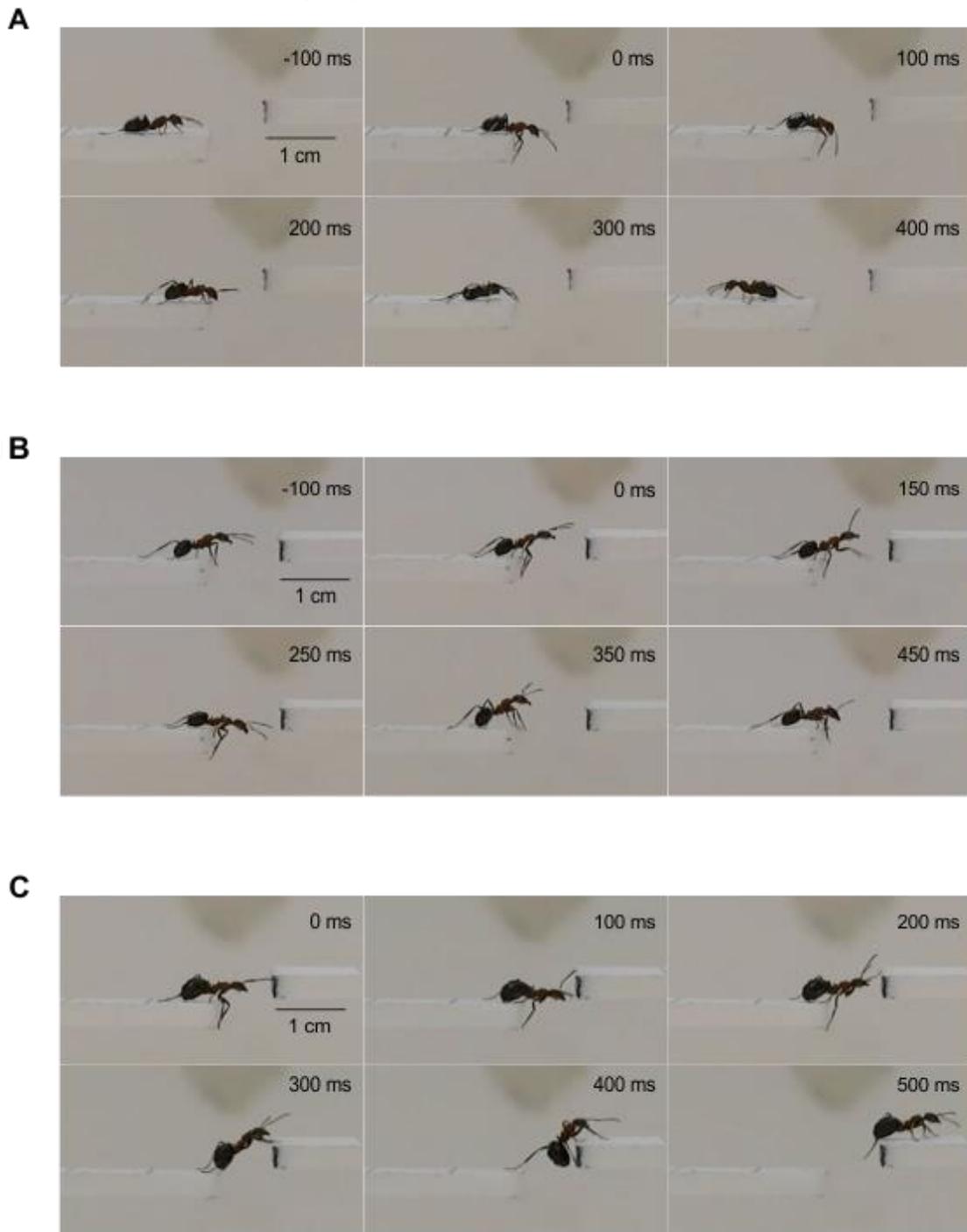


Figure 3.3. Red wood ant behavioural repertoire upon encountering a gap. A. Upon encountering the gap the ant makes no initiation to cross (*no initiation*). **B.** Upon encountering the gap the ant attempts to cross but abandons the gap-cross (*attempt*). **C.** Upon encountering the gap the ant makes a successful gap-crossing (*success*).

When the gap size between the platforms was increased, the ant's ability to cross successfully decreased (Fig. 3.4). At the smallest gaps, just 5 mm wide, 93% of trials were *successes* (green). This percentage decreased as gap width increased until only 2% of trials resulted in a successful crossing at the largest gap width of 10 mm. *Successes* outnumbered *no initiations* (red) and *attempts* (yellow) around 8 mm, before dropping below both of these behaviours at 9 mm. As the gap size increased, more ants unsuccessfully attempted to cross until the gap was 10 mm at which point the number of *attempts* dropped to 7%. The gap width closest to the point at which the percentage of *successes* exceeded the percentage of *no initiations* (red) was chosen for the testing, in this case 8 mm, where 44% of trials resulted in *successes*.

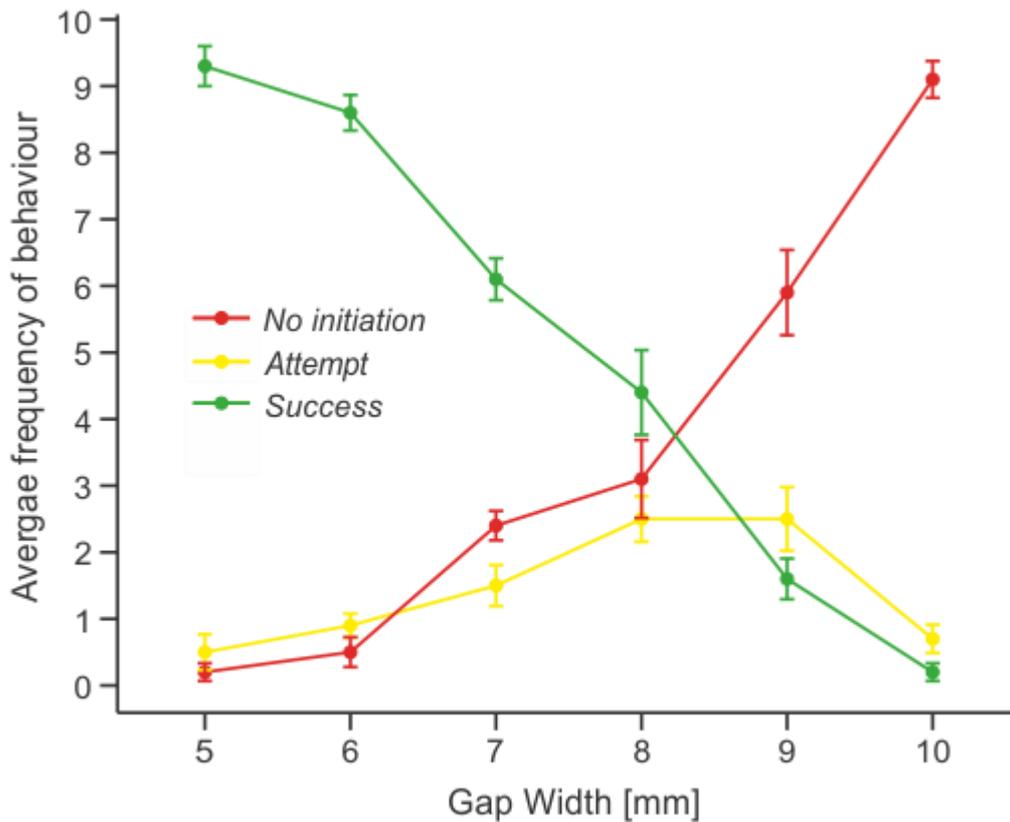


Figure 3.4. Behavioural repertoire of the red wood ant over increasing gap width. As the gap size increases the number of *successes* decrease (green line). As the gap size increases the number of *no initiations* increase (red line). As the gap size increases the number of *attempts* increase until plateauing between 8 mm and 9 mm after which point they decrease until 10 mm (yellow line). Solid circles represent the average frequency of behaviour and the error bars indicate the standard error of the mean (s.e.m).

The patterns of behaviour described above are from all ants taken together, however, we can also see whether they reflect the performance of individual ants (Fig. 3.5). An individual ant's ability to successfully cross a gap decreases smoothly (Fig. 3.5A) as opposed to reaching a gap width at which the success rate sharply declines. All ants tested here appear to conform to this pattern. The frequency of gap-crossing *attempts* generally lies below 25% (Fig 3.5B), except for one individual (#6) that made more *attempts* at the 9 mm gap. Nevertheless, the

ants generally exhibit similar frequency of *attempts* over increasing gap widths. A similar but inverse pattern occurs for the number of *no initiations* (Fig. 3.5C) as that of *successful* crossings. Ants generally display similar behaviour, as the gap width increases the number of *no initiations* smoothly declines.

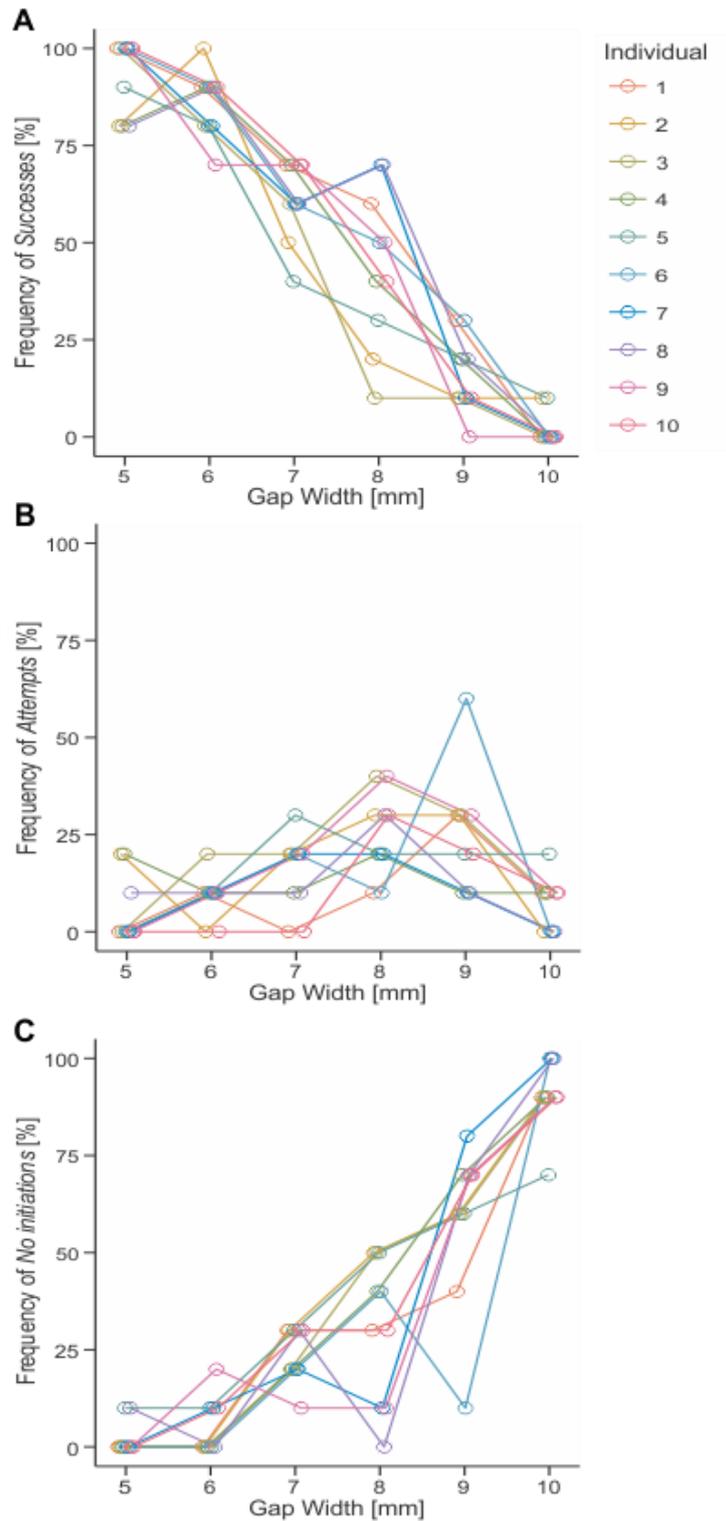


Figure 3.5. Individual variation of behavioural repertoire over increasing gap width. Graphs representing individual variation over increasing gap widths of **A. Successes** **B. Attempts** **C. No initiations**.

3.4.2 Antennal contact influences gap-crossing behaviour

We investigated the contribution of antennal contact prior to each behaviour upon encountering a gap (Fig. 3.6A). Antennal contact was defined as touching the opposite platform with one or both antennae prior to gap-crossing. We assessed the relationship between the number of *successes* and the number of crossings in which contact was made. We found a strong positive correlation between the two (Spearman's rank-order correlation; $\rho = 1$, $N = 12$, $p < 0.001$, $4df$; Fig. 3.6B), which suggests that the number of *successes* is strongly influenced by the number of antennal contacts made before crossing.

We then assessed the relationship between the number of *attempts* and the number of crossings on which contact was made. We found no correlation between the two (Spearman's rank-order correlation; $\rho = -0.041$, $N = 12$, $p > 0.05$, $4df$; Fig. 3.6C), which suggests that the number of *attempts* is unrelated to the number of antennal contacts before crossing.

We then assessed the relationship between the number of *no initiations* and the number of crossings on which contact was made. We found a strong negative correlation between the two (Spearman's rank-order correlation; $\rho = -1$, $N = 12$, $p < 0.001$, $4df$; Fig. 3.6D), which suggests that the number of *no initiations* is strongly, inversely related to the number of antennal contacts before crossing. Taken together these results indicate that antennal contact plays an extremely important role in the ant's ability to cross gaps; when no antennal contact is made with the opposite platform the ant is extremely unlikely to make a successful cross.

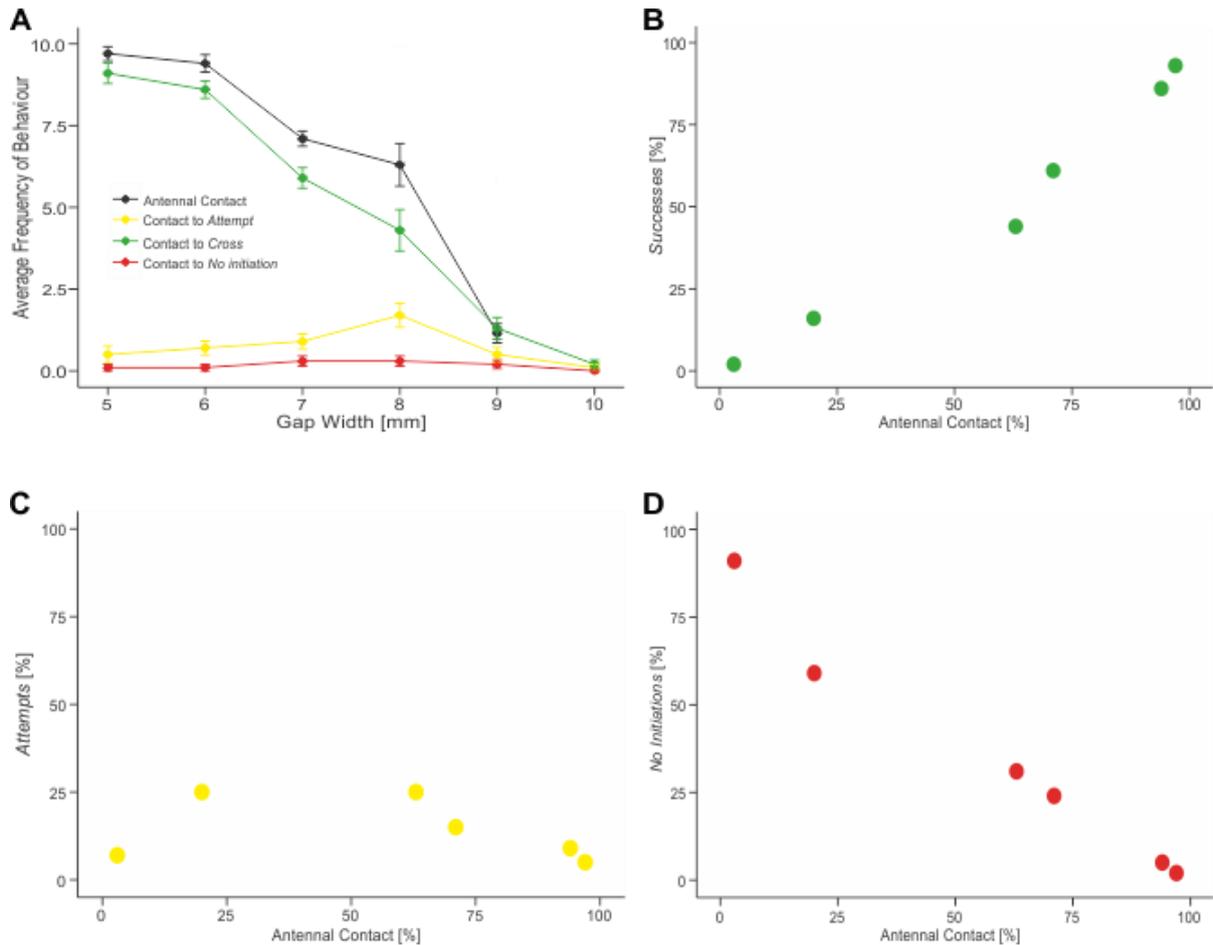


Figure 3.6. Antennal contact influences gap-crossing behaviour. **A.** Line graph showing the contribution of antennal contact prior to each behaviour upon encountering a gap. Solid circles represent average frequency of behaviour. Error bars represent standard error of the mean (s.e.m). **B.** Scatterplot showing the relationship the number of *successes* and antennal contacts. **C.** Scatterplot showing the relationship the number of *attempts* and antennal contacts. **D.** Scatterplot showing the relationship the number of *no initiations* and antennal contacts. All gap widths are 8 mm.

The sequence of behaviours that lead to the three gap crossing strategies can be separated into small sequences, which do not overlap temporally (Fig. 3.7), showing the influence of the antennal contact on the outcome of the trial. We found that, at the 8 mm gap-width, all individuals approached the gap after which a proportion of 0.63 made antennal contact with one or both antennae on the opposite platform. Most of these ants (0.43) went on to make a successful gap cross. In comparison, ants that made no antennal contact (0.37), only 1 (0.01) then made a successful cross, with most (0.28) making no initiation to cross.

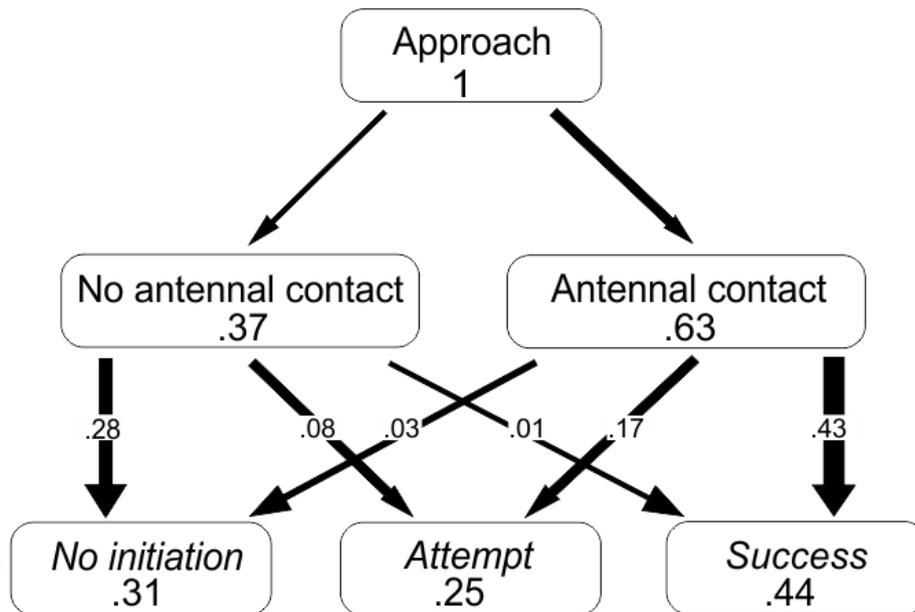


Figure 3.7. Ethogram of red wood ant behaviour upon encountering an 8 mm gap. The boxes represent the behaviour, the numbers within the boxes representing the overall proportion of ants displaying that behaviour. Arrows represent a direct transition from one behaviour to the next. The number on the arrow represents the proportion of ants transitioning from the approach. All behavioural transitions begin with the ant approaching the end of the platform it's walking on (Approach). It can then enter the gap and make contact with the opposite platform with its antennae (Antennal contact) or make simply make no contact with its antennae (No antennal contact). The ant then makes no attempt to cross the gap (*No initiation*), reaches across with its forelimbs (*Attempt*) or successful crosses the gap (*Success*). The sequence represents 10 individual's behaviour upon encountering an 8 mm gap over 10 trials.

3.4.3 Ants prefer the single limbed crossing strategy over 8 mm

To assess preference between single and double forelimb crossing strategies over 8 mm gap widths, we used a second cohort of 10 ants, each of which crossed both the 8 mm gap and the 10 mm gap 10 times. Both strategies always start with the ant approaching the end of the platform and placing both forelimbs into the gap.

Ants can successfully cross a gap using one of two strategies. In the single forelimb strategy, the ant initiates the cross with one of its forelimbs. Once the initial forelimb has reached across and made contact with the opposite platform the remaining legs follow, pulling the body across the gap (Fig. 3.8A). Sometimes the trailing forelimb will leave the initial platform to join the preceding forelimb, contacting the opposite platform at the same time. Alternatively, an ant can achieve a successful gap cross by using both forelimbs to cross the gap (Fig. 3.8B), which we refer to as a double forelimb strategy. In this strategy, the ants thrust both forelimbs into the gap, waving them about until contact is made with the opposite platform, whilst supporting the body with the remaining legs.

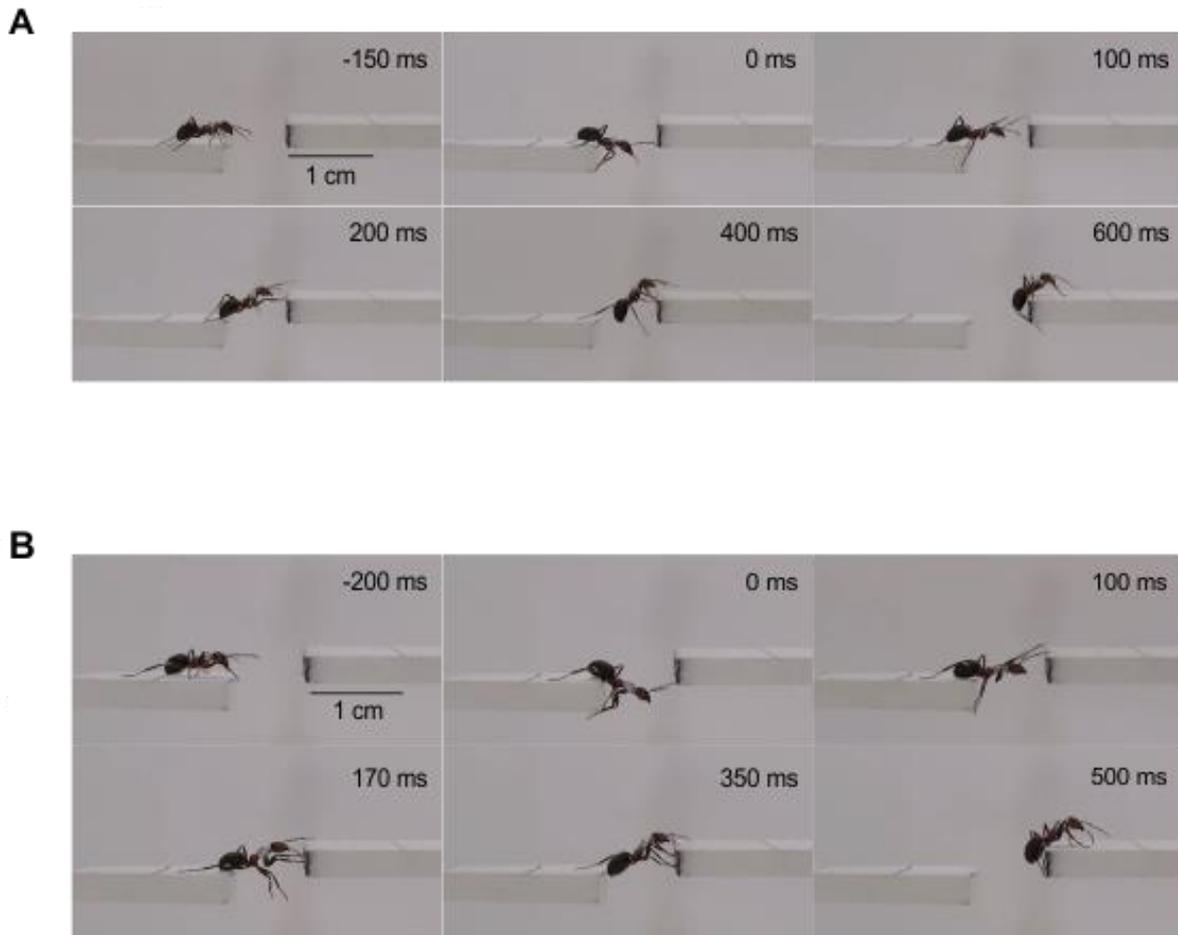


Figure 3.8. High speed video sequences of a red wood ant exhibiting the two successful gap-crossing strategies over an 8 mm gap width. A. A single forelimb gap-cross. The ant targets one forelimb towards the opposite platform's edge, using its remaining 5 limbs as an anchor on the starting platform. The leading forelimb makes contact with the opposite platform before the rest of the body is pulled across. **B.** A double forelimb gap-cross. The ant targets both forelimbs towards the opposite platform's edge, using its remaining 4 limbs as an anchor on the starting platform. The two leading forelimbs make contact with the opposite platform before the rest of the body is pulled across.

To determine the preferred crossing strategy, we compared the number of single forelimb crossings between 8 mm and 10 mm gaps. We found significantly more single forelimb crossings at the 8 mm gap (median = 7.5, Q1 = 6.25, Q3 = 8) than at the 10 mm gap (median = 2.5, Q1 = 2, Q3 = 3.5) (Wilcoxon signed-rank test; $W = 96$, $p < 0.005$; $N = 10$; Fig. 3.9). This suggests that ants prefer to employ the single forelimb strategy whilst crossing gaps but resort to the double forelimb strategy when the gap becomes larger. The remainder of this chapter will assess only ants crossing at 8 mm gap widths.

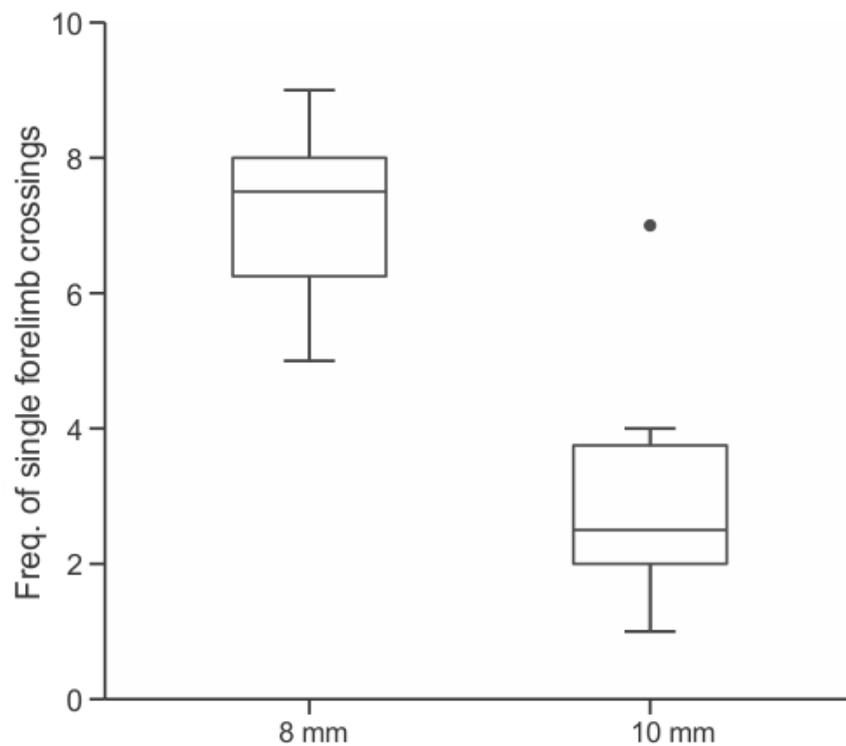


Figure 3.9. Ants prefer to cross 8 mm gaps using the single forelimb strategy. Box and whisker plot displaying the frequency of single forelimb crossings over 8 mm and 10 mm gaps. The horizontal lines within the box denotes the median, the box denotes 25th and the 75th percentiles, and the error bars denote the 95% confidence limits. Solid circles outside error bar limits represent outliers.

3.4.4 Individual ants display variation in limb preference

To investigate whether ants have a forelimb preference, we measured reaching biases whilst gap crossing from 196 individual ants from four separate colonies, Colony #1 (C#1, $N = 50$), Colony #2 (C#2, $N = 50$), Colony #3 (C#3, $N = 50$) and Colony #4 (C#4, $N = 46$). We found individuals vary in their forelimb preference within colonies; individuals differ in the strength and direction of their bias with the colony containing some individuals that were significantly biased towards either their left or right forelimb (Exact Binomial tests, Tables 3.1 - 3.4, Fig. 3.10 - 3.13B).

Table 3.1. Binomial Exact tests of goodness-of-fit for reaches across gap from C#1 ants.

Data from Colony 1; Fig. 3.10; $N = 50$, $n = 10$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Right Forelimb Reaches	2-tailed probability	Number of Ants	Laterality Index
0	0.000977***	1	-1.00
1	0.0117*	0	-0.80
2	0.0654	7	-0.60
3	0.227	12	-0.40
4	0.549	12	-0.20
5	1	8	0
6	0.549	6	0.20
7	0.227	3	0.40
8	0.0654	0	0.60
9	0.0117*	1	0.80
10	0.000977***	0	1.00

Table 3.2. Binomial Exact tests of goodness-of-fit for reaches across gap from C#2 ants.

Data from Colony 2; Fig. 3.11; $N = 50$, $n = 10$. Significant differences are denoted with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.005$).

Right Forelimb Reaches	2-tailed probability	Number of Ants	Laterality Index
0	0.000977***	1	-1.00
1	0.0117*	1	-0.80
2	0.0654	2	-0.60
3	0.227	4	-0.40
4	0.549	5	-0.20
5	1	8	0
6	0.549	10	0.20
7	0.227	11	0.40
8	0.0654	5	0.60
9	0.0117*	2	0.80
10	0.000977***	1	1.00

Table 3.3. Binomial Exact tests of goodness-of-fit for reaches across gap from C#3 ants.

Data from Colony 3; Fig. 3.12; $N = 50$, $n = 10$. Significant differences are denoted with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Right Forelimb Reaches	2-tailed probability	Number of Ants	Laterality Index
0	0.000977***	1	-1.00
1	0.0117*	1	-0.80
2	0.0654	0	-0.60
3	0.227	2	-0.40
4	0.549	9	-0.20
5	1	8	0
6	0.549	7	0.20
7	0.227	12	0.40
8	0.0654	7	0.60
9	0.0117*	2	0.80
10	0.000977***	1	1.00

Table 3.4. Binomial Exact tests of goodness-of-fit for reaches across gap from C#4 ants.

Data from Colony 4; Fig. 3.13; $N = 46$, $n = 8$. Significant differences are denoted with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Right Forelimb Reaches	2-tailed probability	Number of Ants	Laterality Index
0	0.00391*	0	-1.00
1	0.0391*	0	-0.75
2	0.180	1	-0.50
3	0.508	11	-0.25
4	1	9	0
5	0.508	13	0.25
6	0.180	8	0.50
7	0.0391*	3	0.75
8	0.00391*	1	1.00

3.4.5 Ant colonies display a weak forelimb preference

If the strength of forelimb preference is strong within colonies we would expect observations of preferred forelimb use to deviate from a folded binomial distribution (Fig. 3.10A - 3.13A). In C#1 and C#3 we did not observe a significant deviation from the folded binomial distribution (C#1: G -test, $G_{adj} = 4.67$, $2df$, $p > 0.05$, $N = 50$, Fig. 3.10A; C#4: G -test, $G_{adj} = 2.06$, $2df$, $p > 0.05$, $N = 46$, Fig. 3.13A) suggesting the preference was not strong within the colony. In C#2 and C#3 however we found the observed data deviated significantly from the folded binomial expectation (C#2: G -test, $G_{adj} = 14.87$, $2df$, $p < 0.005$, $N = 50$, Fig. 3.11A; C#3: G -test, $G_{adj} = 14.26$, $2df$, $p < 0.005$, $N = 50$, Fig. 3.12A) suggesting these colonies display a strong preference overall. Colonies C#1 – C#4 had MLI scores of -0.18 , 0.13 , 0.17 , 0.16 respectively.

3.4.6 Ant colonies display a directional bias in limb preference

Were colonies of ants unbiased, observations of their forelimb preference should approximate a binomial distribution. Instead, the distribution we observed deviated significantly from the binomial expectation in all four colonies (C#1: G -test, $G_{adj} = 17.65$, $2df$, $p < 0.005$, $N = 50$ Fig. 3.10 B; C#2: G -test, $G_{adj} = 14.06$, $3df$, $p < 0.005$, $N = 50$, Fig. 3.11 B; C#3: G -test, $G_{adj} = 20.12$, $3df$, $p < 0.005$, $N = 50$ Fig. 3.12 B; C#4: G -test, $G_{adj} = 12.61$, $3df$, $p < 0.01$, $N = 46$, Fig. 3.13 B) indicating colonies of ants display a forelimb preference.

If colonies of ants were to exclusively display individual-level lateralisation the number of observations would be split evenly within the colony between left and right handed individuals. Instead, the distributions are shifted towards a particular direction with the majority of individuals sharing the same preference, indicating a bias in the colonies.

We find C#1's distribution is weighted towards the left, with a mean proportion of right forelimb reaches of 0.41 (median = 40), indicating a preference for the left forelimb. In comparison, we find the other three colonies distributions shifted towards the right, with colony C#2 a mean proportion of right forelimb reaches of 0.56 (median = 60), C#3 a mean proportion

of right forelimb reaches of 0.59 (median = 58.6) and C#4 a mean proportion of right forelimb reaches of 0.58 (median = 62.5), indicating a colony-level bias for the right forelimb.

These results indicate red wood ants display a preference for using a particular forelimb whilst gap crossing. In this context, some individual ants preferred to use their right forelimb, others their left and the remainder showed no preference – the hallmark of individual-level lateralisation. Remarkably, the preference differed between colonies, the majority of individuals within a colony showing a preference for using the same forelimb to cross the gap. Thus, wood ants exhibit two forms of lateralisation at the individual-level and at the colony-level. This latter form of lateralisation is entirely novel and previously undescribed.

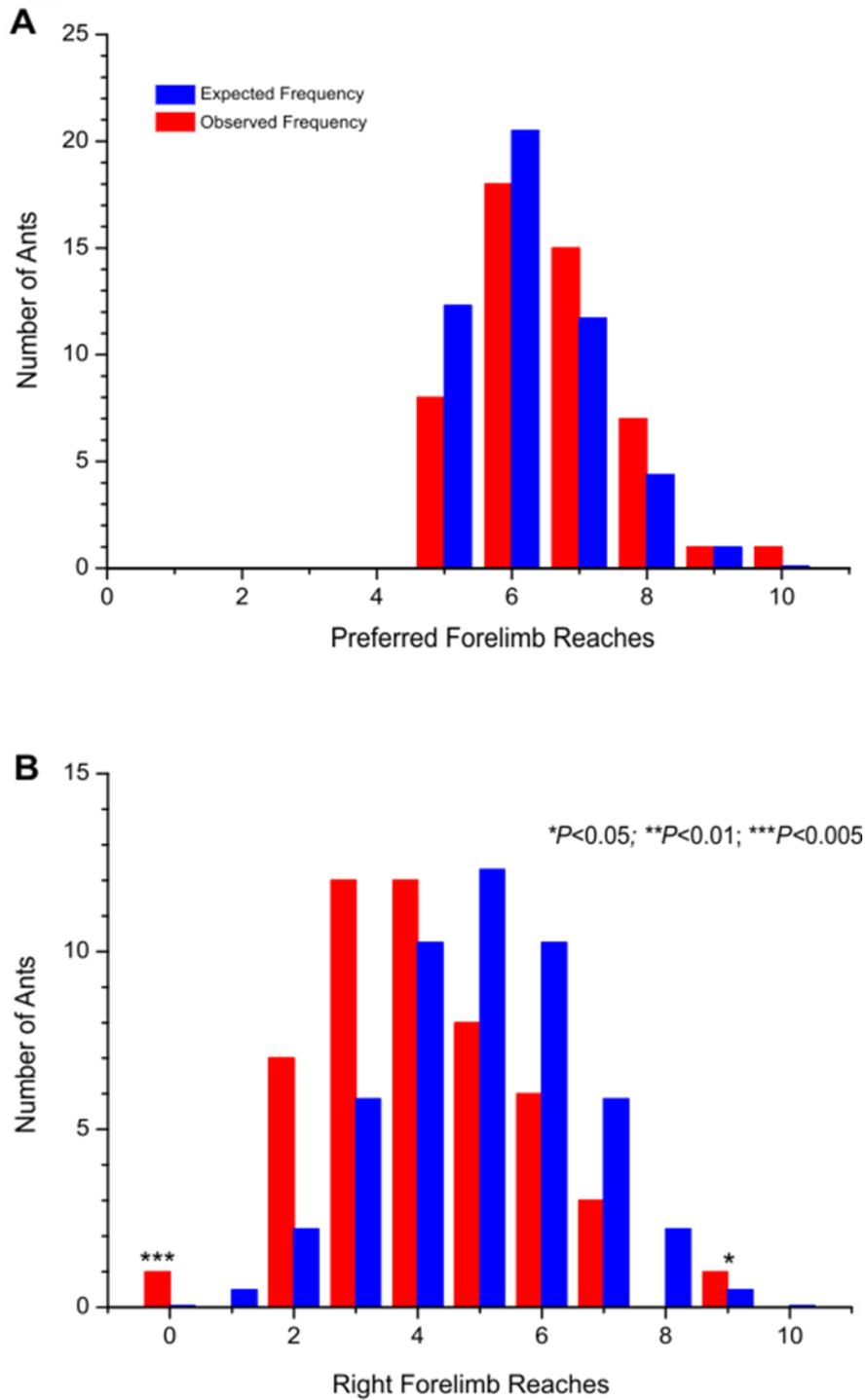


Figure 3.10. Limb preference in targeted forelimb movement during gap crossing in C#1 wood ants. A. The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 50$, $n = 10$) (red). **B.** Frequency distribution of gap crosses initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue).

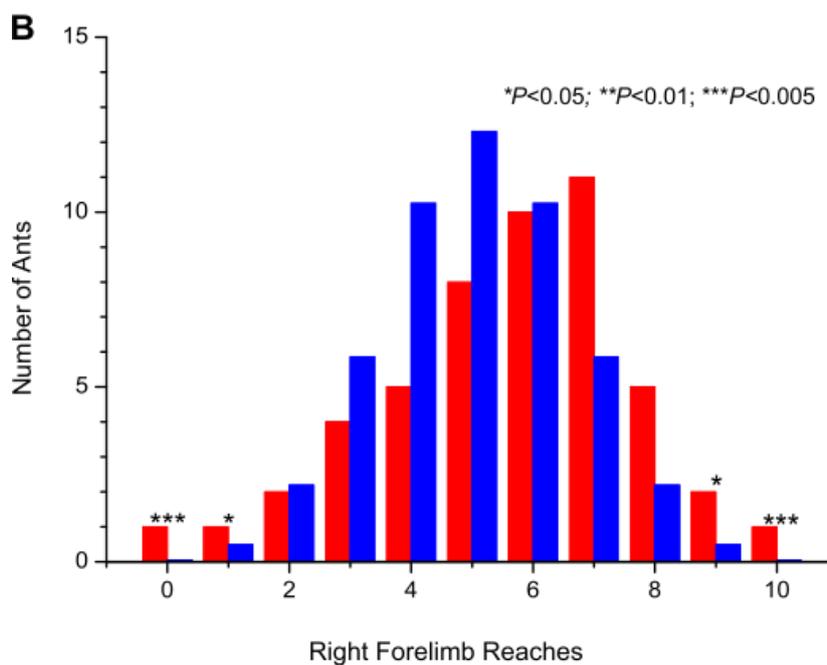
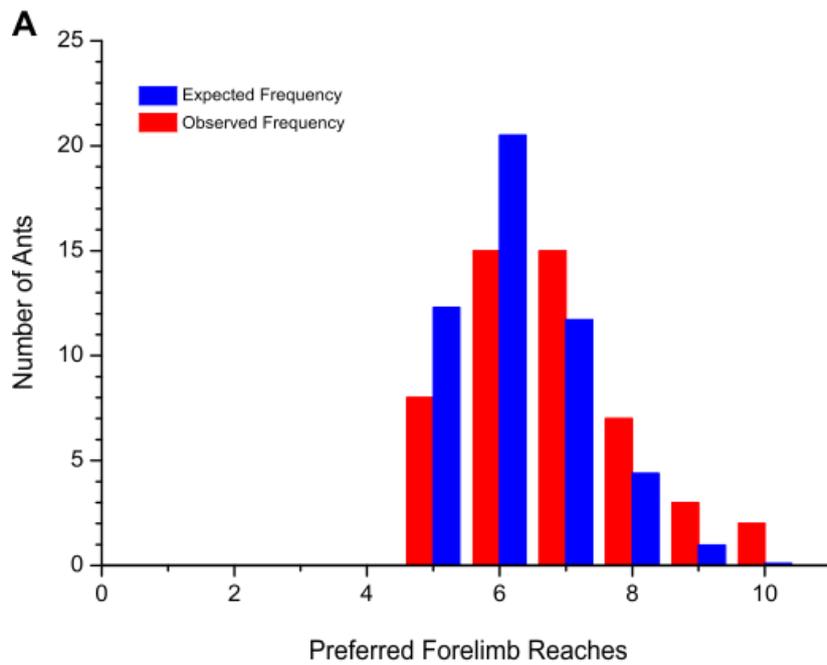


Figure 3.11. Limb preference in targeted forelimb movement during gap crossing in C#2 wood ants. A. The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 50$, $n = 10$) (red). **B.** Frequency distribution of gap crosses initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue). The binomial expectation of no preferred forelimb ($p = 0.05$) (blue) was obtained by mirroring the binomial distribution to incorporate both left and right forelimb use.

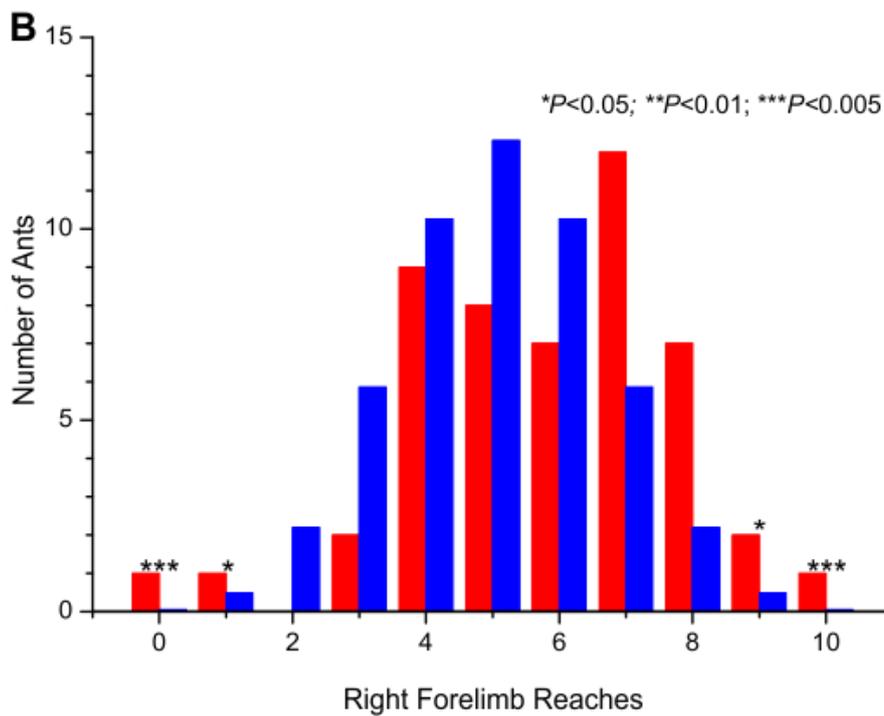
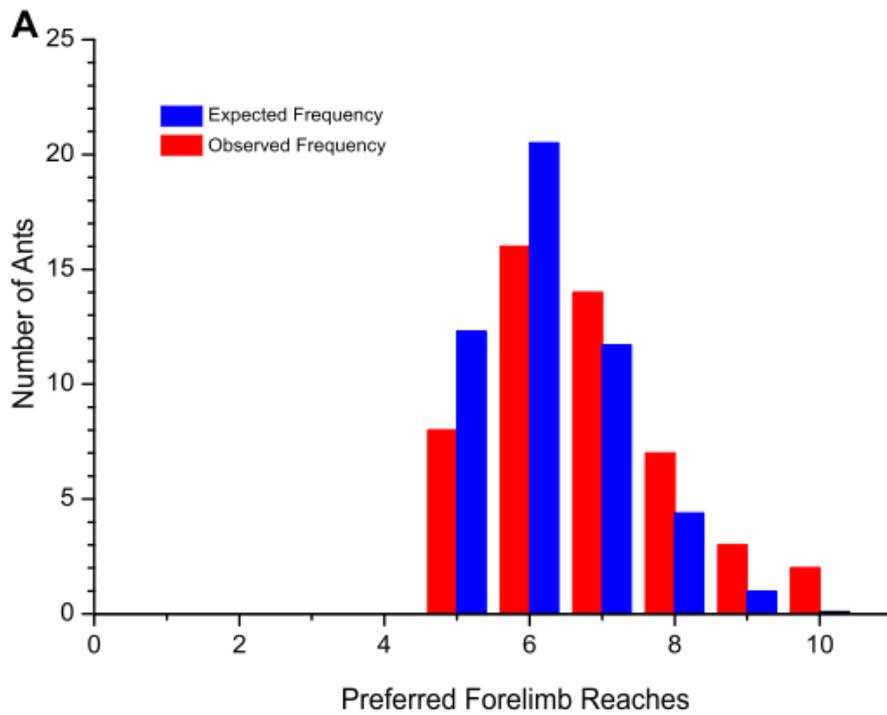


Figure 3.12. Limb preference in targeted forelimb movement during gap crossing in C#3 wood ants. A. The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 50$, $n = 10$) (red). **B.** Frequency distribution of gap crosses initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue).

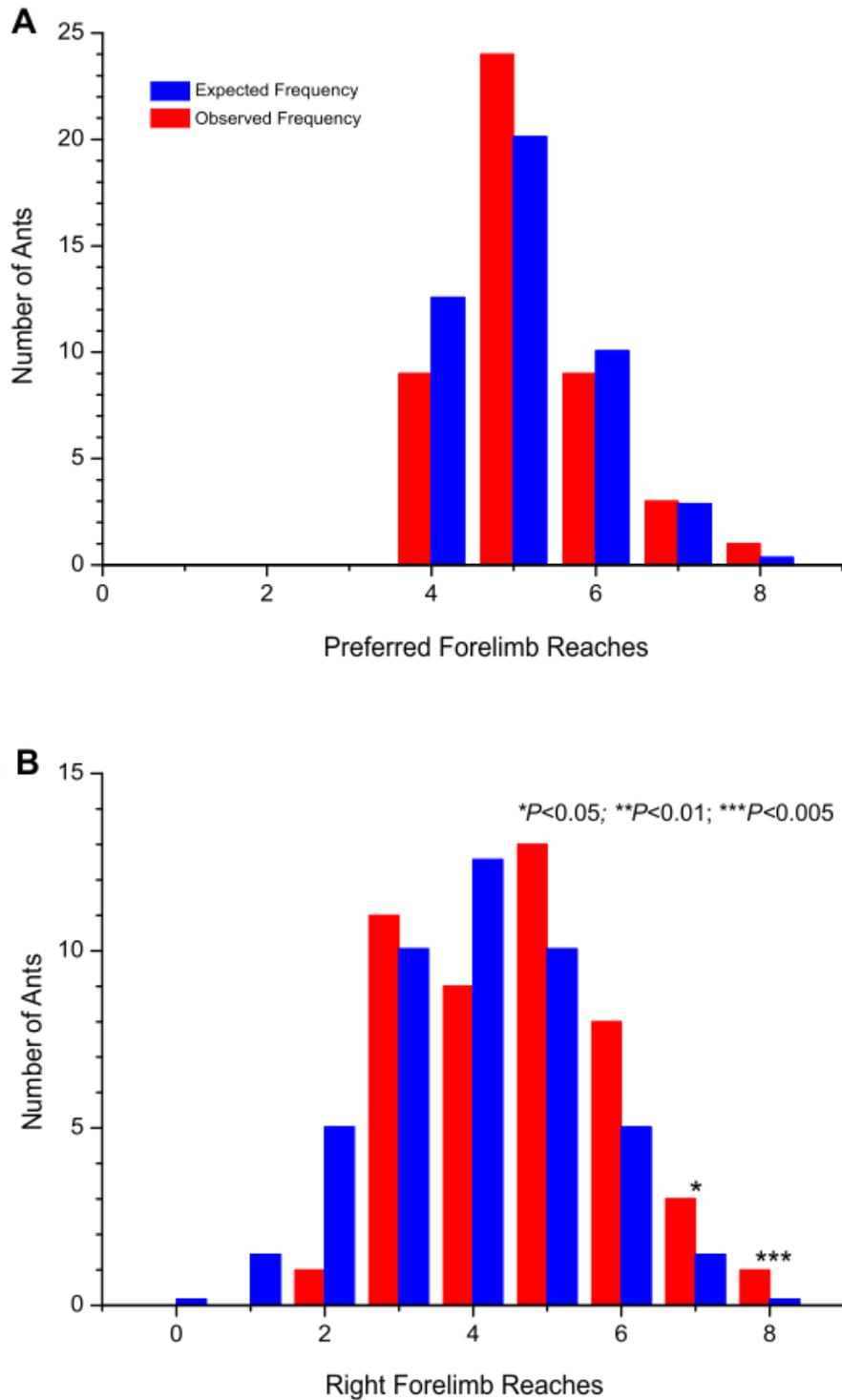


Figure 3.14. Limb preference in targeted forelimb movement during gap crossing in C#4 wood ants. **A.** The frequency distribution of gap crosses initiated by the preferred forelimb ($N = 46$, $n = 8$) (red). **B.** Frequency distribution of gap crosses initiated by the right forelimb (red) compared with the expected binomial distribution ($p = 0.5$) (blue).

3.4.7 Ant colonies display inter-colony variation in limb preference

We compared the effect of colony on right forelimb crossings. We found a statistically significant difference in the number of right forelimb crosses performed between colonies (Kruskal-Wallis; $\chi^2(3) = 28.029$, $p < 0.005$; Fig. 3.15). *Post-hoc* analysis suggests that C#1 caused this significant difference (Mann-Whitney U tests on each pair of groups and adjusted p -values using the Bonferroni method for multiple comparisons; C#1 - C#2, $W = 669$, $p < 0.005$; C#1 - C#3, $W = 585.5$, $p < 0.005$; C#1 - C#4, $W = 603$, $p < 0.005$; C#2 - C#3, $W = 1178.5$, $p > 0.05$; C#2 - C#4, $W = 1171.5$, $p > 0.05$; C#4 - C#2, $W = 1274.5$, $p > 0.05$). These results suggest that colonies vary in the direction of their forelimb preference, specifically C#1 is left biased and C#2, C#3 and C#4 are right biased.

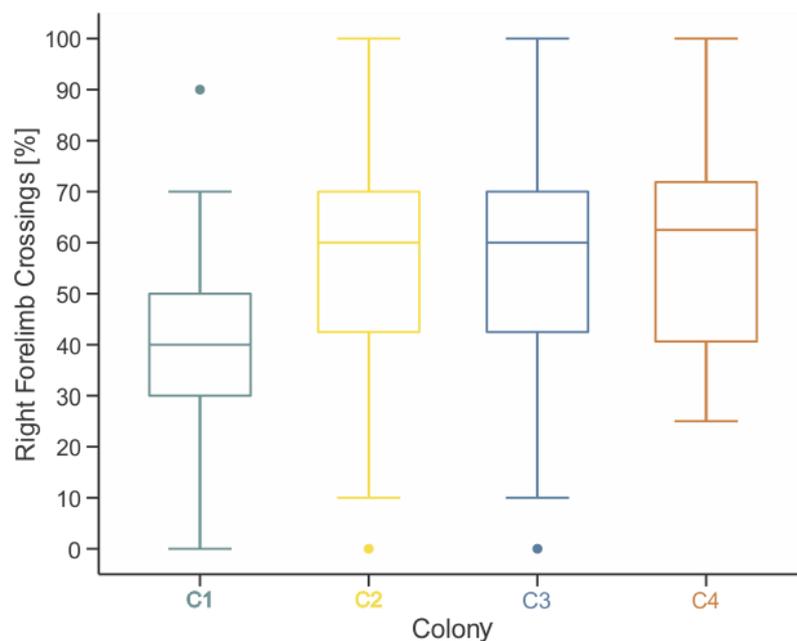


Figure 3.15. Red wood ants display inter-colony variation in forelimb preference. Box and whisker plot displaying the number of right forelimb crosses from four colonies of red wood ants. The horizontal lines within the box denotes the median, the box denotes 25th and the 75th percentiles of the distribution of the measured values, and the error bars denote the 95% confidence limits. Solid circles outside error bar limits represent outliers.

3.4.8 Contribution of gross morphology to limb preference

It is possible that the forelimb preference seen in some ants arises from structural bilateral asymmetry. We measured femur and scape lengths as proxies of leg and antenna length respectively but were unable to detect any gross difference between right and left. We found no significant difference between the length of the left (mean = 1.81 ± 0.14 , $N = 80$) and right (mean = 1.81 ± 0.14 , $N = 80$) legs (Dependent samples t -test; $t = 1.42$, $79df$, $p > 0.05$; Fig. 3.16A). We also found no significant difference between the length of the left (mean = 1.72 ± 0.14 , $N = 80$) and right (mean = 1.72 ± 0.14 , $N = 80$) antenna (Dependent samples t -test; $t = 1.42$, $79df$, $p > 0.05$; Fig. 3.16B). These results suggest that the limb preference observed in individuals was not due to differences in length between left and right legs.

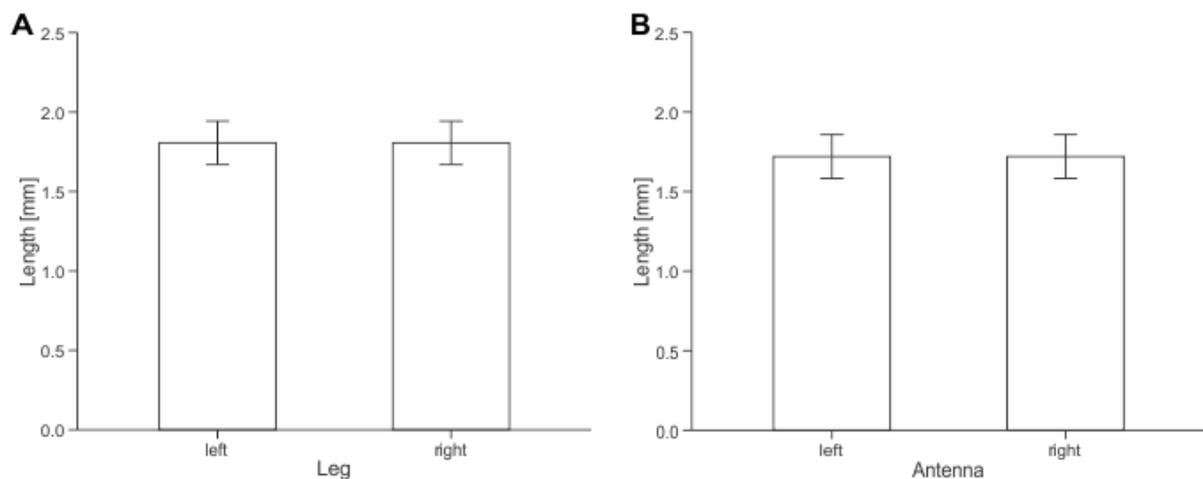


Figure 3.16. Red wood ants display no difference between left and right in legs or antennae length. A. Bar chart showing lengths of left and right legs of red wood ants. **B.** Bar chart showing lengths of left and right antenna of red wood ants. Error bars indicate the standard deviation of the mean.

We then checked for differences in leg length between colonies using the femur as a proxy. The average femur length for each colony was 1.86 ± 0.15 mm ($N = 20$), 1.76 ± 0.12 mm ($N = 20$), 1.79 ± 0.09 mm ($N = 20$), 1.81 ± 0.16 mm ($N = 20$) for C#1 – C#4 respectively. There was no statistically significant difference between the group means (One-way ANOVA; ($F(3, 76) = 2.28, p > 0.05$, Fig. 3.17A) suggesting there was no difference in leg length between colonies. Consequently, the differences in limb preference between colonies are unlikely to be associated with differences in leg length.

We also checked for differences in antennae length between colonies using the scape as a proxy. The average scape length for each colony was 1.79 ± 0.15 mm ($N = 20$), 1.69 ± 0.12 mm ($N = 20$), 1.70 ± 0.10 mm ($N = 20$), 1.71 ± 0.16 mm ($N = 20$) for C#1-C#4 respectively. There was no statistically significant difference between the group means (One-way ANOVA; ($F(3, 76) = 2.28, p > 0.05$, Fig. 3.17B) suggesting there was no difference in antennal length between colonies. Again, the differences in limb preference between colonies are unlikely to be associated with differences in antennae length.

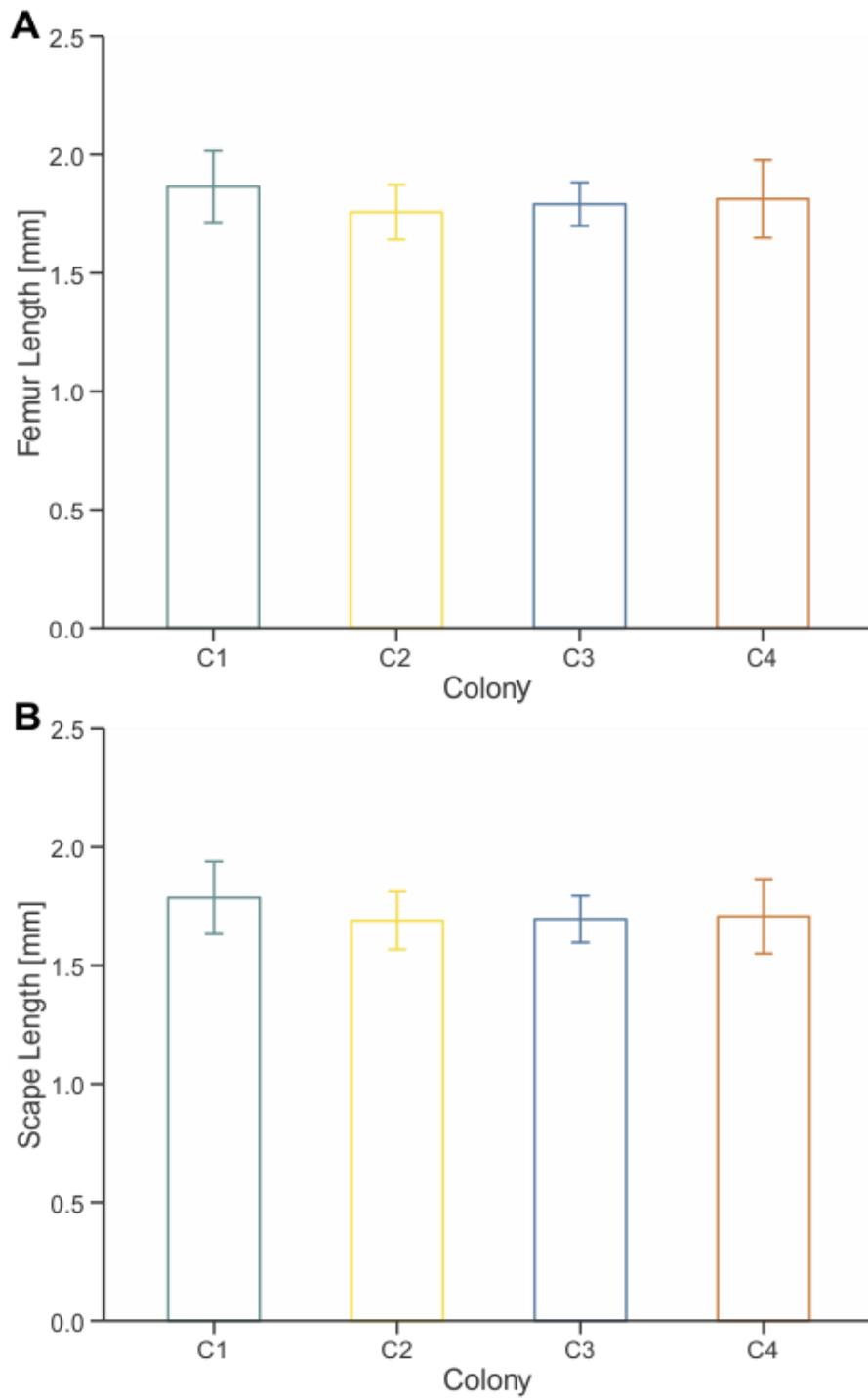


Figure 3.17. Ants display no detectable inter-colony differences in leg or antennal length. A. Bar chart showing inter-colony differences in femur length. **B.** Bar chart showing inter-colony differences in scape length. Error bars indicate the standard deviation of the mean.

3.4.9 No detectable differences in crossing latency between colonies.

We also tested for inter-colony differences in crossing latency. Crossing latency was defined as the period of time elapsed from when the leading forelimb departs from the starting platform until it contacts the opposite platform edge. The average crossing latency for each colony was 190.23 ± 38.0 ms ($N = 20$), 220.85 ± 38.55 ms ($N = 20$), 208.28 ± 61.78 ms ($N = 20$), 194.05 ± 48.27 ms ($N = 20$) for C#1 – C#4 respectively. There was no statistically significant difference between the group means (One-way ANOVA; ($F(3, 76) = 1.73$, $p > 0.05$, Fig. 3.18) suggesting there was no difference in crossing latency between colonies.

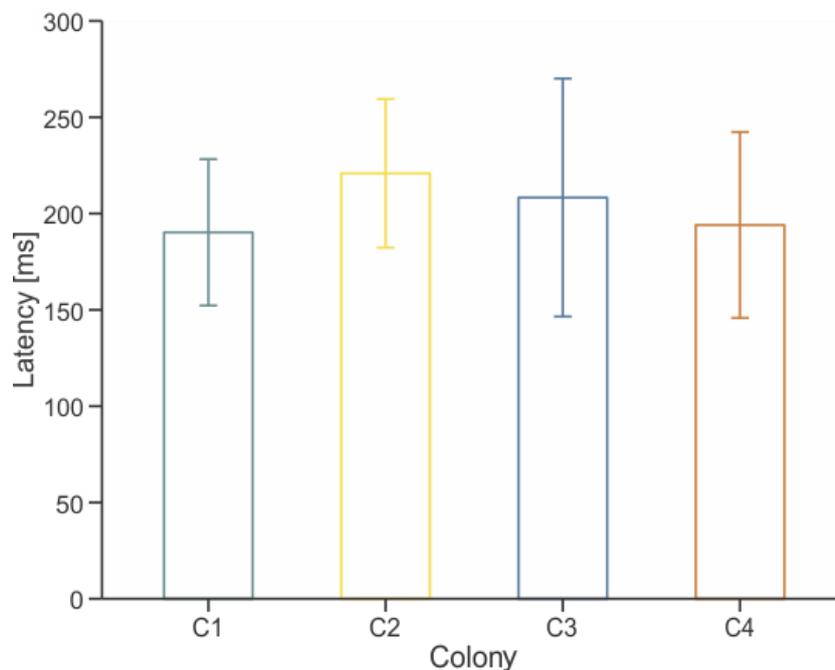


Figure 3.18. Ants display no detectable inter-colony differences in crossing latency.

Bar chart showing inter-colony differences in crossing latency. Error bars indicate the standard deviation of the mean.

3.4.10 Crossing latency is unrelated to strength of preference

We assessed the relationship between the strength of forelimb preference and the crossing latency for each colony. Strength of bias was based on preferred forelimb usage (see methods). We assessed the relationship between the strength of preference and the crossing latency. We found a no correlation between strength of preference and the crossing latency for any of the colonies (Spearman's rank-order correlation; C#1, $\rho = 0.013$, $N = 20$, $p > 0.05$, 18df, Fig. 3.18A; C#2, $\rho = -0.02$, $N = 20$, $p > 0.05$, 18df, Fig. 3.18B; C#3, $\rho = 0.26$, $N = 20$, $p > 0.05$, 18df, Fig. 3.18C; C#4, $\rho = -0.24$, $N = 20$, $p > 0.05$, 18df, Fig. 3.18C), which suggests that the strength of the bias is unrelated to the crossing latency.

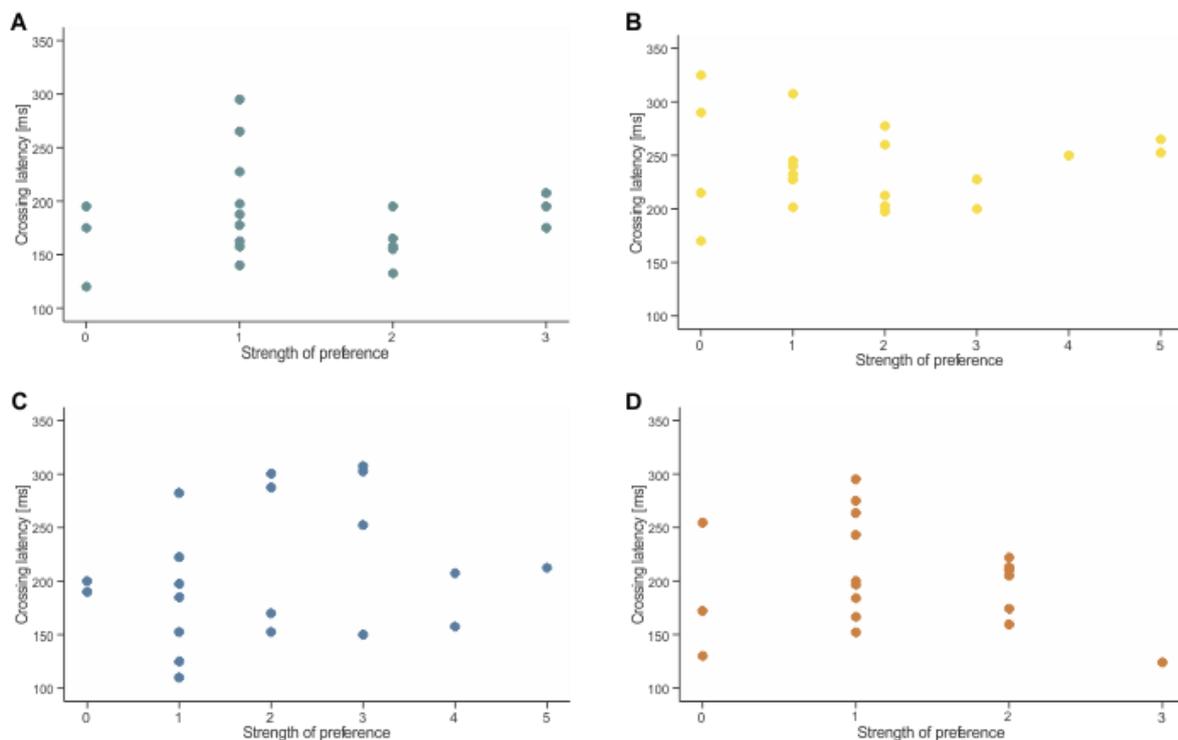


Figure 3.18. Strength of forelimb preference is unrelated to crossing latency. Scatterplot showing the strength of an individual's preference is unrelated to its crossing latency in **A. C#1** **B. C#2** **C. C#3** and **D. C#4**.

3. 5 Discussion

Few cases of motor asymmetries in the nervous systems of invertebrates have been documented (Frasnelli *et al.* 2012b; Frasnelli, 2013). Even less research conducted has focused on preferential forelimb use in insect systems (but see previous chapter and Bell and Niven, 2014, 2016). Here, we set out to investigate whether a relatively small insect nervous system, the red wood ant (*Formica rufa*), is capable of producing a functional, motor asymmetry in the form of forelimb preference. We tested individual ants repeatedly over a gap-crossing paradigm in which they attain a food reward by crossing a gap: reaching with one forelimb across a gap and pulling themselves to an opposing platform.

3.5.1 Behaviour upon encountering a gap

Our results show that the behaviour displayed by an ant upon encountering a gap depends upon the gap width. Strength of lateralisation is thought to be task dependent, increasing as tasks require greater cognitive function (Fagot and Vauclair, 1991) and co-ordination (Hopkins, 1995). It was important, therefore, to find a gap width that was sufficiently large to need precise co-ordination, whilst being small enough to produce a rapid number of crosses within a limited time. Based upon this logic, we chose an 8 mm gap width for our limb preference experiments. Ants displayed a sufficient number of single-forelimb crossings over an 8 mm gap to justify this width. It is unclear, however, whether the ants' limb preference is different at smaller or larger gaps. Similarly, it would be interesting to investigate whether the ants' limb preference is restricted to this context, or whether the limb preferences of ants are consistent in different contexts.

During these initial experiments antennal contact was an extremely important factor in determining whether an ant would make a successful cross: ants use their antennae to detect contact with the far side of the gap, which aids gap-crossing behaviour. Whether they actively direct their antennae towards the opposite platform or whether the contact is produced by coincidentally touching the opposite platform is unknown. Actively directing the antennae would imply visual control, and this scenario would not be improbable given that locusts use vision

to direct their forelimbs over gaps (Niven *et al.*, 2010). However, ants are likely to be much worse at detecting the far side of the gap because they have poorer visual resolution (Catton, W. T., 1998; Schwarz *et al.*, 2011). Fruit flies have been shown to use vision guidance to cross gaps of considerable width relative to their body size (Pick and Strauss, 2005; Triphan *et al.*, 2010) and, similar to the ants in these experiments, avoid initiating insurmountable gaps based on sensory input. It would also be interesting to determine whether visual inputs have an effect on which forelimb used to cross the gap. In general, we need to know more about the sensory inputs and the way they affect gap crossing.

3.5.2 Individual ants display forelimb preference

Individual ants are lateralised in the use of their forelimbs, adding to the growing body of literature that documents lateralisation in the motor output of relatively small insect nervous systems (Heuts *et al.*, 2003; Kight *et al.*, 2008; Cooper *et al.*, 2011; Bell and Niven, 2014; Buchanan *et al.*, 2015). Similar to our observations in locusts (Bell and Niven, 2014, 2016), ants display variation in the strength and direction of their preference. In contrast to locusts however we find that generally ant limb preference is relatively mild, with only a few individuals within each colony showing a strong preference. The general lack of strong lateralisation within the colony suggests there are costs opposing the benefits of strong lateralisation. One potential cost is predictability because competitors and/or predators can exploit predictable movements and/or decisions (Levy, 1977; Ghirlanda and Vallortigara, 2004).

That these small nervous systems are capable of producing behavioural lateralisation is surprising in itself. The fruit fly *Drosophila melanogaster*, a bilaterally symmetrical organism, has a lateralised brain structure that is linked with a specific cognitive function - the formation and recall of olfactory memories (Pascual *et al.*, 2004). It is possible therefore that asymmetric brain structures could underpin the lateralised forelimb preference we observe in ants however, there is now a great deal of detailed anatomy of insect brains and none have reported an asymmetric body or similar lateralised structures elsewhere (Burrows, 1996; Strausfeld, 1976; Kurylas *et al.*, 2008; Ito *et al.*, 2014; Turner-Evans and Jayaraman, 2016).

Our results suggest that future research should aim to establish a more detailed survey of the neuro-anatomy of insect nervous systems in an attempt to elucidate the sources of lateralisation.

3.5.3 Intra-colony variation in the strength and direction of preference

Within-group variation is perhaps best exemplified in the eusocial insects, which often present distinct morphological and/or temporal castes (Oster & Wilson, 1978; Hölldobler & Wilson, 1990), with differences relating to behavioural task. However, like most social insect species red wood ants are monomorphic, i.e. there are no obvious morphological differences between workers except their size (Hölldobler & Wilson, 1990), so it may be surprising to find that red wood ants display intra-colony variation in behavioural preference, with individuals varying in their direction and strength of preference. Social arthropods that lack obvious morphological asymmetries are known to express within-colony variation in behaviour. For example, colonies of dinosaur ants (*Dinoponera*) and primitively social bees and wasps include a queen that is not obviously morphologically different but their behaviour varies greatly. Also, amazonian social spiders display intra-colony variation in social tendency, aggressiveness and boldness without exhibiting any obvious morphological differentiation (Pruitt *et al.*, 2011). Some honeybees preferentially forage for pollen over nectar, and these preferences are associated with different sensory biases, locomotive activity and gene regulation (Page *et al.*, 2012; Page *et al.*, 1995; Linksvayer, 2006, Rüeppell *et al.*, 2004, Rüeppell *et al.*, 2005). Other ant species show variation in the patterns of individual differences within colonies. In *Temnothorax* ants, individuals show different levels of activity, with some workers consistently less active than others (Charbonneau and Dornhaus, 2015).

Inter-individual differences within colonies may have ecologically relevant consequences, for example, the number of individuals with an aggressive behavioural type determines how well the group can defend its nest from social parasites (Pamminger *et al.*, 2012; Scharf *et al.*, 2012) and affects the colony lifespan (Pruitt, 2012, 2013). Given the lack of genetic diversity within these colonies of arthropods it is intriguing that they display such a

range inter-individual variation in behaviour and suggests that the developmental environment plays a large role in producing the variation. Our results highlight a need to consider within-colony variation when documenting behavioural preference in social arthropods because, even in colonies that lack discrete morphological castes or even substantial genetic variation, individuals can boast remarkable variation in traits that could affect group function.

The benefits of lateralised behaviour at the individual-level have been hypothesised (see General introduction) although they fail to explain why some individuals within a group show weak or no preference. The strength of an individual's asymmetry can depend on the ecological pressures inflicted upon it. Brown *et al.* (2004) found that fish (*Brachraphis episcopa*) living under high predation risk displayed more strongly lateralised behaviour than other members of their species living under low predation risk. The strength of preference displayed by individual ants in our study, for example, could reflect their role within the colony. Those individuals that forage more or defend the nest may require greater lateralisation of their forelimbs and, therefore, display stronger lateralisation. Indeed, in our study, we did not differentiate between ants that may have different behavioural roles within the colony. On the other hand, the weak bias found in many of the individuals may not be too surprising given that forelimbs may not be used heavily in predator defence or nest mate interactions. The mandibles are more likely used in predator defence and the antennae in nest mate interactions, although the role of the forelimbs in these behaviours is unknown.

The intra-colony variation in direction of lateralisation may have evolved under frequency-dependent selection in the form of fluctuations of right/left phenotypes over time. Such a scenario is reported in a scale eating fish (*Perissodus microlepis*) in which the frequencies of right and left bending individuals vary cyclically over time with respect to feeding success, suggesting that negative frequency-dependent selection in maintaining the right/left frequency of individuals within the population (Hori, 1993). Theoretically, the variation we observe in direction of preference in our ant colonies could have evolved in the same manner, with right biased individuals becoming less common in the colony as they are selected against through, for example, a parasitoid which reduces the fitness for right biased ants through

unilateral attack. Because our colonies are a sample from only one time point, we would need to retest each colony at multiple time points to establish whether the forelimb preference persists over time in order to test this hypothesis.

Another possible explanation for the variation in bias among individuals, most notably the double peak in right forelimb use when gap crossing (Fig. 3.12B and Fig. 3.14B), could be due to wood ants' nests coming from more than one queen, who are not always necessarily brood sisters. These queens can be mated to unrelated males. This genetic variation could account for sub-populations in the colony who exhibit handedness that is different to the majority.

3.5.4 Ants display colony-level forelimb preference

Social insects have been proposed as a useful model to test the hypothesis (Anfora *et al.*, 2010) about evolution of lateralisation as an ESS due to the clear distinction that can be made between social and non-social species (Frasnelli, 2013). In our study, we find that individuals within the colony align their forelimb preference with that of other ants in the colony. This supports the hypothesis that social animals are likely to align their lateralised behaviour to others in their group with which they find themselves.

One could argue that forelimb preference investigated in this study is not strictly social in nature *i.e.* forelimb usage might not play an important role in social interactions within the group. However, we argue that the original drive for the forelimb preference could be related to preference in other lateralised behaviours such as the side of the path in which they explore new nest sites (Hunt *et al.* 2014), or social interaction with antennae during trophallaxis (Frasnelli *et al.*, 2012a). The forelimb preference, therefore, may have evolved indirectly as a by-product of these other lateralised behaviours that are more likely to have been directly selected as an ESS in social contexts. It should also be noted that population-level lateralisation is not restricted to social species (Frasnelli, 2012b). Evidence from non-social insects documents population-level lateralisation in aggression (Benelli *et al.* 2015a; b; Romano *et al.*, 2015), although interestingly these behaviours are displayed when the insects

congregate in 'near-social' swarms suggesting that social interactions may hold an important role in the development of population-level behaviours.

3.5.5 Inter-colony variation in direction of preference

Eusocial insect colonies can be considered an extended phenotype of the queen (Dawkins, 1982). Individual workers within these colonies have sacrificed their own reproductive success, and are closely related to one another (Wilson, 1971; Hölldobler and Wilson, 1990). In the case of *Formica rufa*, interactions between members of the colony that are beneficial (synergistic interactions), and that could become more efficient if lateralised, occur at the within-colony level of selection. Predators, parasitoids and other ecological factors exert an influence across multiple colonies. For example, predators can learn the direction of lateralisation of ants from multiple colonies, not distinguishing between them. In this way, any colony with a different direction of lateralisation would do better because a predator is less likely to predict its behaviour.

We have some evidence, based on computational modelling, of how the degree of lateralisation found in our colonies can arise and be maintained considering these ecological pressures (Appendix A). This differs from the original Ghirlanda and Vallortigara (2004) model in which selfish individuals form a group and the predator exerts influence at the same level of selection – on the individual. In the case of *Formica rufa*, however, there should be a selective advantage for colonies that are not lateralised in the same direction as all others in the population, producing a frequency dependency that is quite different from the frequency dependency that produces population lateralisation in humans, for example.

Since task dependent limb preference has been demonstrated in this colony of ants, repeating the methods with other colonies would differentiate population-level lateralisation from colony-level lateralisation, something that has not explicitly been done before. As well as this, studying a wider range of behaviours may help understanding of which behaviours are consistent in their biases, and which differ. This would perhaps give understanding into their basis.

Chapter 4

Turning preference of the red wood ant (*Formica rufa*) in a 'Y'-maze.

4.1 Abstract

Lateralisation is often found in movements of the entire body, as well as those of particular limbs, with many animals showing distinct preferences for turning in particular directions. Such preferences are often tested by assessing behaviour within a 'Y'-maze to provide a distinct 'choice' that is easy to score. Here we test red wood ants for innate turning preferences in a 'Y'-maze paradigm. Ants were placed individually within the mazes and allowed to explore over 30 minutes each. Approximately 42% of all individuals tested displayed significant turning biases, with a similar number of individuals biased to the left and right. The turning preference is displayed at the individual-level only, with neither the colony nor the population showing any directional bias. We assessed the temporal correlation of right turns across trials and found that a right turn at one time point predicts subsequent right turns, suggesting that turns are not independent. Close inspection of the ants' behaviour in the 'Y'-maze revealed that their behaviour is dominated by wall-following behaviour, which is likely the source of the non-independence of turns. This calls into question the validity of using the 'Y'-maze paradigm when assessing turning preferences and suggests it is more likely to be assessing the persistence and switching of wall-following.

4.2 Introduction

Turning preferences are a specific form of lateralised motor behaviour in which an animal turns all or part of its body toward one side of space (Hanard and Doty, 1977). These preferences are displayed across a broad range of species: When deprived of any external directional reference humans exhibit a turning preference (Schaeffer, 1928; Souman *et al.*, 2009) that is assumed to be caused by the accumulation of noise in the sensorimotor system (Souman *et al.*, 2009). Other vertebrates also show turning biases that may be linked to asymmetries in

their nervous systems. Horses show a lateralised escape response considered to be under control of the right hemisphere (Austin and Rogers, 2007), whilst rats and mice consistently rotate in a preferential direction, associated with cerebral lateralisation (Glick and Ross, 1981; Kim *et al.*, 1999). Lizards are also lateralised in their turning preference for both escape and predatory behaviour (Bonati *et al.*, 2010; Bonati *et al.*, 2008). Species of fish show lateralisation in escape response (Biasazza *et al.*, 1997; Cantalupo *et al.*, 1995, Heuts *et al.*, 1999) in both aggressive and courtship displays (Cantalupo *et al.* 1996).

Invertebrates too show lateralisation in the form of turning behaviour in branching maze designs. Within the molluscs, cuttlefish show a side-turning preference in 'T'-mazes (Alves *et al.*, 2007), whilst within the insects, ladybirds, giant water bugs, cockroaches, ants and fruit flies all show a turning preference when confronted with a binary choice in a 'Y'-maze (Girling *et al.*, 2007; Kight *et al.*, 2008; Cooper *et al.*, 2011; Hunt *et al.*, 2014; Buchanan *et al.*, 2015). Even the nematode worm, one of the 'simplest' of invertebrates in terms of the numbers of neurons within the nervous system, display a rotatory preference when males have a bias for coiling around the body of females in a particular direction during mating (Downes *et al.*, 2012).

Insights into innate turning tendencies are important to the field of lateralisation research for three main reasons. Firstly, the behaviour is phylogenetically old, with a range of organisms displaying such biases, demonstrating a general feature of nervous systems. However, it is unclear whether this reflects a basic homology (Campbell, 1988) or parallel but independent evolutionary histories (Hodos, 1988; Cantalupo *et al.*, 1996). Secondly, turning preferences are considered important because the directional preference may be coupled with other lateralised behaviours within an organism; right-handed people are more likely to have a dominant left hemisphere for language than left-handed ones (Knecht *et al.*, 2000; Perlaki *et al.*, 2013). These other biases may trigger the preferential turning in one direction, the turning may induce the other lateralised behaviours or they may both be the result of another, underlying cause. Thirdly, the mechanisms by which turning preferences operate may provide a model for understanding how nervous systems co-ordinate more finely modulated left-right

motor activity, in which case unravelling the neural architecture underpinning left/right turning preference could provide crucial insights into the neural basis of limb preference.

We use a 'Y'-maze paradigm to test for innate turning tendencies in red wood ants (*Formica rufa*), adopting a similar experimental design to that used previously to assess turning bias in fruit flies (*Drosophila melanogaster*; Buchanan *et al.*, 2015). We used red wood ants because they provide a suitable model for lateralisation research for reasons already described in the previous chapter, and to permit comparison between different behaviours in terms of lateralisation. Foragers of this species are more active and continuously explore novel areas in search for food or new nest sites (Hunt *et al.*, 2014), behaviour that ensures the number of turns observed will be high in comparison to many other species.

We use three colonies of red wood ant from the previous chapter to determine whether, if colony-level turning biases exist, they can be correlated with the respective colonies' forelimb preference. The high temporal and spatial resolution of the tracking software permitted detailed examination of the ants' behaviour within the 'Y'-mazes. This allowed us to determine the behavioural basis of the observed right/left turns.

Our findings give insights into behavioural turning preference in a relatively simple organism. We show that within 'Y'-mazes red wood ants exhibit lateralised motor behaviour in the form of turning preference, with individuals varying in the strength and direction of their bias. The turning preference of individuals is not aligned with others in the colony. We discuss these findings in relation to the hypothesis that social organisms align their lateralised behaviour with others in the group.

4.3 Materials and Methods

4.3.1 Animals

Red woods ants (*Formica rufa*) (Linnaeus, 1761) from colonies #1 - #3 were used from the previous chapter. Ants were maintained in the same conditions as in the previous stated (see Materials and Methods of Chapter 3).

Ants were selected at random from foragers walking on the surface of the nest. Foragers with damage to their eyes, limbs or antennae were discarded, as were those that showed any signs of lethargy indicative senescence. All the ants selected were relatively large and of similar size. Ants were not returned to the nest after testing.

4.3.2 Experimental setup

A custom made 'Y'-maze was cut from 12 mm thick opaque, black, Acrylic Perspex® using a laser cutter (brightonandhoveplastics.co.uk) to test turning preference in individual ants (Fig. 4.1; Fig. 4.2). Each arm of a maze was 60 mm long and 10 mm wide. A transparent sheet of perforated acetate paper was placed over the mazes. This prevented the ants from escaping whilst allowing air to pass into the maze chambers. A light diffuser made of a sheet of 5 mm thick translucent, acrylic was placed between a white LED array and the maze array to provide uniform illumination. To prevent the ants from flipping upside down within the mazes, the diffuser was lightly roughened with fine-grit sand paper, and the acetate sheet was lubricated with Sigmacote (Sigma). Because each maze was symmetrical and illuminated uniformly from below, the turns were driven by choice and not apparatus design/asymmetry.

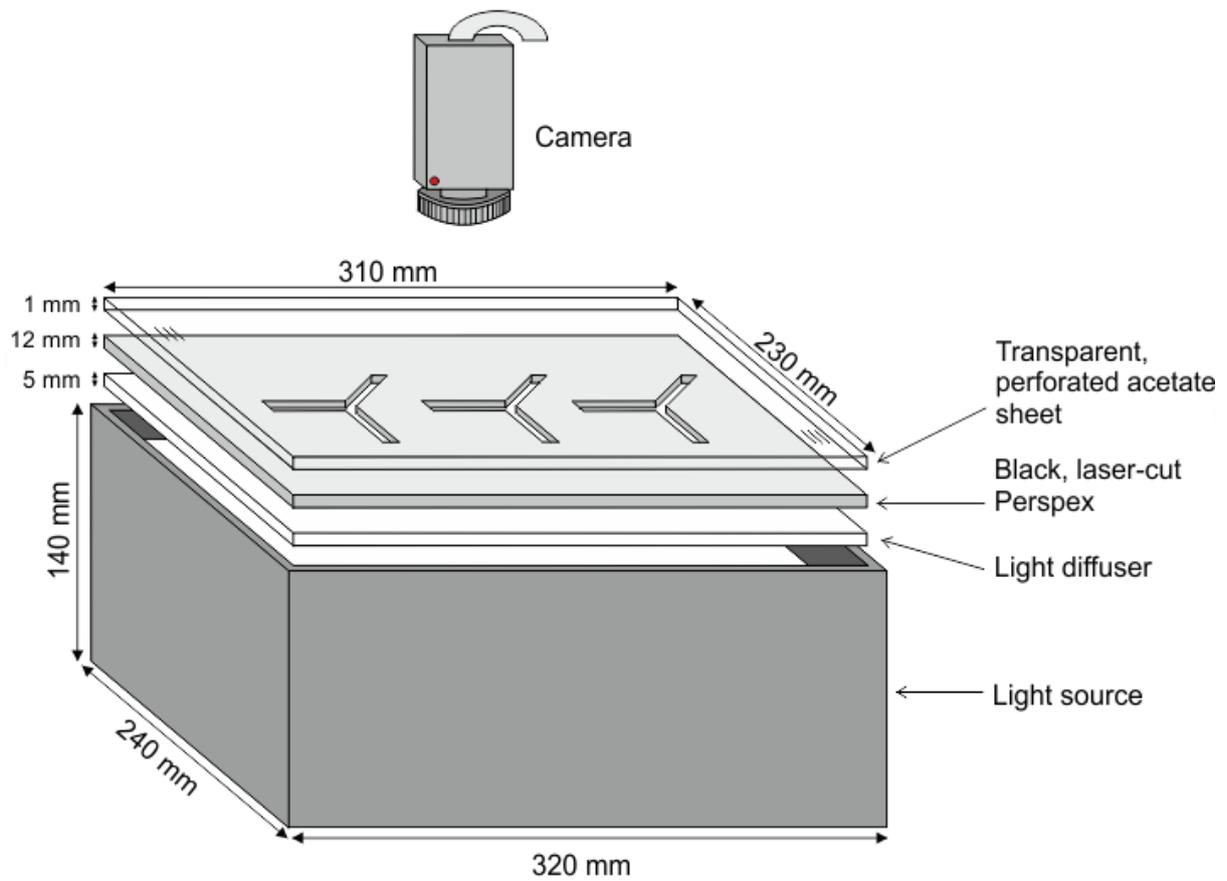


Figure 4.1. A schematic of the 'Y'-maze device used to investigate red wood ant turning preference. A light source was used to uniformly illuminate the mazes from underneath the ants. Laser-cut Perspex mazes allowed ants to make a binary choice after walking along a corridor whilst being filmed from above.

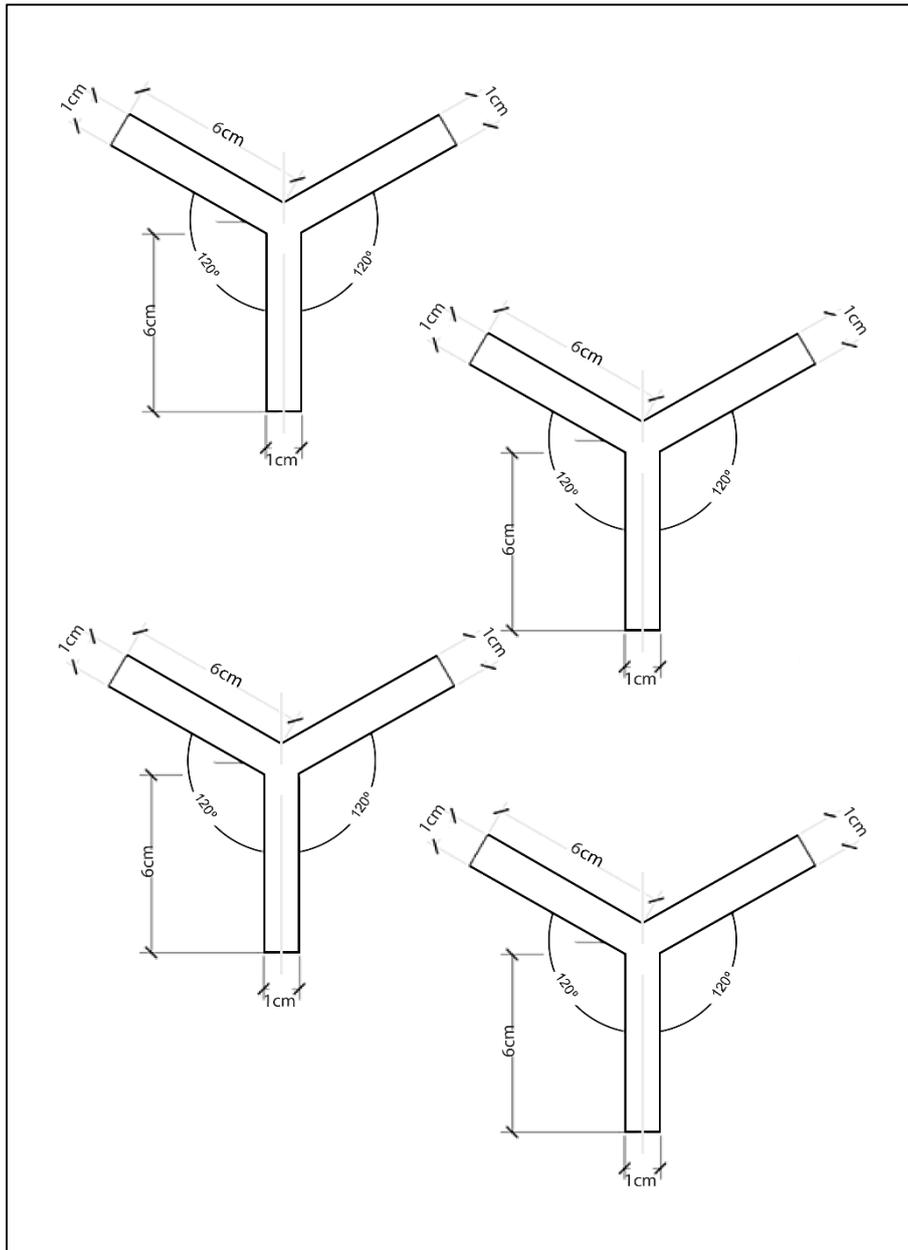


Figure 4.2. 'Y'-maze array of 4 mazes including their dimensions. Small, closely-spaced 'Y'-mazes permitted multiple ants to be filmed simultaneously

4.3.3 Testing

Ants were placed individually into 'Y'-mazes and allowed to walk freely for 30 minutes, during which time they were filmed from above. Testing took place between 13:00 and 17:00 during November 2015. Ants were tested during this time period to ensure standardised activity levels between trials over consecutive days. Eighty-four ($N = 84$) ants in total were tested for turning preference over 28 trials. Ants were tested in cohorts of 3, one ant from each colony #1 - #3 was recorded simultaneously from separate 'Y'-mazes. Ants were free to make as many turns as they wanted over a 30 minute period. Individuals that made fewer than 10 turns during the 30 minute period were excluded from the analysis.

4.3.4 Data collection

The videos for the 'Y'-maze turning experiments were recorded at 60 Hz using a video camera (JVC, GC-PX100) positioned directly above the light box (Fig. 4.1A). Videos of images with a resolution of 1920 x 1080 pixels were saved directly to a computer for later analysis offline in Matlab (Mathworks, version 2014b).

4.3.5 Defining the maze regions

To acquire the position and velocity of each ant in the maze, we needed to identify specific areas within the maze arms. A midline was used to represent a central path as the ants walked along the arm, with maze walls equidistant from this line (Fig. 4.3A). A maze centroid joined the midlines of each arm together, and represented the centre of the maze (Fig. 4.3A). An imaginary 'Start' line was designated as the point at which the ant is deemed to have started or finished a choice (Fig. 4.3B). Further statistics were performed on the ants' position when they are walking in the maze were taken at an imaginary 'Choice' line and 'Centre' line (Fig. 4.3B). The region in which an ant is located after completing a choice and before initiating a new trial was designated as the Outer Zone.

There were two zones in which velocity was measured labelled the 'Inner Zone' and the 'Choice Zone'. The 'Inner Zone' velocity was measured by taking an average of five,

equally spaced data points between the 'Start' line and 'Choice' line. The 'Choice Zone' velocity was measured by taking an average of five, equally spaced data points between the 'Choice' line and 'Centre' line. All velocity data was measured in metres per second (m/s).

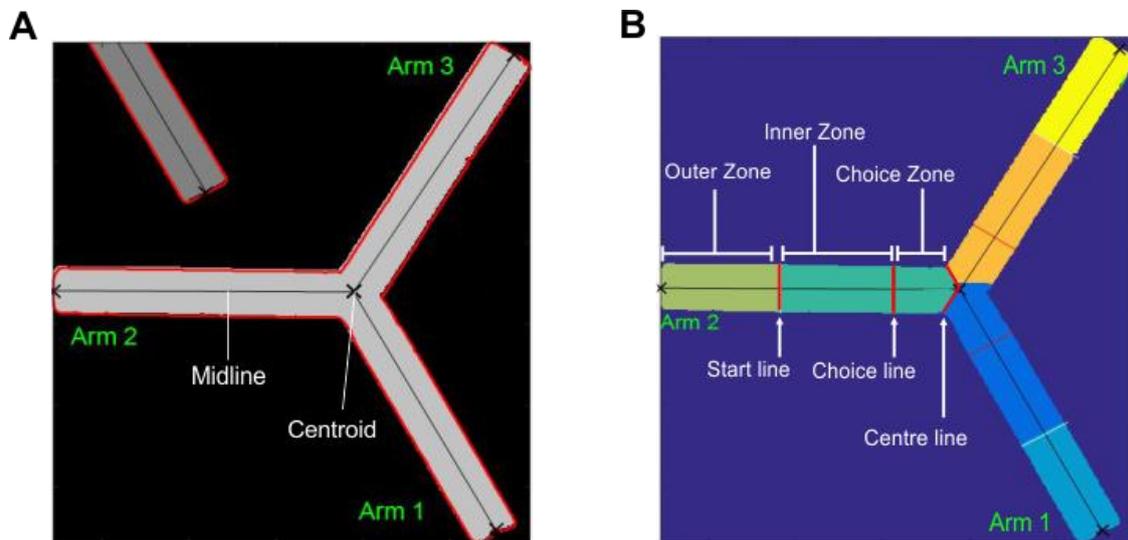


Figure 4.3. Defining the maze regions. A. A maze mask (grey) and background (black). The red line shows the maze perimeter. The arm midlines and maze centroid are shown. Arms are numbered according to their angle from the centre, starting with the maze closest to the x-axis in the lower-right quadrant, and proceeding clockwise from there. The 'Midline' is used to gather statistics on an ant's position within the maze. **B.** A maze showing the different zones of the arms coloured as blue, green and yellow for arms 1, 2 and 3 respectively within a dark blue background. The 'Start', 'Choice' and 'Centre' lines indicate the locations at which an ant's position within the maze was determined. The 'Inner' and 'Choice' zones are on the regions in which velocity was calculated.

4.3.6 Image processing

Custom built software was used to analyse the tracks taken by ants within the mazes. Firstly, the position of the ant in each maze is extracted automatically. These are then checked by eye to ensure there are no gaps in the ant's routes, and that the ant is correctly located within

each maze (Fig. 4.4). If breaks were found within a route it was omitted from analysis. The analysis started again when the ant re-entered the 'Outer Zone' of an arm.

Secondly, the area within the maze in which the ant is deemed to make a left/right choice is located. The method by which a choice is defined is described as follows: An ant starts making a choice when it first crosses from the 'Outer zone' of an arm into the 'Inner Zone' which is 60% between maze centre and arm end. It then makes a left/right choice when it next exits from the 'Inner Zone' of one of the other arms into an 'Outer Zone'. This portion of the track is then deemed the choice portion. If the ant turns and goes back into the outer portion of the same arm it just walked along before making a choice (i.e. before exiting into one of the other arms), no choice is logged and the choice is reinitiated when it re-enters the 'Inner zone' (i.e. re-starts is when it next crosses into the inner zone of that arm).

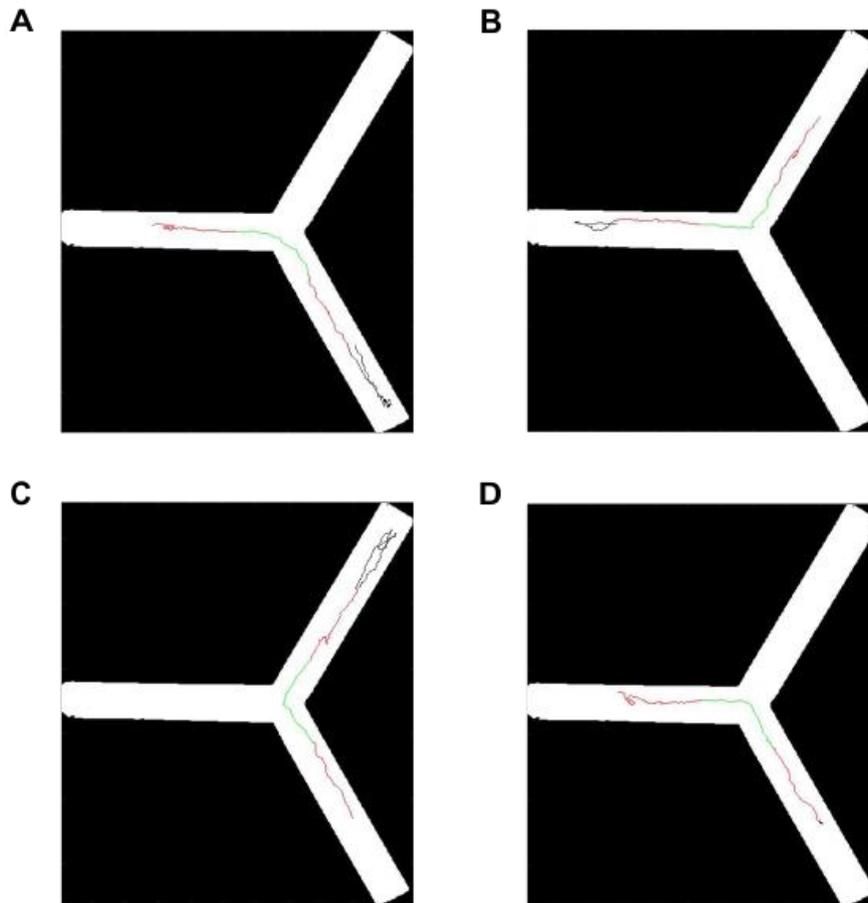


Figure 4.4. Ants make consecutive choices within the ‘Y’-maze. Individual tracks are extracted by the software, checked by eye and divided into left/right choices. The black line within the ‘Y’-maze is the track of the ant between the end of the previous choice (or start of the video for the first choice) and the start of the next. Red/green is the track during the choice. Green is the part of the track within the Choice Zone. **A.** Choice 1: LEFT - The ant starts in the bottom right arm and exits into the left arm. **B.** Choice 2: LEFT - The ant starts in the left arm and exits into the top right arm. **C.** Choice 3: LEFT- The ant starts in the top right arm and exits into the bottom right arm. **D.** Choice 4: LEFT - The ant starts in the bottom right arm and exits into the left arm.

Once individual tracks have been checked the software amalgamates the choices for the 30 minute period, and calculates the total number of left and right turns for each trial from each arm (Fig. 4.5).

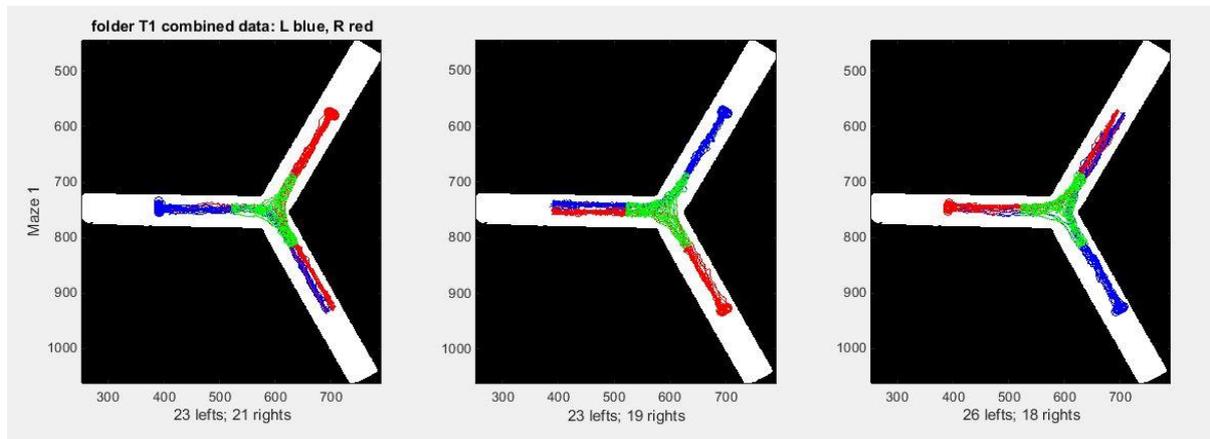


Figure 4.5. Summary plots of ants' tracks during a 30 minute trial. An example summary plot from individual #1 the entire 30 minute trial. Left turns are presented in blue, right with red, with green representing where the choice was made.

4.3.9 Assessing independence of turns

We assessed the temporal correlation of right turns across all individuals using the `autocorrelation` function in Matlab. To determine whether right turns are independent from one another, we assessed the correlation between a right turn at a given step and any right turns that are N steps away in time, where we can pre-define N . For the analysis in this chapter, we pre-define N as 100, which is displayed as the lag on the x-axis. A standardised lag of 100 was used to ensure sufficient replicates for analysis. For comparison, we display the empirical data along with 100 bootstrapped replicates, showing what one would expect if there was true independence of right turns.

4.3.10 Assessing ant position within maze arms

We assessed the positions of ants at three locations (Start, Choice and Centre line) in the maze arm with respect to the left/right decision they made.

4.3.11 Assessing ant velocity within maze arms

We assessed the velocities of ants in two regions (Inner and Choice Zone) of the maze arm. Velocities, measured in metres per second (m/s), were recorded by taking an average on 5 points within each of these regions to get an average.

4.3.12 Statistical analysis

First, individual ants were assessed for their turning preference. The deviation of an individual from the expected binomial distribution ($p = 0.5$) was calculated using a 2-tailed Exact Binomial Test using the method of small P-values. These statistical tests were carried out using custom programs written in Excel (Microsoft). We then grouped all ant colonies together to test for turning preference among the population before splitting the population up and assessing turning preference within each colony. A G-test for heterogeneity (Sokal and Rohlf, 1997) was used to compare turning preference among the ants. To test for heterogeneity among the ants we calculated G heterogeneity (G_H) and two additional statistics, G pooled (G_P) to compare the pooled data across all ants and G total (G_T) the sum of G_H and G_P . In the text G_T is quoted, however, in all cases where G_T is significant this was due to heterogeneity among ants (G_H). The G-test for heterogeneity was carried out using custom written programs in Excel (Microsoft). A LI score was also calculated for each individual (See Materials and Methods, Chapter 2).

The significance bounds used to assess independence of right turns use Box *et al.*'s (1994) approach (equations 2.1.13 and 6.2.2, pp. 33 and 188, respectively) and were computed in Matlab using the `autocorrelation` function in the following way:

$$\text{sigmaNMA} = \text{sqrt}((1+2*(\text{acf}(2:\text{numMA}+1))*\text{acf}(2:\text{numMA}+1)))/N);$$

$$\text{bounds} = \text{sigmaNMA}*(\text{numSTD};-\text{numSTD});$$

where *numMA* is the number of time steps after which one would assume zero autocorrelation. We use 0 because we are assuming no autocorrelation. Where *numSTD* specifies the number of standard deviations of the sample ACF estimation error, in which case we use 2.

The software package R (version 3.0.2) was used for the following statistical analyses (R Development Core Team, www.cran.r-project.org). Independent samples *t*-tests (Baldi and Moore, 2014) were used to compare the mean distance of ant tracks left and right of the midline. A chi-square test of independence (Baldi and Moore, 2014) was used to compare the proportions of tracks left and right of the midline at the 'Start', 'Choice' and 'Centre' lines.

A Pearson's product-moment correlation (Baldi and Moore, 2014) was computed to test for significant relationships between: (i) distances to midline between the 'Start' line and 'Centre' line, 'Start' line and 'Choice' line and 'Choice' line and 'Centre' line; (ii) the average velocities in the 'Inner' and 'Choice' zones; (iii) the average distance to midline between the 'Start' line and 'Centre' line; and (iv) the average speed between the 'Start' line and 'Centre' line. A dependent samples *t*-test (Baldi and Moore, 2014) was conducted to compare the average speed between the 'Inner Zone' and 'Choice Zone'. A Spearman's rank-order correlation (Baldi and Moore, 2014) was computed to test for a significant relationship between the proportion of right forelimb reaches and the proportion of right turns.

4.4 Results

4.4.1 Ants display a turning preference

Eight four ants were assessed for turning preference but eight ants completed fewer than 10 turns and were omitted from all subsequent analysis. The remaining 76 ants made over 7400 turns in total, with each individual averaging 97.4 turns (Fig. 4.6). Of these ants, ~42.1% displayed a significant turning preference (Tables 4.1 – 4.3), 47% of which were left biased and 53% of which were right biased. This population of ants had a MLI of 0. Colony C#1 – C#3 had a MLI of 0.03, - 0.04, 0.02 respectively.

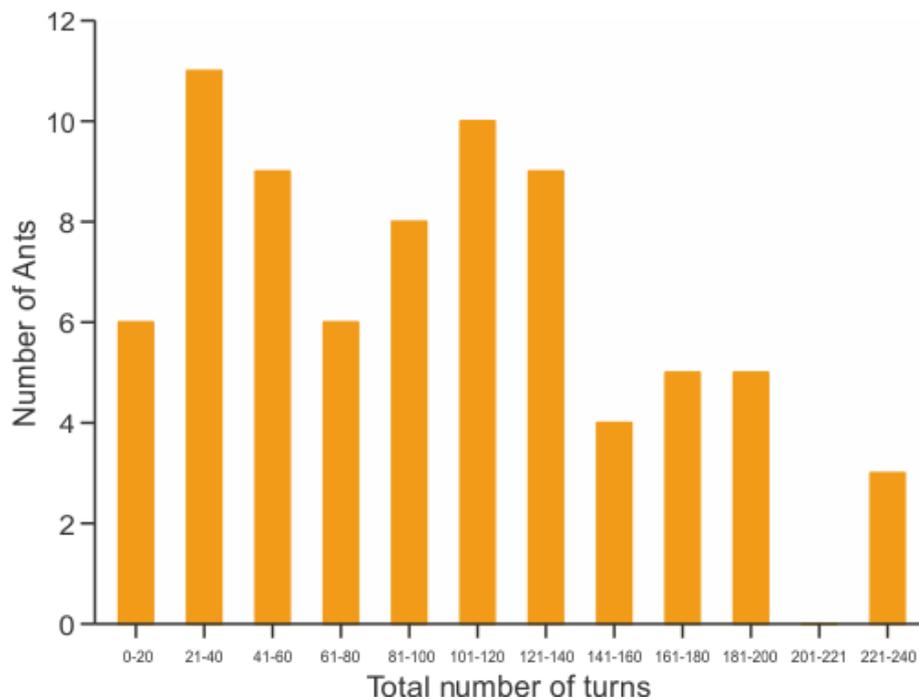


Figure 4.6. Frequency distribution of the total number of turns taken by ants within the 'Y'-maze.

Table 4.1. Binomial Exact tests of goodness-of-fit for right turns for C#1 ants. Data from Figure 4.5A; N = 24. Significant differences are denoted with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Ant	Right turns/ Total turns	2-tailed probability	Laterality Index
1	72/130	0.221	0.11
2	46/94	0.838	-0.02
3	26/94	0.109	-0.20
4	11/16	0.144	0.38
5	16/61	0.000176***	-0.48
6	25/52	0.784	-0.04
7	7/32	0.00132***	-0.56
8	23/27	0.00018***	0.70
9	25/57	0.358	-0.12
10	60/136	0.171	-0.12
11	65/105	0.0148*	0.24
12	44/81	0.44	0.09
13	31/40	0.000431***	0.55
14	37/53	0.00384***	0.40
15	57/80	0.000127***	0.43
16	38/79	0.738	-0.04
17	21/46	0.56	-0.09
18	24/59	0.155	-0.19
19	12/19	0.263	0.26
20	18/36	1	0
21	8/21	0.286	-0.24
22	57/109	0.634	0.05
23	3/15	0.0213*	-0.60
24	8/18	0.648	-0.11

Table 4.2. Binomial Exact tests of goodness-of-fit for right turns for C#2 ants. Data from Figure 4.5B; N = 28. Significant differences are denoted with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Ant	Right turns/ Total turns	2-tailed probability	Laterality Index
1	73/134	0.302	0.09
2	88/162	0.273	0.09
3	45/114	0.0248*	-0.21
4	16/52	0.00549**	-0.38
5	64/132	0.729	-0.03
6	53/123	0.127	-0.14
7	122/184	0.00000861***	0.33
8	18/72	0.0000169***	-0.50
9	72/177	0.0132*	-0.19
10	84/141	0.0231*	0.19
11	39/88	0.289	-0.11
12	67/231	0.00000000101***	-0.42
13	135/234	0.0187*	0.15
14	7/25	0.029*	-0.44
15	56/154	0.000692***	-0.27
16	15/25	0.327	0.20
17	15/32	0.728	-0.06
18	9/14	0.302	0.29
19	83/180	0.298	-0.08
20	95/138	0.00000812***	0.38
21	23/87	0.00000850***	-0.47
22	23/51	0.488	-0.10
23	69/91	0.000000535***	0.52
24	11/23	0.839	-0.04
25	47/111	0.108	-0.15
26	116/196	0.0101*	0.18
27	76/183	0.022*	-0.17
28	7/21	0.134	-0.33

Table 4.3. Binomial Exact tests of goodness-of-fit for right turns for C#3 ants. Data from Figure 4.5C; N = 24. Significant differences are denoted with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Ant	Right turns/ Total turns	2-tailed probability	Laterality Index
1	79/163	0.696	-0.03
2	65/87	0.00000288***	0.49
3	56/106	0.562	0.06
4	65/116	0.195	0.12
5	57/107	0.501	0.07
6	35/69	0.905	0.01
7	26/51	0.89	0.02
8	82/156	0.523	0.05
9	30/38	0.000294***	0.58
10	119/168	0.0000000466***	0.42
11	129/195	0.00000567***	0.32
12	16/41	0.164	-0.22
13	59/114	0.709	0.04
14	50/95	0.610	0.05
15	59/132	0.225	-0.11
16	37/112	0.00031***	-0.34
17	52/117	0.231	-0.11
18	78/130	0.0227*	0.20
19	57/230	0.00000000000000589***	-0.50
20	54/125	0.13	-0.14
21	31/87	0.00734**	-0.29
22	67/148	0.251	-0.09
23	122/199	0.0014***	0.23
24	5/13	0.424	-0.23

Individual ants differed significantly in the strength and direction of their bias (G-test, $G_T = 519.7$, 75df, $p < 0.005$, $N = 76$; Fig. 4.7). However, taken together ants show no significant difference between the overall proportion of left and right turns (G-test, $G_P = 0.06$, 75df, $p > 0.005$, $N = 76$; Fig. 4.7). This suggests that individual ants differ in the strength and direction of their turning preference at the individual-level but that there is no directional turning preference at the colony or population-levels.

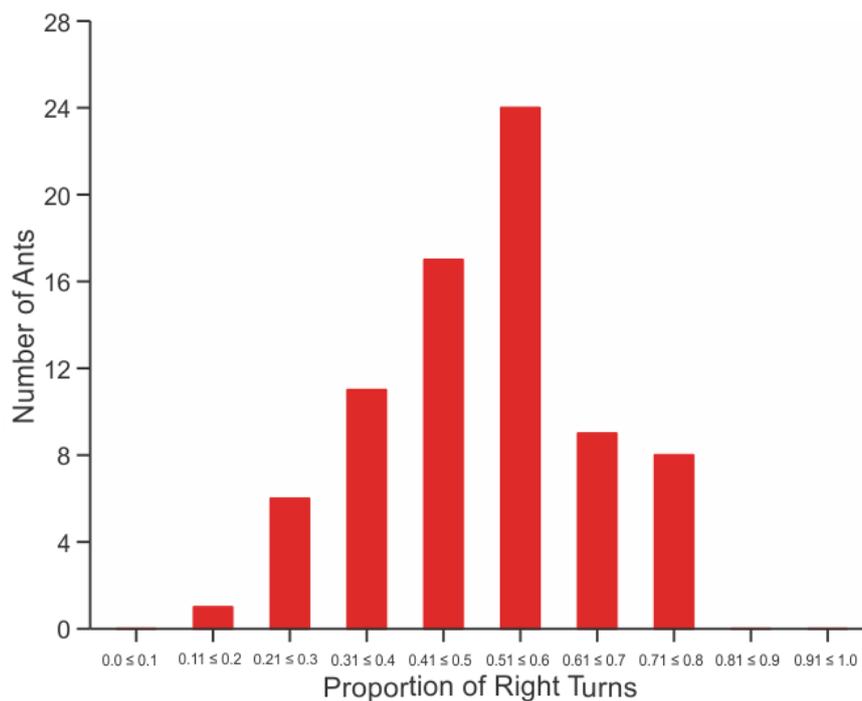


Figure 4.7. Frequency distribution of right turns from all ants. Frequency distribution of the proportion of right turns completed by all red wood ants over a period of 30 minutes ($N=76$).

Within colony #1, 24 ants made on average 59.6 ± 35.9 turns (Fig. 4.7A). This colony displayed significant heterogeneity among individuals (*G*-test, $G_T = 52.16$, $23df$, $p < 0.005$, $N = 24$) suggesting that they differed significantly in their strength and direction of their bias (see also Table 4.1). Again, taken together there was no significant difference between the overall proportion of left and right turns among the ants (*G*-test, $G_p = 0.96$, $23df$, $p > 0.005$, $N = 24$).

Within colony #2, 28 ants made on average 113.4 ± 66.6 turns (Fig. 4.7B). This colony displayed significant heterogeneity among individuals (*G*-test, $G_T = 112.1$, $27df$, $p < 0.005$, $N = 28$) suggesting individuals differed significantly in the strength and direction of their bias (see also Table 4.2). Again, taken together there was no significant difference between the overall proportion of left and right turns among the ants (*G*-test, $G_p = 4.46$, $27df$, $p > 0.005$, $N = 28$).

Within colony #3, 24 ants made on average 116.6 ± 53.3 turns (Fig. 4.7C). Like colony #1 and #2, this colony displayed significant heterogeneity among individuals (*G*-test, $G_T = 98.9$, $23df$, $p < 0.005$, $N = 24$) suggesting individuals differ significantly in the strength and direction of their bias (see also Table 4.3). Again, taken together there is no significant difference between the overall proportion of left and right turns among the ants (*G*-test, $G_p = 1.33$, $23df$, $p > 0.005$, $N = 24$).

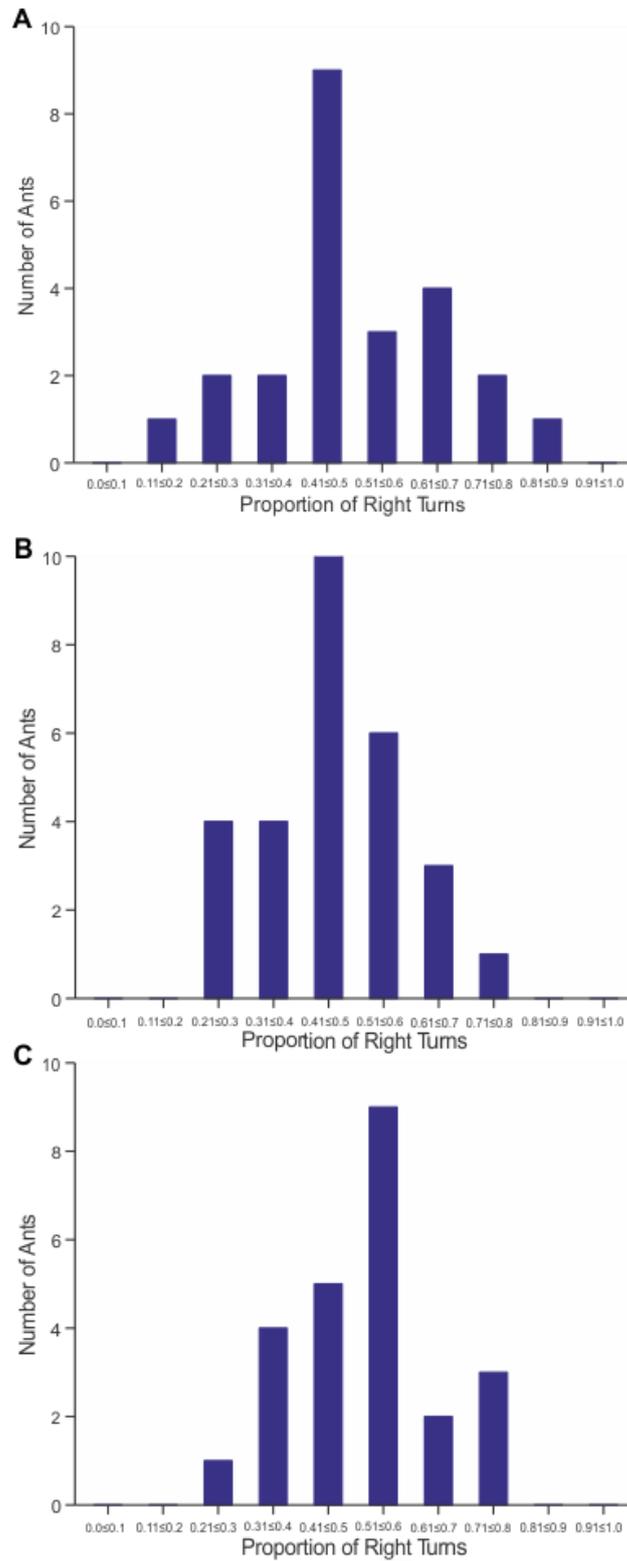


Figure 4.8. Frequency distributions of the proportion of right turns completed by red wood ants from different colonies. **A.** Colony #1 ($N = 24$). **B.** Colony #2 ($N = 28$) **C.** Colony #3 ($N = 24$).

4.4.3 Independence of choices in the 'Y'-maze

Individual ants made a series of left and right turns with each ant varying in the number of total turns during the 30 minute trial (Fig. 4.9).

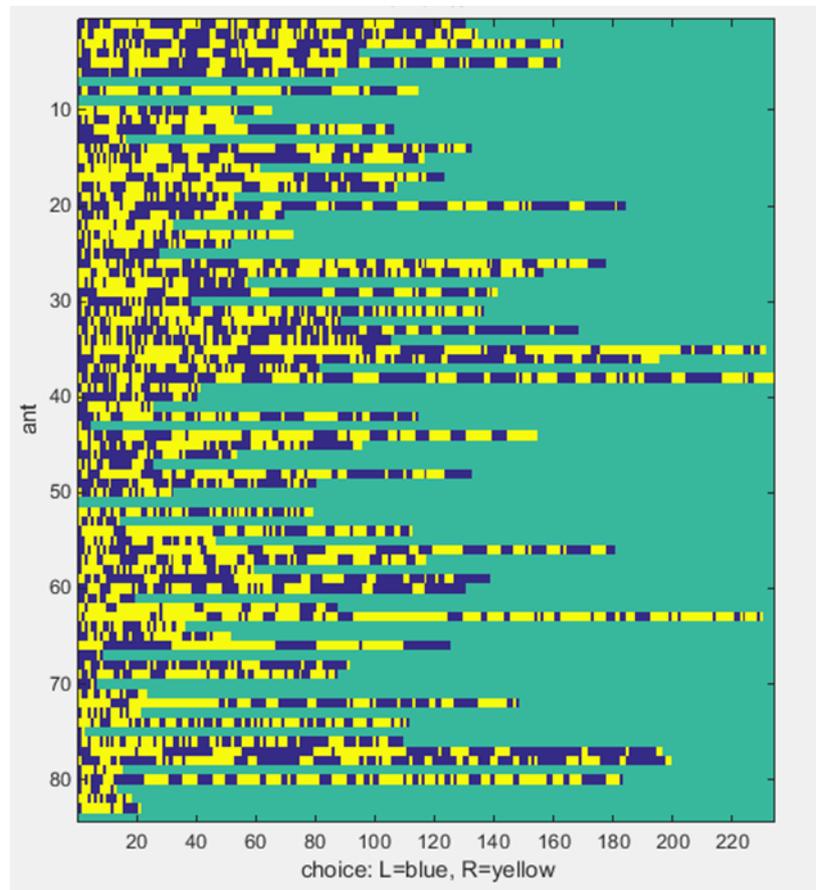


Figure 4.9. Raster plot showing sequence of rights and lefts. A total of 84 ants completed a series of right and left turns within the mazes, with individuals varying in their total number of turns over 30 minutes. The y-axis represents the individual ant and the x-axis represents the number of choices they made. Rights are in yellow and lefts and in blue.

A turn in one direction may influence subsequent turns. We assessed the temporal correlation of turns from each individual to determine whether turns in the sequence are independent from one another. For this analysis, we chose ants that completed more than 100 turns each to ensure that a sufficient number of total turns were available for the analysis. Of the 36 ants that completed more than 100 turns each, 33 displayed a temporal correlation (Fig. 4.10). Significance bounds were calculated for each ant (see Methods) and displayed as dashed horizontal lines. Empirical data falling outside these bounds indicates non-independence of right turns. Typically, there is a significant correlation at lags ± 2 . The autocorrelation functions also showed a characteristic sinusoidal oscillation, which is strongly indicative of an ant making a series of left turns and then switching to make a series of right turns.

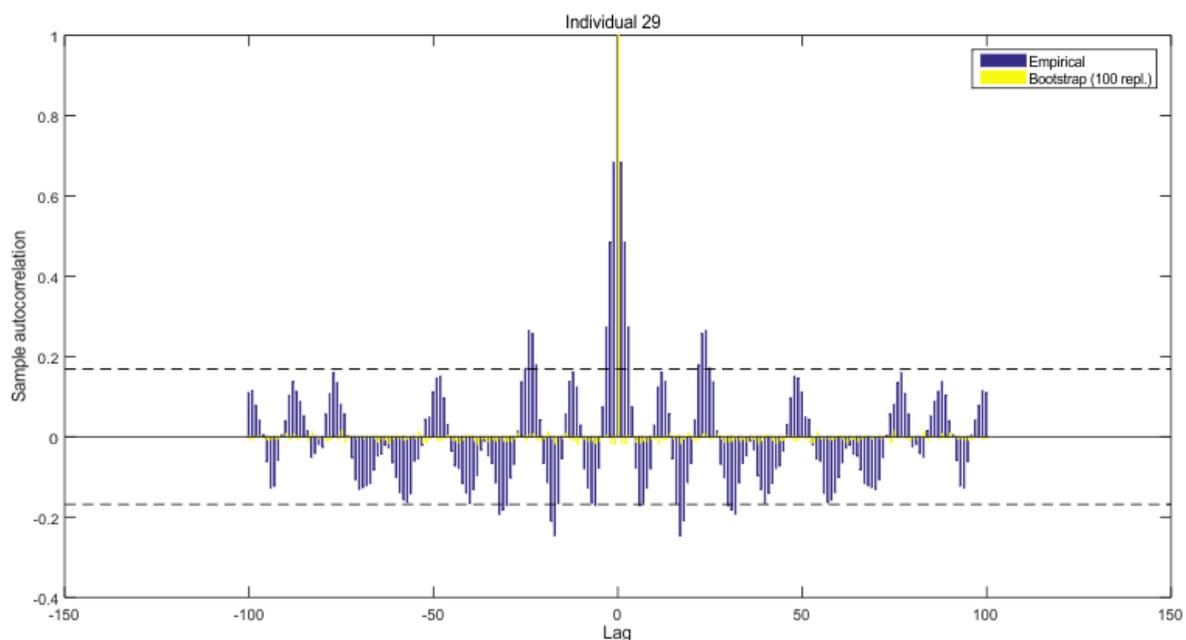


Figure 4.10. Turns are temporally correlated with a single right turn. Individual #29's autocorrelation data. A representative example auto-correlation of right turns with a single right turn reveals that there is a relationship between one right turn and any previous or subsequent right turns. The significance bounds here are indicated by dashed horizontal lines.

Blue bars indicate the empirical data from individual 29 and the yellow bars indicate an expected distribution based on a random sequence generated over 100 replicates.

Only three ants showed no temporal correlation suggesting that their turns were independent from one another (Fig. 4.11).

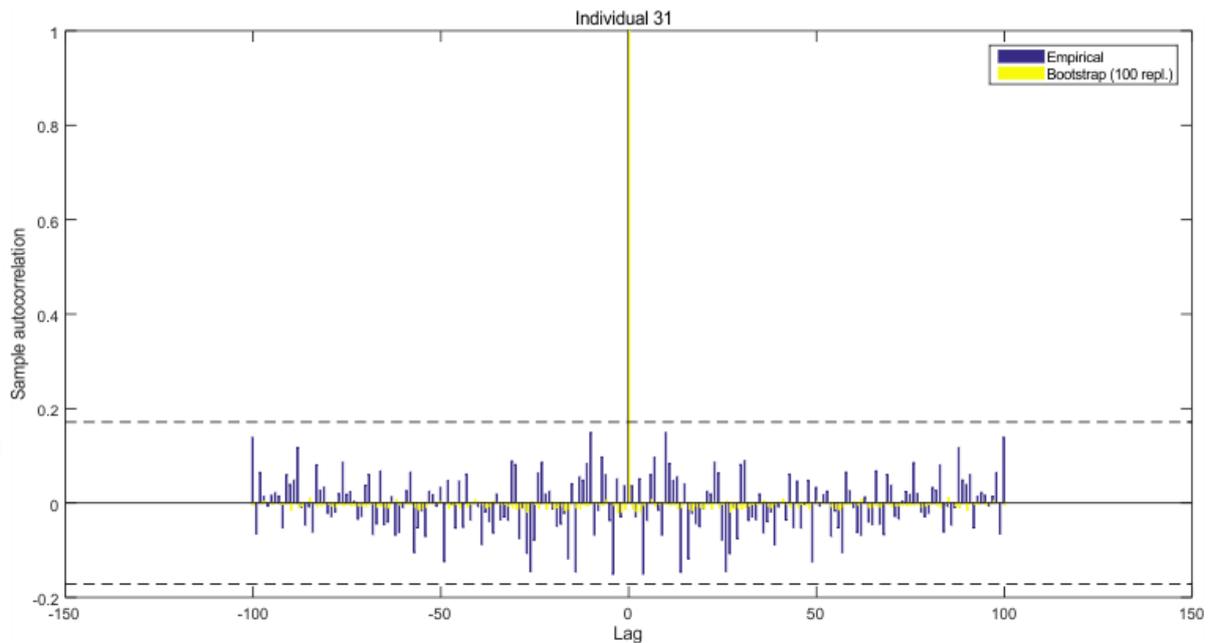


Figure 4.11. A few individual's right turns are not temporally correlated with a single right turn. Individual #31's autocorrelation data. A representative example autocorrelation of right turns with a single right turn reveals that there is no relationship between one right turn and any previous or subsequent right turns. The significance bounds are represented by dashed horizontal lines. Blue bars indicate empirical data from individual 31 and the yellow bars indicate an expected distribution based on a random sequence generated over 100 replicates.

4.4.3 Behaviour within the 'Y'-maze

Ants can assume different positions within the maze whilst walking along arm; they can, for example, remain close to the midline of the maze, with approximately an equal distance either side of their body from the wall (Fig. 4.12A) or, alternatively, they can walk close to the side of the maze wall and maintain this close proximity as they approach the centre (Fig. 4.12B).

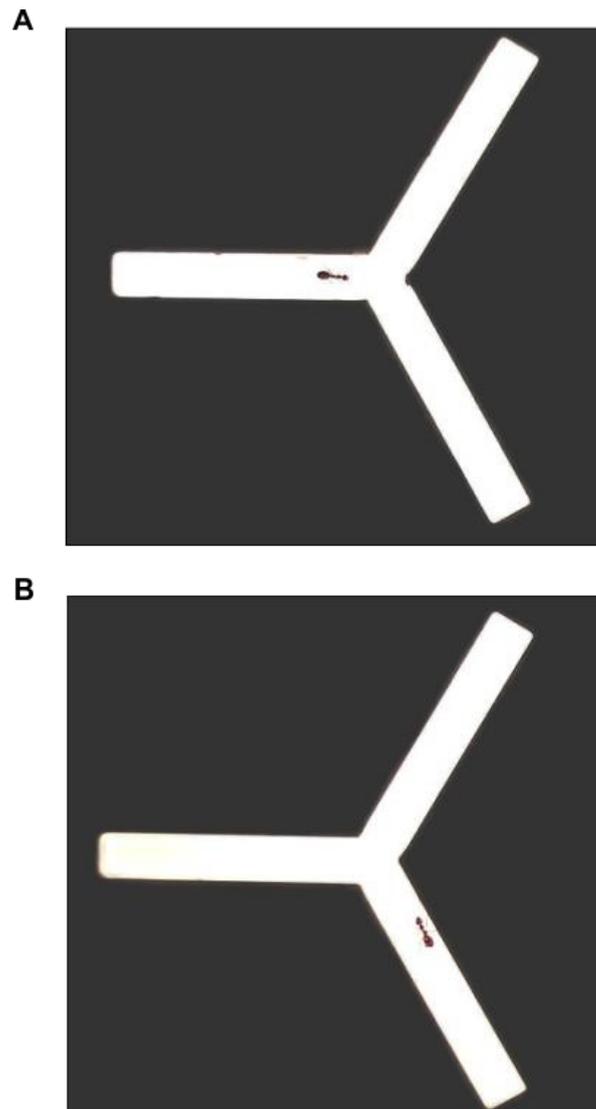


Figure 4.12. Ant behaviour within the maze arm. A. An image showing an ant maintaining a central position with respect to the midline whilst walking along the maze arm **B.** An image showing an ant maintaining close proximity to the maze wall.

Whilst walking along an arm of the maze ant positions differ with respect to the eventual left/right choice they make so that ants' routes are biased from the mid line towards the side they choose (Fig. 4.13). A larger proportion of tracks that resulted in a left can be found left of the midline at the 'Start' (Start, chi-square = 2665.1, 1 *df*, $p < 0.005$), 'Choice' (Choice, chi-square = 2458.8, 1 *df*, $p < 0.005$) and 'Centre' (Centre, chi-square = 4159.3, 1 *df*, $p < 0.005$) lines preceding a left choice than a right (Figs. 4.13 Ab, Bb and Cb respectively).

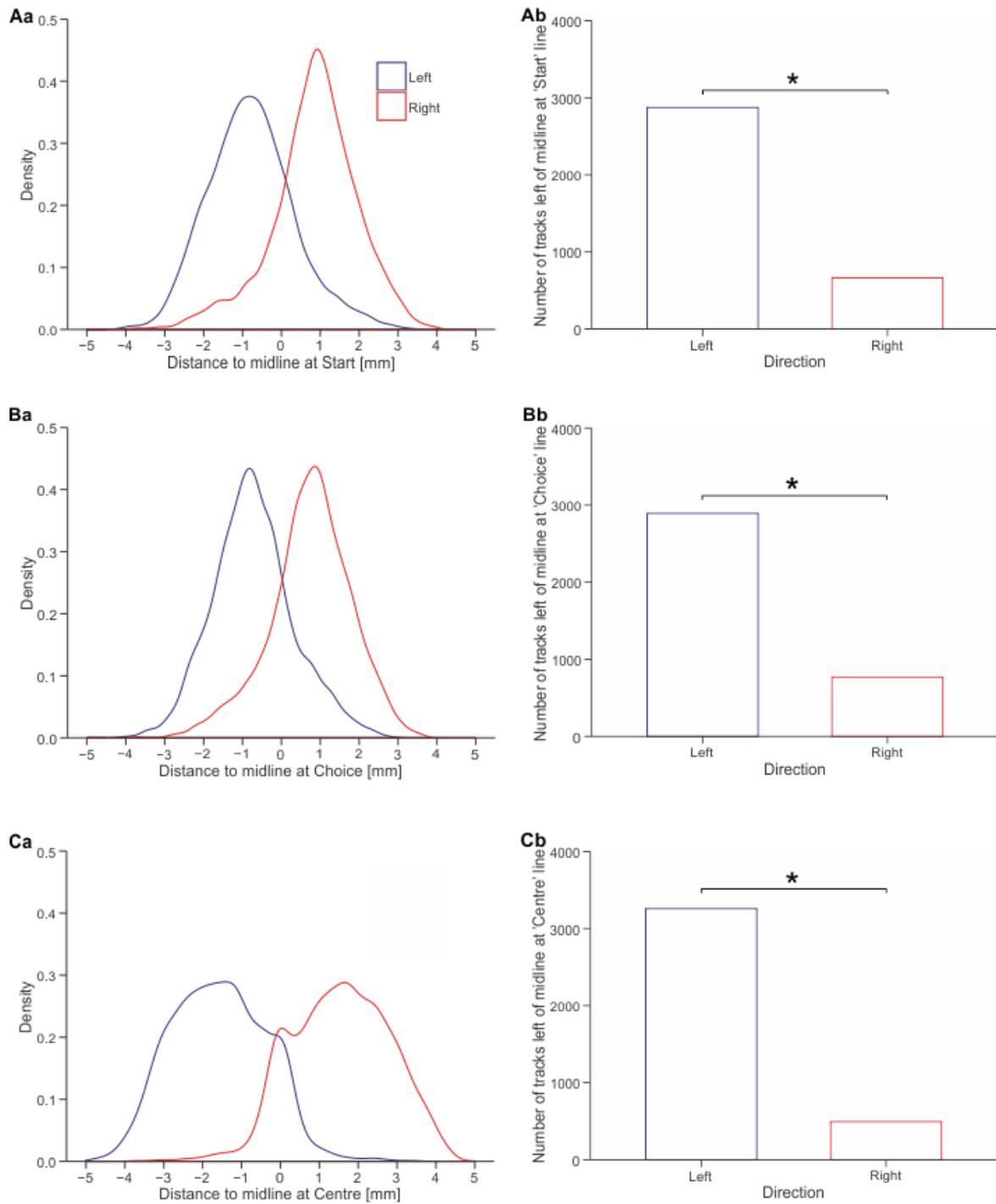


Figure 4.13. Ant positions within the maze arm. Distributions of the distances to the midline when the ants cross the **Aa**. 'Start' line. **Ba**. 'Choice' line. **Ca**. 'Centre' line. Negative values on the x-axis indicate left of the midline and positive vales indicate right of the midline. Bar charts compare the number of tracks left of the midline that result in a left or right at **Ab**. 'Start' line. **Bb**. 'Choice' line. **Cb**. 'Centre' line.

Tracks were positioned significantly further to the right (mean = 1.17 ± 0.77 mm) of the midline compared to the left (mean = 1.12 ± 0.77 mm) when comparing the absolute distances from the midline at the 'Start' line (Independent samples *t*-test; $t = -2.93$, $7402df$, $p < 0.01$; Fig. 4.14A). However, there was no significant difference between the distance to the midline at the right (mean = 1.07 ± 0.72) when compared to the left (mean = 1.05 ± 0.72 mm) at the 'Choice' line (Independent samples *t*-test; $t = -1.2$, $7402df$, $p > 0.05$; Fig. 4.14B). There was also no significant difference between the distance to the midline at the right (mean = 1.67 ± 1.1 mm) when compared to the left (mean = 1.67 ± 1.07 mm) at the 'Centre' line (Independent samples *t*-test; $t = 0.36$, $7402df$, $p > 0.05$; Fig. 4.14C). These results suggest that tracks are positioned further to the right when far away from the maze centre but become equally distant from the mid line as they approach to the maze centre.

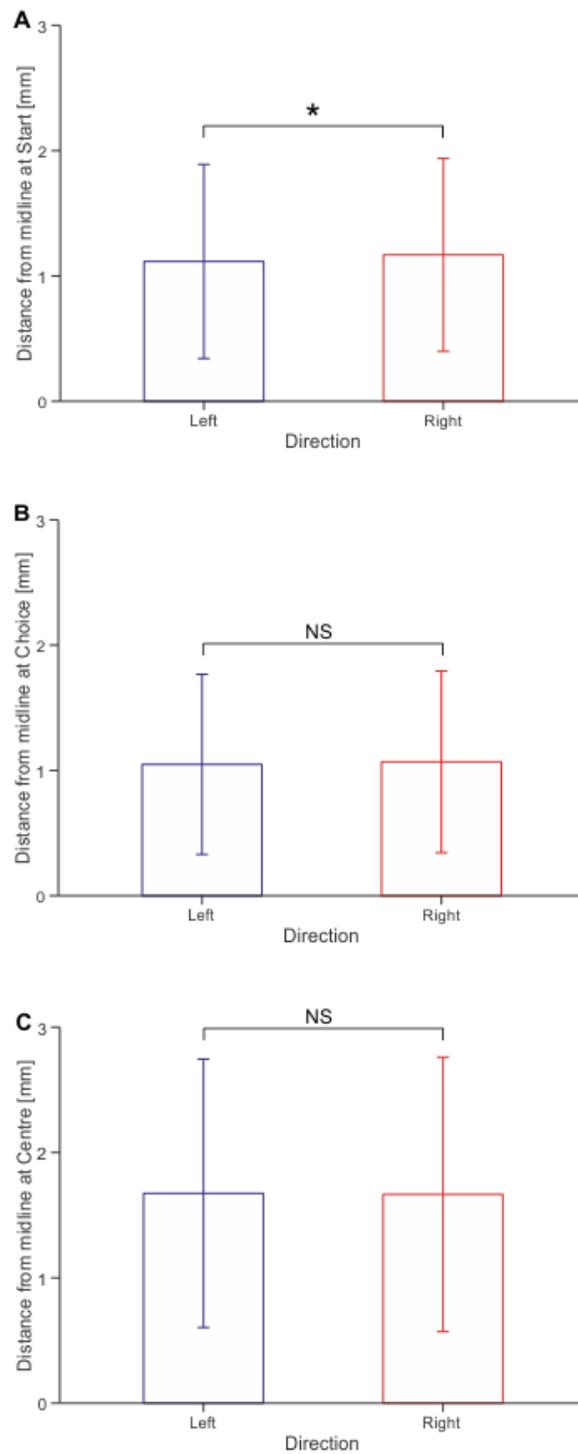


Figure 4.14. Bar charts comparing the absolute distance from the midline between the left and right at the **A.** ‘Start’ line. **B.** ‘Choice’ line. **C.** ‘Centre’ line. Error bars represent the standard deviation of the mean.

The distance from the midline at the 'Start' line was significantly positively correlated with the distance from the midline at the 'Choice' line (Pearson's product-moment correlation; $r = 0.78$, $7403df$, $p < 0.005$; Fig. 4.15A). This suggests that the distance from the midline at the 'Start' line is related to the distance from the midline at the 'Choice' line. Thus, the tracks stay on one side of the midline, between these two points.

We did the same analysis, assessing the relationship between the 'Start' line and the 'Centre' line (Pearson's product-moment correlation; $r = 0.66$, $7403df$, $p < 0.005$; Fig. 4.15B) and 'Choice' line and the 'Centre' line (Pearson's product-moment correlation; $r = 0.7$, $7403df$, $p < 0.005$; Fig. 4.15C) and found a significant relationship in both which suggests tracks tend to stay one side of the midline between these points.

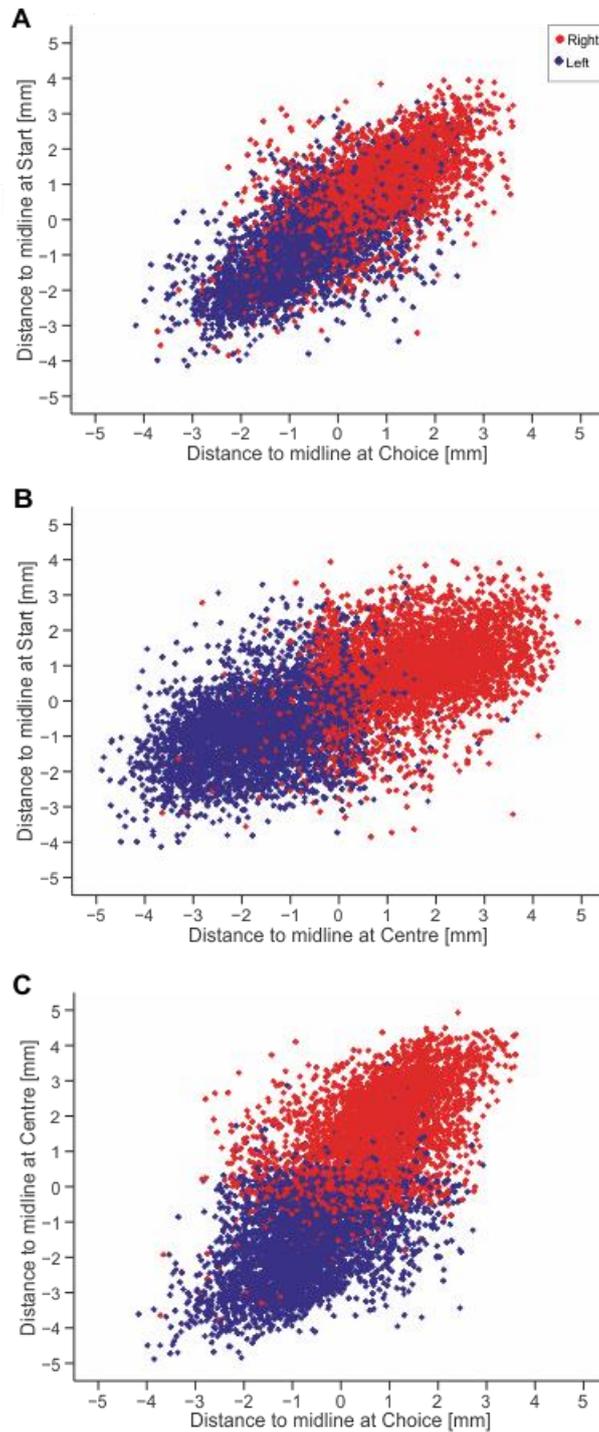


Figure 4.15. Ant tracks along the maze arm are related to one another. A. Distance to midline at ‘Start’ is related to distance to midline at ‘Choice’. **B.** Distance to midline at ‘Start’ is related to distance to midline at ‘Centre’. **C.** Distance to midline at ‘Centre’ is related to distance to midline at ‘Choice’. Negative values on the axis indicate left of the midline and positive values indicate right of the midline.

We determined the velocities of the ants in the 'Inner Zone' and 'Choice Zone' (Fig. 4.16Aa,b). We found a significant difference between the 'Choice Zone' (mean = 0.02 ± 0.009 m/s) and the 'Inner Zone' (mean = 0.019 ± 0.008 m/s) when comparing the speed between the two (Dependent samples *t*-test; $t = 14.76$, $N = 7405df$, $p < 0.05$; Fig. 4.16B), suggesting that ants speed up on approach to the centre of the maze.

We then assessed the relationship between the average velocity along the 'Inner Zone' and the 'Choice Zone'. We found a significant positive correlation between the two (Pearson's product-moment correlation; $r = 0.71$, $7403 df$, $p < 0.005$; Fig. 4.16C) which suggests that the velocity in the 'Inner Zone' is correlated with that in the 'Choice Zone'.

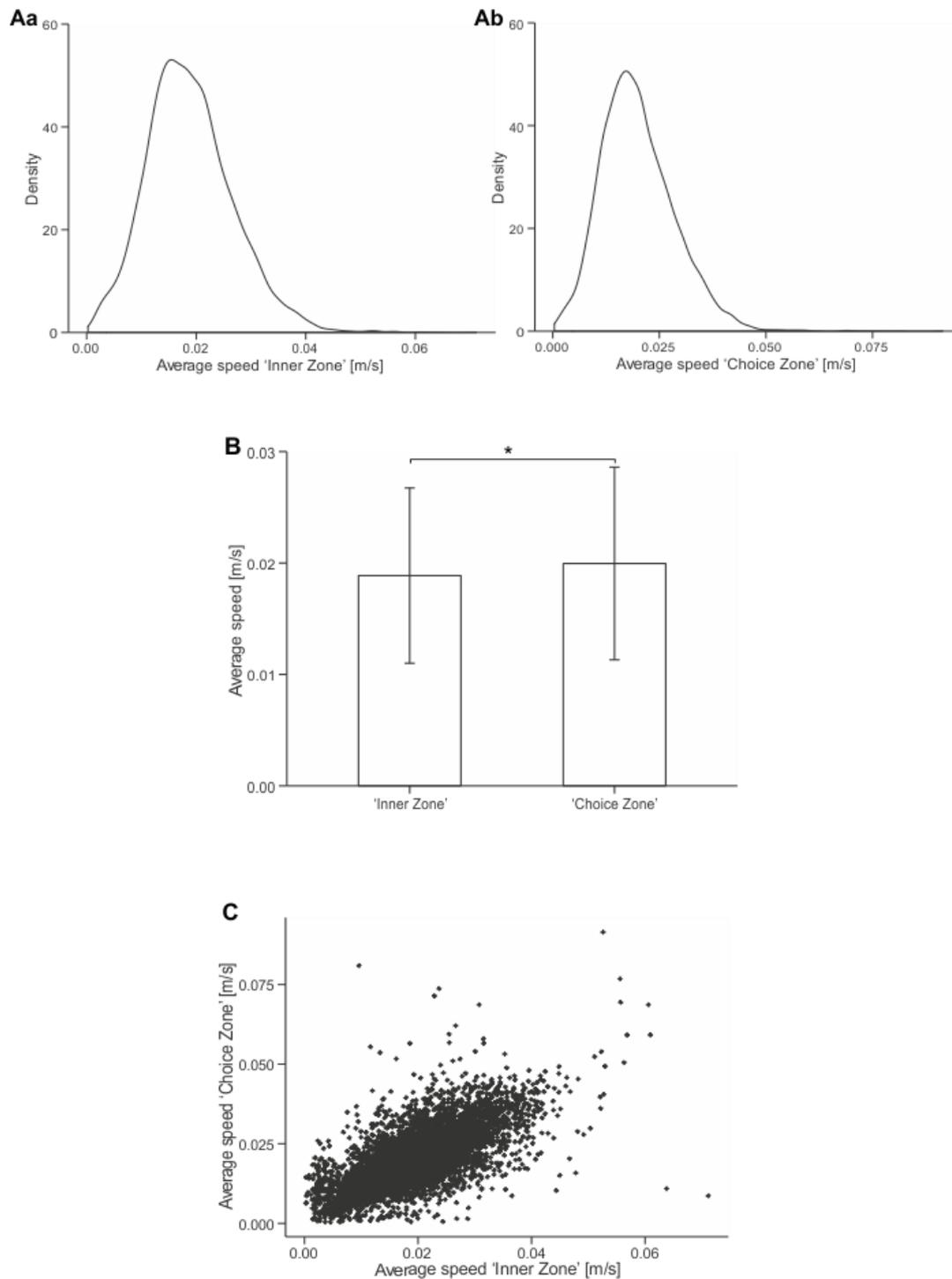


Figure 4.16. Ant speed within the maze arm. Aa. The distribution of track speeds in the 'Inner Zone'. **Ab.** The distribution of track speeds in 'Choice Zone'. **B.** Bar chart comparing the average speed between the 'Inner Zone' and the 'Choice Zone'. Error bars represent the standard deviation of the mean. **C.** Scatterplot showing the relationship between speeds within choice to centre zone and start to choice zone.

We measured the average distance to midline between the Start line to the Centre line (mean = 1.18 ± 0.74 mm; Fig. 4.17Aa, Ba) and the average velocity over the same distance (mean = 0.019 ± 0.008 m/s; Fig. 4.17Ba,b). We assessed the relationship between the two and found no correlation (Pearson's product-moment correlation; $r = -0.001$, $7403df$, $p > 0.91$; Fig. 4.17C), suggesting that where an ant is positioned with respect to the midline is unrelated to how fast it travels along the maze arm.

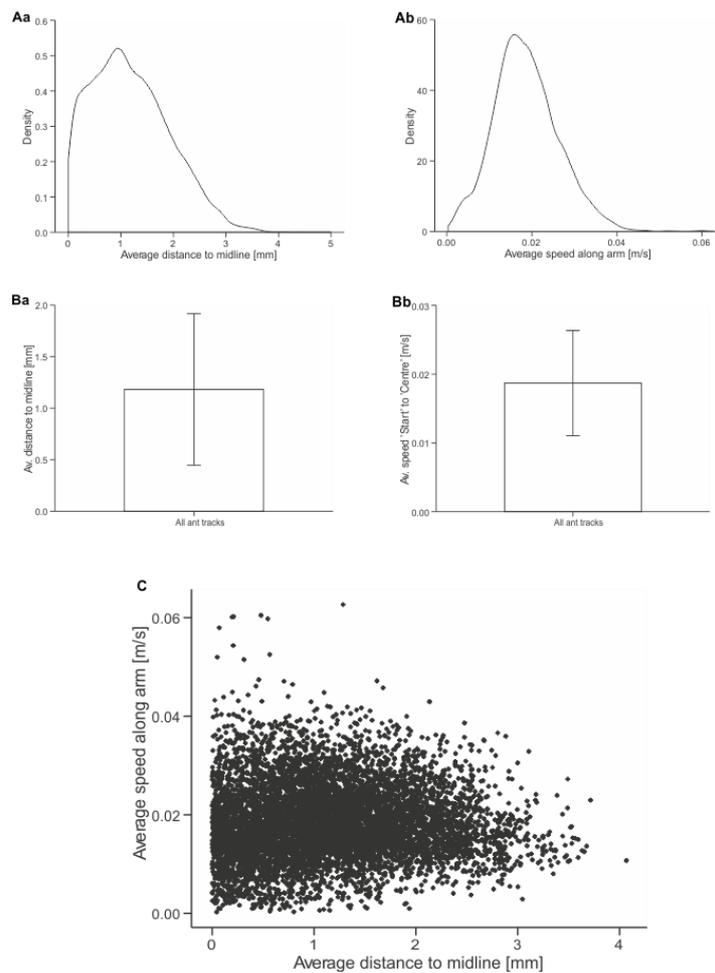


Figure 4.17. Distance to wall is unrelated to speed along maze arm. **Aa.** The distribution of average distance to midline between Start to Centre lines. **Ab.** The distribution of average speed from Start to Centre lines. **Ba.** Average distance to midline from 'Start' line to 'Centre' line **Bb.** Average speed from 'Start' line to 'Centre' line **C.** A scatterplot showing the relationship between A and B.

We then assessed the relationship between the proportion of right forelimb crosses from the previous chapter and the proportion of right turns in this chapter. We found no correlation between the two (Spearman's rank-order correlation; $\rho = 0.71$, $N = 19$, $p > 0.56$, $17df$; Fig. 4.17) which suggests that the direction of forelimb preference is unrelated to the preferred turning direction.

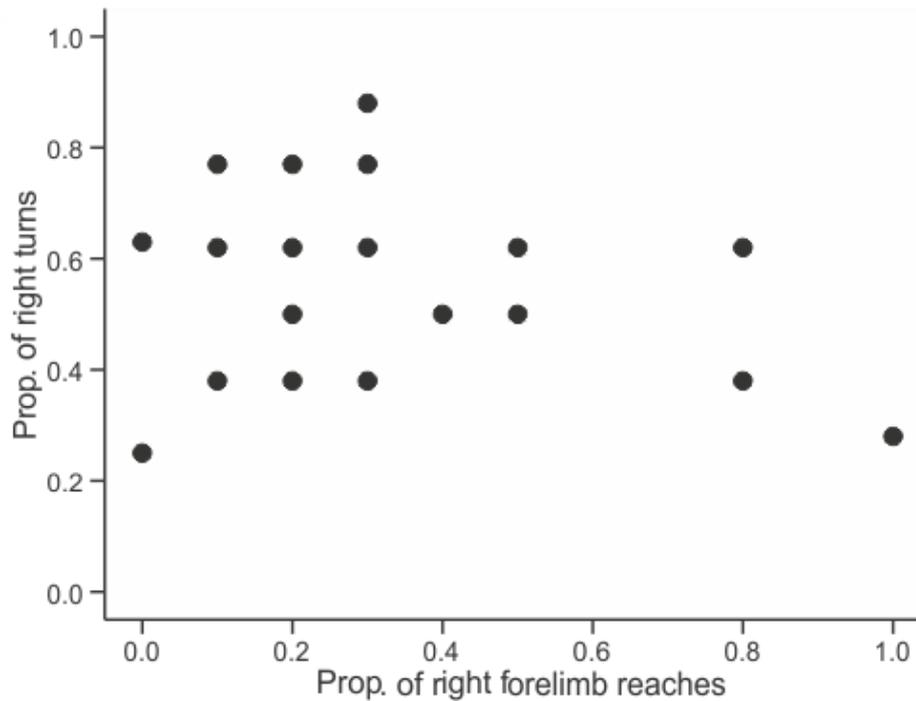


Figure 4.18. Forelimb preference is unrelated to turning preference in wood ants. A scatterplot showing no correlation between ant forelimb preference (previous chapter) and the same individual's turning preference ($N = 19$).

4.5 Discussion

The results demonstrate that individual ants show a turning preference in a 'Y'-maze paradigm, individuals varying in the strength and direction of their preference. The turning preference is exhibited at the individual-level only, with neither colony or population showing an overall directional preference. A temporal correlation of right turns across each individual's trial found that a right turn at one time point predicts subsequent right turns, suggesting that turns are not independent. We assessed the distribution of ant positions at various points within the maze and reveal that their behaviour is dominated by wall-following behaviour, which is likely the source of the non-independence of turns.

When considered in conjunction with the results of the previous chapter, the results suggest that red wood ants present two forms of lateralised motor output, one in the form of forelimb control and the other a turning preference. It is perhaps unsurprising that ants display a turning preference because forelimb and turning preferences are both an expression of the lateralised control of the legs. By directly comparing the results from the previous chapter however, we found no correlation between the two. Similarly, in fruit flies (*Drosophila*), which express both individual-level lateralisation in turning and wing-folding behaviour, there is no correlation between the two (Buchanan *et al.*, 2015). This suggests that there may be different neuronal components responsible for different lateralised behaviours. These results also add to the findings by Buchanan *et al.*, (2015) that genetically identical individuals are capable of displaying variation in the direction and strength of lateralised behaviour.

Ants display forelimb lateralisation at the individual level, with the same direction of preference shared with others in the colony, resulting in a colony-level lateralisation. In contrast, the individual's turning preference in the 'Y'-maze paradigm is not shared with others in the colony and is not expressed at the population level. These results are not consistent, therefore, with the hypothesis that population-level lateralisation is more likely to be found in co-operative, social species (Ghirlanda and Vallortigara, 2004; Anfora *et al.*, 2010). Other insect species provide evidence that is consistent with this hypothesis in 'Y'-maze paradigms. Within social species, Hunt *et al.*, (2014) showed that ants (*Temnothorax albipennis*) exhibit a

leftward turning bias at the population level. In species considered non-social, ladybirds (*Coccinella septempunctata*) and the fruit fly (*Drosophila*) display individual-level lateralisation (Girling *et al.*, 2007; Buchanan *et al.*, 2015). Inconsistent with the hypothesis however, in species considered non-social, the giant water bug (*Belostoma flumineum*) and the american cockroach (*Periplaneta americana*) both display population-level lateralisation for turning preference (Kight *et al.*, 2008; Cooper *et al.*, 2010).

The inconsistencies may arise because, in social species, the lateralised turning behaviour of an individual may not be relevant to others in the group and, therefore, the group need not show the same direction of lateralisation. Behaviours which involve direct contact with other group members, e.g. antennation during trophallaxis (Frasnelli *et al.*, 2012a) may be more likely displayed at the population-level whereas behaviours which only indirectly affect other group members may be less likely to be lateralised at the population-level. This situation is unlikely however given the predictions based on computational modelling in which turning behaviour is relevant to others within the group (Ghirlanda and Vallortigara, 2004), especially in escape response.

It is possible that the 'Y'-maze paradigm is not a viable technique to elucidate innate lateralised turning preference. Indeed, we found that a right turn in one trial is likely to be followed by a subsequent right turn in another. The ants appear to turn one way initially and then switch directions periodically leading to 'bursts' of lefts or rights in the sequence. This observed behaviour could be driven by thigmotaxis (Fraenkel and Gunn, 1961) which is the tendency for an organism to orient itself in space by mechanical contacts, for example through contact with the wall by the legs or antennae. Thigmotaxis can lead to wall-following behaviour which is the trend to move along edges (Creed and Miller, 1990). It is known that ants and termites use structural guidelines created by rocks, crest lines or grooves to orientate themselves (Jander and Daumer, 1974; Klotz and Reid, 1992, 1993; Klotz *et al.*, 2000). To investigate whether the maze walls were influencing the ants' behaviour we tracked their movements within the maze arms to quantify the spatial distribution prior to making a left/right choice. We found a relationship between the proximity to midline of the maze and the

subsequent left/right turn, suggesting ants exhibit positive thigmotaxis, keeping to the wall they are in contact with, which may influence left/right choices. Considering this, the first turn of each ant within the 'Y-maze' may only be appropriate here. In future studies, a range of analyses to both enhance the interpretation of results and to facilitate the validity of comparisons across species is critical.

If the 'Y'-maze paradigm is to be used in future work to investigate innate turning preferences, we encourage an extra level of processing in order to tease apart the mechanisms that may be driving the preference. Firstly, the turning preference could be driven by thigmotaxis which leads to wall-following. Further work could be done to investigate the contribution of the antennae whilst walking along the maze arms. It's possible that sensory lateralisation in the antennae could influence the left/right decision. In addition, given that we have shown that the ants exhibit a forelimb preference the legs could also have an effect on the left/right decision at the maze centre. Moreover, occlusion of the eyes whilst the ants are in the mazes could rule out the influence of visual lateralisation. To eradicate any possibility of thigmotactic behaviour it could be useful to investigate the turning preference in free roaming ants in open areas, recording the turning preference, like that shown in humans (Schaeffer, 1928; Souman *et al.*, 2009) and fruit flies (Buchanan *et al.*, 2015).

Chapter 5

General Discussion

5.1 The importance of lateralisation research

In many respects lateralisation research poses a model problem in biology. Its hierarchical nature entails cellular as well as fine and gross anatomical aspects that can be investigated through numerous experimental techniques including through electrophysiology and pharmacology; due to its presence across phyla its study involves comparative and phylogenetic issues; whilst its developmental origins require genetic and environmental approaches to study them and behaviourally it encompasses issues ranging from the simple to the complex. Lateralisation is pervasive at all levels of biological complexity requiring a multidisciplinary approach to understand both its proximate and ultimate causes.

This thesis approaches the issue of lateralisation behaviourally by testing insects for turning and forelimb preferences. Turning preferences are important because they are considered phylogenetically old, appearing in many species and across phyla/classes. By investigating turning preferences in insects it allows us to consider when this type of lateralised behaviour may have arisen and whether it is a result of homology or arisen independently. Furthermore, turning tendencies are considered to be coupled with other lateralised behaviours so that understanding their basis may aid in the understanding of other, related lateralised behaviours.

The origins and functions of limb preferences have been investigated extensively in humans for centuries (Rogers *et al.*, 2013). More recently, limb preferences have been documented extensively in vertebrates. Despite this, the distribution patterns of limb preferences, their role and functions are hotly debated. Previously, it was difficult to observe the relatively fast movements of small insect species but recent technology has allowed us to resolve high speeds to capture the limb movements of insects. This has allowed us to demonstrate that it is not only humans and vertebrates that possess limb preferences. This

has made insect species candidates for investigating the many aspects of lateralised behaviours.

5.2 The contributions of this thesis

This thesis has produced several contributions to the field of lateralisation research. In chapter two, we show that desert locusts (*Schistocerca gregaria*) are biased in the forelimb they use to reach across a gap in the substrate upon which they are walking. Unlike many other studies of lateralised behaviours, we tested whether the limb preference persisted over various contexts. We found that the limb preference exhibited by locusts is specific to gap-crossing behaviour unlike human handedness, for example, which is maintained over various contexts.

The locusts' preference is also expressed at the individual-level. Again, this contrasts with human handedness, which is expressed at the population-level. The individual-level limb preference of locusts is interesting because social species are predicted to exhibit population-level lateralisation, and even non-social species, such as mosquitos, that form swarms have been shown to produce population-level lateralisation (Benelli *et al.* 2015b). Locusts, which form large swarms but are not considered strictly social, likely maintain their individual-level of lateralisation through considerable selective pressure to avoid other locusts. To our knowledge, this is the first direct evidence of an insect or indeed any arthropod possessing individual-level limb preference despite having otherwise symmetrical limbs.

With our results, we provide evidence to support the idea that small, insect nervous systems are capable of producing sophisticated lateralised behaviour in the form of their limb movements. Models based on small systems of neurons provided by insects offer a unique opportunity to address the question of how symmetrical neuronal assemblies deviate to create behavioural lateralisation (Rogers *et al.*, 2013).

In chapter two, we also show that strongly biased locusts perform better during gap-crossing, making fewer errors with their preferred forelimb. Strongly lateralised locusts make fewer targeting errors than their weakly lateralised counterparts and generally, the number of targeting errors negatively correlates with the strength of forelimb lateralisation. Lateralised

behaviours are widespread in both vertebrates (Rogers and Andrew, 2002; Rogers *et al.*, 2013) and invertebrates (Frasnelli, *et al.*, 2012b; Frasnelli, 2013), suggesting that lateralisation is advantageous. Yet evidence demonstrating proximate or ultimate advantages remains scarce, particularly in invertebrates or in species with individual-level lateralisation. To our knowledge, our study of locust limb preference provides the first evidence that stronger lateralisation confers an advantage in terms of improved motor control in an invertebrate with individual-level lateralisation. With these further results from chapter two we would like to emphasise that there are direct benefits to lateralised behaviours, even in small insect nervous systems that exhibit individual-level lateralisation.

In chapter three, for the first time, lateralisation is considered not just at the individual-level and/or population-level, but also at the colony level. We demonstrate forelimb preference in another arthropod species, the red wood ant (*Formica rufa*) in the same gap-crossing paradigm. As was the case in locusts, we found some individuals preferred to use their right forelimb, others their left and the remainder showing no preference – the hallmark of individual-level lateralisation. Interestingly, the majority of individual ants within a colony show a preference for using the same forelimb to cross the gap. This directional preference differs between colonies, thus, wood ants exhibit two forms of lateralisation at the individual and colony-levels the latter being an entirely novel, previously undescribed form of lateralisation. This emphasises that it is possible to overlook the impact of lateralisation at particular levels of biological organisation, for example the colony, especially when investigating lateralisation in eusocial insects.

In chapter four, we show that red wood ants are lateralised in the movement of their entire body, *i.e.* display a turning preference, within a 'Y'-maze paradigm. In this context, the lateralisation is displayed at the individual-level only, with neither the colony nor the population showing any directional bias. We find no correlation between the lateralisation of limb preference and the lateralised turning of the whole body, suggesting the independence of lateralised neural components. These results emphasise the complex nature of lateralised behaviours. Although an animal may be lateralised in more than one motor output, and that

these behaviours may be coupled, it may require a more comprehensive understanding of the underlying neural components to understand the interplay between the two.

In chapter four, we further analysed the ants' positions within the 'Y'-mazes prior to making left/right choices. Through close inspection of the ants' behaviour in these mazes we reveal that their behaviour is dominated by wall-following behaviour. Therefore, we question the validity of using the 'Y'-maze paradigm when assessing turning preferences and suggest it is more likely to be assessing the persistence and switching of wall-following. This is important because many studies investigating the lateralised turning preferences in animals, especially in invertebrate species, rely on 'Y'-maze paradigms. With the results of this chapter suggest that a great deal of care must be taken to ensure that low-level behaviours do not undermine the interpretation of the choices of the animal under investigation.

In general, with the finding that insect nervous systems are capable of producing both lateralised output in their turning behaviour and limb preference, despite differing in some respects, it seems that insects share the attribute of lateralisation with many vertebrates. This raises the question of whether these lateralised behaviours share a common ancestry or developed independently in the two taxa.

5.3 Lateralisation and social organisation

The hypothesis that lateralisation is an evolutionary stable strategy (ESS) makes a straightforward prediction that social organisms should show lateralisation at the population-level whereas non-social organisms retained lateralisation at the individual-level only. Locusts express polyphenism, an extreme form of phenotypic plasticity, by which major changes in their morphology, physiology and behaviour are produced in response to the density of other locusts (Fusco and Minelli, 2010; Moczek, 2010; Simpson *et al.*, 2011). Consequently, their social organisation relies on the presence of other locusts. Taken alone, the distribution of lateralised locusts from our results are indicative of a non-social animal according to the ESS hypothesis, which naively might be interpreted as inconsistent with the hypothesis. Despite locusts aggregating to form large 'social' groups in their gregarious phase, however, the

distribution of their lateralised behaviour is not necessarily surprising because there is fierce competition within a swarm with individuals being cannibalized by other locusts (Bazazi *et al.*, 2008). Consequently, there may be considerable selective pressure to avoid predictability, maintaining individual-level lateralisation. For future work it would be extremely interesting to compare lateralised behaviour in desert locusts between their solitary and gregarious phases, preferably tracking individuals whilst doing so.

The social organisation of red wood ants forms the most extreme form of sociality in the animal kingdom – eusociality. According to predictions based on computational modelling (Appendix A) these ants would be expected to display a similar direction of lateralisation with other colony members. Our results emphasise this extra level of biological organisation and remarkably, red wood ants share their forelimb preference with other members of the colony. These results are consistent with the ESS hypothesis, that social animals are likely to align their lateralised behaviour to others in their group with which they find themselves. Our results from lateralised turning preference in ants are inconsistent with the hypothesis, however, because the lateralisation is displayed at the individual-level only, with neither the colony nor the population showing any directional bias. An explanation for the inconsistency is that it depends on the selective pressures that are exerted on the lateralised behaviour. Predator or within colony interactions may be weak, therefore there is no guarantee that the lateralised behaviours are aligned with conspecifics, or of the same strength. In addition, we postulate that turning preferences may not be social in nature and, therefore, are unrelated to the interactions with other colony members or that the ‘Y’-maze paradigm may be an unreliable method to test for lateralised behaviours.

The social brain hypothesis (Dunbar, 1998) suggests that living in groups and interactions amongst group members that facilitate group living leads to the evolution of an enlarged brain. In a similar vein, group living is thought to promote the evolution of a lateralised brain. More specifically, the lateralised brains of group members are likely to be lateralised in the same direction as other members of the group. It is likely that these two hypotheses are linked because both require a large number of sophisticated social interactions among many

conspecifics and both result in a large number of conspecifics living in dense groups. Also, the lateralisation allows the specialisation of brain structures that may add to the increased capabilities produced by an enlarged brain.

5.4 The importance of assessing lateralisation against the binomial distribution

The results from this thesis illustrate the importance of using the binomial distribution to assess behavioural lateralisation. Many research papers incorporate statistical tests that supposedly demonstrate lateralised behaviour, but fail to report a null expectation *a priori*, which can result in a type I error. For example, some studies simply report lateralisation as the proportion of rights *versus* lefts, or calculate LI scores and use '0' as the null expectation in the proceeding one-sample *t*-tests. We argue that, when possible, to establish whether true lateralisation exists the binomial expectation should be used to provide a sufficient null expectation. Ideally, many individuals should be tested over multiple trials to generate a distribution that can be compared against the expected binomial distribution. A significant benefit of using this method is that it can incorporate multiple levels of biological complexity. For example, a distribution of right turns/reaches can be built for trials per individual, individuals per colony and colonies per population. This avoids missing lateralisation at different levels of biological complexity. For example, Hunt *et al.* (2014) tested eight ant colonies for turning preference but amalgamated all these prior to testing, preventing them from detecting lateralisation at the colony level is unknown.

5.5 Motor control, sensory inputs and lateralisation

Limb preferences and turning preferences are the two modalities of motor output most frequently reported in lateralisation research. The neural systems underlying each of these lateralised behaviours are poorly understood and thus, the methods by which they interact are unknown. Here, we report no correlation between the two lateralised behaviours. Similarly, Buchanan *et al.* (2015) report no correlation between turning preference and wing folding in the fruit fly (*Drosophila melanogaster*).

We need not expect lateralised motor behaviours to be lateralised in the same direction, however it may be surprising that two motor outputs within an individual aren't related. This could be due to a couple of reasons. Perhaps there are selective pressures acting independently on the two behaviours. For example, synergistic interactions between colony mates could be driving the lateralisation of limb preference in one direction whilst predators could be driving the lateralisation of turning behaviour in the opposite direction. Alternatively, there could be different neural circuits underlying the control of the two motor outputs. Buchanan *et al.* (2015) report that columnar neurons within the central complex are responsible for the lateralised turning behaviour in fruit flies however a different neural circuit could be responsible for their wing folding. Future work should aim to report more than one lateralised behaviour in the same species in order to establish whether motor outputs are generally lateralised in the same direction.

It is thought that bilateral symmetry evolved when organisms adopted an axial orientation to the direction of their movement, and a bilateral nervous system coincided with this evolution (Rogers *et al.*, 2013). It follows that lateralisation in motor output and perceptual abilities evolved simultaneously. When interpreting results of research into motor lateralisation it is difficult to determine whether the lateralised motor output is in itself lateralised *i.e.* efferent pathways are lateralised, of whether it is the sensory inputs that are lateralised *i.e.* the afferent pathways are lateralised, which descend into the motor output pathways causing the lateralised output. For example, in our experiments it is unknown whether the ganglia controlling the output of the legs are lateralised or whether it is the antennae, from which descending information feed into the ganglia, which are lateralised. Similarly, it is difficult to ascertain whether ocular lateralisation could be attributed to the turning preferences we see in ants. Ideally, experiments that involve the occlusion of sensory inputs are required in order to clarify these questions.

5.6 Future directions

Along with the rapidly expanding field of research into lateralisation of insect brains and behaviour, this thesis has brought us further from the notion that only humans and vertebrates could exhibit behavioural lateralisation in motor control. It appears as though the advantages of being lateralised manifest, regardless of brain size and complexity. It is possible that the lateralised behaviours of insects vary in origin and are shaped by different ecological and genetic factors. However, when it is advantageous to coordinate one's lateralised behaviour with other lateralised individuals, similar selection pressures are likely to produce population-level lateralisation.

More behavioural work is needed in order to assemble a more coherent understanding of aspects of lateralisation. More experimental work is needed to test the generality of the ESS hypothesis of lateralisation. It would be interesting to study desert locusts before and after their phase to change to investigate whether the degree of sociality affects the distribution of lateralised individuals. With varying degrees of sociality, Hymenoptera provide a very useful tool for comparative research to test the ESS hypothesis of lateralisation. Do termites show lateralisation in their motor output? And if so, do they co-ordinate this behaviour with the rest of their colony? Do queen ants show behavioural lateralisation, and is it in the same direction as other queens within the nest? Also, do soldiers and workers both show the same direction of lateralisation?

Perhaps one of the greatest unresolved issues, when studying the lateralised brains and behaviours of insects, is an understanding of the neural basis of lateralisation. It seems as though the topic of the mechanistic basis of lateralisation has been neglected relative to the wealth of functional, developmental and evolutionary investigations, with a few exceptions. Is there a functional decoupling of identifiable motor neurons whilst locusts reach to cross gaps? Is unilateral firing responsible for lateralised turning behaviour in the red wood ant? Are there identifiable neural components in the central complex of either of these animals, accountable for the lateralised output? Insect nervous systems offer a great opportunity to investigate these questions by exploring the neural architecture using a combination of

electrophysiological recordings combined with microscopy techniques to identify them functionally and anatomically. Future research should endeavour to apply these methods, establishing a broader approach for the understanding of lateralised brains and behaviour.

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Appendix A

Colony-level lateralization in a eusocial insect

Chapter 2, 'Colony-level lateralization in a eusocial insect', of Paul Calcraft's PhD thesis (P. Calcraft (submitted). 'The evolution of group traits: modelling natural selection on trait prevalence within and between groups'. PhD thesis. University of Sussex) provides theoretical support, based on computational modelling, for degree of lateralisation found in our ant colonies using the empirical evidence for colony-level lateralisation from chapter three in this thesis. Below is the chapter abstract and the relevant figures (Figs. A1-5).

Abstract

Lateralization is the localisation or preference of function on one side of an organism, such as the human tendency to have a dominant left or right hand. This is an example of individual-level lateralization, but populations can be lateralized as well. A population that deviates from a 1:1 ratio of left-lateralized to right-lateralized individuals is said to exhibit population-level lateralization, such as humans' ~9:1 right:left-handed ratio. Individual- and population-level lateralization is very common in vertebrates, and some invertebrate species have recently been identified to exhibit such biases. However, groups or subpopulations (such as colonies) of any species have not previously been shown to possess directional biases of their own, distinct from other conspecific groups. Remarkably, the red wood ant, *Formica rufa*, exhibits such 'colony-level' lateralization in forelimb preference during a gap crossing task. Of the four colonies tested, one colony has more workers with a bias towards the left forelimb, the other three colonies having more workers with a right forelimb bias. Colony-level lateralization is not predicted by established evolutionary theories of population-level lateralization. We offer an evolutionary account of colony-level lateralization in eusocial insects by considering interactions among workers within colonies, between colonies, and between workers and their

predators/competitors. Our model predicts the degree of lateralization seen in wood ant colonies by trading off maladaptive predictability to predators/competitors against adaptive predictability to colony-mates. We consider the impact of this model on existing empirical work on population-level lateralization in eusocial insects, and reveal the conditions under which colony- and population-level lateralization would be expected to occur more generally.

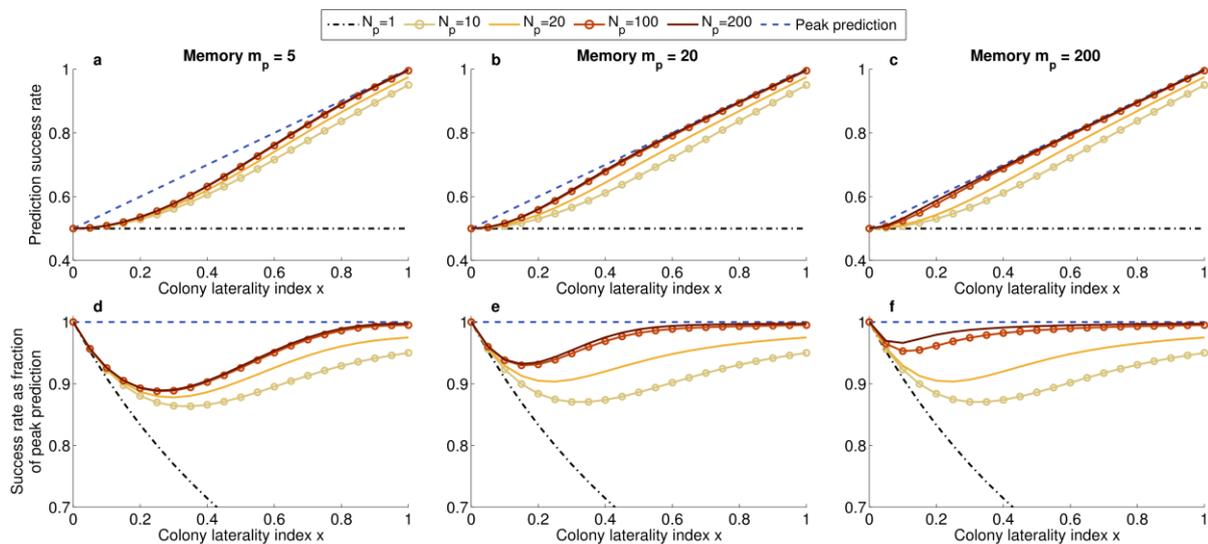


Figure A1. (a-c) Prediction success for guessing the laterality of workers (binary, left or right) from a colony with a given laterality index. Results shown for simulations with varying memory lengths m_p and number of workers encountered N_p . Peak prediction performance, as N_p and m_p tend to infinity, indicated by the dashed blue line. Baseline performance at $N_p = 1$, where memory cannot be utilised, indicated by the dot-dashed line. (d-f) Prediction success as a fraction of peak prediction for each condition.

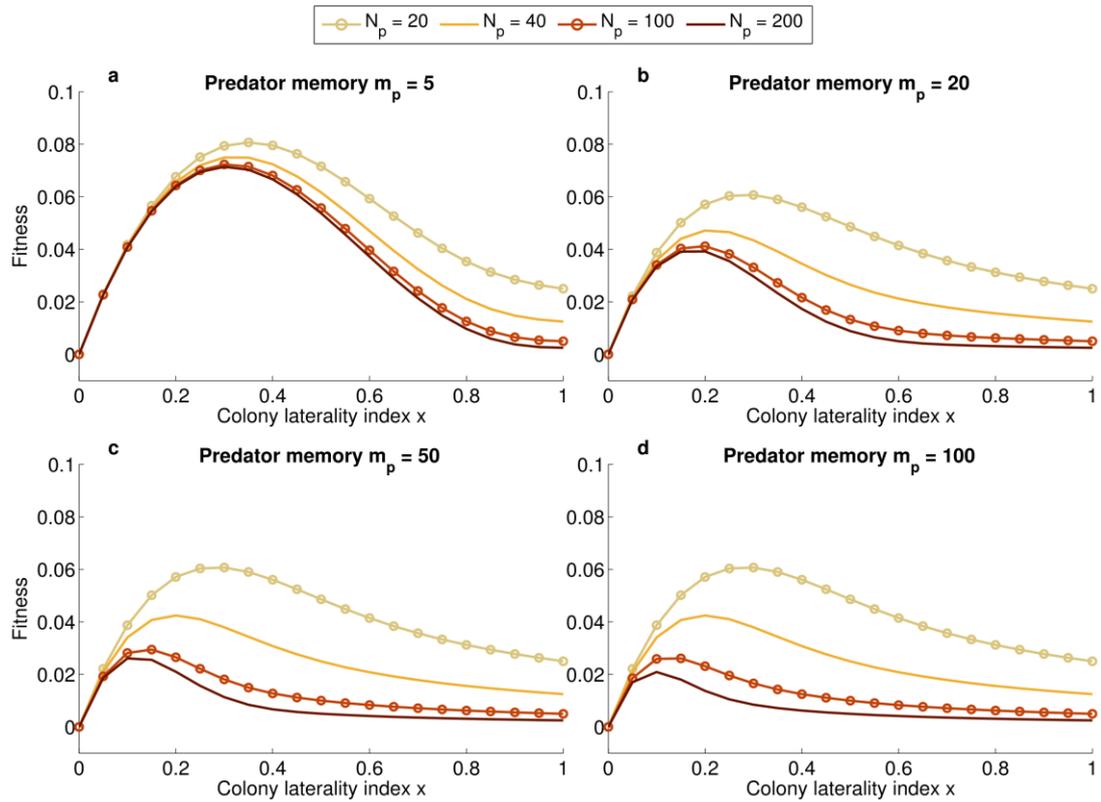


Figure A2. The effect of colony laterality index on colony fitness, where predictability to predators is negative but cooperative interactions are aided by increased laterality. Results shown for varying number of predator encounters N_p (line series) and predator memory lengths m_p (columns). Worker coordination success is a linear function of colony lateralization.

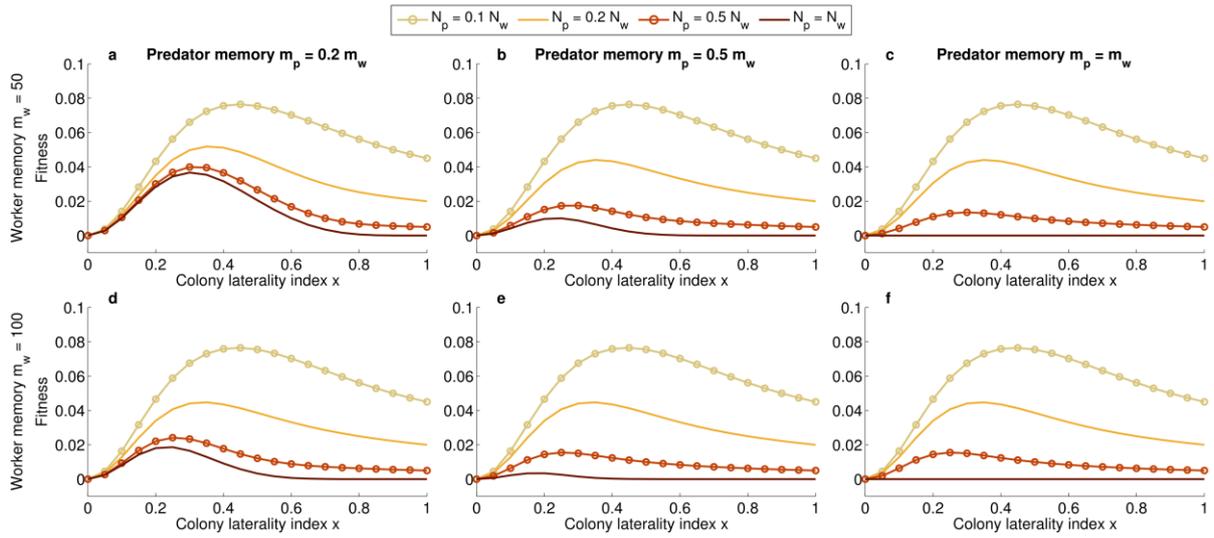


Figure A3. The effect of colony laterality index on colony fitness, where predictability to fellow workers is positive and predictability to predators is negative. Results shown for a fixed number of worker encounters $N_w = 100$, and varying predator encounters N_p (as a fraction of N_w), worker memory lengths m_w (rows), and predator memory lengths m_p (columns, as fraction of m_w). Worker coordination success rate is determined by learning.

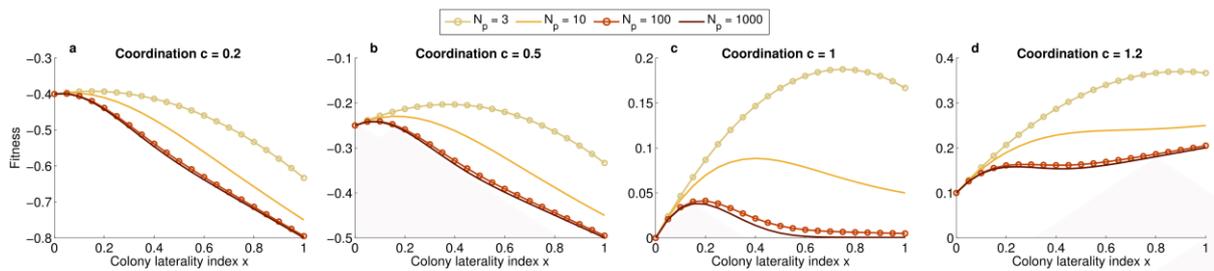


Figure A4. The impact of the importance of coordination c (a fitness multiplier applied to worker coordination success rates) on colony fitness. Results shown for predator memory length $m_p = 25$, and varying predator encounters N_p .

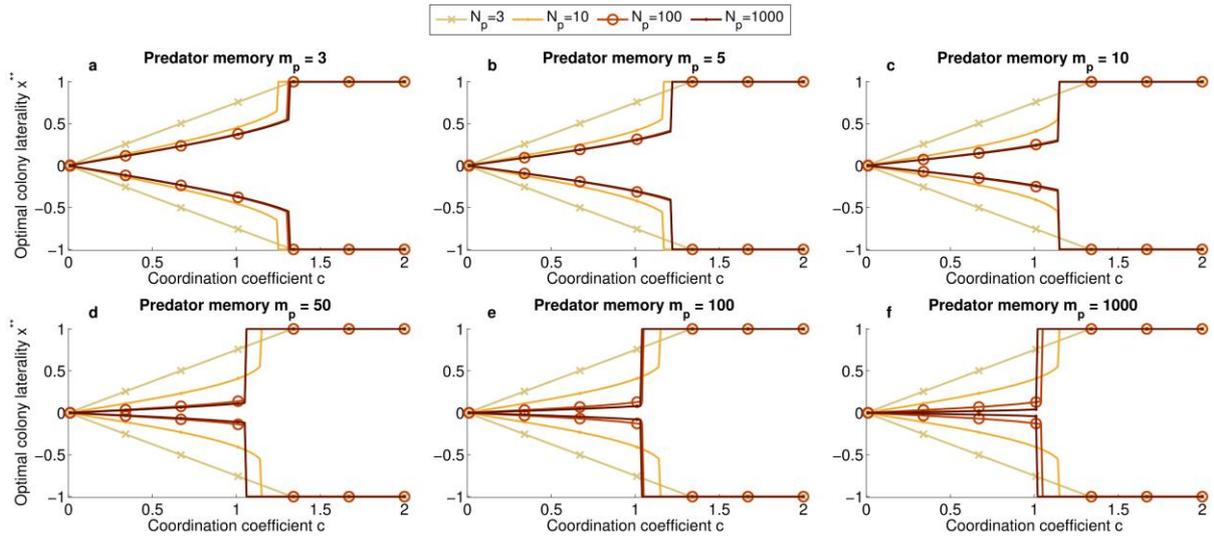


Figure A5. The impact of coordination coefficient c on optimal colony handedness. Results shown for different numbers of predator encounters N_p and memory lengths m_p .