



## Research report

## Functional MRI and functional connectivity of the visual system of awake pigeons

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## H I G H L I G H T S

- ▶ First study to image the whole brain of awake pigeons.
- ▶ We show a habituation program for MR-sessions under awake and head-fixed conditions.
- ▶ Movement under these conditions is minimal.
- ▶ First measurement of functional connectivity in a non-mammalian species.
- ▶ Analyses of BOLD-response and functional connectivity are feasible.

## A R T I C L E I N F O

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## A B S T R A C T

At present, functional MRI (fMRI) is increasingly used in animal research but the disadvantage is that the majority of the imaging is applied in anesthetized animals. Only a few articles present results obtained in awake rodents. In this study both traditional fMRI and resting state (rsfMRI) were applied to four pigeons, that were trained to remain still while being imaged, removing the need for anesthesia. This is the first time functional connectivity measurements are performed in a non-mammalian species. Since the visual system of pigeons is a well-known model for brain asymmetry, the focus of the study was on the neural substrate of the visual system. For fMRI a visual stimulus was used and functional connectivity measurements were done with the entopallium (E; analog for the primary visual cortex) as a seed region. Interestingly in awake pigeons the left E was significantly functionally connected to the right E. Moreover we compared connectivity maps for a seed region in both hemispheres resulting in a stronger bilateral connectivity starting from left E then from right E. These results could be used as a starting point for further imaging studies in awake birds and also provide a new window into the analysis of hemispheric dominance in the pigeon.

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## 1. Introduction

Functional MRI (fMRI) studies of awake animals are methodologically challenging but also more rewarding since functional imaging data obtained from awake animals are more comparable with brain activity under normal cognitive test conditions. Anesthesia significantly affects the physiological state including the regulation of cerebral circulation throughout the brain [1–6]. Only a small number of fMRI studies under awake conditions have

been published in small mammals such as, e.g. rats [7–9] and mice [10]. Moreover since it is shown that both in human [11] and non-human primates [12] brain state during rest is not fully comparable with the anesthetized state, new studies increasingly start to study rsfMRI in awake rodents [13–17].

Along with rats, pigeons are known to be standard animal models for studies on the mechanisms of learning. Having the ability to perform MRI in awake pigeons would clearly open the door for a substantial list of interesting studies. Pigeons are used to study mechanisms of learning and memory, i.e. reinforcement [18], matching [19], interval timing [20], sequence acquisition [21] and equivalence learning [22]. Also, pigeons have traditionally served as a model organism to study perception and its neurobiological and anatomical basis in birds, with a similar wealth of literature as for learning and cognition. Again mentioning just a few areas of research, these studies examined, e.g. color perception [23], optic illusions [24] and magnetoperception [25]. Awake functional MRI of pigeons would allow many of these ‘learning and memory’

*Abbreviations:* BOLD, Blood Oxygenation Level Dependent; E, Entopallium; FC, functional connectivity; GLd, nucleus geniculatus lateralis pars dorsalis; ROI, region of interest; rsfMRI, resting state functional magnetic resonance imaging; Rt, nucleus rotundus; TE, echo time; TR, repetition time.

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scientific experiments while simultaneously looking at activity of the whole brain.

Resting state fMRI aims at detecting low frequency fluctuations of less than 0.1 Hz in the Blood Oxygenation Level Dependent (BOLD) signal measured during rest. Using this technique, functional connectivity (FC) is defined as the temporal correlation of these fluctuations between different brain regions [26]. Coherence of low frequency fluctuations of the BOLD signal has been shown in different mammalian species including mice [27], rats [28], monkeys [29], and humans [26]. This paper shows the first application of rsfMRI in a non-mammalian species, a pigeon. Birds have a forebrain organization that importantly differs from mammals. Their telencephalon is homologous to that of mammals but displays no cortical lamination [30]. Instead, functional entities are structured as clusters that lack a visible anatomical subdifferentiation [31]. Given the substantial difference between the cerebra of birds and mammals, rsfMRI analysis could provide a new comparative window into the functional architectures of mammalian and avian forebrains.

Thus, the aim of the present study was to establish a habituation protocol in pigeons to test birds under awake but motionless imaging studies under as low stress conditions as possible. In addition, we aimed to use this approach to compare BOLD signal strengths and functional connectivities of the tectofugal system in the left and right hemisphere. Since the visual system of birds in general and the pigeon in special represents a well-known model for brain asymmetry [32–35], imaging in awake birds could open a new approach to study brain asymmetries.

## 2. Material and methods

### 2.1. Animals

For this study adult pigeons (*Columba livia*) of the Valentia Figurita breed ( $n=4$ ; 165–200 g) were used which were kept individually in cages of 35 cm × 40 cm × 45 cm in the animalarium of the Bio-Imaging Lab in a 12 h/12 h dark/light cycle. Food and water was provided ad libitum. Experimental procedures were in agreement with the European guidelines for the care and use of laboratory animals (86/609/EEC) and were approved by the Committee on Animal Care and Use at the University of Antwerp, Belgium (2012-04).

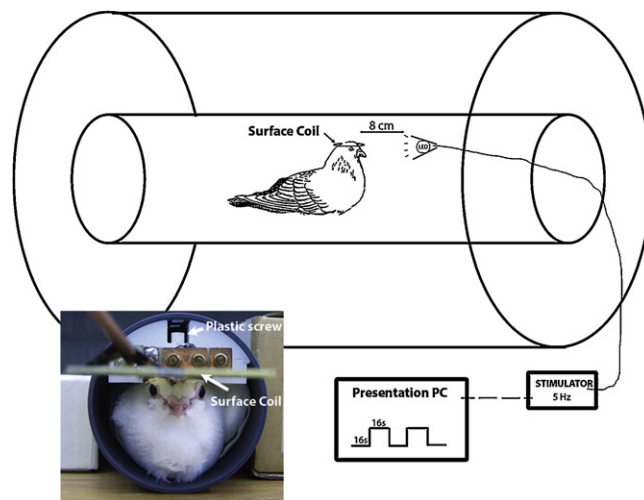
### 2.2. Surgery

Figuritas, the smallest breed of rock pigeons, were used here to fit into the bore of the Bruker 7T Pharmascan (Bore diameter: 16 cm). Four weeks before the start of the training, the animals were deeply anesthetized with ketamine/xylazine (0.12 ml/100 g body weight) and fixed in a stereotactic apparatus. After removing feathers from the skull and suturing the skin, a small plastic head holder was glued to the skull with dental cement. The head pedestal had a tapped hole that was later used to fix the head of the animal to the testing tube. The pigeons were allowed to recover for four weeks. During the first two days, they received 2 mg/kg Rimadyl (Caprofen) as an analgesic.

### 2.3. Pre-MRI acclimation training

In the current study, each animal was subjected to three distinct experimental procedures in order to train the animals with a habituation protocol to stay calm and relaxed during an fMRI procedure of up to 1 h.

- (1) Habituation to the test environment: The animals were first habituated to the general test environment. For this, they were transported to a laboratory within the imaging center, were placed in a tight cloth jacket and were gently positioned into a plastic tube with an inner diameter of 77 mm. The cloth jacket covered the body of the animal and left the head, the neck and the tail of the animal free. The size of the jacket could be adjusted with Velcro bands to prevent the bird from flapping with its wings. The plastic tube was open at both ends. The birds were placed for 10 min into the tube the first day, 30 min the second, and 60 min the third day. None of the birds displayed any signs of discomfort during this procedure.
- (2) Habituation to head fixation: It is essential that the heads of the pigeons do not move during scanning. To achieve this, the pigeons were slowly accustomed to be fixed via their head pedestal to the plastic tube. This tube had a groove into which the pedestal fitted. In addition, the pedestal was secured with a plastic screw that matched its tapped hole. The pigeons were first placed into the cloth



**Fig. 1.** Schematic drawing of the experimental setup. The awake pigeon was positioned in the middle of the magnet with the surface coil positioned over the brain. Two LEDs were positioned 8 cm from the pigeon's head. The LEDs were connected to the stimulator which was set to a frequency of 5 Hz. The stimulator itself was gated (during functional MRI measurements) using a PC with Presentation software connected via a fiber optic wire. During each ON period of 16 s the stimulator powered the LEDs. The inset shows a picture of the pigeon inside the tube (and the surface coil in position) with its head fixed to the tube using the pedestal and plastic screw.

jacket, placed into the plastic tube and were then secured via their pedestal to the groove. The animals were habituated first for 5 min to this condition. This length was prolonged every day (10, 20, 40 min) until the animals were kept for 60 minutes under these conditions. On the very first day the pigeons made attempts to move the head within the first few minutes. They were then very calm for the entire remaining habituation procedure.

- (3) Habituation to sound: The noise during MR scans occurs during the rapid alterations of currents within the gradient coils. Sound level can be high and reach 103 dB on the linear scale [36]. We recorded the sounds emitted by the Pharmascan system during an fMRI session and measured an average sound level of 67 dB, although few peak values of 68 dB were recorded for short periods of time. This acoustic recording was then used for the last step of the habituation protocol. In this last step, the pigeons were fixed via their pedestal to the plastic tube and two loudspeakers were positioned on either side of the tube. On the first day, we played the recorded sounds of an fMRI session for 1 h at a sound level of 45 dB. This was slowly increased daily by steps of about 10 dB until the animals remained head-fixed in the tube for 1 h per day at a sound level of 68 dB. This was then repeated for 1 week until the real scanning procedure started.

### 2.4. Awake BOLD fMRI under activity induced and resting state conditions

The head pedestal of one pigeon had broken off after the first measurement (binocular condition, see below) and this subject was therefore omitted from further measurements of the study (mono-ocular conditions, see below). It is important to note that this happened not during a scanning procedure but when the animal was in its home cage. For the scanning procedure, the plastic tube together with the head-fixed animal was inserted into the bore of the Pharmascan. During MRI data collection, the animals remained within a dark environment (lights were turned off within the MRI scanner room).

#### 2.4.1. Stimuli and stimulation device

The visual stimuli were presented using two green 3 mm LED's (Agilent Technologies; Dominant wavelength = 569 nm; intensity 2.3 millicandela (mcd)) that were positioned 8 cm from the bird's head. LED's were connected to a stimulator and were lit with a frequency of 5 Hz during visual stimulation (duration one light pulse = 100 ms). The stimulator was positioned inside the Faraday cage and was connected to the presentation computer using a fiber optic wire (Fig. 1). Stimulus presentation was controlled by Presentation software 0.76 (Neurobehavioral Systems).

#### 2.4.2. Experimental design

The pigeons were measured three times under three different conditions: binocular stimulation ( $N=4$ ), right mono-ocular stimulation ( $N=3$ ) and left mono-ocular stimulation ( $N=3$ ). Mono-ocular conditions were made possible by covering one of the eyes with a patch attached to a Velcro ring, which was held in place using non-lasting glue ("UHU Bastelkleber" solvent free). Each fMRI measurement consisted of an ON/OFF block design alternating visual stimulation periods (ON blocks) with resting periods/darkness (OFF blocks). Each block (ON and OFF) lasted 16 s, which

corresponds to the acquisition time of 2 images. The ON stimulus type was presented 63 times, resulting in the acquisition of 126 images during ON periods per measurement.

Resting state fMRI scans were acquired twice for the three different conditions. For each condition one scan was acquired in the dark and one was obtained while two green LED's that were positioned 8 cm from the bird's head where continuously flickering (5 Hz).

#### 2.4.3. Image acquisition

Imaging was performed on a horizontal MR system (Pharmascan 70/16 US, Bruker Biospin, Germany) with a magnetic field strength of 7 T. A custom-made circular RF surface antenna (24 mm) was positioned around the head and served for transmission and acquisition of radio frequency pulses. BOLD fMRI data were acquired using a  $T_2$ -weighted Fast Spin Echo sequence [echo time (TE)/repetition time (TR): 60/2000 ms] [37]. Each whole-brain volume contained 15 axial slices, 1 mm thick, with a gap of 0.066 mm between slices. Slices were acquired along the Frankfurt zero plane. In-plane resolution was 0.34 mm  $\times$  0.34 mm and matrix size was 64  $\times$  64 voxels.

Anatomical three-dimensional (3D) images required for localization of the functional data (see below) were obtained for each bird using a FISP sequence with TE/TR: 4/8 ms; scan repetition time 1200 ms. Voxel size was 0.085 mm  $\times$  0.085 mm  $\times$  0.170 mm and matrix size was 256  $\times$  256  $\times$  128 voxels.

RsfMRI images were acquired using a RAREst sequence with TE/TR: 16/2000 ms. Voxel size was 0.17 mm  $\times$  0.34 mm and matrix size was 128  $\times$  64. Fifteen slices of 0.8 mm were acquired 150 times resulting in a scan-time of 5 min per resting state scan.

To compare movement in the scanner between awake (trained) and anesthetized state, two of the four birds were re-measured again under anesthesia. Birds were anesthetized with an intramuscular injection in the chest of 5.7 ml/kg of a mixture containing 10 ml of medetomidine (1 mg/ml, Domitor, Orion, Finland) and 0.5 ml of ketamine (50 mg/ml, Ketalar, Parke-Davis, Belgium). Body temperature was continuously monitored with a cloacal temperature probe and maintained at 41.5  $\pm$  0.5  $^{\circ}$ C by a feedback controlled warm air heating system (SA-Instruments). Respiration rate and amplitude were constantly monitored with a small pneumatic sensor (SA-Instruments) positioned under the bird. Image acquisition (fMRI) was exactly the same as the awake measurements.

#### 2.5. Data analysis

##### 2.5.1. Image (pre-)processing

Intra-individual head motion was corrected using a six-parameter rigid body spatial transformation using the Statistical Parametric Mapping toolbox, version 8 (SPM8; Wellcome Trust Center for Neuroimaging; Department of Cognitive Neurology, London, UK; <http://www.fil.ion.ucl.ac.uk/spm/>). The realigned fMRI images for each subject were then co-registered to each individual anatomical 3D dataset. In parallel, the 3D dataset was spatially normalized, using SPM8, to the pigeon brain MRI atlas that we developed in our lab [38]. The transformation matrix of this spatial normalization was then applied to the realigned and co-registered functional data, resulting in functional data precisely co-registered to the atlas dataset. Finally, functional data were smoothed with a 0.68 mm width Gaussian kernel. Resting state data were smoothed with a 0.34 mm  $\times$  0.68 mm width Gaussian kernel. Next, resting state time data were linearly de-trended and filtered (0.01–0.1 Hz) using the Resting-State fMRI Data Analysis Toolkit (REST 1.7; <http://www.restfmri.net/>). Using REST time courses were extracted for two seed regions of which one was located in the left entopallium, one in the right entopallium. Positioning of the seed regions was done very securely in the same location in both hemispheres. Functional connectivity (FC) maps were generated in SPM8 by comparing the seeds time course with time courses of all other voxels within the pigeons brains using linear regression with the time course of the seed as covariate. Resulting statistical maps showed significantly connected voxels [39]. Both motion parameters, resulting from the realignment, as a global signal time course were regressed out during this analysis to improve the specificity of the FC [40].

#### 2.6. Statistical analyses

##### 2.6.1. Activity induced BOLD changes (fMRI)

Statistical voxel-based analyses were performed using a mass-univariate approach based on the General Linear Model, implemented in SPM8. Data were filtered with a high-pass filter of 96 s. Model parameters were then estimated using a classical restricted maximum likelihood algorithm.

A subject-level analysis was first performed in which the main effect of the visual stimulus versus rest was computed. The statistical group analysis was restricted to the entopallium and the visual Wulst as a priori defined regions of interest (ROI) using the delineations of the pigeon MRI brain atlas [38]. The entopallium and the visual Wulst are primary visual telencephalic areas with thalamotelencephalic input from the visual tecto- and the thalamofugal systems, respectively. The analyses revealed a BOLD signal in the bilateral entopallium. In order to investigate differences in BOLD response between the different conditions (binocular, left eye open, right eye open), we first identified voxels that displayed a differential response to the different conditions in a one-way repeated measures ANOVA in the predefined

ROI. In order to identify the differences between conditions, post hoc *t*-tests were then performed (paired *t*-tests) only on the voxels found to be significant in the one-way ANOVA. Because statistical tests were performed on a voxel basis, resulting in numerous tests, *p* values were adjusted to the number of independent tests performed. This was done using the Family Wise Error method. This method uses the Random Field Theory to calculate the number of independent tests, taking into account the number of voxels but also the amount of auto-correlation among data.

##### 2.6.2. Hemispherical comparison

Finally, in order to compare responses obtained in both hemispheres, we first calculated for each subject the differential effect between activation and rest. This was done in each entopallium. Then, these differential effects were compared across hemispheres using two-tailed paired *t* tests. Differences were considered as statistically significant when *p* < 0.05. A lateralization index was calculated using mean *T*-values of entopallium, (Left – Right)/(Left + Right)  $\times$  100.

#### 2.7. Resting state BOLD fluctuations (rsfMRI)

##### 2.7.1. Hemispherical comparison

To compare local FC in E in both hemispheres, cluster size and  $T_{\max}$  (maximum *T*-value) derived from the single subject FC maps with both seed regions are compared. Next to compare inter-hemispheric FC starting from E, cluster size and  $T_{\max}$  of the cluster at the contralateral side are compared. This was done in SPSS using a non-parametric test of related samples (Wilcoxon signed rank test).

### 3. Results

#### 3.1. Movement during scanning

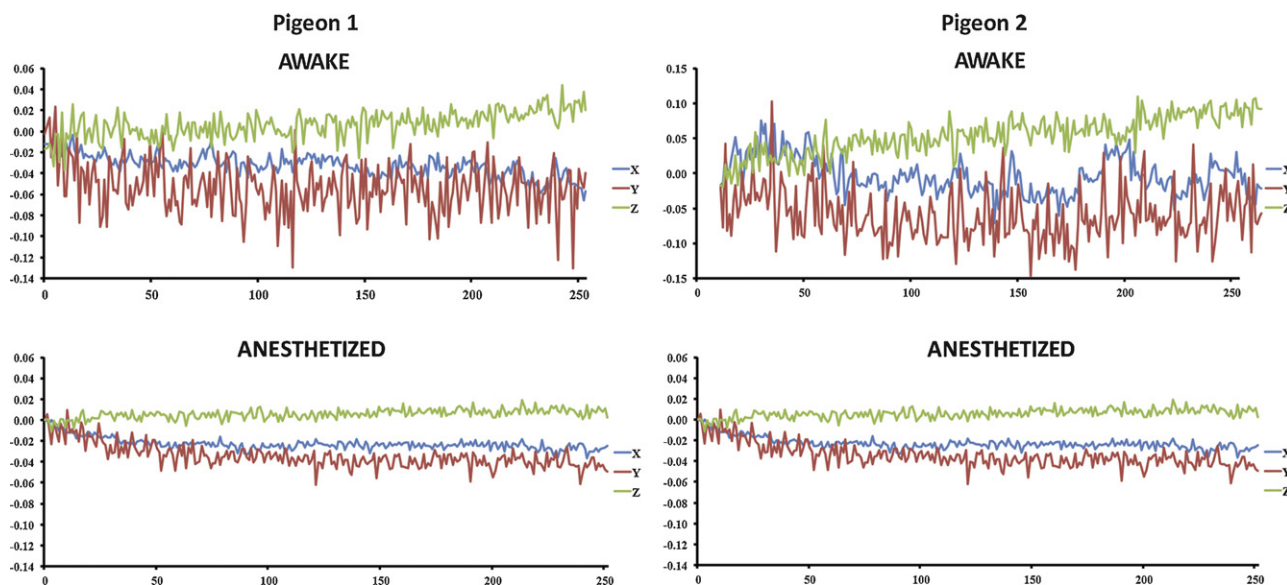
After habituation training, none of the animals displayed any signs of discomfort when being handled for the next scanning session. All birds were completely calm. This is evident in Fig. 2, which shows examples of the movement from two pigeons who were measured twice, once awake and once again anaesthetized. As visible, movement is very small under both conditions. Although small jerks are visible when the pigeon is not anaesthetized, these minute movements were never beyond one voxel (0.34 mm) in any direction for all four pigeons.

#### 3.2. Awake binocular stimulation

All 4 pigeons showed a clear BOLD response in the telencephalic entopallium (Fig. 3) when visually stimulated, however, a large variance in amplitude of the BOLD response was observed between animals (mean *T*-value right E: 3.48; SD: 2.32; coefficient of variation: 0.67; mean *T*-value left E: 3.36; SD: 1.23; coefficient of variation: 0.37). No statistically significant difference was detected between the two hemispheres in BOLD responses (paired *T*-test left vs. right: *p* = 0.85, *N* = 4). The lateralization index was therefore also very small and around zero, with a large variation between the individuals (mean: 2.64%; SD: 14.5%).

#### 3.3. Unilateral vs. binocular stimulation

We compared the fMRI outcome upon visual stimulation in awake animals between the different conditions (binocular, left eye open and right eye open) using a one-way repeated measures ANOVA (*N* = 3). A statistical difference could be observed in a cluster in the left entopallium ( $F_{\max}$ , i.e. voxel presenting the maximal *F* value among all significant voxels of the cluster = 48.60; *p* = 0.026), while the equivalent difference in the right entopallium was not significant ( $F_{\max}$  = 20.77; *p* = 0.072). Post hoc analysis of the cluster in left E revealed that there is a significantly greater neural activity when the pigeon has both eyes open (binocular) compared to when it has only its ipsilateral left eye open ( $T_{\max}$ , i.e. voxel presenting the maximal *T* value among all significant voxels of the cluster = 9.15; *p* = 0.001), and also a significantly greater activity when the contralateral right eye is open compared to the ipsilateral left ( $T_{\max}$  = 7.60; *p* = 0.004) (Fig. 4).



**Fig. 2.** Movement parameters of two pigeons measured both during an awake and an anaesthetized measurement. Translation parameters are derived from the realignment procedure of SPM8. Motion of the brain (head) was always confined within one voxel ( $0.34 \text{ mm} \times 0.34 \text{ mm} \times 1 \text{ mm}$ ). Colors (blue, red and green) correspond each to a different direction of movement. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 3.4. Functional connectivity of the entopallium

Single subject functional connectivity (FC) maps starting from a seed region both in the left and the right E were made for all animals. Fig. 5 shows the location of the seed regions (A and B) and an example of a single subject map starting from both seeds in a data set that was acquired in an awake pigeon. FC maps show not only local correlation with the seed region but also that left E is functionally connected to right E (C). This can be confirmed by placing the seed region in the other hemisphere (D).

### 3.5. Lateralization of functional connectivity

Six resting state scans were acquired in awake condition for 3 different birds. To estimate laterality first of all local FC was estimated. This was done by calculating the cluster size and  $T_{\max}$  in the entopallium in which the seed was placed and by comparing this outcome for both hemispheres. These values were not significantly different for both cases ( $p=0.074$  and  $p=0.711$  resp.) indicating a comparable local FC in the entopallium at the left and the right side.

Next, the cluster size and  $T_{\max}$  were compared for the contralateral hemisphere which were both significantly higher at the right side ( $p<0.001$  and  $p=0.002$  resp.) indicating that the FC from left to right is higher than the other way around (Fig. 5). An overview of these results is shown in Table 1.

**Table 1**  
Statistical outcome comparing functional connectivity for the left and right entopallium.

Comparison	Seed location		$p$ -value for $T_{\max}$	$p$ -value for clustersize
	Left	Estimated hemisphere		
Local FC	Left	Left	0.074	0.711
	Right	Right		
Inter-hemispheric FC	Left	Right	<0.001	0.002
	Right	Left		

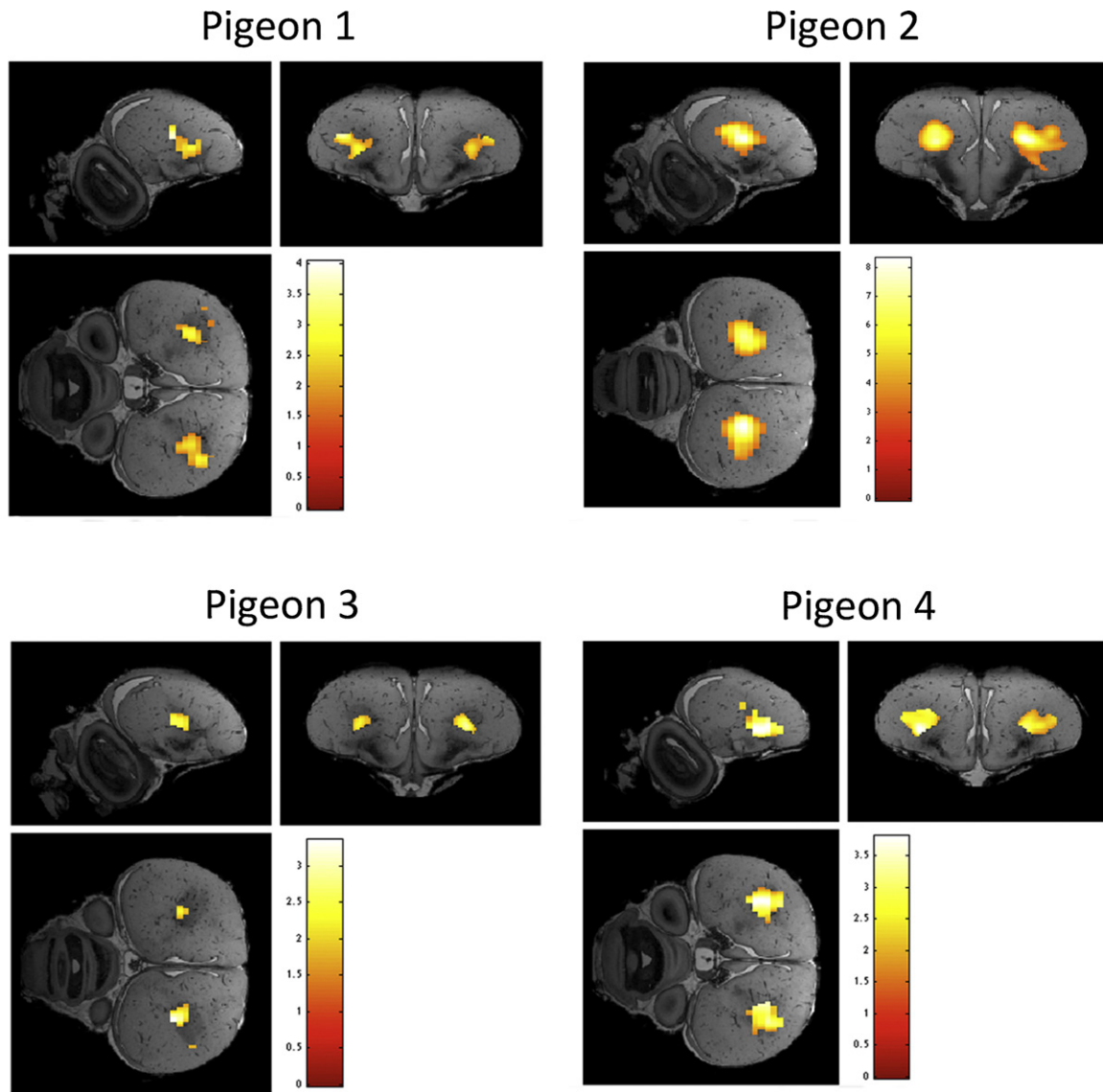
## 4. Discussion

Our study shows that a habituation program with pigeons for lengthy MR-sessions under awake and head-fixed conditions is possible. Our results reveal that movement under these conditions is minimal and that studies that analyze BOLD-response and the functional connectivity of various neural components are feasible. We regard this finding as a door opener for future MRI-studies with awake pigeons and possibly even other bird species. Additionally, we could show, that visual stimulation during an fMRI experiment reveals asymmetries of the tectofugal system. In the following, we will discuss these two points.

### 4.1. MRI of awake pigeons

A large number of studies in various mammalian species reveal differences in the result patterns of (rs)fMRI studies under awake and anaesthetized conditions [4,7,11,12]. Since different bird species represent interesting models for the analyses of neural plasticity, song production, learning, and cognition, it is necessary to develop and test a procedure for awake bird MRI. The habituation program for pigeons outlined here takes a mere three weeks of training. As shown in the result section, maximal movement amplitudes during 1 h scanning session never exceeded one voxel.

A part of this success is due to peculiarities of the bird skull. During the evolution of birds a gradual expansion of pneumatized spaces within some bones occurred [41]. At the same time, avian skulls become much more dense than those of similarly sized mammals, even when compared to bats, the only flying mammalian order [42]. Increased bone density in birds possibly reflects adaptations for maximizing bone strength and stiffness while minimizing bone mass and volume [43]. Given the association between bone density and material properties, the crania of birds are stiffer and have a lower ductility than those of, say, rodents [42]. This implies that head-fixing birds as done in this study, ensures a higher stability of the brain during scanning than in mammals. However, when birds would panic in the scanner, they still possibly could torque their skull a bit or could even activate sufficient strengths to detach the pedestal by shearing forces. Thus, both for scientific and for animal welfare reasons, a solid habituation program is necessary.

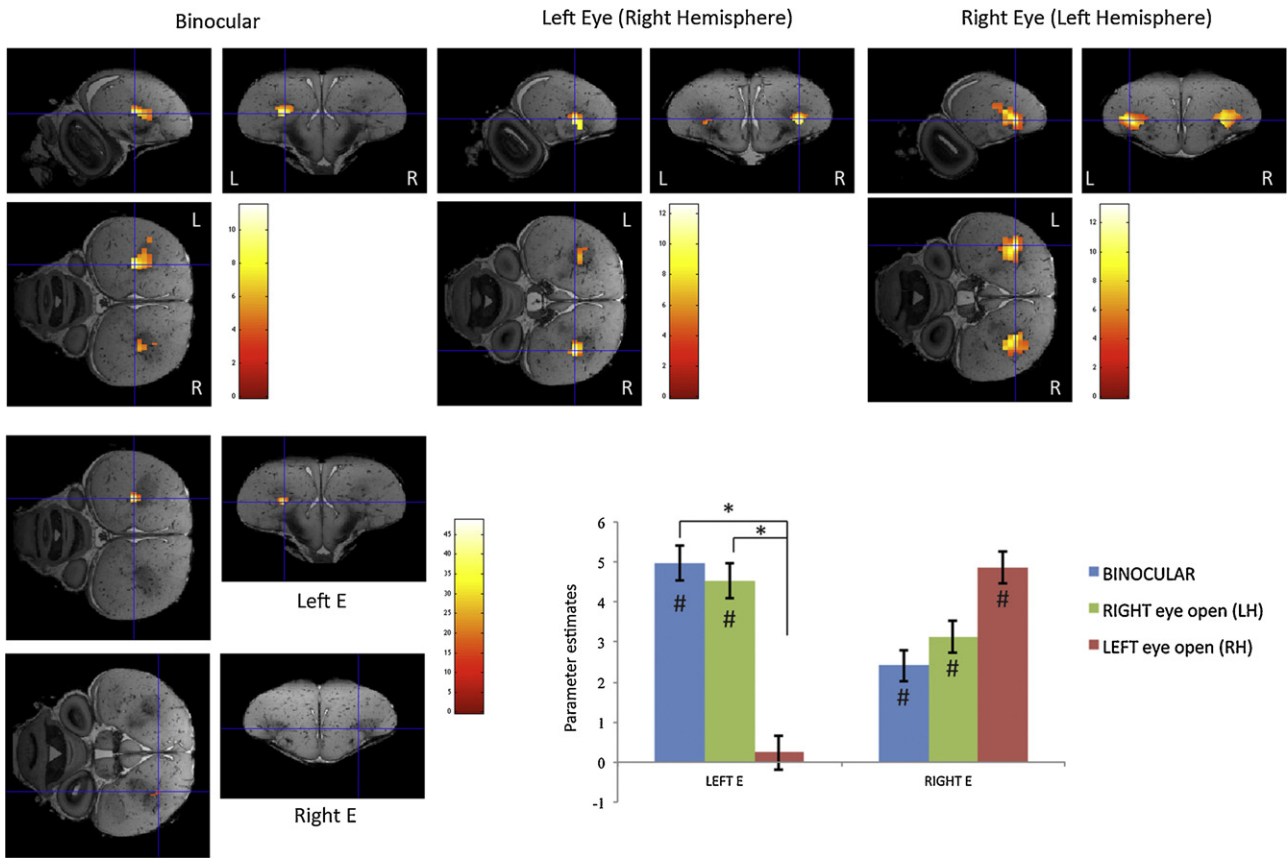


**Fig. 3.** Activations induced in awake pigeons by the visual stimuli (vs. rest) per individual. The statistical parametric maps (unilateral one sample  $t$ -test) are superimposed on anatomical images coming from the high-resolution pigeon atlas [38]. They illustrate the bilateral activation of the telencephalic entopallium (E).  $T$  values are color coded according to the scales displayed on the right side of the figure. Only voxels in which the  $t$ -test was found significant ( $p$  value  $<0.05$ , corrected for multiple comparisons at the whole brain level) are displayed.

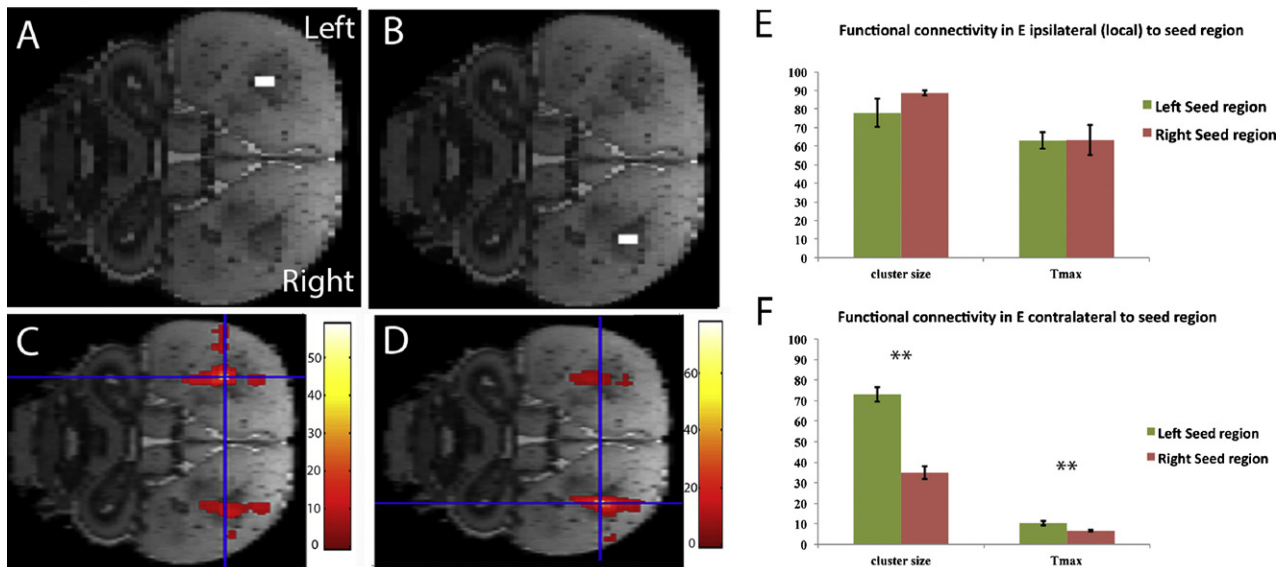
A possible concern could be the stress level of the birds while being scanned. Birds typically become motionless when they are highly stressed and may not show any extrinsic signs of discomfort. However, studies show that restraint for up to 18 h in a confined space such as a transport crate does not cause significant elevations of oxidative stress as measured in serum levels of oxidative damage and of antioxidant capacity in pigeons [44]. Conversely, pigeons that had flown around 200 km displayed increased markers of oxidative stress [44]. Therefore, we are inclined to believe that our subjects were not only calm but also experienced low levels of stress. Future studies could confirm this by measuring, e.g. the heart rate before and during scanning.

A possible problem for awake bird MRI studies is the ability of at least some bird species to sense the magnetic field. In fact, birds have a vision-based magnetic compass that perceives the inclination of the earth's magnetic field and thus derives directions from the axial course of the field lines and their inclination in space [45]. This unusual functional mode arises from the underlying physical processes: the avian magnetic compass is based on a radical pair mechanism [46] in the eye, where Cryptochrome 1a,

the most likely candidate receptor molecule for mediating directional information, is located along the disks of the outer segments of the UV-receptors [47]. There is evidence that this light-based magnetic system is at least to some extent a part of the image-forming visual pathway [48]. In addition, the ophthalmic branch of the trigeminal nerve is activated by a changing magnetic-field stimulus and relays this information to the bird brainstem [49]. It is likely that also pigeons sense the magnetic field in terms of a magnetic compass and/or local magnetic deviations [25,50,51]. In this case, our pigeons possibly perceived a visual and a trigeminal stimulation that accompanied the changes of the strong magnetic field within the scanner. It was impossible to prepare the birds for this input during the habituation training but yet the pigeons were completely calm when placed the first time into the bore. Thus, although we believe that the magnetic stimulation must have been sensed as a strong sensory input, it did not arouse the animals. Obviously, the neural effects of the magnetic stimulation were not visible as a BOLD signal, since the magnetic field (7 T) was the same during the whole measurement (including small magnetic fields generated by switching of the gradients inherent to the sequence)



**Fig. 4.** Neural substrates for visual stimulation between different eye conditions. TOP: Superimposition of the statistical fMRI results (one-sample *t*-test, *N* = 3) to the high-resolution pigeon MRI atlas [38]. Results are of those voxels displaying a significant activation during the respective condition (binocular, left eye open, right eye open). *T* values are color coded according to the scale displayed on the right side of each panel. BOTTOM LEFT: Results are of those voxels displaying a significant difference between conditions (One way repeated measures ANOVA), a statistically significant difference was seen in left E. *F* values are color coded according to the scale displayed on the right side of the panel. BOTTOM RIGHT: Estimates of the relative response amplitude (+/– SEM) of neural activations elicited during the different conditions in the left and right entopallia (the values have been extracted from the voxel with the maximum *T* value). The zero level corresponds to the mean activation level during rest periods (exposure to darkness). Hashes indicate statistically significant differences between stimulation vs. rest. Stars indicate statistical significance of comparison between conditions (*p* < 0.05, FWE corrected).



**Fig. 5.** Functional connectivity for pigeon 1 for the seed regions shown in left E (A) and right E (B). C and D show the maps resulting from data acquired in an awake pigeon for the respectively seed regions. All maps are overlaid on atlas images [38]. Comparison of *T*<sub>max</sub> and cluster size (+/– SEM) of (E) left hemispheric cluster starting from seed in left hemisphere vs. right hemispheric cluster starting from seed in right hemisphere and (F) left hemispheric cluster starting from seed in right hemisphere vs. right hemispheric cluster starting from seed in left hemisphere. Comparison made based on single subject maps. Double stars indicate statistical significance of comparison between hemispheres (*p* < 0.01).

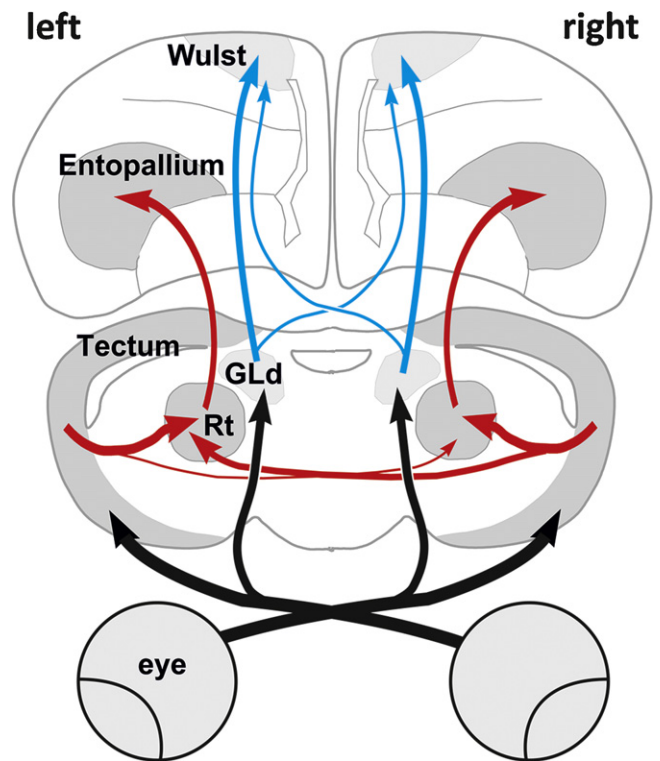
and did not follow an ON/OFF paradigm, statistical analysis of the possible BOLD response to magnetic stimulation could therefore not be done.

We could not detect a significant BOLD response in the visual Wulst although it receives visual input via the thalamofugal pathway. We assume that this is due to the geometry of our tube into which the pigeons were confined. Pigeons have two retinal areas of enhanced vision; one pointing into the binocular frontal area, the other looking monocularly sideways. The LEDs were positioned in front of the animals within their binocular area and, when activated, emitted a green light of 2.3 mcd intensity. This light source was also reflected within the dark gray and was visible to the lateral visual field of the animals with a drastically reduced luminance. It is known that the frontal visual field of pigeons is primarily represented in the tectofugal and less in the thalamofugal system [52,53]. Consequently, lesions of tecto- or thalamofugal structures primarily cause deficits in the frontal and the lateral visual field, respectively [54,55]). Thus, we possibly did not detect a Wulst activation since the luminance of the light source for the lateral visual field was much lower than for the frontal field.

#### 4.2. Visual asymmetry

Brain asymmetries are a ubiquitous vertebrate trait that possibly represents an ancient brain organization [35]. Presently, the most thoroughly studied animal model for cerebral asymmetries at the neural and the behavioral level is the visual system of birds [56]. In this group of animals, optic fibers almost completely cross at the optic chiasm. By simply restricting vision to one eye, diverse studies could show that the left hemisphere is superior for visual feature discrimination and categorization [57–59]. In birds, visual information is processed by thalamofugal and tectofugal pathways that are equivalent to the mammalian geniculocortical and extrageniculocortical systems, respectively [60]. The thalamofugal pathway consists of fibers projecting from the retina to the n. geniculatus lateralis pars dorsalis (GLd) and then to the visual Wulst in the telencephalon [61]; Fig. 6). The tectofugal pathway consists of fibers projecting from the retina to the tectum. From there, neurons ascend bilaterally to the thalamic n. rotundus (Rt), which in turn projects ipsilaterally onto the telencephalic entopallium [60].

Fig. 6 demonstrates the connective asymmetry of the tectofugal pathway. The ascending fibers that cross from the tectum to the contralateral rotundus are more numerous from the right tectum to the left thalamus than vice versa [62]. Consequently, the entopallium of the left hemisphere receives a massive input from the contralateral right and, via crossing tectothalamic fibers, also from the ipsilateral left eye. Due to the asymmetries of the crossing tectothalamic fibers, the right entopallium mainly represents the contralateral left eye only. This anatomical finding has meanwhile been verified by single and multiple cells recordings at rotunda [63] and entopallial level [64]. Additionally, lesion studies [54,65] and behavioral experiments on lateralized interhemispheric transfer [66] are in accord with the connective asymmetry of the tectofugal pathway. These findings can explain left–right differences of functional connectivity. The left rotundus receives input from tectal neurons of both half brains (Fig. 6). As a result, the left tectofugal system is functionally coupled to the right tectofugal one. Functional connectivity is expectedly lower when starting from the right hemisphere, since only few tectal neurons recross from the left tectum to the right rotundus. Thus, functional connectivity provides information about lateralized connective patterns within systems and is able to verify anatomical [62] and behavioral findings [66].



**Fig. 6.** Schematic diagram of the ascending visual pathways in pigeons. The thalamofugal pathway is depicted in blue, the tectofugal system in red. Note that the tectorotundal pathway is asymmetrically organized such that the projection from the right tectum to the left rotundus is larger than that from the left tectum to the right rotundus. As a result, the left sided tectofugal structures integrate information from both eyes to a larger extent. Abbreviations: GLd: n. geniculatus lateralis pars dorsalis; Rt: n. rotundus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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