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Brain Asymmetry of Structure and/or Function

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Lesley J. Rogers

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About the Special Issue Editor

Lesley J. Rogers, Professor, is a Fellow of the Australian Academy of Science and Emeritus Professor at the University of New England, Australia. After being awarded an Honours degree by the University of Adelaide, she received a Doctor of Philosophy and later a Doctor of Science from the University of Sussex, UK. Her publications, numbering over 500, include 17 books and over 250 scientific papers and chapters, mainly on brain and behaviour. In the 1970s her discovery of lateralized behaviour in chicks was one of three initial findings that established the field of brain lateralization in non-human animals, now a very active field of research. Initially, her research was concerned with the development of lateralization in the chick, as a model species, and the importance of light stimulation before hatching, then it compared lateralized behaviour in different species spanning from bees to primates and, more recently, it has focussed on the advantages of brain asymmetry.

Preface to "Brain Asymmetry of Structure and/or Function"

Left–right asymmetry is an important characteristic of the brain, as we now know from studies of many vertebrate species and, more recently, from studies of some invertebrate species. The commonality of this feature of the brain suggests that it provides advantages in cognitive processing by, for example, allowing parallel and different processing of inputs on each side (or in each hemisphere) of the brain, and by controlling different sets of outputs from each side.

In some species, asymmetry of brain structure is quite obvious, as long known to be the case in humans, whereas in other species structural asymmetries are not known to be present even though behavioural asymmetries are clearly evident. These behavioural asymmetries, also referred to as lateralization, are manifested as left–right differences in response to the same stimulus (including visual, auditory and olfactory stimuli, as well as magnetic directional information), in attention to novel stimuli and in processing of spatial and social information. They are also revealed as asymmetries in motor behaviour, such as hand and foot preferences and turning preferences.

Research on asymmetry in a broad range of species, including humans, is presented in the chapters of this book. The various contributions report on and discuss behavioural lateralization, asymmetries in sensory receptors and neural organisation and the role of genes, hormones and environmental factors in the expression of lateralization. The likely advantages or disadvantages of having brain asymmetry are addressed in some chapters. Also included are chapters focusing on the development of brain asymmetry, showing the influence of inputs from the environment. The evolution of brain asymmetry is also covered.

The first seven chapters report research on non-human species and the last four chapters are concerned with asymmetry in humans. Most chapters report new research findings but two chapters are reviews summarising research and taking a new perspective on published evidence.

Lesley J. Rogers
Special Issue Editor

Review

Lateralized Functions in the Dog Brain

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Abstract: Understanding the complementary specialisation of the canine brain has been the subject of increasing scientific study over the last 10 years, chiefly due to the impact of cerebral lateralization on dog behaviour. In particular, behavioural asymmetries, which directly reflect different activation of the two sides of the dog brain, have been reported at different functional levels, including motor and sensory. The goal of this review is not only to provide a clear scenario of the experiments carried out over the last decade but also to highlight the relationships between dogs' lateralization, cognitive style and behavioural reactivity, which represent crucial aspect relevant for canine welfare.

Keywords: dog; lateralization; emotion; behaviour; physiology

1. Introduction

Brain hemispheres specialise to process and analyse information in an asymmetrical way: this is a phenomenon widely reported in the animal kingdom [1,2] and, as shown by the increasing scientific study over the last decade, it is now well manifested also in canine species. Based on findings derived from experiments carried on different animal models, clear evidence exists that basic lateralized neural mechanisms are very similar across vertebrate brains with a specialisation of the left hemisphere in the control of routine behaviours, responding to features that are invariant and repeated, and with the specialisation of the right hemisphere in detecting novelty (unexpected stimuli) and in the expression of intense emotions, such as aggression and fear [3,4].

In this review, our first aim is to provide a comprehensive overview of the experiments carried out in dogs providing extensive evidence of hemispheric asymmetries in function, structure and behaviour. Our second aim in this paper is to analyse lateralized patterns specifically involved in emotional processing by the dog brain and how the study of emotional lateralization could represent a valid and interesting tool to contribute to the improvement of canine welfare and management.

In dogs, deepening the knowledge of cerebral lateralization with particular regard to emotional processing is particularly interesting since behavioural asymmetries which indirectly reflect lateralized cognitive processing of emotions can be easily detected (e.g., paw preference, nostril use, and tail wagging) and can give insight into the different valences of an emotion felt by the animal. The latter is crucial not only for a better understanding of canine cognition but also for the improvement of dogs' training and handling during several activities within the human community (e.g., animal-assisted therapy, police and rescue work, and guides for vision impaired people).

2. Sensory Lateralization

The complementary specialisation of dogs' brain hemispheres is clearly apparent at different sensory levels, including vision [5], hearing [6–10] and what is considered to be the most relevant sensory domain for canine species, namely olfaction [11,12].

Asymmetries of dogs' visual sensory channels have been observed by studying their asymmetrical head-turning response to bidimensional visual stimuli presented during feeding behaviour [5]. The experimental set-up consisted of the presentation of black silhouette drawings of different animal models (a dog, a cat and a snake) to the dog's right and left visual hemifields using two retro-illuminated panels. When stimuli were presented at the same time in the two visual hemifields, dogs preferentially turned the head with their left eye leading in response to alarming stimuli (the snake silhouette that is considered to be an alarming stimulus for most mammals [13] or the cat silhouette displaying a defensive threat posture). Given that, in dogs, neural structures located in the right hemisphere are mainly fed by inputs from the left visual hemifield and vice versa (crossing of fibres at the optic nerve level is 75% [14]), left head turns in response to threatening stimuli are consistent with the specialisation of the right side of the brain for expressing intense emotion including fear (snake) and aggression (cat with an arched lateral displayed body and erected tail). The latter specialisation of the right hemisphere has been reported in several animal models (reviewed in [1,2]).

It is interesting to note that left head turns (right hemisphere activation) lead to shorter latencies to react and longer latencies to resume feeding (i.e., higher emotional response). Moreover, during monocular presentation, higher responsiveness to stimuli presented in the left visual hemifield was observed, and this was irrespective of the type of stimulus. Overall, these results support the hypothesis that in canine species, as well as in other mammals, the neural sympathetic mechanisms controlling the "fight or flight" behavioural response are mainly under the activation of the right hemisphere [15]. In dogs, it is interesting to note that both in vivo [16] (Computed Tomography (CT) brain scanning) and post mortem techniques [17] have revealed a right-biased hemispheric asymmetry with the right hemisphere greater than the left; the latter could reflect the right hemisphere specialisation for intense emotional activities like fight or flight reactions, which are related to aggressive and defensive-escape behaviours.

As in dogs, a number of animals exhibit aggressive and defensive behaviours when the right hemisphere is active. Chicks, for example, respond strongly to a potential predator (silhouette of a predatory bird) seen in their left visual field (right hemisphere) [18,19]); very similar results were reported in toads, which showed stronger avoidance responses when a model snake was presented on their left side than when it was on their right side [20]. In domestic animals, horses approached by a potential threatening stimulus (a human opening an umbrella) reacted more (i.e., moving further away) when the approach was from their left side than when it was from their right side [21].

There is now evidence that the auditory sensory system in the dog brain also works in an asymmetrical way depending on the type of acoustic stimulus [6,8,9]. Specifically, during feeding behaviour, dogs' head orienting responses to different sounds played at the same time from two speakers placed symmetrically with respect to the subjects' head were recorded [6] (see Figure 1A). When thunderstorm playbacks were presented, dogs consistently turned the head with their left ear leading and, given that the direction of the head turn is an unconditioned response indicating a contralateral hemispheric advantage in attention to the auditory stimulus [22], this result supported the right hemisphere specialization in processing alarming stimuli. In a way similar to that previously reported about vision, in this experimental condition, left-head orienting turns also led to longer latencies to resume feeding from the bowl. On the other hand, dogs consistently turned the head with their right ear leading in response to playbacks of canine vocalizations ("disturbance" and "isolation" calls) supporting the role of the left hemisphere in the analysis of familiar conspecific calls, as reported in other species (non-human primates [23], horses [24], cats [25] and sea lions [26]). Nevertheless, in dogs, conspecific vocalizations are not always processed by the left hemisphere, since the right hemisphere is used for processing vocalizations when they elicit intense emotion [6,7].
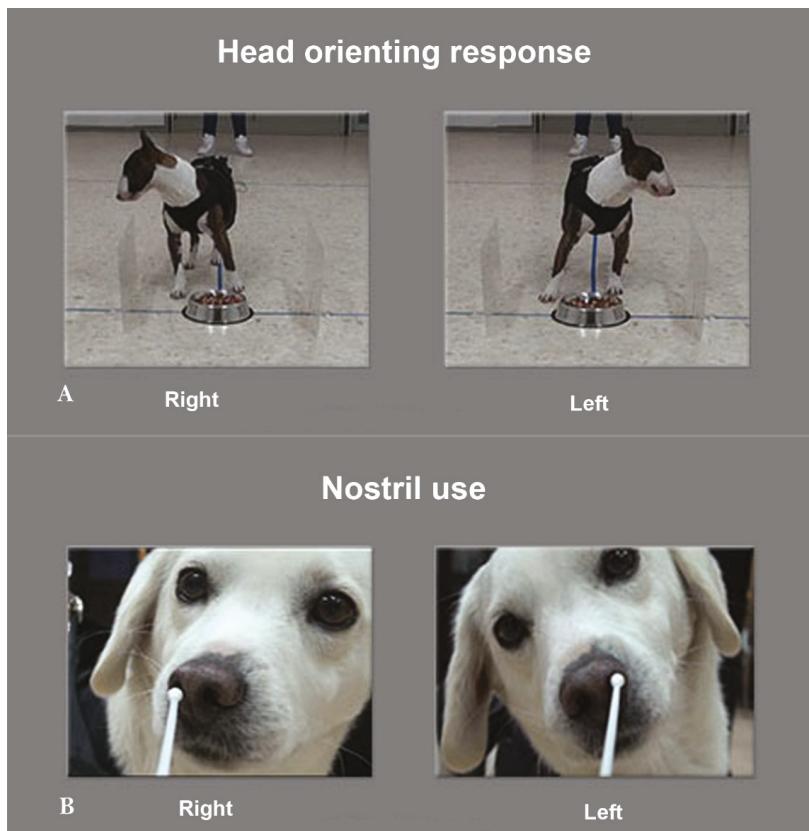
Figure 1. Behavioural techniques used to study functional lateralization in dogs: (**A**) head-orienting response used to study auditory lateralization; and (**B**) left and right nostril use during sniffing of different olfactory stimuli.

In dogs, the left hemisphere advantage in processing vocalizations of familiar conspecifics seems dependent on the calls' temporal features, since the presentation of the reversed version of the same canine call caused the loss of the right bias in the head turning response [27].

Head orienting response methods have been used in dogs to study possible lateralized neural mechanisms in processing human speech [8]. Results revealed that dogs consistently turned their head to the right during presentation of human spoken commands with artificially increased segmental cues (i.e., higher salience of meaningful phonemic components); moreover, a significant left-turning bias was observed in response to manipulated commands with increased supra-segmental vocal cues (i.e., higher salience of intonation component). These results have been confirmed by recent neuroimaging studies and overall suggest a convergent lateralized brain specialisation between canine and human species for processing speech [9].

Regarding olfaction, asymmetries in nostril use have been observed during free sniffing behaviour of odorants that differ in terms of emotional valence [11,28]. Briefly, cotton swabs installed on a digital video camera were used to present odorants to dogs (see Figure 1B). The camera was installed on a tripod in the centre of a large silent room. A frame-by-frame analysis of nostril use video footages revealed a clear right nostril bias during sniffing of clearly arousing odours for dogs (e.g., adrenaline and veterinary sweat). Given that, in dogs, the olfactory nervous fibres, which drive odour information from

peripheral receptors to the olfactory cortex, are uncrossed, right nostril use indicates a prevalent right hemisphere activation [29]. The latter was consistent with the previously reported right hemisphere involvement in analysing alarming/threatening stimuli and had direct implication for dogs' welfare and training since, for example, the constant use of the right nostril during olfactory inspection of a human being could reveal an increased arousal state of the animal, even in the absence of clear behavioural signs (this could be useful in those activities like animal-assisted therapy in which dogs must possess advanced behavioural control skills in order to help them handle high arousal situations and consequently it is not always easy to detect stress increase directly from behavioural signs).

When non-aversive stimuli were presented (e.g., food, lemon, and canine vaginal secretions), right nostril use was observed only during the first presentations indicating the initial involvement of the right hemisphere in the analysis of novelty (this bias was not evident for initial sniffing of food probably because of its reduced valence as a novel stimulus). Furthermore, a shift from the right to the left nostril use was observed with repeated stimulus presentations, indicating the prevalent control of sniffing behaviour by the left hemisphere when routine responses to odour stimuli emerge as a result of familiarization [1,2,30,31]. Left hemisphere specialisation in routine tasks has been observed in pigeons [32], wild stilts [33], toads [34] and chickens [35]. In the latter case, during a routine task of finding food, chicks using the right eye (left hemisphere) and not the left eye learn to find food grains scattered on a back-ground of distracting pebbles (similar to the grains).

There is now evidence that dogs' olfaction works in an asymmetrical way for processing both conspecific and heterospecific odours collected during different emotional events [12]. In particular, during sniffing of canine odours collected in a stressful situation (i.e., an "isolation" situation in which dogs were isolated from their owners in an unfamiliar environment), a consistent use of the right nostril was observed (right hemisphere activity). Moreover, when human odorants were presented to dogs, a significant left-nostril bias (left hemisphere activation) was found for sniffing olfactory stimuli collected from humans during a fearful situation (emotion-eliciting movies) and physical stress. The observed opposite nostril use pattern in response to conspecific and heterospecific odorants suggests that dog's olfaction uses different sensory pathways to extract emotional cues from canine and human chemosignals. Furthermore, an interesting hypothesis about the left nostril use during sniffing at human sweat collected during a fear situation and physical stress is that these heterospecific chemosignals (probably produced during the escape behavioural response to a predator) could elicit dogs' prey drive (i.e., approaching behavioural tendencies) to the stimuli through the selective activation of the left hemisphere. The evidence that, in dogs [36], as in other animal models (e.g., toads [34] and birds [33]), neural structures on the left side of the brain are involved in the control of predatory behaviour supports this hypothesis.

3. Paw Preferences

Asymmetries of motor functions have been widely reported in various vertebrate and invertebrate species, including the dog [1,2]. There is now a growing body of literature on motor lateralization in dogs, focused mainly on behavioural lateralization in the form of forelimb preferential use. In recent studies, paw preference has been assessed using several tasks: removal of a adhesive plaster from the eye [17,37] or of a piece of tape from the nose [38–42], removal of a blanket from the head [43], retrieval of food [44,45] from a toy object (namely the "Kong", see Figure 2) [46–50] or a metal can [43], paw-shaking [43], first foot placed forward to depart from a standing or sitting position [49,51] or during a run [52] and stabilization of a ball [39] and hindlimb raising behaviour during urination [53].

Figure 2. Motor lateralization: right paw use during stabilization of a food object (namely the "Kong").

The existence of motor asymmetries at a population level is currently a subject of wide debate. It has been reported in several species, including humans [54], non-human primates [55,56], rats [57], humpback whales [58] and common European toads [59] but studies on other animals, as for example marmosets [60], sheep [61,62], cats [63] and horses [64,65], has shown a motor bias only at the individual-level. However, the same species may also display a limb preference at the level of population or at the individual level depending on the task, as found in monkeys [66,67], cats [68] and sheep [69].

Motor lateralization in dogs is stable between breeds and over time [41,46] but variable between sexes. Although a few studies have reported an association between paw preference and sex at a population level but in opposite directions, with males showing a left-paw and females a right-paw preference [29,43,47], this seems to be inconsistent with other findings, which describe no population bias [17,39,41,46,51]. These conflicting results suggest that sex hormone status could be influential on the development of individual motor laterality but further investigations are necessary to accurately determine if this is the case.

There have been several recent studies that revealed an interesting association between emotional functioning and limb preference in animals, including dogs. It is well established that in primates motor bias is associated with differences in the behaviour of individuals and their emotional states. In particular, left-handed/pawed animals displayed more fear responses, higher stress levels and levels of reactivity than right-handed/pawed animals [4,70,71]. The latter, instead, were more likely to approach new objects and showed more social behaviours to capture a prey (chimpanzees: [72], marmosets: [73,74]). These behavioural differences match the known specialization of the hemisphere involved in the control of motor functions (contralateral to the preferred limb). Therefore, the limb preferential use could be indicative of the subject's personality type and its likelihood of expressing a positive or negative emotional functioning. Recent studies have reported indeed that left-handed marmosets have a negative cognitive bias compared to right-handed marmosets, which display a positive cognitive bias [75]. Concerning dogs, Branson and Rogers [46] showed that dogs with weaker motor lateralization were more reactive when exposed to potentially threatening stimuli (thunderstorm and fireworks sounds) since they displayed more stressed behaviours than lateralized subjects. Dogs

with stronger paw preference are otherwise more confident and relaxed in an unfamiliar environment and when presented with novel stimuli [76]; on the contrary, they are less able in a problem-solving task, to manipulate and explore a new object to obtain food than ambilateral subjects [76].

Given these findings, preferential limb use could be employed as a measure to assess vulnerability to stress and welfare risk in animals [4] and also in dogs. Consequently, it is essential to correctly categorize subjects as left- or right-pawed, choosing a motor test that provides reliable information about dogs' dominant paw, in order to make inference about dogs' dominant hemisphere and their ability to cope with stress. Wells et al. [48] recently investigated whether dogs use their dominant paw in the most common motor test employed in this species, namely the Kong test. They found that dogs use their non-dominant paw to stabilize the Kong to obtain food and their dominant paw for postural support. These findings need to be considered for correct implications on animals' welfare and emotional vulnerability.

Therefore, the evaluation of paw preferential use could provide notable information regarding a dog's predisposition to solve future behavioural problems or about its suitability for work. It has been demonstrated, indeed, that the direction of laterality is predictive of success in a Guide Dog Training Programme; in particular, right-pawed dogs were more successful in completing the training than left-pawed and ambilateral subjects [77].

Considering that behavioural differences in dogs' response to different situations are linked with motor lateralization and that dogs' temperament plays an important role in the selection of dogs (for working or adoptions), Schneider and colleagues [50] examined the relationship between paw preference and temperament. They found no differences between lateralized and non-lateralized dogs in the score obtained by a questionnaire completed by owners, aside from stranger-directed aggression scale, where lateralized subjects registered higher scores than the ambilateral ones. This may suggest the existence of a lateralized component in that particular type of aggressive response but further investigations are required. Moreover, recent findings show that behavioural signs of fear and distress displayed in a given situation and motor laterality are not associated with cortisol concentration in saliva samples [42].

However, it would be interesting in the near future to deepen our understanding of the relationship between motor laterality and emotional functioning since knowing the direction of paw preference of a dog we could correctly assess the strategy to be employed to preserve and improve its welfare.

Motor laterality is also associated with the analysis of visuospatial information, as we recently found in our research. Specifically, agility trained dogs with weaker paw preference were less attentive in performing agility exercises and displayed greater latency in the wave poles task (i.e., dogs' ability to work around pole obstacles that are secured in a straight line to a metal base) when the owner was positioned in its left visual field [78]. These results clearly show that stimuli with high emotional valence (the owner) could influence specific cognitive abilities, particularly when the right hemisphere processes them. In a more recent study, we reported that visuospatial attention is strictly related to motor lateralization since left-pawed dogs exhibited left visuospatial bias, right-pawed dogs a reversed rightward bias, while ambilateral dogs displayed no bias [79]. The existence of such a relationship has significant implications for animal welfare since it establishes a basis on which to develop new therapies for the rehabilitation of visual attention during pathological conditions (namely, unilateral spatial neglect); it could also help humans to improve canine training techniques, choosing the correct side to handle dogs and how to capture their attention easily.

The importance of paw preference assessment as a useful tool to preserve animal welfare derives also from the evidence of a direct relationship between dogs' motor laterality and immune response via an asymmetrical modulation exerted by the autonomic nervous system [38,80–82]. Right-pawed and left-pawed dogs exhibit different patterns of immune response, in particular the former displayed higher granulocytes percentage, number of γ-globulins [38], anti-rabies antibody titres and interferon gamma (IFN-γ) serum level [80] while the latter showed higher lymphocytes number [38] and higher

expression of specific interleukin genes (IL-2 and IL-6) after immune challenge [81]. Furthermore, ambidextrous dogs exhibit a significantly higher increase of catecholamine levels after immunization with rabies vaccine than lateralized subjects [82].

The direction of dogs paw preference is also related to anatomical asymmetries of the brain. Aydınlıoğlu et al. [45] found a variation in callosal size, particularly in its posterior segment (namely the isthmus) that was larger in right-preferent dogs than left-pawed subjects. Post mortem analyses also showed morphological asymmetries in canine hippocampi, which were associated with both sex (males larger than females) and paw preference. Female left-pawed dogs showed indeed larger hippocampi than the right ones [44]. In light of this evidence, motor lateralization may be considered as a direct consequence of brain structural asymmetries that could be, more broadly, the likely cause of cerebral specialization of functions.

4. Tail-Wagging as a Tool to Study the Asymmetrical Representation of Emotional Processing in the Dog Brain

Tail wagging represents an interesting model to study competition or cooperation between brain hemispheres in the control of behavioural response to emotional stimuli mainly for two reasons:

(1)   Dogs move their tails in an asymmetrical way in response to different emotional stimuli [83].
(2)   Studies on behavioural asymmetries associated with lateralized brain functions have usually focused on asymmetric use of paired organs (e.g., forelimbs) but not of a medial organ (i.e., the tail). In order to test asymmetries in tail wagging behaviour, family pet dogs of mixed breeds were placed in a large rectangular wooden box with an opening at the centre of one of its shorter sides to allow subjects to view the different stimuli (see Figure 3). Different emotional stimuli were presented as follows: the dog's owner; an unknown person; an unfamiliar dog with agonistic approach behaviour; and a cat. Tail wagging was analysed frame by frame from video footages recorded through a video camera placed on the ceiling of the box (see Figure 3).
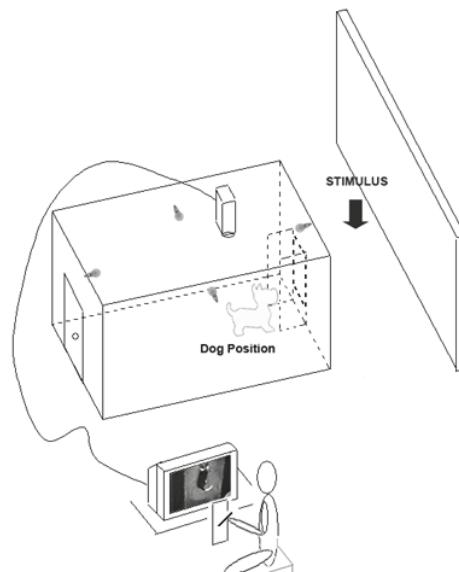

Figure 3. Schematic representation of the testing apparatus used to study asymmetric tail-wagging behaviour.

Results revealed that both direction and amplitude of tail wagging movements were related to the emotional valence of the stimulus. Specifically, when dogs looked at stimuli with a positive emotional valence (e.g., their owner), there was a higher amplitude of tail wagging to the right. On the other hand, during presentation of negative emotional stimuli (an unfamiliar dog with a clear agonistic behaviour), a left bias in tail wagging appeared. Given that the movement of the tail depends on the contralateral side of the brain [84], results are consistent with Davidson's laterality-valence hypothesis about the specialization of the left hemisphere for the control of approaching behavioural responses (right-wag → positive stimulus) and the dominant role of the right hemisphere for the control of withdrawal responses (left-wag → negative stimulus) [85]. In dogs, similar results were reported in the work of Racca et al. [86] in which subjects presented with pictures of expressive dog faces exhibited a left gaze bias (right hemisphere activation) while looking at negative conspecific facial expressions and a right gaze bias (left hemisphere activation) when looking at positive ones. The amplitude of tail-wagging movements is also a determinant cue for estimating "quantitatively" the level of arousal elicited by different emotional stimuli: during presentations of an unfamiliar human being, dogs significantly wagged their tails to the right side of their bodies but with less amplitude than towards the owner, whereas the sight of a cat once again elicited right side tail-wagging movements with less amplitude than towards the unfamiliar human being. The right side tail-wagging bias observed during cat presentations would probably reflect the tendency of dogs to approach the stimulus under the left hemisphere control of prey-drive behaviour.

In order to test whether or not dogs detect this asymmetry, in a more recent experiment, 43 dogs of various breeds were shown movies of other dogs or black silhouettes manipulated in order to display prevalent right or left sided tail-wagging or no wagging at all [87]. In addition, dogs' emotional response to movies were evaluated by measuring the subjects' behaviour and cardiac activity. Results revealed that when dogs saw movies of a conspecific exhibiting prevalent left-sided tail wagging, they had an increased cardiac activity and higher stress behaviours. Moreover, when observing movies of conspecific with right-sided tail wagging movements, dogs exhibited more relaxed behaviours with a normal cardiac activity (i.e., heart rate values similar to those of the dogs during resting) suggesting that the canine species is sensitive to the asymmetric tail movement of conspecifics, which has direct implication for understanding dog social behaviour. Different results were reported in a previous study in which the approach behaviour of free-ranging dogs to the asymmetric tail wagging of a life-size robotic dog replica was recorded [88]. Results revealed a preference to approach the robotic model (i.e., without stopping) when its tail was wagging to the left side. Authors reported that a possible explanation for the stop response during the approach to the model moving its tail with a clear bias to the right may originate when tested dogs are presented with a signal that would otherwise be positive (right wag) yet is not accompanied by additional reciprocal visual or acoustical responses by the robotic model. Another possible explanation for the different results between the two experiments is that, in the first experiment, tail movements were taken by real dogs (i.e., biological movements) while in the second they were artificially reproduced by a robotic model (even in the presence of a good dog-replica robotic movements are not properly biological).

5. Conclusions

Overall, there is clear evidence that functional lateralization has profound connections with cognition in dogs. A greater understanding of this association may certainly contribute to improve dog welfare and the relationship between dogs and humans. Non-invasive techniques of measuring lateralization (e.g., paw preference or tail wagging) could constitute a reliable, simple and direct tool of evaluating dogs' cognitive style and emotional affective states, providing elements that could enhance every-day management practice and improve both dogs' welfare and behavioural medicine.

Author Contributions: The authors contributed equally to this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Audition and Hemispheric Specialization in Songbirds and New Evidence from Australian Magpies

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Abstract: The neural processes of bird song and song development have become a model for research relevant to human acquisition of language, but in fact, very few avian species have been tested for lateralization of the way in which their audio-vocal system is engaged in perception, motor output and cognition. Moreover, the models that have been developed have been premised on birds with strong vocal dimorphism, with a tendency to overlook species with complex social and/or monomorphic song systems. The Australian magpie (*Gymnorhina tibicen*) is an excellent model for the study of communication and vocal plasticity with a sophisticated behavioural repertoire, and some of its expression depends on functional asymmetry. This paper summarizes research on vocal mechanisms and presents field-work results of behavior in the Australian magpie. For the first time, evidence is presented and discussed about lateralized behaviour in one of the foremost songbirds in response to specific and specialized auditory and visual experiences under natural conditions. It presents the first example of auditory lateralization evident in the birds' natural environment by describing an extractive foraging event that has not been described previously in any avian species. It also discusses the first example of auditory behavioral asymmetry in a songbird tested under natural conditions.

Keywords: auditory perception; auditory lateralization; song production; extractive foraging; visual laterality; memory; Australian magpie

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1. Introduction

Field studies of behavioural laterality in birds are still relatively rare, but the few undertaken so far have shown that laterality may play a role in vigilance behaviour [1,2], in predation and sexual behaviour [3,4] and even in tool manufacture, as shown in the New Caledonian crow, *Corvus moneduloides* [5]. In fact, in the special case of tool use and manufacture by crows, the activity appears to be strongly lateralized because birds were seen to use their right eye even when this posed some difficulties [6].

Asymmetries in avian species have been found in visual processing from sensory input to motor output, admittedly largely in domestic chickens [7,8] and pigeons [9]. Lateralized foot use has been shown in pigeons [10,11], the New Zealand kākā [12], some songbirds (sittellas and crested shrike-tits [13]), Japanese jungle crow [14] and also in cockatoos and some parrots [7,15–17]. This paper will explore whether such lateralities, as shown in the visual behavior of many vertebrate species [18], may also be present in auditory abilities and their behavioral expressions in birds.

Without a doubt, vision and audition are the most well-developed sensory abilities both in birds and in humans, and they are often used in conjunction: for example, there is plenty of evidence that learning is particularly effective and often more powerful when vision and audition are coupled [19,20]. In many oscine birds, song learning occurs in a visual context, suggesting that both auditory and visual perceptual systems could be involved in the acquisition process. Hultsch et al. [21] examined, in male juvenile nightingales, whether song performance improved after coupling visual with auditory stimuli. It did and did so convincingly [21]. In a study on chickens, Van Kampen and Bolhuis [22] demonstrated that learning is improved through compound training with simultaneous exposure to visual and auditory stimuli, showing that either modality has some facilitating effects on the memorization of features from the other modality. Such coupling has also worked in the combination of visual with aversive olfactory stimuli [23]. Additionally, there is evidence from research on zebra finches that visual stimuli activate auditory brain areas, e.g., the HVC, formerly called high vocal centre, now called HVC and used as a proper noun (see Figure 1 below) [24]. Given this interaction between auditory and visual processing and, since visual lateralization is widespread in avian species, it could be that auditory processing is also lateralized.

The importance of asymmetry in song production was identified early by Nottebohm [25]. He found that when the HVC in the left hemisphere was lesioned, male canaries could not produce song. When the HVC in the right hemisphere was lesioned, it had no effect on song production [25]. However, such lateralization does not apply to song production in all species, since it has been shown in zebra finches that some perceived manifestations of lateralization in the HVC during song production proved to be rapid switches between hemispheres and that the overall contributions of both sides were actually equal [26].

In research on memory formation, hemispheric dominance has been found in zebra finch males. Gobes and Bolhuis [27] showed that tutored-song memory and a motor program for the bird's own song have separate neural representations in the songbird brain. Lesions to the caudomedial nidopallium (NCM) of adult male zebra finches impaired tutor-song recognition, but did not affect the males' song production or their ability to discriminate calls. Lesions were bilateral, so any potential lateralization could not be measured. Moorman and colleagues [28] recently measured neuronal activation during sleep in juvenile zebra finch males that were still learning their songs from a tutor. They found that during sleep, there was learning-dependent lateralization of spontaneous neuronal activation in the NCM. Birds that imitated their tutors well were left dominant, whereas poor imitators were right dominant, similar to language proficiency-related lateralization in humans. Indeed, interest in comparative work in song production and perception and human speech [29] has increased substantially in the last decade, finding important similarities in the role of specific auditory nuclei between humans and birds [30,31].

Limitations: Species Investigated

The species most often chosen for detailed neurobehavioral research on auditory perception/song performance is the zebra finch. The choice makes sense on a number of levels: the song of this species is relatively simple and has a defined learning period, the birds are easy to keep in a laboratory setting (opportunistic breeders as they are, they reproduce easily in captivity and over short periods of time).

However, research of song in zebra finches has some limitations. The zebra finch is a sexually dimorphic bird in which only the male sings. This is not the case in all avian species. In fact, the zebra finch has model character only for songbirds with credentials similar to itself [32,33]; these include, for instance, migratory songbirds of high latitudes that need to fit a complete reproductive time-table into the shortest possible time frame: find a mate in spring, breed, raise offspring and migrate in autumn. Under such circumstances, offspring have to become independent rapidly. Juvenile males have to be taught how to be able to compete and win a female, relying on recall and a perfect memory of the song that an adult male tutor may have taught them in the previous year [34,35].

Having chosen such a model for research on bird song may have implied a questionable underlying assumption that song in all songbirds is purely a male activity (be this for courtship or territorial display) and may be exclusive to the breeding season. The zebra finch model also implies that song is mostly or always crystallized early in development with limited or non-existent ability for any ongoing learning/brain plasticity. However, as has always been known, there is a considerable number of songbirds with vast and flexible repertoires [36], and some of these live in complex social groups. Burish et al. [37] argued that telencephalic volume is strongly correlated with social complexity. This correlation, so they show, accounts for almost half of the observed variation in telencephalic size, more than any other behavioral specialization examined, including the ability to learn song. Moreover, female song is widespread and ancestral in birds [38–41]. In other words, as was recognized some time ago, relying on the zebra finch model in terms of broader questions of behavior could lead to ignoring the importance of social learning in non-reproductive contexts [42,43], the significance of variability in avian communication outside the breeding context and the possibility of different underlying mechanisms of brain activity [44–46] for hearing and vocal production, of which lateralization may be an important manifestation.

Since the discovery of mirror neurons in birds by Prather and colleagues [47], we also know that birds can learn song without being actively supervised and instructed by an adult. Tchernichovski and Wallman [48] explain that, on input, the motor signal is delayed, and this implies that the mirror neurons are providing a 'corollary discharge' signal: that is, a neural representation of the song being heard is available to the bird on first hearing it, and the bird can now check the encoded version against the song it later sings; or expressed differently, the bird has the same neuron activation whether it sings or just listens and gets a copy of the song in its memory against which it can judge its own output (performance) of the song.

Importantly, the mirror neurons identified by Prather et al. [47] belong to a population of neurons that is not replaced, as other neurons in the song system are [49], but is stable across song development. It is this stability that enables the juvenile to improve its song as the memory trace of the correct version remains present and can be accessed. It was established decades ago that amongst the network of nuclei involved in song perception and production, some are essential and some are not essential for song production [50], as discussed below.

2. Song Control System, the Auditory System and Lateralization

Song development and song production entail a set of complex interactions between neurological, physiological and behavioral events, and it has taken more than thirty years of research to begin to understand the nature, type and dynamics of these interactions.

Songbirds possess a network of interconnected nuclei in the fore-, mid- and hind-brain used in the perception and production of vocalizations (see Figure 1) [51]. Furthermore, feedback loops are essential for vocal learning, and these are found only in passerines and parrots (and two species of humming birds), cetaceans and humans. The HVC, the robust nucleus of the arcopallium (RA) and the tracheosyringeal component of the hypoglossal nucleus (nXIIts), are necessary for the acquisition and expression of learned song [50], whilst Area X and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) are important feedback loops [52–54]. These main nuclei and some important auxiliary nuclei (Figure 1), represented on both sides of the brain, have been tested for lateralized expression. The budgerigar, a psittacine species, capable of vocal learning, but not classed as a songbird, has multiple forebrain areas for vocal production, but some of these, it appears, are not homologous to those of songbirds [55].

A link has been made sometimes between size of song nuclei and song complexity. It is said that song nuclei tend to be larger in those species that have more complex songs, and the HVC is larger in individuals with larger repertoires [56]. However, the relationship between presence and size of nuclei and actual song performance is not always matched. Gahr et al. [57] found that the male and the female of the African duetting bush shrike, *Laniarius funebris*, produce songs of similar complexity, but the HVC is, nevertheless, sexually dimorphic (larger in the male than in the female). The Australian magpie, *Gymnorhina tibicen*, also duets, and these findings are therefore relevant here. Gahr et al. [57] argued that their results show how misleading it can be to assume a causal relation between sex difference in vocal behaviour and in the size of brain areas involved in song production and learning.
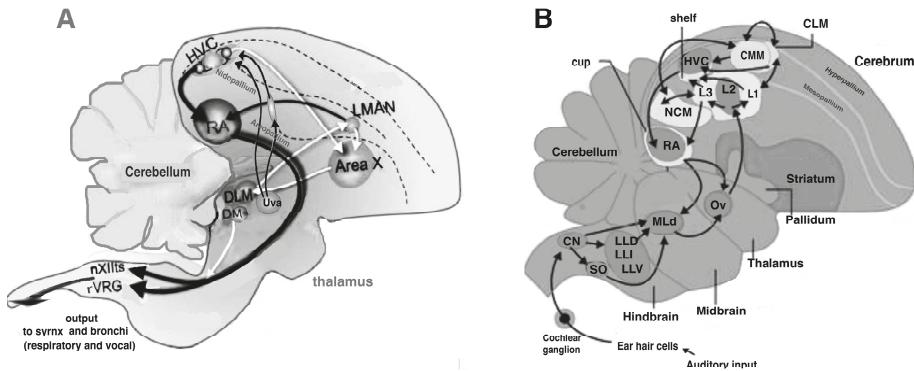
Figure 1. Song control system and auditory pathways. (**A**) The song control system. (**B**) Auditory pathways; simplified-arrows indicate flow of activations; right lateral view. (**A**) Song output via the main nuclei, the HVC of the nidopallium; RA, robust nucleus of the arcopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; Area X of the striatum; DLM, medial subdivision of the dorsolateral nucleus of the anterior thalamus; DM, dorsomedial subdivision of nucleus intercollicularis of the mesencephalon; Uva, nucleus uvaeformis; nXIIts, tracheosyringeal portion of the nucleus hypoglossus (nucleus XII); rVRG, rostral ventral respiratory group. (**B**) Auditory input HVC of the nidopallium with HVC shelf (lightly shaded); CLM, caudolateral mesopallium; CMM, caudomedial mesopallium; Field L, large area (light grey) subdivided into L1, L2 and L3; NCM, caudomedial nidopallium; RA, robust nucleus of the arcopallium; Ov, nucleus ovoidalis; MLd, nucleus mesencephalicus lateralis, pars dorsalis; LL, lateral lemniscus subdivided into: LLD, dorsal nucleus; LLI, intermediate nucleus; LLV, ventral nucleus; CN, cochlear nucleus; SO, superior olive (adapted from [58]).

However, our own investigations of the song control system in magpies do not confirm those of Gahr and colleagues [57], as is summarized below. Moreover, unlike model species such as zebra finches, *Taeniopygia guttata*, Australian magpies do not use song as part of a reproductive strategy. Both males and females sing [59], and song in both males and females declines, not increases, during the breeding season and does not appear to play any known role in mate choice [60].

Exciting research in recent years has focused on specific areas of the brain and found intensity invariant neurons in Field L, important for distant conspecific recognition (temporal resolution of 30 ms) and noise invariant neurons for individuals at closer distance with a temporal resolution of just 10 ms [61]. While these areas (NCM and CM) were once just considered secondary auditory areas, they have now been recognized as important loci for conspecific song discrimination and individual song recognition and, as such, have behavioural significance [62–67]. Indeed, Woolley and colleagues [68] identified all nine functional areas in the forebrain and midbrain of the zebra finch

(four in the midbrain alone), each of which was shown to play a specific role in extracting distinct complex sound features [68]. With the importance of these areas now identified, it should also be possible to ask whether any of these specific sound inputs activate neurons differentially in the left or the right hemisphere.

Indeed, a study by Poirier et al. [69] using functional magnetic resonance imaging (fMRI) discovered that, in zebra finches, the mid-brain shows neural activation in song recognition of both individual (own) and conspecific song, which is a crucial auditory and cognitive ability. These nuclei, called MLd (dorsal part of the lateral nucleus of the mesencephalon), are located in the midbrain, a subcortical region that, not so long ago, had been considered non-plastic and even 'primitive' [69]. They showed that there was a distinct right-side bias in the MLd, confirming a complex topography across the forebrain regions [70]. In other words, in perception of song, as distinct from song production, robust evidence is now emerging of lateralization of the mechanisms involved. In research on starlings, behaviourally-relevant song stimuli were used to test whether the NCM might be a site for categorizing complex communication signals, and it was indeed confirmed, largely on the right side of the brain [71,72].

There is no need here to catalogue all the various nuclei with lateralized functions in avian auditory perception and song output; in their review on memory-related brain lateralization [73], Moorman and Nicol (2015) published a very useful table listing nuclei concerned, together with the species and lateralized functions. Suffice it to say that avian species that are lateralized do not necessarily have the same side bias: chaffinches, song sparrows and canaries were found to be left lateralized for control of song, whereas the zebra finch is largely right lateralized [51]. The point is rather that the number of species tested is relatively limited and, except for the starling, they belong to a group of birds that are sexually dimorphic, may be short-lived, limited in repertoire and of varying brain plasticity. Each may have its own specific architecture with respect to how and what is lateralized.

It is a contention of this paper that lateralization may well be different, probably stronger and show more functional separation, the more complex a repertoire is and the greater the ability to learn. It is further a contention that in cases of functional changes of song, one might also expect changes in brain activation and different adaptations, particularly in species that, more like humans, show the same vocal capacities in male and female and are life-long learners. Although this hypothesis cannot be fully tested or confirmed in one paper, it would seem an important and necessary task to establish research on such a species, especially for comparative purposes with human vocal development. Australian magpies satisfy these criteria.

3. A Life-Long Learner as a Model Species

This paper reports new data and summarizes previous research obtained both in the laboratory and in the field concerned with auditory and visual hemispheric specialization in the Australian magpie, a species native to Australia. The magpie is one of Australia's foremost songbirds apart from the lyrebird. It is territorial, and residents consist of pairs with long-term bonds, their immediate offspring of one year and sometimes those of previous years.

The main reasons why magpies make a very useful model for perceptual research and memory formation is that in both males and females, song does not crystallize. With a lifespan of 25 or more years, they readily add new elements and sequences to their song, and they are also excellent mimics [74,75]. In these qualities, there are substantial overlaps with parrots and specifically with Australian cockatoos, as well as with ravens and crows. We know that they are amongst the most cognitively complex and long-lived birds (sulphur-crested cockatoos: 100 years; galahs: 80 years) [76]. These attributes are not odd anomalies in avian species, as may once have been believed, but may be significant in that these specific characteristics appeared early in avian evolution.

Some researchers concerned with hemispheric specialization have especially raised the question of evolution [77–79], but so far, little has been made of the geographic origin of modern birds. It has been known since the 1980s, but generally scientifically accepted since 2004, that a number of bird lineages and all modern songbirds in the world today arose in East Gondwana, now Australia [80–82], seemingly the only location where lineages survived the mass extinction events of 65 mya, including galliformes and anseriformes [83], to name a few among the precocial birds, although taxonomists still argue about dates [84], and all (altricial) songbirds. Songbirds radiated out from Gondwana to the rest of the world, a process that took tens of millions of years [85,86]. For reasons of similar climate and vegetation, those species that only went as far as the subtropical and tropical islands to the north of the supercontinent and to the tropical regions of northern hemispheric mainlands (the Indian subcontinent was once part of Gondwana) could presumably keep some of the traits they had acquired in Gondwana. Cockatoos probably arose in the Cretaceous [87,88], i.e., belong to the most ancient lineages of altricial land birds, and their highly lateralized footedness and its connection with complex cognition, a link that has been made only recently [16], gains significance given its very ancient origin.

As to songbirds, Sibley's and Ahlquist's broad taxonomical subdivision into Corvida and Passerida [89], although not necessarily used by taxonomists now, is still very useful to explain certain broad commonalities and traits. Corvida contain overwhelmingly birds with complex cognitive abilities (from problem solving, tool use, to measurably larger brain to body ratios) than the Passerida. Zebra finches (a native Australian species) belong to the Passerida, smaller songbirds that were the ones identified as among the main, probably first, 'escapees' from the Gondwanan continent. Magpies and crows belong to the Corvida, the group consisting of many species, in which we find most extant examples of complex vocal behaviour, learning and problem-solving abilities, qualities that significantly and overwhelmingly are present in species forming long-term bonds and/or engaging in cooperative breeding [76,90]. Most of these lineages, including magpies and lyrebirds [74,91,92], are capable of substantial and accurate mimicry. In summary, brain plasticity, large repertoires and often sophisticated vocal communication may require special architectural features in the brain. One could speculate that avian brains of songbirds of ancient lineages, and even of non-songbirds as cockatoos, might also be highly lateralized and be so for other functions [68].

3.1. Song Production in Australian Magpies

Magpies have an extraordinarily large repertoire. Strangely, 'repertoire size' in the literature, with a few exceptions [65], tends to mean the number of syllables in a song or total number of identifiably different songs a bird might sing, and is measured as such rather than as the sum total of vocalizations, not only song. To establish the true range of brain asymmetry or the lack thereof, it would seem important to consider the entire range of a bird's utterances (see Figure 2), since these are likely to represent different contexts and functions and may be under different neural control. In addition, there is the question of where and how the brain gets engaged when vocalizations are a matter of affect or are learned and/or intentional, such as in referential signalling [93]. To my knowledge, there is little to no research that has been done on any of these aspects, including any lateralization of their perception or production.

My own fieldwork on magpie vocal behavior identified as many as 27 different alarm calls [94], falling roughly into six distinct types, recognizable in sonograms as highly specific in profile. Field studies playing back alarm calls established that at least one of these calls is a referential alarm call, signalling the presence of an eagle [95]. We then also established the stability of such referentiality in different magpie subspecies and very different locations [96].

It would seem important to learn whether several categories of vocalizations have greater left or right hemisphere activation and what this might tell us. We already know from studies of song learning in zebra finches that new songs learned are memorized in the right hemisphere while the original song (long-term memory) is retrieved from and shows neural activity in the left hemisphere [27]. However, according to the results reported by Olsen et al. [97], direction and strength of laterality depend on how well each song is learned and by whom: The greater the retention of song from their first tutor, the more right-dominant the birds were when exposed to that song; but the more birds learned from their second tutor, the more left-dominant they were when exposed to the first song [97]. Lateralized memory strengthens the performance of well-learned song and presumably enables the bird to be competitive for females in the coming season. Since magpies are improvisers and have no tutors [75], it is likely that the quality of learning and recall determines whether the sounds are stored in long-term memory (left hemisphere) [98].
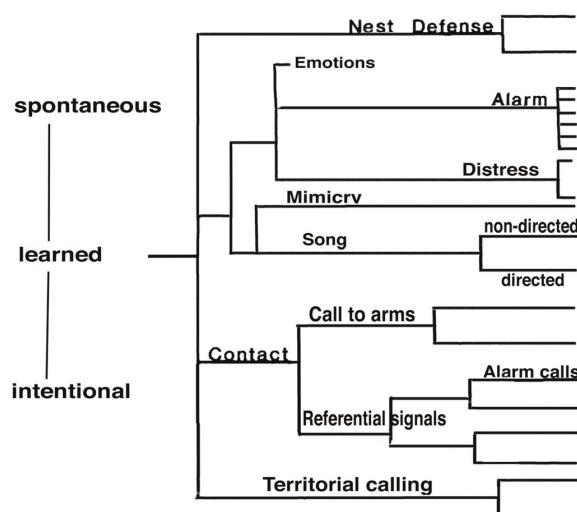
Figure 2. Range of vocalizations expressed by Australian magpies. These categories can roughly be subdivided as those that are a matter of affect, such as distress, fear and anger, but alarm calls, while also short, may involve forebrain regions (as in referential calls) or even in mobbing calls. Learned vocalizations in song, while not tutored, may have elements that are territorial or regional markers, and all mimicry is of course learned. Intentional vocalizations can be long or short, but they must have stereotyped characteristics, be uttered only in the presence of conspecifics and would usually lead to a change of behaviour in others (see [93,95]).

3.2. Song Control System in Magpies

When we sectioned magpie brains, albeit in a small sample (*N* = 9), we found that the female and male song nuclei of the magpie are about equal in volume and well developed and also well-developed in juvenile magpies (2–3 months post fledging), which is consistent with the vocal competence of juvenile magpies [99]. We also found the same song control nuclei and in the same topographical position in the forebrain of the Australian magpie, as present in canaries and zebra finches [50,52,99,100].

Our results indicate that, from juvenile to adult age, the volume of RA increases (10%), and the volume of the Area X decreases (19%). No such age-dependent change occurred in the HVC or LMAN (see Figure 1). The volume of mMAN (the medial magnocellular nucleus located adjacent to LMAN) was 40% smaller in juvenile females compared to a juvenile male and an adult female, but the volume of RA in the juvenile male was some 36% smaller than that of the juvenile females, suggesting that there may be both sex- and age-dependent differences in these nuclei. Interestingly, juvenile female magpies showed a fully-developed RA nucleus 2–3 months after fledging, whereas RA was developmentally delayed in the juvenile males, and the reverse applied to the nucleus mMAN [99]. Since all of the measurements were made on coronal sections and only one side of the brain was measured, no data examining lateralization were collected.

The Syrinx

The primary sound-producing organ in a bird is the syrinx, and the secondary system aiding sound production consists of the larynx, mouth, tongue and laryngeal muscles. Opening and closing of the beak may also affect the song produced [101–103].

The musculature controlling the syrinx is considered such a crucial anatomical feature that songbirds have been classified as such according to the absence or presence of these muscles [104]; or rather, the definition of a true songbird is based on the identification of the number of muscles present in the syrinx. Some avian species do not have a syrinx and produce sounds via clavicular sacs, and suboscines may have a syrinx with just one or two pairs of syringeal muscles. Certain suboscines, e.g., Tyranni, such as pittas, have a mesomyodian syrinx with either no or just one pair of syringeal muscles [105].

In the true oscines, as are magpies, the syrinx is equipped with four or more pairs of syringeal muscles, typically five pairs, important in the production of song. More recent research suggests that the syringeal muscles have mainly a modulatory function [106]. Furthermore, as some writers about psittacine vocalization have pointed out, the complexity of sound and a rich vocal repertoire may belie the simplicity of the sound-producing apparatus [107].

In early 19th century studies of the function of the syrinx, it was assumed that both sides of the syrinx always act together to produce one sound; but since the development of spectrographs, it could be shown that this was not the case, and birds could produce harmonically unrelated sounds simultaneously on both sides of the syrinx, giving rise to the 'two-voice' theory of song. Nottebohm [25] lesioned the hypoglossal nerve leading to the left side of the syrinx of male canaries, the consequence of which was that the bird's song was severely affected, losing the majority of its syllables, but sectioning the right side alone had relatively little effect on the postoperative song, a finding that was confirmed by testing other small songbirds, such as several species of sparrow and chaffinch [50,108]. The experiments have shown that the neural control of the syrinx is lateralized, with the left side being dominant. However, to assume that neural control and physiological adaptations come only in a fixed model for all songbirds would be incorrect.

Birds vocalize by expelling air over the elastic membranes of the syrinx housed within the inter-clavicular sac, an air sac in the pleural cavity. In songbirds, the syrinx consists of two parts, one in each bronchus, and each is innervated separately [50]. For a long time, sound was seen as being produced by the actions of lateral and medial labia, as well as the medial tympaniform membranes in the syrinx (see Figure 3). The actual sound-generating mechanism, however, appears to be located in the lateral tympaniform membranes (LTM) and not, as believed in classic theories, the medial tympaniform membranes (MTM). Goller and Larsen [106] showed in his sample of songbirds (a female crow, *Corvus brachyrhynchus*, wild-caught male Northern cardinals, *Cardinalis cardinalis*, and brown thrashers, *Toxostoma rufum*) that even the removal of the MTM did little to alter song performance. Instead, they concluded on experimental evidence that, since sound production is always accompanied by vibratory motions of both labia, the vibrations of the labia had to be the actual sound source. The onset and termination of vocalization (called phonation) is usually controlled by the syringeal

muscles that open or close the lumen on each side of the syrinx. The elasticity and complexity of the membranes may determine the quality of sounds. The air pressure, the muscles and the internal membranes can interact to produce near pure tones (single frequency and similar to human whistles).
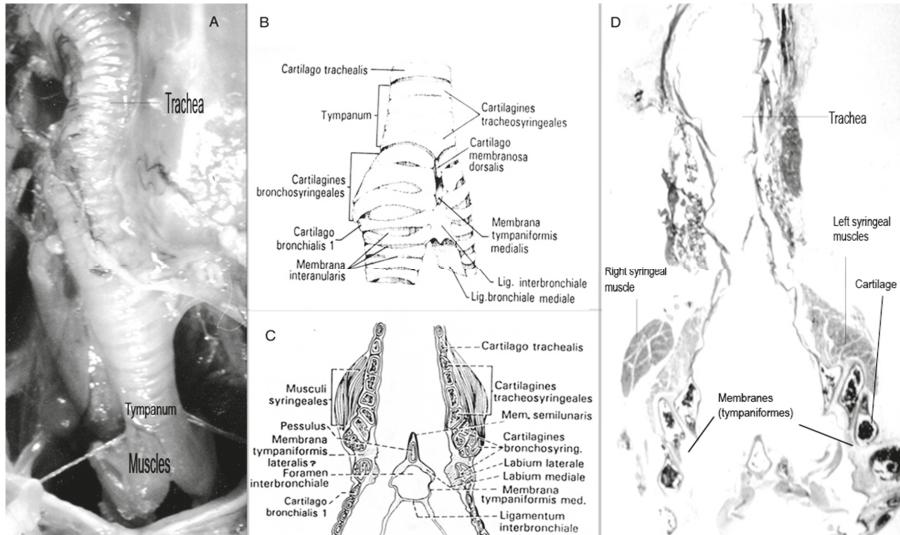

Figure 3. Syrinx anatomy. The syrinx of *Gymnorhina tibicen* (**A**,**D**). (**A**) The first panel shows the exposed syrinx deep in the chest of the Australian magpie (requiring sectioning the sternum), autopsied and photographed by the author. The two lips (musculature) at the bottom of the image are at the point of dividing into the two bronchial branches. The syrinx is connected to the trachea and the bronchial tubes below, but at the most vibratory section, just above the thick muscle belt, there are sinews and ligaments. (**B**) shows the syringeal cartilage, dorsal view (as (**A**)), of the European black-billed magpie, *Pica pica*, a relative in name only of the Australian magpie, which was named after the European magpie. However, both are songbirds and of about equal size. In *Pica pica*, the four tracheosyringeal cartilages are fused to form the tympanum. The photograph of the Australian magpie syrinx in (**A**) shows the trachea, the tympanum and the tracheosyringeal cartilage. Where the cartilage splits into its bronchosyringeal arms, this is covered in the photograph by a layer of muscle flaps (inversely heart-shaped). (**C**) presents a diagram of a syrinx (horizontal plane) of a male European blackbird, *Turdus merula*, one of the most common European songbirds, diverse and musical in its song. (**D**) is a histologically-prepared horizontal cross-section of a syrinx of an adult male Australian magpie prepared by the author. Note the similarities of details of (**C**) with (**D**). The syrinx of the blackbird and the Australian magpie is arranged very similarly, particularly in the medial and lateral labia, the lateral and the medial tympaniform membranes and the asymmetrical arrangement of the syringeal muscles [75,108,109].

The production of sounds depends on a number of additional physiological features, called the peripheral auditory system. The length of the trachea is important since formant frequencies are inversely proportional to the length of the vocal tract; i.e., if this were halved, the formant frequencies would be doubled [110]. Nowicki's paper of 1987 [111] showed that not just the syrinx, but the vocal tract contributed to the sound quality, at least in filtering sound [112], although, as a singular tube, it would not contribute to our understanding of lateralization, but can explain certain auditory characteristics [113,114]. Indeed, Hoese and colleagues [101] provided evidence experimentally of an important coordination between beak and sound output (Figure 4), showing that restricting beak movement or closed beak vocalizations [115] changed the tonal quality of song and caused

frequency-dependent changes in amplitude that may alter the message and, thus, require some instructional cues from the forebrain, and these may indeed be lateralized.
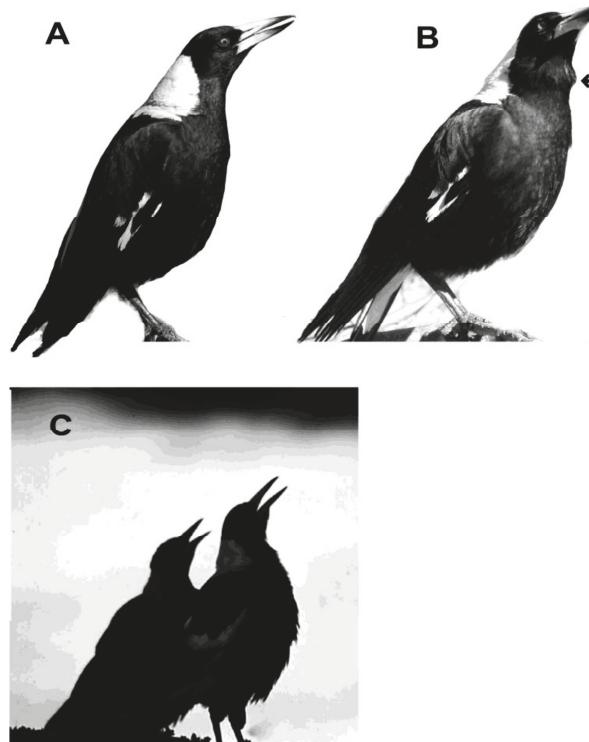
Figure 4. Body postures for specific phonations/song types in Australian magpies. The bird (**A**) is producing a low-level alarm call; posture erect and vigilant, and head raised slightly, beak open. (**B**) The same bird quietly singing. Note the bird is erect, but relaxed, and the beak is closed. The arrow points to the laryngeal area, and movement of feathers is clearly visible while the bird sings. (**C**) A pair carolling (i.e., using the territorial call). The birds arch their backs, extend their necks and throw their heads back, opening the beak widely to produce this loud and specialized call; chest and belly feathers tend to be ruffled as if major muscle groups are also involved in sustaining the call. Body posture and beak movement thus substantially differ from postures adopted in alarm calls or song.

3.3. Sound Production in the Magpie

Having identified the anatomy of the magpie's syrinx, our laboratory then proceeded to test phonation in wild magpies [116]. As in other songbirds, magpies have a tracheobronchial syrinx in which the cranial end of each primary bronchus contains a pair of vibratory structures, the medial and lateral labia, which vibrate in response to aerodynamic forces and produce sound when adducted into the expiratory airstream of the bronchial lumen (Figure 3 above). The muscles on each side of the syrinx are innervated by the ipsilateral tracheosyringeal branch of the hypoglossal nerve so that each side of the syrinx is under independent motor control by ipsilateral motor neurons that are in turn controlled by the central song system predominantly on the same side [116].

Lateralization of song production at the level of the syrinx (i.e., the contribution of the left and the right side of the syrinx) is relatively easy to ascertain either by syringeal nerve section or by measuring airflow on the left and right sides. If there is no airflow through one side of the syrinx, this

indicates that the labial valve on the ipsilateral side of the syrinx is closed and silent. Vocalizations must therefore be generated by airflow through the contralateral side of the syrinx, and this was true of some magpie vocalizations, as described below [116].

We discovered during our investigation (see the details of the method in [116]) that in magpies, the left and right sides of the syrinx can simultaneously generate different, harmonically unrelated frequencies during some of these bilaterally-produced vocalizations. At first glance, this result fit into the 'two-voice' theory. However, in magpies, it was not a matter of syllables being produced on one side and some others on the other, but the distribution of activation was according to the frequency of sound. The higher frequency was consistently produced on the left side. The left/right distribution of frequencies explains why magpies can drop three or even four octaves of sound from one note to the next. Moreover, this lateralization of frequency range is in the opposite direction from other songbirds with very complex song or large repertoires studied previously, in which the right side of the syrinx produces the highest fundamental [116].

Another finding was that magpies sometimes sang syllables unilaterally while maintaining bilateral airflow through the syrinx. This motor pattern is rare in other songbirds so far studied, which nearly always silence the contralateral side of their syrinx during unilateral phonation [117]. The results also showed a number of nonlinear phenomena (such as biphonation, deterministic chaos, etc.) in which the two acoustic sources of the syrinx interact. Nowicki and Capranica [118] had found these in the black-capped chickadees, *Parus atricapillus*, and identified them as heterodyne frequencies (not harmonics), resulting from cross-modulation between the two syringeal sides. In magpies, we found such nonlinear phenomena in begging calls, and here, they were a prominent feature. Still, the workings of the syrinx in its detailed functions suggests that further investigation in species differences of lateralization may be important. Brenowitz [119] argued that revision may even be necessary especially for large songbirds or when songbirds with substantially larger song repertoires are being examined and concern the role hemispheric specialization may play.

Indeed, lateral specialization for different frequency ranges may, in fact, increase the range of frequencies that the bird can sing. There is some evidence of the advantages of lateralized control in so far as the magpies' patterns of syringeal lateralization are more similar to those in the brown thrasher, *Toxostoma rufum*, the grey catbird, *Dumetella carolinensis*, and the northern mockingbird, *Mimus polyglottos*, all of the family Mimidae, than to the motor patterns of other species that have been studied. In the northern mockingbird, two-voiced singing is achieved from a single side of the syrinx unlike the magpie's dual use of different frequency ranges on each side of the syrinx [120]. The comparison with the Mimidae species is useful because they are amongst the most prolific singers and thus invite comparison with the Australian magpie. We know of none of these prolific singers, including our own study, as to whether they are lateralized consistently in one direction or whether lateralization changes over time since the method permits only seven days of testing of awake and relatively confined birds. The thermistors that had been implanted were removed after a week and the birds released [116]. Perhaps even more important is the possibility that, if the syringeal activation is lateralized consistently in the same direction, one might surmise that this could contribute to versatility and complexity in song repertoire.

4. Testing Sound Perception and Laterality in Field and Laboratory Studies

So far, some of the areas of interest in lateralization in song/vocal production have been raised. The last section of this paper will now be devoted to auditory perception in magpies as gleaned from scores in field observations and some specific elements of foraging behaviour, pertinent to lateralization, discussed.

We have a good and representative sample of hearing ranges of non-songbirds, raptors and songbirds [121], and one can infer from the magpie's own vocalizations that their auditory range is likely to fit in well into the average range of hearing in songbirds so far tested (see Figure 5).

It is important to know this hearing range well because without this biological evidence, it would be difficult to argue for auditory perception and lateralization at extreme upper and lower ends of hearing capabilities, unless there is some evidence, as one of the following field observations will show.

Audible sounds perceived by magpies may range from 0.5 kHz to 7 kHz, requiring higher sound pressure levels (SPL) for the very low frequencies (below 1 kHz), as well as for sounds above 5 kHz, at least judging by the range of sounds they can produce. At the low frequency end is a call that magpie females make. It is a particularly low frequency call emitted near or in the nest and typically directed at offspring (see Figure 5C). Its function seems to be both affiliative, as well as mildly punitive. The latter has been recorded in contexts when the offspring were still begging for food in the nest after the mother had fed them; a reassuring 'growl' (sometimes referred to as purrs) immediately stopped all begging (personal observation).
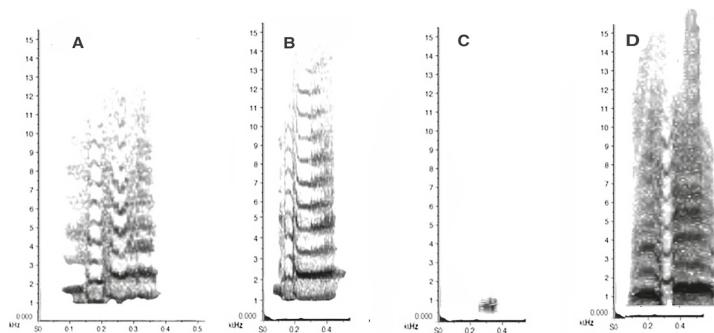

Figure 5. Magpie range of vocalizations. The figure shows the wide range of frequencies produced in magpie vocalizations, not included here is an actual song/warble sequence typically in the range of 1.5–2.2 kHz. y axis: frequencies in kilohertz (kHz); x axis: time in seconds. (**A**) is a complex single alarm call (type that is often a precursor to the eagle alarm call); (**B**) a sharp high amplitude alarm call; (**C**) a 'purr', discussed below; (**D**) is a mobbing call, containing a good deal of noise (grey); note that the mobbing call, stretched here for better visibility, has a characteristic midsection, which clearly distinguishes this category of call from alarm calls; that midsection being of less than 1 ms can at best be identified by a human ear as a faint 'click' sound, but with better temporal resolution of hearing in birds, it is likely to be unmistakable for conspecifics. Note that (**A,B,D**) are very high amplitude sounds, and (**A,B**) have frequency ranges (audible harmonics with considerable energy, darker horizontal lines/regular intervals) from 2 to 6 kHz and in some special calls, as (**B**), even maintaining some energy at 7–8 kHz. (**C**) By contrast, a very low amplitude 'purr' vocalization, is even lower (400–500 Hz) than the fundamentals of alarm calls and below the magpie's typical song and is usually delivered at 35–40 dB. Every example presents just one sound, but the darker harmonics indicate that the call has some energy at that frequency level, well above the first formant (**A**) at 6 kHz; (**B,D**) at approximately 5–6 kHz), and accordingly, one may assume that magpies can also hear most of the sounds they produce, even if the very upper limit harmonics (at 7 kHz and beyond) may become inaudible to magpies).

Anatomical differences between mammalian and avian audition have often been called upon to possibly explain differences in perception. Cohen [122] suggested that the hearing threshold of humans is generally about 18 dB lower than that of passerines, and the lesser hearing capacity in songbirds has been attributed to some main factors, although they have been questioned. King and McLelland [103] had shown that the basilar membrane of the cochlea of birds is restricted in size by head size. In pigeons, for example, this membrane is a mere 3 mm long, less than a tenth of that in the human ear. However, while this membrane carries the neuro-epithelial receptor cells, cells are far more densely packed in avian than in human ears, and so, King and McLelland [103] point out that the 'crista basilaris', in its cross-section, has about ten-times more receptor cells than the mammalian organ

of Corti. A counter-argument made by Henry and Lucas [123] is that the avian middle ear has just a single ossicle, the columella, that transfers acoustic energy to the cochlea, while mammals possess three middle ear ossicles, and these ossicles improve high-frequency efficiency. Several studies of columellar middle ear systems indicate that efficiency is greatest from 2 to 3 kHz and declines sharply above 3–4 kHz (reviewed in [124]).

However, there is apparently another level at which avian audition is different and, in this case, arguably better than the human ear, and this is in the temporal resolution of sounds, which, according to King and McLelland [103], was alleged to be 10-times faster in songbirds than in human ears, but if true, would provide a substantial auditory advantage and possible specialized ability to focus on specific sounds. By 2002, a study by Dooling and colleagues [125] tackled this question of temporal resolution. They found that birds were capable of discriminations between two sounds that differed in fine structure over time intervals as small as 1 ms, much faster than any estimate of the monaural temporal resolution capacity of humans. The researchers were thus able to demonstrate that the temporal resolution in the processing of acoustic communication signals in birds was well beyond the limits typically reported for humans; with the correction of King and McLelland's [103] claims, however, that a bird's discrimination of the temporal fine structure of complex sounds is two- to three-times, not ten-times, better than the limits shown for humans [125]. Henry and Lucas [123] speculated that taxa with lower temporal resolution may compensate for this with greater frequency resolution. They base this on theoretical models of cochlear tuning that predicts a trade-off between temporal resolution and frequency resolution [126].

Whether or not any of these very specific aspects of audition in birds are lateralized remains largely unchartered territory. Studies in temporal resolution have been undertaken mostly on aquatic mammals [127]. Interest had also been particularly consistent with respect to localizing sound by establishing interaural time differences (ITDs) and interaural level differences (ILDs). The puzzle is how birds with small heads can identify the direction of sounds [128–130]. A more recent study suggests that budgerigars may be able to localize pure tones as high as 4 kHz based solely on ITD information and that small birds generally may be able to enhance directional hearing by using the acoustic coupling of the middle ear cavities and so perform well above expectations [131]. In larger birds, one suspects that head turning, studied in the context of visual perception, may be useful to identify sounds, and these could reveal side biases.

Indeed, several such studies of auditory laterality have been undertaken by placing the sound sources behind the test birds, some purely for establishing threshold levels [122]. The playback method, placing specific auditory stimuli to the side or behind an animal, is a technique that is usually used in larger animals as, for instance, a study on dogs that tested hemispheric specializations for processing auditory stimuli [132]. Dogs turned their head to the right side (left hemisphere) in response to conspecific vocalizations, but to the left side (right hemisphere) in response to the sound of a thunderstorm. In birds, because of their small heads, it usually becomes a little more difficult although not insurmountable to test auditory responses. One study, for instance, tested experienced and young, inexperienced harpy eagles and exposed them to sounds of pure tones, of a bird (tinamous) and of a potential prey item (howler monkey calls) and of a conspecific from a speaker placed behind the bird. Both young and adult harpy eagles turned their head to the left when exposed to irrelevant sounds, such as pure tones or peeps of the tinamous, and both turned right on hearing the calls of another harpy eagle. On hearing the calls of the howler monkey, however, the captive young harpy eagle without hunting experience oriented to the left, whereas the eagle experienced in hunting oriented significantly to the right, clearly an example of purely auditory orienting asymmetry [133]. This suggests that socially-relevant information and potential food items are identified by sound alone and processed by the left hemisphere.

In humans, a behavioral method used to establish hemispheric dominance in auditory perception is dichotic listening in which subjects have earphones in both ears and similar sounding consonants (such as Da/Ta) are delivered to each ear separately and simultaneously, and the subjects then tell the

experimenter which consonant/syllable they mostly heard. Research in those cases have shown a clear right ear/left hemisphere dominance [134,135].

The same method (in principle) has been successfully employed in studying the ability of budgerigars to identify cues of interaural time differences (ITDs) and interaural level differences (ILDs) by implanting headphones [131], a technique also used to test left-right identification of sounds [136]. Interestingly, in humans, ITD performance drops off markedly for frequencies above 1.5 kHz, but budgerigars maintained sensitivity up to 4 kHz. The method could be used to also establish ear preference. Possible methods of auditory lateralization testing for lateralized brain functions have recently (2017) been discussed by Rogers and invite further study [137].

5. Field Studies Concerning Audition in Australian Magpies

5.1. Introduction

Very few field studies have shown lateralization of auditory processing in birds. There has been one study that meticulously established that some prey search by magpies is based purely on audition. Floyd and Woodland [138] hypothesized that magpies can forage for scarab larvae purely by listening to the chewing sounds they make in the soil. These sounds are so faint that the experimenters were unable to hear what the magpies heard under the same field conditions.

Magpies feed regularly on scarab larvae, and they are a prized food owing to their size (2–3 cm) and the high protein content and fluids they provide. Some studies confirmed that, in some cases, grubs retrieved from below the surface could be found by visual means. In heavily infested areas in England, rooks, *Corvus frugilegus*, and starlings, *Sturnus vulgaris*, feeding on scarab larvae, *Phyllopertha horticola*, were able to do so because of visual cues, for instance when turf had died off, i.e., had changed colour, or the soil surface was loose and could be lifted and pulled aside [139]. The American robin, *Turdus migratorius*, was also shown to use visual surface cues (worm casts) for locating earthworms [140]. Similarly, it was known that in Australia the currawong, *Strepera graculina*, closely related to the Australian magpie, both belonging to the family of Artamidae, used a similar visual guidance system in years of severe infestation of scarab beetles of the species *Seriesthis pruinosa* [141].

However, not all scarab larva species leave identifying marks on the surface. Floyd and Woodland [136] wanted to know how magpies could find larvae that leave no visual cues. First, they established that there were no visual or other cues by which the magpies could identify where the larvae were, and they then conducted a series of auditory tests, finally pre-recording the chewing sounds the larvae made while feeding underground and playing back these sounds to magpies through micro-speakers. Under well-controlled experimental conditions, they could then test whether the magpies found the sound source. They did.

The speakers they used for playback in the field had a frequency response of 50–12,000 Hz [138]. Most of the sounds played backed to the magpies were at frequencies between 50 and 800 Hz, but there was a small high frequency component in the 1700–3000-Hz range. The scarabs produced sounds at an intensity of 30–38 dB. As tape hiss intensity was 30 dB, the subjects were offered a choice of playback of scarab noises or tape hiss alone (at 30 dB); the former resulted in immediate and successful responses; the latter did not elicit responses. Playback intensities were measured at 2 cm above ground level [138].

5.2. Foraging for Scarab Beetles by Magpies Is Lateralized

My own field observations on foraging behaviour in magpies (specifically for scarab larvae) are based on recordings made over a three-year period using several of our well-established research field sites on the Northern Tableland, near the city of Armidale, New South Wales (30°32′ S, 148°29′ E). All sites were permanent magpie territories of 3–7 residents, consisting of one breeding pair, juveniles and also some young adults (daughters from the previous year). Each territory was at least 2.5 hectares in size, flat grassland dotted with the occasional mature gum trees, some pine trees and shrubs, an

environment in which scarab larvae flourish. Two of the territories were adjacent to each other while the visits to two others were separated from each other by at least 2 km and 5 km, respectively.

On this Northern Tableland, largely sheep-grazing country, at altitudes of about 1000 m, three species of scarab beetles were strongly represented [142]. The larvae may pupate and emerge as beetles any time between November and March, i.e., larvae reach their full size at exactly the time when magpie offspring fledge (around September, sometimes earlier-depending on weather conditions) and make the greatest protein and food demands on the parent birds.

Magpies feed exclusively on the ground, and they walk, putting one foot before another, while foraging, sometimes referred to as 'walk-foraging' [143]. Their ground feeding habits make them easy to watch and follow their foraging in open fields especially. Moreover, magpies forage very systematically and according to a time-plan. They will reliably be at one specific transect of their territory at a certain time of day and will generally walk diagonally and in half a meter to meter distance from one another (Figure 6). No matter how large the territory, once their habits and time frame were known, observations could be made at set times in the morning and in the afternoon (changing the hour of day weekly to cover the times of their most vigorous foraging in the morning and the later afternoon).

Figure 6. Directionality and spacing in magpie foraging. Magpies tend to walk slowly and steadily in a direct line and in parallel to each other, taking transect after transect in a methodical way.

Each territory was visited daily for five days a week between September and March for three consecutive seasons, and all observable incidents of extractive foraging were recorded. Individual magpies could not be identified.

In the first weeks of watching foraging behavior closely, it became clear that the steps in all successful extractive foraging events were the same; the foraging bird was: (1) scanning the ground walking slowly; (2) then stopping and seemingly looking closely at the ground binocularly; (3) holding absolutely still; (4) in the last moment, turning the head so that the left side of the head/ear was close to the ground; (5) straightening up, the bird then executed a powerful jab into the ground; (6) then retrieving a large scarab larva from the grassy surface; and (7) expertly removing the hard head and the biting mandibles before swallowing it or feeding it to an offspring. Steps 3–7 typically lasted less than 30 s.

5.3. Results: Extractive Foraging

The sheer consistency of the foraging sequence and the changed posture of the bird observed made it possible to recognize the special extractive foraging strategy and made it clear, especially in some years with greater abundance of scarab larvae, that this was not an unusual and rare event, but a seasonal and integral part of the foraging behaviour of the territorial magpies, at least in a region where scarab larvae were often abundant.

A total number of observations accounted for 446 attempts at extractive foraging, but only 135 observations were ultimately included. One reason for the exclusion of a substantial number of seemingly successful extractions was the consequence of the behaviour of juveniles. Young juveniles (a month old or less post fledging) walked with the parent bird, but had the tendency to intervene in the process of foraging, by posting themselves in front of the adult to block the path, just so as to ensure that the morsel was fed to them as shown in Figure 7.

Figure 7. Parent feeding scarab larvae to magpie juvenile. Female magpie feeding a larva to a young fledgling. Such an example was not included in the analysis, and this method of feeding, the juvenile right in front of the parent bird, was limited in time and dependent on the juvenile's development. Blocking the path of the female walking was observed only in juveniles one month post-fledging. By two months post-fledging, most juveniles walked next to the adult (usually on the right side) and actively started observing the processes of the adult's food acquisition.

The most common reason for exclusion, however, concerned problems for the observer regarding distance or terrain. The most obvious problem occurred when the magpies foraged with their backs turned towards the observer and often at some distance, and in such cases, it made it difficult to be certain of the direction of head movements prior to extraction. Hence, such sequences were excluded even when the actual retrieval of larvae was seen.

The instances included were based on the foraging data obtained from four different territories. In an area of over 18 hectares traversed daily, the total number of resident magpies observed seems small ($N = 16$), and hence, it is very possible that, in some cases, the same magpies were scored repeatedly if they happened to be the successful ones in extracting the larvae, and this may partially account for the consistency of the findings. Relatedness is unlikely to be an issue in these results since juveniles forced out by the parents tend to roam in bachelor groups for at least four years and feed in non-dedicated, usually inferior, sites before some of them succeed in finding a suitable territory and a partner. There is no evidence that a daughter or son might secure a neighbouring territory.

Equally, the number of juveniles observed, at least in the first month of the season (September), typically made no contribution to extractive foraging, but were keen consumers: they often did not commence making successful extractions of larvae on their own until nearly the middle of the

observation period. Hence, although some magpies may have contributed several scores of extractions of larvae over the observation period of three months, this does not invalidate the observations because each event was a new event and an individual magpie could have approached the excavation site differently on each occasion.

Most incidents of successful extractive foraging were observed in October and November, the observed incidents sharply declining after mid-December when the ground became very dry and compacted and most scarab beetles might have emerged (see Figure 8).
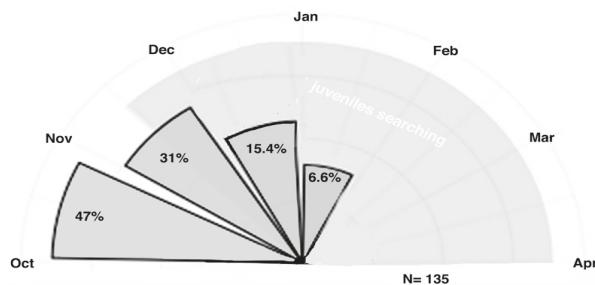
Figure 8. Walk-foraging and successful extractive foraging events. The majority of scarab larvae were retrieved in October, decreasing substantially by December and found only scarcely thereafter and not at all by February (percentage figures refer to successful retrievals counted). The large light-shaded semi-circle shows the months and hours when juveniles started searching for scarab larvae on their own, mostly with relatively little success.

All 135 recorded sequences showed the same left ear preference: the bird being observed tilted the head so that the left ear was held closer to the ground before straightening up and delivering the successful jab of its beak into the soil (Figure 9). This tilting of the head to a left position was clearly visible in each of the incidents. One would expect to find that not all scores of extractive foraging used the left ear (i.e., at least some magpies might have tilted the head in the other direction), but this was not so. Even though some of the scores were likely repeats for the same individual, the total absence of right ear use means that the bias is significant at the population level.

Figure 9. Lateralized auditory detection of prey item. The image of the magpie shows Step 4 in the extractive foraging sequence, moving the head from a 90° angle, binocular viewing, to a 45° angle, moving the beak to the right and up so that the left ear is closer to the ground.

5.4. Discussion

To my knowledge, this is the first example of auditory lateralization in the field describing an extractive foraging event that has not been described in any avian species. It is also the first example of auditory behavioural asymmetry under natural conditions.

The point made here is that the foraging strategy was not based on visual scanning, but crucially on auditory examination of a potential prey item and that it was consistently performed by the left ear. In the image shown above (Figure 9), the bird is walking leftwards, and the right ear would have been nearer for auditory inspection than the left, but the bird turned the head right around in order to listen to the underground larva with its left ear. In all cases included in the sample, the birds turned to position their left ear close to the ground.

It seems highly unlikely that this head tilt related to improving visual scanning. The visual field of magpies in the binocular field at close range is about 28–34° [4], and any fixation of a potential prey item is therefore most accurate when the beak points at about 90° to the ground (for binocular viewing). Since scarab larvae create no visual surface cues, as Floyd and Woodland (1981) had so convincingly shown [138], the only way magpies are able to identify the location of the underground prey item is by auditory means. Hence, regarding the head tilt prior to grasping the grub, we are left with only one explanation, namely that the bird obtained confirmation of the presence of a scarab larva exclusively by aural means.

In retrospect, watching magpie groups combing through their territories in such an orderly fashion (Figure 6 above) and doing so grid-by-grid every day may well be a result of having acquired the skill of extractive foraging. Clearly, the sounds that larvae make are so faint that they would be easily missed unless a group spaces out in such a way that every part of the ground can actually be scanned by listening to sounds at very close proximity.

5.5. Additional Field Results in Magpie Foraging

The results of foraging raise the question why this auditory behaviour is left-biased (right hemisphere) and significant at the population level and how this may fit the results in other and related studies we had conducted.

The extractive foraging results of lateralized listening follow from the results obtained on lateralized foraging behaviour in magpies in a series of additional field studies conducted by members of our laboratory [4,144]. One tested head turning during foraging (visual scanning); another scored eye preferences for tracking moving prey (for both, see [4], called Study 2 in the summarizing table below); and a third scored the side of begging behaviour of juveniles walk-foraging with a parent bird [144] (see the results summarized in Table 1 below).

Head turning during visual foraging (pecking food from the ground) was found to favour the right eye/left hemisphere. There was a slight, but significant bias at the population level for the bird to turn its head so that the right eye monocular field was directed towards the ground.

In a third study (eye preference for moving prey [4]), we supplied the magpies with food by purposely throwing mince-meat pieces in their direction and then scoring which eye they last used before taking and consuming it. Of 155 scores, 97 percent were left-eye dominant, meaning they involved left-eye viewing the moving target before food retrieval.

Later that year (also published in [4]), we had the opportunity to observe magpies dealing with moving prey items and watching the magpies trying to capture them. There was a locust plague, and locust were either jumping or flying up from the grass. Under natural conditions, we received the same results as in the food-supplementation experiment, finding a strong left-eye/right hemisphere preference. The results are consistent with use of the right hemisphere processing spatial information as known from studies in chicks [145].

In another field study (called Study 4 here; see also Table below [144]), it was recorded on which side juveniles approached the parent birds and begged for food while walk-foraging, and a significant group-level bias for begging on the right side of the parent was found. Juveniles were 2.46-times

more likely to beg on the right side than on the left [144]. By begging on the right side of a parent, a juvenile uses its left eye to view the adult and is in the parent's right visual field. Hoffman et al. [144] pointed out that visual inputs from the right visual field are processed by the left hemisphere, which is known to inhibit conspecific aggression, as found in chickens [146]. By approaching in the right hemifield, a juvenile magpie may also avoid being scolded by the parent bird [144]. Alternatively, and more likely, as a recent comparison across species indicates [147], the infant is positioning itself so that it can monitor the parent's behavior using its left visual field and right hemisphere, specialized for processing social behaviour.

Table 1. Hemispheric specializations in five field studies on foraging and vigilance in magpies.

Study	No. (Subjects)	No. Scores (Behavioral) Total/Bracket: Majority of Responses	Left Eye or Ear/Right Hemisphere	Right Eye or Ear/Left Hemisphere	Authors
(1) Extractive foraging	16	135 (135)	Left ear dominant		Kaplan, this paper,
(2) Head-turning during foraging	20	266 (116)		Right eye dominant	Rogers and Kaplan 2006 [4]
(3) Tracking moving prey	12	159 (155)	Left eye dominant		Rogers and Kaplan 2006 [4]
(4) Begging position of juveniles during foraging	6 parent-juvenile pairs	16/64 scores	Left eye dominant (begging juveniles)	Right eye dominant (feeding adult)	Hoffman et al. and Rogers 2006 [144]
(5) Inspecting predator	55	270 (compound score/various behaviors)	Left eye dominant		Koboroff, Kaplan and Rogers 2008 [148]

Brackets give the number of subjects/behavior showing eye/ear bias.

A fifth field study, not on foraging, but on eye preference in magpies when viewing a predator, scored eye use when presented with taxidermic models of a potential predator, a lace monitor [148]. We established by scoring monocular fixations from video footage that magpies used their left eye in the majority of instances while inspecting the potential predator, such as jumping (73%), prior to circling (65%), as well as during circling (58%) and for high alert inspection of the predator (72%), and we concluded that mobbing and perhaps circling are likely agonistic responses controlled by the left eye/right hemisphere [148].

The results of the second field study are consistent with preferred use of the left hemisphere and right eye in control of feeding responses as has also been shown in other species, including the zebra finch [149]. In the third field study, magpies show a left eye/right hemisphere preference reflecting a specialization for spatial information using global cues and also for rapid responding. It is thus noteworthy that of the three foraging tasks, two were controlled by the right hemisphere or expressed differently; it would be odd if two foraging tasks, looking for prey on the ground and listening for prey under the ground, were managed by different hemispheres. One is consistent with feeding responses generally, while the other method (extractive foraging using the left ear) is based on spatial information and auditory cues. Hence, these two foraging methods do not only require different strategies, but are also under the control of different hemispheres. While three of the findings for four field studies relate to visual lateralization in magpies (see Table 1), there may also be an auditory element to them.

My field study of foraging for scarab larvae showed a very strong bias towards the left ear to pinpoint the larvae's presence under the ground, leaving the right ear free to respond to the begging or other calls of an offspring. This may allow the magpie to attend to two tasks at once. Rogers et al. [148] showed in chicks that the performance of two tasks simultaneously, such as foraging and attending to a predator overhead, is undertaken effectively in strongly-lateralized chicks in which visual search is processed by the left hemisphere and predator detection by the right hemisphere [150].

Furthermore, agonistic responses are processed by the right hemisphere, consistent with research results in chicks [146] and other species [151]. Chicks also use the left eye to examine novel objects and

the details of a stimulus detecting small changes in familiar stimuli, whereas the right eye detects large changes that represent categories rather than details [152]. It is conceivable and even probable that the same hemispheric specializations that apply to eye use apply also to ear use.

6. Conclusions

This paper has presented evidence of lateralized behaviour in phonation and listening in one songbird species. Motor output and the way magpies produce song were shown to involve an entire range of techniques that enable an individual magpie not only to maintain singing for hours, but allow for a range of extraordinary modulations at a wide range of frequencies by using unusual techniques of lateralized frequency use (higher on left, lower on right side of syrinx). Paradoxically, so far, specific functions for their varied song have not been discovered. It is clear that their song can identify individuals one from another [75], but such individual recognition is conceivably achieved by just listening to their territorial call, referred to as carolling. There appears to be no territorial advantage for having a larger or smaller repertoire. It is possible, given that magpies form auditory maps of other species in their territory (they mimic only heterospecific sounds pertaining to their territory [74,76]), that the auditory memory, in this case of heterospecific sounds, is lateralized on the left side, as in other songbirds, but this has not been studied. It is also possible that such auditory 'maps' may be linked to other brain regions.

The substantial and innovative neuroscientific research in avian vocal production and vocal perception over the last decades notwithstanding, it pertains largely to a few small songbird species. Ocklenburg and Güntürkün in their paper [153] published a telling 'cladogram' showing that we have no information at all on lateralization in vocal production (central and peripheral) and vocal perception on any of the 28 clades of extant non-songbirds. Although Passeriformes are just one clade in this cladogram [153], Passeriformes, i.e., the true songbirds, actually make up the majority of all extant birds (over 5000 species). Additionally, while we know plenty about the zebra finch and a few other songbird species in this regard, there is little to no information available on almost all other extant songbirds either. It would help to understand whether large repertoires and flexible/plastic brains have developed other or additional neural mechanisms for song production and perception and whether this is achieved via specific hemispheric specializations. The magpie is certainly a representative of this kind of songbird. With an evolutionary history of likely more than 20 million years and in an evolutionary context of substantial speciation pre- and post the mass extinction of 65 mya, the emergence of a major songbird at that time may be as fascinating genetically as it is in its current performance.

Here, results of several field studies were presented. The results of lateralization in the field have been telling us that there are behaviours that are clearly highly lateralized in magpies. Extractive foraging has a particular place in ethological-cognitive research and, in primates, has been identified as one of the very complex cognitive behaviours and, when reported, relies usually on vision or on experience, but not purely on audition (the very specialized adaptations of the aye-aye being one of the few known exceptions).

This is the first paper that reports this auditory behaviour in a songbird and, furthermore, shows that the success of it may depend on a highly lateralized neuronal aspect in the auditory system. The results of the other field studies on foraging behaviour make a powerful point that the bird has to handle very different experiences and tackle potential dangers while foraging or encountering predators. Here, it has been shown that these key functions are lateralized, which may have substantial advantages for survival.

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symmetry

Article

Distribution of Antennal Olfactory and Non-Olfactory Sensilla in Different Species of Bees

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Abstract: Several species of social bees exhibit population-level lateralization in learning odors and recalling olfactory memories. Honeybees *Apis mellifera* and Australian social stingless bees *Trigona carbonaria* and *Austroplebeia australis* are better able to recall short- and long-term memory through the right and left antenna respectively, whereas non-social mason bees *Osmia rufa* are not lateralized in this way. In honeybees, this asymmetry may be partially explained by a morphological asymmetry at the peripheral level—the right antenna has 5% more olfactory sensilla than the left antenna. Here we looked at the possible correlation between the number of the antennal sensilla and the behavioral asymmetry in the recall of olfactory memories in *A. australis* and *O. rufa*. We found no population-level asymmetry in the antennal sensilla distribution in either species examined. This suggests that the behavioral asymmetry present in the stingless bees *A. australis* may not depend on lateral differences in antennal receptor numbers.

Keywords: lateralization; asymmetry; bees; antennal sensilla; olfaction

1. Introduction

The different functional specialization of the right and left sides of the nervous system (lateralization) is a feature shared by many vertebrates and also invertebrate species (see [1,2]). Lateralization manifests itself in a substantial range of behaviors and cognitive tasks, and mediates distinct sensory, motor and cognitive processes. In several species, behavioral asymmetries such as, for example, a side-bias in turning in one direction or a preferential use of one eye, ear or nostril to respond to specific stimuli, have been associated with corresponding asymmetries in the anatomical substrates of the nervous system (see [1]). These anatomical differences can be present at different levels: (i) in macroscopic anatomy, such as, for example, the Sylvian fissure of the lateral sulcus in humans that, in most people, is longer in the left hemisphere [3]; (ii) in the different size of the fibers that connect sensory reception and motor afference, such as the Mauthner cells responsible for the lateralization in the C-start bending reaction to danger in fishes [4]; or (iii) at the cellular level, such as in the different arrangement of synapses for specific neurotransmitters between the right and the left side of specific cerebral structures (e.g., the glutamate *N*-methyl-D-aspartate (NMDA) receptor, implied in learning and memory, in the left and right hippocampus of rodents [5]).

Several species of social bees exhibit population-level lateralization in learning odors and recalling olfactory memories. The first evidence comes from a study by Letzkus and colleagues [6], showing that honeybees *Apis mellifera* trained with only one antenna in use to associate an odor with a sugar reward in the proboscis extension reflex (PER) paradigm performed better in a recall test 5–6 h after training when they used their right antenna. Letzkus et al. [6] also looked at the distribution of one type of olfactory sensilla (the *sensilla placodea*) on the antennae and found that the right antenna had more *sensilla placodea* compared to the left antenna, and they linked this result with the better performance

of the bees in the PER. Since the bees were both trained and tested with only one antenna in use, it is very difficult to establish whether the behavioral asymmetry observed related to the learning phase or to the recall of the olfactory memory.

Access to unilaterally acquired memories for odors is transferred to the other side of the brain in honeybees [7] and this transfer seems to occur from the right to the left side of the brain. Specifically, Rogers and Vallortigara [8] showed that there is a time-dependence in the behavioral asymmetry in the PER. Specifically, when honeybees are trained in a PER paradigm with both antennae in use, they are better at recalling the olfactory memory 1–2 h after training using their right antenna, and 8–12 h after training using their left antenna [8,9]. The same pattern of lateralization in the recall of short- and long-term olfactory memories has been found in the Australian social stingless bees *Trigona carbonaria*, *Trigona hockingsi* and *Austroplebeia australis*—the three species are better able to recall short- and long-term memories through the right and left antenna respectively [10]. It is important to underline that the results of the study conducted by Rogers and Vallortigara [8] and those of the following studies [9–13] investigated asymmetry in the recall of olfactory memories and not in the learning phase as the bees were trained with both antennae in use. Recent evidence has confirmed that in honeybees trained with only one antenna in use during olfactory learning, the left hemisphere is more responsible for long-term memory and the right hemisphere is more responsible for the learning and short-term memory [14]. Moreover, the gene expression in the brain of these honeybees was also asymmetric, with more genes having higher expression in the right hemisphere than the left hemisphere [14].

Interestingly, the non-social mason bees *Osmia rufa* are not lateralized in this way for the recall of short-term memory since they can retrieve it both through the circuits of the right and left antenna [11]. However, when tested for electroantennographic (EAG) responsivity to different odors, most mason bees showed individual lateralization (seven and eight individuals out of 21 showed significantly stronger responses respectively with the right and the left antenna), whereas honeybees show population-level lateralization with higher EAG responses on the right than on the left antenna [11].

Bumble bees *Bombus terrestris* trained on the PER paradigm with only one antenna in use and tested one hour after training show the same asymmetrical performance favoring the right antenna as do honeybees and the three species of Australian stingless bees. However, in bumble bees EAG responsivity is not lateralized at the population level, as it is in honeybees. In fact, as with mason bees, most bumble bees show individual lateralization (nine and three individuals out of 20 showed significantly stronger responses respectively with the right and the left antenna) [12].

In honeybees, the population-level asymmetry in the recall of olfactory memories may be partially explained by a morphological asymmetry at the peripheral level—the right antenna has about 5% more olfactory sensilla than the left antenna [6,13]. However, this does not exclude that the right antenna may also have a more important role than the left antenna in learning the association between an odor and a sugar reward in the PER paradigm [6]. As a consequence, it is possible that the morphological asymmetry observed in the number of olfactory sensilla influences the learning process and not the recall of the olfactory memory.

Moreover, honeybees with only the right antenna in use are better at discriminating a target from a background odorant in a cross-adaptation experiment (i.e., when a target odor is superimposed on the same or a different background odor), and this behavioral performance is not due to different discrimination of changes in odor concentration, nor to different learning abilities during odor discrimination [15]. Indeed, Rigosi et al. [15] showed that odor representations in the projection neurons of the right and left antennal lobes (ALs) are different with higher Euclidian distances between activity patterns in the right AL compared to the left. Interestingly, it is the odor representation in the right and the left ALs that is different. In fact, the functional activity patterns elicited by stimulation with different odors (both pheromones and environmental odors) in the right and the left AL of the same honeybee are bilaterally symmetrical [16]. In addition, at 14 days post-emergence the levels of neuroligin-1 expression, a protein involved in learning and memory, are higher in honeybees with only their right antenna compared to honeybees with only the left or both antenna [17].

In bumble bees *Bombus terrestris*, morphological counting of the olfactory and non-olfactory sensilla show a predominance in the number of only one type of olfactory sensilla, the *s. trichodea type A*, in the right antenna [12]. In the Australian stingless bee *T. carbonaria*, the right and the left antenna present the same number of olfactory and non-olfactory sensilla [18].

The antennae of female wasps *Anastatus japonicus* Ashmead (Hymenoptera: Eupelmidae), a non-social parasitoid, present more *s. placodea* on the right antenna than on the left antenna. Interestingly, in this species the distribution of *s. trichodea* and *s. basiconica* is asymmetrical between the antennae, but it depends on the segment. In fact, these sensilla are more abundant on the third flagellum antennomere of the right antenna than on the corresponding flagellum of the left antenna—the reverse results were observed for *s. trichodea* on the scape, pedicle, and fourth to fifth flagellum antennomeres, and for *s. basiconica* on the seventh flagellum antennomere and the third clava antennomere— suggesting that the asymmetry between the antennae can vary depending on the segment [19].

Here we looked at the possible correlation between the distribution of antennal sensilla in those species mentioned above that have been previously studied for behavioral asymmetry in the recall of olfactory memories, specifically the social Australian stingless bee *Austroplebeia australis* and the non-social mason bee *Osmia rufa*.

2. Materials and Methods

2.1. Subjects

Female adult mason bees *Osmia rufa* were obtained as they emerged from over-wintering cocoons collected at Crevalcore (Bologna, Italy) during spring 2011. Australian stingless *A. australis* foragers ($N = 14$) of unknown age were caught as they exited a well-established hive located in Valla, NSW, Australia within the natural range of the species in summer 2014.

2.2. Types of Sensilla

The different types of sensilla were identified on the basis of previous studies conducted on other Apoidea species [11,12,15]. For both species, we distinguished three types of putative olfactory sensilla (Figure 1)—*s. placodea*, *s. trichodea type A* (thick), and *s. coeloconica*—and two types of non-olfactory sensilla (Figure 1)—*s. trichodea type B* (thin) and *s. ampullacea* (Figure 2a)—clearly distinguishable from the putative olfactory *s. coeloconica* (Figure 2b) as they are smaller in size. Interestingly, the antennae of *A. australis* also present two more types of sensilla which we could clearly recognize, the non-olfactory *s. coelocapitulum* (Figure 1) and the putative olfactory *s. basiconica* (Figures 1 and 2c), exactly as with honeybees [13] and *T. carbonaria* [18]. Bumble bees *B. terrestris* also possess *s. basiconica*, but not *s. coelocapitulum* [12].

We also observed other types of sensilla in *O. rufa*, the *s. basiconica thick* (Figure 2d)—which is bigger than the standard *s. basiconica* (Figure 2c) and presents only lateral pores—and the *s. trichodea type C* (Figure 2e)—characterized by lateral pores, rifling, and an apical pore, which may have a double taste (because of the apical pore) and olfactory (because of the lateral pores) function. Finally, in *A. australis* we saw another type of *s. trichodea type D* (Figure 2f), with no pores (and thus probably non-olfactory), which given the lack of curvature could be easily recognized and distinguished from the olfactory *s. trichodea type A* (Figure 2f). We decided not to count these three new types of sensilla (i.e., *s. basiconica thick*, *s. trichodea type C* and *s. trichodea type D*) since we were not sure of their function. Thus, we limited our analyses to the sensilla we had already observed in other Apoidea species and which we could clearly distinguish based on previous studies.
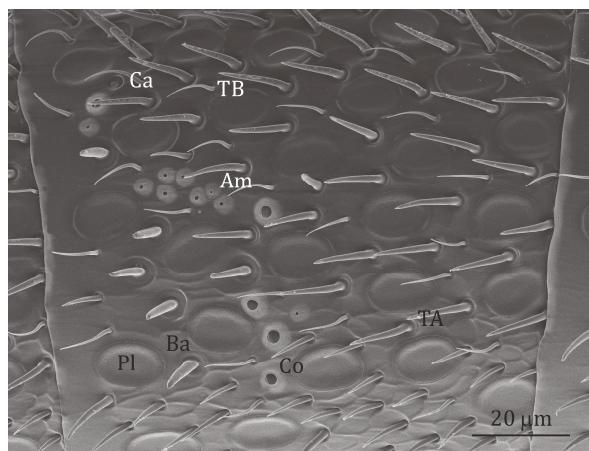
Figure 1. Scanning electron micrograph of the 9th segment of the left antenna of an *A. australis* forager (left view, i.e., imaging of the left antenna side). In black the putative olfactory *s. placodea* (Pl), *s. trichodea type A* (TA), *s. coeloconica* (Co) and *s. basiconica* (Ba); in white the non-olfactory *s. trichodea type B* (TB), *s. ampullacea* (Am) and *s. coelocapitulum* (Co). All the sensilla mentioned above are also present in *O. rufa* females apart from *s. basiconica* and *s. coelocapitulum*.
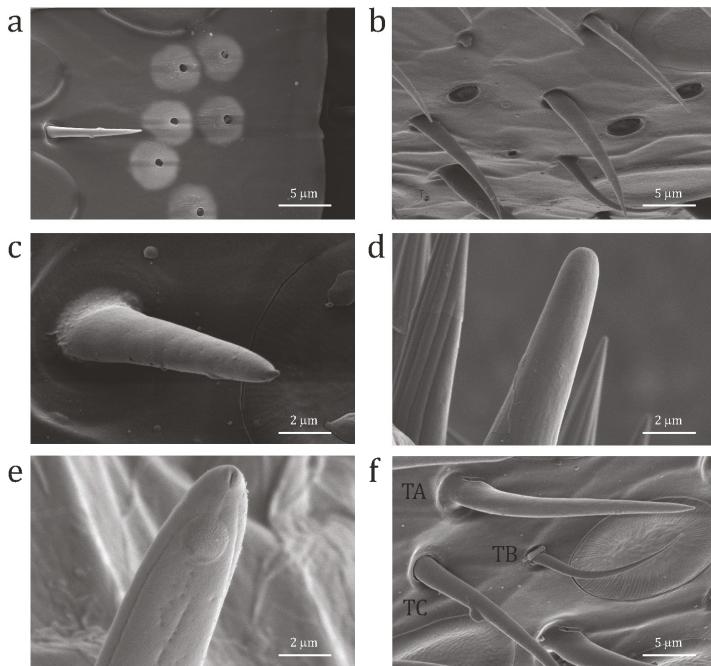
Figure 2. Scanning electron micrographs of details of (**a**) *s. ampullacea* (Am) in *A. australis*; (**b**) *s. coeloconica* (Co) in *A. australis*; (**c**) standard *s. basiconica* (Ba) in *A. australis*; (**d**) *s. basiconica thick* in *O. rufa*; (**e**) *s. trichodea type C* in *O. rufa*; and (**f**) *s. trichodea type D* (TD), *s. trichodea type A* (TA), *s. trichodea type B* (TB) in *A. australis*.

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The mason bees *O. rufa* were preserved in a freezer in Trento before being taken to the Department of Medicine Laboratory, Azienda Provinciale per i Servizi Sanitari (APSS), Trento, Italy for preparation and imaging of the sample. There the antennae of the bees were removed and cleaned using ultrasound in a bath of acetone. The right and left antenna of each bee were then attached to a circular stub by double-sided conductive tape (TAAB Laboratories Equipment Ltd., Aldermaston, UK) and gold-coated to guarantee electrical conductivity during imaging with a XL 30, field emission environmental scanning electron microscope (FEI-Philips, Eindhoven, The Netherlands). Each antenna was imaged from four different viewpoints—ventral view (sample positioned at 0°), right view (sample tilted at −75°, imaging of the right antenna side), left view (sample tilted at +75°, imaging of the left antenna side), and dorsal view (following removal of the antenna from the stub and placing it upside down)—as done previously for honeybees [13], bumblebees [12] and *T. carbonaria* [18].

The same procedure was adopted for the *A. australis* bees with the difference that these bees were preserved in a freezer in Australia before being transported to the Department of Medicine Laboratory in Trento, Italy. Since *A. australis* bees are much smaller in size that mason bees, the whole heads of the bees rather than just the antennae were removed, as previously done for *T. carbonaria* [18]. Then, they underwent the same sample preparation and imaging as mason bees. As there are no olfactory receptors on the first two segments of the mason bee flagellum, only the third to tenth segments were scanned. Each segment from the third to ninth was scanned longitudinally at a magnification of 600 times. A magnification of 800 times was used for the tenth smallest segment (apex). For the same reason, only the second to tenth segments were scanned for *A. australis*. Given that the antennae of this species are smaller than the antennae of mason bees, each segment was scanned longitudinally at a larger magnification of 1000 times rather than 600 times.

2.4. Sensilla Counting and Statistical Analyses

Each sensilla was tagged and counted in all acquired images using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). We conducted analysis of variance (ANOVA) with the antennae (two levels), segments (eight levels for *O. rufa* and nine levels for *A. australis*), and type of sensilla (five and seven levels respectively for *O. rufa* and *A. australis*) as within-subjects factors, using Greenhouse–Geisser values of probability when sphericity was violated. Further analyses were conducted by grouping and separating the putative olfactory from the non-olfactory sensilla. Two-tailed binomial tests were used to evaluate individual differences in the number of olfactory and non-olfactory sensilla between the right and the left antennae.

3. Results

The results for both species are shown in Figure 3. For *O. rufa*, the overall ANOVA revealed significant main effects of segment (Greenhouse–Geisser, $F_{2.841,36.930} = 734.905$, $p < 0.0001$) and sensilla type (Greenhouse–Geisser, $F_{1.416,18.410} = 976.911$, $p < 0.0001$), but no effect of the antenna (left versus right) (sphericity assumed, $F_{1,13} = 4.217$, $p = 0.061$), although there was a tendency towards more sensilla on the left antenna. There was a significant interaction between segment $\times$ sensilla type (Greenhouse–Geisser, $F_{3.877,50.399} = 261.403$, $p < 0.0001$) but no significant interaction with antenna (antenna $\times$ type, Greenhouse–Geisser, $F_{2.154,28.006} = 2.031$, $p = 0.147$; antenna $\times$ segment, Greenhouse–Geisser, $F_{2.005,26.066} = 0.602$, $p = 0.555$; antenna $\times$ type $\times$ segment, Greenhouse–Geisser, $F_{3.734,48.538} = 1.269$, $p = 0.296$).

Similarly, for *A. australis*, the overall ANOVA revealed significant main effects of segment (Greenhouse–Geisser, $F_{2.002,26.027} = 458.141$, $p < 0.0001$) and sensilla type (Greenhouse–Geisser, $F_{1.545,20.091} = 1982.535$, $p < 0.0001$), but no effect of the antenna (left versus right) (sphericity assumed, $F_{1,13} = 0.045$, $p = 0.835$). There was a significant interaction between segment $\times$ sensilla type (Greenhouse–Geisser, $F_{4.941,64.230} = 244.914$, $p < 0.0001$) but no significant interactions with

antenna (antenna $\times$ type, Greenhouse–Geisser, $F_{1.324,17.206} = 1.380$, $p = 0.267$; antenna $\times$ segment, Greenhouse–Geisser, $F_{3.480,45.243} = 1.405$, $p = 0.251$; antenna $\times$ type $\times$ segment, Greenhouse–Geisser, $F_{5.609,72.911} = 0.917$, $p = 0.483$).

We then summed up all the olfactory sensilla and all the non-olfactory sensilla, and conducted separate ANOVAs with sensilla type (olfactory vs. non-olfactory—two levels) as within-subjects factors to see whether there was any significant antenna $\times$ sensilla type interaction. For *O. rufa* (Figure 3a) we found a significant main effect of segment (Greenhouse–Geisser, $F_{2.841,36.930} = 734.905$, $p < 0.0001$) and sensilla type (sphericity assumed, $F_{1,13} = 6102.170$, $p < 0.0001$), but no effect although a tendency of the antenna (sphericity assumed, $F_{1,13} = 4.217$, $p = 0.061$). Again, there was a significant interaction between segment $\times$ sensilla type (Greenhouse–Geisser, $F_{2.835,36.855} = 533.080$, $p < 0.0001$) but no significant interaction with antenna (antenna $\times$ type, sphericity assumed, $F_{1,13} = 3.337$, $p = 0.091$; antenna $\times$ segment, Greenhouse–Geisser, $F_{2.005,26.066} = 0.602$, $p = 0.555$; antenna $\times$ type $\times$ segment, Greenhouse–Geisser, $F_{2.499,32.488} = 1.040$, $p = 0.378$). Likewise, ANOVA of the data for olfactory vs. non-olfactory sensilla for *A. australis* (Figure 3b) revealed significant main effects of segment (Greenhouse–Geisser, $F_{2.002,26.027} = 458.141$, $p < 0.0001$) and sensilla type (sphericity assumed, $F_{1,13} = 2269.510$, $p < 0.0001$), but no effect of the antenna (sphericity assumed, $F_{1,13} = 0.045$, $p = 0.835$). There was a significant interaction between segment $\times$ sensilla type (Greenhouse–Geisser, $F_{3.160,41.079} = 98.505$, $p < 0.0001$) but no significant interactions with antenna (antenna $\times$ type, sphericity assumed, $F_{1,13} = 1.893$, $p = 0.192$; antenna $\times$ segment, Greenhouse–Geisser, $F_{3.480,45.243} = 1.405$, $p = 0.251$; antenna $\times$ type $\times$ segment, Greenhouse–Geisser, $F_{3.086,40.112} = 1.029$, $p = 0.391$).
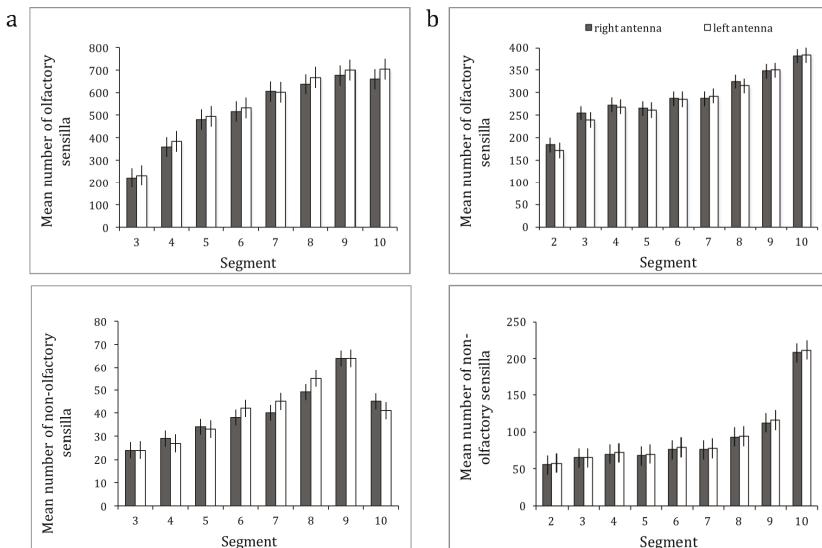
Figure 3. The mean number of olfactory and non-olfactory sensilla with the respective standard error (SE) in function of the segment number for the right antenna (dark grey bars) and for the left antenna (white bars) of (**a**) *O. rufa* females ($N = 14$) and (**b**) *A. australis* foragers ($N = 14$).

We also looked at possible differences between the right and the left antenna when the segment was not considered as a factor. For *O. rufa*, there was no significant effect of antenna (sphericity assumed, $F_{1,13} = 4.217$, $p = 0.061$) nor the antenna $\times$ type interaction (Greenhouse–Geisser, $F_{2.154,28.006} = 2.031$, $p = 0.147$), but only the effect of the sensilla type was significant (Greenhouse–Geisser, $F_{1.416,18.410} = 976.911$, $p < 0.0001$). For *A. australis*, the differences between the left and right

antennae were not significant (sphericity assumed, $F_{1,13} = 0.045$, $p = 0.835$) nor the antenna $\times$ type interaction (Greenhouse–Geisser, $F_{1.324,17.206} = 1.380$, $p = 0.267$), but only the effect of the sensilla type was significant (Greenhouse–Geisser, $F_{1.545,20.091} = 1982.535$, $p < 0.0001$).

Interestingly, when we looked at possible individual differences between the number of olfactory and non-olfactory sensilla on the two antennae, we found that most individuals of both species showed individual-level asymmetry (Tables 1 and 2). Ten out of 14 individual *O. rufa* showed a significantly higher number of olfactory sensilla (estimated by the two-tailed binomial test, $p < 0.05$) either on the right (two individuals) or the left (eight individuals) antenna (Table 1). Nine of 14 mason bees, six of which were the same individuals that showed individual asymmetry for the olfactory sensilla, had more non-olfactory sensilla either on the right (four individuals) or the left (five individuals) antenna (Table 1).

Table 1. Individual-level lateralization in *O. rufa*. The number of olfactory and non-olfactory sensilla on the left and right antennae of individual mason bees *O. rufa* ($N = 14$) is reported with the corresponding *p*-values and z-scores of two-tailed binomial tests. The direction of asymmetry is indicated as L (left) or R (right) when statistically significant. The asterisks indicate significant difference: n.s. for $p > 0.05$; * for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$; **** for $p \leq 0.0001$.

Mason Bees *O. rufa*									
Olfactory Sensilla					Non-Olfactory Sensilla				
Left	Right	2-Tailed Binomial Test	z-Score	Asymmetry Direction	Left	Right	2-Tailed Binomial Test	z-Score	Asymmetry Direction
4559	3715	<0.0001 ****	9.27	L	372	433	0.035 *	−2.11	R
3833	4139	0.0006 ***	−3.42	R	260	371	<0.0001 ****	−4.38	R
4364	4144	0.013 *	2.48	L	316	324	0.779 n.s.	−0.28	
4647	4548	0.307 n.s.	1.02		407	323	0.0021 **	3.07	L
4517	4219	0.0014 **	3.18	L	403	397	0.857 n.s.	0.18	
4473	4077	<0.0001 ****	4.27	L	298	312	0.596 n.s.	−0.53	
4227	4014	0.019 *	2.34	L	329	231	<0.0001 ****	4.10	L
4506	4183	0.00056 ***	3.45	L	337	225	<0.0001 ****	4.68	L
3958	3966	0.936 n.s.	−0.08		172	304	<0.0001 ****	−6.00	R
4335	4126	0.024 *	2.26	L	408	402	0.857 n.s.	0.18	
3839	4164	0.0003 ***	−3.62	R	350	223	<0.0001 ****	5.26	L
4672	4477	0.042 *	2.03	L	390	270	<0.0001 ****	4.63	L
4071	4104	0.726 n.s.	−0.35		327	333	0.849 n.s.	−0.19	
4378	4291	0.357 n.s.	0.92		266	360	0.0002 ***	−3.72	R

Table 2. Individual-level lateralization in *A. australis*. The number of olfactory and non-olfactory sensilla on the left and right antennae of individual Australian stingless bees *A. australis* ($N = 14$) is reported with the corresponding *p*-values and z-scores of two-tailed binomial tests. The direction of asymmetry is indicated as L (left) or R (right) when statistically significant. The asterisks indicate significant difference: n.s. for $p > 0.05$; * for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$; **** for $p \leq 0.0001$.

Australian Stingless Bees *A. australis*									
Olfactory Sensilla					Non-Olfactory Sensilla				
Left	Right	2-Tailed Binomial Test	z-Score	Asymmetry Direction	Left	Right	2-Tailed Binomial Test	z-Score	Asymmetry Direction
2199	2090	0.099 n.s.	1.65		683	680	0.960 n.s.	0.05	
2639	2662	0.764 n.s.	−0.30		855	826	0.497 n.s.	0.68	
2648	2639	0.912 n.s.	0.11		846	868	0.610 n.s.	−0.51	
2601	2677	0.303 n.s.	−1.03		823	875	0.215 n.s.	−1.24	
2531	2510	0.779 n.s.	0.28		805	818	0.764 n.s.	−0.30	
2674	2576	0.180 n.s.	1.34		819	764	0.174 n.s.	1.36	
2712	2840	0.089 n.s.	−1.70		904	930	0.562 n.s.	−0.58	
2673	2787	0.126 n.s.	−1.53		937	917	0.659 n.s.	0.44	
2524	2756	0.0015 **	−3.18	R	892	910	0.689 n.s.	−0.40	
2831	2394	<0.0001 ****	6.03	L	874	676	<0.0001 ****	5.00	L
2080	2373	<0.0001 ****	−4.38	R	703	711	0.849 n.s.	−0.19	
2765	2523	0.0009 ***	3.31	L	1000	812	0.0001 ****	4.39	L
2406	2711	<0.0001 ****	−4.25	R	876	893	0.704 n.s.	−0.38	
2715	2972	0.0007 ***	−3.39	R	808	856	0.250 n.s.	−1.15	

Six out of 14 individual *A. australis* showed a significantly higher number of olfactory sensilla (estimated by two-tailed binomial test, $p < 0.05$) either on the right (four individuals) or the left (two individuals) antenna (Table 2). The same two individuals that had more olfactory sensilla on the left antenna, also had significantly more non-olfactory sensilla on the left antenna (Table 2).

4. Discussion

We showed that both *O. rufa* and *A. australis* do not show differences at the population level in the number of olfactory and non-olfactory sensilla on the right and the left antennae. However, about half of the individuals of both species presented individual-level asymmetry—some bees had more olfactory (and/or non-olfactory) sensilla on the right antenna and others had more sensilla on the left antenna.

Our results seem to be partially in line with previous findings. In fact, although previous studies found that the right antenna presents on average (i.e., at the population level) a higher number of at least one type of olfactory sensilla compared to the left antenna in the species *A. mellifera* [13], *B. terrestris* [12] and *A. japonicas* Ashmead [19], no significant differences in the number of either olfactory or non-olfactory sensilla between the left and the right antennae were found in *T. carbonaria* [16].

As both *T. carbonaria* and *A. australis* show population level lateralized behavior in the recall of olfactory memories [10], as do honeybees [8], we are tempted to conclude that this behavioral asymmetry cannot be explained by a different number of sensilla on the right compared to the left antenna. Indeed, for honeybees the difference between the number of olfactory receptors between the right and the left antenna is only 5% [13]. It is likely that behavioral asymmetry in the learning and recall of olfactory memories through the circuits of the right and left antenna is due to other functional asymmetries in the way these memories are represented and processed in the brain, as shown in the antennal lobe of *A. mellifera* [15]. Moreover, lateralized behavior may be due to asymmetries in the expression of specific neurotransmitters between the two sides on the nervous system, as suggested by a study by Biswas and colleagues [17], showing that levels of neuroligin-1 expression are higher in honeybees with only their right antenna compared to honeybees with only the left or both antennae [17].

It is also important to consider that the asymmetrical processing of odors may be advantageous to the single individual as this would allow, for example, the learning of new odors with one hemisphere and the keeping of memories of old odors in the other hemisphere [9]; this does not necessarily imply that all the individuals should be aligned in the same direction. Indeed, in our study we showed that about half of the individuals in the population (depending on the species considered) are lateralized at the individual level. The individual asymmetry that we observed in the current study of *O. rufa* matches well with previous findings of individual-level lateralization in the EAG responses [11]. In fact, 15 individuals out of 21 (71%) were found to have significantly stronger responses with either the right (seven individuals) or the left antenna (eight individuals) [11]. Here, 10 out of 14 (71%—exactly the same proportion) of mason bees showed significant asymmetry in the number of olfactory sensilla, and nine out of 14 individuals in the non-olfactory sensilla, regardless of the direction.

An alignment of lateralization within the population has been suggested to be a consequence of social pressure [20] and to arise as an evolutionarily stable strategy when individually asymmetrical organisms must coordinate their behavior with that of other individually asymmetrical organisms within the same species [21,22]. Recently, evidence has started to emerge suggesting that also so-called "non-social" species of insects are lateralized at the population level when biases in social interactions are considered. This is the case for *O. rufa*, a species that does not show behavioral asymmetry in the recall of short-term olfactory memory, but shows population-level lateralization in aggressive displays [23], similarly to eusocial honeybees [24] and social stingless bees *T. carbonaria* [18]. Thus, it is plausible that *A. australis* would also exhibit population-level biases in competitive and/or cooperative interactions with other individuals.

Clearly, it is the possibility of being engaged in interactions with other individuals rather than the way in which the species nests (socially or not) that may affect lateralization. Nonetheless the reason why some species show individual-level or population-level lateralization at the peripheral level, i.e., in the antenna, and what, if any, is the functional significance of this remains at present unexplained. It is possible that morphological asymmetry in the number of olfactory sensilla on the antennae influences the learning process of odors rather than the recall of the memories associated with these odors. Likewise, an asymmetrical distribution of non-olfactory sensilla may influence other sensory processes and allow parallel processing of different kinds of information in the two sides of the brain.

Author Contributions: E.F. and G.V. conceived and designed the experiments; E.F. performed the experiments; E.F. analyzed the data; E.F and G.V. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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A Matter of Degree: Strength of Brain Asymmetry and Behaviour

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Abstract: Research on a growing number of vertebrate species has shown that the left and right sides of the brain process information in different ways and that lateralized brain function is expressed in both specific and broad aspects of behaviour. This paper reviews the available evidence relating *strength* of lateralization to behavioural/cognitive performance. It begins by considering the relationship between limb preference and behaviour in humans and primates from the perspectives of direction and strength of lateralization. In birds, eye preference is used as a reflection of brain asymmetry and the strength of this asymmetry is associated with behaviour important for survival (e.g., visual discrimination of food from non-food and performance of two tasks in parallel). The same applies to studies on aquatic species, mainly fish but also tadpoles, in which strength of lateralization has been assessed as eye preferences or turning biases. Overall, the empirical evidence across vertebrate species points to the conclusion that stronger lateralization is advantageous in a wide range of contexts. Brief discussion of interhemispheric communication follows together with discussion of experiments that examined the effects of sectioning pathways connecting the left and right sides of the brain, or of preventing the development of these left-right connections. The conclusion reached is that degree of functional lateralization affects behaviour in quite similar ways across vertebrate species. Although the direction of lateralization is also important, in many situations strength of lateralization matters more. Finally, possible interactions between asymmetry in different sensory modalities is considered.

Keywords: functional asymmetry; strength of lateralization; direction of lateralization; advantages; disadvantages; vertebrate species; limb preference; eye bias

1. Introduction

A number of papers have reviewed the evidence for functional asymmetry of the brain, citing research showing that it is present in a growing list of vertebrate species [1–4], as well as more recent research demonstrating its presence in invertebrate species (summarized in [5]). The ubiquity of functional asymmetry suggests that it confers selective advantages [6], and some evidence in support of this deduction has been found by comparing the performance, within a species, of individuals with strongly versus weakly lateralized brains. By summarizing the research on different species, this paper attempts to arrive at a conclusion about the benefits versus deficits of strong versus weak lateralization.

The first obstacle encountered in an attempt to bring the research together is that different measures of the strength of laterality have been used [7]. Strength of paw or hand preference has been used as the axiom of strength of laterality in humans and other primates, although other techniques are now being used. In birds, strong versus weak or no laterality of visual responses has been generated by incubating eggs in the light or in darkness during the final days before hatching [4,8], and a similar method has been used to manipulate strength of lateralization in fish [9].

Although it is recognized that using different measures of the strength of lateralization could lead to different results, at this juncture it is worth taking a broad perspective to see how these disparate measures of laterality may be related to cognitive performance. The hypothesis considered is that cognitive ability is enhanced by having a strongly lateralized brain. That is, general cognitive performance may be enhanced by having a brain that is largely, if not entirely, subdivided to process information differently on the left and right sides (i.e., with distinctly separate computational processes being carried out in the left and right hemispheres [10,11]).

As summarised previously [3,4], in a range of vertebrate species the left hemisphere is specialized to categorize stimuli (e.g., food from inedible objects, general characteristics shared by all conspecifics versus those of other species), to focus attention and attend to specific targets and cues, to control established/learnt patterns of behaviour under relaxed conditions and to sustain responding by inhibiting fleeing and inhibiting attention to extraneous stimuli. The right hemisphere has broad attention, used to monitor the surrounds for the presence of predators and attend to other distracting stimuli, and also to detect novel stimuli. The right hemisphere also attends to social cues and, as part of that, recognizes faces of conspecifics, controls aggressive and sexual behaviour, as well as fear responses, and assesses multiple properties of stimuli. As an example of the right hemisphere's control of social responding, pigeons display more rapid social reactions to members of the flock on their left side, processed by the right hemisphere [12]. Also, as shown in a wide range of vertebrate species, infants are positioned more commonly on their mother's left side [13], thus being monitored by her right hemisphere. The right hemisphere also comes into play when the animal is under stress, and in these circumstances it has a dominant role in controlling responses [4].

Left-analytic encoding versus right-global encoding has been demonstrated clearly in chicks using tests similar to those designed for testing humans [14]. These subdivisions of function have been determined by testing a range of non-human species, primarily but not exclusively domestic chicks [15], pigeons [16], zebra fish [17], sheep [18] and dogs [19]. Observation of some species in the wild has confirmed that these asymmetries are seen not only in laboratory settings but also in natural habitats; for example, in Australian magpies responding to a predator [20] and in cetaceans feeding with a right side bias [21].

Similar or the same left-right specialisations are present in humans. A body of research has shown ([1], for example) that the majority of humans use the left hemisphere when they perform established or routine patterns of behaviour and, when using this hemisphere, their attention is focused. By contrast, the right hemisphere of humans has a broad attention used in detecting and responding to unexpected stimuli and responding to affective stimuli [22]. The right hemisphere of humans is also used to recognize faces, especially their emotional expressions [23], and to process other aspects of social information. Not surprising therefore, the right hemisphere is specialized for expressing anger and hostility and for processing of speech with emotional prosody [24] and it also has a role in depression (see later).

The question addressed in this paper is: does cognitive performance depend on the *degree* of lateralization of all or any of these respective hemispheric specializations present in vertebrate species?

2. Limb Preference and Performance

Not surprisingly, since each limb is controlled by its contralateral hemisphere [25], a good deal of research on humans has investigated associations between hand preference and cognitive performance. In general, left-handed subjects excel in tasks requiring cognitive functioning and behaviour associated with the right hemisphere, such as visuospatial ability [26,27] and arithmetic ability [28], whereas right-handed subjects excel on tasks associated with the left hemisphere, such as verbal tasks [29]. It is noted, however, that the association between handedness and cerebral asymmetry is not strong and, as Badzakova-Trajkov et al. [30] found, there is no correlation between handedness and spatial attention, measured in a line-bisection task and as memory of faces.

A study by Denny [31] conducted on a very large population of people from various European countries found that left-handers were significantly more likely to have depressive symptoms than were right handers. Non-right handers, meaning either ambidextrous or left-handed (also referred to as mixed handedness), are more prone than right-handers to suffer from a range of conditions, including schizophrenia [32,33], psychosis [34] and post-traumatic stress disorder [35]. Also, as found in a large sample of 11-year-old children [36], ambidextrous handedness is associated with poorer verbal, nonverbal, reading and mathematical skills compared to either left- or right-handers. This finding has been supported by the results of follow-up studies conducted on children of various ages and adolescents [37,38].

Many studies have compared right-handed with non-right-handed subjects and not weak handedness with strong handedness; this may not be the best way to categorise subjects. A study by Tsuang et al. [39] classified subjects into three categories (left-, right- and mixed handedness) and reported that schizotypy is associated with mixed handedness only, thus making the point that classification into right- versus non-right handed groups is not sufficient to reveal significant relationships. One study found heightened anxiety in strongly left- and right-handed people compared to mixed-handers [40]. Another study reported higher incidence of health problems, including heart disease, thyroid disorders, allergies and epilepsy, in individuals with inconsistent handedness, or ambidexterity [41]. Along these lines, research linking handedness to the development of dyslexia is now progressing and dyslexia-candidate genes have been discovered to play a role in the biological mechanisms that establish left-right asymmetry of the body and influence handedness [42]. Nevertheless, as Ocklenburg et al. [43] point out, the ontogenetic relationship of handedness to lateralization of language (and, by extrapolation, dyslexia) is multifactorial and complex.

Some studies have calculated hand preference using several tasks for scoring the hand used and then categorized subjects into consistent versus inconsistent left- or right-preferring. For example, using this method Hardie et al. [44] found that social anxiety was highest in the inconsistent left-hand preferring group. This result exemplifies the need for precise measurement of hand preference as a finer approach in future studies.

Using another measure of laterality, Johnson et al. [45] have reported that weak lateralization of auditory perception is more common in humans with dyslexia. Neuroimaging studies can also reveal lateral asymmetries, as for example hypoactivity in the left extrastriate cortex in dyslexic subjects compared to controls [46]. These are just some examples from the quite extensive body of research on functional lateralization and behaviour in humans. Handedness has commonly been used as the proxy measure of lateralization but more recent studies have used more direct measures of cerebral asymmetry [47,48].

In nonhuman primates, as in humans, strength of hand preference has been used as a proxy measure of strength of brain lateralization. An early report of association between strength of hand preference and performance in chimpanzees was made by McGrew and Marchant [49] and it concerned termite fishing. This behaviour involves tool use: the chimpanzee holds a stick in one hand and inserts it into the termite nest. The termites attack, and they remain clinging to the probe as the chimpanzee withdraws it, thus allowing the chimpanzee to consume them, usually after rubbing them off the stick by running it over their other hand or arm. Chimpanzees with a stronger preference to insert the probe repeatedly using the same hand were more successful in gaining termites to consume than were those with weaker hand preferences. Some chimpanzees preferred to use their right hand and some their left hand but direction of hand preference did not determine success in termite fishing. It was the strength of hand preference that counted.

Even though this review is focused on vertebrate species, it is worth mentioning a study of motor performance in desert locusts showing that locusts with strong limb preferences make fewer errors when they cross a gap than do locusts with weak limb preference [50]. To my knowledge, this is the only study, so far, investigating strength of lateralization and performance in invertebrates.

Strength of hand preference is associated with ability to attend to two tasks simultaneously, as shown in common marmosets [51]. The marmosets had to forage for food and at the same time respond to a model predator. First their hand preferences were determined by scoring the hand used to pick up pieces of food and take them to their mouth, scored 100 times per subject over several days. They were also trained to search for mealworms, a favourite food, presented in blue bowls at different locations within a room furnished with branches at various angles and heights and to avoid green bowls, placed next to the blue bowls and not containing mealworms. Hence, they were trained to use a win-shift strategy. On testing they were released into the room to search for mealworms and, once they had commenced searching, one of three model predators was presented. One predator was a taxidermic specimen of a kestrel moved overhead using a fishing line and a system of pulleys. Another was a model snake pulled across the floor. The third was a wooden carving of two frogs, resembling rearing snakes, also moved across the floor, and chosen because previous research had shown that the marmosets mobbed this stimulus consistently [52]. The stronger the hand preference the shorter was the latency to detect/react to the predator and the negative correlation between latency and strength of hand preference was significant for the test using the kestrel and the test using the frog carving. Marmosets utter phee calls when aroused and tsik calls when they mob a predator, and the number of these calls correlated positively with strength of hand preference. In summary, marmosets with stronger hand preferences, regardless of whether their preference was for the left or right hand, detected the predator sooner and reacted to it more strongly. Since in control trials, in which the predator was presented but the marmosets were not required to search for food, no significant relationship was found between hand preference and latency to detect the predator or number of calls, it can be concluded that strength of laterality has an effect only when the two tasks have to be undertaken simultaneously. Given that marmosets with weaker laterality are less able to perform the two tasks simultaneously, one can predict that they would be more vulnerable to predation in the natural environment.

The above results did not depend on whether the left or right hand was preferred by the marmosets. However, a number of other studies on primates have found behavioural differences between left- and right-hand preferring animals. Left-handed marmosets are generally more fearful that right-handed marmosets: they are less likely to touch novel objects [53], less likely to sample novel foods and react more strongly to calls made by a natural predator [54], and they are more likely to have a negative cognitive bias [55]. Left-handed marmosets are less responsive to social group influences than are right-handed ones [56]. Similar left- versus right-hand differences in behaviour have also been reported for chimpanzees [57] and rhesus macaques [58] and at least one study of humans has shown that left-handed subjects are more cautious in a novel problem-solving task than are right-handed ones [59].

A number of studies have examined relationships between paw preference and general performance [60–64]. One measure of paw preference in dogs involves scoring repeated trials in which the dog holds steady a Kong with one paw while it licks inside it to obtain a favourite food. A study using this measure found that dogs with no significant preference to use one paw over the other expressed more fear on hearing the sounds of thunderstorms than did dogs with either a left- or right-paw preference [60]. Other studies have found that dogs without a significant paw preference are more excitable when exposed to novel stimuli or environments [61] and are less aggressive to strangers [62]. Dogs without a significant paw preference also show shorter latency to obtain food from a novel puzzle-box than do either left- or right-pawed dogs [63]. In contrast, Siniscalchi et al. [64] found no association between paw preference and the reactivity of dogs to hearing thunderstorms although the dogs used their right hemisphere to respond to the sounds.

In conclusion, and despite some reported differences in behaviour between subjects with significant left- and right-forelimb preferences, the above studies show that subjects without a preference, or with a relatively weak preference, to use one hand/paw consistently are more fearful

and excitable, less able to perform two tasks simultaneously, less responsive to novel stimuli and less responsive to social group influences.

A recent report on wild elk [65] supports some aspects of this conclusion: viz., elk with weaker forelimb preferences were more reactive to predator-like chases by humans than were those with stronger forelimb preferences (cf. similar findings in dogs). Other results obtained in this study (e.g., elk with stronger limb preferences were more likely to migrate) have not yet been matched by similar studies of group behavior in other species.

3. Strength of Lateralization and Performance in Birds

A strong body of experimental data demonstrates the presence of lateralization of visual processing and behaviour in the avian brain (summarized in [66]). Although the focus of this research has been on lateralization in domestic chicks and pigeons, laterality has been reported for visual behaviour in other avian species (e.g., zebra finches [67]; Australian magpies [20]; parrots [68]) and for production and processing of song (e.g., canaries [69]; and see paper by Kaplan in this special issue).

Lateralization of control of visual behaviour in the avian brain was first reported in 1979 [70], but it was not until some twenty years later that the potential function of the strength of lateralization, which varies between individuals, was examined. The first paper was published by Güntürkün et al. [71] and it reported a significant correlation between strength of lateralization in pigeons and success in discriminating grain from inedible grit. Pigeons were tested in three conditions: left-eye covered, right eye covered and both eyes uncovered. A laterality index for each bird was determined by the comparison of left- versus right-eye performance, the absolute value of which gave the strength of asymmetry. This value was then correlated against binocular performance and it revealed that the stronger the asymmetry, the more successful was the binocular performance (better at avoiding pecking at grit). Since most birds performed better on this task when they used the right eye (and left hemisphere) [72], as found previously to be the case in chicks [70] (also summarized in [66]), on this task the right eye is dominant. The authors suggested that asymmetry of the visual system enhances computational speed of object recognition by confining to one hemisphere the particular processing necessary to categorize grain as separate from grit (actually in the left hemisphere) and preventing conflicting information from the other hemisphere.

Experiments using domestic chicks have tested this hypothesis by manipulating conditions during development in order to produce groups of chicks that are lateralized for a range of visual functions and groups not lateralized for these same functions. The two types of chicks were generated by either exposing the developing embryos to light in the final days before hatching or by keeping them in darkness until after hatching (summarized in [66]). As a consequence of embryos being oriented in the egg so that the right eye is next to the shell and the left eye is next to the body and thus occluded, light exposure during this critical period stimulates only the right eye and causes asymmetrical development of the visual pathways [73]. In the absence of light exposure no such asymmetry develops and this difference persists throughout the first few weeks of life. Hence, it is possible to test the advantages of having (or not having) brain asymmetry for visual processing by comparing light- versus dark-incubated chicks, usually during the first and second week of post-hatching life. Clear differences in performance have been found.

Rogers et al. [74] tested the hypothesis that lateralization would enhance performance when two tasks had to be performed simultaneously, one relying on processing by the left hemisphere and the other on processing by the right hemisphere. One task was to search for grains of food scattered on a floor to which pebbles had been adhered: chicks learn to avoid pecking at pebbles using their right eye and left hemisphere [75]. The other task was to detect, and respond to, a model predator (a silhouette of a hawk) moved over the top of the cage, a function of the left eye and right hemisphere [76]. The light-exposed (lateralized) chicks performed well on both tasks, whereas the non-lateralized, dark-incubated chicks performed poorly on both tasks and their performance deteriorated as the task continued. Not only were they unable to avoid pecking at the pebbles, but also they were slow to detect

the model predator and, once they had detected it, they became less and less able to peck at grains of food and avoid pebbles. They became very disturbed. This result was confirmed by Dharmaretnam and Rogers [77], who additionally found that the dark-incubated chicks made more distress calls during the dual task than did the light-exposed chicks. This was also confirmed more recently by Archer and Mench [78], who found that the effect extends to at least six weeks post-hatching. Since monitoring for predators while searching for food is a common demand in the natural environment, the results of these experiments demonstrate a survival-relevant function of having a lateralized brain.

Chiandetti et al. [79] compared the performance of chicks exposed to light in ovo during the last three days before hatching and chicks incubated in the dark on a task in which grains of food were given to them in small paper cones with either a striped pattern or a checked pattern. The cones were placed along the walls of a rectangular arena, those with one type of pattern to the chick's right side and those with the other pattern on its left side. First the chicks were trained to expect food only in the cones with one of the patterns. Then they were tested either monocularly or binocularly with the cones, now empty, on the opposite sides (position reversed) and the choice made by the chicks was determined to see whether they chose the cones that they expected to contain food using object-specific cues (pattern) or position-specific cues (place). The dark-incubated (not visually lateralized) chicks chose pattern and largely ignored place: they attended to object-specific cues only. The light-exposed (lateralized) chicks chose either pattern or place, meaning that they attended to both possible cues specifying the location of food. Since the left-hemisphere attends to object-specific cues and the right hemisphere to position [80,81], it appears that the light-exposed chicks were able to use both hemispheres, whereas the dark-incubated ones could use only their left hemisphere. In other words, having a lateralized brain permits use of both hemispheres and thereby allows the chick to take into account more of the cues specifying food.

Later Chiandetti and Vallortigara [82] extended this research to show that, whereas light-exposed chicks could discriminate the left from right side, dark incubated chicks could not do so. The former could discriminate between a bowl of food placed in the corner of a cage with a blue wall on the right side from one placed with the blue wall on the left side. Dark-incubated chicks treated both bowls as the same.

In a study of eight species of Australian parrots, Magat and Brown [68] found that strength of laterality was associated with performance on a task requiring discrimination of pebbles from seed and another task requiring the bird to obtain a food reward suspended from its perch on the end of a string. On the pebble-seed discrimination task, individuals with stronger lateralization (measured as eye preference) scored better than individuals with weak lateralization, and performance of those with strong left-eye preference did not differ from those with strong right-eye preference. However, this relationship did not hold for lateralization measured as foot preference, which was contrary to the prediction made from a later paper by the same researchers showing, in 11 out of 16 species of parrot, that eye and foot preferences were correlated [83]. On the string-pulling task, strength of foot, but not eye, preference was associated with performance and, again, direction of foot preference had no significant association with performance. Overall, this study supports the previous research with chicks and pigeons in that performance is better in more strongly lateralized individuals, although it raises an issue about what behaviour is chosen to measure lateralization.

In birds, therefore, the evidence is clear that strength of lateralization is significantly related to performance.

4. Strength of Lateralization and Performance in Aquatic Vertebrates

In studies of species without limbs, laterality can be measured using eye preferences. One method is to determine the eye preferred by the test animal to view its image in a mirror [84]. Using such a measure of laterality and linking this to a test for "boldness", measured in terms of time to emerge from a dark box into an unfamiliar illuminated environment, Brown and Bilbost [85] found that rainbowfish not displaying an eye preference in the mirror test emerged sooner (were more "bold") than fish with

significant eye preferences. Since it is currently impossible to say what cognitive abilities underlie this test, one can only speculate that earlier emergence could depend on attention to fewer cues by the non-lateralized fish and hence the expression of less fear. Other possible explanations for the result were discussed by Brown and Bilbost [85]. For the purpose of this review, it is a question of whether shorter emergence time is advantageous or not, and that would depend on the potential presence of predators. Indeed, it has been shown that strongly lateralized fish respond to predators more rapidly than do non-lateralized fish [86], and exposing fish to higher levels of predation increases the strength of lateralization, irrespective of whether it is to the left or right side [87].

In an experiment designed to replicate that of Rogers et al. [74] but using fish, Dadda and Bisazza [88] tested gathering of prey by weakly versus strongly lateralized fish in the presence of a predator. Similar to the result obtained by testing chicks, the strongly lateralized fish obtained the prey in a shorter time than did the weakly lateralized fish, and they did so by attending to the prey with one eye and the predator with the other eye. Sailfish show a similar advantage of being lateralized, as shown in a recent study [89]. Sailfish attack schools of sardines by slashing with their bill to the left or right side and they have individual side preferences. The study found that prey capture is more successful in fish with strong biases than in those that are weakly biased [89].

Along similar lines, Sovrano et al. [90] found that lateralized fish (assessed by turning preference) displayed superior performance compared to non-lateralized fish on a task requiring them to orient using either geometric or non-geometric spatial cues. Within the lateralized group the direction of lateralization had no effect. Once again, strength but not direction of lateralization has been found to be important.

Lateralized tadpoles (*Lithobates sylvaticus*), determined using a swimming test and scoring clockwise versus anticlockwise rotation, are better at learning to recognize a predator's odour than are non-lateralized ones [91]. In this study, however, there was also a difference within the lateralized group: those with clockwise rotation learnt to recognize the threat associated with a predator's odour, whereas those with anticlockwise rotation were less able to do so. In other words, laterality in one particular direction enhanced predator detection and, in this aspect, these results diverge from the ones discussed immediately above.

Empirical evidence obtained by testing aquatic species indicates that stronger lateralization of the brain has advantages over weaker lateralization but there is some contrary evidence also. Dadda et al. [92] have found that fish (*Girardinus falcatus*) with weaker lateralization (determined from preferred eye used to monitor a predator behind a barrier) perform better than those with stronger lateralization on a task requiring them to enter a tank via a middle door in an array of nine doors: fish with stronger lateralization made more mistakes by swimming through doors to the left or right of the middle door. In addition, when these fish had to choose to join one of two shoals, each seen with a different eye, weakly lateralized fish were more likely to choose the high quality (larger) shoal, whereas strongly lateralized fish choose the shoal seen with the eye dominant for social behaviour regardless of the quality of the shoal.

5. Interhemispheric Communication and Lateralization

Communication between the left and right sides of the brain is essential for a lateralized brain. In humans this is achieved primarily via the large corpus callosum and, in other mammalian species, by a less well-developed corpus callosum. In birds a small anterior commissure connects the hemispheres and the tectal and posterior commissures (TC and PC) connect each side of the midbrain. In addition, the avian brain has a decussation that crosses the midline of the brain to allow left-right sharing of information [93,94]. Known as the supraoptic decussation (SOD), it is comprised of neural projections from the thalamus on one side of the brain to the hyperpallial region of the hemisphere/forebrain on the other side. In the SOD of chicks, more projections cross from left to right than from right to left [93,95]. This structural asymmetry correlates with some functional asymmetries: sectioning the SOD of chicks aged two days post-hatching removes the lateralization of visual search

performance normally present in the second week of life [96]. Sham operated control chicks tested monocularly on a search task requiring them to find grain scattered amongst pebbles (for details of the task see [97]) learn to avoid pecking pebbles when tested with a patch over their left eye but they cannot learn if the patch is on their right eye. This asymmetry is weakened or absent in chicks with a sectioned SOD: in these chicks performance is poor when using either the left or the right eye [83]. Furthermore, chicks tested binocularly after sectioning of the SOD are unable to perform the task, compared to excellent learning in the sham-operated controls. This result demonstrates the importance of thalamofugal visual projections that cross the midline of the brain. In the intact brain, and when both eyes are able to see, these midline-crossing projections enable learning by limiting it to the right eye/left hemisphere system.

Birds have two sets of visual projections: one involving the thalamus and SOD, discussed above, and the other involving the optic tecta and projecting to the entopallial region of the forebrain hemispheres. The optic tecta on each side of the brain are linked by a tectal commissure (TC) and crossing the midline right alongside the TC is the PC. Sectioning the TC/PC commissural system of the chick brain, on day two post-hatching, leads to lateralization of one particular type of visual behaviour [98]. When, on day five or six after hatching, the chicks were tested monocularly by presenting them with a small red bead, which stimulates pecking, unoperated chicks and sham-operated chicks pecked at the bead and did so on average only once each time it was presented (for 15 s and on eight times to each eye): no lateralization was apparent. Chicks with their TC/PC sectioned behaved in the same manner when tested using their left eye but, when they were tested using their right eye, they pecked at the bead more and more each time it was presented: the group data showed that there was a linear increase in pecking to over four pecks in 15 s on the eighth presentation. They appeared to find the bead more attractive each time they saw it. Such dishabituation suggests that, in intact chicks, the TC/PC commissure must transmit information from one optic tectum to the other in order to suppress continued and increasing responding to novel and attractive stimuli, such as the red bead. It is likely that this relies on a firm categorical memory of the red bead, which intact chicks tested using the right eye access via the TC/PC. Denied access to this memory the chicks using their right eye and without a TC/PC may be forced to use an imperfect memory, which makes the bead more attractive each time it is seen.

Interhemispheric communication is more effective in strongly lateralized brains, as found by comparing pigeons hatched from eggs that had received exposure to light with pigeons hatched from eggs incubated in the dark [99]. In a task reliant on use of both hemispheres together, Manns and Römling [99] tested pigeons that had been hatched from eggs either incubated in the dark or exposed to light. The task, known as transitive inference, required monocular training in which one eye was presented with red and blue keys, only the red being rewarded, and then blue versus green keys, only the blue being rewarded. This established a hierarchy of red preferred over blue and blue over green. A similar hierarchy was established when the bird could see using the other eye, except that, for this eye, two of the colours were different (green versus yellow, with green rewarded, and yellow versus pink, with yellow rewarded). Then in testing the birds were binocular and they were confronted with pairs of colours that they had not seen previously (e.g., blue versus yellow). Light exposed chicks could combine their training to choose, for example, blue over yellow, showing that they could integrate information stored in both hemispheres. Dark-incubated pigeons were unable to integrate the information from both hemispheres even though they we able to learn the combinations in the monocular condition just as well as could the light-incubated birds. Thus, binocular (normal) performance of tasks requiring integration of information on both sides of the brain, and depending on interhemispheric communication, is not possible in a non-lateralized (or weakly lateralized) brain. Since this research did not extend to investigation of what pathways might be involved in the transfer of information between hemispheres, it is not possible to say whether the communication is indirect via, for example, the TC/PC or, perhaps, occurs at the hemispheric level via the anterior commissure.

Although in its infancy, research on functional lateralization and interhemispheric communication at both the behavioural and neural levels promises to be a fruitful way of progressing our understanding of lateralized brain function.

6. Multiple Modality Laterality and Future Research

So far, lateralization has been discussed as a unitary phenomenon, involving processing of all information in the same way and to the same degree. However, it is possible that brains may be lateralized for processing, say, visual information, and not for processing auditory or olfactory information. We know, for example, that light exposure of chick embryos establishes lateralization for certain sorts of visual processing (discussed above) but this treatment has no effect on lateralization of olfactory processing [100] or on decision making about approach to familiar versus unfamiliar stimuli [101].

What does it mean to be strongly lateralized for some types of processing but weakly lateralized for others? Moreover, is there any concordance of lateralization of the brain and lateralization of the viscera? According to studies on zebra fish, some neural asymmetries are concordant with visceral asymmetry, since they are reversed together in *fsi* mutants, but not all behavioural asymmetries are concordant with visceral asymmetry and this appears to lead to the emergence of new patterns of behaviour [102].

Research examining the relationship of laterality across modalities and how they interact should provide a rich field of study and enhance knowledge of cognitive processing. So far we have very little information on the interaction between lateralization in different sensory modalities but fascinating evidence for the interaction between light exposure and birds' ability to orient using their magnetic compass has been discovered [103], and see the paper by Gehring and colleagues in this Special Issue, showing that monocular light stimulation influences the lateralization of processing magnetic compass information.

This raises another important aspect of lateralization: viz., that it is not fixed but can change in strength over an individual's lifespan. From research on chicks, we know that visual lateralization changes markedly over early, and critical, stages of development (see [4,104], p. 120) and that it can be modulated by steroid hormones [105–107] and environmental stimulation (e.g., light exposure, discussed above). A recent review by Hausmann [108] considers the influence of sex hormones on lateralization in humans and points out the difficulties in drawing conclusions from the research on humans. Future research on non-human species promises to shed light on all of these issues.

To conclude, the evidence indicates that brain lateralization is advantageous because it allows parallel processing in the two hemispheres and it suggest that greater efficiency is achieved by confining the neural circuits used in different types of processing to separate hemispheres, thereby reducing conflict and redundancy.

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Early- and Late-Light Embryonic Stimulation Modulates Similarly Chicks' Ability to Filter out Distractors

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Abstract: Chicks (*Gallus gallus*) learned to run from a starting box to a target located at the end of a runway. At test, colourful and bright distractors were placed just outside the starting box. Dark incubated chicks (maintained in darkness from fertilization to hatching) stopped significantly more often, assessing more the left-side distractor than chicks hatched after late (for 42 h during the last three days before hatching) or early (for 42 h after fertilization) exposure to light. The results show that early embryonic light stimulation can modulate this particular behavioural lateralization comparably to the late application of it, though via a different route.

Keywords: attention; functional lateralization; cerebral lateralization; embryo; light; fish; chicks; birds

1. Introduction

It is now well established that environmental light stimulation interplays with a genetic cascade of events in promoting brain specialization in two different classes of vertebrates, fish and birds (reviews in [1–3]).

A complex chain of developmental steps leads to brain lateralization in zebrafish starting with an asymmetrical expression of a gene network that controls the development of structural left-right differences within the epithalamus, including asymmetric parapineal migration [4–7]. As a secondary consequence, in the transparent eggs of the zebrafish an early action of light prompts functional brain asymmetries including motor and sensory processing. Fry hatched from eggs exposed to the photic input during the first week after fertilization prefer to attend to conspecifics with the left eye, whereas fries whose embryonic development happened in darkness do not display the same asymmetry [8]. If the light fails to reach the embryos in two distinct moments within the first week post-fertilization, the normal development of some lateralized behaviours is either compromised or prevented. For instance, darkness during the first day results in an inversion of the reaction to a dummy predator: after normal light regimes, zebrafish avoid the predator appearing on the left side, whereas after darkness they respond more intensely to a predator coming from the right side [9]; in contrast, darkness during the third day prevents the appearance of any asymmetric response to the predator [10]. However, the role of light stimulation on brain structural asymmetry has not been conclusively clarified in zebrafish, as no effect of light has been shown on the asymmetry of molecular markers [11], and some behaviours are lateralized while some others are not, independently of the neuroanatomical asymmetries [12].

Embryonic light application influences also the neurodevelopment of cerebral lateralization in the avian brain, but via a different pathway, i.e., by the asymmetric stimulation of one eye. For more than three decades, it has been repeatedly shown that an asymmetrical embryonic positioning before hatching (due to unilateral expression of Nodal signals responsible for the body torsion [13]) allows light penetration of the egg during the final days of incubation to act selectively on one side of the chick's head and to trigger anatomical and functional brain asymmetries via right eye stimulation [14]. In response to the asymmetric light input to the retinal cells, brain regions in the left hemisphere fed by the right eye develop earlier than their counterparts in the right hemisphere and a higher number of fibers crosses from the left side of the thalamus to the right hemisphere via the supraoptic decussation [15–17]. Such structural asymmetry is functionally detectable in several visually-guided behaviours, such as the advantage of the right eye in preventing pecks to not edible elements when searching for food [18]. Furthermore, chicks presenting such an asymmetry outperform chicks hatched in darkness in dual tasks [19] or when they have to combine different kinds of information to master a correct discrimination [20]. The multifaceted role of light is apparent in the fact that light exposure affects not only abilities related to the stimulated right eye but also functions of the left eye related to attack, copulation, predator detection [15], and visuospatial abilities [21]. Moreover, reversing the eye exposed to light by untwisting the embryo's head and applying a patch to the right eye causes the pattern of asymmetries to be inverted [15,22]. Note, however, that although light exerts such an important role in the establishment of lateralization, some forms of asymmetries as those associated with unilateral eye used during sleep [23] or with the neural mechanisms of social recognition and imprinting [24–26] develop even if the incubation process takes place in darkness [27,28].

The depicted scenario shows a composite set of mechanisms at play in the development of brain asymmetries and the common thread to birds and fish seems to indicate that, following two different anatomical routes, light moulds a similar functional cerebral specialization in the two taxa [29]. Broadly speaking, the right hemisphere orchestrates a form of primitive avoidance and wariness while the left-hemisphere complements brain specialization with the control of routine behaviours of feeding and analysis in familiar contexts, counteracting distraction and irrelevant response to novelty credited to the right hemisphere [1,30]. Thus, apparently similar behavioural asymmetries can be generated by different neural asymmetric systems [31,32].

To check whether a different critical period for the application of the light input could be part of the asymmetric neurodevelopment of cerebral functions also in the chick, Chiandetti et al. [33] exposed eggs to light for a brief period after fertilization, when other photosensitive regions are developing but no retinal photoreceptors have been differentiated yet [34,35], and thereafter maintained them in the dark. Chicks hatched under this condition performed in a comparable fashion to chicks hatched from eggs light-stimulated in the canonical time-window, i.e., during the last three days of incubation [33]. The testing condition required chicks to avoid an obstacle placed midway between the starting box and the target at the other end. In such a situation, the two light-stimulated groups showed no preference to detour the obstacle by circumventing it well as much from the left as the right side. By contrast, chicks hatched in darkness showed a pronounced bias to detour the obstacle systematically on the left side. In that case, a motoric difference between stimulated and unstimulated individuals was insufficient to explain the pattern of behaviour observed because all the chicks showed the same motor bias to run slightly toward the left side of the environment when tested without any obstacle on their way to the target (for the specificity of light effect, see reviews in [2,31]). Rather, unstimulated chicks' selective bias emerged only when an obstacle was on the way to the target. It is possible that, when freely running, chicks previously kept in darkness are less able to sustain attention toward the target and need the right eye to view the obstacle in order to keep track of it while running toward the target, and avoiding the obstacle.

Here, in the attempt to widen the comprehension of when and how light stimulation is effective in shaping visually-driven asymmetric responses, we assessed stimulated (early and late) and unstimulated chicks and compared the performance of the three groups of animals in a further testing situation. Briefly, two days old domestic chicks (*Gallus gallus*) first learned to run from one end of a runway to a target located at the opposite end; then, at day 5 of age, colourful and bright distractors were placed close to the starting area and we scored whether the chicks pecked at the distractor, how many times and whether preferentially on the one positioned on the left, assuming that the novelty would have engaged mainly the left eye (right hemisphere). The task was chosen as a replication of a previous one with a change in the type of distractor that could provide an incremental knowledge about the observed phenomenon.

2. Materials and Methods

The study was carried out in compliance with the European Community and the Italian law on animal experiments by the Ministry of Health, under the authorization of the Ethical Committee of the University of Trieste (protocol number 385 pos II/9 dd 16.03.2012).

2.1. Subjects

Chicks of the Ross 308 (Aviagen) broiler strain hatched in our laboratory under controlled conditions. The eggs were collected from a local commercial hatchery immediately after fertilization and, thereafter, kept in a FIEM snc, MG 100 H incubator under controlled temperature (37.7 °C) and humidity (about 50–60%) conditions, in a darkened room so that no further incidental light could reach the eggs. Fifty eggs were incubated in complete darkness from the arrival to the laboratory and until the hatching day (Di-chicks = 38); fifty eggs were exposed to light from their arrival to the lab and for 42 h and thereafter remained in the dark (EarlyLi-chicks = 39); fifty eggs were maintained in darkness and exposed to light from day 18 and for 42 h before hatching (LateLi-chicks = 36). A 60 W incandescent light bulb or 15 LEDs (18 lumens per LED) provided homogeneous light of about 250 lux within the incubator. As reviewed in [2], high intensity and prolonged exposure to light can exert various effects (from hatchability to interlimb coordination) and this applies to LEDs too; however, the light regime adopted in our protocol is not proven to have comparable side-effects (and see [33] for an analysis of the identical running trajectories in Di-, EarlyLi-, and LateLi-chicks). Immediately after hatching, each chick was reared singly in a metal home-cage (28 cm wide × 32 cm high × 40 cm deep) illuminated by LED (12 L: 12 D cycle) and located in a separate room at 30 °C. Food and water were available *ad libitum*.

2.2. Apparatus

A white rectangular enclosure (40 cm wide × 50 cm high × 160 cm deep) with sawdust (5 cm in depth) on the floor served as training apparatus. A red conspicuous plastic beacon was placed at the middle of the smaller end of the apparatus and 7 cm above the floor, indicating the presence of a plastic feeder (target) exactly below it. Two lamps of 50 W centered on the top of the smaller ends provided uniform illumination to the apparatus. For the testing, two slanted walls were adjusted close to the starting point on both the left and the right (see Figure 1) and decorated with salient shiny beads placed at about chick's head height, functioning as distractors.

In order to keep track of the chick's movements within the apparatus, a black removable sticky paper was temporarily attached on the chick's back. The behaviour was videorecorded from above and scored offline by an independent observer blind with respect to the hatching conditions of the animals.
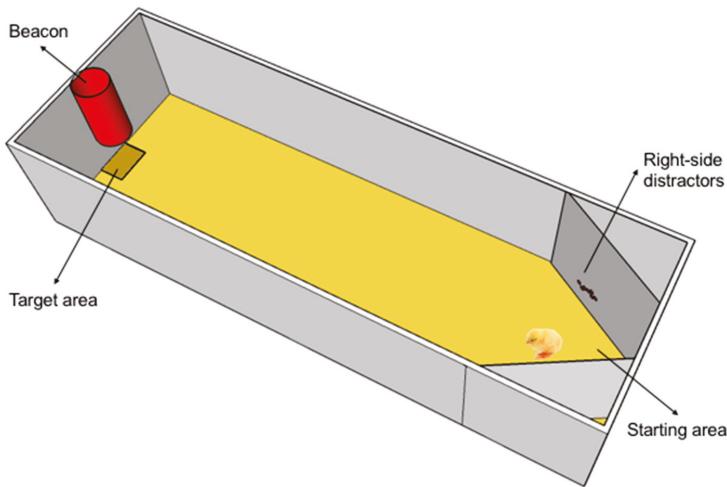
Figure 1. Schematic layout of the experimental apparatus as prepared for the test, with exemplifier distractors placed on both the left and the right side of the starting point and the red beacon signalling the presence of the plate with the mealworms (available only during training). A chick is inspecting the distractors located on the left of the starting point.

2.3. Procedure

On day 2 of age, after 3 h of food deprivation, each chick was first accustomed to the training apparatus by letting it free to explore the environment for about 30 min and reach the target where some mealworm larvae (*Tenebrio molitor*) were placed. The next two days, each chick was placed within the apparatus at the opposite end in front of the target and left free to run toward the feeder. This procedure was repeated 20 times (10 times per day).

On day 5, each chick was given one trial as used during training to reinstate motivation and immediately after it was tested only once with the distractors. In the single testing trial, no mealworm was available under the target. This procedure was chosen to rule out any potential influence of the presence of the reward. The trial ended as soon as the chick reached the feeder. The positions of the starting point and the target were counterbalanced between subjects in order to control for any undesired asymmetry within the environment. As a dependent measure, we scored the number of pecks directed at right and left distractors.

3. Results

After having verified that the assumption of homogeneity of variances was not satisfied with the Levene's test, we ran the non-parametric test Kruskal-Wallis on the overall number of pecks, which showed a significant difference between the three hatching groups ($\chi^2_{(2)} = 10.194$, $p = 0.006$): Di-chicks were more distracted than the two stimulated groups on the pecks toward the left distractor ($\chi^2_{(2)} = 9.352$, $p = 0.009$), but not to the right distractor ($\chi^2_{(2)} = 2.844$, $p = 0.241$), as visible in Figure 2. Di-chicks pecked more at the distractor placed on the left side than both EarlyLi- ($Z = -2.271$, $p = 0.023$) and LateLi-chicks ($Z = -2.588$, $p = 0.010$), whereas no difference emerged between the two light-stimulated groups ($Z = -0.368$, $p = 0.713$, Mann-Whitney Post Hoc test).
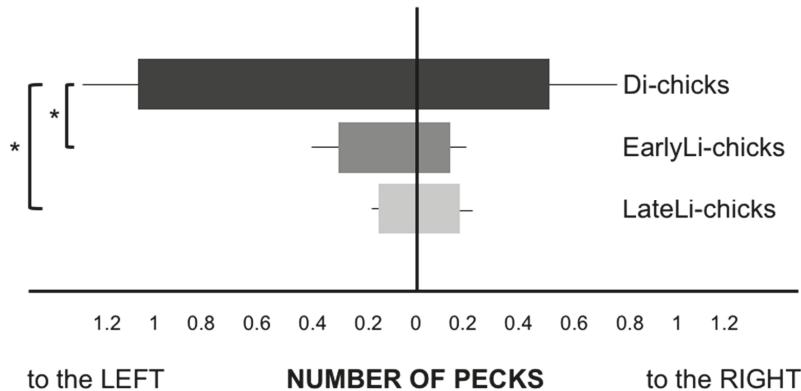
Figure 2. Plot of the performance (average number of pecks and S.E.M.) of the three groups of chicks in the presence of the distractor (* $p < 0.05$).

4. Discussion

In this investigation of the time windows in which embryonic light stimulation affects the development of functional brain asymmetries, we replicated previous findings showing that chicks hatched from eggs exposed to environmental illumination for 42 h, at either an early or a late stage of embryonic development, display a comparable behaviour. Specifically, both EarlyLi- and LateLi-chicks were not distracted by the novel elements placed in proximity of the starting area. Conversely, Di-chicks, hatched from eggs maintained for the whole developmental period in complete darkness, were significantly attracted by the novel elements and could not restrain from pecking at these items before reaching the target, and especially at those placed on their left side. Note that the procedure used here matches the one used in our previous work [33], where we showed a specific effect of light stimulation on hemispheres' functionality with no detriment of dark incubation condition on a typical motor and cognitive development (see also [36] for comparable results on pigeons).

The observed pattern of chicks' performance confirms that embryonic application of light stimulation modulates the ability to sustain attention. EarlyLi- and LateLi-chicks ignored the novel elements and focused on the target, directly approaching it in a routine-like behaviour as learned during the familiarization trials without distractors. Both the left and the right eyes seemed equally good in targeting the goal and avoiding the salient distractor elements presented at test. By contrast, Di-chicks were strongly biased toward the distractors. The fact that the distractors placed on the left side resulted more attractive than those placed on the right side, uncovers the brain asymmetry at play in Di-chicks: the right eye is engaged in sustaining attention to the target, while the left eye mediates attention deployment to the novel and salient elements located on the left side. Our findings suggest that in Di-chicks the separation of the two hemispheres also maintains segregated the processing of the target and the distractor. While the left hemisphere would control the routine running behaviour toward the target, the right hemisphere is engaged by novelty and the chicks stop their running to assess the distractor located on the left, as they do when they monitor the predator [19,37].

In LateLi-chicks, instead, it appears that the right hemispheric involvement in response to novelty is modulated by the asymmetric embryonic light stimulation. The cross-talk between the two halves of the brain makes the left hemisphere capable of inhibitory control over the compulsory attention directed toward the novelty, similarly to the testing situation in which the right eye (left hemisphere) inhibits pecks at irrelevant elements spread among grains [19].

What remains to be understood is how light induces a comparable performance in LateLi- and EarlyLi-chicks, considering that only on LateLi-chicks light acts asymmetrically on the fully-formed eye. In zebrafish, the involvement of both habenulae in the control of behaviour is shown by the fact

that selective inactivation of these nuclei induces a persistent freezing response [38]; furthermore, the use of the right eye to target the food implies that the left hemisphere is engaged in sustained control, with the enrollment of the left lateral habenulae, reducing the probability of being distracted [39,40]. Although the fact that there is no clear evidence that the role of light on the lateralized behaviour depends directly on a stimulation of the parapineal, this could account for the performance observed in both fish and EarlyLi-chicks. This hypothesis requires further investigation: indeed, in fish, other photosensitive areas than the eye participate in determining lateralization [41,42] and one may wonder whether the involvement of the same regions could be extended to explain chicks' performance, since analogous cells are developing in the chick embryo at the early stages when we applied the light stimulation [43]. Due to the common differentiation of the diencephalic areas in birds and fish, the involvement of the ephyphysis-habenula axis could be the target for a further window in which light may be operating in chicks as well. A further complication may derive from the fact that the habenular nuclei are asymmetric in several species [44,45] and hence might be differentially stimulated by the action of light. By contrast, in birds the habenulae are assumed to be symmetric, despite the fact that one study on chicks showed that there can be individual asymmetries and males tend to present a larger right medial habenula [46]; unfortunately, there was no mention of the incubation condition in this study and hence whether it applies to our results or not is open to speculation. On the basis of atlases of different avian species, it appears that the pineal gland, projecting to the habenulae, is larger in absolute size in chicks than, for instance, in pigeons. The cytochemical characterization of the avian pineal organ demonstrates many structural, functional and biochemical analogies between the retinal and the pineal photoreceptors [47,48]. Furthermore, other brain regions involved in lateralization may have been simply overlooked in previous histological assessments of light stimulation effects. There might also be a further extra-retinal photoreceptive candidate in birds outside the pineal gland. As shown in quails, in the avian ventral thalamus and septal region there are so-called deep photoreceptors that seem to participate in the regulation of seasonal cycles of reproduction [49,50]. These further photosensitive receptors respond to light in the quail and might be activated in domestic chicks as well. Certainly, this hypothesis paves the way to further investigations addressing specifically the neural substrates enrolled by light at precocious stages of the chick's embryonic development.

5. Conclusions

Here we documented that an early application of light during incubation modulates a particular functional asymmetry in chicks in a similar way to the well-known late stimulation. Light seems to operate on a genetically determined asymmetry by mediating a better cooperation between the two hemispheres. The asymmetrical light stimulation experience does not simply affect hemispheric specialization (like a left-hemispheric dominance of visuomotor control (discussed for instance in [32])) but also how efficiently the hemispheres can interact or cooperate [36,51].

The mechanisms responsible for the early modulation remain to be investigated, however a broader consideration on the effects of light stimulation before birth is worth discussing. Despite it is controversial whether the human foetus is reached by asymmetric light to one eye (it is attested that 2/3 of the embryos are rotated with the right eye toward the external abdominal wall in the latest stages of gestation), in principle the light reaches the intrauterine environment [52]. Indeed, at about 36–40 weeks, the foetus responds to flashes of light to the maternal abdomen with an increment in cardiac frequency and eye and body movements [53]. There are also indications that light can affect the development of cerebral lateralization in human foetuses by modulating the available hormonal levels [54]. Although assessing this hypothesis has proven to be very difficult, seasonal anisotropy has been recently shown with respect to the distribution of handedness as related to gender: longer photoperiods experienced during the first 14–18 weeks are associated with left-handed males [55]. Hence, at present, a hormonal modulation cannot be ruled out.

Asymmetries induced by genetic factors are shaped by environmental illumination, as indicated by previous results on zebrafish and chicks, but here we showed that in an avian species this takes place

in two different time-windows. If light entails two different processes in the two time-windows, a more sensitive test could reveal a specific involvement of each hemisphere. For instance, an investigation of the performance under monocular testing condition [56] could reasonably refine the enrolment of each hemisphere depending on the specific genetic-environment route, since monocular and binocular performances could differ profoundly (e.g., [57]).

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Lateralization of the Avian Magnetic Compass: Analysis of Its Early Plasticity

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Abstract: In European Robins, *Erithacus rubecula*, the magnetic compass is lateralized in favor of the right eye/left hemisphere of the brain. This lateralization develops during the first winter and initially shows a great plasticity. During the first spring migration, it can be temporarily removed by covering the right eye. In the present paper, we used the migratory orientation of robins to analyze the circumstances under which the lateralization can be undone. Already a period of $1^1/_2$ h being monocularly left-eyed before tests began proved sufficient to restore the ability to use the left eye for orientation, but this effect was rather short-lived, as lateralization recurred again within the next $1^1/_2$ h. Interpretable magnetic information mediated by the left eye was necessary for removing the lateralization. In addition, monocularly, the left eye seeing robins could adjust to magnetic intensities outside the normal functional window, but this ability was not transferred to the "right-eye system". Our results make it clear that asymmetry of magnetic compass perception is amenable to short-term changes, depending on lateralized stimulation. This could mean that the left hemispheric dominance for the analysis of magnetic compass information depends on lateralized interhemispheric interactions that in young birds can swiftly be altered by environmental effects.

Keywords: avian magnetic compass; lateralization; right eye/left brain system; plasticity; commissures; Cryptochrome 1a

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1. Introduction

In most vertebrates studied up to now, several perceptual, cognitive, and motor systems display a left–right difference of neural processing [1,2]. This ubiquity of functional brain asymmetries is probably the result of some fundamental benefits. Indeed, various studies could demonstrate in several species, ranging from fish to humans, that those individuals that are more strongly lateralized in a certain function also display higher performances when this function is tested [3–5]. This is possibly due to three mechanisms. First, asymmetries can selectively increase the perceptual or motor learning effect in one hemisphere. This is the case for, e.g., birds where the eyes are so laterally placed that most of the visual input derives from monocular vision. Thus, increased perceptual training of one eye can result in higher discrimination ability with this side [6]. The second mechanism for an advantage of asymmetry is directly related: increased learning with one perceptual or motor system also decreases reaction times, resulting in a time advantage of the dominant side [7,8]. The third mechanism of an advantage is parallel and complementary processing during task execution. If, for example, lateralized and non-lateralized chicks are tested in a foraging task that requires them to find grains scattered

among pebbles and, at the same time, monitor overhead for a flying model predator, the strongly lateralized birds can conduct both tasks efficiently and in parallel [9]. Thus, hemispheric specialization seems to increase parallel processing by enabling separate processing of complementary information into the two hemispheres [10].

An important function that has been found to be lateralized is the avian magnetic compass. Information is obtained in the right eye and processed in the left hemisphere of the brain: with only their right eye open, birds could use their magnetic compass in the normal way, whereas they were disoriented when they had to use their left eye alone [11–15]. The reception of magnetic directional information is associated with the visual system. The Radical Pair Model, proposed by Ritz and colleagues [16], assumes that magnetoreception is based on spin-chemical processes in specialized photopigments; the eye was suggested as the site for magnetoreception, with cryptochromes as molecules forming the crucial radical pairs. Experimental evidence supports this model: radio-frequency fields in the MHz-range, a diagnostic tool for radical pair processes [17] disrupt magnetoreception (e.g., [18–22]). Furthermore, Cryptochrome 1a was found in the retina of birds, located along the disks of the outer segments of the UV/V cones [23], activated by light of the short wavelengths that allows birds magnetic compass orientation [24].

The first behavioral experiments documenting a lateralization of the magnetic compass in favor of the right eye/left hemisphere were performed with migratory birds, European Robins, *Erithacus rubecula* (Turdidae), and Australian Silvereyes, *Zosterops l. lateralis*, making use of their spontaneous directional preferences during the migratory phase [11,12]. Later studies, however, questioned these findings, reporting that migratory birds, among them European Robins, were oriented in their migratory direction even if they had to rely on their left eye alone [25–27]. Yet, there were marked differences between these studies, an important one being a difference in the test season: the tests documenting the lateralization of the magnetic compass had been spring experiments with birds returning to their breeding grounds whereas the tests not finding a lateralization were predominantly autumn experiments with birds mainly following an innate course (see [28]). A follow-up study testing the same robins consecutively during the first three migration seasons indicated that lateralization of the magnetic compass was not present in very young robins, but develops only after the first autumn migration [29]: initially, the magnetic compass was not lateralized and the birds could orient with their right as well as their left eye. During the following spring migration and the second autumn migration, in contrast, the same birds could no longer orient with their left eye alone—the magnetic compass had become lateralized in favor of the right eye/left brain hemisphere. Yet, in the beginning, the lateralization proved to be flexible with considerable plasticity: during spring migration, covering the right eye for 6 h prior to the orientation tests could temporarily restore the ability for magnetic compass orientation to the left eye. During the subsequent autumn migration, however, the same treatment no longer had any effect. These results strongly indicate that the magnetic compass asymmetry requires a developmental period: while it is absent during the first autumn and subsequently susceptible to change during the first spring, it becomes more strongly fixed beginning with the second autumn.

The flexible phase during spring migration is of particular interest because it allows some insights into the processes leading to the lateralization of the avian magnetic compass. Here, we report behavioral experiments during the first spring migration of migratory European Robins, designed to analyze in more detail the time-span required to restore magnetoreception to the left eye, the extent and the duration of this effect and the circumstances under which it takes place.

2. Results

The various test conditions are listed in Table 1. The results are summarized in Table 2, indicating significant differences between various treatments and the respective binocular controls; for the data of the individual birds, see Tables S1–S3a,b in the Supplementary Material.

2.1. The Effect of Monocular Pre-Exposure

Our previous study [29] had shown that, during the first spring migration, covering the right eye for 6 h had temporarily restored the ability for magnetic compass orientation to the left eye. This raised the question of how this treatment affected the right eye. The respective data are given in Figure 1: after having the right eye covered for 6 h, the robins could orient with their right eye (6hpeL-R) as well as with their left eye (6peL-L). Obviously, disrupting the input from the right eye for 6 h had no adverse effect on ability of the right eye/left hemisphere to process magnetic compass orientation; it just seems to remove the lateralization.

Table 1. Definition of the test conditions and their abbreviations.

Abbreviation	Test Condition
Bi	binocularly tested, control
L	monocularly left-eyed
6peL-L	6 h pre-exposed monocularly left-eyed, tested left-eyed
6peL-R	6 h pre-exposed monocularly left-eyed, tested right-eyed
1^1/$_2$peL-L	1^1/$_2$ h pre-exposed monocularly left-eyed, tested left-eyed
1^1/$_2$peL/1^1/$_2$-L	1^1/$_2$ h pre-exposed monocularly left-eyed, then 1^1/$_2$ h without eye cover, tested left-eyed
3peRFBi-Bi	3 h binocularly pre-exposed in an RF field (1.314 MHz, 480 nT), tested binocularly
3peRFL-L	3 h monocularly left-eyed pre-exposed in a RF field(1.314 MHz, 480 nT), tested left-eyed
3pe92R-92R	3 h right-eyed pre-exposed in a 92 µT field, tested right-eyed in the 92 µT field
3pe92L-92L	3 h left-eyed pre-exposed in a 92 µT field, tested left-eyed in the 92 µT field
3pe92L-92R	3 h left-eyed pre-exposed in a 92 µT field, tested right-eyed in the 92 µT field

If not indicated otherwise, the birds were pre-exposed and tested in the geomagnetic field. RF field: radio frequency field.

Table 2. Orientation after various lengths and modes of monocular pre-exposure. Twelve birds were tested in all test conditions (48 birds altogether, see Tables in Supplementary Material).

Year	Condition	Test Magnetic Field	n	Med. r_b	α_N	r_N	ΔBi	ΔX
2011	Bi	geomagnetic field	4	0.79	354°	0.80 ***		
"	6peL-L	"	3	0.81	21°	0.92 ***	+27° *d	X$_1$
"	6peL-R	"	3	0.91	28°	0.66 **	+32° n.s	+7° *s
2012	Bi	"	3	0.45	15°	0.62 **		
"	L	"	3	0.48	(12°)	0.22 n.s.	(−3°) *s	X$_2$
"	1^1/$_2$peL-L	"	3	0.77	11°	0.85 ***	−4° n.s.	(−11° *s)
"	1^1/$_2$peL/1^1/$_2$-L	"	3	0.60	(322°)	0.22 n.s.	(−53°) *d	(−50° n.s.)
2013 I	Bi	geomagnetic field	3	0.92	351°	0.65 **		
"	3pe92R-92R	92 nT	3	0.90	22°	0.74 ***	+31° n.s.	
2013 II	Bi	geomagnetic field	3	0.82	10°	0.95 ***		
"	3peRFBi-Bi	"	3	0.44	15°	0.59 *	+5° *s	X$_3$
"	3peRFL-L	"	3	0.41	(180°)	0.18 n.s.	(+170°) ***s	(−165° *s)
"	3pe92L-92L	92 nT	4	0.50	354°	0.80 ***	−16° n.s.	X$_4$
"	3pe92L-92R	92 nT	3	0.83	(287°)	0.21 n.s.	(−103°) ***	(−67° *s)

For the definition of the test conditions, see Table 1. n, tests per bird; med-r_b, median vector length per birds; α_N, r_N, direction and length of grand mean vector, with asterisks indicating significant directional preference by the Rayleigh test [30] (α_N in parentheses if not significant). ΔBi, difference to the respective binocular control, and ΔX, difference to the X-sample above, with asterisk indication a significance of the difference by the Mardia Watson Wheeler test (d) and the Mann–Whitney U-test (s). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s.: not significant.
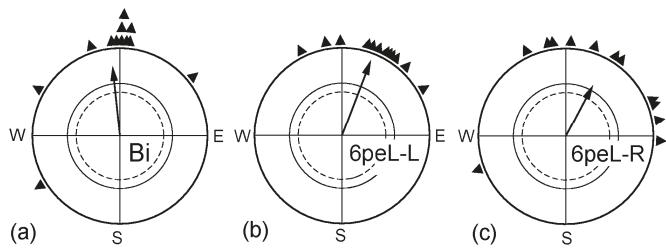
Figure 1. Effect of covering the right eye for 6 h prior to the test. (**a**) Untreated binocular control; (**b**) Birds tested monocularly left-eyed after having the right eye covered for 6 h; (**c**) Birds tested monocularly right-eyed after having the right eye covered for 6 h. The triangles at the periphery of the circle indicate the mean headings of the individual birds; the arrow represents the grand mean vector in relation to the radius of the circle = 1, and the inner circles mark the 5% (dotted) and the 1% significance border of the Rayleigh test [30].

Another question concerned the duration of the interval required to restore the ability to use information from the left eye and how long the effect would last. The data are given in Figure 2. When the right eye was covered immediately before the test (L), the monocularly left-eyed birds were disoriented, documenting lateralization in favor of the right eye. If the birds had been monocularly left-eyed already $1^1/_2$ h before the tests began ($1^1/_2$peL-L), they showed normal orientation with their left eye, not different from when they were tested as binocular controls (Bi). However, this effect of removing the lateralization proved to be rather short-lived: when the birds had been monocularly left-eyed for $1^1/_2$ h, followed by a binocular period of another $1^1/_2$ h and were then tested left-eyed immediately afterwards ($1^1/_2$peL/$1^1/_2$-L), they were disoriented (Figure 2d)—lateralization had set in again.
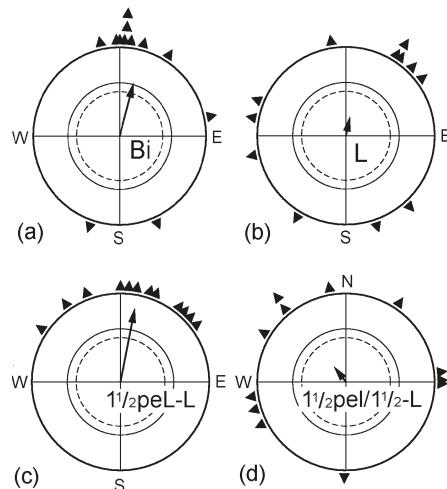

Figure 2. Time required for removing the lateralization in favor of the right eye. (**a**) Untreated binocular control; (**b**) Birds tested monocularly left eyed; their right eye was covered immediately before the tests began, indicating the lateralization in favor of the right eye; (**c**) Birds tested monocularly left eyed after having the right eye covered $1^1/_2$ h before the beginning of the tests; (**d**) Birds tested monocularly left eyed after having the right eye covered for $1^1/_2$ h, and then the cover was removed for $1^1/_2$ h before the beginning of the tests. Symbols as in Figure 1.

The previously described tests showing a temporary removal of lateralization as an effect of covering the right eye had been performed in the local geomagnetic field. With the following treatments, we tested for possible effects of the magnetic conditions during monocular deprivation.

First, we pre-exposed birds binocularly to a radio-frequency field that had been shown to disrupt magnetic orientation [19]. This meant that the birds did not receive interpretable magnetic information during a period 3 h immediately before the tests began (3peRFBi-Bi). Immediately after the pre-exposure, these birds were significantly oriented in their migratory direction in the geomagnetic field (see Figure 3b), even if the distribution of their mean headings shows a certain increase in scatter. Birds that were exposed monocularly left-eyed to the radio frequency field for 3 h immediately before they were tested left-eyed (3peRFL-L), in contrast, were disoriented in the geomagnetic field immediately afterwards (Figure 3c). Covering the right eye for a period of 3 h, twice as long as the one used in the previous series, should have been sufficient to enable the birds to use their left eye for obtaining magnetic compass orientation, yet in this case, it did not work. Obviously, receiving only visual information from the left eye could not remove the lateralization. Our data clearly show that interpretable directional information from the magnetic field is essential for allowing the processing of magnetic information by the left eye again.
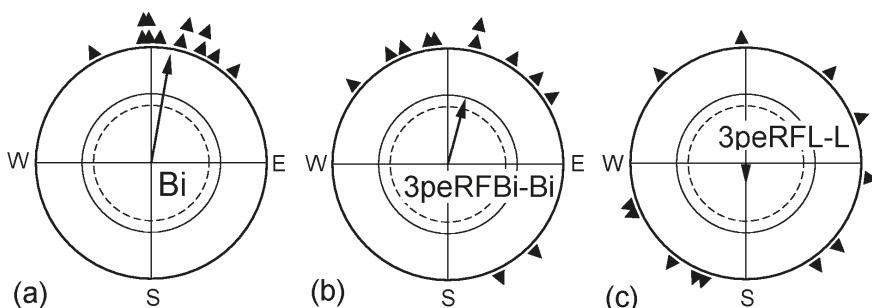

Figure 3. Effect of denying the birds interpretable magnetic information before the tests. (**a**) Untreated binocular control; (**b**) Birds exposed binocularly to a radio frequency for 3 h prior to being tested binocularly in the local geomagnetic field; (**c**) Birds exposed monocularly left-eyed to the radio frequency field for 3 h prior to being tested monocularly left-eyed in the geomagnetic field. Symbols as in Figure 1.

In a next step, we exposed the birds prior to the tests for 3 h to a magnetic field of 92 μT, twice as strong as the local geomagnetic field. Robins cannot spontaneously cope with such field strengths, but become able to orient in it if they had a chance to adjust to this intensity before the tests. In a previous study, 1 h pre-exposure to such a strong field had proven sufficient to allow orientation [31]. We tested two different groups of birds: Group I was pre-exposed with the right eye open and subsequently tested monocularly right-eyed (3pe92R-92R). Group II was pre-exposed and tested monocularly left-eyed (3pe92L-92L). The results are given in Figure 4: both groups of birds were oriented in the strong magnetic field. However, there was a difference between the groups: while the right-eyed birds were oriented in their migratory direction right away, with the headings of the three tests not different from each other, the left eyed birds were first disoriented and oriented only from the third test onward, with only the distribution of the forth headings significantly different from the disoriented first round (see Table 3). When left-eyed, the birds thus required more time to adjust to the stronger field.
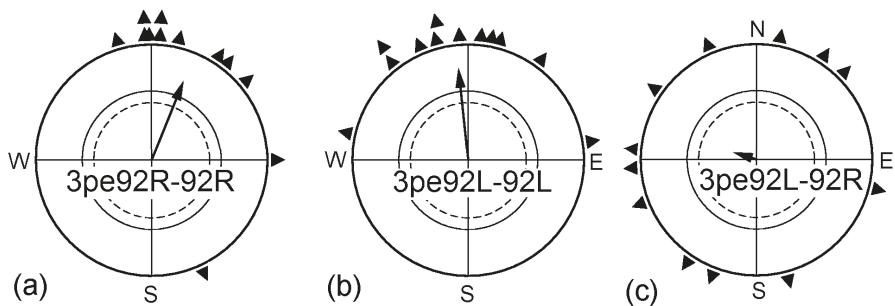
Figure 4. Adjusting to higher magnetic intensities. (**a**) Birds of group I pre-exposed monocularly right-eyed for 3 h in a magnetic field of 92 µT, twice the intensity of the geomagnetic field, and then were tested in that field; (**b**) Birds of group II pre-exposed monocularly left-eyed for 3 h in the strong magnetic field then tested in that field; (**c**) Birds of group II pre-exposed monocularly left-eyed for 3 h in the strong magnetic field and then tested monocularly right-eyed in that field. For the orientation of the untreated control birds, see Figure 3a and Table 2. Symbols as in Figure 1.

Table 3. Orientation of the robins pre-exposed and tested in the 92 µT-field.

Test Round	Monocularly Right-Eyed Birds				Monocularly Left-Eyed Birds			
	N	α	r	$\Delta_{\text{Round 1}}$	N	α	r	$\Delta_{\text{Round 1}}$
1	12	8°	0.87 ***		11	(304°)	0.38 n.s.	
2	11	(20°)	0.46 n.s	+12° n.s.	12	(10°)	0.36 n.s.	+66° n.s.
3	10	22°	0.64 **	+14° n.s.	11	3°	0.54 *	+59° n.s.
4					12	13°	0.74 ***	+69° *

N, number of birds contributing; α, r, direction and length of mean vector, with asterisks at r indicating a significant directional preference by the Rayleigh Test [30] (Batschelet, 1981). The column $\Delta_{\text{Round 1}}$ gives differences to the behavior in the first test round, with asterisks indicating significance; symbols as in Table 2.

Birds that had been pre-exposed monocularly left-eyed to the 92 µT field for 3 h were tested monocularly right-eyed in the same 92 µT field; however, they were disoriented (Figure 4c). While covering the left eye per se did not interfere with the ability of the right eye to mediate magnetic directional information (see Figure 1c), the right eye/left hemisphere could not cope with the increased intensity if it had not experienced the respective magnetic condition before. For adjusting to higher field strengths, processing of such input in the respective hemisphere seems to be required—there appears to be no transfer from the right to the left hemisphere where the adjustment to higher magnetic intensities is concerned.

3. Discussion

Our data show that, during the first spring migration, the lateralization of the magnetic compass in favor of the right eye/left hemisphere of the brain can be easily undone by covering the right eye for a short time. However, this activation of the left eye/right hemisphere system does, in turn, not seem to affect the right eye. Interpretable magnetic information mediated by the left eye is essentially required to remove the lateralization. When the left eye system is activated, it can adjust to intensities outside the functional window of the magnetic compass (see [32]), but if it does, this ability is not transferred to the right eye system.

The directional information from the magnetic field originates in the retina and is transmitted by the visual nerve to higher centers in the brain (e.g., [33–35]). There are two main ascending visual systems in the bird brain that reach the telencephalon: one is the tectofugal system that runs from the retina via the optic tectum to the thalamic *nucleus rotundus*, which, in turn, projects to the

entopallium. The second is the thalamofugal system that ascends from the retina via a thalamic link to a telencephalic area called wulst [36]. Anatomical and physiological studies in pigeons could demonstrate that both systems display asymmetries with a dominance of the left hemisphere. The tectofugal system is characterized by diverse anatomical and physiological asymmetries along its route [37–41]. Consequently, left-sided tectofugal lesions result in more severe visual deficits than right-sided ones [42,43]. In the thalamofugal system, the left- but not the right-sided wulst is able to importantly modify activity patterns of the tectofugal pathway [44]. Thus, both ascending visual pathways are lateralized with a superiority of the right eye and constitute a leading role of the left hemisphere, e.g., in recognizing and categorizing objects [45,46]. This could also be the reason for the normal dominance of the right eye/left hemisphere system in magnetoreception. At the same time, several commissural fibers that run through the tectal and posterior commissures are asymmetrically organized in birds such that the left tectum is less inhibited by its right counterpart than vice versa [47]. As a consequence, the dominant left hemisphere is able to inhibit the subdominant right. Inhibitory interactions are possibly crucial when only one function for which one hemisphere is dominant has to be executed [48]. Against this background, we will discuss our findings.

3.1. Fast Re-Activation of the Left Eye/Right Hemisphere System for Sensing Magnetic Directions

A mere $1^{1}/2$ h of covering the right eye enabled the robins to orient with their left eye. This extends the findings of our previous study [29] and shows that the lateralization of magnetic compass orientation is still amenable to changes during the first spring migration. Ninety minutes is probably too short for major anatomical changes within the visual pathway [49]. It is more likely that the causal mechanisms for re-activation of the left eye system are related to the short-term synaptic plasticity of the strength of commissural synapses. As the dominant left hemisphere is able to inhibit the subdominant right [47,48], the functional asymmetry of the magnetic compass could be constituted via asymmetrically organized inhibitory interactions between the two hemispheres. Both the left and the right hemispheres are obviously able to do the task during the first spring migration, but, under normal conditions, the dominant left hemisphere (right eye) would inhibit the subdominant right half brain (left eye). This asymmetrical inhibition is abolished after forcing the animal to use the left eye alone for $1^{1}/2$ h. However, these birds return to their left hemispheric dominance after just another further $1^{1}/2$ h of binocular vision. Thus, left hemisphere (right eye) dominance seems to be the normal state of the magnetic compass system. In young birds, this condition is subject to plasticity after the accumulation of left eye experience, but it returns to its previous status once the right eye can be used again.

The short time of $1^{1}/2$ h required to enable birds to use their left eye during spring migration could also explain some of the seemingly controversial findings in the literature. Engels and colleagues [27] reported that they did not find lateralization of the magnetic compass in their spring experiments with robins. In their method section, the authors quote previous studies [25,50] that imply that the covering of the right eye occurred at least 2 h before the tests began, often earlier—this would have allowed sufficient time for the neural circuit to process information from the left eye again.

3.2. Conditions Required for the Re-Activation of the Left Eye/Right Hemisphere System

The findings of the second part of our study are more difficult to interpret because they touch the still open question of whether the reception of magnetic directions is an integrated part of vision or magnetic information is processed more or less independently as a sense of its own.

Learning visual discrimination tasks takes longer with the left eye/right hemisphere than with the right eye, and in several cases of bilateral learning, the right hemisphere did not share the knowledge, but had to be trained separately (e.g., [51,52]; for review, see [53]). It is unclear whether the longer time required for the left eye/right hemisphere system to adjust to higher magnetic intensities represents a parallel case. We exposed the robins prior to the tests for 3 h to a higher magnetic field of 92 µT. As shown previously [31], robins need about 1 h to orient in this field strength. Robins allowed to use their right eye were oriented right away, but this was not so for the left-eyed birds; they took

considerably longer. This appears to be in accordance with the results of discrimination studies mentioned above. The adjustment to higher magnetic intensities means that the birds become able to interpret a slightly different activation pattern on the retina (see [16,31]); it can start only after the ability to process magnetic information has been restored to left eye/right hemispheres. However, this alone can probably not account for the longer delay of the left eye system, as our experiments show that it requires only $1^{1}/2$ h, possibly less. It means that the left eye system is indeed considerably slower in performing the adjustment, requiring much longer than the 1 h observed in binocular birds [31].

Once the left eye system could orient in the stronger magnetic field of 92 µT, this ability was not directly transferred to the right eye system. This is contrary to the results of most visual discriminations tasks, which found a more efficient transfer of visual discrimination from the left eye to the right eye [43,51,52]. This is assumed to be due to the more bilateral left hemispheric visual representation in the tectofugal pathway that enables the right eye system to swiftly access left eye information [38]. The fact that our result pattern runs contrary to these data from visual pattern discrimination studies indicates that magnetic compass information is processed differently from visual patterns. It could also be related to the thalamofugal wulst system where cluster *N* was suggested to be a central hub of magnetic compass processing ([35], but see [54]). At least in pigeons, the wulst is known to modify lateralized activity patterns of the tectofugal pathway [41,44] and to thereby affect interhemispheric exchange of information [43].

An important finding in the experiments with left-eyed birds is that exposure to a radio-frequency field that disrupts magnetic orientation [18,19] did not result in an ability to orient with the left eye, although this eye had had access to visual information. Obviously, the change in asymmetry concerning magnetic compass information is not the result of mere right-eye monocular occlusion. What is required for this kind of change is the ability to sense interpretable magnetic compass information with the left eye during periods of absence of right eye input—if this specific requirement is not met, the normal left hemispheric (right eye) dominance prevails. This, too, seems to indicate that magnetic directional information is processed differently from visual input, with the specific magnetic stimulus necessary to overcome the normal lateralization in favor of the right eye system. However, it is also conceivable that magnetic compass information is just a specific kind of visual input within the visual system and that disruption of this input through a radio-frequency field makes it impossible for the left eye/right hemisphere system to regain the ability to process this specific input class. Details of how magnetic compass information is processed have to be analyzed in further studies.

3.3. Lasting Flexibility in the Avian Magnetic Compass?

Our results clearly show that, during the first spring migration, both hemispheres are in principle able to process magnetic compass information. Indeed, in a histological study [23], Cry1a, the putative receptor molecule, was found in both eyes alike in robins almost a year old, i.e., after spring migration was finished. Older robins were not examined, but at least in Domestic Chickens, *Gallus gallus*, Cry1a was still present in both eyes when they are more than two years old [55]. This suggests that magnetic directional information could still be provided by the left eye and processed in the right hemisphere, but that it is actively suppressed by the left hemisphere—information from the left eye is no longer processed as long as corresponding information from the right eye is transmitted to the brain. However, if this is interrupted, it can be replaced by that from the left eye. The observation that the right eye system is not affected by being temporarily covered and that the re-gained ability to use the information from the left eye is lost rather quickly when the right eye is open again demonstrates the dominance of the right eye/left hemisphere in processing magnetic directional information.

For this study, we used young robins during their first spring migration, i.e., birds less than one year old; older birds roughly $1^{1}/2$ years old had proven less flexible in an earlier study [29]. It seems possible that changes of asymmetry are easier during early ontogeny and less flexible in adult individuals. Indeed, Lesley Rogers [46,56] pioneered studies on the ontogenetic establishment of visual asymmetries in chicks and could demonstrate that both functional and anatomical lateralized systems

can be easily modified in early ontogeny, with a similar effect also observed in young pigeons [57,58]. In Japanese quails, a life-long potential for plasticity has been observed [7]. Hence, we cannot exclude that in robins and other birds the left eye/right hemisphere system can still be activated in later years, but this appears to require more time than the six hours for which we tested during the second autumn migration [29]. Possibly, if a bird is injured and loses its right eye, its brain proves flexible enough to eventually restore magnetoreception to the left eye system. Studies in humans make it likely that asymmetries that depend on lateralized commissural interactions can retain their plasticity up to late adulthood [59].

4. Material and Methods

The experiments were performed during spring migration of the years 2011 to 2013 in the garden of the Zoological Institute of the University of Frankfurt am Main (50°08′N, 8°40′E).

4.1. Experimental Birds

The test birds were European Robins, a passerine species that is distributed all over Europe. The northern populations are nocturnal migrants and spend the winter in the Mediterranean region. In September, juvenile birds were caught using mist nets in the Botanical Garden of Frankfurt am Main, right next to the test sites and were identified as transmigrants of Scandinavian origin by their wing lengths. They were housed in individual cages in a photoperiod simulating the natural one until early December, when it was decreased to L:D 8:16. Around New Year, it was increased in two steps to L:D 13:11. This induced premature migratory activity and allowed us to conduct spring experiments already in January and February. After the end of the experiments, the birds were released in the Botanical Garden in the beginning of April when the photoperiod outside had reached 13 h.

4.2. Covering One Eye

With all experimental test series, we ran control tests with the same individual birds, testing them binocularly (Bi) without any treatment, because previous tests proved any unspecific effects from covering one eye to be negligible. The methods used to cover one eye for monocular testing was identical with those used in earlier studies with migratory birds [11,12,29]: a small non-magnetic aluminum cap was placed over the eye to be covered, fixed with adhesive tape (Leukoplast), as shown in Figure 1 in [11]. This was done either immediately before the tests started or at predetermined intervals before tests began; in some treatments, the eye-cover was removed before tests started and the other eye was covered instead. The various test conditions are defined in Table 1. Immediately after each test, any eye cover was removed and the birds were returned to their housing cages.

4.3. Test Performance

Testing followed our standard procedure, see, e.g., [11,18,29]: the test sites were wooden houses in the garden of the Zoological Institute where the geomagnetic field (46 µT, 66° inclination) was largely undisturbed. The birds were tested individually once per day in funnel-shaped cages lined with thermo-paper [60] where they left scratches as they moved. The cage was lit with green light, our standard control light. Each test lasted about 1 h. The individual birds were mostly tested three times in each test condition, in one condition four times (see Table 2).

In two test conditions, the birds were exposed to a radio-frequency field of 1.315 MHz (the local Larmor frequency) and 480 nT for 3 h before they were tested. This field was produced by a coil antenna consisting of a single winding of coaxial cable with 2 cm of the screening removed. This antenna was mounted horizontally on a wooden frame and was fed by oscillating currents from a high frequency generator, generating the oscillating field vertically, i.e., at a 24° angle to the vector of the geomagnetic field (for details, see [18,19,61]); four birds at a time were exposed in this field in all-plastic housing cages. In another test series, the birds were pre-exposed to, and tested in a magnetic field of 92 µT, twice the strength of the local geomagnetic field. This field was produced by Helmholtz coils (2 m

in diameter and 1 m clearance) arranged in the way that the induced field added to the geomagnetic field, increasing the intensity, but not altering magnetic North and inclination [31].

4.4. Data Analysis and Statistics

After each test, the thermo-paper was removed from the funnels, virtually divided into 24 uniform sectors, and the scratches in these 24 sectors were counted by a person blind to the test conditions. Tests with less than a total of 35 scratches were considered to be of too little activity and were discarded; these tests were repeated with the same bird at the end of the test period. From the distribution of the scratches, the heading of the bird in the respective test was determined. The headings of each bird in each test condition were added to calculate a vector with the heading α_b and the length r_b. From these headings α_b, we calculated second order grand mean vectors for the various test conditions, which were tested for significant directional preference using the Rayleigh test [30]. The data of monocular treatments were compared with the binocular control data and the data from the same birds in different treatments with the Mardia Watson Wheeler test for differences in distribution, and the Mann–Whitney U test was applied to the differences of the birds' mean bearings from the grand mean for differences in variance [30]. From the individual vector lengths r_b, medians were calculated for each test condition; they reflect the intra-individual variance.

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Ethical Statement: The experiments were performed according to the rules and regulations for Animal Welfare in Germany, under the permit V54-19c20/15-F104/54 issued by the Regierungspräsidium Darmstadt (regional council) of the State of Hessen on 22.12.2010, extended on the 12.9.2012 until 31.12.2013.

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How Ecology Could Affect Cerebral Lateralization for Explorative Behaviour in Lizards

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Abstract: As recent studies have shown a left-eye preference during exploration in *Podarcis muralis*, which could be strictly related to its territoriality, we tested the same behaviour in a similar species, but one living in different habitats and showing a different ecology. In particular, we assessed the preferential turning direction in adults of a non-territorial lizard, *Zootoca vivipara*, during the exploration of an unknown maze. At the population level, no significant preference emerged, possibly for the lack of the territorial habit and the characteristics of the natural environment. Nevertheless, females turned to the left more frequently than males did. We hypothesize this as a motor bias, possibly due to a necessity for females to be coordinated and fast in moving in the environment, because of their viviparous condition and the resultant reduction of physical performance during pregnant periods, which are likely to increase vulnerability to predators.

Keywords: brain asymmetry; eye preference; lateralization; lizard; motor bias; territoriality

1. Introduction

Scientists have collected a large amount of evidence supporting behavioural bias spread across vertebrates, and even invertebrates [1–3]. This is even clearer if we consider that being lateralized could bring benefits, hence, affecting the fitness of individuals that present it [4–6].

Brain asymmetries can be manifested and studied as behavioural visual asymmetries, or the preferential use of a specific eye for looking at a type of stimulus, with the latter being especially easily evident in animals with laterally-placed eyes [7,8]. We know that different reactions to right-and left-placed stimuli have been ascertained in several species, verifying the specialization of the brain to perceive information with the left or right eye and in elaborating it with the contralateral hemisphere, according to the nature of the cue (for review, see [1,4,8]).

Being so lateralized could be advantageous by allowing better processing of two tasks at the same time, each one perceived with an eye, and then elaborated by the contralateral hemisphere [4,8,9]. This can enhance a lateralized individual's cognition to simultaneously attend to multiple cues [4,10,11]. As the behaviours involved, i.e., lateralized, are generally usual and important for survival, such as feeding and vigilance, it could be extremely advantageous that they can be performed simultaneously [12–14].

Recently, the scientific interest about lateralized species has increased for mammals, as well as for birds [15–18]. Lizards are an interesting model for studying visual lateralization because they have almost complete decussation of the optic chiasma and they lack the larger number of interhemispheric connections present in mammals, allowing cues perceived with one eye to be processed almost

entirely with the contralateral half of the brain [19,20]. Each visual system could then work largely independently [21]. Some previous works focused on *Podarcis muralis*, a lizard species widespread in Europe, highlighting that this species is lateralized for some crucial daily behaviours [22]. According to the bibliography, this species shows preferences in using the left eye in predatory tasks (detailed observation of stimuli), and the right eye in vigilance and exploratory tasks (global attention and spatial processing of stimuli), also in the wild [23–27]. Speculations hypothesized that the lateralization present in this species could be related to its strong territoriality [28,29] and consequent habit of exploring with a high vigilance level during its activities; hence, laterality in this lizard could be evolved as an adaptive character in response to specific environmental needs [22,30]. In this work, we attempted to investigate an eventual form of lateralization in a non-territorial and elusive lizard species, *Zootoca vivipara*, with different life habits [31], environment, and needs than to *P. muralis*, so as to compare results for both species, and attempt to understand the importance/weight of ecological conditions on the manifestation of behavioural and cerebral biases. Starting from the study conducted by Csermely et al. [32], we focused on exploration, an activity closely related to life in the natural environment and biology.

2. Materials and Methods

2.1. Subjects and Housing

From June to July we collected 10 wild adult *Zootoca vivipara* lizards, five males and five females, from Ampola Lake, a biotope in the southwestern area of Trentino, near the town Tiano di Sopra (TN). We obtained the required administrative permit for capturing the lizards from the wild from Comunità Alto Garda e Ledro (prot. 11083/11.4, 25 May 2012). Captures were made by noosing or hands; the lizards were put in cloth bags immediately after and carried to the terraria. Behavioural observations were carried out in the research and the didactical station SperimentArea, situated in Rovereto (Trento, Italy). Here the lizards were housed in $80 \times 50 \times 40$ cm glass terraria or $40 \times 40 \times 30$ cm plastic cages, under the natural Italian summer photoperiod (16:8 h light/dark cycle) and temperature (25–35 °C) regulated with artificial lighting, if necessary. Each terrarium had a floor covered with a sand substratum with the addition of soil, bark, and musk, other than rocks and bricks for refuge and/or basking. The lizards were fed daily with multivitamin powder-dusted mealworm larvae (*Tenebrio molitor*) and crickets; water was provided *ad libitum*. In order to maintain the correct substratum humidity, the terrain was adjusted daily with water vaporization, if necessary. Once entering the terrarium, the lizards were allowed to accustom themselves to the new environment for seven days before the tests started. At the end of the experiment lizards were released at the same site of capture; none of them was harmed by the experiment, which was carried out under license from Italian authorities.

2.2. Apparatus

In order to compare the explorative behaviour of *Zootoca vivipara* with that of *Podarcis muralis*, we employed the same experimental apparatus previously used in Csermely et al. [32] (Figure 1), modified, consisting of a 54×66 cm PVC base maze with 10 cm high sides. Thirteen $12 \times 10 \times 6$ cm blocks were scattered regularly on the base at the distance of 6 cm each other; four additional $6 \times 10 \times 6$ cm blocks were located against two sides of the base; their length was limited to one-half of that of the others so as to maintain the regular reciprocal distance among the blocks. The blocks' presence had to induce the exploring lizard to continuously change direction when it arrived at the T-crossroads, then forcing it to decide to go either to the left or to the right. The blocks were attached to the base with adhesive tape. They were made of a series of commercial Duplo® bricks (Lego A/S, Billund, Denmark) and covered with plastic adhesive paper with marble coloration to prevent the lizards from climbing them. Experiments were conducted without transparent cover, for possible interferences of the reflectance of neon light placed on the top of the maze. The apparatus was located in a circular tub

(110 cm of diameter, 50 cm high), necessary to contain animals in case of escape and to avoid possible surrounding influences on the individual behaviour. In addition, four black corrugated honeycomb panels were collocated all around the apparatus, working as screens for the operator.
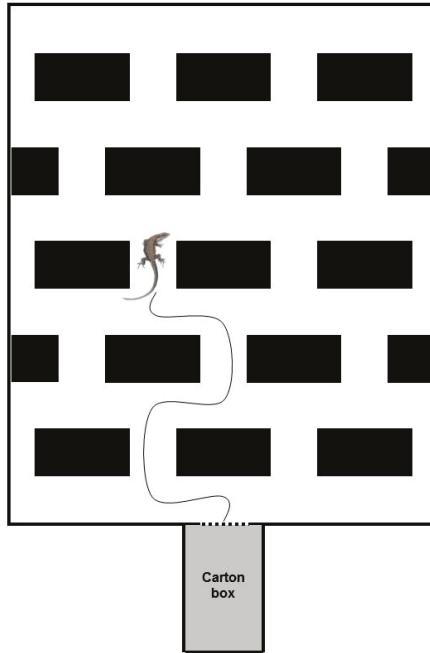

Figure 1. Schematic 2D representation of the experimental apparatus (adapted from [32]).

2.3. Procedure

Before the beginning of the tests, we allowed lizards to thermoregulate at least 30 min under the light of a 50-W halogen lamp, allowing them to reach the temperature for maximal locomotor performance, necessary to express correct exploring behaviour. Experiments were conducted in the same place as the lizard housing, hence, with the same light and temperature conditions. Afterwards, a lizard was gently removed from the terrarium and placed in a 15 × 9 × 6.5 cm carton box external to the maze, but attached to it. The lizard remained in the box for 5 min to acclimatize; thereafter, the operator, located behind a black Poliplak® screen (RÖHM GmbH, Sontheim/Brenz, Germany), using a thin cable, lifted up the PVC gateway that had prevented the lizard from entering the maze through the opening before the beginning of the test. The test started when the lizard entered the maze. After 20 min, if the lizard did not spontaneously enter it, the operator beat, with a small stick, the distal part of the box containing the lizard to encourage it to move out. The lizard could move freely within the maze for 20 min. During the experiment, the gateway remained open; hence, the lizard could come back to the box. At the end, it was returned to its terrarium and the maze floor and walls were cleaned with ethyl alcohol to prevent any possible effect of chemical cues on subsequent individuals.

The tests were carried out when the air temperature was within the 27–35 °C range. Light was homogeneous and both natural and artificial by a neon lamp placed on the experimental apparatus. We considered the following behaviour parameters: when the subject entered the maze, we assessed (1) the rotation of the head to the left or the right, and (2) the frequency of direction of the turn (leftward or rightward). Afterward, while the lizard was exploring the maze, we observed (3) the delay time (duration of hesitation) at each T-crossroads, and (4) the duration of each turning, (5) the total frequency

of direction of turning; and (6) when passing a T-crossroad for the first time (excluding any possible olfactory influence).

All tests were recorded with a digital mini DV colour video camera Sony "Handycam" DCR-SR58 (Sony, Tokyo, Japan) $17.0 \times 9.0 \times 8.0$ cm placed above the maze. Frame by frame analysis of the footage was possible by the Windows Live Movie Maker 6.0 video software (Microsoft Corporation, Redmond, WA, USA).

2.4. Statistical Analyses

We used the binomial test to compare the number of turns to the left or to the right performed by each lizard and the individual preference in turning the head. The Wilcoxon matched-pairs signed ranks test (T+) was used to compare the number of turns to the left or to the right and the preference in turning the head in the group as a whole. For comparing durations, we used the Mann–Whitney U test (U). Calculations have been performed using the SPSS 18.0 for Windows software [33]. Means are ± Standard Error (SE) and the probability, set at α = 0.05, was two-tailed throughout, unless otherwise stated.

3. Results

Although all lizards hesitated in entering the maze, they all moved out of the box and completed the experiment. Some lizards, before exiting, required a stimulation by gently tapping on the starting box with a stick. During experiments lizards did not appear frightened but explored the environment, walking inside it and turning around the blocks. They walked both in the central and lateral routes of the maze; sometimes they tried to climb the blocks or the maze walls. During the exploration some individuals arrived near the entrance box and entered it, but they shortly moved back out to the maze. During the experiment lizards moved for 619.15 ± 71.45 s and froze for 580.85 ± 57.82 s, without any difference inside the group and between sexes for both the time of movement and immobility.

Immediately after entering the maze six lizards out of 10 (four males and two females) rotated the head to the right (binomial test; $p = 0.289$), two lizards (both females) to the left, and two lizards (one male and one female) did not rotate the head before entering.

Three lizards (one male and two females) out of 10 performed the first turn immediately after entering the maze on the left and seven lizards (four males and three females) out of 10 on the right (binomial test; $p = 0.3438$).

The subsequent movements of the lizards were in various directions, moving progressively further from the entering point. The delay time for turning at each T-crossroad showed similar results for both the left and right directions (2.53 ± 0.90 s and 2.00 ± 0.72 s, respectively; z = −0.227; $p = 0.821$), and also between sexes (males left: 3.96 ± 1.12 s; females left: 1.10 ± 0.24 s; U = −0.522; $p = 0.690$; males right: 3.23 ± 0.89 s; females right: 0.78 ± 0.09 s; U = −1.567; $p = 0.117$). During each T-crossroad turning, lizards kept the head right-turned for 4.62 ± 0.88 s and left-turned for 6.19 ± 0.23 s (U = −0.076; $p = 0.940$); no differences emerged between sexes in keeping the head right-turned (males: 4.74 ± 0.97 s; females: 4.51 ± 0.90; U = −0.522; $p = 0.6$) and left-turned (males: 8.66 ± 3.26 s; females: 3.73 ± 0.52 s; U = −0.83; $p = 0.4$).

The average number of turns per lizard per test was 27.70 ± 3.19, with no significant differences between sexes (males: 25.80 ± 2.75, females: 29.60 ± 4.01; U = 11.500; $p = 0.834$). Statistical analyses did not reveal any population-level bias for turning to the left or to the right among the lizards (13.2 ± 2.30 and 14.5 ± 1.68, respectively; T+ = −0.153; $p = 0.878$) and between sexes for the right turning (T+ = −0.674; $p = 0.500$), but females showed a bias in turning left compared with males (T+ = −2.032; $p = 0.042$). Males performed 9.80 ± 1.40 left-turns and 16.0 ± 2.00 right-turns (T+ = −1.826, $p = 0.068$) and females performed 16.60 ± 2.67 left-turns vs. 13.0 ± 1.342 right-turns (T+ = −1.214, $p = 0.225$). If we consider the average number of turns that lizards performed when encountering a T-crossroad for the first time (i.e., without any olfactory influence) there emerged a preference in turning right (8.5 ± 1.02) compared with the left direction (5.9 ± 0.78; T+ = −1.963, $p = 0.050$) in the population.

This result is due to the males' choice (right: 8.5 ± 1.02; left: 4.4 ± 0.52) more than the females' choice (right: 8.0 ± 0.72; left: 7.4 ± 0.72; Table 1). Moreover, females turned more frequently than males to the left (T+ = −2.032; p = 0.042) than to the right (T+ = −0.412; p = 0.680).

Considering the total number of turns per lizard to the left or to the right, 2 individuals of the 10 tested showed a preference for turning right (Binomial test; p = 0.029 and p = 0.035), both males (Table 2).

Table 1. Number of first turns for lizards when encountering a T-crossroad for the first time. p-values refer to binomial test comparisons.

Lizard	Sex	Left	Right	Tot	p
1	M	4	15	19	0.0192
2	M	5	10	15	0.3018
3	M	7	9	16	0.8036
4	M	3	6	9	0.5078
5	M	3	5	8	0.7266
6	F	5	5	10	1.2461
7	F	9	6	15	0.6072
8	F	8	12	20	0.5034
9	F	10	9	19	1.0000
10	F	5	8	13	0.5811

Table 2. Number of turns for lizard for test. p-values refer to binomial test comparisons.

Lizard	Sex	Left	Right	Tot	p
1	M	7	19	26	0.0290
2	M	13	23	36	0.1325
3	M	16	15	31	1.0000
4	M	6	17	23	0.0347
5	M	7	6	13	1.0000
6	F	11	7	18	0.4807
7	F	17	12	29	0.4583
8	F	30	18	48	0.1114
9	F	17	16	33	1.0000
10	F	8	12	20	0.5034

By the number of first turn performed by each lizard it emerged that only one individual (a male) showed a preference, in particular in turning rightward (binomial test; p = 0.0192).

4. Discussion

Overall, our lizards resulted in not showing any evident bias or side preference in exploring a novel environment. Hence, the explorative behaviour of *Zootoca vivipara* lizards does not seem to be controlled by a form of lateralization. This is interesting as this result is in strong contrast with what was found by Csermely et al. [32] in *Podarcis muralis*. In fact, although experiments were conducted in the same way, and with the same experimental apparatus, *P. muralis* evidenced a strong bias in turning left, that the authors associated to a visual guided bias during exploration, i.e., a visual lateralization [32]. As such, these results suggest that differences emerging between these species are probably due to their remarkably different ecology, although, at present, there is no evidence of a clear explanation for the differential lateralization of the two species.

As a first point, we observed that although during the experiment almost all *Z. vivipara* individuals gave good clear signals of exploration, they all showed hesitation in entering the maze and the time they spent in exploring was similar to the time they spent in freezing. This poor activity and the overall low level of confidence in the maze could be related to the secretive behaviour of *Z. vivipara* and, in particular, likened to the thermally-heterogeneous habitats where this species is

commonly found, allowing less active movements in general and, at the same time, more time spent in thermoregulation [34,35].

Results on behavioural lateralization highlighted in *P. muralis* have been explained by the authors with its strong territoriality and its consequent natural high predisposition to explore [32]. A support of this is the fact that, in Csermely et al. [32], an evident higher frequency of turning emerged, especially in males, mainly motivated by the need to defend their own area. In contrast, our *Z. vivipara* lived in a wild, cold-climate environment, characterized by the presence of unique ecological factors potentially influencing the explorative behaviour (therefore, the *Z. vivipara* lateralization). For example, the human impact and presence in such areas is generally low, in contrast with the high anthropic level locations, where *P. muralis* was studied by Csermely et al. [32] lived. This could force individuals to maintain a high level of attention and vigilance, pushing towards a stronger lateralization in the explorative behaviour.

Nevertheless, an overall female bias in turning left emerged here, referring also to the first and more spontaneous encounter with a T-crossroad. Whereas the lack of turning preferences in the overall T-maze could be due to the low sample used in these experiments, the turning bias in the first T-crossroad could be explained either as a visual lateralization or a motor lateralization. As a visual lateralization, the direction of choice is consistent with that found in *P. muralis*, and with previous studies, which appointed to the right hemisphere the capability of processing global aspects of the environment [14,32,36]. However, it is in strong contrast with the evidence that, in *P. muralis*, the turning bias is found in males, not in females [32]. As this previous result could be linked with males' territorial attitude, we suggest that *Z. vivipara* females' visual preference could be related to the viviparous nature of several populations of this species, which constrains female individuals in having a longer reproductive period in respect to oviparous females, and a consequently higher level of attention compared with male individuals [34]. During gestation, females must be more vigilant to guarantee the offspring's survival, hence, to increase their fitness. However, gestation incurs some costs, such as a shift in thermoregulatory needs and locomotor impairment [34,35,37–39]. Pregnant individuals are physically limited by their body increase which may affect and reduce their fleetness and speed, thus, with locomotor costs. Being lateralized, in particular for the same direction in the same population, could be advantageous for the possibility of coordination in behaviour between individuals, in particular for anti-predatory tasks [4]. This is especially true for social/gregarious species [4,40,41]. Although there is no evidence of gregarious habits in *Zootoca vivipara*, the absence of territoriality allows tolerance between individuals, and a coordination in moving may become, for these lizards, one of the evolutionary strategies for contrasting costs of viviparity. This could become a hypothesis of explanation of the necessity for female lizards to be specialized in vigilance as a group, especially in moving.

Movement, particularly in exploratory behaviour, is preceded by a high-level observation that probably guides the subsequent choice of direction. There are several indications of left-eye processing in using the environmental layout to guide locomotion to a target site using spatial information [42]. However, we emphasize that the female leftward preference in turning, which emerged at T-crossroads is not supported by the head rotation durations we measured during each T-crossroad turning. These comparisons, easily indicative of visual system involvement, did not show any significance, not sustaining a visual influence in the choice of direction. It is therefore possible to advance the hypothesis in this context, that the left-turning females' bias highlighted by this work may be evidence of footedness, hence, a motor bias more than a visual one.

Very differently to *P. muralis*, closely related to dry and bare environments, *Z. vivipara* is strictly dependent on habitats, as wetlands, where the vegetation cover is prominent and could become a visual impediment between individuals. Thus, it could be difficult for these individuals to maintain a visual link in groups of conspecifics. This is also true during thermoregulatory exposure. In fact, because of their viviparity, female lizards preferentially used the half-basking behaviour (partially hidden), although basking in the open is more efficient [34]. This allowed these lizards to significantly

reduce the risk of facing exposure to predators, optimizing the trade-off between predation risks and basking efficiency [34,43,44]. In this context, it could be disadvantageous or simply necessary to visually coordinate the behaviour, but it may help to synchronise a motor response.

All this contributes to explaining the different response *Z. vivipara* provide compared with *P. muralis*, i.e., the main absence of lateralization and the different sex evidence, also underlining the importance and close relationship between the living environment and conditions and the evolution of biases.

In conclusion, our results show, that in general *Z. vivipara* is not lateralized in exploring a new environment. However, females showed a bias for turning left during exploration, possibly more easily explained as a motor bias. As these results are in contrast with what emerged in *P. muralis* individuals in previous studies, which showed a visual lateralization especially in males, we propose it could be related to the different ecology of the species, in particular, with differences of the territorial and viviparous natures. Moreover, this is a confirmation of the crucial role of the real-life environment and habits in the emergence and evolution of cerebral lateralization, supporting its advantageous nature, which contributes in its manifestation in different contexts.

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Effects of Handedness and Viewpoint on the Imitation of Origami-Making

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Abstract: The evolutionary origins of the human bias for 85% right-handedness are obscure. The Apprenticeship Complexity Theory states that the increasing difficulty of acquiring stone tool-making and other manual skills in the Pleistocene favoured learners whose hand preference matched that of their teachers. Furthermore, learning from a viewing position opposite, rather than beside, the demonstrator might be harder because it requires more mental transformation. We varied handedness and viewpoint in a bimanual learning task. Thirty-two participants reproduced folding asymmetric origami figures as demonstrated by a videotaped teacher in four conditions (left-handed teacher opposite the learner, left-handed beside, right-handed opposite, or right-handed beside). Learning performance was measured by time to complete each figure, number of video pauses and rewinds, and similarity of copies to the target shape. There was no effect of handedness or viewpoint on imitation learning. However, participants preferred to produce figures with the same asymmetry as demonstrated, indicating they imitate the teacher's hand preference. We speculate that learning by imitation involves internalising motor representations and that, to facilitate learning by imitation, many motor actions can be flexibly executed using the demonstrated hand configuration. We conclude that matching hand preferences evolved due to socially learning moderately complex bimanual skills.

Keywords: social learning; imitation; handedness; laterality; origami; evolution

1. Introduction

Handedness is a behavioural lateralization, defined as a species-level bias to use a certain hand configuration for most tasks. It is expressed in *Homo sapiens* as a species-universal behavioural bias (70–90%) towards using the right hand for fine manipulations and the left hand for stabilising actions [1–3]. Among vertebrates, 61 species (out of 119 measured) show population-level limb preferences, of which 25 species are mammals, 30 birds, and 6 amphibians, reptiles, and fish [4]. Some other animal species also have behavioural hand biases up to 90% at the species level [5–8], and many other species have individually stable hand preferences [9,10]. However, within our evolutionary clade, humans are the only great ape that shows strong, species-universal biases towards one direction of handedness. Non-human great ape hand preferences are characterized by high variability in their direction and a low magnitude of expression [4,11]. In particular, humans have much higher ratios of the dominant to non-dominant hand preference, compared to other apes [4,11,12]. Among the other great apes there are groups of individuals with a majority of right-handers, but there are also groups with a majority of left-handers [13]. In contrast, despite much cross-cultural research, no human group has been found with more than 30% left-handers [14]. The origins of handedness probably lie in brain lateralization [7]. The advantages to having lateralized brain functions, not only in the direction of laterality, but also

in the strength of laterality, are ubiquitous in vertebrates [4,15]. In that case, we should ask why other ape species who engage in bimanual manipulations do not show a universal bias to either right- or left-handedness.

The obvious question concerning this bias to right-handedness is why most humans are lateralized in the same direction. Our recent ancestors had a similar majority of right-handers as today, judging by the proportion of right and left handprints and hand stencils in cave art [16,17]. Data on older, prehistoric handedness, from asymmetries in fossil brain endocasts, arm bones, and tooth cut-marks, show that 67–69% of hominins between 3 million and 30,000 years ago were right-handed with only 5–11% left-handed [18–20]. Interestingly, the same data also show a rate of 12% mixed-handedness in Neanderthals (a parallel species with whom we share a common ancestral species around 600,000 years ago). In contrast, present-day estimates for our species are around 4% mixed-handers [21,22]. Thus, *Homo sapiens* is characterised by a reduction of mixed-handers, and an equivalent increase in right-handers, compared to the Neanderthals.

A possible evolutionary driver of this directional bias in *Homo sapiens* is given by the Apprenticeship Complexity Theory [13,23,24]. This emphasizes the importance of the social learning environment of prehistoric hominins as they acquired complex tool manipulations [25]. It [26,27] proposes that group-level handedness biases in humans evolved to facilitate faster learning through imitation of complex tool manufacture. Stone tool-making changed over time towards requiring longer sequences and more subgoals, from the pre-Oldowan to the Late Acheulean [24,28,29]. Throughout the Pleistocene, as stone crafts and other essential survival tasks increased in difficulty, so did the pressure on children to learn those skills [24]. According to the Apprenticeship Complexity Theory, selective pressure for learning efficiently (i.e., quickly and accurately) favoured learners whose hand preference matched that of their teachers. This pressure was probably a factor for all individuals given that, for example, the archaeological evidence suggests that, for most of hominin prehistory, functional stone knapping skills were learned by all individuals in a group; the emergence of craft specialisation was a very recent invention linked to artistic production, reduced mobility, and complexification of human societies from the Mesolithic and Neolithic onward [30–35].

The nature of the social learning environment can also affect the efficiency of skill transmission. There is a broad spectrum of social learning strategies across human cultures and across animal species, ranging from unsupervised observation to interactive teaching [36–41]. Prehistoric hominins could have used any of these learning strategies [28,42–44]. Before the emergence of teaching, hominin children would have engaged in social learning through observation. Imitation is a form of observational learning that does not involve any teacher input, where the learner copies the actions of others.

Imitation is often seen as a goal-directed process involving knowledge and understanding on the part of the learner, rather than merely copying of a sequence of actions. Imitation requires learners to form an internal representation of the teacher's action sequence in order to reproduce it [45,46]. This action representation involves the mirror neuron system in Broca's area [47,48]. Imitation also relies on the learners' ability to adopt the visual perspective of others, in order to understand their actions [49–51]. Children's learning by imitation in various human societies has a wide range of forms [52] such as helping with daily tasks [53] or third-party observation [40]. The learner's position with respect to the demonstrator(s) will vary in different situations. For example, for participatory tasks, such as hunting, the child is most likely to be behind, or alongside, the demonstrator. In contrast, during observatory tasks, such as basket weaving, the child is more likely to be opposite, facing the demonstrator.

Viewing position during imitation tasks has attracted particular focus due to the transformation of visual perspective required to map between the reference frame of the imitator and the demonstrator [46]. When positioned beside a demonstrator (egocentric viewpoint), the observer's viewpoint matches that of the demonstrator. In this case no mental transformation is needed to interpret the demonstrated action. However, when positioned opposite a demonstrator (allocentric viewpoint), the observer must compensate for the discrepancy between their viewpoint and that of the demonstrator. In this case a

mental transformation of the input may be necessary, regardless of hand preferences. The brain processes actions differently depending on whether they are observed from beside, versus opposite, the viewer, with greater activation in the contralateral hemisphere in the former case but the ipsilateral hemisphere in the latter case [54], and with greater activity in the sensory-motor system in the former than the latter case [55]. In addition, visual object recognition is affected by the object's orientation [56,57]. People are better at action prediction for images of tools viewed from the perspective that they would see when using them [58]. Furthermore, in haptic (active touch) tasks, recognition is easier when objects are explored from the orientation typically used to manipulate them ([59], but see [60]). However, none of the previous studies on viewpoint also tested the interaction with handedness. In sum, there is some evidence showing that imitation is harder if there is a discrepancy in viewpoint that needs to be compensated for (though such viewpoint effects have not always been observed; for example, as described below, Reference [61] found no difference in an observer's ability to reproduce knot-tying dependent on their viewpoint). Furthermore, it is not known how handedness might affect the mental transformation required to process different viewpoints.

The Apprenticeship Complexity Theory predicts that the transmission of complex tasks is more efficient when the demonstrator and learner have the same hand preference. We define "complexity" here as motoric and conceptual difficulty, reflected in the learning time needed to acquire the skill [62]. In this sense, higher complexity is found in tasks with long learning times, narrow error tolerances, many components, and/or many steps in a sequence [63–65]. In particular, complementary bimanual tasks with asynchronous digit use rank high on the manipulative complexity scale [66] and thus are excellent models for testing the interplay of handedness and learning. We expected that folding origami, as used in the present study, would be a good example of such a task.

Previous research on handedness and skill learning is very limited. Two studies found that a congruent hand preference between the demonstrator and observer resulted in more efficient learning than incongruent hand preferences. Reference [27] taught knot-tying to groups of right-handed and left-handed observers, with right- and left-handed demonstrators. They showed faster learning from same-handed demonstrators in both groups. Similarly, Reference [67] showed that same-hand demonstrations result in higher accuracy and speed for targeted hand movements than opposite-hand demonstrations (in right-handed observers). In early child development, imitation of the demonstrators' hand configuration is apparent. Infants are heavily influenced by the demonstrator's hand used when manipulating objects. When tested experimentally, although most infants showed right-hand preferences when first grasping an object, an action demonstrated by a left-handed researcher led all infants to subsequently use their left hand [68]. However, object recognition in the haptic modality is not affected by the hand used to explore the object, which suggests a constancy in object representations that generalises across the hands [60]. Thus, more work is needed to determine how learning and handedness are related.

Few studies have tested the effect of viewpoint on imitation. Reference [69] showed that imitation of body movements is easier when standing behind a teacher rather than opposite a teacher. Reference [70] found more accurate mirror imitation of body movements when the teacher faced the observer. Two other studies used a knot-tying task where participants reproduced knots after viewing demonstrations from different visual angles. Consistent with the body movement studies, Reference [71] showed that knots demonstrated in videos were learned more effectively if the teacher was shown from a position beside, rather than opposite to, the learner. Another study [61] used live knot-tying demonstrations from three different viewpoints (beside, opposite, and at right angles to the demonstrator) and found no difference in the mean number of trials to successfully replicate each knot. Reference [61] did, though, find that participants preferred to reduce the discrepancy in viewing angles by sitting beside the demonstrator when given the choice. These conflicting results mean that more work is needed to determine when viewpoint affects the learning of complex bimanual skills.

In order to disentangle the variables of hand preference and viewpoint on the acquisition of manual skills, we conducted a bimanual learning study involving making origami. We chose origami folding because it is a complex task that has been used in previous studies of learning [41,72,73]. Origami has several important advantages over "live" stone tool-making experiments: (1) the starting raw materials can be perfectly controlled so that all participants receive identical blank origami papers; (2) people already possess the motor skill to fold paper, so they can concentrate on learning the folding sequence, which was the focus of our experiment; (3) folding origami is clean and safe and does not require special equipment; (4) our experiment is reproducible by any scientist, not just the few who have access to specialist flint knapping resources. Following the method of [74], we showed participants demonstration videos of a teacher making nine different origami figures ranging from easy to hard. Learners were required to reproduce each figure by imitating the demonstrated actions. We manipulated the videos to show left-handed and right-handed teachers, and for the teachers to have either the same perspective as the participant (beside view) or a 180-degree rotated perspective (opposite view). We predicted that demonstrations of the origami figures would be harder to imitate if they showed an incongruent hand configuration, and if they showed the teacher opposite, rather than beside, the learner.

2. Materials and Methods

2.1. Participants

We recruited thirty-two participants (9 male) aged between 18 and 22 years. Participants were University of Liverpool undergraduate students, with no restriction on hand preference. All subjects gave their informed consent before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the University of Liverpool (Project identification code PSYC-1011-075).

2.2. Stimuli

Videos were created demonstrating nine asymmetric origami figures being folded. A right-handed teacher folded all of the origami figures using a 21 cm^2 piece of orange paper. The teacher was video recorded from above, at a height of 42 cm from the desk (see Supplementary Video S1 for full videos). Four versions of each of these nine videos were created varying the hand configuration and viewpoint. Following [74], we rotated each video 180 degrees in the picture plane to create the alternate viewing positions and mirror-flipped the video to create a left-handed version in both viewpoints (Figure 1). The camera was placed directly above the teacher's hands to ensure both that the same visual information was available for the two viewing positions and that both viewing positions could occur in actual viewing (this would not be the case if an angled camera angle had been used).

The first video shown to participants was an easy practice stimulus and it was the only one that required the use of scissors. Each participant created all eight origami figures once. They created two figures in each of the four viewpoint conditions (Left-Handed Opposite, Left-Handed Beside, Right-Handed Opposite, and Right-Handed Beside), so that each participant received all conditions. The assignment of the two figures to each condition, and the order of presentation of conditions, was counterbalanced using a Latin Square procedure to reduce order effects. Two participants were assigned to each of the 16 counterbalancing conditions.
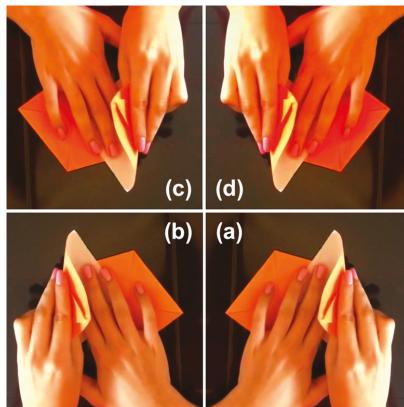
Figure 1. Still images from each of the four conditions created for the "Fish" video showing (**a**) right-handed beside; (**b**) left-handed beside; (**c**) left-handed opposite; and (**d**) right-handed opposite teachers.

2.3. Procedure

Participants initially read an information sheet that stressed that they had to reproduce the origami figures as similarly as possible to the example shown in the video. They were not informed about the different experimental conditions. Participants were given nine 21 cm^2 pieces of paper (one was orange for the practice trial; with two red, yellow, purple, and green papers each assigned to each experimental condition). The demonstration videos were presented to participants on an Apple MacBook Pro 13" using QuickTime player. Participants were free to interact with the video while they reproduced the figures, by pausing with the pause button, and rewinding by dragging the progress bar backwards.

Participants were first shown the practice demonstration video. After the practice reproduction, any queries were resolved before the experiment began. Participants then did the eight experimental trials. While participants were watching the demonstration videos, the experimenter discreetly recorded the number of times the video was paused and was rewound, and also recorded the time taken to complete the figure, using a stopwatch.

After all nine figures were completed, participants were asked a series of questions:

1. Do you know what the experiment is testing?
2. Did you notice anything about the position of the hands in the videos?
3. Did you notice anything about the hands themselves in the videos?
4. Did you find any of the trials particularly hard?

Participants then completed a Short Form Edinburgh Handedness Inventory [75].

2.4. Analyses

Performance at origami folding was coded with respect to preferred hand dominance. For left-handed participants, left-handed demonstrations were coded as congruent and right-handed demonstrations as incongruent. The reverse coding was used for right-handed and mixed-handed participants. We included mixed-handers in the right-handed category because they all had positive scores (range +25 to +62.5) on the Short Form Edinburgh Handedness Inventory (left-handers had scores ranging from −50 to −100). Since human hand performance is not categorical but rather on a continuum [76], we focus here on hand preference, that is, the categorical choice of hand dominance when doing a task.

The efficiency of reproducing origami figures was measured in two ways: first, the time taken to complete the figure divided by the length of the demonstration video; second, the total number of pauses and rewinds for each video.

The accuracy of reproducing origami figures was also measured in two ways. First, subjective ratings were made of the completed origami figures. Two naïve raters who did not take part in the experiment independently rated the similarity of the produced origami figures to the originals. As training, the two raters were initially shown all 32 practice figures and were asked to rate these using the full scale from 1 for figures which looked 'unlike the original' to 10 for figures which looked 'exactly like the original'. Zero was given to incomplete figures. Raters were required to assign at least three figures to each number on the scale to ensure that they used the full scale. Raters then rated each set of eight experimental figures created by each participant using the same scale. The eight demonstration figures were laid out in front of them together with the set of eight figures from one participant. After completing ratings for all 32 participants, the figures from the first two participants that they had rated were re-presented (without informing the raters) for rating to check for the stability of ratings. The raters completed their ratings of participants in reverse orders with respect to each other.

Second, the experimenter recorded the symmetry of all eight figures produced by each of the 32 subjects in turn. Each figure was categorised as having matching asymmetry to the original (MA), having the opposite asymmetry to the original (OA), as being incorrectly folded to be symmetrical rather asymmetrical (IS), or as being uncodeable due to the poor quality of the reproduction.

3. Results

The Short Form Edinburgh Handedness Inventory scores revealed three left-handed participants (scores -100 to -50), five mixed-handers (scores $+25$ to $+62.5$), and 24 right-handed participants (scores $+75$ to $+100$). Our sample was similar to the usual human distribution of hand dominance with 9% left-handed, 16% mixed and 75% right-handed participants.

Participants were asked questions prior to debrief in order to assess whether they were aware of the aims of the experiment. None of the 32 participants reported the correct overall aims of the experiment. When prompted, only two participants had noticed that the hand configurations in the video changed in some way, and only three noticed that the viewpoint of demonstrations changed.

For the subjective ratings of figures' similarity to originals, inter-rater reliability between the two raters revealed a fair agreement between the two raters, k = 0.35 ($p < 0.001$), 95% CI (0.414, 0.284), as well as moderate agreement within rater 1, k = 0.56, ($p < 0.001$), and substantial agreement within rater 2, k = 0.64, ($p < 0.001$).

3.1. Effect of Viewpoint and Handedness of Demonstrator on the Efficiency of Making Origami Figures

The data were analysed using an ANOVA with two within-subjects variables, viewpoint (Beside or Opposite) and handedness of demonstrator relative to the participant (Congruent, where, for example, a right-handed participant watched a right-handed demonstrator; or Incongruent, where, for example, a left-handed participant watched a right-handed demonstrator) and reaction time as the dependent variable. There was no effect of viewpoint, $F(1,31) = 0.06$, $p = 0.8$, partial $\eta^2 = 0.002$, with similar time to complete figures for Beside (Mean = 231 s, SD = 10.9) and Opposite (Mean = 225 s, SD = 10.2) videos. There was also no effect of handedness, $F(1,31) = 1.16$, $p = 0.3$, partial $\eta^2 = 0.04$, with similar time for Congruent handedness (Mean = 227 s, SD = 10.9) and Incongruent handedness (Mean = 229 s, SD = 11.66) videos. Finally, there was no interaction between viewpoint and handedness, $F(1,31) = 0.35$, $p = 0.6$, partial $\eta^2 = 0.01$.

Repeating this ANOVA using the sum of pauses and rewinds as the dependent variable revealed the same pattern of results. Again, there was no effect of viewpoint, $F(1,31) = 0.07$, $p = 0.8$, partial $\eta^2 = 0.002$, with a similar number of pauses and rewinds for Beside (Mean = 7.9, SD = 0.53) and Opposite (Mean = 7.8, SD = 0.66) videos. There was also no effect of handedness, $F(1,31) = 0.40$, $p = 0.5$, partial $\eta^2 = 0.01$, with a similar number of pauses and rewinds for Congruent handedness

(Mean = 7.7, SD = 0.59) and Incongruent handedness (Mean = 8.1, SD = 0.67) videos. Finally, there was no interaction between viewpoint and handedness, $F(1,31) = 0.03$, $p = 0.9$, partial $\eta^2 = 0.001$.

3.2. Effect of Viewpoint and Handedness of Demonstrator on the Accuracy of Making Origami Figures

The ANOVA was repeated with ratings of accuracy of reproduction of the origami figure as the dependent variable. Here, there was no effect of viewpoint, $F(1,31) = 3.06$, $p = 0.09$, partial $\eta^2 = 0.09$, with similar ratings for figures produced from Beside (Mean = 6.2, SD = 0.37) and Opposite (Mean = 5.8, SD = 0.44) videos. There was also no effect of handedness, $F(1,31) = 1.46$, $p = 0.2$, partial $\eta^2 = 0.05$, with similar ratings for figures in the Congruent handedness (Mean = 6.2, SD = 0.42) and Incongruent handedness (Mean = 5.8, SD = 0.42) conditions. Finally, there was no interaction between viewpoint and handedness, $F(1,31) = 0.002$, $p = 0.9$, partial $\eta^2 = 0.00$.

3.3. Check That Results Were Not Influenced by the Inclusion of Participants Who Were Not Right-Handed

In order to ascertain that the results reported above were not contaminated by the inclusion of the three left-handed participants and five mixed-handed participants, these three ANOVAs were repeated but only including the 24 right-handed participants. These ANOVAs produced the same pattern of results, with no signficant main effects of viewpoint or of handedness congruency and no significant interactions between these two factors.

3.4. Analysis of Symmetry of Reproduced Origami Figures

Participants produced similar numbers of figures with matching asymmetry to the original (MA; 90 figures, 35%), as figures with the reverse asymmetry to the original (RA; 76 figures, 30%). The remainder of figures were incorrectly reproduced as symmetrical (IS; 30 figures, 12%) or were too poorly reproduced to be coded (60 figures, 23%). The IS and uncodeable responses occurred at similar rates across the four conditions (5–10/condition for IS; 13–17/condition for uncodeable).

Of primary interest was the proportion of figures produced with matching (MA) versus reverse (RA) asymmetry across the four conditions. Considering only these two types of responses, for the Beside viewpoint, when handedness was congruent, as expected most figures had matching asymmetry (MA = 38/46 responses, 83%). Critically, when handedness was incongruent, most figures continued to have matching asymmetry (MA = 29/40 responses; 73%). Thus participants continued to fold using the same hand configuration as the teacher even when the teacher had the opposite handedness as them. A similar, but weaker, pattern occurred for the Opposite viewpoint. Here, when handedness was congruent most figures had, as expected, matching asymmetry (MA = 26/39 responses, 67%). Crucially, when handedness was incongruent most figures again continued to have matching asymmetry (MA = 26/41 responses; 63%). Thus people usually used the same hand configuration to fold as shown by the teacher, whether or not the teacher had the same hand preference as them, with this preference being rather weaker for demonstrations shown from the Opposite viewpoint.

4. Discussion

Contrary to our predictions, there was no effect on performance of either viewing position of the participant relative to the teacher or of handedness of the participant relative to the teacher. Performance was assessed in terms of efficiency of reproduction of each figure, as measured both by the amount of time taken to complete the figures and by the number of pauses and rewinds of the demonstration videos needed to finish the figures. Performance was also assessed in terms of the quality of reproduction of each figure using subjective ratings of the figures' similarity to the originals and coding of the asymmetry of each figure relative to that of the original.

Our experiment provides no evidence for an effect of viewing position on performance, with no difference between viewing demonstration videos from the same visual perspective as the demonstrator, or from a 180 degree rotated viewpoint. This result is in line with [61] who found that a change in viewing position did not perturb imitation performance. It goes against [56], that visual

object recognition is faster for objects viewed from a beside orientation. One possible explanation is that our participants were able to interpret the opposite viewpoints by mentally rotating their body relative to the object, rather than by mentally rotating the origami figures themselves. Mental object rotation is more difficult than mental body rotation [77] and is more sensitive to angle of rotation. Therefore, it is possible that people imitate origami folding using the easier strategy of copying the hand movements, rather than by trying to reproduce the shape of the origami figures directly. Future work could test this by investigating whether viewpoint affects performance in the visual recognition of origami figures. In addition, for our video stimuli we elected to use the overhead view, rather than the angled views that would be more characteristic of live demonstrations, in order to better control for visual differences across the four conditions. It is possible that an angled view would give learners a stronger cue to the differences in hand configurations, as most of our participants did not notice any change in the hands between conditions. Again, this possibility could be tested in future studies.

Our results also show that participants were no more efficient at completing the task when observing the folding demonstration by the congruent-handed teacher compared to the incongruent-handed teacher. This contradicts previous studies by [27,67] which both showed improved learning with congruent handedness. Our study is, instead, consistent with the results of [58] that handedness congruence had no effect on performance in a tool action prediction task. Our findings are also in line with [60], who suggest that handedness does not affect object representations. Further support for this conclusion comes from the analysis of the asymmetry of the figures produced. This indicated that participants preferred to fold using the hand configuration demonstrated by the teacher whether or not the teacher had the same hand preference as them. We do not know if participants achieved matching symmetry in origami figures by imitating the teacher's hand configuration itself, or by imitating the direction of folding only. Future work could test this directly by video-recording the participants to see exactly how their hands move during the task. Follow-up studies should also recruit more left-handed and mixed-handed participants in order to test a balanced sample of groups with a range of hand dominances.

We interpret our findings as indicative that learning by imitation involves internalising observed motor representations in order to execute them, as [78] found in their study of brain activation during imitation of Mousterian stone knapping actions. An earlier study [73], presented origami folding instructions as a sequence of written instructions with pictures of the folding shown from the Beside viewpoint. They found that when people learn to fold origami from such pictorial-verbal instructions, they creatively interpret the instructions by reformulating them into spatial terms and adding information. Hand configurations were not specified in this study, but the direction of folding was (e.g., "fold the tip diagonally to the left"). They measured folding success by subjective ratings of figure similarity, and found that people who read fewer instructions aloud were more successful [73]. This result suggests that internalising visual representations is a key process in action imitation [78], and that verbal instructions can actually hinder learning. During learning by imitation alone, as in the present study, no explicit teaching occurred and there were no verbal instructions, so the learner was not instructed to use a particular hand configuration. In this case, the internalised motor representations allowed the learner to use any hand configuration. Our results suggest that these motor representations are hand-independent, consistent with what has previously been found by [60]. This enabled learners to flexibly match their hand preference to that used by the demonstrator.

Our finding that incongruent handedness between demonstrator and learner did not affect performance, was thus, we argue, because learners effectively preferred to remove the handedness incongruence by adopting the hand configuration of the demonstrator. Support for this claim comes from our finding that learners preferentially produced origami figures that matched the direction of asymmetry demonstrated by incongruent-handed teachers. The conflicting results found in previous studies could be explained by participants finding it harder to reproduce stimuli created by incongruent-handed teachers, but being able to compensate for this by changing their handedness to be congruent with that of the teacher.

If, as we have suggested, the preferred configuration of hand use is flexible when people learn a complex bimanual task, this indicates that social learning by imitation can be a powerful determinant of handedness. Despite having established hand preferences, our adult participants behaved the same as the infants in [68] who imitated the hand configuration of the demonstrator. We speculate that an ability to facilitate learning by rapidly and temporarily adopting a demonstrator's hand configuration evolved due to the pressure for efficient social learning of complex tasks. Similarly, [79] proposed that hominins were the only species who engaged in sufficiently complex manual tasks to trigger the expression of a handedness bias.

Our evolutionary scenario is as follows: prior to the evolution of complex tool manufacture, hand preferences were evenly distributed among human populations, as is the case in other species of living apes, which we take to reflect the ancestral condition. Thus, some groups would have had a left-hand bias, some groups a right-hand bias, and other groups no bias. During social learning of simple skills such as many foraging tasks like picking fruit or pounding nuts, there was no need to conform to any particular hand configuration. This was because the tasks were simple enough to allow easy mental transformations so that each individual could use their preferred hand. This situation still exists among non-human apes. We propose that human imitation of manual skills began to require congruent handedness only once a certain level of tool complexity was reached. At this stage, learners found it beneficial to flexibly adopt the hand configuration of their teachers, in order to minimise the difficulties of mental transformation for such tasks.

Importantly, if, as we have argued, learners can efficiently imitate their teacher's hand preference, then we would have no reason to expect a drive for dominance of any particular hand preference. Thus, our species-level directional bias towards right-handedness still needs to be explained. We suggest that a combination of, first, functional brain laterality and, second, task expertise effects could be the evolutionary driver for the dominance of right-handedness in *Homo sapiens*.

First, regarding brain laterality, the two brain hemispheres have specialised, complementary roles in controlling the contralateral hand and arm [2]. Evidence from vertebrate lateralization indicates an evolutionarily ancient hemispheric specialisation was already in place before the hominin lineage emerged [5,79,80]. We believe the persistence of 10% left-handers in humans today is due to both a minority advantage in combat (Fighting Hypothesis) [20] and to atypical functional brain lateralization patterns which occur naturally in the population [81].

Second, this hemispheric specialisation could be critical when undertaking a highly complex bimanual task such as stone tool-making. Specifically, the brain's motor control specialisations mean that typically the dominant (right) arm controls movement direction and shape, while the non-dominant (left) arm maintains a stable position [2,82,83]. During direct, hand-held percussion for stone tool-making, these are precisely the bimanual complementary roles required of the two hands [13,17]. The non-dominant hand firmly holds the stone core at an appropriate angle while the dominant hand strikes the core with a fast, accurate, aimed motion. In this task, the hand role differentiation is extreme. In contrast, our origami task required quite similar and sometimes overlapping roles for the two hands. It is likely that the behavioural asymmetries were not salient in this task, as evidenced by the fact that most participants did not notice the change in conditions. Furthermore, the differentiation in difficulty between the motor skill required to, for example, hold the paper flat on the table (typical for the non-dominant hand) while folding one corner (typical for the dominant hand) was much less than for stone tool-making. In addition, task learning is much faster, in part because there is ongoing visual feedback and the error tolerances are much wider than in stone knapping. We suggest that the relative difficulty of the movements required by the two hands during origami-making is likely not enough to cause differential activation of the brain's hemispheric specialisations, as previously proposed by [7]. In contrast, given that the acquisition of stone tool-making skills was both essential for survival and very difficult, we speculate that the most efficient learning strategy used the brain's existing hemispheric specialisations. For example, archaeological evidence from Neanderthals living 80,000 years ago, at the site of Buhlen in Germany,

suggests that they experienced childhood pressure to become right-handed through social learning of extremely difficult "Keilmesser" stone tool manufacturing techniques [84].

We thus argue that the level and nature of manual expertise required to master a task may be crucial in determining whether the handedness of a demonstrator influences task performance. In experimental psychology, origami-folding and knot-tying are considered complex tasks. Indeed, 23% of origami figures produced by our participants were poorly formed, indicating that the task was challenging. However, humans can learn to reproduce a particular knot or origami figure in about an hour. For this type of task, flexible imitation of incongruent handers may be sufficient to support successful performance. In contrast, proficient Oldowan stone knapping needs weeks of practice and most stone tool types take years to master [85–87]. While the basic gestures and concepts of stone tool production can be learned in an hour with active teaching ([42], N. Uomini pers. obs.), learning this task by imitation alone is much less efficient [28]. The non-verbal social learning of stone knapping skills requires close attention to fine details of hand postures, stone core geometry, selection of where to strike, and striking direction and speed. It is likely that these essential details are easier to perceive and to reproduce when watching a congruent hander. This prediction could be tested by measuring the frequency of handedness congruence between masters and their apprentices learning modern-day crafts such as stone carving (we are not aware of any published data).

Our experimental findings are consistent with the evolution of concordant hand preferences due to social learning by imitation of complex bimanual skills such as stone tool manufacture. In our study, learners flexibly adapted their hand configuration to match that of the demonstrator, resulting in no decrease of performance for the incongruent-handedness conditions or from viewpoints requiring mental transformation. This result partly supports the Apprenticeship Complexity Theory, at least for origami imitation. We suggest that our origami task was not sufficiently challenging to cause obligate use of the brain's preferred right/left hand specialisations. As a consequence, using an incongruent hand configuration did not disrupt learning. Future work should focus on acquiring difficult craft skills that require extended learning periods and precise motor control. This would allow a check of whether there is a cost to incongruent-handedness and viewpoint on more complex and ecologically valid tasks. More work is also needed to establish how objects are represented during tool creation tasks that require mental transformation, particularly for difficult sequential bimanual actions such as stone knapping.

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Effects of Emotional Valence on Hemispheric Asymmetries in Response Inhibition

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Abstract: Hemispheric asymmetries are a major organizational principle in human emotion processing, but their interaction with prefrontal control processes is not well understood. To this end, we determined whether hemispheric differences in response inhibition depend on the emotional valence of the stimulus being inhibited. Participants completed a lateralised Go/Nogo task, in which Nogo stimuli were neutral or emotional (either positive or negative) images, while Go stimuli were scrambled versions of the same pictures. We recorded the N2 and P3 event-related potential (ERP) components, two common electrophysiological measures of response inhibition processes. Behaviourally, participants were more accurate in withholding responses to emotional than to neutral stimuli. Electrophysiologically, Nogo-P3 responses were greater for emotional than for neutral stimuli, an effect driven primarily by an enhanced response to positive images. Hemispheric asymmetries were also observed, with greater Nogo-P3 following left versus right visual field stimuli. However, the visual field effect did not interact with emotion. We therefore find no evidence that emotion-related asymmetries affect response inhibition processes.

1. Introduction

Hemispheric asymmetries, i.e., functional and structural differences between the left and the right brain hemisphere, affect behaviour and cognition in all vertebrate classes [1–5]. One of the least well understood asymmetric systems is the one that supports emotion processing [6–8]. First reports that the hemispheres might differ in emotional processing are often attributed to Hughlings-Jackson [9], who noted that aphasic patients (with left hemisphere lesions) often had preserved emotional language. Later patient studies showed that left hemisphere lesions are more commonly associated with depressed or catastrophic reactions, whereas right hemisphere lesions are more likely to trigger inappropriate euphoria [10]. These early studies have since been complemented by research using a range of methodologies, including the testing of patients with unilateral lesions [11], asymmetric EEG activity [12,13], visual half-field and dichotic listening techniques [14,15], and functional imaging [16,17], to show that the hemispheres differ in how they process emotional information, and in the emotional responses they generate.

Two asymmetric systems have been proposed to account for hemispheric differences in emotional processing. The first (described by the "right-hemisphere hypothesis") suggests that the right hemisphere is specialised for the processing of all emotions. The other (described by the "valence" hypothesis) proposes lateralisation as a function of valence, with the left specialised for positive (or approach-related) emotions, and the right for negative (or withdrawal-related) emotions. For decades, researchers have tried to determine which hypothesis better explains emotional asymmetry [18–21]. Rather than pitting these hypotheses against each other, recent research has been based on the premise that both might be correct, and has instead focused on the situations in which emotion-based or valence-based processing might arise [22–24].

There is an emerging consensus that the right hemisphere hypothesis applies largely to emotion perception, regardless of modality, and reflects lateralisation of the right posterior cortex. Such asymmetries may depend largely on the emotional nature of the stimulus, and emerge in a bottom-up fashion. Valence-based processing is more closely tied to emotional experience or expression, and appears to reflect asymmetries in the dorsolateral prefrontal cortex (dlPFC). This asymmetry has been linked to both emotional valence (positive/negative) [25,26] and motivational direction (approach/withdrawal) [27], although valence-related asymmetries are sometimes also reported in perception. Schepman and colleagues have argued that valence effects should emerge when tasks include top-down processing (for example, related to expectancies) that engage frontal emotional processing networks [22,23]. Grimshaw and Carmel [28] have further argued that prefrontal asymmetries in emotional processing may not reflect emotional experience, but the top-down control of emotional information, with the left hemisphere specialised for the inhibition or control of negative information, and the right for inhibition or control of positive emotion. Their rationale is based largely on the fact that prefrontal mechanisms (and particularly those localised to lateral PFC) play a key role in both cognitive and/or attentional control on the one hand, and on emotion regulation on the other [29]. Like Schepman, they argue that valence-based effects are most likely to be observed when tasks involve the use of top-down control mechanisms.

As yet, few experimental studies have directly assessed asymmetries in inhibition or control processes, and so it is unknown whether such control processes show emotional asymmetries, and if so, whether they are in line with either a right hemisphere or valence-based explanation. To address this gap in the literature, we used a lateralized version of the classic Go/Nogo Task in which we manipulated the emotional nature of the stimuli. In this type of task, two categories of stimuli are presented to the subjects: the more frequent Go stimuli to which subjects are asked to respond, e.g., by button press, and the less frequent Nogo stimuli to which participants are asked to withhold responses [30,31]. When Nogo stimuli are rare, participants adopt a prepotent response that must be inhibited when the imperative Nogo signal appears. Poor inhibitory control is indicated by errors of commission on Nogo trials. These behavioural measures are complemented by two neural measures revealed by event-related potentials (ERPs): the Nogo-N2 and the Nogo-P3 [32,33]. These components are assumed to reflect different sub-processes of the response inhibition. The earlier of the two components is the Nogo-N2, a negative component that is thought to reflect either pre-motor inhibition [32] or monitoring of response conflict [34]. The later of the two components is the Nogo-P3, a positive deflection that has been linked to the evaluation of successful inhibition, given that it peaks too late after the response to directly reflect inhibition processes [35–38].

In our version of the task, Go and Nogo stimuli were presented tachistoscopically in the left (LVF) or right visual field (RVF). This ensures that initial visual and emotional processing of the stimulus is lateralized to the contralateral hemisphere [39–42]. To examine emotional processing, we used pictures of emotional and neutral scenes as Nogo stimuli and unidentifiable scrambled versions of the same pictures as Go stimuli. Participants were asked to press a button in response to scrambled stimuli and to refrain from responding whenever intact stimuli were presented. Half of the (intact) Nogo stimuli had a neutral valence, while the other half showed emotional pictures, taken from the International Affective Picture System (IAPS) [43]. We manipulated the valence of the emotional images between

subjects; for half of the participants, emotional pictures were negative (mutilations), and for the other half positive (erotic scenes). In this task, the emotional content of the Nogo stimuli was not the imperative aspect for the subjects' responses, but instead served merely as a task-irrelevant distraction; that is, participants withheld their response whenever an intact image appeared, regardless of its content. This is an important aspect of the design, as it allows us to assess the effects of the emotional nature of the stimulus to be controlled, independent of any effects of emotion on the generation or inhibition of the response itself (for example, positive and negative stimuli are associated with approach and avoidance responses, respectively).

To our knowledge, no studies have assessed the effects of lateralised emotional stimuli on Go-Nogo task performance. However, a number of studies have manipulated the emotional nature of imperative stimuli in central vision, while still keeping emotion itself incidental to the task [44–49]. The effect of emotion on behavioural measures is mixed across these studies, with some showing more errors on Nogo trials in emotional contexts [50], but most showing no behavioural effects [44,45,49]. However, more consistent findings are reported in the ERP measures. Most studies show no effect of emotion on the Nogo-N2 [44,45,49,51], although some have reported an attenuation of the N2 for emotional relative to neutral stimuli [46,47]. In contrast, emotional stimuli consistently enhance the Nogo-P3 [46,47,51], with effects sometimes greater for positive than for negative stimuli [44,45]. This enhanced Nogo-P3 is typically interpreted as a more effortful or less efficient inhibitory control in emotional contexts [44,45,48]. Our question concerns the effects of lateralisation of the emotional stimuli on response inhibition processes. Importantly, emotional information can be extracted from peripherally-presented complex scenes, even with very brief stimulus presentations [52,53]. We therefore determined whether emotion affected either behavioural or electrophysiological measures of the response inhibition. Based on the right hemisphere hypothesis, we would expect emotion effects to be stronger for imperative stimuli presented in the LVF. However, because response inhibition depends on top-down control processes, it might be sensitive to emotional valence [22,23,28]. If so, we predict stronger effects of negative stimuli in the LVF, and stronger effects of positive stimuli in the RVF.

It is also possible that our experiment will show that response inhibition processes are affected by asymmetries in stimulus processing that are not specific to emotion. Using verbal stimuli, we previously showed that magnitudes of both Nogo-N2 and Nogo-P3 were affected by hemispheric asymmetries in stimulus processing [54]. When lateralised stimuli were presented tachistoscopically, the Nogo-N2 and related delta frequency band power were stronger when response inhibition was applied to stimuli presented in the LVF, implying greater response conflict generated by stimuli presented to the non-dominant hemisphere. A similar, albeit weaker, effect also reached significance for the P3 [54].

2. Materials and Methods

2.1. Subjects

Forty-two healthy adults (22 women, 20 men) participated in the present study. Subjects were recruited at the Institute of Cognitive Neuroscience, Department of Biopsychology at Ruhr-University Bochum by public advertisement and received course credit or monetary reimbursement (20 €) for participation. The mean age was 24.5 ± 4.3 years (range 18 to 34 years). Exclusion criteria were current or past psychiatric or neurological disorders, current psychotropic medication, colour blindness, mixed- or left-handedness, and ages younger than 18 or older than 35 years. Furthermore, subjects were required to be heterosexual due to the nature of the stimulus material (see below). All subjects were right-handed (mean laterality quotient 86.5 ± 16.1; range 37.5–100), as assessed with the Edinburgh Handedness Inventory [55], had normal or corrected-to normal vision, and were naïve to the study's intent.

Written informed consent was obtained from all subjects prior to participation. The study conforms to the Declaration of Helsinki and has received ethical clearance by the Ethics Board of Faculty of Psychology at Ruhr-University Bochum, Germany.

2.2. Experimental Task

The task was a tachistoscopic adaptation of a classic Go/Nogo task [30,54] that included emotional and neutral pictures as imperative stimuli. Pictures were taken from the International Affective Picture System (IAPS) [43]. Stimulus presentation and timing was controlled by Presentation software (Neurobehavioural Systems, Inc., Berkeley, CA, USA). Figure 1 provides a schematic illustration of the sequence and time course of stimulus presentation. At the beginning of each trial, a fixation cross was presented in the centre of the screen for 1000 ms. Subsequently an imperative stimulus (emotional or neutral; intact or scrambled picture) was presented in the left (LVF) or right visual field (RVF), with the inner edge of the stimulus located at 3 degrees of visual angle from the fixation cross. Stimuli had a width of 4 degrees of visual angle and a height of 3.5 degrees of visual angle. Stimuli were presented for 185 ms. Stimulus presentation was followed by the fixation cross for 525 ms. Trials were separated by inter-trial intervals jittered between 750 ms and 950 ms. There was a short break in fixation between the end of the inter-trial interval and the beginning of the next trial in which the fixation cross disappeared and reappeared again.
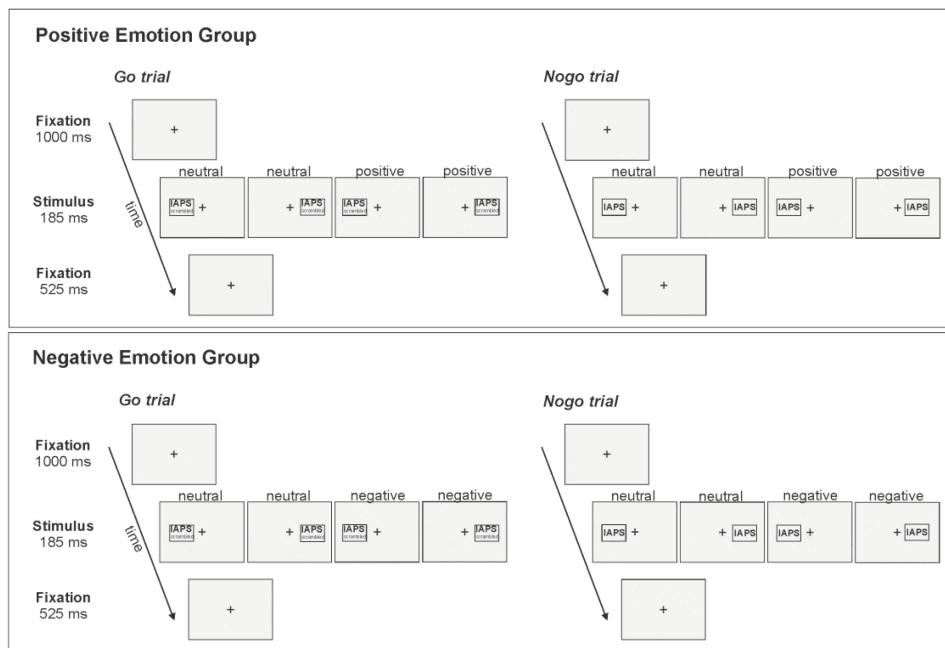
Figure 1. Schematic illustration of the sequence and time course of stimulus presentation. Half of the stimuli for each condition (Go/Nogo) and each valence (positive/neutral or negative/neutral) were presented in the left, the other half in the right visual field. Nogo stimuli were intact IAPS pictures; Go stimuli were scrambled versions of the same IAPS pictures. Trials were separated by inter-trial intervals jittered between 750 ms and 950 ms.

Stimuli were presented on a 17 inch CRT monitor in a dimly lit room, with subjects seated at a viewing distance of 57 cm. Note that at this viewing distance 1 cm on the screen equals 1° of visual

angle. Head position was stabilized with a chin rest. Subjects were instructed to keep their gaze on the central fixation cross throughout the entire task, and to press the arrow up key on a key board as fast and as accurately as possible whenever a Go stimulus was presented. Participants responded with their right hand on all trials. Only the right hand was used to respond in order to maximize trial numbers in critical conditions. Although contralateral movement-related potentials can arise prior to the imperative stimulus and potentially affect nogo-ERPs, these do not interact with stimulus lateralization, and should therefore have equivalent effects on left and right visual field stimuli [56,57]. Furthermore, subjects had to withhold their response whenever a Nogo stimulus was presented. Nogo stimuli were intact (i.e., non-scrambled) emotional or neutral pictures, while Go stimuli were scrambled images of the same emotional or neutral pictures (see Figure 1). For half of the subjects, emotional pictures had a negative valence (negative emotion group). There were 12 images in each category. Negative emotional pictures depicted bodily mutilation, injury, or dead bodies. For the other half of the participants, emotional pictures had a positive valence and depicted erotic (heterosexual) couples (positive emotions group). Neutral images all depicted people engaged in common activities. Comparison of normative ratings [8] revealed that positive images were rated as more pleasant and more arousing than neutral images, and negative images were rated as more unpleasant and more arousing than neutral images (all $p < 0.001$). Positive and negative images did not differ in arousal ($p = 0.13$). The same picture sets were used for male and female participants, as men and women rate these images similarly. There were 12 female and 10 male participants in the negative emotion group and 10 female and 10 male participants in the positive emotions group. There were no significant differences in gender composition between the two groups ($p = 0.77$).

In total, the task comprised 672 trials, with 29% (192) Nogo and 71% (480) Go trials. For each trial type (Go/Nogo), half of the stimuli were emotional (Go: 240; Nogo: 96) and half were neutral, and of those, half were presented in the LVF (Go: 120; Nogo: 48) and half in the RVF. Note, however, that because Go stimuli were scrambled images, they were emotional only in that they were derived from emotional images; they had no emotional meaning of their own. Stimulus presentation was randomized. Task completion took approximately 16 minutes. Accuracy (percentages of correct responses), false alarms (that is, responses on Nogo trials), misses (that is, non-responses on Go trials), and mean response times (RTs) for correct responses were assessed.

2.3. Electrophysiological Recordings

EEG was recorded from 64 channels using an actiCAP electrode system with Ag-AgCL electrodes and a standard BrainAmp amplifier, and the corresponding recording BrainVision Recorder software (Brain Products, Gilching, Germany) at a sampling rate of 1000 Hz. Electrodes were arranged according to the International 10-20 system (FCz, FP1, FP2, F7, F3, F4, F8, FC5, FC1, FC2, FC6, T7, C3, Cz, C4, T8, TP9, CP5, CP1, CP2, CP6, TP10, P7, P3, Pz, P4, P8, PO9, O1, Oz, O2, PO10, AF7, AF3, AF4, AF8, F5, F1, F2, F6, FT9, FT7, FC3, FC4, FT8, FT10, C5, C1, C2, C6, TP7, CP3, CPz, CP4, TP8, P5, P1, P2, P6, PO7, PO3, POz, PO4, PO8). Electrode FCz was used as a primary reference. Impedances were kept below 10 kΩ, mostly ranging from 5 to 10 kΩ. Initially, 44 subjects were tested, but in two subjects (1 male, 1 female), not enough electrodes could be recorded throughout the task due to technical problems. Data from these subjects were excluded from all analyses, rendering a sample of N = 42 for the study, as described above.

EEG data were processed off-line using BrainVision Analyzer 2 (Brain Products, Gilching, Germany). Raw data were first down-sampled to 500 Hz and filtered with 0.5 Hz low cutoff and 20 Hz high cutoff (48 dB/oct). The filtered data were visually inspected and all trials containing gross technical artefacts were rejected. Horizontal and vertical eye movements as well as pulse artefacts were then corrected using infomax independent component analysis (ICA), which was applied to the unepoched data. Epoched data were subjected to automatic artefact rejection applying the following rejection criteria: maximum voltage steps of more than 50 μV/ms, maximum value differences of 200 μV in 200 ms intervals, or activity below 0.1 μV. The overall number of trials rejected by this procedure was below 5% of all trials for each condition and channel.

Data analyses (peak and latency quantification) were performed after the calculation of current source density (CSD) of the signal in order to ensure reference-free evaluation [54]. CSD transformation replaces the potential at each electrode with the CSD, thereby eliminating the reference potential. The algorithm applies the spherical Laplace operator to the scalp distribution of the potential. Since this distribution is only known for electrodes that were actually used, spherical spline interpolation is applied to calculate a continuous potential distribution [58].

Data were segmented into 1200 ms epochs starting 200 ms before and ending 1000 ms after onset of Go or Nogo stimuli, respectively. Baseline correction was applied based on the 200 ms directly preceding the stimulus onset. Segments were averaged according to condition (Go/Nogo), emotionality (emotional/neutral), and side (LVF/RVF). Note that only trials with a correct Go or Nogo response were included. Electrodes used for amplitude and latency quantification were chosen based on the typical scalp topographies for the N2 and P3, as well as on careful visual inspection of grand-average ERPs in the present data set. N2 amplitude and latency were measured based on the maximum negative peak occurring in a time window from 190 to 390 ms after stimulus onset at electrode FCz. P3 amplitude and latency were measured based on the maximum positive peak occurring in a time window from 300 to 600 ms after stimulus onset at electrode Pz.

2.4. Statistical Analysis

Statistical analyses were performed using IBM SPSS 23 software (IBM Corporation, Armonk, New York, USA). With regard to behavioural performance and accuracy (percentage of correct responses, that is, button presses in the Go condition, and non-responses in the Nogo condition); RTs for correct responses in the Go condition were also analyzed. The significance level was set to $p < 0.05$. Effect sizes are provided as the proportion of variance accounted for (partial η^2). Mean amplitudes are provided together with the standard error of the mean (SEM as measure of variability).

3. Results

3.1. Behavioural Data

Overall, participants were significantly more accurate when responding to Go stimuli (98.10%) as compared to when withholding their responses to Nogo stimuli (87.75%) ($t_{(41)} = 8.39$; $p < 0.001$). Subsequent analyses were carried out only for Nogo trials (see Figure 2), as the factor emotionality could only be interpreted for these trials because stimuli were scrambled on Go trials. Also, as indicated by very high accuracy on Go trials (>98%), there was possibly a ceiling effect in the Go condition.
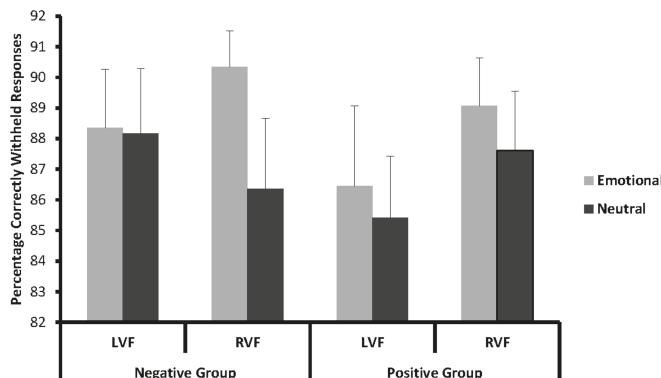

Figure 2. Mean percentage of correctly withheld responses in the Nogo condition for emotional and neutral stimuli in left (LVF) and right (RVF) visual field in the positive and negative emotion groups.

Accuracy data for the Nogo condition was analysed with a $2 \times 2 \times 2$ repeated measures ANOVA with the within-subjects factors side (LVF/RVF) and emotionality (emotional/neutral), and the between-subjects factor valence (positive/negative). Here, the main effect of emotionality showed a strong trend towards significance ($F_{(1,40)} = 3.89$; $p = 0.056$; partial $\eta^2 = 0.09$), indicating that participants were more accurate in withholding their responses to emotional (88.55%), as compared to neutral, pictures (86.89%). All other effects failed to reach significance (all $p > 0.13$).

3.2. EEG Data

3.2.1. N2

N2 Amplitude

For N2 amplitude (see Figure 3), analysis revealed a more negative N2 in the Nogo ($-13.82\ \mu V \pm 1.28$) compared to the Go condition ($-9.25\ \mu V \pm 0.30$), as was to be expected for a Go/Nogo task ($t_{(41)} = 4.47$; $p < 0.001$). Subsequent analyses of emotion effects were carried out within the Nogo trials. N2 amplitudes in the Nogo condition were analysed with a $2 \times 2 \times 2$ repeated measures ANOVA with the within-subjects factors side (LVF/RVF) and emotionality (emotional/neutral), and the between-subjects factor valence (positive/negative). In this analysis, all effects failed to reach significance (all $p > 0.20$).
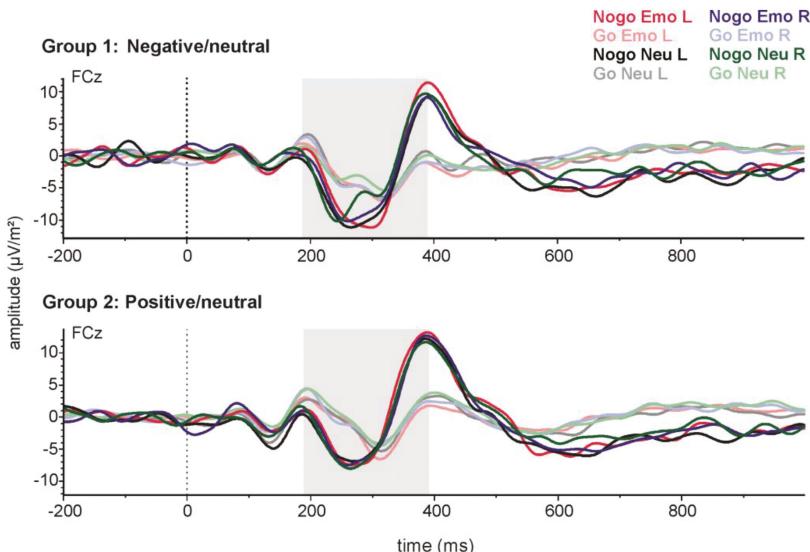
Figure 3. Stimulus-locked grand-average event-related potentials (ERPs) for emotional and neutral Go and Nogo stimuli presented on the left (**L**) or right (**R**) side at electrode FCz. The upper panel shows data from the negative emotion group and the lower panel data from the positive emotion group.

N2 Latency

With regard to latency, the N2 emerged earlier in the Nogo (291.29 ms $\pm$ 5.12) than in the Go condition (297.28 ms $\pm$ 5.84) ($t_{(41)} = 3.44$; $p < 0.01$). Subsequent analyses of emotion effects were carried out within the Nogo trials. N2 latencies in the Nogo condition were analysed with a $2 \times 2 \times 2$ repeated measures ANOVA with the within-subjects factors side (LVF/RVF), and emotionality (emotional/neutral) and the between-subjects factor valence (positive/negative). In this analysis, all effects failed to reach significance (all $p > 0.18$).

3.2.2. P3

P3 Amplitude

For P3 amplitude (see Figure 4), greater amplitudes were observed in the Go (17.27 µV ± 1.64) as compared to the Nogo condition (12.43 µV ± 1.60) ($t_{(41)}$ = −5.01; $p < 0.001$). Subsequent analyses were carried out only for Nogo trials, to evaluate effects of emotion. P3 amplitudes in the Nogo condition were analysed with a 2 × 2 × 2 repeated measures ANOVA with the within-subjects factors side (LVF/RVF) and emotionality (emotional/neutral), and the between-subjects factor valence (positive/negative). Here, the main side effect reached significance ($F_{(1,40)}$ = 4.12; $p < 0.05$; partial η^2 = 0.09), indicating a more positive P3 after stimulus presentation in the LVF (13.31 ± 1.55) as compared to the RVF (11.71 ± 1.53). Moreover, the main effect of emotionality ($F_{(1,40)}$ = 16.32; $p < 0.001$; partial η^2 = 0.29) reached significance, indicating that emotional stimuli lead to a higher P3 (14.01 ± 1.65) than neutral stimuli (11.01 ± 1.41). This effect of emotionality on P3 amplitude was modulated by stimulus valence, as indicated by a significant valence × emotionality interaction ($F_{(1,40)}$ = 11.28; $p < 0.01$; partial η^2 = 0.22). Post-hoc tests revealed no difference between emotional and neutral stimuli in the group of subjects who saw negative emotional images ($p = 0.65$). However, for those who saw positive emotional images, P3 amplitude was greater for emotional stimuli (16.86 ± 2.39) than for neutral stimuli (11.37 ± 2.07) ($p < 0.001$). Importantly for our hypotheses, the effects of side and emotion did not interact.
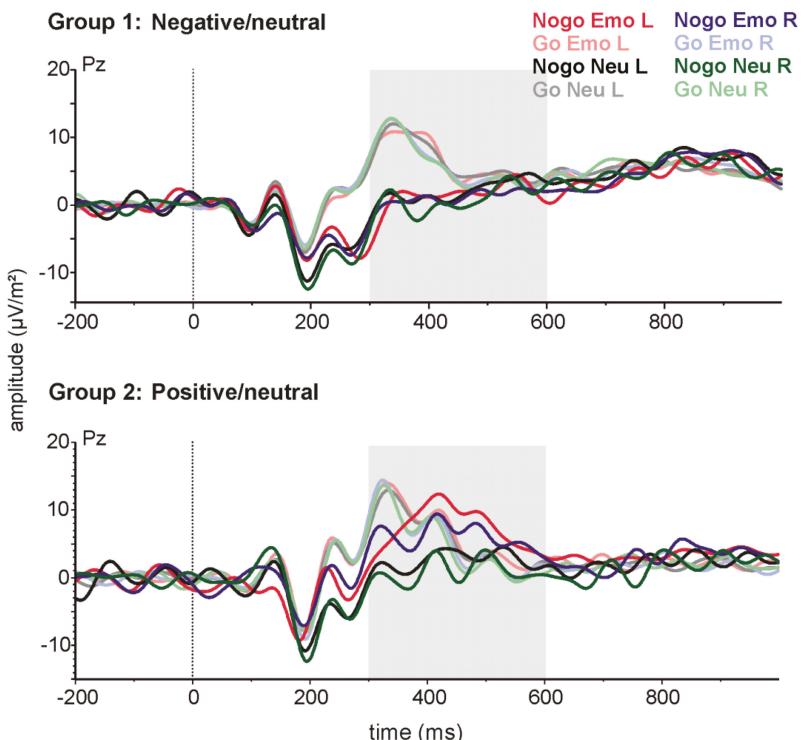

Figure 4. Stimulus-locked grand-average ERPs for emotional and neutral Go and Nogo stimuli presented on the left (**L**) or right (**R**) side at electrode Pz. The upper panel shows data from the negative emotion group and the lower panel data from the positive emotions group.

P3 Latency

For latency, the P3 emerged earlier in the Go (372.53 ms $\pm$ 9.00) as compared to the Nogo condition (404.61 ms $\pm$ 8.41) ($t_{(41)} = -2.84$; $p < 0.01$). Subsequent analyses were carried out only for Nogo trials, to evaluate emotion-related effects. P3 latencies in the Nogo condition were analysed with a $2 \times 2 \times 2$ repeated measures ANOVA with the within-subjects factors side (LVF/RVF) and emotionality (emotional/neutral), and the between-subjects factor valence (positive/negative). In this analysis, all effects failed to reach significance (all $p > 0.22$).

4. Discussion

Functional hemispheric asymmetries are one of the core organizational principles underlying many cognitive functions in the human brain, including emotion processing [6–8], but their interaction with prefrontal functions, e.g., executive control, is not well understood. The present study was designed to elucidate the neurophysiological basis of this relationship by recording N2 and P3 ERP components during a divided visual field Go/Nogo task with scrambled (Go) or intact (Nogo) pictures with emotional or neutral content that was incidental to the task. Based on right-hemisphere dominance for emotional processing, we would expect emotion effects to be stronger for imperative stimuli presented in the left visual field than in the right. Alternatively, valence-based models lead to the prediction of stronger effects of negative stimuli in the left visual field, and stronger effects of positive stimuli in the right visual field.

We observed expected differences between Go and Nogo trials in the accuracy data, indicating that our task effectively elicited response conflict and response inhibition [30,32]. Additionally, accuracy on both Go and Nogo trials was high (98% and 88%, respectively), showing that participants were able to distinguish intact from scrambled images even though they were presented only briefly in the periphery. High accuracy also meant that sufficient trials were available for analysis of N2 and P3 responses.

We observed effects of emotion that are broadly consistent with those reported in other studies that have manipulated the emotion of imperative stimuli in Go/Nogo tasks. Behaviourally, emotional stimuli were associated with marginally better performance on Nogo trials, consistent with a "freezing" effect of emotion, perhaps driven by attentional prioritisation of emotional stimulus processing [50]. Turning to the ERP measures, we found no effect of emotion on the Nogo-N2, consistent with other studies [44,45]. Collectively, these studies suggest that conflict monitoring processes are not sensitive to emotion. Also consistent with other studies, we observed that Nogo-P3 was enhanced in the presence of positive emotional stimuli [44,45]. The most common interpretation of emotional potentiation of the P3 is that sustained attentional engagement with positive emotional stimuli affects the execution or evaluation of inhibitory processing.

Emotion effects were observed even though stimuli were only briefly presented in peripheral locations, meaning that our experimental paradigm should be sensitive to hemispheric differences in emotional processing if they exist. Nonetheless, neither emotionality nor valence interacted with the visual field on any measure. Our findings therefore do not support either a right hemisphere or valence-based interpretation of emotional processing in the context of response inhibition. This complete lack of asymmetry in emotional processing was unexpected, given robust findings of emotional asymmetry on perceptual processing [6–8]. It is possible that asymmetries were not observed because emotion was incidental to the task, which required participants only to distinguish intact from scrambled stimuli. Many studies that have produced evidence for emotional asymmetries use explicit emotional identification or judgments [6–8], or involve tasks in which emotion is relevant to response [53]. We purposefully made emotion itself task-irrelevant so that we could observe the effects of emotion on control processes independent of any effects on motor execution (e.g., approach and avoidance tendencies that might have been activated by positive and negative stimuli, respectively) [7]. Further studies on the task dimensions that influence emotional asymmetries would be necessary to evaluate this hypothesis.

Although emotion did not interact with visual field, hemispheric differences were still observed, in that the Nogo-P3 was enhanced for the left visual field relative to right visual field stimuli. This finding is partially consistent with our previous study in which both Nogo-N2 and Nogo-P3 were enhanced for verbal stimuli presented in the left visual field [54]. The fact that similar effects have been observed for both verbal and pictorial stimuli suggests that this effect might not be stimulus-specific, and might instead reflect asymmetry in the application of inhibitory control more broadly. Notably, response inhibition mechanisms have been localised to the right hemisphere [59], and specifically to right inferofrontal cortex [60,61].

The use of lateralised stimuli in Go/Nogo task (especially with ERP measures) is not common in the literature, and further studies are needed using a broader range of stimulus modalities before drawing strong conclusions about how hemispheric asymmetries in stimulus processing affect response inhibition processes. For example, in order to render findings on emotional lateralisation in inhibition-related ERPs more comparable to those found for verbal stimuli [54], it would be an interesting follow-up study to use the emotional content of the stimuli not as a distracting task-irrelevant feature but as the Go/Nogo indicator. Here, emotional stimuli with reduced stimulus complexity should be used. While IAPS pictures are widely used and thus constitute a well-validated stimulus set in emotion processing research, they typically are presented for longer intervals than in the present tachistoscopic experiment when participants must make judgments of valence based on stimulus content. This brief presentation may lead to reduced accuracy when using them as Go/Nogo stimuli. Less complex emotional stimuli, e.g., emotional faces, might be better suited for such tasks (see [62] for a tachistoscopic EEG study that successfully used emotional faces as imperative stimuli). Moreover, in the present study, although it was task-irrelevant, emotion was a stimulus property of the Go/Nogo stimuli. To further disentangle stimulus- and distractor-related effects, it would be informative to use a paradigm with central presentation of non-verbal Go/Nogo stimuli without emotional content (e.g., red and green coloured squares). The distractors (IAPS pictures or emotional faces) would then be presented in the LVF or RVF. By comparing distractor trials to trials without distractors, a more fine-tuned assessment of the effects of left- or right-hemispheric stimulus processing on prefrontal inhibition processes would be possible. Moreover, we used a between-subjects design in the present study, with one group of subjects tested with positive and neutral stimuli and the other group with negative and neutral stimuli. A within-subjects design in which each participant is tested in both conditions would have greater statistical power and should be used in future studies addressing ERP-correlates of emotional lateralization. Additionally, the Go/Nogo task is only one of several paradigms that allow investigation of prefrontally mediated inhibition processes. Other paradigms targeting prefrontal functions that involve inhibition, e.g., the stop-signal task [63], stop-change task [64], or the task-switching paradigm [65] could yield further evidence for a relationship between emotional lateralisation and prefrontal inhibition.

5. Conclusions

Emotional asymmetries are commonly observed in perception and in emotional expression and experience. We manipulated the emotional nature of the Nogo stimulus in order to distinguish between right hemisphere and valence-based explanations of emotional asymmetry. P3 amplitudes were enhanced when stimuli were presented in the left visual field, suggesting that evaluative processes involved in response inhibition are sensitive to hemispheric differences in stimulus processing. P3 amplitudes were also potentiated by positive images, showing that the evaluative processes reflected by the P3 are sensitive to emotional content. However, contrary to both right-hemisphere and valence models, we found no evidence that emotional effects depended on the hemisphere to which those stimuli were presented.

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Asymmetry for Symmetry: Right-Hemispheric Superiority in Bi-Dimensional Symmetry Perception

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Abstract: A right-hemispheric superiority has been shown for spatial symmetry perception with mono-dimensional stimuli (e.g., bisected lines). Nevertheless, the cerebral imbalance for bi-dimensional stimuli is still controversial, and the aim of the present study is to investigate this issue. Healthy participants and a split-brain patient (D.D.C.) were tested in a divided visual field paradigm, in which a square shape was presented either in the left or right visual field and they were asked to judge whether a dot was placed exactly in the center of the square or off-center, by using the left/right hand in two separate sessions. The performance of healthy participants was better when the stimuli presented in the left visual field (LVF) were on-center rather than off-center. The performance of D.D.C. was higher than chance only when on-center stimuli were presented in the LVF in the left hand session. Only in this condition did his accuracy not differ with respect to that of the control group, whereas in all of the other conditions, it was lower than the controls' accuracy. We conclude that the right-hemispheric advantage already shown for mono-dimensional stimuli can be extended also to bi-dimensional configurations, confirming the right-hemispheric superiority for spatial symmetry perception.

Keywords: perceptual symmetry; cerebral hemispheres; split-brain patient; spatial processing; bi-dimensional stimuli

1. Introduction

Symmetry is easily detected by the visual system, and the way in which humans and other animals process visual symmetry is a central issue both in psychology and neuroscience. In fact, several models have been proposed in the attempt to explain how symmetry is detected and analyzed by the brain (e.g., [1–7]). Among the most acknowledged models, the perceptual rules proposed by Gestalt psychologists suggested that our preference for symmetric configurations ("symmetry bias") could be considered as a consequence of the perceptual preference for regularity and balance, compared to randomness and imbalance, by the human visual system. A debated point in this context concerns the possibility that such a regularity is extracted automatically, or it calls attentional processes into play (see [3] for a review). In support of the first point of view, it has been shown that patients with hemispatial neglect ("blind" for the left visual field as a consequence of a right-hemispheric lesion) show a preference for symmetrical arrangements in both visual fields, confirming that preattentive processes are responsible for figure-ground organization [8–10]. Other important results in this context come from those patients who have undergone surgical resection of callosal fibers, in the attempt to avoid the spread of epileptic foci between the two cerebral hemispheres, the so called "split-brain

patients" [11]. In a series of experiments carried out with a split-brain patient, Funnell, Corballis, and Gazzaniga [12] showed that the right disconnected hemisphere is superior to the left hemisphere in perceptual matching tasks with mirror-reversed stimuli. By presenting stimuli consisting of either color pictures of nameable objects, black-and-white line drawings, or abstract geometrical forms, the authors concluded that the left hemisphere is specialized in pattern recognition, whereas the right hemisphere is specialized in spatial processing (see also [13]). The split-brain patients' literature is strongly linked to that of perceptual symmetry, because a number of authors suggested that at least when talking about vertical symmetry perception, this mechanism is due to the symmetrical morphology of our brain. In other words, it has been suggested that the preferential activation of two homologue areas in the left and right hemispheres is the basis for the automatic detection of symmetry in the physical world. In this "callosal hypothesis", the detection of symmetry may be favored by the activity of two specular areas in the left and right halves of the brain, which are connected by means of the fibers constituting the corpus callosum [1,2,14–16]. In this frame, it has been suggested that both the left and the right hemispheres are capable of low-level perceptual processing, and that hemispheric asymmetries arise at later stages of visual processing, in associative areas representing the two sides of visual space [17]. It has to be highlighted, however, that contrasting models have been recently suggested [18,19]. The fact that higher order cortical areas are involved in the detection of symmetrical patterns was confirmed in a functional magnetic resonance imaging (fMRI) study [20]: it was shown that independently of the size and the geometrical configuration of the stimuli, as well as independently of the recruitment of attentional control, symmetrical arrangements activated associative visual areas, in particular V3, V4, V7, and lateral occipital areas (for similar results see also [21,22], for a review see [23]).

A right-hemispheric superiority for symmetry detection has been found in healthy participants by means of the divided visual field paradigm. Wilkinson and Halligan [24] presented lines which could be either perfectly divided into two halves (bisected lines) or divided into two asymmetrical segments (misbisected lines), either in the left visual field (LVF) or in the right visual field (RVF), and participants were asked to judge whether each stimulus was symmetrical or asymmetrical. The performance of participants was better in terms of both accuracy and response times when bisected lines, but not misbisected lines, were presented in the LVF, concluding in favor of a right-hemispheric preference for symmetry. Besides the advantage of the right hemisphere in geometrical processing, the authors explained their results also by referring to the differential hemispheric specialization for low and high spatial frequencies. In particular, they concluded that the cerebral asymmetry they found could also be due to the fact that the right hemisphere is more strictly linked than the left hemisphere to the magnocellular visual pathway. This pathway is more sensitive to the low spatial frequencies of the stimuli, which are processed faster than the high spatial frequencies [25], and this could be intended as a further reason for the faster detection of symmetry by the right hemisphere. According to the authors, the short stimulus exposure used in the divided visual field paradigm (tachistoscopic presentation), together with the lateralized presentation of the stimuli (eccentricity), may facilitate the low spatial frequency analysis and thus the right-hemispheric processing. The same right-hemispheric superiority for low spatial frequencies has been confirmed also by means of complex visual stimuli, both in healthy participants and in split-brain patients (e.g., [26–28]). Nevertheless, in the same study Wilkinson and Halligan [24] failed to find a cerebral imbalance in the detection of symmetry when "double axes stimuli" (squares in which a circle could be placed on-center or off-center) were presented, explaining this finding as possibly attributable to the fact that square bisection activates bilateral networks. In a following fMRI study, Wilkinson and Halligan [29] found that the cerebral substrate of the LVF advantage for detecting the presence/absence of symmetry in lines is the right anterior cingulate gyrus. Bertamini and Makin [30] found that symmetry processing induced occipital alpha Event Related Desynchronization (ERD) in the right hemisphere, confirming at the electrophysiological level the stronger right- than left-hemispheric involvement in symmetry detection (see also [31,32]). In another electroencephalographic (EEG) study, Makin and colleagues [33] also showed that the

Sustained Posterior Negativity is stronger for reflection than for rotation and translation, and that this is true when participants were explicitly required to detect the presence of regularity in the stimuli.

The right-hemispheric causal involvement in symmetry detection has been demonstrated by means of transcranial magnetic stimulation (TMS) studies: Bona and colleagues [34] applied TMS over the left or right lateral occipital cortex while participants were asked to distinguish symmetrical from asymmetrical random dot patterns. The authors found that both hemispheres are involved in the task, but that the right-hemispheric stimulation leads to a stronger disruption of symmetry detection with respect to the left-hemispheric stimulation (see also [35]). TMS was also exploited together with an adaptation paradigm, revealing that when applied between adaptation and test stimuli, TMS applied over the dorsolateral extrastriate cortex, but not over V1/V2, reduced adaptation effects to dot patterns [36].

Only in one study among those reviewed above, has the cerebral asymmetry for double axes figures been investigated [24], and the authors failed to find significant differences in the ability to detect symmetry between the left and right hemispheres. Starting from this evidence, the main aim of the present study is to further assess this issue both in healthy participants and in a split-brain patient. In particular, in a divided visual field paradigm, participants were presented with stimuli consisting of a square containing a dot placed either in its exact center or slightly off-center. They were asked to judge whether the circle was/was not placed exactly in the center of the square. We hypothesized that, as found in previous works with single axis stimuli, a LVF superiority may be observed also for double axes stimuli, starting from the several studies of a right-hemispheric superiority for symmetry detection. To this aim, we almost quadrupled the number of healthy participants with respect to the study of Wilkinson and Halligan (from 12 to 44 participants), we further shortened the tachistoscopic presentation time (from 170 ms to 150 ms), and we also tested a complete callosotomy patient in order to obtain data from each surgically disconnected hemisphere. Additionally, in contrast to Wilkinson and Halligan, we asked participants to take part in two separate sessions, differing from one another in the hand used to respond, in order to consider the hand of the response as a further within-subject factor in the statistical design. Specifically, we did not expect to find differences in healthy participants according to the hand used to respond (the use of one hand does not allow us to test the unilateral responses in the intact brain), but we expected that the use of one hand in the split-brain patient would ensure the contralateral hemispheric involvement (due to the contralateral organization of the motor pathways; e.g., [37–39]). By using these changes, we expected to confirm that the performance is better when symmetrical (on-center) stimuli were presented in the LVF, both in healthy participants and—importantly—in the patient. The right-hemispheric superiority for spatial processing in split-brain patients has already been shown [12,13], but it has not been investigated for symmetry detection. In a study involving two split-brain patients, including the patient tested here, Corballis et al. [40] found a right-hemispheric superiority when patients were asked to distinguish between canonical and mirror-reversed letters (F and R), concluding that this task depends on matching to an exemplar (the canonical oriented letter), for which the right-hemisphere is dominant (as opposed to the left-hemispheric superiority in letter naming). In contrast to that study, in the present study we presented geometrical shapes, for which no comparison with a "model" is required, and thus we aimed at investigating the pure hemispheric imbalance in symmetry detection.

2. Materials and Methods

2.1. Participants

D.D.C. is an Italian male patient suffering from medically intractable epilepsy, who has had the corpus callosum (CC) surgically sectioned in the attempt to avoid the spread of epileptic foci between the cerebral hemispheres. He underwent the first partial section of CC in 1994, when he was 18-year old, and the complete section in 1995; the anterior commissure was also resected (see Figure 1). D.D.C. was 38 years old at the time of the test, his postoperative IQ was 83, and his laterality quotient

was +40 (for more details, see [41]). The patient declared that he wrote with his left hand until he was 10, and then he was forced to use the right hand. D.D.C. is free from perceptual or motor impairments, and he has intact linguistic skills in both hemispheres [41]. He was tested at the Epilepsy Center of the Polytechnic University of Marche (Torrette of Ancona), during a pause between routine neurological examinations.
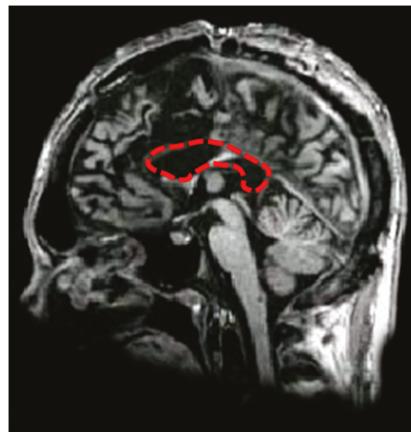

Figure 1. Midsagittal MRI of patients: the figure shows D.D.C.'s brain, showing the complete absence of callosal fibers (in the area delimited by the red-dashed line).

The control group was composed of 44 healthy volunteers (22 female; age: M = 25.5 ± 0.89). All were right-handed as assessed by the Edinburgh Handedness Inventory ([42]; M = 67.31 ± 2.28), had normal or corrected-to-normal vision, and none of them had any neurological or psychiatric history. These participants were tested at the Psychobiology Laboratory of the University of Chieti.

Informed consent was obtained from all participants prior to the experiment and the experimental procedures were conducted in accordance with the guidelines of the Declaration of Helsinki.

2.2. Stimuli

Stimuli consisted of a square designed by means of the software Microsoft PowerPoint 2007 (Microsoft Corp., Redmond, WA, USA). The square shape had black contours and it encompassed a white area measuring 7.6 × 7.6 cm (width × height; 4.2 × 4.2 degrees of visual angle, seen at a distance of 72 cm, on a screen with a resolution of 1280 × 768 pixels). The perimeter of the figure measured 0.3 mm in thickness. A black circular dot with a diameter measuring 0.9 cm was placed within the square. The dot was placed perfectly in the center of the square in half of the stimuli (On-Center condition), whereas in the other half it was placed 3 mm (0.18° of the visual angle) away from the center (Off-Center condition: 25% above, 25% below, 25% left, and 25% right, with respect to the center of the square).

2.3. Procedure

Each trial started with a vertical red line, measuring 2 mm in thickness and 16 cm in height, presented in the center of a white screen for 1000 ms. In the following 150 ms the red line remained visible, and a stimulus was presented in the center of the left or right half of the screen (the center of the square was placed at 3.7° of the visual angle from the center of the screen, with the innermost edge placed at 1.6° of the visual angle). Finally, a white screen was presented until the participant gave the response, and then the next trial started.

Each participant took part into two sessions, each composed of 96 trials. In each session, the stimulus was presented 48 times in the left visual field (LVF) and 48 times in the right visual field (RVF). For each visual field, 24 trials consisted of the square containing the dot in the center (On-Center condition), and 24 trials consisted in the square containing the dot moved away from the center (Off-Center condition, with 6 trials for each position: up, down, left, right; see Figure 2). The presentation order of the trials was randomized within and among participants.
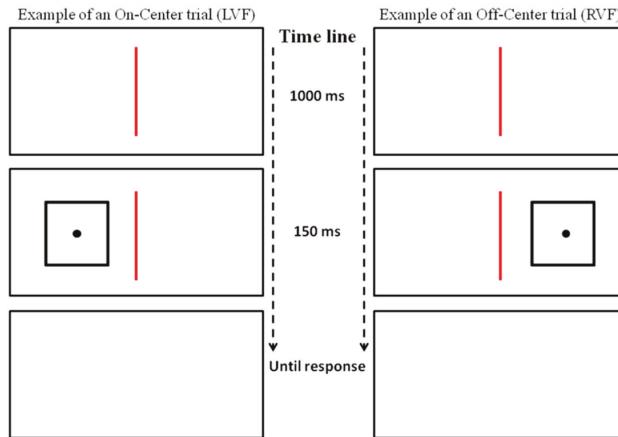

Figure 2. An example of (1) an On-Center trial, in which a symmetrical stimulus is presented in the Left Visual Field (LVF, **left panel**) and (2) an Off-Center trial, in which an asymmetrical stimulus is presented in the Right Visual Field (RVF, **right panel**).

Participants were tested in isolation. Before the beginning of the experiment, 4 trials were presented to allow the participants to become familiar with the task. They were asked to maintain their gaze in the center of the screen for the whole task, on the red line (a red line was used instead of the most conventional central fixation cross, in order to avoid possible cues concerning the exact center of the screen on the horizontal plane), and to evaluate in each trial the position of the dot inside the square. Specifically, they were asked to press one key when the dot was perfectly placed in the center of the square, and a different key when the dot was not in the center. They were also informed that the dot position could be a few millimeters away from the exact center of the square, in any direction (up, down, left, right). In the two experimental sessions, participants were required to carry out the task using either the left hand or the right hand, and the order of the two sessions was balanced among the participants. D.D.C. started with the right hand session.

The paradigm was controlled by means of E-Prime software (Psychology Software Tools Inc., Pittsburgh, PA, USA), and lasted about 15 min.

3. Results

3.1. Control Group

Data were analyzed by means of an analysis of variance (ANOVA), in which the Inverse Efficiency Score (IES) was the dependent variable. The IES was calculated by dividing the response times obtained in the correct responses by the proportion of correct responses in each condition. Reaction times were excluded when they were lower than 150 ms and higher than 1500 ms (3.11% of the trials). In a first ANOVA, Order of sessions (first session: Left hand, Right hand) and Sex of participants (Female, Male) were considered as between-subjects factors, and Hand of response (Left, Right), Visual field of presentation (Left, Right), and Condition (On-Center, Off-Center) were considered as within-subjects

factors. Neither Order of sessions, nor Sex of participants were significant, nor did they interact with the other factors, thus they were excluded from the main analysis.

The ANOVA was carried out considering three within-subject factors: Hand of response (Left, Right), Visual field of presentation (Left, Right), and Condition (On-Center, Off-Center), and the IES was considered as the dependent variable. When required, the Duncan test was used for post-hoc comparisons. The main effect of Condition was significant ($F_{(1,43)} = 4.060$, $p = 0.050$, $\eta_p^2 = 0.09$), showing that the performance of participants was better in the On-Center condition (922.76 ± 101.46), than in the Off-Center condition (1423.06 ± 132.46). Importantly, the interaction between Condition and Visual field was significant ($F_{(1,43)} = 6.196$, $p = 0.017$, $\eta_p^2 = 0.13$). Post-hoc comparisons revealed that when stimuli were presented in the LVF, the performance of participants was better in the On-Center condition than in the Off-Center condition ($p < 0.001$). Moreover, in the On-Center condition, the performance was better when stimuli were presented in the LVF than in the RVF, and the opposite was true for the Off-Center condition, even if both comparisons failed to reach statistical significance (On-Center: $p = 0.088$; Off-Center: $p = 0.083$; Figure 3). Other main effects and interactions were not significant.
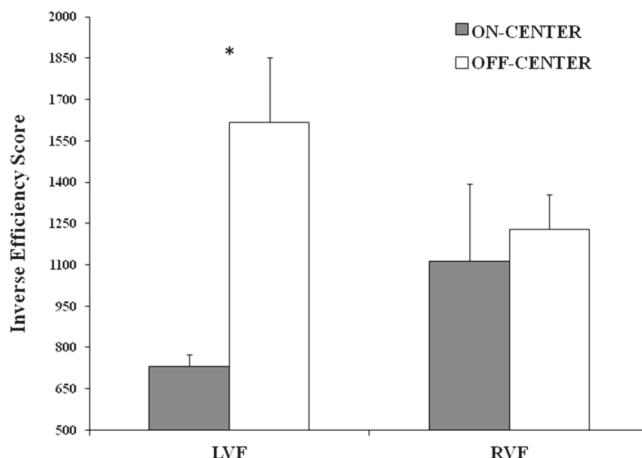
Figure 3. Interaction between the Visual field of presentation (Left, Right) and Condition (On-Center, Off-Center) on the Inverse Efficiency Score (response times of the correct responses divided by the proportion of correct responses) in healthy participants. Bars represent standard errors and the asterisk shows the significant comparison.

3.2. D.D.C.

The results of D.D.C. were analyzed by using a binomial distribution analysis and chi-square tests (as in [39]). The binomial distribution was computed considering the frequency of correct responses in each condition (Hand of response × Visual field × Condition). The results showed that the patient's responses were given at the chance level (50%) in any condition in the Right hand session, and in the On-Center condition-RVF in the Left hand session. In the Left hand session, his performance was significantly below the chance level for the Off-Center condition in both LVF and RVF, whereas it was significantly above the chance level only in the On-Center condition-LVF (Table 1).

Table 1. The frequencies of correct responses of patient D.D.C., and respective probabilities in the binomial distribution, in the Left hand session (upper panel) and in the Right hand session (lower panel), for stimuli presented in the Left Visual Field (LVF) and in the Right Visual Field (RVF), in the On-Center and Off-Center condition. Significant results are represented in bold.

Session	Results	LVF		RVF	
		On-Center	Off-Center	On-Center	Off-Center
Left hand	Correct responses	20	6	13	3
	Binomial: p	**<0.001**	**0.008**	0.149	**<0.001**
Right hand	Correct responses	11	9	13	12
	Binomial: p	0.149	0.078	0.149	0.161

Chi-square tests were used to compare the frequency of correct responses in the LVF vs RVF, as well as in the On-Center vs Off-Center conditions, in each session (Left hand, Right hand). Only the comparison between the On-Center and Off-Center conditions in the Left hand session was significant, showing that the patient correctly categorized the stimuli more frequently in the On-Center condition (Table 2). Thus, in order to better investigate this effect, chi-square tests were also computed for all of the interactions between VF and Condition. In the Right hand session, the comparisons were not significant. In the Left hand session, the comparisons between LVF and RVF were not significant either for the On-Center or for the Off-Center conditions, but D.D.C. categorized stimuli better in the On-Center condition than in the Off-Center condition, when presented with both in the LVF ($\chi^2 = 7.54$, $p = 0.006$) and in the RVF ($\chi^2 = 6.25$, $p = 0.012$; see Table 2).

Table 2. The frequencies of correct responses of patient D.D.C., Chi-square values and respective significance levels in the Left hand session and in the Right hand session, for stimuli presented in the Left Visual Field (LVF) and in the Right Visual Field (RVF), in the On-Center and Off-Center condition, as well as their interactions. Significant results are represented in bold.

Results	LEFT HAND				RIGHT HAND			
	LVF	RVF	On-Center	Off-Center	LVF	RVF	On-Center	Off-Center
Correct responses	26	16	33	9	20	25	24	21
Chi^2	2.38		13.71		0.55		0.2	
p	0.122		**<0.001**		0.456		0.655	
	On-Center		Off-Center		On-Center		Off-Center	
	LVF	RVF	LVF	RVF	LVF	RVF	LVF	RVF
Correct responses	20	13	6	3	11	13	9	12
Chi^2	1.485		1		0.167		0.428	
p	0.223		0.317		0.683		0.513	
	LVF		RVF		LVF		RVF	
	On-Center	Off-Center	On-Center	Off-Center	On-Center	Off-Center	On-Center	Off-Center
Correct responses	20	6	13	3	11	9	13	12
Chi^2	7.54		6.25		0.2		0.04	
p	**0.006**		**0.012**		0.655		0.841	

3.3. Control Group vs. D.D.C.

The mean percentage of correct responses for each condition obtained in the control group was compared with the percentage of D.D.C.'s correct responses, by means of exact *t*-tests, using the percentage of responses by the patient as a reference value for each session (Left hand and Right hand), separately (as in [39,43]).

The results showed that healthy participants gave more correct responses than D.D.C. in all conditions and in both sessions (for all comparisons: $3.39 < t_{(43)} < 18.21$, $p \leq 0.001$), with the exception

of the On-Center condition-LVF in the Left hand session, where no difference between the patient's performance and the control group's performance was observed ($t_{(43)} = -1.23$, $p = 0.225$; Figure 4).
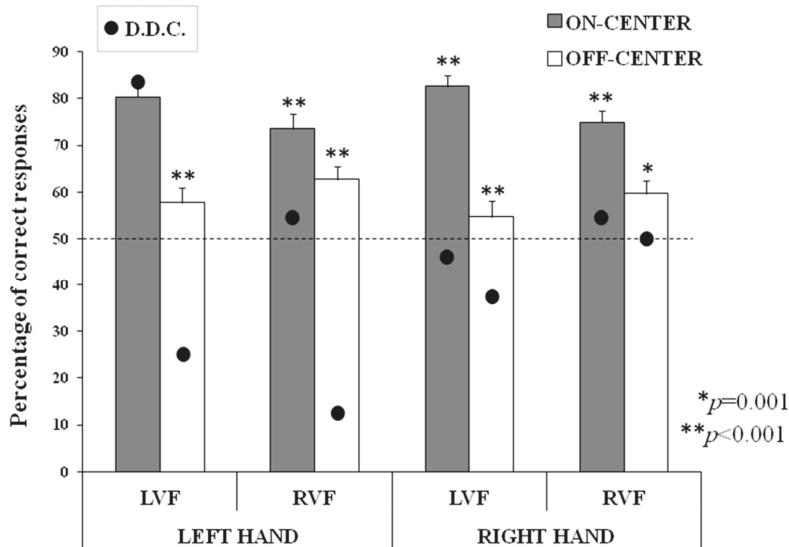

Figure 4. The percentage of correct responses in the left hand session (on the left) and in the right hand session (on the right), for On-Center stimuli (gray columns) and Off-Center stimuli (white columns) presented in the Left Visual Field (LVF) and in the Right Visual Field (RVF). The columns represent the results of healthy participants (bars represent standard errors), and black circles represent the results of D.D.C. Dashed lines represent chance levels (50%). Asterisks show the significant comparisons between the performance of D.D.C. and healthy controls.

4. Discussion

The main aim of the present study was to investigate the possible hemispheric imbalance in the processing of visual symmetry for double-axes stimuli. As reviewed in the Introduction, a right-hemispheric superiority has been found in symmetry detection when mono-dimensional stimuli are presented, but no asymmetries have been found using bi-dimensional stimuli [24]. Nevertheless, we found that the right hemisphere is superior compared to the left hemisphere in the detection of bi-dimensional symmetry, both in healthy participants and in a callosotomized patient.

It has to be highlighted that in the sample of healthy participants, we did not find significant differences depending on the hand used to respond. Although, we did not hypothesize that the use of one hand would lead to statistical differences in the healthy sample, we did expect that in the patient with complete callosal section, the use of one hand would highlight the effects of the activity of the contralateral hemisphere. Indeed, in the split-brain literature the collection of responses given with one hand has been repeatedly exploited as a tool to test the contralateral hemispheric activity (e.g., [37–39]). We found that D.D.C.'s performance was at the chance level in all of the conditions when he provided the responses using his right hand, suggesting that the left disconnected hemisphere is not capable of discerning symmetry from asymmetry. Moreover, in the left hand session, the performance of the patient was below the chance level when off-center stimuli were presented, suggesting that the right disconnected hemisphere cannot correctly judge asymmetry or, alternatively, that a right-hemispheric bias for symmetry influences this result. A possible alternative explanation could be that the patient shows a simple response bias for on-center stimuli, but the fact that the percentage of correct responses for on-center stimuli presented in the LVF is 54% (random level), together with the fact that such a bias

is present only when the left hand is used, makes this possibility less likely. Moreover, in the left hand session a main effect of symmetry confirmed that a "symmetry bias" is present in the right hemisphere (on-center stimuli were better categorized than off-center stimuli), for stimuli presented both in the LVF and in the RVF. Finally, the comparison between the performance of D.D.C. and the control group confirmed that only when the left hand was used and symmetric (on-center) stimuli were presented in the LVF was the comparison not significant, meaning that D.D.C. had the same accuracy level as the controls, whereas in all of the other conditions his performance was largely below that of the control group. Considered together, these results show that the right disconnected hemisphere of the split-brain patient can correctly process symmetry in bi-dimensional stimuli. The fact that the right (disconnected) hemisphere is superior to the left hemisphere in a number of tasks requiring spatial processing [12,13] is not a possible explanation for the present results. In fact, if it were the case, we should find a better performance in D.D.C. with both symmetrical and asymmetrical stimuli presented in the LVF. Similarly, a possible hemispheric difference in visual processing has to be discarded: by using the binocular rivalry paradigm, it has been shown, in two split-brain patients, that both hemispheres are able to process simple and complex stimuli (colored disks and faces), revealing a typical degree of binocular rivalry in both hemispheres [44–46]. Moreover, by means of binocular rivalry paradigms there is evidence of a redundancy effect [47], meaning that both hemispheres processed visual stimuli similarly and a callosal dysfunction can constitute an advantage in response times only when stimuli presented in both visual fields must be compared to each other [48]. The evidence of a right-hemispheric superiority which is specific for symmetry allows us to conclude that the symmetry bias is lateralized in the right hemisphere.

Regarding the healthy participants, the first crucial result found here is that they were better at categorizing symmetrical (on-center) rather than asymmetrical (off-center) stimuli, independently of the visual field of presentation. This evidence confirms the "symmetry bias" [23], that is the preference for symmetrical over asymmetrical configurations already proposed by the Gestalt theory. Some evidence has been collected concerning such a bias, showing that the human visual system detects symmetry more easily than asymmetry. First of all, symmetry detection is faster than asymmetry detection [49] and it affects the performance of observers even when it is not crucial for the task [50], as well as when the symmetrical arrangement constitutes the distracters during a visual search task [51]. Finally, it has been shown that symmetry is detected before eye movements are made towards a symmetric object, meaning that symmetry detection occurs also in the absence of overt attention [52]. This bias can have evolutionary roots, since symmetry is a relevant cue in the biological and physical world, so much so that it has been verified that it is innate and it is present in human infants (e.g., [53]), as well as in other species (e.g., [54]). The callosal hypothesis suggests that the symmetry bias could be due to the activity of two homologous areas in the two hemispheres, connected by means of the callosal fibers [1,2,14–16]. Starting from this view, it is predicted that when two patterns are presented in the two visual fields, they should be detected faster when they are symmetrical than when they are asymmetrical. Similarly, concerning split-brain patients, it can be expected that the absence of callosal connections should reveal no difference between the bilateral presentation of symmetrical or asymmetrical configurations. This prediction has been confirmed in split-brain and acallosal patients [55]. In the present study, we did not present stimuli bilaterally, and thus we did not aim at further exploring this issue, but we decided to present bi-dimensional stimuli in each visual field separately, in order to assess the specific propensity of each disconnected hemisphere at detecting bi-dimensional symmetry. To our knowledge, this is the first time a right-hemispheric superiority for bi-dimensional symmetry detection has been shown in a callosotomized patient. Moreover, the right-hemispheric bias for symmetry found in D.D.C. has been also confirmed in our control group: the results showed that when stimuli were presented in the LVF, the performance was better in the on-center than in the off-center condition, suggesting that the right hemisphere "prefers" symmetrical patterns also in healthy participants. The same conclusion might be suggested by the almost significant result showing a better discrimination of the on-center condition in the LVF than in the RVF. Moreover,

a slightly better discrimination of the off-center condition in the RVF than in the LVF seems to suggest a complementary superiority in the two hemispheres in healthy brains, with a right-hemispheric preference for symmetry processing and an opposite left-hemispheric preference for asymmetry processing. Nevertheless, further investigations are needed in order to verify this hypothesis.

The right-hemispheric superiority in bi-dimensional symmetry detection are in accordance with the results by Verma et al. [56]. By presenting symmetric and asymmetric geometric figures in the periphery of each visual field, the authors found that both right-handed and left-handed participants with left-hemispheric speech dominance were more accurate when symmetrical stimuli were presented in the LVF (whereas contrasting cerebral asymmetries were found in participants with right-hemispheric dominance for speech). Differently from the results of the present study and from the results by Verma and colleagues [56], Wilkinson and Halligan [24] did not find a hemispheric imbalance in healthy participants, by using a divided visual field paradigm and double-axes stimuli. As reviewed above, however, we manipulated a number of parameters which could explain the difference in the results between the two studies, e.g., we lowered the presentation time of the stimuli. Also the eccentricity of the stimuli differed between the two studies, although it has been shown that this parameter did not influence symmetry detection [57]. Moreover, we tested a number of participants: Wilkinson and Halligan, in fact, divided their whole sample into different subgroups, each carrying out a different task. Thus, in that study 12 participants carried out the task with the double-axes stimuli (for more details on their study see the Introduction). Nevertheless, the results of the present study confirm those by Wilkinson and Halligan with single-axis stimuli; in fact, we found that the right hemisphere is superior to the left hemisphere in correctly judging symmetrical patterns. In single axis conditions, Wilkinson and Halligan exploited the "classical" bisection paradigm to assess symmetry perception, in which participants are asked to divide a line into two equal segments or to evaluate a pre-bisected line as composed of two symmetrical or asymmetrical segments. By using this paradigm, a number of studies confirmed the presence of "pseudo-neglect" in healthy participants (i.e., the systematic trend to bisect a line leftward than at its real center), and the opposite bias in patients suffering from neglect, who "ignore" the left hemispace and bisect the line rightmost rather than at the veridical center (for a review see [58]). A central issue in this context is the difference found in the performance of neglect patients according to the mono- or bi-dimensional stimuli they were required to bisect: in fact, if on one hand the rightward bisection is considered a "landmark" of hemispatial neglect, on the other hand, no such bias has been found when neglect patients were asked to find the central point of bi-dimensional stimuli. For instance, this dissociation has been evidenced by Halligan and Marshall [59] with a neglect patient: the patient showed the bias in horizontal and vertical line bisection, but he did not show biases when required to place a dot in the center of a square or of a circle. Conversely, however, MacDonald-Nethercott and colleagues [60] found the same magnitude of pseudoneglect (leftward bias) in healthy participants by using both lines and elliptical shapes.

A possible dissociation has been suggested between vertical and horizontal spatial processing: Churches and colleagues [61] have recently shown that independent of the shape of the bi-dimensional images, a consistent correlation exists between the biases within each dimension (vertical, horizontal) across different shapes, but that there is no correlation between the vertical and horizontal bias when the two dimensions are compared to one another. The authors concluded that the parietal ("where") route is involved in vertical plane processing, and that the occipital ("what") route is responsible for horizontal plane processing. This suggestion is in line with other studies, showing that the parietal cortex is mostly involved in line bisection (mono-dimensional stimuli), whereas the occipital extrastriate cortex is mostly involved in bi-dimensional stimuli activating the object-based route instead of the parietal space-based route (e.g., [62]). This is also a possible explanation for the dissociation found with neglect patients between mono- and bi-dimensional stimuli, in whom the spatial deficit is mainly due to a parietal or temporo-parietal lesion [58], and it is also confirmed by TMS evidence in healthy subjects. As reviewed above, in two different studies Bona and colleagues [34,35] showed that a right-hemispheric stimulation disrupted symmetry detection in a stronger fashion than a

left-hemispheric stimulation, when TMS was applied over the lateral occipital cortex. The involvement of the occipital cortex in multi-dimensional symmetrical patterns has been also confirmed in other fMRI and EEG studies [21,22].

We can conclude that the occipital, object-based visual route is responsible for the processing of bi-dimensional stimuli, and that this is also the cerebral substrate for symmetry detection when stimuli are bi-dimensional [36,62]. The present results confirm the "symmetry bias" consisting of the preference for symmetrical rather than asymmetrical configurations [23], and also show that such a bias is right-lateralized in the human brain. These speculations are based on the results we collected with healthy participants, but also—and importantly—with a patient with a complete section of the corpus callosum. This evidence is in line with other results collected before, by using different paradigms [60], and by exploiting both neuroimaging and electrophysiological measurements [21,22], as well as brain stimulation techniques [34,35]. We can also hypothesize that the same cerebral substrate could be the basis for the processing of three-dimensional stimuli, and thus for the detection of symmetry in the real world, but further work is needed in order to assess these hypotheses.

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The Genetics of Asymmetry: Whole Exome Sequencing in a Consanguineous Turkish Family with an Overrepresentation of Left-Handedness

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Abstract: Handedness is the most pronounced behavioral asymmetry in humans. Genome-wide association studies have largely failed to identify genetic loci associated with phenotypic variance in handedness, supporting the idea that the trait is determined by a multitude of small, possibly interacting genetic and non-genetic influences. However, these studies typically are not capable of detecting influences of rare mutations on handedness. Here, we used whole exome sequencing in a Turkish family with history of consanguinity and overrepresentation of left-handedness and performed quantitative trait analysis with handedness lateralization quotient as a phenotype. While rare variants on different loci showed significant association with the phenotype, none was functionally relevant for handedness. This finding was further confirmed by gene ontology group analysis. Taken together, our results add further evidence to the suggestion that there is no major gene or mutation that causes left-handedness.

Keywords: handedness; hemispheric asymmetries; genetics; ontogenesis; consanguineous marriage

1. Introduction

Handedness is a heritable trait [1] and, historically, it was thought that left-handedness was determined by a major gene effect [2]. This idea was based on the statistical distribution of the phenotype, but has since been refuted by molecular studies. In particular, the fact that genome-wide associations studies (GWAS) consistently failed to identify a gene that explains enough phenotypic variance to qualify as a single-gene explanation has disproven single gene theories [3,4]. Thus, most authors today agree that handedness is likely to be a multifactorial trait that is determined by several different genetic and non-genetic factors (e.g., [5–8]). A number of contributing loci have been identified by GWAS and candidate gene studies using handedness questionnaires or hand skill tests like the pegboard test as phenotypes, e.g., *LRRTM1*, *PCSK6* and *AR* [9–15]. However, the general understanding is that there is likely a large number of yet unidentified genetic contributions to handedness [5]. Besides replication of published loci, identification of new candidate genes therefore is one of the major aims of current research on handedness genetics. Since GWAS in healthy cohorts are unlikely to identify

rare genetic variants relevant for handedness, other methods to identify candidate genes should also be considered.

One possible way to increase statistical power to detect relevant candidate genes for handedness without the need for overly large cohorts is testing population isolates with reduced genetic heterogeneity and overrepresentation of left-handedness. For example, Somers et al. [16] performed a genome-wide genetic linkage study of left-handedness and language lateralization in a sample of 368 subjects from a population isolate in the Netherlands. Due to the geographical isolation of the town that the subjects were recruited from, as well as a genetic bottleneck event in the early 17th century, founders in the sample of Somers et al. [16] showed lower genetic heterogeneity than random samples from the Dutch population. The sample was deliberately enriched for left-handedness, as the authors only selected families that had left-handed subjects in at least two generations, with at least two left-handed family members per generation. This resulted in a sample in which 24% of participants were left-handed, roughly 2.5 as many as in the general population. While Somers et al. [16] did not observe any genome-wide evidence for linkage in handedness, there was at least suggestive evidence for linkage for left-handedness in the 22q13 region. Somers et al. [16] argued that the absence of any significant linkage indicates that there is no major gene coding for handedness and it is likely to be a polygenic complex trait.

In addition to testing populations that show lower genetic heterogeneity than the general population due to a genetic bottleneck in the past and a more or less isolated way of living, another methodological option to detect genetic variants that influence handedness is to test families with a history of consanguineous marriage and an overrepresentation of left-handedness. This method has for example been used by Kavaklioglu et al. [17]. These authors used whole exome sequencing in 17 members of an extended family from Pakistan that practiced consanguineous marriage and had an overrepresentation of non-right-handed members (about 40%). Neither multipoint linkage analysis across all autosomes nor single-point analysis of exomic variation resulted in any clear candidate genes or mutations, leading Kavaklioglu et al. [17] to conclude, similar to Somers et al. [16], that handedness is a polygenic complex trait and not driven by a major gene or single mutation.

Although neither of these studies observed any significant effects, this does not necessarily imply that rare mutations could not affect handedness in other samples. Thus, more research in similar samples in other regions is needed. Also, previous studies in bottleneck populations analyzed handedness as a dichotomous variable (e.g., right-handedness/non-right-handedness). However, it is commonly measured as a continuous variable using a lateralization quotient (LQ) [18], ranging from −100 (consistent left-handedness) to +100 (consistent right-handedness). Interestingly, findings from a recent PCSK6 candidate gene study on handedness showed that the direction and degree of handedness might underlie differential genetic influences [9]. Thus, using the LQ as a phenotype instead of differentiating between left- and right-handers could potentially yield interesting insights into the genetics of handedness. To this end, we performed whole exome sequencing in nine members of an extended Eastern Turkish family that practices consanguineous marriage and has an overrepresentation of left-handedness. We then conducted a quantitative trait analysis with handedness LQ as a trait. Our hypothesis was that if there was indeed a major gene effect of a rare variant in this cohort, this variant should be significantly related to handedness LQ. If no such association was found, this would further confirm the idea that handedness is not driven by a major gene effect.

2. Materials and Methods

2.1. Participants

All participants were from Turkey, specifically from the vicinity of Şanlı Urfa, a city in the east of Turkey. This area was chosen as it has a higher prevalence of kin marriage compared to other regions of Turkey. The study was approved by the ethics committee of Dokuz Eylül University, Faculty of Medicine, İzmir, Turkey. All participants were treated in accordance with the declaration of Helsinki.

All participants gave written informed consent, and in case of participants younger than 18 years, the parents also gave written informed consent. Subjects were compensated for participating in the experiment with a gift of high quality Turkish sweets, as they refused to take money as reimbursement. Nine members of the family, two female and seven male, with a mean age of 29.33 (SD = 13.07; range: 11–46 years) agreed to participate in the study (Figure 1). Verbal interviews confirmed at least four consanguineous marriages between living family members and a family history of previous consanguineous marriages. None of the participants had a history of any psychiatric diseases or neurological diseases.
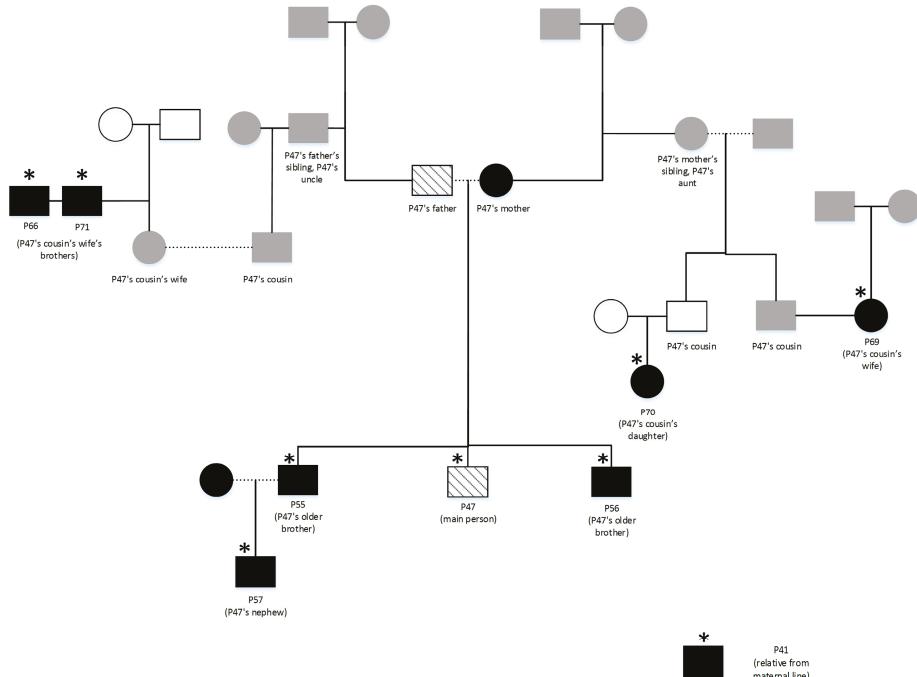
Figure 1. Family tree for the investigated cohort. Squares indicate male family members, circles indicate female family members. Asterisks indicate family members that participated in the present study. For these family members, handedness was determined using the Edinburgh Handedness Inventory (EHI). For other family members shown in the figure, handedness was assessed by verbal report. Black indicates left-handedness, white right-handedness and white with black shading ambidexterity. For family members with grey symbols, no information about handedness could be obtained. Consanguineous marriages are indicated by dotted lines. Consanguineous marriages were also performed by several family members of earlier generations not shown in this figure, as confirmed by verbal report.

2.2. Phenotyping

2.2.1. Edinburgh Handedness Inventory

Handedness was assessed with a Turkish translation of the EHI [18]. In this questionnaire, participants have to indicate whether they prefer to use left or right hand for ten different activities which are hand preference in writing, drawing, throwing a ball, using scissors, a toothbrush, a knife (without fork), a spoon, and a broom (upper hand), striking a match, and opening a box. An individual LQ can be calculated using the Formula LQ = $[(R - L)/(R + L)] \times 100$ (R = the number of right-hand

preferences; L = the number of left-hand preferences) as based on participants' answers. The LQ has a range between +100 and −100. Positive values indicate right-handedness and negative values indicate left-handedness. At the same time, higher absolute values indicate more consistent handedness and lower absolute values indicate more inconsistent handedness or ambidexterity.

2.2.2. Pegboard Test

In addition to questionnaires like the EHI that assess hand preference, hand skill can be assessed with motor tasks such as placing dots in squares or circles on a sheet of paper as quickly as possible [19,20], or picking up matches placed on a table as quickly as possible [19]. The most commonly used measure is the so-called "pegboard task" (e.g., [15,21,22]) that was also utilized to determine participants manual hand skills in the present study. The test consists of measuring the time taken by the subjects to move, with each hand separately, a row of 10 pegs on a board from one location to another. The test is repeated three times for each hand. The measure of relative hand skill (PegQ) is calculated as the difference between the average times for the left hand (L) and the right hand (R), (L − R), divided by the average time for both hands combined, (L + R)/2 [15]. A positive PegQ demonstrates superior relative right-hand skill, and a negative PegQ demonstrates superior relative left-hand skill.

2.2.3. Dichotic Listening Task

The Dichotic Listening Task is a noninvasive behavioral test to determine language lateralization. During a dichotic listening test, two different consonant-vowel (CV) syllables are presented to participants simultaneously using headphones, one to the right ear and one to the left ear. The syllables used in the present study were "BA, DA, GA, KA, PA, TA" [23]. Participants are instructed to indicate the syllable which they heard best by pressing a button [23]. Overall, 72 stimulus pairs were presented with Sony stereo headphones type MDR-ZX100 using Presentation software (https://www.neurobs.com/). The stimuli consisted of two times presenting all possible 36 combinations of the six syllables, including homonyms (e.g., BA-BA). Syllables were spoken by a native Turkish speaker and were provided by Dokuz Eylül University, Faculty of Medicine, Biophysics Department. Voice-onset times were controlled for.

2.3. Collection of DNA Samples

For the non-invasive collection of high quality DNA, saliva samples were collected using Oragene-DNA OG-500 saliva self-collection kits. These kits were used since they ensure DNA sample stability at room temperature for a prolonged time, which was essential since data collection took place in a field study without permanent access to refrigeration. From each participant, 2 mL of saliva were collected.

2.4. Whole Exome Sequencing

DNA was extracted from saliva samples and purified according to the kit protocol. All samples passed initial quality control with OD260/OD280 ratios between 1.6 and 2.0, and were then shipped to GATC Biotech AG (Konstanz, Germany), a service provider for DNA sequencing and bioinformatics (www.gatc-biotech.com). In addition to the nine samples from the family, we also included one sample of an unrelated right-hander from Turkey, to differentiate possible regional exome variation from true rare variants specific for the family, in addition to comparison against other reference genomes (see below). All samples passed a second DNA quality control performed by GATC. "INVIEW HUMAN EXOME" (http://www.gatc-biotech.com/de/produkte/inview-applikationen/inview-human-exome.html) was chosen as the whole exome sequencing platform. The array used was an Agilent Genomics SureSelectXT All Exon V5 (Agilent Technologies, Santa Clara, CA, USA). Mapping to the UCSC Genome Browser *Homo Sapiens* reference genome (hg19) was performed using BWA (Burrows-Wheeler Aligner; http://bio-bwa.sourceforge.net/ [24], with default parameters.

On average, 99.13% of high quality reads were mapped to the reference genome (see Table S1 for mapped read metrics for all samples). Removal of polymerase chain reaction (PCR) duplicates was conducted using Picard (http://broadinstitute.github.io/picard/) and local realignment using GATK (Genome Analysis Toolkit; https://software.broadinstitute.org/gatk/) [25]. On average, 93.99% of the exome was covered with a sequence depth read of at least 10× (see Table S2 for the depth of coverage summary). single-nucleotide polymorphism (SNP) and InDel calling was performed using GATK's UnifiedGenotyper (https://software.broadinstitute.org/gatk/documentation/tooldocs/current/org_broadinstitute_gatk_tools_walkers_genotyper_UnifiedGenotyper.php) [25], with a Bayesian genotype likelihood model. Subsequently, variant annotations were performed using snpEff (http://snpeff.sourceforge.net/) [26]. Further analysis of exome data and quantitative trait analysis was performed using "QIAGEN Ingenuity Variant Analysis" (http://www.ingenuity.com/products/variant-analysis) (see results for analysis pathway). The quantitative trait test that was used represents a continuous version of the Sequence Kernel Association Test (SKAT) where each sample is associated with a continuous quantity (in our case handedness LQ) instead of a case and control label. The underlying test is a variance component score test, based on a linear mixed effects model where the impact of rare variants is taken into account as random effects and co-variants are included as fixed affects. The quantitative trait test determines asymptotic p-values that are calculated approximately using Kuonens saddlepoint method. Furthermore, Gene ontology (GO) analysis was performed using the webtool WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/). This was done in order to identify whether associated gene variants were involved in GO groups with functional significance for handedness development (e.g., left-right axis differentiation or nervous system development). The minimum number of genes included in each GO group was set to five, and analyses were corrected for hypergeometric testing ($p < 0.001$) using false discovery rate (FDR) correction [27].

3. Results

3.1. Phenotyping

All nine family members investigated were left-handed according to EHI results (mean LQ: −84.44, standard deviation: 26.51; range: −100 to −20). The person from whom the control sample was obtained was right-handed (LQ: 100). Analysis of pegboard data showed that seven family members showed superior left hand skill and two family members slightly superior right hand skills (mean PegQ: −0.17, standard deviation: 0.15; range: −0.45 to 0.04). The control person showed superior right hand skills (PegQ: 0.19). For the dichotic listening data, three family members showed a left ear advantage (33.33%) and six showed the typical right ear advantage (66.66%). Dichotic listening data were analyzed non-parametrically due to the small sample size. In absolute number, family members on average reported more syllables presented to the right ear (35.67, standard deviation: 9.72) than to the left ear (29.56, standard deviation: 6.50), but this difference failed to reach significance (Z: −1.31, $p = 0.19$). To determine whether this nonsignificant result was indicating a real absence of an effect or rather was an artefact due to the small sample size, we also analyzed the data with a bootstrapped t-test for dependent comparisons with 5000 iterations. As this comparison also failed to reach significance ($p = 0.26$) it is likely that family members indeed did not show the typical right ear advantage found in the population.

3.2. Sequencing Results

Overall, the analysis detected 299,431 variants on 19,576 genes in family members that were non-identical to the reference genome. As a first step, variants with a call quality less than 20 and all variants in highly variable exonic regions were excluded, narrowing down the number of variants to 235,339 on 19,075 genes. We then excluded all variants that were present in less than at least seven of the nine family members (77.78%), resulting in 9714 variants on 4376 genes. This was done in order to include only variants that were consistently typical for the sample. Furthermore, all variants

with a frequency higher than 3% in the 1000Genomes project (http://www.1000genomes.org/) were excluded, as we focused on detecting rare variants. This step resulted in 810 variants on 411 genes left in the analysis. Afterwards, only variants likely to cause loss of function of a gene were included using the "Predicted deleterious" filter, resulting in 116 variants on 69 genes. This was done to only include causal genetic variants that affect protein function. As a last step, quantitative trait analysis was performed to include only variants that showed significant relations with handedness LQ with p-values of at least $p < 0.01$. This analysis revealed 49 variants on 26 genes that were significantly associated with the phenotype (see Table 1). Most of these genes were involved in general cellular processes and only very few were associated with the brain or neuronal processes specifically.

Table 1. Rare gene variants statistically associated with the phenotype. IDs from the Single Nucleotide Polymorphism Database (dbSNP) are given when available. Likely gene functions were determined using PubMed (http://www.ncbi.nlm.nih.gov/gene). (Chr. = chromosome).

Chr.	Gene	dbSNP ID	Likely Function
2	ANKRD36C	202102082	Ion channel inhibitor activity
3	MUC20	2688539 3828408	Cellular protein metabolism
4	ZNF595	-	Regulation of DNA transcription
4	FRG1	199978807 201142987	Associated with facioscapulohumeral muscular dystrophy
7	MUC3A	71540917 775174499 747768677 759956700 796070497 796719496 796627084 796799995 796422604 796558082 796345426 796976589 62483696	Cellular protein metabolism
10	FRG2	200347477	Protein coding in the nucleus
11	MUC6	770290437 34490696 200644196 796934918 111641154 112301388 78265558	Cellular protein metabolism/ production of gastric mucin
11	MUC5AC	74390930 749291344	Cellular protein metabolism
11	TRIM49	74584169	Protein-protein interactions, preferentially expressed in testis
14	HOMEZ	148005528	Regulation of DNA transcription
15	GOLGA6L2	76062343	Protein binding
16	CBFA2T3	71395351 71395352	Transcription corepressor activity

Table 1. *Cont.*

Chr.	Gene	dbSNP ID	Likely Function
17	CCDC144NL	73298040	Affects blood copper, selenium and zinc
17	KCNJ12	77987694 80335301	Encodes an inwardly rectifying K+ channel in neurons, heart and muscle cells.
17	RECQL5	142406301	DNA helicase activity
18	CNDP1	10663835	Encodes a member of the M20 metalloprotease family that is specifically expressed in the brain
19	MUC16	4992693	Cellular protein metabolism
19	ZNF443	62114866	Regulation of DNA transcription
19	SIGLEC11	9676436 78673790	Anti-inflammatory and immunosuppressive signaling
21	BAGE2	9808647	Melanoma antigen
21	BAGE5	113315187	Melanoma antigen
X	RBMX	76876438 74463481 74667874 35899675 77794331	RNA binding

Gene ontology (GO) analysis showed that the identified genes were significantly enriched within nine GO groups. The majority of these GO groups were related to protein glycosylation (see Table 2). The remaining GO group was "Golgi lumen".

Table 2. Results of the GO group analysis. *p*-values are Benjamini-Hochberg corrected.

GO Group	Genes	Adjusted *p*-Value
O-glycan processing	5	0.0000002
Protein O-linked glycosylation	5	0.0000005
Post-translational protein modification	5	0.00005
Protein glycosylation	5	0.0001
Macromolecule glycosylation	5	0.0001
Glycosylation	5	0.0001
Glycoprotein biosynthetic process	5	0.0002
Glycoprotein metabolic process	5	0.0005
Golgi lumen	5	0.0000007

4. Discussion

Handedness is a trait that has been related to both cognitive ability [28] and psychopathology [29], making the identification of genetic factors underlying its ontogenesis highly interesting for cognitive neuroscientists and clinical psychologists alike. Here, we performed whole exome sequencing in nine members of an extended Eastern Turkish family with a long history of consanguineous marriage and an overrepresentation of left-handedness. For the first time, we used quantitative trait analysis in such a cohort in order to identify rare genetic variants that were associated with handedness.

The results from the EHI clearly revealed that all nine tested family members were left-handers and, for most family members, these findings were also supported by the results of the pegboard test. Family members showed reduced language lateralization. While in the general population about 95% of individuals show left hemispheric language dominance, in our sample only 66.66% of individuals showed a right-ear advantage during dichotic listening and there was no significant right-ear advantage. This number is however only slightly lower than the 70–80% observed in

left-handed samples [30]. Given the small sample size of the present study, we would assume that our data are within the normal range for left-handed populations.

The quantitative trait analysis revealed rare variants on 49 loci on 26 genes that were significantly associated with the EHI LQ. However, the biological significance of these genes for handedness remains unclear. As handedness represents a functional asymmetry between the left and right motor cortices in controlling for fine motor skills [6], one would expect genes involved in shaping this phenotype to be specifically expressed in the brain or spinal cord. Moreover, they should have functional relevance for left-right axis development or nervous system development or function in the broadest sense. Almost all of the genes that were associated with handedness LQ in the present study did not meet these criteria, as they were involved in general cellular or regulatory processes not specific for nervous tissue. Furthermore, some genes clearly were relevant for function in body parts other than the brain, making an involvement in handedness development highly unlikely. Only two out of 26 genes showed a functional relevance for neuronal functioning in the broadest sense. The first of these genes, *KCNJ12* (potassium voltage-gated channel subfamily J member 12), encodes a functional inward rectifier potassium channel [31]. Functionally, most studies have linked it to the heart (e.g., [32]) or muscle [33] function, but also tumerogenesis [34]. While a recent study suggested that protein-protein interactions between a G protein-gated inwardly rectifying potassium channel (Kir3), G proteins and G protein-coupled neurotransmitter receptors might be functionally relevant for GABA-B receptors [35], direct evidence linking *KCNJ12* to a specific function in the central nervous system is sparse. While Stonehouse et al. [36] could show that the inwardly rectifying potassium ion channel encoded by *KCNJ12* in humans can be localized in sections of rat hindbrain and dorsal root ganglia tissue, there is no evidence for a functional link to handedness development so far. The second gene, *CNDP1* (carnosine dipeptidase 1), encodes a member of the M20 metalloprotease family which acts as carnosinase. While it is expressed in the brain, most studies have linked it to susceptibility for diabetic nephropathy in human diabetic patients (e.g., [37]), with no evidence for a direct functional link to handedness. Thus, the analysis of functionally relevant rare variants did not result in any evidence for a major gene or mutation determining handedness in our cohort.

This interpretation was further supported by the result of the GO analysis. Out of nine GO groups that reached significance, seven were linked to glycosylation, an enzymatic process that attaches glycans to other molecules. Glycosylation represents an important post-translational modification of proteins in a vast number of different tissues. While congenital disorders of glycosylation have been shown to affect central nervous function [38], glycosylation has also been related to the development and progression of several different types of cancer and other diseases unrelated to the brain [39]. Interestingly, it has been shown that inbreeding in human populations strongly affects the glycosylation of human plasma proteins, potentially leading to the increased prevalence of tumors that has been reported in certain isolated populations as well as other phenotypic changes [40]. Thus, it is likely that the significant effects for glycosylation-related GO groups were an effect of inbreeding and only by happenstance were associated with the handedness phenotype. The other three significant GO groups also were unlikely to affect handedness, as they either represented processes unrelated to the brain or were too general ("Golgi lumen" "post-translational protein modification") to specifically be involved in the formation of the functional motor cortex asymmetry underlying handedness.

The present study contains several methodological aspects that have the potential to be optimized in future studies. Clearly, testing a larger group of family members with a consanguineous background would be ideal. Unfortunately, we were only able to recruit left-handed family members in the present study, but for future studies including both left-and right-handers from the same family would by optimal. Also, for quantitative trait analyses, larger cohorts would be favorable, if recruitment is possible. This would be particularly important as the GATK protocol used for variant calling in the present study gives optimal results with sample sizes of 30 or larger. Moreover, in our cohort there was the possibility that some of the individuals (e.g., P69, see Figure 1) married in with potentially their own forms of left-handedness, and do not necessarily share a genetic basis with the other members

of the family. This could have confounded the analysis and should be controlled for when recruiting cohorts for future studies. Moreover, the test used to determine quantitative trait association did not account for different degrees of relatedness, but for a weakly heritable trait this is unlikely to bias the results. As rare variants might be highly cohort-specific, more studies in cohorts with diverse ethnic backgrounds are needed to completely exclude a possible influence of major rare variants on handedness. Another possible criticism of our data could be that it is unclear to what extent an overrepresentation of left-handedness is a specific characteristic of the sample that was investigated in our study or the general population it comes from. While there is no specific published data on handedness in the vicinity of Şanlı Urfa, studies in Turkish samples indicate that the frequency of left-handedness in Turkey is between 6% and 11% [41–43], which is in line with what has been found in other populations worldwide (around 10%). Tan reports the incidence of familial left-handedness in Turkey to be around 28.4% [43], which is lower than the 39.3% that has been reported in a large American sample [44]. Thus, the over-representation of left-handedness observed in our sample is typical for this family, not the general population in Turkey.

5. Conclusions

Taken together, both the analysis of single rare variants and the analysis of GO groups revealed no indication for a rare variant that could realistically determine handedness. Thus, our analysis in a Turkish cohort with lower genetic heterogeneity than the general population independently replicates previous findings from similar studies in Dutch [16] and Pakistani [17] cohorts. Thus, our study supports the conclusions of these studies that handedness is likely to be determined by complex polygenic and/or epigenetic factors [45].

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