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Social predispositions and underlying neural mechanisms in chicks (*Gallus gallus domesticus*)

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GENERAL INTRODUCTION

Core knowledge: Agents and Actions

Research in the developmental cognitive sciences has put forward the hypothesis that cognition is based on a set of specialised processes (core knowledge systems) that help infants to shape and structure the otherwise chaotic sensory input immediately after birth (Leslie 1994; Spelke 2000). According to this view, complex cognitive skills may depend on a set of four domain systems. One is devoted to the representation of approximate numerosities, and basic numerical relationships; a second one represents surfaces in the environment and continuous spatial relationships among them; a third one concerns inanimate objects and their motion, with representations of physical causality in the domain of the so-called naïve physics; a fourth system represents animate agents with their actions, belonging to the domain of naïve psychology (Leslie 1994; Spelke 2000). These systems emerge early in human ontogeny and, probably, phylogeny, and are limited in the domain and task specificity (Spelke 2000; Spelke and Kinzler 2007). Based on this core architecture, young vertebrates are able to categorize and learn from the continuous flow of sensory information.

A crucial skill for survival among all vertebrate species is to rapidly detect and monitor the presence of other creatures in the environment, being those social companions, predators or preys. The discrimination between animate and inanimate objects is thus an essential ability, probably largely inborn, that sets the stage for the development of mature social cognition (Leslie 1994).

Static and dynamic cues: evidence for social predispositions in vertebrates

Social predispositions represent a set of rudimental knowledge about animate beings that helps young vertebrates to survive and to structure their experience in order to be able to learn from it. In order to detect the presence of a living creature in the environment, an organism can rely on both static features, *e.g.* the head region, and dynamic features, *e.g.* the way it moves. Many studies showed that newborns preferentially look at face-like stimuli

(Morton and Johnson 1991; Turati et al. 2002; Farroni et al. 2005; Figure 1(a)), and the same phenomenon can be observed in infant monkeys (*Macaca fuscata*; Sugita 2008). Also in monkeys, it was found that the face preference is not species-specific before the experience occurred (Sugita 2008). Earlier studies conducted on newly hatched domestic chicks revealed that they prefer to approach a stuffed hen over an artificial object or the same hen but scrambled (Johnson and Horn 1988). Their preference is mainly driven by the features' configuration of the head and neck region. Noteworthy, their preference is not species-specific. In fact, the preference for head-neck configuration is preserved when the stimulus is a gadwall duck or even a polecat (see Figure 1(b)). This non-species-specific preference have been confirmed in chicks showing that they also have a spontaneous preference to approach schematic face-like stimuli and even photos of human faces (Rosa-Salva et al. 2010, 2011, 2012b). It is important to underline that chicks undergo the process of filial imprinting. Therefore, it is extremely important for a visually naïve chick to approach an animate rather than an inert object, in order to subsequently imprint on it.

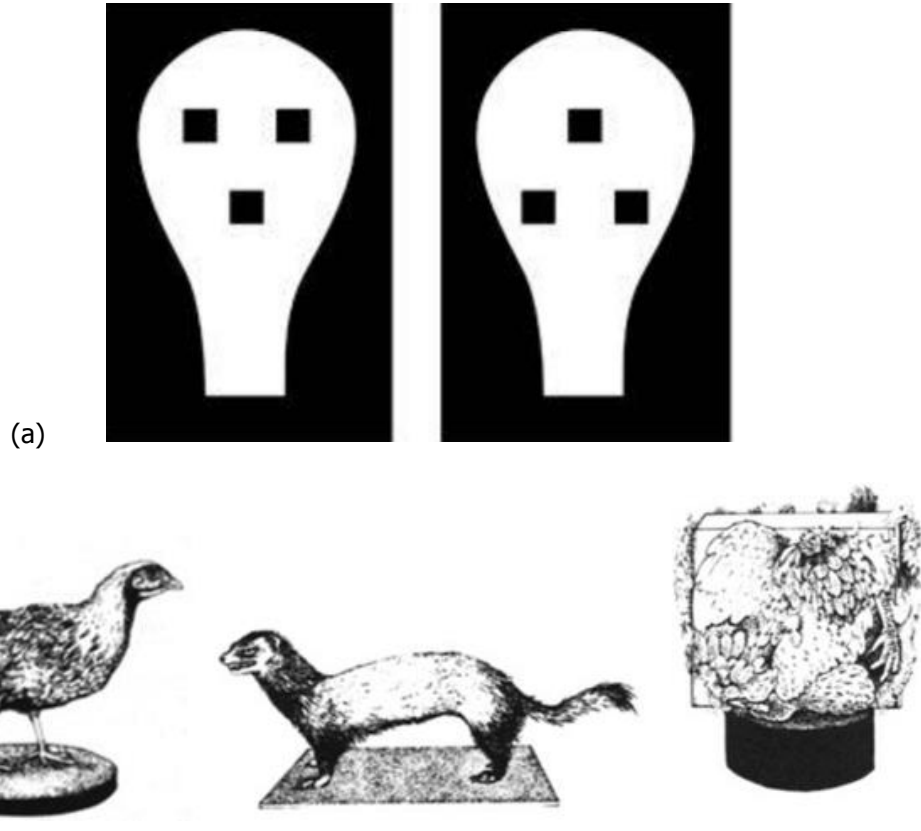


Figure 1: Static cues eliciting social predispositions. (a) A couple of stimuli used by Farroni and colleagues to investigate newborns' preference for faces: the first image (face-like) was preferred over the second one, in which the same features are simply inverted (Farroni et al. 2005). (b) Schematic representation of the stimuli originally used by Johnson and Horn. From left to right: a taxidermied jungle fowl, a polecat and an identical jungle fowl cut in pieces and reassembled in a scrambled fashion (Johnson and Horn 1988). The fowl and the polecat were preferred over the scrambled fowl box, both in fact present the head-neck configuration.

In general, moving objects should be preferred over static ones because they are more easily discriminated from background. Moreover, the movement attracts attention because it is biologically relevant to attend and monitor objects that are in a continuous state of transition. However, not every kind of movement has the same efficacy in capturing and re-directing attention. This has been extensively studied in the case of biological motion using point-light displays (PLD). In the biological motion pattern, the points are moving in a semi-rigid manner because they are located in correspondence of the joints of a moving

animal. Any other visual morphological cue apart from the moving configuration of those points is removed (see Figure 2). The first evidence for a spontaneous preference for biological motion patterns over rigid or random motion has been found with visually naïve chicks (*Gallus gallus*; Vallortigara et al. 2005). Later it has been shown that also newborn babies (Simion et al. 2008), marmosets (*Callithrix jacchus*; Brown et al. 2010), and fishes (*Oryzias latipes*; Nakayasu and Watanabe 2013; Schluessel et al. 2015) prefer the biological motion pattern. As in the case of face-like stimuli, also for biological motion there is no preference for the species-specific movement. Indeed, chicks not only prefer a PLD depicting a walking hen to rigid or random motion, they also prefer one depicting a walking cat. Noteworthy, no preference was found for either the hen stimulus neither the cat when were presented simultaneously (Vallortigara et al. 2005; Figure 2).

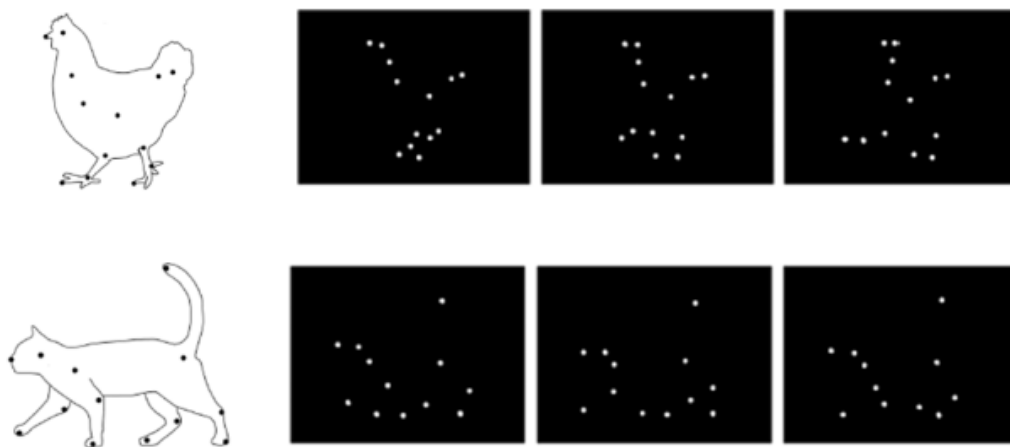


Figure 2: Dynamic cues eliciting social predispositions.

Schematic representation of PLDs used to study biological motion's preference in visually naïve newly hatched chicks. Images on the left represent where the points were located to create the animations. In the first line is depicted the hen stimulus, in the second line the cat stimulus (Vallortigara et al. 2005).

A few studies have focussed on the elementary features (*e.g.* changes of speed, self-propulsion) that distinguish animate from inanimate motion. One of these features is the presence of an internal energy source within the moving object. This can be inferred if an object starts to move from rest alone, or if it needs a physical contact with another object to start moving. Human infants discriminate objects that starts to move on their own, and they generate coherent expectations about those objects (Gelman 1990; Leslie 1994; Luo

et al. 2009). Newly hatched visually naïve chicks prefer to imprint on an object that visibly starts from rest over another one, which is set in motion by physical contact with the first object (Mascalzoni et al. 2010; Figure 3). Similarly, human newborns share with chicks the same preference for self-generated motion (Mascalzoni et al. 2013; Di Giorgio et al. 2016b).

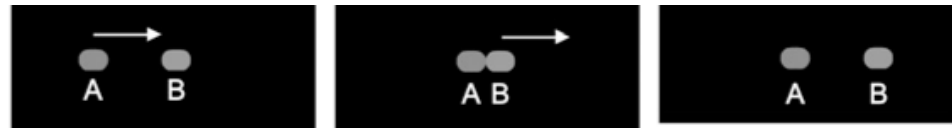


Figure 3: Start from rest and self-propulsion.

Schematic representation of the animation used for imprinting by Mascalzoni and colleagues: object A starts from rest alone, stops moving near object B, which starts to move immediately after (Mascalzoni et al. 2010). Subsequently, in a preference test chicks preferred to approach object A (therefore preferential imprintability).

Other cues to self-propulsion – *e.g.* change in direction, violation of gravity, changes in speed – have been found to automatically attract attention of human adults (Tremoulet and Feldman 2000; Abrams and Christ 2003; Gyulai 2004; Szego and Rutherford 2007, 2008). Interestingly, infants seem to prefer changes in speed rather than changes in direction (Frankenhuis et al. 2013), and the same do chicks as it is demonstrated in the first study of the present thesis (see Study 1: Rosa-Salva et al. 2016). In this study, we demonstrated that newly hatched visually naïve chicks spontaneously prefer a simple object that accelerates and decelerates over one that moves at constant speed. Chicks' preference was driven by the two moments of visible speed change in the motion pattern. Indeed, when such moments were not visible, any preference disappeared.

Underlying neural mechanisms

CONLERN and CONSPEC: cortical and subcortical structures

The CONSPEC and CONLERN model has been proposed as a two-process theory to account for infants' face recognition (Morton and Johnson 1991). CONSPEC was hypothesised as a domain-specific face-detecting mechanism already active at birth, guiding the preference for face-like stimuli in newborns. A second mechanism, CONLERN, would be responsible for learning the features of those objects, toward which CONSPEC has oriented infant's

attention, mainly conspecifics' characteristics. Studies with newborns support the idea that CONSPEC would be a subcortical mechanism receiving input from the retinotectal pathway (Simion et al. 1998; Johnson 2005). Indeed, the visual behaviour of newborns until two months of age seems to be mainly guided by subcortical structures, since the retinogeniculo-cortical pathway is not fully developed (Atkinson 1983; Braddick et al. 1985; Atkinson and Braddick 1989; Atkinson et al. 1992; Braddick et al. 1992; Kraemer and Sjöström 1998; Braddick et al. 2003). This subcortical route hypothesis postulates the involvement in human adults of a subcortical pathway comprising superior colliculus, amygdala, and pulvinar, which should process faces rapidly, but not accurately (Johnson 2005). Moreover, this subcortical route may be responsible for the residual face detection ability in adult patients with cortical lesions, who are unable to process face identity (Vuilleumier 2000; Vuilleumier and Sagiv 2001; Morris et al. 2001; DeGelder et al. 2003). On the other hand CONLERN, a domain-relevant system that, under typical circumstances, comes to specialize in faces recognition, was postulated to be a cortical network (Johnson et al. 2015).

Behavioural evidence reviewed in the previous section highlights the need to use an experimental model, in which visual experience can be precisely controlled for. Indeed, it is extremely important to ensure the complete absence of specific visual learning prior to the test. Moreover, direct evidence is needed to support the hypotheses on neural correlates of social predispositions. For both ethical and practical reasons human newborns and, in general, the offspring of altricial species are not a suitable model to test these hypotheses. The domestic chick belongs to a precocial nidifugus species, autonomous soon after hatching. This allows testing it immediately after birth, while applying strict control of its sensory experience. Indeed, chicks can hatch in artificial incubators in the darkness and isolated from conspecifics. They have shown a wide range of perceptual and cognitive skills immediately after hatching, proving to be a valuable tool for the investigation of innate (not learned) factors. Chicks have been used extensively in comparative developmental research in parallel with human infants. This research has revealed the presence of remarkable similarities in the fundamental predispositions and cognitive mechanisms available early in life in these two phylogenetically distant species (Johnson and Horn 1988; Morton and Johnson 1991; Farroni et al. 2002; Vallortigara et al. 2005; Vallortigara and Regolin 2006; Rosa-Salva et al. 2007; Simion et al. 2008; Mascialzoni et al. 2010; Di Giorgio et al. 2016b).

Given such characteristics, chicks may serve as an excellent model for the study of neural correlates of social predispositions.

Avian brain compared to mammalian brain

Quite recently avian brain nomenclature underwent a review process due to improved knowledge on vertebrate brain evolution (Reiner et al. 2004). According to the classical view, based on the old concept of *Scala naturae*, evolution would be progressive and linear from fishes, to amphibians, to reptiles, to birds and mammals (and, among mammals, to primates and eventually to humans). According to this view, vertebrates' brains were organized progressively from the less complex of fishes to the most complex of mammals. Hence, the avian brain was believed to be mainly responsible for instinctive and more primitive behaviour, except for a small part of telencephalon thought to be pallial and involved in learning and *intelligent* behaviour (Edinger 1888, 1896, 1908; Northcutt 2001). Thanks to the initial observations that the thalamus sends visual, auditory and somatosensory input to the telencephalon in all vertebrates as the mammalian one sends to the neocortex (Karten 1969; Zeier and Karten 1971; Karten and Shimizu 1989; Shimizu and Hodos 1989; Mello and Clayton 1994; Vates et al. 1996). Thanks also, to the discovery of new methods to study brain connectivity (Karten and Dubbeldam 1973; for a review see Karten 2015), the anatomical profiles of gene products (Northcutt 2001), and embryo development (Puelles et al. 2000), evidence has been found for drawing homologies among many telencephalic regions in birds and cortical regions in mammals. The avian hyperstriatum, neostriatum and archistriatum – respectively hyperpallium, nidopallium and arcopallium according to the revised nomenclature – should be homologous to the mammalian pallial regions (Reiner et al. 2004; Jarvis et al. 2005; see Figure 4). However, one-to-one homologies between striatal regions and pallial ones are still under debate.

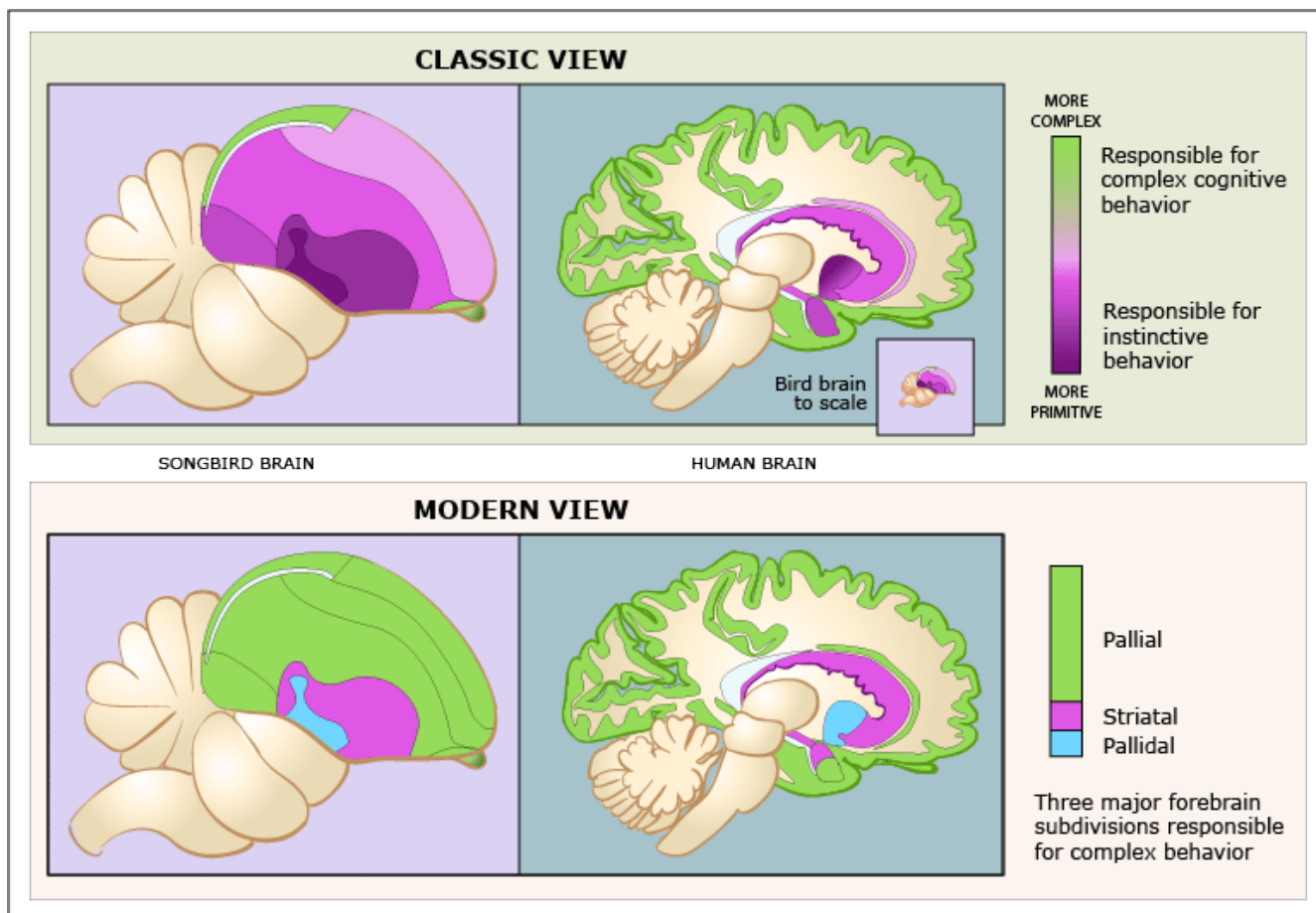


Figure 4: Avian brain compared to human, classic and modern view.

A schematic representation of an avian brain on the left side is compared with a human one on the right side of the image. In the first row is visible in different tones of violet, how the classical view interpreted a great part of avian telencephalon as responsible for instinctive behaviour. In contrast, on the second row the telencephalic regions now believed to be homologue of mammalian pallium are depicted in green (image taken from: http://avianbrain.org/new_terminology.html).

According to the CONSPEC and CONLERN model, in order to find the neural substrate for social predispositions in birds, candidate structures could be sub-pallial regions for CONSPEC and pallial telencephalic regions for CONLERN. The sub-pallial route in birds should be composed of the optic tectum, homologue to the mammalian superior colliculus, the arcopallium and the nucleus Taeniae, supposed to be respectively the pallial and sub-pallial avian homologue for the mammalian amygdaloid complex (Cheng et al. 1999; Reiner et al. 2004; Jarvis et al. 2005; Yamamoto and Reiner 2005; Yamamoto et al. 2005; Moreno and González 2007; Martínez-García et al. 2008) and the nucleus rotundus homologue to

the pulvinar complex (Jarvis et al. 2005). On the other hand, a plausible substrate for CONLERN in birds could be the intermediate medial mesopallium (IMM, according to the new nomenclature; IMHV, Intermediate medial hyperstriatum ventrale according to the old nomenclature; Reiner et al. 2004; Jarvis et al. 2005). The IMM is a small telencephalic region widely described as being involved in learning the features of the imprinting object (Horn 1998, 2004).

The social behaviour network and the mesolimbic reward system

Apart from the CONSPEC and CONLERN model theorised to account for the development of social skills in young vertebrates, a more specific brain network was postulated to account for complex social behaviour in adult individuals – the so-called social behaviour network. Newman (1999) first described the social behaviour network in mammals as a large integrated network that controls several different social hormone-regulated behaviours composed by amygdala, septum, preoptic area, midbrain and hypothalamus. All nodes of this network contain by definition sex steroid hormone receptors and are essential for many complex social behaviours such as mating, sexual, parental, and aggressive behaviours. Although homologies among vertebrates' brain networks should be drawn carefully (Goodson and Kingsbury 2013), the social behaviour network has been extended to all vertebrates (O'Connell and Hofmann 2011) and combined with the mesolimbic reward system in a larger integrated social-decision making network (O'Connell and Hofmann 2011; Figure 5). In fact, the salience of the stimulus has to be evaluated before emitting the appropriate behaviour. Moreover, to be adaptive, a social behaviour has to be rewarding (O'Connell and Hofmann 2011). The mesolimbic reward system is a dopaminergic pathway mainly involved in the evaluation of the salience of the stimuli, in reinforced and motivated behaviours (Alcaro et al. 2007). These two systems share two nodes: the septum and the amygdala. As stated in the previous section, the amygdala is a node shared also with the subcortical route for the face processing of CONSPEC. The septum is a sub-pallial mesolimbic region highly conserved among vertebrates (Puelles et al. 2000; Lanuza and Martínez-García 2009). It is a heterogeneous structure rich in arginine vasotocin, the avian homologue of arginine vasopressin in mammals, which is known to mediate parental care and parent-offspring bonding (Martinez-Vargas et al. 1976; Barfield et al. 1978; Watson and Adkins-Regan 1989; Balthazart et al. 1992; Goodson and Bass 2001; Baeyens and

Cornett 2006). In fact, the septum is involved in establishing the parent-offspring affiliation from the parents' side in zebra finches (*Taeniopygia guttata*; Goodson et al. 2009). Evidence in various avian species also shows its involvement in consummatory sexual behaviour (Taziaux et al. 2006), in gregariousness (Goodson et al. 2009; Kelly and Goodson 2014) and in aggressive and submissive behaviours (Nishizawa et al. 2011).

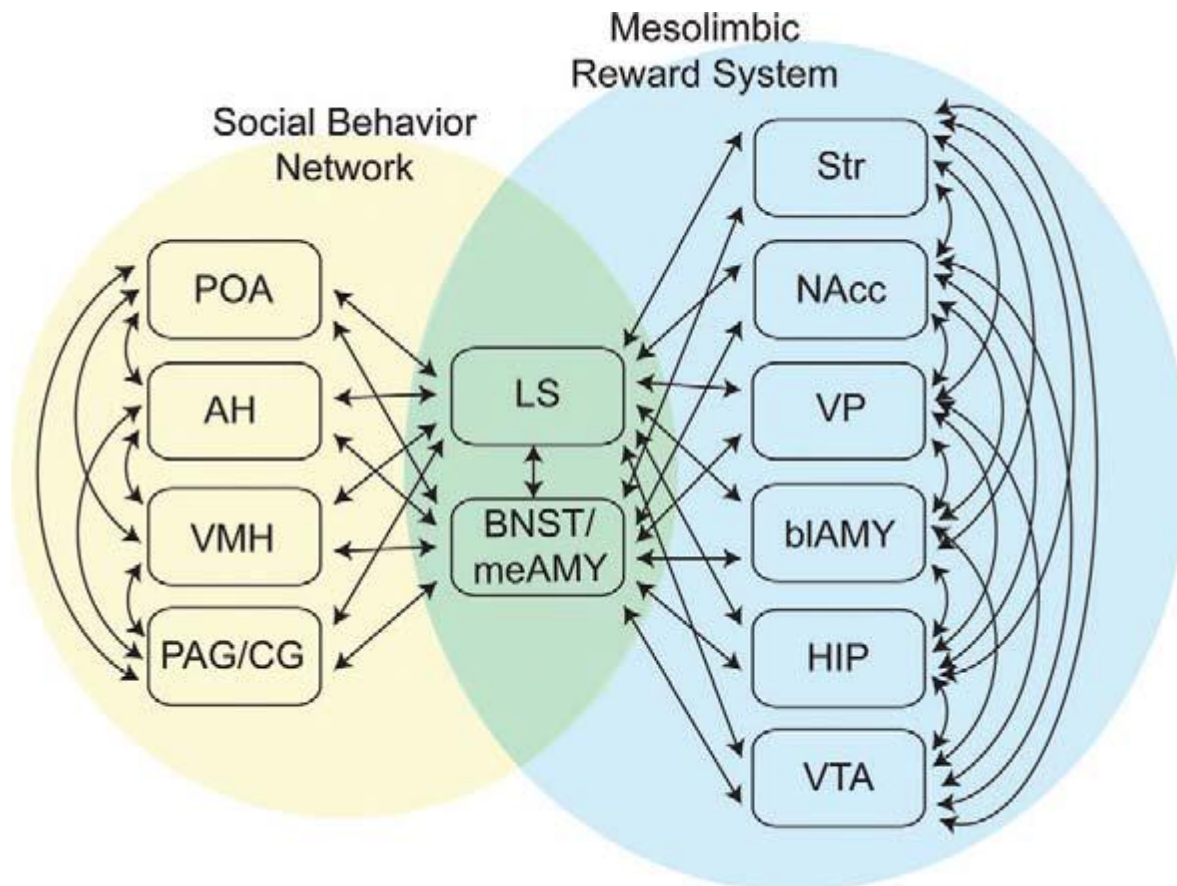


Figure 5: Social decision-making network.

Schematic representation of the social decision-making network proposed by O'Connell and Hofmann in 2011. In yellow on the left are the brain regions of the social behaviour network, on the right in blue areas of the mesolimbic reward system and in the centre areas involved in both systems (green). Black arrows indicate the anatomical connections within each system in mammals. POA, preoptic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; PAG/CG, periaqueductal grey/central grey; LS, lateral septum; BNST/meAMY, bed nucleus of the stria terminalis/medial amygdala; Str, striatum; NAcc, nucleus accumbens; VP, ventral pallidum; bIAMY, basolateral amygdala; HIP, hippocampus; VTA, ventral tegmental area (image taken from O'Connell and Hofmann 2011).

Remarkably, two previous studies with newly hatched visually naïve chicks (*Gallus gallus domesticus*) showed an involvement of septum in the perception of movement of an alive conspecific (Mayer et al. 2017b, a). Interestingly in the first study visually naïve newly hatched chicks were individually exposed either to an alive conspecific (another same age chick) or to an empty chamber (see Figure 6(a)). Septum and arcopallium (supposed the avian pallial homologue for the mammalian amygdala) were differentially activated in the two groups. Chicks exposed to the alive companion showed more activity in these areas when compared to the control chicks exposed to the empty chamber (Mayer et al. 2017b). In the second study, the control group were individually exposed to a stuffed rotating chick, in order to counterbalance the static features of the social companion, but not the peculiar pattern of movement (see Figure 6(b)). A different involvement of septum was observed between the two groups, chicks exposed to the alive conspecific had higher activity in the right septum (Mayer et al. 2017a). Whereas, no difference was found between the two groups in arcopallium. These data suggests arcopallial involvement in processing of static configuration of features such as face-like stimuli in line with what was hypothesised in humans (Johnson 2005). Instead, septum seemed to be more selective for motion cues. Therefore, the aim of the present study is to investigate neural correlates of social predispositions in newly hatched visually naïve chicks with particular reference to the role of septum, toward which we had strong *a priori* expectations when investigating animate motion.

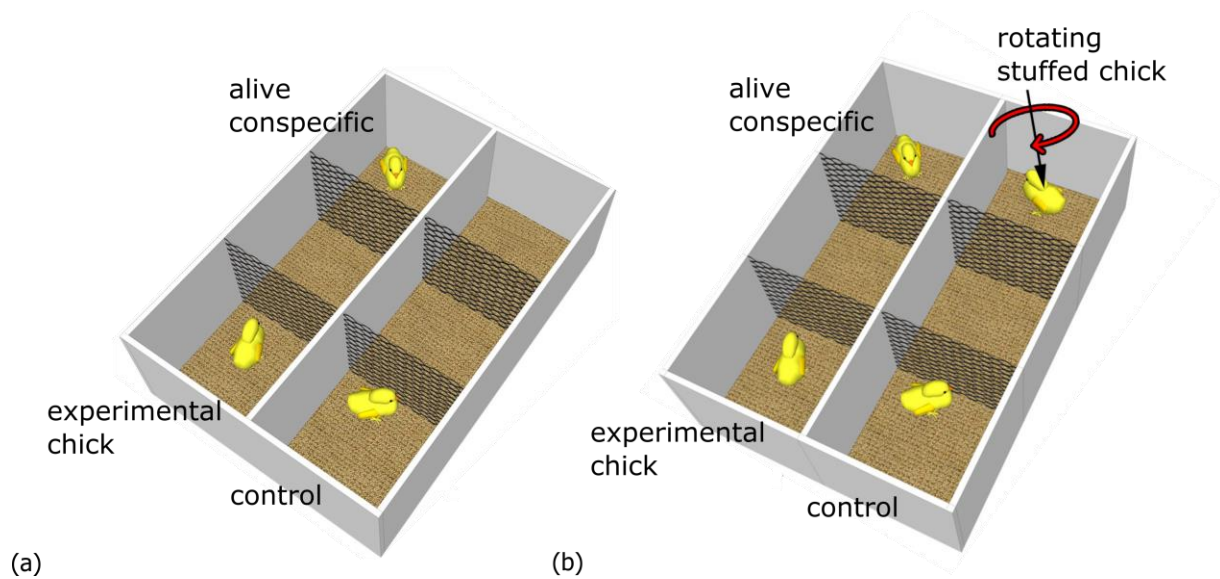


Figure 6: First exposure to an alive conspecific. Experimental setups used from Mayer and colleagues to test the visual neural correlates of the first exposure to an alive conspecific in chicks. (a) In a first study, the experimental chicks saw an alive conspecific, whereas the controls saw an empty chamber. (b) In the second study, the treatment for the experimental group remained constant, while the control group saw a stuffed chick rigidly rotating. In both studies, the acoustical and olfactory stimulations were balanced between groups. Images adapted from Mayer et al. 2017b, a.

Immediate Early Genes (IEGs)

Immediate early genes (IEGs) are a class of around 30-40 genes that are rapidly activated in response to an increase of neuronal activation. They are supposed to play an important role in neuronal plasticity related to learning (Lanahan and Worley 1998; Jones et al. 2001; Guzowski 2002; Fleischmann et al. 2003; Kubik et al. 2007). IEGs' expression enables the visualisation of complete patterns of neuronal activity during learning keeping intact neuronal networks and functioning (Miyashita et al. 2008; Barry and Commins 2011). Their expression is very low or absent in non-activated cells, but it rapidly increases in response to *trans*-synaptic signalling between neurons (Sheng and Greenberg 1990). Among them 10-15 are classified as regulatory transcriptional factors regulating the expression of downstream genes (Lanahan and Worley 1998; see Figure 7). Their products have been successfully used to detect neuronal activity in mammals as well as in birds (Milbrandt 1987; Cole et al. 1989; Mello et al. 1992; Smulders and DeVoogd 2000; Mayer et al. 2010; Mayer and Bischof 2012; Mayer et al. 2016a). In Figure 7 is exemplified how regulatory transcription factor IEGs are related to neuronal activation. When the neuron is activated, a protein cascade is generated. This protein cascade activates IEGs expression inside the nucleus of the neuron. Thanks to the mRNA IEG's expression is translated in the production of specific proteins, product of IEGs' expression. Once the protein product of these genes are translated, they enter back inside the neuronal nucleus where they regulate the transcription of late-response genes (Barry and Commins 2011). Late-response genes' expression will then directly affect cell structure and function (Tischmeyer and Grimm 1999). Among them c-Fos is a widely used IEG product known to be involved in plastic changes of neuronal networks related to learning (Kaczmarek 1993). A common method used to detect IEGs' expression is an immunohistochemical procedure using specific antibodies against IEGs' products, proteins. In Figure 7 is reported an example of an immunohistochemical staining for c-Fos from our lab. In the enlarged portion of the photomicrograph, black nuclei are clearly visible against the background stained green neurons. Black stained nuclei are c-Fos-ir (immunoreactive) neurons. Eventually, IEGs expression and in particular *c-fos* has been shown to be higher during the first stages of learning and memory formation (Anokhin and Rose 1991), making it an optimal methodology to study neuronal correlates of social predispositions in newly hatched vertebrates.

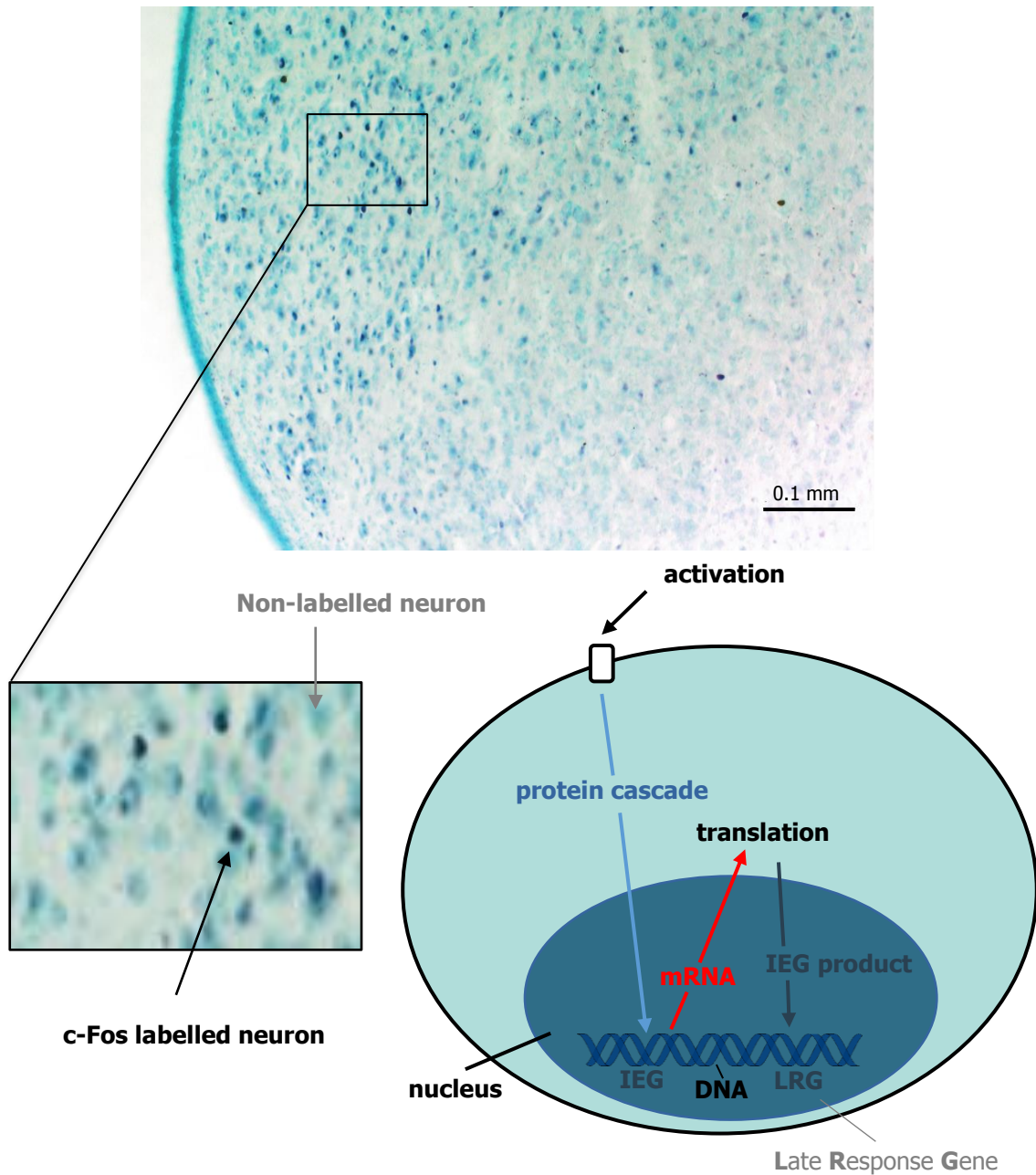


Figure 7: Immediate Early Genes.

Photomicrograph of a coronal section of a chick brain at the level of IMM (intermediate medial mesopallium) and a detail of it at higher magnification. c-Fos-ir nuclei are stained in dark (black arrow) clearly discernible from the methyl green counterstained background and non-labelled cells (grey arrow). Schematic representation of how IEGs are related to neuronal activation. An action potential reaches the neuron generating a protein cascade, which is activating IEGs. IEGs, thanks to mRNA, translate rapidly into proteins (IEG product), which enter back the nucleus modulating the activity of other late-response genes (LRG).

Aim of the thesis

The aim of the present work was to investigate different types of social predisposition in newly hatched visually naïve chicks and their neural correlates using behavioural as well as neurobiological techniques. The experimental part that follows is subdivided in two main sections. A first section about social predispositions and underlying neural mechanisms in chicks, itself subdivided in two parts focussing respectively on dynamic cues and static features eliciting predispositions. A second section about spontaneous lateralisation of the brain. Despite the apparent oddity, this second section and the relative study reported came as a natural consequence to shed some light on the lateralised results obtained in the brain studies of Section 1 and in other experiments in our laboratory.

The aim of Study 1 was to investigate whether some very elementary motion cues such as speed and direction changes are able to elicit alone spontaneous preference in newly hatched visually naïve chicks. Moreover, we wanted also to validate a very simple and controlled set of stimuli for future brain investigations. The hypothesis behind was that speed changes as well as direction changes could be preferred over constant velocity and trajectory patterns of motion because they exhibit visible cues of self-propulsion, implying an internal energy source to the simple moving object.

Once established a stable behavioural preference between a couple of simple stimuli differing only in the presence of one elementary motion cue related to self-propulsion, we designed Study 2. We wanted to investigate the neural substrates involved in the perception of simple animate motion cue that chicks spontaneously prefer immediately after hatching prior to any other visual experience and learning. We hypothesised an involvement of some areas of the social decision making network such as septum, preoptic area and amygdala homologues and of an area known to be involved in learning the features of the imprinting object, intermediate medial mesopallium (IMM).

The aim of Study 3 was to replicate an early finding (Johnson and Horn 1988) about spontaneous preference in chicks for a naturalistic configuration of features over the same features in a less naturalistic arrangement (stuffed hen *vs.* scrambled pieces of the stuffed hen). Once replicated the preference, the neural correlates of this preference were investigated. We hypothesised differential activation in optic tectum, the avian homologue

of the mammalian superior colliculus and in IMM, as social predispositions in chicks may act by directing attention toward more naturalistic objects for subsequent imprinting.

As mentioned above the scientific question of Study 4 was a direct consequence of the lateralisation pattern observed in Studies 2 and 3 and in other experiments in our lab. We frequently observed the presence of a spontaneous asymmetry in favour of the left hemisphere unrelated to the different experimental manipulations and without any embryo light stimulation in a critical window known to trigger asymmetries in chicks visual projections from the thalamus to the forebrain (Deng and Rogers 1997). We designed three conditions: absence of any visual stimulation (dark-dark), embryo light stimulation and no visual experience after hatching (light-dark), no embryo stimulation and symmetrical featureless visual stimulation after hatching (dark-light). We hypothesised to find a spontaneous left lateralisation trend in all three conditions, with a more pronounced effect in the embryonic stimulated group (light-dark) and a decreased asymmetry in the group with the visual experience after hatching (dark-light).

EXPERIMENTAL PART

SECTION 1: Social predispositions and underlying neural mechanisms

Dynamic cues eliciting social predispositions

The detection of animate creatures is fundamental in animal species. Simple shapes moving in a self-propelled fashion implying the presence of an internal energy source are spontaneously perceived as animate and engage attention since birth: *e.g.*, autonomous changes in speed are associated with animacy perception (Abrams and Christ 2003; Gyulai 2004; Szego and Rutherford 2007; Frankenhuys et al. 2013). Here we investigate whether such an elementary motion cue can attract visually naïve domestic chicks. We also investigate the neuronal basis of this phenomenon by staining the immediate early gene product c-Fos as neuronal activity marker.

Study 1: Spontaneous preference for visual cues of animacy in naïve domestic chicks: The case of speed changes¹

The present study was partially performed during my internship and became the topic of my master thesis (Experiments 1 to 4). The last two experiments were instead run at the beginning of my PhD program (Experiments 5 and 6). A summary of the experiments presented for my master degree is provided at the end of the introduction.

Abstract

Animacy perception arises in human adults from motion cues implying an internal energy source to the moving object. The internal energy of the object is often represented by a change in speed. The same features cause preferential attention in infants. We found that speed changes affecting adults' animacy ratings elicit spontaneous social preferences in visually-naïve chicks. Human observers evaluated the similarity between the movement of a red blob stimulus and that of a living creature. The stimulus entered the screen and moved along the azimuth; halfway through its trajectory it could either continue to move at a constant speed or linearly increase in speed. Animacy ratings of humans were higher for accelerating objects. Naïve chicks were then tested for their spontaneous preference for approaching the stimulus moving at a constant speed and trajectory or an identical stimulus, which suddenly accelerated and then decelerated again to the original speed. Chicks showed a significant preference for the 'speed-change stimulus'. Two additional controls showed that matching the variability of the control 'speed-constant' stimulus to that of the 'speed-change stimulus' did not alter chicks' preference for the latter. Chicks' preference was suppressed by adding two occluders on both stimuli, positioned along the object trajectory in such a way to occlude the moment of the speed change. This confirms that, for chicks to show a preference, the moments of speed change need to be visible. This is the first demonstration of social predispositions for speed changes in any naïve model or non-human animal, indicating the presence of an attentional filter that orients attention toward one of the general properties of animate creatures. The similarity with human data suggests a phylogenetically old mechanism shared between vertebrates.

¹ Please note that data here reported have been published on *Cognition* in 2016 (Rosa-Salva et al. 2016).

Introduction

From the pioneering studies of Heider and Simmel (Heider and Simmel 1944) and of Michotte (1963) took root a number of studies focussing on the perception of simple animations that elicit in the observer the impression of animate objects acting in the scene. Interestingly, human adults presented with these kind of cartoons reported the perception of functional relations between the objects moving that are not present. For example, in the study of Heider and Simmel the participants usually reported that the small circle was *escaping* from the big triangle (1944; Figure 8), inferring the presence of an agency in the movement of the 2-dimensional shapes simply by looking at their movements. Thanks to the different studies performed, it is now known that some kinematic patterns elicit automatic and fast perceptions of animacy, causality and agency (Tremoulet and Feldman 2000). Some of these findings have been replicated also with human infants and newborns (Luo and Baillargeon 2005; Csibra 2008; Luo et al. 2009; Luo 2011; Frankenhuys et al. 2013; Mascalzoni et al. 2013; Di Giorgio et al. 2016b). The mechanisms behind these phenomena have been hypothesised as acting implicitly in the visual system, by categorising the perceived world into animate and inanimate beings (Scholl and Tremoulet 2000).

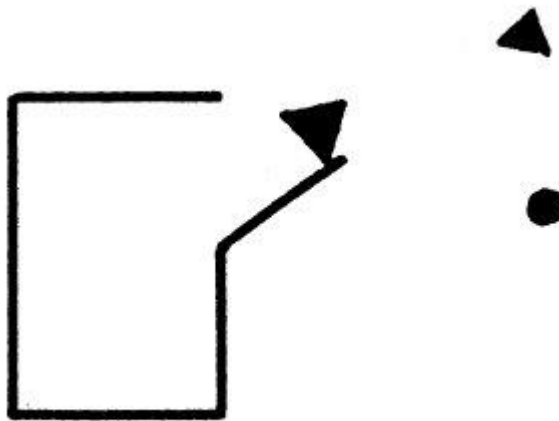


Figure 8: Heider and Simmel.

A photogram from the film originally used by Heider and Simmel in 1944. The three objects move around the scene coherently to the constraint of the "house" and generating in human observers the impression of animate beings interacting.

Recently, a series of comparative studies started to address the developmental and phylogenetic origins of this mechanism (animate vs. inanimate), by investigating the

perception of biological motion and faces in visually naïve animals and human newborns (Vallortigara et al. 2005; Vallortigara and Regolin 2006; Simion et al. 2008; Rosa-Salva et al. 2010, 2011, 2015). Thanks to the use of a precocial animal model with its embryonic development *ex-utero*, the role of nature and nurture in social predispositions can be systematically assessed from both a behavioural and a neural perspective (Spelke 2000; Spelke and Kinzler 2007; Carey 2009; Vallortigara 2012).

In the present study, we investigated the role of simple motion cues that induce the perception of self-propulsion in visually naïve newly hatched chicks. In fact, only one study directly investigated the role of self-propulsion in chicks, by presenting them with the classical Michotte's launching effect (Figure 9). Two simple shapes of different colours are presented; one is spontaneously starting to move from rest, the other one apparently moves after *being pushed* by the other. In a subsequent preference test between the two different objects, chicks preferred to approach the self-propelled one (Mascalzoni et al. 2010). For the first time here, we investigated the role of self-propulsion in visually naïve animals using a single object. Crucially, this set of experiments could understand whether this cue of animacy is inborn or experience dependent, thanks to the use of the chick as an animal model.

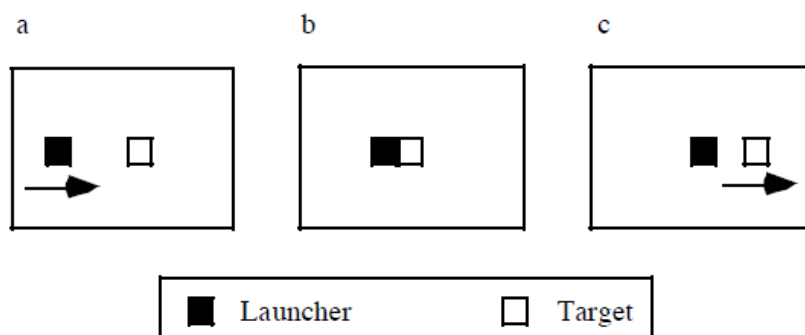


Figure 9: Michotte's launching effect.

Schematic representation of the typical Michotte's launching effect adapted from Hubbard and Ruppel 2002. (a) A black object already in motion approaches a stationary white object. (b) The black object contacts the white and becomes stationary. (c) The white moves away after contact.

In this first set of experiments, we were able to clearly show a spontaneous behavioural preference for speed changes in visually naïve chicks. In a first experiment, visually naïve

chicks did not show any significant preference between a stimulus changing direction and speed simultaneously and a stimulus moving at constant speed. In order to understand the absence of chicks' preference for two cues that increased animacy perception in human observers in previous studies (Tremoulet and Feldman 2000), we showed stimuli similar to those used with chicks to human subjects. To disentangle the role of speed change and direction change, human observers were asked to give animacy ratings to four types of stimuli: constant moving (*inanimate*), direction change and speed change together, speed change alone and direction change alone. Participants' evaluations revealed a significant main effect of speed change, regardless of the presence of direction changes. Taking advantage of these findings, the *animate* stimulus in the following experiments performed only visible changes in speed. The red circle of the *animate* stimulus suddenly accelerated at one-third of its trajectory and at two-thirds decelerated back to the initial speed (Figure 10). The *inanimate* stimulus was moving at constant speed, average speed between the two stimuli was identical. Chicks showed a significant preference for the *animate* stimulus.

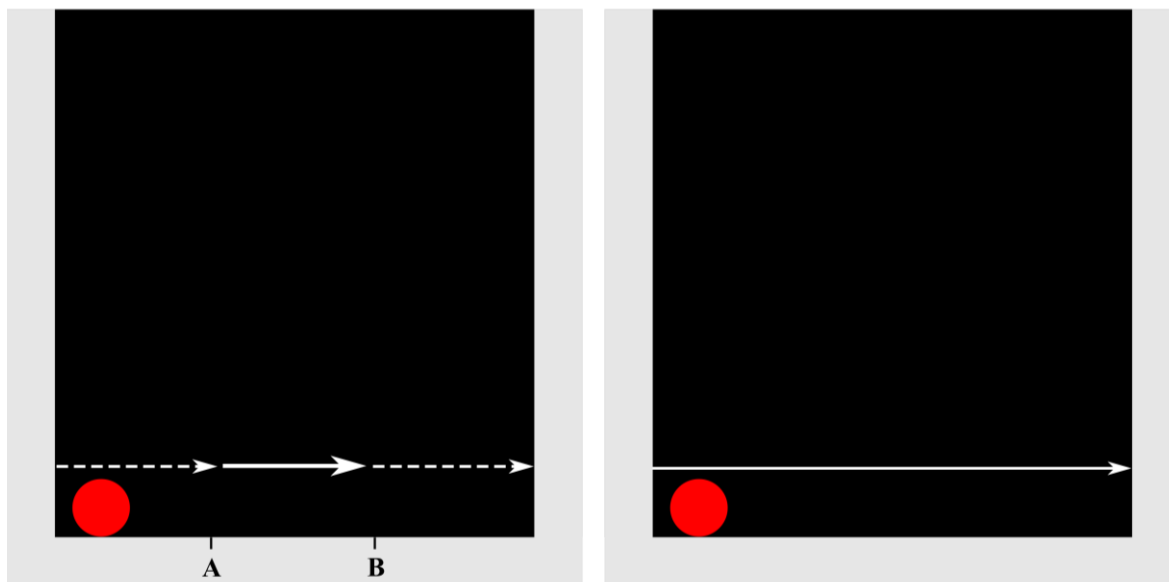


Figure 10: Stimuli eliciting spontaneous preference for speed changes. Schematic representation of one couple of stimuli used for this set of experiments (Exp. 2-4 of the original paper). On the left side, the animate experimental stimulus increase its speed in point A accelerating and decelerates back to initial velocity in point B. On the right side, the inanimate control stimulus is moving at constant speed (Rosa-Salva et al. 2016).

Since the *animate* stimulus was differing from the *inanimate* one also for the overall variability, the level of variability could also account for chicks' preference. In order to control the amount of variability present in the *animate* stimulus, two additional experiments were designed. The *animate* stimulus was identical to the previous experiment, whereas the *inanimate* one was moving at two constant speeds, alternating a constant-slow and a constant-fast velocity. The change in speed of the new *inanimate* object happened when not visible, behind the lateral grey walls. In one experiment, the average speed over one complete cycle was kept identical between *animate* and *inanimate* object. In another experiment, the two constant speeds were manipulated in order to match the relative difference between the two speeds of the *animate* stimulus. Chicks maintained the preference for the *animate* stimulus.

A control condition covering the two moments of speed change was added to verify whether the preference was driven by the cues to self-propulsion. A last experiment was run to clarify the role of direction changes, by implementing a more naturalistic pattern of trajectory change. A detailed description of the last two experiments follows.

Experiment 1 (Exp.5 of the original manuscript)

In the previous experiments, we were able to establish a stable preference for the *animate* stimulus, visible speed-changes, over other stimuli of comparable intrinsic variability, but without visible changes in speed. We thus decided to test whether chicks' preference was exactly driven by the two moments of visible speed change in the *animate* stimulus. In order to control for this hypothesis, we covered the two moments of speed change in the *animate* stimulus. Our prediction was a drop in chicks' preference for it.

Subjects

Fifty-one chicks (26 males) were employed for this experiment. The subjects employed for the entire present work were domestic chicks (*Gallus gallus domesticus*). The "Hybro" strain, a local variety derived from the White Leghorn breed, was employed. We obtained fertilised eggs from a local commercial hatchery (Agricola Berica, Montegalda (VI) - Italy). Eggs were incubated and hatched within a Marans P140TU-P210TU incubator at a temperature of 37.7°C, with 60% humidity. The incubator was kept in complete darkness, preventing any visual experience during incubation and hatching. This was done to prevent any visual experience prior to the experimental manipulation. Subjects that underwent behavioural tests were immediately after the end of the test, housed in groups in standard home cages, with food and water available ad libitum and a natural day-night cycle and afterwards donated to local farmers.

Apparatus

Experimental apparatus was a white hallway (85x30x30 cm) with two monitors (LCD Monitor BenQ XL2410T) at its ends, which played the stimuli (Figure 11). The hallway was virtually parsed in three sectors: one central (45 cm long) and two identical lateral sectors (each 20 cm long), comprising the area directly in front of the two monitors. Two steps (1.5 cm high) delimited the central sector. The chicks were placed in the centre, to enter the lateral sectors chicks had to climb on one of the two steps. To record chick's behaviour during the test a video camera was placed above the apparatus. It was connected to a screen in the same room, allowing for on-line scoring. Only the two monitors playing the stimuli provided the illumination to the apparatus.

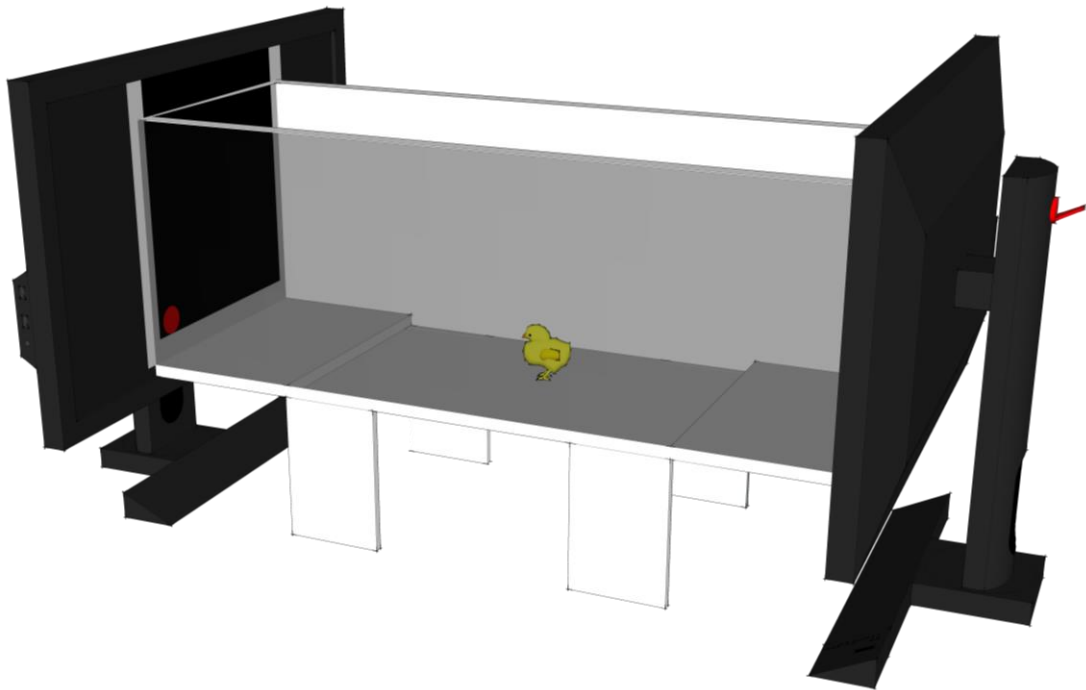


Figure 11: Test apparatus Study 1 and 2.

Schematic representation of the experimental apparatus used in Study 1 and 2 of the present work. One of the two long walls is depicted translucent for demonstrative reason. The chick is placed in the central sector. On the two opposite ends of the corridor two monitors playing the stimuli. Two small steps delimit the central sector, which the chick had to climb to approach the stimuli (adapted from Lorenzi et al. 2017).

Stimuli

Experimental stimuli were videos depicting a simple red circle moving, created with MATLAB R2012b with the Psychtoolbox-3 extension (Kleiner et al. 2007). In each stimulus, a red object was moving linearly on a dark background in loop for six minutes. A couple of stimuli was used, composed of the *animate* experimental stimulus and the *inanimate* control one. The side of presentation of the experimental stimulus was randomised between subjects. The portion of screen visible to the chick was 30cm long, delimited by an U-shaped light grey frame, composed of two lateral walls (visible 2.5cm wide) and a floor over which the red object was moving. The object always entered the view already in motion, appearing from behind one of the two lateral grey walls. Similarly, once completed its motion it disappeared from view while still moving, slipping behind one of the lateral walls.

The *animate* stimulus (see Figure 10) was accelerating at one-third of its way and decelerating back to initial velocity at two-thirds (the slower and the faster speed being respectively of ~ 3.37 and ~ 19.64 cm/s, with a slower-to-faster speed ratio of ~ 0.171). Two grey occluders (5.6 cm wide) were positioned at one- and two-thirds of the path, occluding the exact two moments of speed change (Figure 12). The *inanimate* stimulus alternated two different velocities (~ 15.83 and ~ 2.95 cm/s, slower-to-faster speed ratio of 0.18) and two identical grey occluders were identically located also in it.

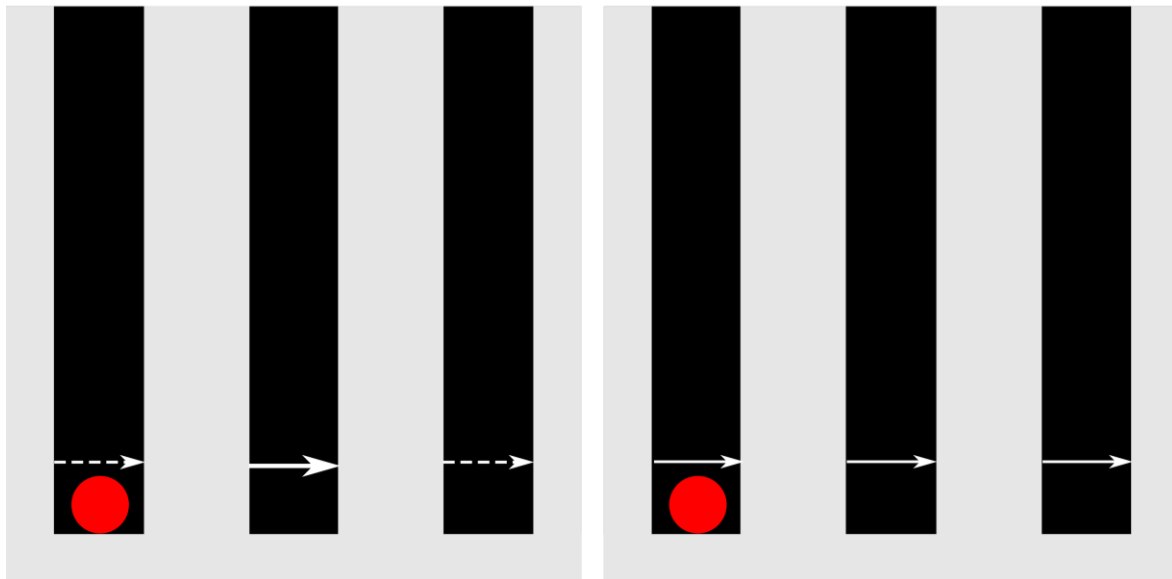


Figure 12: Control stimuli for self-propulsion Experiment 1. Schematic representation of the couple of stimuli used in Experiment 1. On the left side, the *animate* object accelerates and decelerates behind the two grey occluders. Single moments of self-generated speed change are hidden. On the right, the *inanimate* object is moving at two constant speeds (constant-fast and constant-slow), two identical grey occluders are placed to balance the amount of grey in both stimuli (Rosa-Salva et al. 2016).

Procedure

The test was performed between first and third day post-hatching. Subjects were individually taken from the incubator in complete darkness and carefully transferred into a dark box in the experimental room. Each chick was placed at the centre of the central sector facing one of the two long walls and its behaviour observed for six minutes. Chicks that remained in the central sector (no preference) were excluded from further analyses.

Whereas, entrance in one of the two lateral sectors was considered as a preference for the stimulus nearby. An event recorder in the room allowed the experimenter to score the seconds spent by the subject in each of the three sectors during the entire duration of the test. Behavioural measures adopted were first stimulus approached and the *ratio* of time spent near the *animate* stimulus (speed-changing) over the total time spent near both stimuli calculated as follows:

$$ratio = \frac{time\ spent\ near\ animate}{time\ spent\ near\ the\ animate + time\ spent\ near\ the\ inanimate}$$

Values could range from 1 (full choice for the animate) to 0 (full choice for the inanimate) and 0.5 representing no preference.

All subjects were scored on-line. In order to estimate the inter-coder reliability, a second scorer (blind to the position of the two stimuli) scored again off-line 10% of all subjects. Overall, 30 videos were randomly chosen from all the experiments and coded again (Pearson's correlation of 0.987, $p < 0.001$ between the ratio calculated based on the original and on the off-line coding).

Statistical analyses

The Pearson's chi-square of independence was used to compare the number of chicks that first approached the *animate* or the *inanimate* stimulus. A one-sample two-tailed *t*-test was used to compare the *ratio* to the expected chance level (0.5). For every *t*-test Cohen's *d* was calculated as a measure of effect size with the software G*Power version 3.

Results

Coherently with our predictions, when the moments of visible speed change were occluded, the preference for the animate stimulus disappeared. No preference for any of the stimuli was observed, neither for the dependent variable first approach ($X^2=0.49$, $p=0.484$, see Figure 13 (a)), nor for the dependent variable *ratio* (mean=0.44, S.E.M.=0.07, $t_{(50)}=-0.844$, $p=0.403$, $d=0.12$, see Figure 13 (b)).

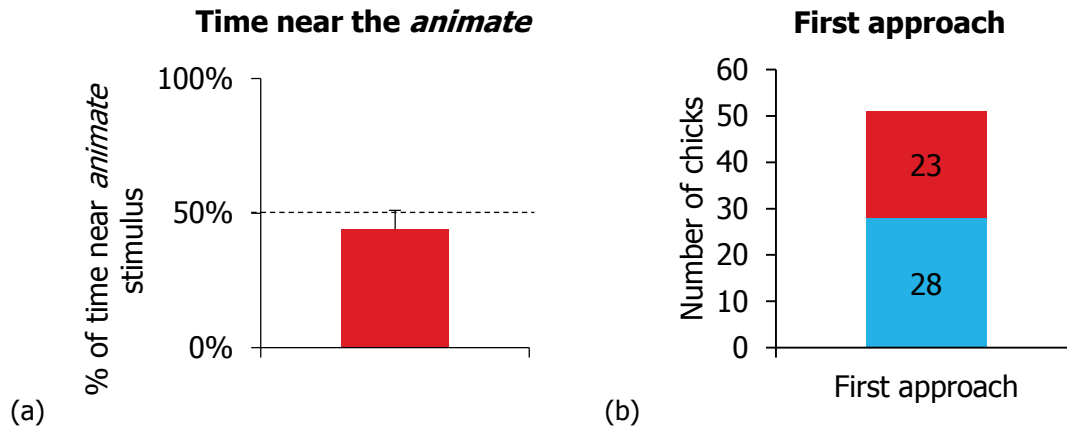


Figure 13: Results Experiment 1.
 (a) Percentage of the ratio of time spent near the *animate* stimulus over the total choice time, dashed line represents the chance level. (b) Number of chicks that first approached the *animate* stimulus (23, in red) versus the *inanimate* one (28, in blue) is plotted. Error bar indicates S.E.M..

Experiment 2 (Exp. 6 of the original manuscript)

Results obtained with chicks in a previous experiment investigating both changes in speed and changes in direction revealed a paradoxical effect, namely that not only the presence of direction changes was insufficient to elicit preferential approach in chicks, but also detrimental. In fact, no preference was observed for the *animate* stimulus despite the fact that it also contained a speed change. Therefore, the aim of Experiment 2 was to determine whether this detrimental effect could be due to the unnatural combination created by the speed and direction change. In fact, when animate beings invert their direction of motion, they decelerate, stop and accelerate again moving in the new direction. On the contrary, in a previous experiment, the red circle suddenly inverted its direction without any deceleration nor pause, and immediately accelerated away. This could cause the impression of an inanimate object bouncing against an invisible obstacle. Here we replicated the previous experiment with chicks, implementing the naturalistic speed-profile of changing direction expected for animate beings.

Subjects

Fifty-nine domestic chicks (26 males) were employed for this experiment.

Stimuli

Stimuli were gradually decelerating and a briefly stopping (0.25s), before the inversion of direction in the *animate* stimulus. The inversion of direction happened halfway and was followed by a gradual increase in speed. Average speed was identical to that of the *inanimate* constant moving stimulus.

Results

Chicks revealed a significant preference for the stimulus displaying speed and direction changes, confirming and extending the results of Experiment 1. This was evident both for the dependent variable first approach ($X^2=4.898$, $p=0.027$, see Figure 14 (b)) and for the *ratio* (mean=0.63, S.E.M.=0.06, $t_{(58)}=2.219$, $p=0.030$, $d=0.28$, see Figure 14 (a)).

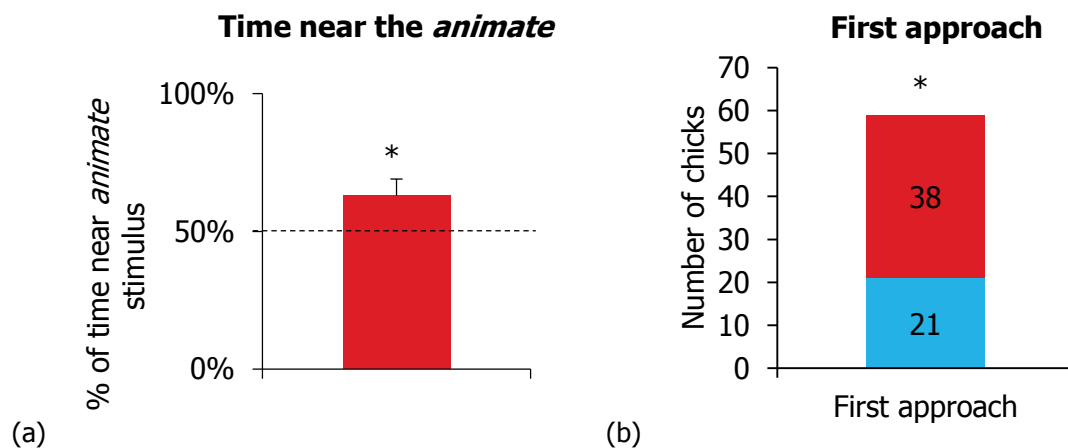


Figure 14: Results Experiment 2.

(a) Percentage of the ratio of time spent near the *animate* stimulus over the total choice time, significantly different from chance level (50%, dashed line). (b) A significantly higher number of chicks that first approached the *animate* stimulus (38, in red) versus the *inanimate* one (21, in blue) is plotted. Asterisks indicate significance ($\alpha \leq 0.05$). Error bar indicates S.E.M..

Discussion

We were able to demonstrate the presence of unlearnt preferences in naïve newborn animals for an elementary motion cue of animacy. This is the first evidence that naïve animals prefer those cues when exhibited in the motion of a single object, without any interaction with a second one. This adds and extends previous results that newly-hatched chicks preferentially imprint on objects characterised by another elementary motion cue that elicits animacy perception in humans (Mascalzoni et al. 2010). In fact, here we show that chicks respond also to changes in speed as a cue for self-propulsion in addition to “start from rest” investigated by Mascalzoni and colleagues. These results are also in line with the evidence in infants’ and adults’ perception of chasing displays, which underlined the importance of speed changes (Frankenhuis et al. 2013). Moreover, these findings were extended to zebra fishes strengthening the observation that some kind of rudimental knowledge about animate creatures is shared among phylogenetic distant species of vertebrates (*Danio rerio*; Nunes 2016).

Nevertheless, our results demand for careful interpretation. First, the paradigm employed tests only the relative preference between two stimuli (and in numerous other studies with chicks Rosa-Salva et al. 2011, 2010; Vallortigara & Regolin 2006; Vallortigara et al. 2005; non-human primates, Sugita 2008; and human infants Rochat et al. 1997; Farroni et al. 2005; Simion et al. 2008; Frankenhuis et al. 2013). Therefore, it is impossible to determine the absolute level of preference for one stimulus. Indeed, it is likely that all stimuli employed are on their own quite attractive for chicks. In fact, all stimuli have features that are socially attractive for these animals (appropriate size and shape, red colour, presence of movement). Importantly, this reflects the reality of visual processing: multiple stimuli are often present in the visual field, each characterised by various features. The preferential processing of one stimulus over another depend on the relative weight of multiple relevant properties.

Another note of caution concerns the disambiguation between spontaneous preference and imprinting learning. We believe that the adaptive role of predispositions is also to direct subsequent learning mechanisms toward appropriate stimuli (for a similar argumentation regarding the human species and the predisposition for faces see Johnson 2005). It is likely that preferential approach of one kind of stimulus by naïve chicks will end up ensuring filial

imprinting for the preferred stimulus, due to the increased exposure to it. However, these are for now only speculations, since the preference that we observed did not automatically translate into animacy perception or preferential imprinting (indeed the current study was purposely designed to test spontaneous approach preference, rather than preferential imprintability). For example, we cannot know whether the moving objects attract chicks because they perceive them as *animate*. Even assuming that preferential imprinting will indeed occur, we do not know whether this will result from a direct social preference for that motion attribute or simply from an increased level of attention to it (an attentional orienting mechanism is believed to be at the basis of face preferences in humans: Morton and Johnson 1991; Simion et al. 1998; Johnson 2005; Tomalski et al. 2009). This hypothesis could be tested in future studies. Investigating the role of speed changes in attentional orientation, by implementing speed changes in visual search, learning or other tasks not based on spontaneous social preferences (*e.g.*, using stimuli that are too small to be social companions, and thus categorised as potential food items).

The present study offers a clear-cut demonstration of sensitivity to one fundamental perceptual property of animacy and it investigates the evolutionary and developmental origins of this trait. Among the most important contributions of the present paper is that a preference for a property associated with human animacy perception has been obtained in a phylogenetically distant animal model and in the absence of any prior visual experience. This allows us to investigate the origins of this trait in a way that is impossible when studying mammal newborns, for which a certain amount of previous visual experience is unavoidable. Studies on infants with months of visual experience cannot inform us on the innate predispositions that characterise the initial state of the developing system and represent the building blocks for further cognitive development (Spelke 2000; Spelke and Kinzler 2007; Carey 2009; Vallortigara 2012).

In fact, a remarkable aspect of animacy perception is its automatic and irresistible nature, which raises questions on its developmental and phylogenetic origins. Overall, evidence from human adults and infants suggests the presence of core-knowledge mechanisms responding to the motion cues indicating the presence of animate beings (Spelke and Kinzler 2007). Along these lines is also the view (Frankenhuis et al. 2013) that babies employ “a coarse, property-based attentional filter that navigates their attention toward social interactions. This filter could be phylogenetically old, shared with other vertebrates, and

respond to general properties of animate (anti-gravitational acceleration)". Moreover, children with autism have difficulties in learning to identify as animate abstract shapes based on the above mentioned motion cues, suggesting a failure of the corresponding core-knowledge mechanism (Rutherford et al. 2006). However, claims of unlearned core-knowledge mechanisms need to be supported by evidence from other animal species and especially from naïve models, allowing both to uncover the phylogenetic history of a given trait and to assess claims on its innate versus cultural origin, disentangling the role of inborn and experience-dependent factors in animacy perception. Our data offers direct support to this hypothesis showing remarkable similarity between the responses of 4-month-old human infants (Frankenhuis et al. 2013) and visually naïve domestic chicks to elemental motion properties that induce animacy perception in adult observers. Most importantly, the present study provides the first evidence that naïve animals respond to this elementary motion cue of animacy, which can be conveyed by the motion of a single object, indicating the presence of a mechanism to orient toward one of the general properties of animate creatures. This is also the first demonstration of a predisposition for speed changes in any non-human animal.

Another note of caution involves the relationship between speed-changes and other simple animacy cues, such as start from rest (Mascalzoni et al. 2010) and direction-changes. Both speed changes and start from rest are clearly effective cues for eliciting chicks' social preferences. However, the available evidence does not allow any prediction on the hierarchy and interaction between these two cues, whose effects have been demonstrated in two independent studies with different methodologies. Thus, it is currently unknown whether they can have additive effects and whether one is more powerful than the other.

As regard direction changes, our initial results, initially suggested that the presence of direction changes was insufficient not only in eliciting the perception of animacy in human adults or preferential approach in chicks, but also detrimental. In fact, we did not observe any preference for the direction-change stimulus despite the fact that it also contained a speed change. In Experiment 2, we tested whether this detrimental effect could be due to the unnatural combination of the profile of speed and direction changes of previous experiment's stimuli. In fact, when animate creatures invert their motion direction they decelerate, stop and then accelerate again when moving away in the opposite direction. On the contrary, in Experiment 1 the object abruptly inverted its motion direction, without any

deceleration or pause, and suddenly accelerated away. This could have originated the impression of an inanimate object bouncing toward an invisible obstacle (this has been reported by some human observers exposed to the stimulus). In Experiment 2 we replicated the previous experiment, implementing the natural speed profile expected for animate creatures inverting their motion direction, finding a significant preference for the natural configuration (speed and direction changes). We can thus exclude that direction changes have a detrimental effect on chicks' preference for speed changes. However, it is unclear whether they can elicit chicks' preferences when presented alone (without speed changes). Contradictory results on this regard come from human studies. On the one hand, adults showed increased animacy ratings for pure direction changes (Tremoulet and Feldman 2000). On the other hand, infants' response to changes in speed did not depend on their level of engagement with the stimuli, contrary to what was the case for changes in trajectory (Frankenhuis et al. 2013). This can be interpreted as evidence for automatic orientation responses to speed changes, and endogenous engagement for trajectory changes. Similarly, adults' animacy ratings are more influenced by acceleration rather than direction changes (Tremoulet and Feldman 2000).

Here we established a direct test of spontaneous preference that can detect chicks' sensitivity to speed changes, without the need to test for differential imprintability (*e.g.* Mascalzoni et al. 2010). This makes testing more practical and efficient, reducing the manipulation level of the animals, as well as the time and the sample size needed to perform an experiment. This also opens new possibilities for investigating the neural bases of animacy preferences (see Study 2: Lorenzi et al. 2017). This was not feasible with the paradigm of Mascalzoni and colleagues (2010), based on the Michotte's launching effect (1963), in which both the *animate* and the *inanimate* object needed to be present simultaneously on screen and to interact with each other, making it difficult to design a well-controlled brain study with that stimulus. On the contrary, with the present stimuli it is possible to stimulate selectively one group of chicks only with the *animate* stimulus and one only with the *inanimate* one and then to compare them. Moreover, stimuli are identical (*e.g.* colour, shape, average speed) but differ only in the independent variable (motion pattern). A power analysis based on the effect size obtained in the experiment finding the preference for speed changes ($d=0.62$) indicates that a sample of 18 subjects would be sufficient to reveal a significant preference in a replica of this condition (one sample *t*-test against

chance level, one tailed, $\alpha=0.05$, $\beta=0.8$). This is significantly less than what required for most of these studies (*e.g.*, corresponding analysis based on Experiment 1 from Mascalzoni et al. 2010, indicates a required sample size of 45 individuals), making it more attainable and ethical to conduct invasive studies of neural correlates. Thus, using the stimuli validated by the present work, future studies could verify which of the candidate brain areas suggested by the literature are indeed involved in the processing of this powerful motion cue of animacy. This was done in Study 2 that will follow this discussion, by staining immediate early genes' products to map neuronal activity in chicks exposed either to *animate* or *inanimate* stimulus.

From a behavioural perspective, future studies with naïve animal models should extend the present findings to other vertebrate species. Taking advantage from the approach validated in this study to other motion cues related to animacy perception in human observers could be investigated with visually naïve chicks (see Introduction; see also Rosa-Salva et al. 2015 for a review). For example, the alignment between the object's major symmetry axis and the trajectory of motion, in relation to changes in direction, and a stable *front-back* arrangement.

Study 2: Dynamic features of animate motion activate septal and preoptic areas in visually naïve chicks (*Gallus gallus domesticus*)²

Abstract

The septum is an evolutionarily well-conserved part of the limbic system. It is known to be involved in many aspects of social behaviour and is considered a key node of the social behaviour network, together with the preoptic area. Involvement of these two brain regions has been recently observed in newly hatched chicks exposed to the natural motion of a living conspecific. However, it is unknown whether these areas respond also to simple motion cues that elicit animacy perception in humans and social predispositions in chicks. For example, visual objects that appear to change spontaneously their speed (an index of self-propulsion, typical of animate creatures) attract visually naïve chicks. Here we show that the right septum and the preoptic area of newly hatched visually naïve chicks exposed to speed changes has higher neuronal activity (revealed by c-Fos expression), compared with that of chicks exposed to constant motion. We thus found an involvement of these two areas in the perception of motion cues associated with animacy in newly hatched chicks without any previous visual experience. This demonstrates their early involvement in processing simple motion cues that allow the detection of animate creatures and elicit social predispositions in this animal model, as well as preferential attention in human infants and the perception of animacy in human adults.

² Please note that data here reported have been published on *Neuroscience* in 2017 (Lorenzi et al., 2017).

Introduction

The detection of animate creatures, such as conspecifics, preys or predators, is crucial for survival and can be based on dynamic cues typical of animate motion, as opposed to that of inanimate objects (for a review see (Rosa-Salva et al. 2015). Studies conducted in visually naïve chicks (*Gallus gallus domesticus*), human newborns and infant monkeys demonstrated similar preferences for stimuli displaying both static and dynamic cues of animacy (Sugita 2008; Simion et al. 2008; Mascalzoni et al. 2010; Rosa-Salva et al. 2010, 2011, 2012b, Versace et al. 2016, 2017). These social predispositions are active since birth and, at least in animal models, emerge in the absence of any specific learning experience. One of the adaptive functions of social predispositions might be to guide the action of learning mechanisms (*e.g.*, filial imprinting) towards appropriate social objects, channelling the subsequent development of neural mechanisms specialised for sophisticated processing of social information (Johnson 2005; Rosa-Salva et al. 2015; Versace and Vallortigara 2015; Miura and Matsushima 2016; Di Giorgio et al. 2017).

The present study focusses on responses to specific features of animate motion. Naïve chicks and human newborns show a preference for point-light displays depicting semi-rigid biological motion, in which some points maintain always a fixed distance between each other, but vary their distance with respect to other points, the typical gait pattern of legged animals (Vallortigara et al. 2005; Vallortigara and Regolin 2006; Simion et al. 2008; Miura and Matsushima 2012). Animate motion can also be recognised because it is self-propelled, revealing the presence of an internal energy source to the moving object. Different motion patterns associated with self-propulsion increase the perception of animacy in human observers (Tremoulet and Feldman 2000) and are preferred by newborn babies (Di Giorgio et al. 2016b), infants (Frankenhuis et al. 2013) and domestic chicks (Mascalzoni et al. 2010; see Study 1: Rosa-Salva et al. 2016). In the first study of the present work, we investigated naïve chicks' responses to speed changes, another index of self-propulsion. We found a spontaneous preference for a simple object that autonomously changes its speed, accelerating and then decelerating, to an identical one that moves at constant velocity. The preference disappeared when the two moments in which the object was changing speed were occluded from view, indicating that chicks were indeed responding to this visual cue (Rosa-Salva et al. 2016).

The neural correlates of these social predispositions are mostly unknown. We are conducting a series of experiments to investigate this issue in chicks, by mapping neuronal activities through immediate early genes expression (Mayer et al. 2016b, 2017a, b). In these studies from our group, the natural motion typical of conspecifics stimulated differential activation, in the amygdaloid, septal and preoptic areas (Mayer et al. 2017a, b), as well as does the static configuration of features in intermediate medial mesopallium (IMM; see Study 3: Mayer et al. 2016b).

As regards the last structure, IMM is a telencephalic area of the mesopallium involved in filial imprinting learning (Horn 1979; Bolhuis 1991; Ambalavanar et al. 1993; McCabe and Horn 1994; Horn 1998, 2004; McCabe 2013). Although IMM is not needed for the expression of predispositions (Horn and McCabe 1984), we found differential activation of this region after approach to a stimulus whose static configuration of features respects that of a social companion (stuffed hen) compared to a scrambled version of it, please for more detailed information read Study 3 of the present thesis (Mayer et al. 2016b).

Interestingly, the other three areas identified in our previous studies (septal, amygdaloid and preoptic nuclei) are implicated in adult social behaviours, being part of the so-called "social decision making network" (Balthazart et al. 1998b, a; Gahr 2001; O'Connell and Hofmann 2011; see Figure 5). This network is shared among all vertebrates and comprises the social behaviour network (Newman 1999; Goodson 2005), consisting of interconnected areas rich in sex steroid receptors, and the mesolimbic reward system (O'Connell and Hofmann 2011).

Septum is a well-conserved part of the limbic system in all vertebrate groups (Northcutt 1981; Puelles et al. 2000), it is vertically traversed by the tractus septopallio-mesencephalicus, which connects the visual Wulst with the optic tectum (Karten et al. 1973), it receives important connections from hippocampus and it is interconnected with preoptic area and arcopallium (Montagnese et al. 2004, 2008; Xin et al. 2017). Septum is involved in multiple social functions. In mammals it has been implicated in agonistic and mating behaviour (Kollack-Walker and Newman 1995, 1997), in pair bonding (Liu et al. 2001), in dominance hierarchies (Ferris et al. 1990) and in attack-defence responses (Albert and Chew 1980). In birds, it is involved in sexual behaviour (Taziaux et al. 2006), gregariousness (Goodson et al. 2009; Kelly and Goodson 2014), songbirds' vocalisations,

aggressive and submissive behaviours (Goodson 1998; Nishizawa et al. 2011). The avian preoptic area (POA) of the hypothalamus plays a conserved role in aggression, parental care, male sexual behaviour as well as in appetitive and consummatory behaviour (Akerman et al. 1960; Balthazart et al. 1990; Slawski and Buntin 1995; Balthazart et al. 1998a; Riters et al. 1998; Ruscio and Adkins-Regan 2004; Taziaux et al. 2006; Bharati and Goodson 2006; Taziaux et al. 2008).

Finally, the amygdala has been theorised to support early social responses in humans (e.g., attention to faces; Johnson 2005). The mammalian amygdala has been implicated, among other things, in fear responses (Hamm and Weike 2005), olfactory social communication (Petrulis 2009), maternal behaviour (Sheehan et al. 2001) and innate reproductive and defensive behaviours (Choi et al. 2005). Avian homologues for mammalian amygdala are still debated (Cheng et al. 1999; Reiner et al. 2004; Jarvis et al. 2005; Yamamoto and Reiner 2005; Yamamoto et al. 2005; Martínez-García et al. 2008). The nucleus taeniae (TnA), in the posterior and medial arcopallium, is considered homologue to the subpallial, medial amygdala (Cheng et al. 1999; Yamamoto and Reiner 2005; Yamamoto et al. 2005). Homologies for the pallial amygdala are more controversial, but most agree that at least part of the arcopallium is homologue to the pallial amygdala (Butler et al. 2011). In birds, the arcopallial region is involved in agonistic behaviour (Phillips and Youngren 1971) and fear responses (Phillips 1968; Martin et al. 1979). TnA is also implicated in sexual behaviour (Thompson et al. 1998; Ikebuchi et al. 2009). In an altricial species, the zebra finch, TnA can already be delineated at post-hatching day one (Ikebuchi et al. 2012), suggesting that its early development may be involved in early social control.

Given the importance of these areas for social functions in adult animals, it is surprising that so far very few studies investigated their involvement in early social behaviours of newborn animals. Early social behaviours are crucial for the ontogenesis of social cognition (Johnson 2005; Sugita 2008; Di Giorgio et al. 2016a). In our recent studies, arcopallium responded to the first exposure to an alive conspecific compared to baseline (*i.e.*, exposure to an empty chamber Mayer et al. 2017b). However, this area did not show a difference between chicks exposed to a living conspecific and those exposed to a taxidermised individual of identical appearance rigidly rotating on its axis (Mayer et al. 2017a). This suggests that arcopallium could be more responsive to the static features that characterise conspecifics than to their motion. On the contrary, septum was activated in both conditions

(Mayer et al. 2017b, a). Finally, while POA was not investigated in the first study, this area showed higher activity in response to the natural motion of the living chick, compared to the rotating one (Mayer et al. 2017a). This suggests that these two areas should be sensitive to the type of natural motion emitted by a live conspecific. However, it remains unclear which features of the motion of the alive conspecific (*e.g.*, semi-rigidity, speed changes, starts from rest etc.) elicited the effect and whether any of these motion features presented in isolation would be sufficient to activate septum and POA, if displayed by an agent whose morphology does not resemble that of a conspecific. In the current study, we thus compared brain activation (immediate early gene expression) in septum, POA, arcopallium and IMM of visually naïve chicks exposed either to a stimulus characterised by speed changes (*animate*) or to a control constant-speed stimulus (*inanimate*; stimuli from Study 1: Rosa-Salva et al. 2016). This allowed us to use very well controlled stimuli to test the neural correlates of chicks' social predispositions for animate motion cues.

Materials and Methods

Subjects

Subjects were 24 domestic chicks (*Gallus gallus domesticus*). For any other detail on subjects please see the section Subjects of Study 1.

Apparatus

The test apparatus (see Figure 11) was identical to that used in Study 1. One screen placed at one side of the corridor showed one of the stimuli (which one depended on the experimental condition). The second screen remained off, its presence serving only to counterbalance the visual appearance of the two sides of the corridor.

Stimuli

The stimuli were identical to those used in Study 1 to find the preference for speed changes (Figure 10). The animate stimulus was changing its speed twice along a constant trajectory, increasing at one-third and decelerating back at two-thirds. The inanimate stimulus was moving at constant speed and trajectory.

Test session for c-Fos Labelling

Chicks were tested individually during the first day after hatching. The animals were taken from the incubator in complete darkness and transported, inside a closed dark box, to the experimental room. Each animal was placed in the middle of the central sector of the corridor (Figure 11), facing one of the two long walls (left-right orientation with respect to the long walls was counterbalanced between subjects). The chick could freely move inside the apparatus for the whole duration of the test (6 minutes). Doing so, it could spontaneously approach the screen with the stimulus. This involved climbing on the step on the stimulus side of the apparatus, which allowed a precise definition of a successful approach. Birds that failed to do so were excluded from further procedures. After the behavioural exposure, the subjects were carefully placed in the transportation box and carried back to the dark incubator, where they remained until perfusion. In order to distinguish individual subjects also in the darkness, but to keep the auditory environment as it was before, they were placed individually within the same incubator as the other chicks.

Overall, we collected 12 experimental group chicks that approached the speed-changing stimulus and 12 control group chicks that approached the speed-constant stimulus. Two-to-six subjects were tested per week, equally subdivided in the two groups. In this way, the groups were always balanced with regards to the hatching batch and the staining procedure.

Immunohistochemistry

Seventy-five minutes after the end of the experimental manipulation, subjects were overdosed by an intramuscular injection of 0.05ml Ketamine/Xylazine Solution (1:1 Ketamine 10mg/ml + Xylazine 2mg/ml) per 10g of body weight. They were perfused transcardially with cold phosphate-buffered saline (PBS; 0.1mol, pH = 7.4, 0.9% sodium chloride, 5°C) and paraformaldehyde (4% PFA in PBS). The skull with the brain was transferred to 4% PFA, where it was post-fixed and stored until processing. To ensure correct orientation (45°) for the coronal sections, procedures described in the chick brain atlas of Kuenzel and Masson 1988 were followed. The brains were then carefully removed from the skulls under a dissecting microscope. The left and the right hemispheres were separated and processed separately. Each hemisphere was covered with a 7% gelatine in PBS containing egg yolk (4.2g gelatine + 60ml PBS + 1 egg yolk at 40°C), after cooling they were post-fixed in 4% PFA/PBS/20% sucrose for approximately 48h at 5°C, and then transferred to 30% Sucrose/0.4%PFA/PBS for further 48–72h. The brains were frozen at -80°C in plastic moulds, covered with O.C.T. (Tissue-Tek freezing medium). Six series of 40µm coronal sections were cut on a Cryostat (Leica CM1850 UV) at -20°C and collected in PBS. For free-floating immunostaining only the sections of the first series were used, whereas the other series were kept as backup. Between every of the following steps the sections were washed in PBS. After incubation in 0.3% H₂O₂/PBS for 20min. the sections were treated for 30min with 3% normal goat serum (S-1000, Vector Laboratories, Burlingame, CA). The first antibody reaction was carried out for 24-36h at 5°C on a rotator using c-Fos antibody in PBS solution (1:2000; rabbit, polyclonal K-25, Santa Cruz, CA) containing 0.1% Bovine Serum Albumin (BSA, SP-5050, Vector Laboratories). Section were incubated in the secondary antibody reagent for 60min at room temperature (biotinylated anti-rabbit in PBS, 1:200, BA- 1000, Vector Laboratories). The ABC kit (Vectastain Elite ABC Kit, PK-6100, Vector Laboratories) was used for signal amplification and the VIP kit (SK-

4600, Vector Laboratories) for the visualisation of the c-Fos containing neurons. After serial mounting on gelatine-coated slides, sections were dried at 50°C and counterstained with methyl green (H-3402, Vector Laboratories). They were gradually dehydrated in ethanol (70%, 80%, 90%, 99% ethanol, for 3min each then placed in xylene) and cover slipped with Eukitt (FLUKA).

Analyses

Brains Analyses

Brain sections were examined under a microscope at a magnification of 200x (Zeiss Axio Examiner) and a digital camera (Zeiss AxioCam MRC 5). Counting of the c-Fos immunoreactive (-ir) cells was performed blind to the experimental condition. Contrast and exposure time of the camera were adjusted so that the image on the screen (ZEN Imaging software, Zeiss) matched the view under the microscope. Successful immunostaining produced dark, purple-black stained nuclei, which were easily distinguishable from the non-activated cells, which were stained green (see Figure 7). For counting, a rectangle enclosure 150x250µm², was placed over the different sample areas in a way such that it covered as many activated cells as possible while keeping a minimum distance from the border of a neighbouring subdivision and the edge of the brain section. Every activated c-Fos-ir cell within each sample area was marked on the screen with the "event marker" of the ZEN software, which automatically computed the total number of marked c-Fos-ir cells. To estimate labelled cell density in the different brain areas evaluated sections were selected by the shape and anatomical landmarks that would correspond to the Kuenzel and Masson atlas (Kuenzel and Masson 1988). It is noteworthy to highlight that in the atlas the coordinates were estimated in two weeks old chicks (average body weight 300-325g). Whereas, the newly hatched chicks used here weight about 46g and have a different anterior coordinate.

To estimate labelled cell density in septum two to eleven sections of each hemisphere were selected by the shape and anatomical landmarks that would correspond to the A(nterior)9.4 to A8.4 (Kuenzel and Masson 1988). The septum of each section was parsed into six subdivisions: dorsal, dorso-lateral, dorso-medial, ventro-lateral, ventro-medial and medial

portion of the ventro-medial septum. Typical placements of the counting enclosure are depicted in Figure 15.

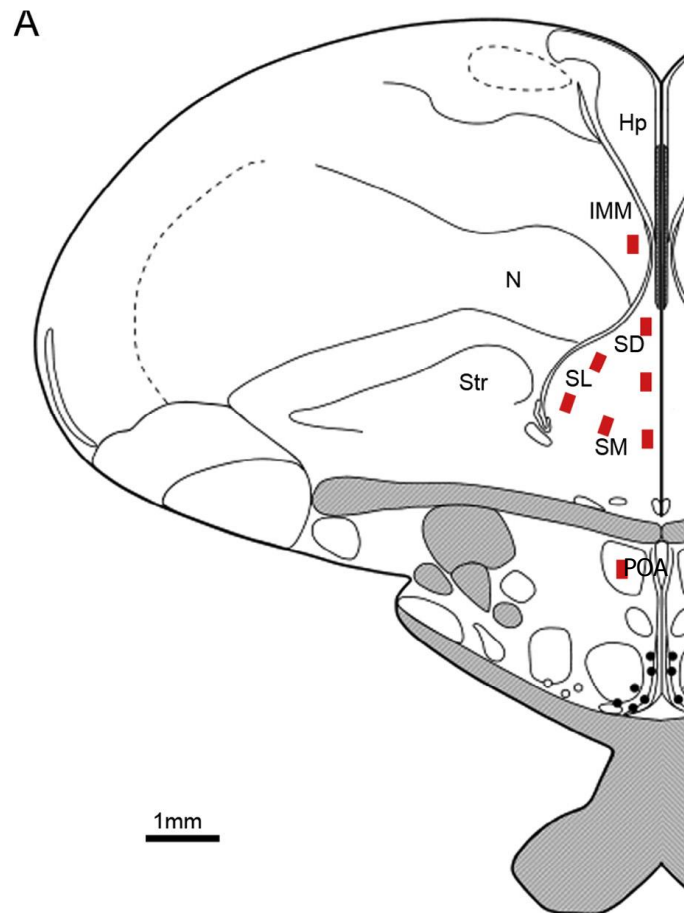


Figure 15: Counting areas in intermediate medial mesopallium, septum and preoptic area.

Schematic representation of a coronal section (adapted from Kuenzel and Masson 1988) showing typical placement of the counting areas (red rectangles) in IMM, POA and septum. Hp-hippocampus, N-nidopallium, Str-striatum, SD-dorsal septum, SL- lateral septum, SM-medial septum.

To estimate labelled cell density in the intermediate medial mesopallium (IMM) we relied on previous descriptions of this region (Liu et al. 2001; Klatt and Goodson 2013). McCabe and Horn (1994) report IMM to be located at the anterior coordinate A7.6 of the Kuenzel and Masson atlas (1988). Seven to eight brain slices, from a region where the shape of IMM was corresponding to that observed between A8.6 and A7.6 of Kuenzel and Masson (1988), were selected for the analysis. The rectangular enclosure was positioned inside IMM as in Ambalavanar et al. (1993), see Figure 16. To estimate cell densities within the arcopallium

and TnA, three to eight sections of both hemispheres were selected from the region extending from A7.6 to A6.4 in Kuenzel and Masson (1988). Arcopallium is a telencephalic region delimited in its upper boundary by the lamina arcopallialis dorsalis, whereas TnA in the ventral part can be visually distinguished by the different cell densities (see Figure 17). Recently, arcopallium has been reported to be functionally subdivided in medial and lateral regions (Xin et al. 2017). However, arcopallial subdivisions are still heavily debated in the literature (Reiner et al. 2004; Hanics et al. 2017). Our aim was not to investigate the internal subdivisions of this structure; thus, we took the most conservative approach (Karten and Hodos 1967) and we parsed arcopallium into dorsal and ventral parts for counting (see Figure 17).

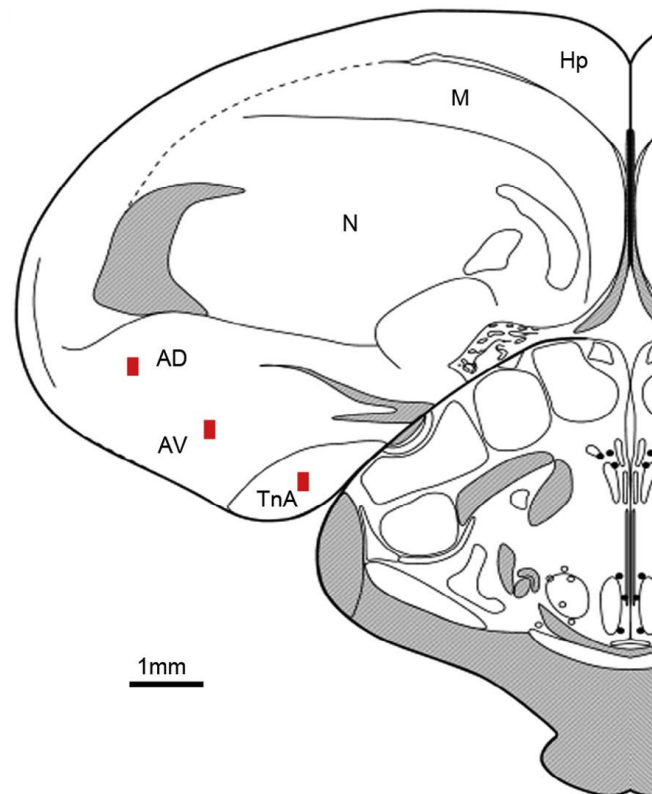


Figure 16: Counting areas in arcopallium and nucleus Taeniae. Schematic representation of a coronal section (adapted from Kuenzel and Masson 1988) showing typical placement of the counting areas (red rectangles) in arcopallium and TnA. Hp-hippocampus, N-nidopallium, AD-dorsal arcopallium, AV-ventral arcopallium.

After completing the cell counts, the mean values derived from the sections were initially calculated for each of the subdivisions independently and cell densities were standardised to 1mm^2 . Cell counts, pooled from the six subdivisions in septum, were further averaged to

estimate overall septal activity. Also, the cell counts pooled from the two subdivisions in arcopallium and the counting in TnA were averaged to estimate overall activity in this amygdala equivalent. This was done for the two hemispheres separately. The resulting individual bird means were considered overall indicators for the number of c-Fos-ir cells in the three regions of interest (septum, arcopallium, IMM) and were employed for further statistical analysis.

After the completion of the analyses of these three brain areas, we decided *post-hoc* to additionally analyse data from a sub-region of the preoptic area (POA), which showed responsiveness to natural motion in another study from our lab (Mayer et al. 2017a). Counting was thus done on one section selected from the region where the anterior commissure was apparent A8.2 (Kuenzel and Masson 1988). The counting area was positioned beneath the anterior commissure (Figure 15). This region was not initially analysed because, due to damage occurring during the separations of hemispheres, counting of POA was not feasible in few individuals and in some individuals, the area was available only in one of the two brain hemispheres. However, given the involvement of this area in the motion perception of a living conspecific evidenced by our previous study (Mayer et al. 2017a), we decided that it was of interest to investigate it all the same. Thus, POA data for either one or both hemispheres were available overall for 19 subjects (10 experimental and 9 controls). Specifically, data for the left hemisphere were available for 10 experimental and 6 control animals, whereas for the right hemisphere for 7 experimental and 6 control animals. For the overall estimation of the neuronal activity in the POA the measurements from two hemispheres, if available, were averaged, otherwise the estimation was based on the measurement from the only hemisphere available. The cell densities were standardised to 1mm².

Behavioural analyses

The video recordings of the behavioural procedure were analysed off-line. For every animal, we measured the latency of the first approach to the stimulus (seconds) and the ratio of time spent near the stimulus (*i.e.*, above the platform adjacent to the screen) over six minutes. This was calculated by the following formula: number of seconds spent near the stimulus/360 seconds. Mathematically the values obtained by this formula can range from 0 to 1. High values of the ratio indicate a higher proportion of test time spent near the

stimulus, and vice versa for low values. Moreover, the frequency of different behaviours was quantified every 10sec for the entire 6min period. The behaviours measured were indices of motor activity, vocalisations and head orientations. Motoric activity indices scored were walking and pecking. The different type of vocalisations measured were soft calls, distress calls and contact calls. Head orientation was measured with respect to the stimulus and could range from 0° to 315° with 45° as unit (see Figure 17). At 0° the beak was oriented straight to the stimulus whereas, at 180° the beak was directed toward the black opposite screen. Head orientation 0° was interpreted as binocular episode, whereas, head orientations 45°, 90°, 270° and 315° were scored as monocular episodes (45° and 90° represented left monocular episodes, whereas 270° and 315° were right monocular episodes).

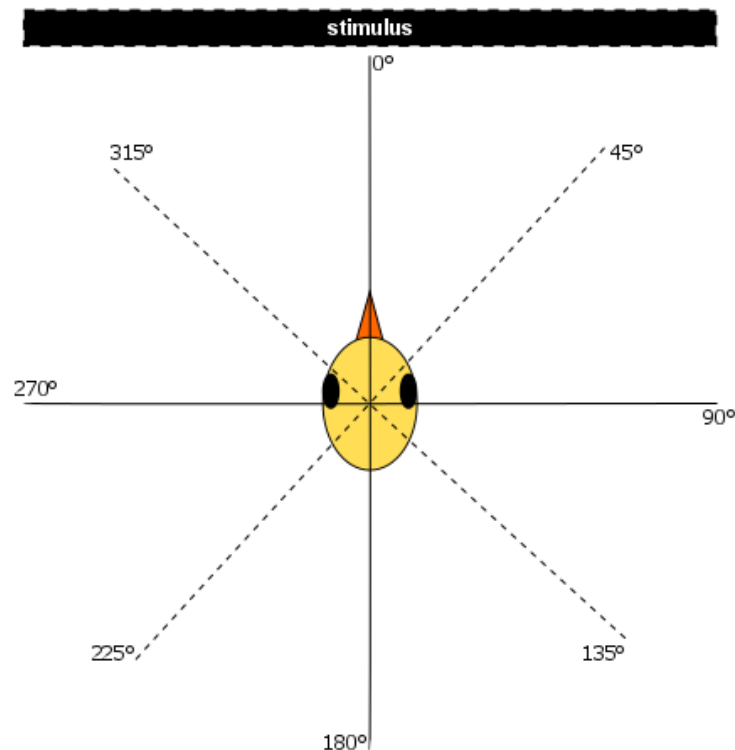


Figure 17: Head orientation.

Schematic representation of how head orientation was scored. Head orientation 0° were scored as binocular episodes, 45° and 90° as monocular left episodes and 270° and 315° as monocular right episodes.

A binocularity index was computed for each subject as follows:

$$\text{binocularity index} = \frac{\text{binocular episodes}}{\text{binocular episodes} + \text{monocular episodes}}$$

Moreover, an eye lateralisation index was computed for each subject as follows:

$$\text{eye lateralisation index} = \frac{\text{right monocular episodes}}{\text{right} + \text{left monocular episodes}}$$

Scores of both indices ranged from 1 (*e.g.*, for the eye lateralisation index this value was obtained if only the right eye was used for monocular episodes), to 0 (only the left eye was used). Being computed this way, the values of the eye lateralisation index were directly comparable with the brain lateralisation index (see above). Because of a complete decussation of the optic fibre in birds (Walls 1942), each eye projects mainly to the contralateral brain hemisphere making it particularly interesting to investigate the relationship between eye use and lateralised brain activity.

Statistical Analyses

The presence of a difference in the density of c-Fos-ir cells in IMM, septum and arcopallium was tested by a repeated measurement ANOVA, with a between-subjects factor "group" (2 levels: experimental and control) and two within-subjects factors: "area" (3 levels: septum, IMM and arcopallium) and "hemisphere" (2 levels: left and right). For *post-hoc* analyses of the differences between the groups, two-tailed *t*-tests were performed for each brain area. The lateralisation within the groups was tested by a two-tailed paired *t*-test. To test lateralisation differences between the groups, independent samples two-tailed *t*-tests were performed for each lateralisation index. No Bonferroni correction was applied on the *t*-tests, since our experimental design was based on strong *a-priori* expectations about the pattern of activity in the various areas (Fay and Gerow 2013), derived from our previous studies (Mayer et al. 2016b, 2017a, b). After the completion of the statistical analysis of the other three brain areas, the densities of c-Fos-ir cells in the POA were estimated as additional, independent *post-hoc* and compared between the groups with a two-tailed *t*-test. Lateralisation was not tested and measurements were pooled between the two hemispheres (see above).

Each behaviour was compared between the two groups with a two-tailed t -test for independent samples. The binocular and the eye lateralisation indices were compared between the two groups with an independent samples two-tailed t -test. The eye lateralisation index was compared also to chance level (0.5) for the two groups separately with a one-sample two-tailed t -test. In order to verify whether behavioural differences could account for the differences in c-Fos-ir cells densities, correlations between each behaviour and each brain area were tested with Pearson's correlation analysis. Finally, Pearson's correlation analysis was run also to test correlations between eye preference (eye lateralisation index) and brain lateralisation. All statistical analyses were performed with the software IBM SPSS Statistics (v. 20). For every t -test Cohen's d was calculated as a measure of effect size with the software G*Power version 3.

Results

Behaviour

Overall, no significant differences were present between the two groups for any of the behavioural measurements. The approach latency to the stimulus was 121.92 ± 23.405 in the experimental group and 157.58 ± 26.743 in the control group ($t_{(22)} = -1.004$, $p = 0.326$, $d = 0.41$), the ratio of time spent near the stimulus was 0.661 ± 0.0649 in the experimental and 0.523 ± 0.072 in the control ($t_{(22)} = 1.434$, $p = 0.166$, $d = 0.58$). Also the motoric activities were not different between the two groups (walking: experimental 7.25 ± 0.986 , control 7.42 ± 1.317 , $t_{(22)} = -0.101$, $p = 0.920$, $d = 0.04$; pecking: experimental 7.83 ± 1.272 , control 6 ± 1.255 , $t_{(22)} = 1.026$, $p = 0.316$, $d = 0.42$). In both groups several call types were emitted with similar frequencies (soft calls: experimental 15.75 ± 1.793 , control 12.58 ± 1.479 , $t_{(22)} = 1.362$, $p = 0.187$, $d = 0.56$; distress calls: experimental 1.75 ± 1.067 , control 3.08 ± 1.221 , $t_{(22)} = -0.822$, $p = 0.420$, $d = 0.33$; contact calls: experimental 3.92 ± 0.753 , control 5.58 ± 0.949 , $t_{(22)} = -1.375$, $p = 0.183$, $d = 0.56$). Furthermore, no eye preference for looking at the stimuli was detectable in any of the two groups. In both groups the eye lateralisation index was not different from chance level (0.5), in the experimental group it was 0.515 ± 0.0428 ($t_{(11)} = 0.349$, $p = 0.734$, $d = 0.10$) and in the control group it was 0.453 ± 0.056 ($t_{(11)} = -0.845$, $p = 0.416$, $d = -0.24$). Consistent with this, the eye lateralisation index was not different between the two groups ($t_{(22)} = 0.883$, $p = 0.387$, $d = 0.36$) and also the binocular

index did not show any differences between the two groups (experimental 0.2557 ± 0.025 , control 0.224 ± 0.013 $t_{(22)}=1.123$, $p=0.274$, $d=0.46$).

Immunohistochemistry

All 12 brains from the experimental and 12 brains from the control group were successfully stained for c-Fos. Due to the methyl-green counterstaining nuclei of all neurons were stained green, whereas the nuclei of c-Fos-ir cells were stained black after the immunohistochemical procedure and background staining was minimal. Thus, c-Fos-ir cells could be easily discerned from other neurons. All birds showed individual distributions of c-Fos-ir cells within the measured areas and the two hemispheres. As already mentioned in the methods, not all the subjects provided an intact POA. In some individuals, this area was damaged and not analysable in at least one hemisphere. However, if the area was intact, the labelled cells were easily distinguishable from the background.

The repeated measurement ANOVA on septum, IMM and arcopallium revealed significant main effect of area ($F_{(2,44)}=7.528$, $p=0.002$) and hemisphere ($F_{(1,22)}=4.595$, $p=0.043$). Moreover a significant interaction of area x hemisphere x group ($F_{(2,44)}=3.800$, $p=0.030$), revealed significant differences in densities of c-Fos-ir cells between the experimental and control group in a brain region and hemisphere-dependent fashion. Statistical *post-hoc* analysis for the three areas are reported below. The number of c-Fos-ir cells differed considerably in the right septum between the experimental and the control group ($t_{(22)}=2.242$, $p=0.035$, $d=0.92$; Figure 18). Experimental birds showed more than twice as much c-Fos-ir cells within the right septum (742.9 ± 135.4 cells/mm²) compared to the controls (343.8 ± 115.6 cells/mm²). Such significant difference between the two groups was not present in the left septum (experimental: 818.4 ± 172 cells/mm², control: 604.4 ± 149.9 cells/mm²; $t_{(22)}=0.938$, $p=0.359$, $d=0.38$).

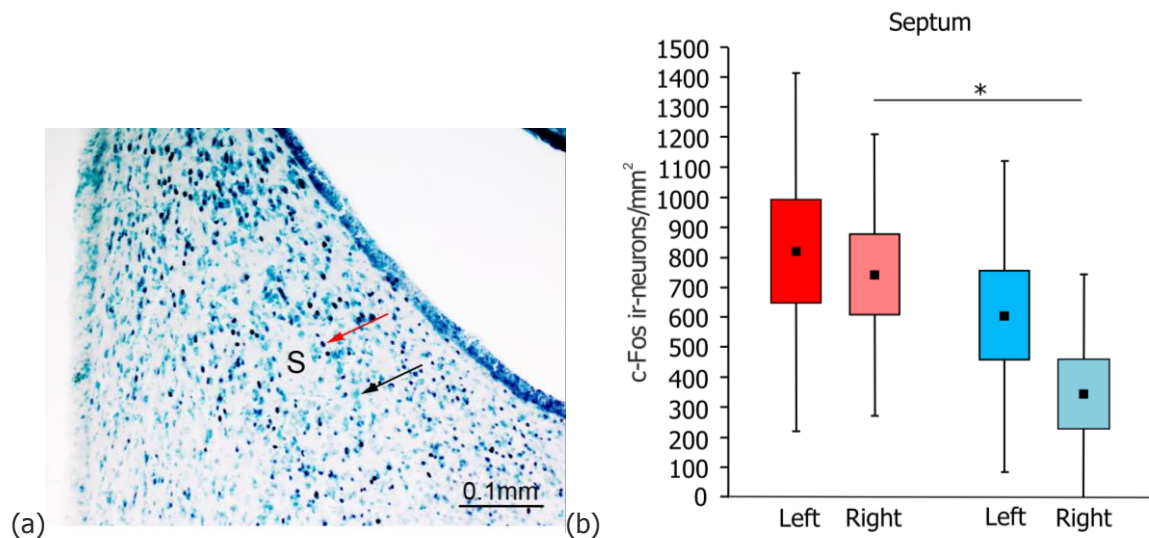


Figure 18: Results in septum.

(a) Photomicrograph of a coronal section through dorsal septum (S) of an experimental chick's brain. c-Fos-ir-cell nuclei are stained dark (red arrow) and are easily discernible from the methyl-green counterstained cells (black arrow). (b) Estimated c-Fos-ir cell densities in septum for the two groups for the two hemispheres. Significantly higher density of c-Fos-ir cells in the right septum of experimental chicks (animate-red) compared to controls (inanimate-blue). Remarkably, left hemispheres of both groups show a non-significant higher number of c-Fos-ir cells than the right. Graph-plot: mean (black square), S.E.M. (box), S.D. (whiskers), * indicates $p \leq 0.05$. On the Y-axis are represented c-Fos-ir cells per mm^2 .

Differences between the two groups were also not present in the left IMM (experimental: $1123.9 \pm 189.4 \text{ cells/mm}^2$, control: $952.5 \pm 222.9 \text{ cells/mm}^2$; $t_{(22)} = 0.586$, $p = 0.564$, $d = 0.24$; Figure 19) nor in the right IMM (experimental: $785.8 \pm 179 \text{ cells/mm}^2$, control: $876.9 \pm 210.9 \text{ cells/mm}^2$; $t_{(22)} = -0.330$, $p = 0.745$, $d = 0.13$).

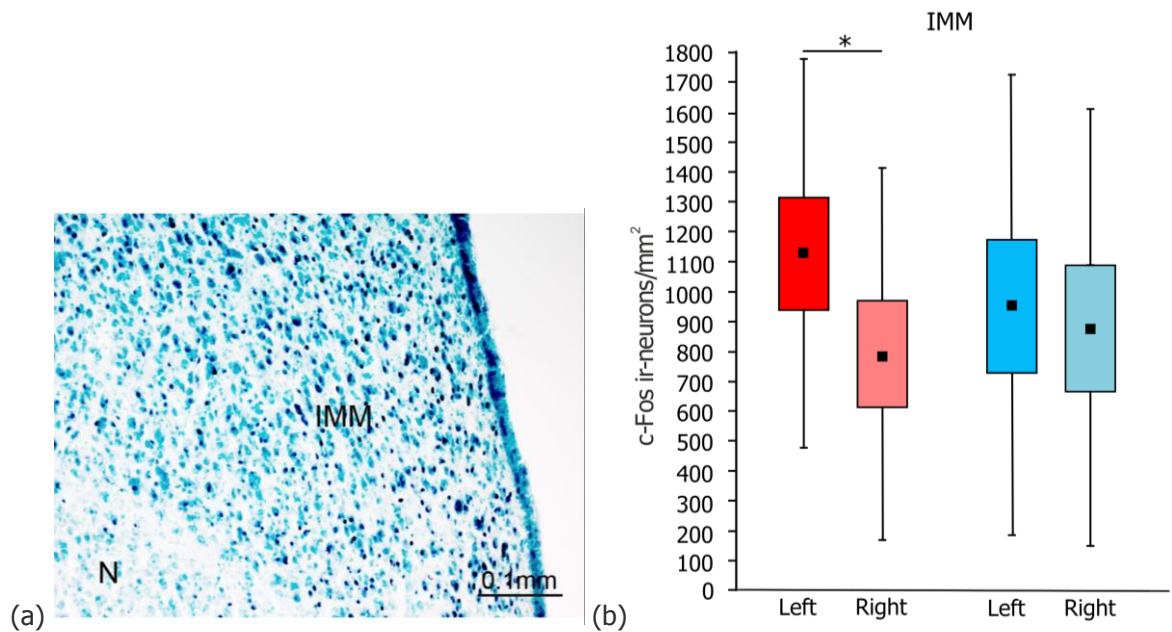


Figure 19: Results in intermediate medial mesopallium.

(a) Photomicrograph of a coronal section through IMM of an experimental chick's brain. N-nidopallium. (b) Estimated c-Fos-ir cell densities in IMM for the two groups for the two hemispheres. Significantly higher density of c-Fos-ir cells in the left than in the right IMM of experimental chicks (animate-red). Similarly, higher density was present but non-significant in the left than the right hemisphere of the control group (inanimate-blue). Graph-plot: mean (black square), S.E.M. (box), S.D. (whiskers), * indicates $p \leq 0.05$. On the Y-axis are represented c-Fos-ir cells per mm^2 .

Also the arcopallium did not show any difference between the two groups (experimental left: $869.8 \pm 129.4 \text{ cells/mm}^2$, control left: $926.2 \pm 101.5 \text{ cells/mm}^2$; $t_{(22)} = -0.343$, $p = 0.735$, $d = 0.14$; experimental right: $697.8 \pm 112.4 \text{ cells/mm}^2$, control right: $645.6 \pm 132.4 \text{ cells/mm}^2$; $t_{(22)} = 0.301$, $p = 0.766$, $d = 0.12$; Figure 20).

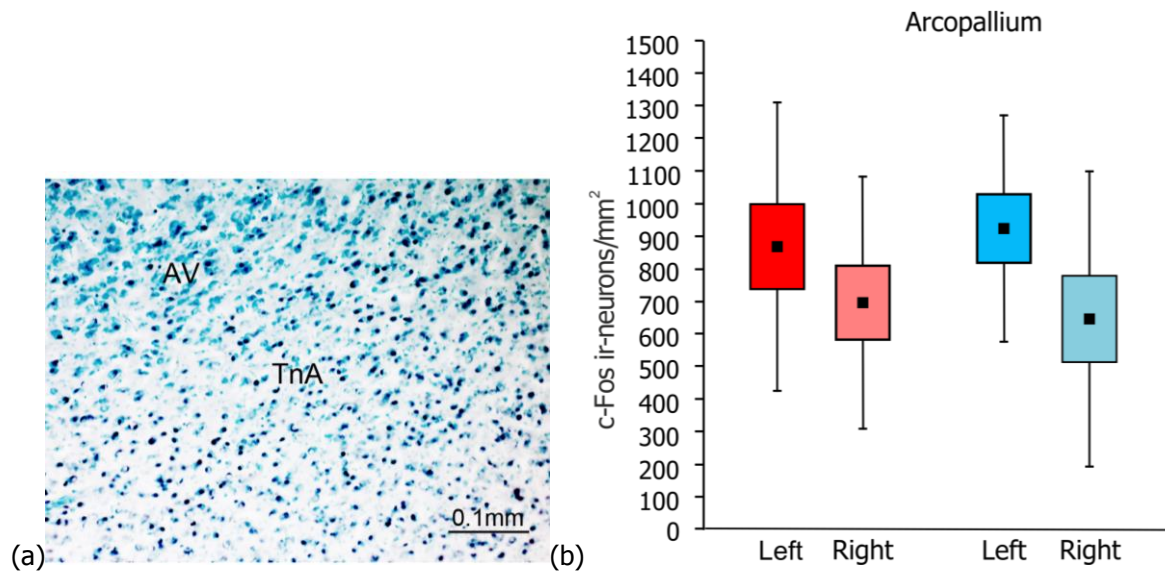


Figure 20: Results in arcopallium.

(a) Photomicrograph of a coronal section through arcopallium of an experimental chick's brain. AV-ventral arcopallium. (b) Estimated c-Fos-ir cell densities in arcopallium and TnA for the two groups for the two hemispheres. No difference was present in the number of c-Fos-ir cells in arcopallium. A non-significant trend for higher density in the left than in the right hemisphere was present in both groups. Graph-plot: mean (black square), S.E.M. (box), S.D. (whiskers), * indicates $p \leq 0.05$. On the Y-axis are represented c-Fos-ir cells per mm^2 .

Overall, a left lateralisation trend was visible in all measured brain areas of both groups (see Figure 18, Figure 19, Figure 20). To examine the presence of significant lateralisation we performed within group comparisons of brain regions of the left hemisphere with the corresponding brain regions of the right hemisphere. In the experimental group we found a significant lateralisation only in the IMM ($t_{(11)}=2.809$, $p=0.017$, $d=9.38$), whereas the differences were not significant in septum ($t_{(11)}=0.658$, $p=0.524$, $d=0.59$) nor in the arcopallium ($t_{(11)}=1.626$, $p=0.132$, $d=2.62$). The lateralisation of the control group was not significant in any of the brain areas (IMM, $t_{(11)}=0.349$, $p=0.734$, $d=1.82$; septum: $t_{(11)}=1.817$, $p=0.096$, $d=2.19$; arcopallium: $t_{(11)}=1.653$, $p=0.126$, $d=2.63$). Counting of the POA revealed higher c-Fos-ir density in the experimental group compared to the control: experimental birds showed more than twice as much c-Fos-ir cells compared to the controls (experimental $n=10$: $473.3 \pm 104.7 \text{ cells/mm}^2$, control $n=9$: $216.3 \pm 53.9 \text{ cells/mm}^2$; $t_{(17)}=2.1090$, $p=0.050$, $d=0.78$; see Figure 21).

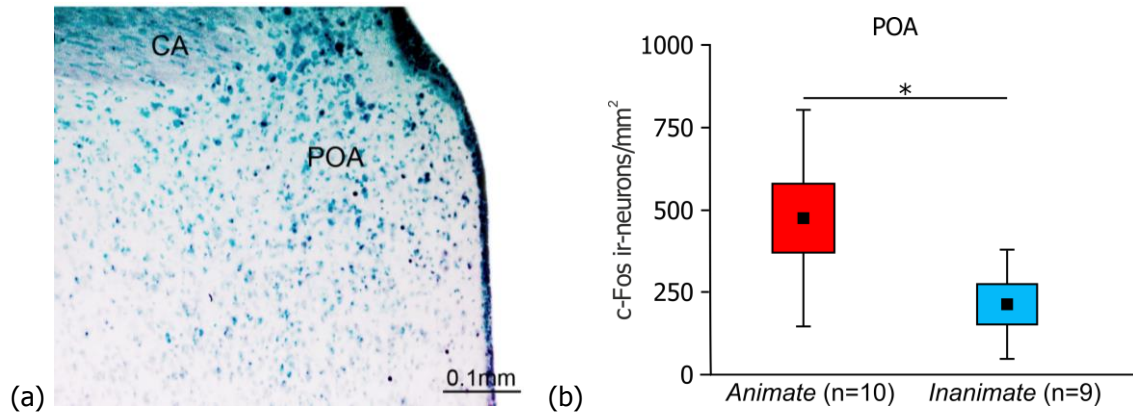


Figure 21: Results in preoptic area.

(a) Photomicrograph of a coronal section through POA of an experimental chick's brain. CA - anterior commissure. (b) Estimated c-Fos-ir cell densities in POA for the two groups experimental (animate-red) and control (inanimate-blue). Due to damage during the staining, counting of POA was not feasible in few individuals and in some individuals, the area was available only in one of the two brain hemispheres. Therefore, data for the two hemispheres separated are not available and the size of each group are reported in the plot. Significantly higher density of c-Fos-ir cells in POA of experimental chicks compared to controls. Graph-plot: mean (black square), S.E.M. (box), S.D. (whiskers), * indicates $p \leq 0.05$. On the Y-axis are represented c-Fos-ir cells per mm^2 .

Correlations of neuronal activity with behaviour

We performed correlation analyses between measured brain activities and behavioural parameters without distinguishing between groups, since we did not observe relevant differences between the groups in this respect. We found two significant correlations on two related behaviours, which we report here below, because it might be informative for future studies. Pearson's correlation test revealed a significant positive correlation between latency to approach the stimulus and density of c-Fos-ir cells in the left arcopallium ($r=0.505$, $n=24$, $p=0.012$) and a significant negative correlation between the ratio of time spent near the stimulus and c-Fos-ir cellular density in the left arcopallium ($r=-0.465$, $n=24$, $p=0.022$; see Figure 22).

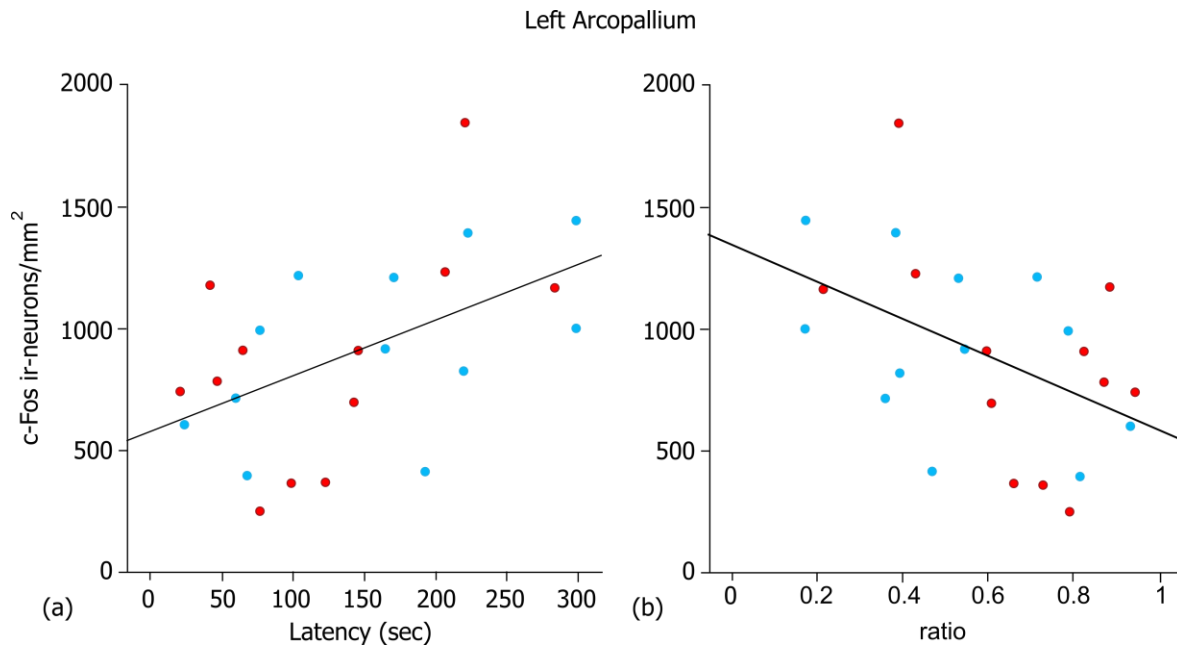


Figure 22: Brain behavioural correlations.

Scatterplots showing behaviour correlations with the number of c-Fos-ir cells within the left arcopallium (Y-axis). (a) Positive correlation with the latency to first approach the stimulus on the X-axis. (b) Negative correlation with the ratio of time spent near the stimulus on the X-axis. Experimental chicks are depicted in red (animate), and control chicks in blue (inanimate).

These correlations were not significant for the right hemisphere (latency: $r=-0.243$, $n=24$, $p=0.253$; ratio: $r=0.162$, $n=24$, $p=0.451$). Noteworthy, the latency to approach a stimulus and the ratio of time spent near it are two inversely proportional measures of the preference for the stimulus. In fact, in the current test a lower latency to approach one stimulus was usually associated with a higher time spent close to it during the test.

Discussion

The current study is the last of a series of three separate experiments demonstrating septal activation in response to the movement of alive conspecifics, and the second experiment to reveal activation of the preoptic area (Mayer et al. 2017b, a). In the present study, septal activity in the right hemisphere was higher after exposure to the predisposed speed-change *animate* stimulus, compared to chicks exposed to the control speed-constant *inanimate* stimulus. Moreover, also in the POA we found higher number of c-Fos-ir cells for chicks exposed to the *animate* stimulus, compared to the control group. The observed differences were region specific and not due to the overall activity of the brains, since the densities of c-Fos-ir cells in the arcopallium and IMM were not different between the groups. In addition, the differential activation of the septal nuclei and of the POA can be explained only by the visual stimulation provided by the experimental stimuli: both groups of chicks were exposed to the same visual environment, and no behavioural differences could be observed between them. This was probably facilitated by the fact that both the test stimuli present various features that are socially attractive to chicks: movement itself, the red colour, the appropriate shape and size to elicit optimal filial imprinting (see Study 1). It should also be noted that the procedure employed in this experiment was specifically designed to avoid behavioural differences between the two groups, which could potentially complicate the interpretation of the brain results. In fact, the preference for the *animate* had been already established in the previous behavioural study (see Study 1). Since we obtained our measurements from chicks exposed only to either one of the two stimuli, rather than from chicks that showed different preferences between the two, the focus of the investigation was not on the brain structures involved in the expression of the choice, rather on those involved in the processing and response of the predisposed and non-predisposed stimuli.

We thus demonstrated selective responsiveness to this preferred motion pattern that also elicits animacy perception in human observers (Tremoulet and Feldman 2000). This adds to the recent evidence that nodes of the social behaviour network (Newman 1999; O'Connell and Hofmann 2011; Goodson and Kingsbury 2013) might be already involved in early social responses of naïve animals exposed for the first time to an alive conspecific (Mayer et al. 2017b, a) and among distant vertebrate species in the perception of animacy motion cues (Carreira et al. 2017).

The difference in septal and POA activation could be associated with the different social responses elicited by the motion of the two stimuli. We assume that the exposure to the *animate* stimulus had a social valence for the experimental subjects, which are compelled to look for an imprinting object at this stage of development (Bateson 1966). The test exposure represented chicks' first encounter with a salient visual object (in general the first visual experience), whose appearance is optimal to elicit imprinting. In addition, our task was based on a social affiliative response (filial approach). Moreover, the same pattern of activation was found in chicks exposed to an unquestionably social stimulus, a living conspecific (Mayer et al. 2017b, a). Finally, this is also consistent with the widespread involvement of these two brain areas in social functions (Goodson 1998; Liu et al. 2001; Taziaux et al. 2006; Nishizawa et al. 2011; Klatt and Goodson 2013). Septum and POA could also be involved in establishing the emotional valence of the stimulus, as suggested by the fact that septum belongs to the mesolimbic reward system that process the valence of external stimuli (O'Connell and Hofmann 2011).

Social predispositions are believed to be adaptive in that they direct subsequent filial imprinting toward appropriate objects, prioritising attention towards animate creatures (Rosa-Salva et al. 2015). It is unknown so far, if septum or POA are involved in the learning process of filial imprinting, since no research has been conducted on the role of these structures in early social development. However, their role in pair bonding and parental care is well established in adult mammals and birds (Liu et al. 2001; Balthazart and Ball 2007; Goodson et al. 2009). Those social behaviours are mediated by the neuropeptides arginine vasopressin and oxytocin in mammals (Liu et al. 2001; Lim and Young 2006; Carter et al. 2008; Leng et al. 2008) and by their homologues arginine vasotocin and mesotocin in birds (Baeyens and Cornett 2006; Goodson et al. 2009; Klatt and Goodson 2013), for which these areas are rich of receptors (Newman 1999; Panzica et al. 2002; O'Connell and Hofmann 2011; Goodson and Kingsbury 2013). Also, the anatomical location where we counted inside POA (medial preoptic area) is dense of aromatase expressing cells, a signature of testosterone action in the brain (Balthazart and Ball 2007). Interestingly, the levels of testosterone in the IMM are associated with the preference for predisposed stimuli in chicks (Bolhuis et al. 1986). Therefore, the presence of sex steroid hormonal receptors within septum and POA may also suggest their involvement in imprinting on predisposed stimuli. Future studies could investigate which kind of hormonal receptors are expressed in

septal and preoptic neurons activated by the predisposed stimulus and whether the learning process of filial imprinting modulates septal and POA activity differently in relation to the presence of predisposed features in the imprinting stimulus.

Since the activity difference in septum was caused by exposure to visual cues of animacy, another question arises: what is the source of visual or visually modulated information that influences septal activity? So far there is no evidence that septum is directly devoted to visual processing. Visual input to septum could reach septum through its interconnection with the hippocampus (Atoji et al. 2002; Montagnese et al. 2004; Atoji and Wild 2004; Montagnese et al. 2008), which receives visual information from the visual Wulst (Atoji and Wild 2006). This is the telencephalic terminal of the thalamofugal visual projection and is considered the avian homologue of the primary visual cortex (Medina and Reiner 2000; Wild and Williams 2000). This is also compatible with a possible role of septum in modulating the imprinting process, since both visual Wulst and hippocampus have been implicated in imprinting (Güntürkün et al. 1993; Sadananda and Bischof 2004; Maekawa et al. 2007; Aoki et al. 2015).

It is still unclear which might be the specific functional consequences of the activation of septum and preoptic area in this context. However, we can propose some speculations based on c-Fos action on several late response genes involved in modulation of connectivity (Sheng and Greenberg 1990). The activity we measured could be related to rewiring in the brain areas of interest, reflecting processes associated with the tuning of the network of areas that control social responses, such as filial approach, towards the most appropriate social objects. The plastic changes might reflect the adjustment associated with the first coming online of some components of the social-decision-making network, concurrently with the first socially relevant experience of the naïve animals.

The tractus septopallio-mesencephalicus, which runs through septum in the dorso-ventral direction, connects the visual Wulst back to the optic tectum (Karten et al. 1973; Reiner and Karten 1983; Manns et al. 2007). The optic tectum is an important structure for orienting toward stimuli (Hodos and Karten 1974; Jarvis 1974) and for the perception of motion (Frost and Nakayama 1983; Frost et al. 1988, 1990), with some tectal neurons of domestic chicks responding specifically to changes in speed (Verhaal and Luksch 2016). An involvement of the optic tectum in social predispositions was also postulated from our group

(Rosa-Salva et al. 2015). However, the tractus septopallio-mesencephalicus has never been shown to terminate in septum and thus it is unclear if it can contribute to the septal activation.

Finally, it is important to consider that sub-telencephalic regions might play important role in innate complex stimulus recognition in chicks (Zachar et al. 2008; Rosa-Salva et al. 2015) and some of them, such as the pretectal nucleus, sends projections to the septum (Montagnese et al. 2008). Thus, it is also possible to hypothesise a contribution of sub-telencephalic regions to the septal activation found in the present study.

We also considered which visual features caused the observed effects. The two stimuli were balanced for most low-level features, such as colour, size, distance travelled and average speed. However, they necessarily differed in the maximum speed reached by each object. In principle, we cannot exclude that the differences observed in this study were due to this factor, but we consider this highly unlikely. Study 1 clearly demonstrated that chicks' social preference for the *animate* stimulus is elicited by the presence of visible speed changes, and not by speed itself (Rosa-Salva et al. 2016): occluding the moments of speed change abolished the preference. Given the involvement of septum and POA in social behaviours, it is reasonable to assume that the activation of these areas is driven by the same visual cues that affect chicks' affiliative responses. Moreover, identical patterns of activation in the right septum and in the POA have been found in our previous studies (Mayer et al. 2017b, a). Notably, in these studies chicks were exposed to a clearly social dynamic stimulus (a living conspecific), which obviously differs markedly from the speed-change stimulus in its low-level perceptual features, including its speed of motion.

Another interesting aspect of our results is that the effect found in septum was restricted to the right hemisphere. Higher right hemisphere activation has been reported for human adults (Grossman et al. 2000) and infants (Ichikawa et al. 2010) viewing point-light displays of biological motion, another kind of stimuli associated with animacy perception that elicit social predisposition in chicks (Vallortigara et al. 2005). Likewise, in chicks the left eye-system (right hemisphere) is preferentially used to monitor this kind of stimuli (Rugani et al. 2015). Overall, this is in line with the preferential involvement of the right hemisphere in social responses, social recognition and the rapid recognition of emotional stimuli (Rogers et al. 2013; Vallortigara and Versace 2017). In chicks the left-eye system is more reactive

to emotionally charged stimuli (Rogers and Anson 1979), is dominant for cognitive abilities involved in the formation of the dominance hierarchies (Daisley et al. 2010), is involved in early testosterone-induced courtship behaviour (Rogers et al. 1985), individual recognition of social companions and social learning by observation (Vallortigara and Andrew 1991; Vallortigara 1992; Vallortigara et al. 2001; Deng and Rogers 2002a; Rosa-Salva et al. 2009, 2012a).

To understand the lateralised effect observed in septum, it is important to consider the overall lateralisation pattern that emerged from our results. Regardless of the experimental condition, all brain areas showed a trend for higher activity in the left hemisphere compared to the right one: this may represent a baseline lateralisation effect, independent from the experimental stimuli. A similar trend for a spontaneous higher activation of the left hemisphere has been reported in our previous study on septal activation in chicks exposed to a living conspecific. Also in this case, the effect observed in septum was limited to the right hemisphere, resulting in a similar level of activation of the left and right septum in chicks exposed to the conspecific, while the septum of the control group showed the usual spontaneous left lateralisation, as did other areas whose activation was not different between the groups (Mayer et al. 2017a). Activation in the right septum could be more easily modulated by exposure to visual cues typical of social stimuli than in the left septum, due to the masking effect caused by the left hemisphere's spontaneous higher level of activity (*e.g.*, a ceiling effect). In order to understand whether this trend for left lateralisation was spontaneous, we specifically designed an IEGs experiment investigating the pattern of c-Fos-ir cells without any stimulation reported in Study 4 of the present work. It is unclear why this general trend for left lateralisation was significant only in the IMM of chicks exposed to the speed-change stimulus, even though the same trend was visible also in the control group. This could be associated with the fact that biochemical and morphological changes associated with imprinting are generally more marked in the left IMM (Horn 2004), which might enhance the general leftward trend especially for those chicks exposed to the preferred stimulus (a more attractive imprinting object). However, lateralisation in imprinting learning is more complex than that, with interactions between the different types of information to be stored and the time course of memory formation (*e.g.* Vallortigara and Andrew 1994; Solomon et al. 1997, 1998; Andrew 1999; Mayer et al. 2016b).

The same general trend for left lateralisation was present in arcopallium for both groups. Moreover, left arcopallium activity correlated positively with latency of first approach. Since arcopallium is involved in fear responses (Phillips 1968; Martin et al. 1979), this could reflect individual variation in the level of cautiousness: latency to approach the stimulus will be influenced by the duration of the initial freezing response in the novel environment. However, usually the control of fear responses is lateralised in favour of the right arcopallium (Phillips and Youngren 1986; Rosa-Salva et al. 2009). Here the correlation regarded the activation of arcopallium of the left hemisphere, making an interpretation in terms of simple fear responses less straightforward. A more plausible interpretation involves the left hemisphere specialisation for response control mechanisms (Bullock and Rogers 1986; McKenzie and Andrew 1996; Andrew 2009): *e.g.*, the left hemisphere is especially involved in deciding whether to approach a potential imprinting object (McKenzie et al. 1998). We hypothesise that the more reluctant a subject was of moving in the environment, the more the left hemisphere was activated by the task of controlling (inhibiting) the emission of the approach response.

Overall, the results of the present paper demonstrate an involvement of right septal nuclei and preoptic area in early social responses of visually naïve animals, in line with previous studies showing that they are activated by the first exposure to a living conspecific (Mayer et al. 2017b, a). This confirms that these important nodes of the social behaviour network are already engaged in social responses at birth and that learning experiences associated with social companions are not needed for their involvement in this function. Moreover, we also demonstrated for the first time a sensitivity of these brain areas to the very same elementary motion cues that are typical of animate creatures and elicit chicks' social predispositions for filial approach (Study 1: Rosa-Salva et al. 2016), human infants' preferential attention (Frankenhuis et al. 2013) and adults' perception of animacy (Tremoulet and Feldman 2000).

Static features eliciting social predispositions

The detection of animate creatures can be triggered by static configurations of features. Particularly, the configuration of head and neck is a powerful cue related to the presence of a living creature. In an early set of studies, it was found that newly hatched chicks prefer a taxidermised hen over a similar hen cut into small pieces and reassembled in scrambled order on a box, which did not preserve the head-neck configuration (Johnson and Horn 1988). Here we try to replicate the original findings in our laboratory. Moreover, we investigate the neural correlates of the spontaneous preference for predisposed stimuli resembling naturalistic configurations of features by visualising c-Fos expression in three different brain areas.

Study 3: Social predisposition dependent neuronal activity in the intermediate medial mesopallium of domestic chicks (*Gallus gallus domesticus*)³

Abstract

Species from phylogenetically distant animal groups, such as birds, and primates including humans, share early experience-independent social predispositions that cause offspring, soon after birth to attend to and learn about conspecifics. The behaviour of newly hatched visually naïve chicks that preferentially approach a stimulus resembling a conspecific (a stuffed fowl) rather than a less naturalistic object (a red box or a scrambled version of the stuffed fowl) provide one example of this phenomenon. However, the neuronal mechanisms underlying this behaviour are mostly unknown. Here we analysed chicks' brain activity by an immunohistochemical staining c-Fos. In a spontaneous choice test, we confirmed a significant preference for approaching the stuffed fowl over a texture fowl (a fowl that was cut in small pieces attached to the sides of a box in scrambled order). Comparison of brain activation of a subgroup of chicks that approached either one or the other stimulus revealed differential activation in an area relevant for imprinting intermediate medial mesopallium, suggesting that a different level of plasticity is associated with approaching predisposed and non-predisposed stimuli. A similar trend was observed also in the intermediate layers of optic tectum, a candidate area for processing early orienting toward social stimuli.

³ Please note that data here reported have been published on *Behavioural Brain Research* in 2016 (Mayer et al. 2016b).

Introduction

The ability to identify animate creatures rapidly and from very early in life is biologically relevant for species phylogenetically distant as birds and primates (Johnson 2005; Sugita 2008; Rosa-Salva et al. 2015). This is particularly true for domestic chicks, that are subject to filial imprinting, a learning phenomenon that restricts subsequent social behaviour to an object experienced shortly after hatching (McCabe 2013; Bateson 2015). In controlled laboratory settings, imprinting can be obtained for a variety of artificial objects. Nevertheless, imprinting is not constrained completely to its object. Of particular interest, domestic chicks are facilitated to imprint on naturalistic objects, such as a mother hen or a stuffed red jungle fowl (*Gallus gallus spadiceus*, the wild ancestor of domestic chicks, Zeuner 1963), over artificial objects (Salzen and Meyer 1968; Cherfas and Scott 1981; Kent 1987; Bolhuis and Trooster 1988). This suggested an interaction of two independent mechanisms: a learning process of imprinting and a prewired predisposition to approach certain kinds of visual objects that emerges in the first days of life. A series of seminal studies conducted by Gabriel Horn and his collaborators described a preference to approach a stuffed red jungle fowl hen with respect to highly salient artificial stimuli, such as an illuminated red box, in visually naïve chicks (*e.g.* Bolhuis et al. 1985, 1989; Johnson et al. 1985; Johnson and Horn 1986, 1988; Davies et al. 1992; Hampton et al. 1995; Bolhuis and Horn 1997; Horn 2004; see Figure 1 (b)). These authors also demonstrated a crucial role for the configuration of features contained in the head and neck of a hen (Johnson and Horn 1988; Rosa-Salva et al. 2015). Notably, in one of these studies the canonical fowl was preferred over the so-called “texture fowl” (a jungle fowl that was cut in small pieces attached to the sides of a box in scrambled order). Studies conducted by our group further refined the behavioural characterisation of chicks’ approach preferences. In line with what has been observed in newborns of human and non-human primates (Johnson 2005; Sugita 2008), naïve chicks have a preference for face-like schematic stimuli and photographed faces over control stimuli matched for low-level properties (Rosa-Salva et al. 2010, 2011, 2012b). However, despite the amount of work done on the behavioural characterisation of the social predispositions displayed by this model organism, very little is known about the underlying neural mechanisms. The only attempt to investigate the neuronal basis of this social predisposition in naïve chicks (Egorova and Anokhin 2003) focussed on the neural correlates of non-specific priming stimulations that cause the emergence of the

predisposition (Bolhuis et al. 1985; Johnson et al. 1985; Bolhuis et al. 1989; Davies et al. 1992; Hampton et al. 1995; Bolhuis and Horn 1997; Egorova and Anokhin 2003). In this study, they were stimulating chicks with either a motoric or an acoustical non-species specific priming before the preference test for predispositions. Behavioural results showed that both groups that received a priming exhibited a strong preference for the predisposed stimulus, whereas non-stimulated chicks that remained in the darkness until testing did not show the preference for the predisposed stimulus. Brain results may indicate an involvement of the medial part of the caudal nidopallium (neostriatum according to the early nomenclature; see Jarvis et al. 2005 for nomenclature change), an area involved in the recognition of species-specific communication (Horn and McCabe 1984; Stripling et al. 1997; Ribeiro et al. 1998; Duffy et al. 1999). Despite its interesting approach, the study of Egorova and Anokhin (2003) has severe limitations, such as the small number of subjects used to investigate brain activity (3–5 per condition). Moreover, the activity in the caudal medial nidopallium could simply represent an effect of the increased number of calls emitted by stimulated chicks, compared to the unstimulated controls, which were kept in the darkness. This makes it difficult to draw firm conclusions from their results, calling for further investigation. To shed light on these issues, we performed an experiment on the neuronal basis of domestic chicks' predisposition to approach hen-like stimuli, using the immediate early gene product c-Fos as a neuronal activity marker. Social predispositions are bound to interact with imprinting by directing chicks' attention towards appropriate social objects and imprinting is indeed facilitated for naturalistic objects compared to artificial ones (Salzen and Meyer 1968; Cherfas and Scott 1981; Kent 1987; Bolhuis and Trooster 1988). Thus, in the present study we focussed on the intermediate medial mesopallium, IMM an area crucially involved in filial imprinting (McCabe et al. 1981; Horn and McCabe 1984; Johnson and Horn 1986; Horn 1986, 1990; Bolhuis and Honey 1998). Although it is known that the preference for hen-like objects is not suppressed by bilateral IMM lesions (Horn and McCabe 1984), it has never been investigated whether neuronal mechanisms related to imprinting respond differently to naturalistic and artificial stimuli. We hypothesised that activity within the IMM would differ between chicks that spontaneously approached a stuffed hen or a texture fowl. As regards the direction of the effect, we expected higher activation for the individuals that approach the stuffed hen. The second brain region of interest in this study was the optic tectum (TeO), which represents the avian

homologue of the mammalian superior colliculus. In a recent review we have summarised the existing literature identifying candidate brain areas relevant for social predispositions (Rosa-Salva et al. 2015), with particular regard to some sub-pallial (homologues of subcortical) structures. These structures include the optic tectum, which was hypothesised by some scholars to be crucial for early orienting toward social stimuli in both chicks and human newborns (Johnson 2005). As for IMM, we thus expected differential activation of TeO in chicks that approached the two stimuli. As a last region of interest we selected the hyperpallium apicale (HA, Hyperstriatum accessorium, old nomenclature), a part of the visual Wulst which is homologue to the visual cortex in mammals (Karten et al. 1973; Medina and Reiner 2000). We expected to find no difference in the activation of this area, since all chicks were exposed to the same visual environment and the stimuli were well balanced for the low-level perceptual properties.

Material and methods

Subjects

Seventy-six domestic chicks were employed for this study. Eggs were hatched in individual compartments (12×8cm) separated by thin plexiglas walls, in dark incubators. Approximately 24h after hatching the temperature of the incubators was set to 33°C. To estimate individual hatching time-points, each incubator was equipped with an infrared LED lamp and a camera (CCD Board camera 8.47mm, 1/3"). Photos were captured digitally every 20min with a time-lapse software (Super Viewer, Somagic Inc) starting at least 24h before the expected hatching time. Chicks were treated according to two fundamental procedures (Figure 23). The main difference between the two was that in the first procedure (upper line of Figure 23) chicks did not receive any visual experience prior to the moment of the preference test, whereas in the second procedure (lower line in blue of Figure 23) chicks were habituated to light for some hours before undergoing the preference test. Thirty chicks underwent the first procedure and were used only for behavioural observations to confirm the presence of a predisposition to approach a stuffed fowl.

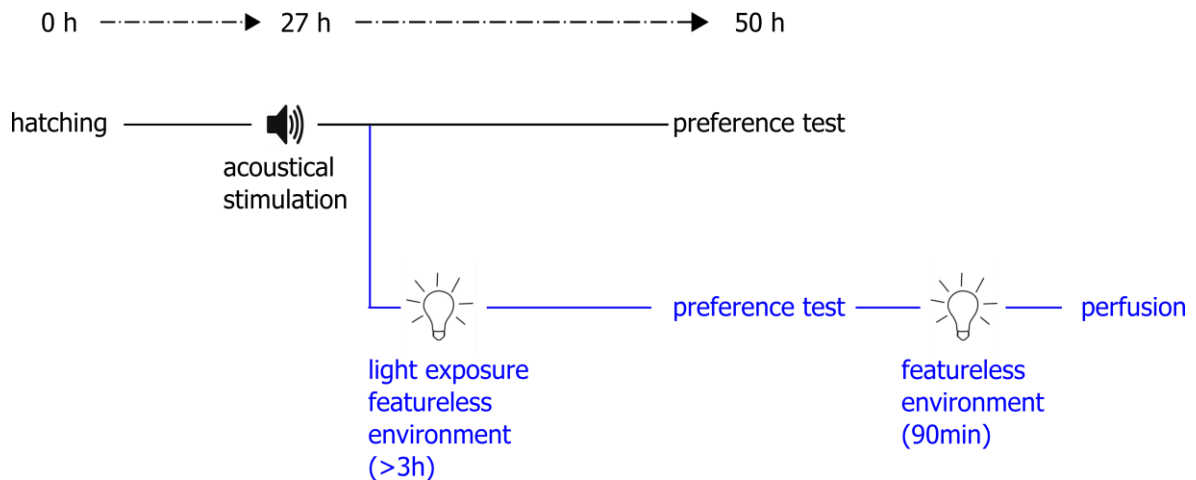


Figure 23: Different conditions in Study 3.

Schematic representation of the experimental flow in the two different conditions. All chicks were stimulated acoustically at around 27h of age. In the first condition (black upper line), chicks were kept in the darkness until test at around 50h after hatching. Chicks that underwent the second condition (lower blue line), were light exposed in a featureless environment and then tested around 50h of age. Chicks that were used for brain studies were put back to the featureless environment until perfusion.

Thirty-eight chicks underwent the second procedure; a preference test between the same two stimuli tested in the first procedure. Their behavioural data were analysed as for the first procedure. Of these 38 chicks, 23 animals were selected for the study of brain activity. Only these individuals were sacrificed. The 23 animals used for the study of brain activity were selected because at the preference test they expressed an absolute preference for one of the two stimuli (*i.e.* they approached only one of the two, spending all their choice time near that stimulus, without alternating between the two). Fourteen of these chicks approached the stuffed fowl and 9 the texture fowl. One brain from the stuffed fowl preference group was damaged during processing and was thus excluded from further analysis, bringing the final sample to 22 (stuffed fowl N=13, texture fowl N=9). Finally, an additional group of 8 chicks was used to create a baseline condition for the brain activity study. These subjects were treated according to the second procedure, with the only difference that they were never exposed to the two stimuli. These chicks were also sacrificed at the end of the procedure to investigate brain activation. No behavioural data was collected from them.

Testing apparatus

The experimental apparatus employed was a black corridor (220x44x45cm, Figure 24). The walls and the floor of the corridor were covered with black non-reflective material. The two ends of the corridor were delimited with a black metal grid, behind which the experimental stimuli were visible against a black background. The stimuli were placed on two rotating platforms (5 rpm).

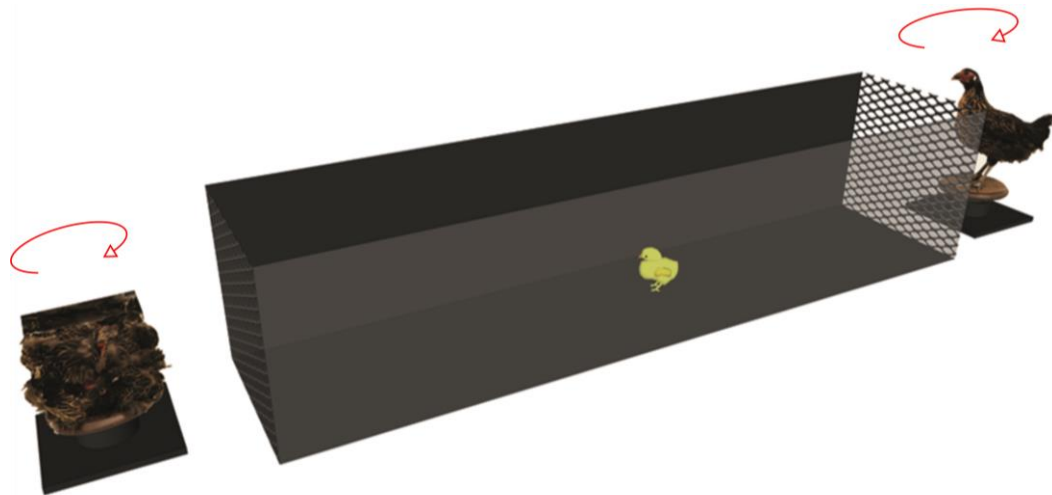


Figure 24: Test apparatus Study 3.

Schematic representation of the experimental apparatus employed. For demonstrative reason one lateral long wall is depicted translucent. Chicks were positioned in the centre and were free to approach either the texture fowl or the stuffed fowl, which were rotating behind a grid at the two opposite ends of the choice runway.

On one side of the corridor chicks could see a stuffed fowl hen ("stuffed fowl"), whereas on the other side there was a "texture fowl" (a 9×18cm box, with all side facing surfaces covered with small pieces cut from the pelt of a second identical fowl, attached in scrambled fashion; see Figure 25 and Johnson and Horn 1988). The stuffed fowl hens were acquired from a local taxidermist and selected to resemble as closely as possible the jungle fowl hen used in previous studies on chicks' social predispositions (Johnson and Horn 1988). These two stimuli were balanced for low-level visual properties such as luminance, colour, visual texture and movement, but they differed in the spatial configuration of the local features that characterise the stuffed fowl (Figure 25).



Figure 25: Photographs of the two stimuli employed in Study 3.
(a) A stuffed jungle fowl. (b) A texture fowl, a similar fowl was cut in small pieces and stuck in scrambled order to the sides of a box.

The left-right placement of the stimuli in the corridor was counterbalanced between subjects. The stimuli were illuminated from above (120cm). Light (45W, warm light) was diffused by placing a semi-translucent white plastic sheet under the bulb. Lamp placement was such that most of the corridor was only dimly illuminated, except for the two end-ports of the corridor that were directly adjacent to the stimuli. These two illuminated areas (each 30 cm long) represented the “choice sectors” used to score chicks’ approach behaviour toward the stimuli. Behaviour was recorded through a digital camera suspended centrally above the arena and then analysed off-line.

Acoustical stimulation procedure

According to the protocol we wanted to replicate, approximately 24 h after hatching (mean age=27h, S.E.M.=0.49) we acoustically stimulated chicks. This stimulation was described to elicit the emergence of the predisposition for the fowl (see Egorova and Anokhin 2003). They were positioned in individual cardboard compartments (10×10cm), inside a different dark incubator (33°C) with a loudspeaker. All handling and transportation of the chicks occurred in the dark. Non-species-specific sound stimulation was provided using a digitally constructed audio-file composed of non-repeating rhythmic segments of music. For this purpose, 15 stereo tracks containing music from different genres were layered and played simultaneously. The 3h audio-file was divided into fragments and rendered to a single

mono-file. The frequency of sound oscillations in the fragments varied from 100 to 12000Hz and the loudness of the sound varied from 50 to 98dB. The duration of individual musical fragments varied from 10 to 60s and the duration of intervals between them varied from 30 to 90s. Stimulation lasted a total of 180min (four 45min sessions with 15min intervals between sessions). After the end of the stimulation, chicks participating to the first procedure were maintained in individual compartments within a dark incubator until the test. For chicks participating to the second procedure, in order to reduce brain activity caused by the first exposure to light, the chicks were allowed to habituate to light before the test. For this purpose, each chick was exposed for at least 3h to a featureless environment (a rectangular cage of 28×40×32cm, with non-reflecting white walls and floor) that was illuminated from above with a light source (led 240lx, colour temperature 3000K).

Test of the behavioural preference for the stuffed fowl

Chicks of the first procedure were individually tested for their spontaneous preference between the two stimuli (mean age at the time of test=50.1h, S.E.M.=0.89). Each subject was placed in the centre of the choice corridor (facing one of the two long walls). Chicks were free to move in the corridor for the entire duration of the test (8min), while their behaviour was recorded. Whenever a chick entered one of the two "choice sectors", this was considered as an approach toward the adjacent stimulus. The chick's starting position with respect to the two long walls, as well as the left-right position of the two stimuli within the apparatus, were counterbalanced across animals. This test procedure was adapted from the works of Gabriel Horn and his collaborators (Bolhuis et al. 1985; Johnson et al. 1985; Johnson and Horn 1986, 1988; Bolhuis et al. 1989; Davies et al. 1992; Hampton et al. 1995; Bolhuis and Horn 1997; Horn 2004; McCabe 2013).

Test session for c-Fos labelling

Also for the second procedure, after at least 3h the preference test was conducted as described above (mean age at test=52.9h, S.E.M.=0.86). The chicks to be included in the sample for brain activity measurements were selected because they expressed an absolute preference for either stimulus (approaching one stimulus and spending the totality of their choice time near that stimulus, without alternating between the two). Immediately after the end of the test, those chicks were placed back in their familiar 'featureless' cages, where

they remained until the time of perfusion. According to our experimental design, chicks were divided in two “choice-groups” based on the stimulus they chose to approach during the spontaneous preference test (either the stuffed fowl, or the texture fowl). We also performed an additional *post-hoc* baseline condition. Chicks in the baseline condition were treated exactly according to the same procedure as the other animals used for brain measurements. The only difference was that, instead of undergoing the preference test between the two stimuli, baseline chicks were placed for an identical amount of time (8min) in the empty apparatus (no stimuli present). The rationale behind this condition was to measure the baseline activity caused in IMM by aspects of the procedure other than the exposure to the two stimuli, such as light exposure in the featureless environment and exposure to the test set-up. In these baseline brains, in addition to IMM we also measured the activity in the HA as a test of region-specificity.

Immunohistochemistry

Please see section Immunohistochemistry in Study 2, the procedure here employed was identical.

Analyses

Brains analyses

Several brain structures showed c-Fos immunoreactivity, including areas of the hyperpallium, mesopallium, nidopallium, hippocampus, septum, olfactory bulb and striatum, together with several mesencephalic and diencephalic structures. In order not to lose statistical power, we measured the density of c-Fos-ir cells only in the three areas of interest: the intermediate medial mesopallium (IMM), the optic tectum (TeO) and the hyperpallium apicale (HA). To estimate labelled cell density in the IMM 5 brain slices were selected from a region where the shape of IMM was corresponding to what is depicted in plate A7.6 of the Kuenzel and Masson atlas (1988). The rectangular enclosure (150x250 μm^2) was positioned inside the IMM according to the drawings of Ambalavanar et al. (Ambalavanar et al. 1993) exactly how it was done in Study 2 of the present work (see Figure 15). Labelled cells in the HA were counted from 5 sections of each hemisphere, which were selected in accordance to the region extending from A13.0 to A11.0 of the atlas

(Kuenzel and Masson 1988). One counting rectangle was positioned within the dorso-medial HA of each section (Figure 26).

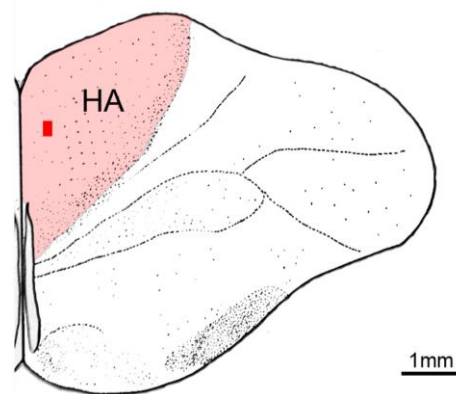


Figure 26: Counting area in hyperpallium apicale. Schematic drawing of a coronal section through frontal telecephalon. The red rectangle in the typical placement of cell counting within the dorso-medial hyperpallium apicale (HA) painted in light red. Adapted from Kuenzel and Masson atlas (1988).

Counting within the TeO of each hemisphere was performed within the dorsal, medial and ventral parts separately (Figure 27). In each of these subdivisions we further distinguished between outer layers 1–9, intermediate layers 10–12 and inner layers 13–15 (for the definition of these layers, see the supplementary plate A4.6 in the Kuenzel and Masson 1988). The counting rectangle was positioned in each of the 9 subdivisions of each hemisphere. After completing the cell counts for each animal, mean values from the five sections were calculated per hemisphere and cell densities were standardised to 1mm^2 . Thus the calculated neuronal activity of an individual in the IMM and HA was based on 5 counted areas for each hemisphere. Cell counts pooled from the different subdivisions of TeO (dorsal, medial, ventral) were further averaged to estimate overall activity in the TeO. Initially, this was done separately for the outer, intermediate and inner layers. However, because no significant differences were found between these subdivisions, the measured values from all 9 subdivisions on 5 slices of each hemisphere were pooled for further analysis. Thus, the overall estimate TeO activity in one hemisphere of an individual bird was based on an average of 45 counted areas. The resulting means for individual birds were considered overall indicators for the number of c-Fos-ir neurons and were employed for further statistical analysis.

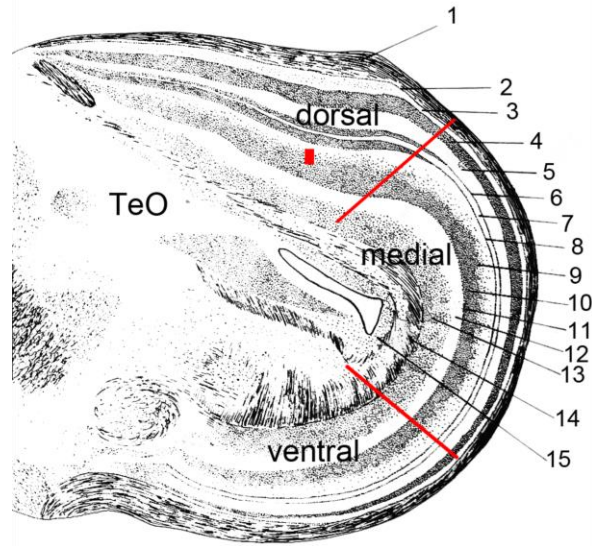


Figure 27: Counting areas in optic tectum.

Schematic drawing of a coronal section through optic tectum (TeO) showing the 15 layers and its portioning into dorsal, medial and ventral (red lines). The red rectangle in a typical placement for cell counting. Adapted from Kuenzel and Masson atlas (1988).

Statistical analyses

In order to assess chicks' behavioural preferences, the total time spent in each choice sector was used to compute a *ratio* as preference index according to the formula:

$$ratio = \frac{time\ near\ the\ fowl}{time\ near\ the\ fowl + time\ near\ the\ texture\ fowl}$$

Significant departures from chance level (0.5), which indicated a preference for the predisposed (>0.5) or the control stimulus (<0.5), were estimated by one-sample two-tailed *t*-test. The presence of difference in the density of IEG-ir neurons was tested by a mixed-design ANOVA, with a between-subject factor "choice group" (2 levels: fowl and texture fowl) and a within-subject factor "area*hemisphere" (6 levels: 3 areas: HA, IMM, TeO per 2 hemispheres: left and right). To correct for violation of sphericity a Greenhouse-Geissler correction was applied. For *post-hoc* analyses, *t*-tests (two tailed) were carried out for each area. To correct for multiple measurements the Bonferroni correction for 12 comparisons was applied. For every *t*-test Cohen's *d* was calculated as a measure of effect size with the software G*Power version 3.

Results

Behavioural

The choice behaviour of the subjects from the first procedure revealed a clear preference for approaching the stuffed fowl with respect to the texture fowl. In fact, the proportion of time spent near the stuffed fowl during the test was significantly different from chance level ($t_{(29)}=2.846$, $p=0.008$, $d=0.52$), with an average preference score of 0.73 (S.E.M.=0.08; see Figure 28 (a)). This means that on average chicks spent 73% of their total choice time in the sector adjacent to the stuffed fowl.

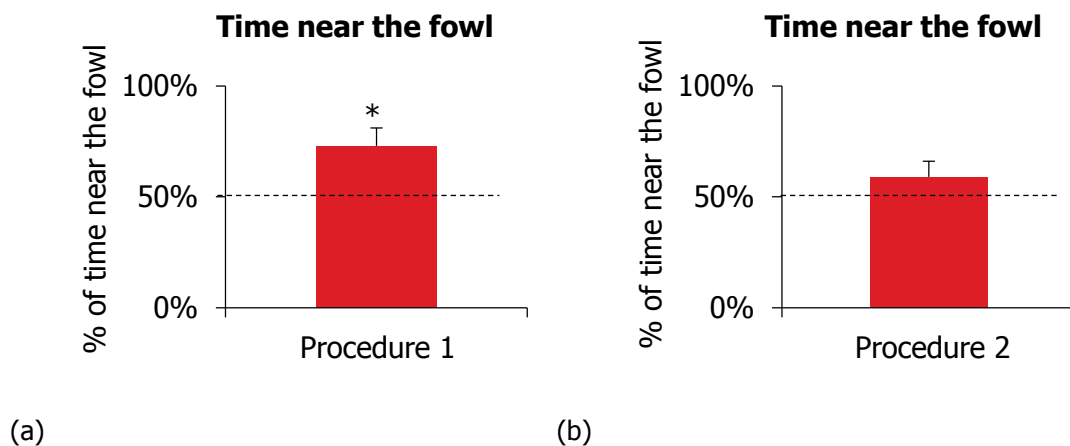


Figure 28: Behavioural results of Study 3.

Percentage of the ratio of time spent near the stuffed fowl over the total choice time. (a) Significantly different from chance in the first procedure. (b) A similar non-significant trend in the second procedure. Asterisk indicate significance ($\alpha \leq 0.05$), dashed line the chance level (50%). Error bar indicates S.E.M..

A similar, although non-significant, trend was observed in the chicks treated according to the second procedure, from which we obtained the subjects employed for the neuroanatomical investigations. The average preference score of 0.59 (S.E.M.=0.07; see Figure 28 (b)), was not significantly different from chance level ($t_{(37)}=1.252$, $p=0.219$, $d=0.21$). However, a higher number of chicks approached the stuffed fowl rather than the texture fowl, resulting in an imbalance between the sample sizes of the two groups in the brain study. The chicks to be included in the sample for brain activity measurements were selected because they expressed an absolute preference for either stimulus (having a preference score of either 0, absolute preference for the texture fowl, or 1, absolute preference for the stuffed fowl). These birds were divided in two groups according to the

choice they made: chicks that approached the stuffed fowl and chicks that approached the texture fowl.

Immunohistochemistry

The ANOVA revealed significant differences in the number of c-Fos-ir neurons between the two groups in a region-dependent fashion (interaction of area*choice-group: $F_{(2,645,52.895)}=5.236$, $p=0.004$). Importantly, IMM counting showed significant differences in the density of c-Fos-ir neurons between the two experimental groups (Figure 29).

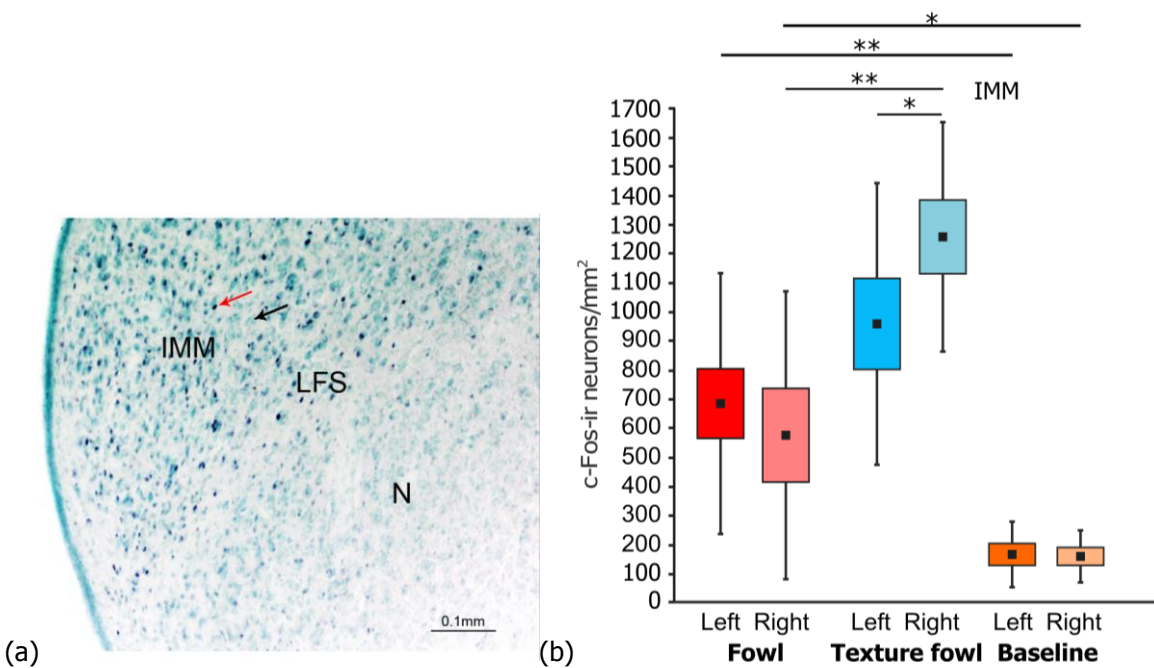


Figure 29: Results in intermedial medial mesopallium.

(a) Photomicrograph of a coronal section through right IMM of a chick that approached the texture fowl. A high number of c-Fos-ir cells (red arrow) is clearly discernible from green-stained non-reactive neurons (black arrow). N-nidopallium, LFS-lamina frontalis superior. (b) Estimated c-Fos-ir cell densities in IMM for the three groups for the two hemispheres (red-fowl; blue-texture fowl; orange-baseline). Significantly higher density of c-Fos-ir cells in the right than in the left IMM of the texture fowl group and in the right of the fowl group. Significantly higher density was present in the fowl group than in the baseline group for both hemispheres. Graph-plot: mean (black square), S.E.M. (box), S.D. (whiskers), * indicates $p \leq 0.05$, ** indicates $p \leq 0.001$. On the Y-axis are represented c-Fos-ir cells per mm^2 .

The density of labelled neurons in the right IMM of birds that approached the texture fowl (mean±S.E.M.: 1259.3 ± 131.7 c-Fos-ir neurons/ mm^2) was two-fold higher compared to the right IMM of the stuffed fowl preference chicks (mean±S.E.M.: 576.4 ± 136.7 c-Fos-ir

neurons/mm²). *Post-hoc t*-test comparison between the right IMM of birds that approached the texture fowl with those that approached the stuffed fowl: $t_{(20)}=-3.452$, $p=0.003$, $d=1.54$ (after a Bonferroni correction for 12 measurements: $p=0.036$). Such group difference was not present in the left IMM: $t_{(20)}=-1.191$; $p=0.247$, $d=0.52$. The IMM activity in birds that approached the texture fowl showed also a significant lateralisation (Figure 29). *Post-hoc t*-test: Right IMM (texture fowl) vs. Left IMM (texture fowl): $t_{(8)}=-3.316$; $p=0.011$, $d=-3.33$ (Bonferroni correction for 12 measurements: $p=0.132$). The difference within IMM was region specific and not due to the overall activity of the brains, since the activation in HA (visual Wulst) was nearly identical in both groups (Figure 30).

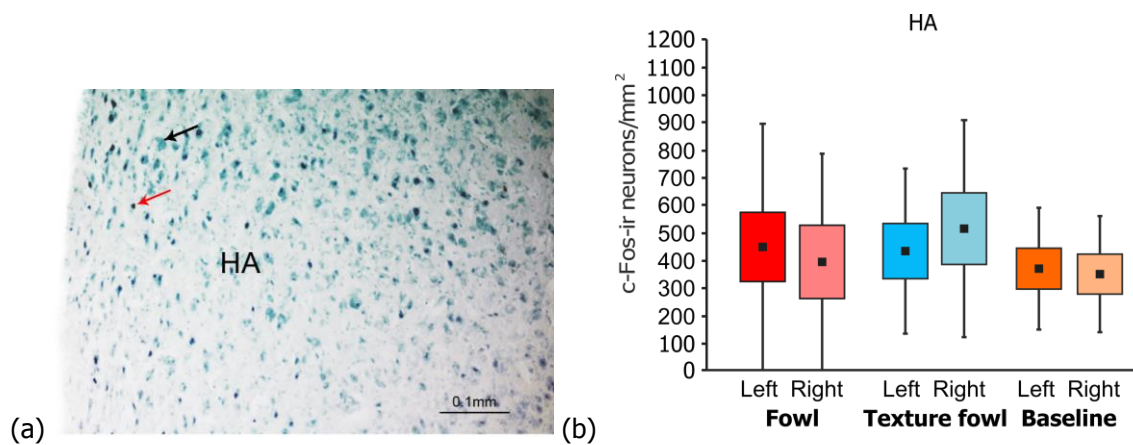


Figure 30: Results in hyperpallium apicale.

(a) Photomicrograph of a coronal section through the right frontal telencephalon of a chick that approached the texture fowl. A high number of c-Fos-ir cells (red arrow) is clearly discernible from green-stained non-reactive neurons in HA (black arrow). (b) Estimated c-Fos-ir cell densities in HA for the three groups for the two hemispheres (red-fowl; blue-texture fowl; orange-baseline). Graph-plot: mean (black square), S.E.M. (box), S.D. (whiskers). On the Y-axis are represented c-Fos-ir cells per mm².

A non-significant trend for enhanced activity was also present within the TeO of the right hemisphere (Figure 31), in the group of chicks that approached the texture fowl (mean±S.E.M.: 403.15±136.6 c-Fos-ir neurons/mm²) compared to chicks that approached the stuffed fowl (mean±S.E.M.: 245.0±88.8 c-Fos-ir neurons/mm²).

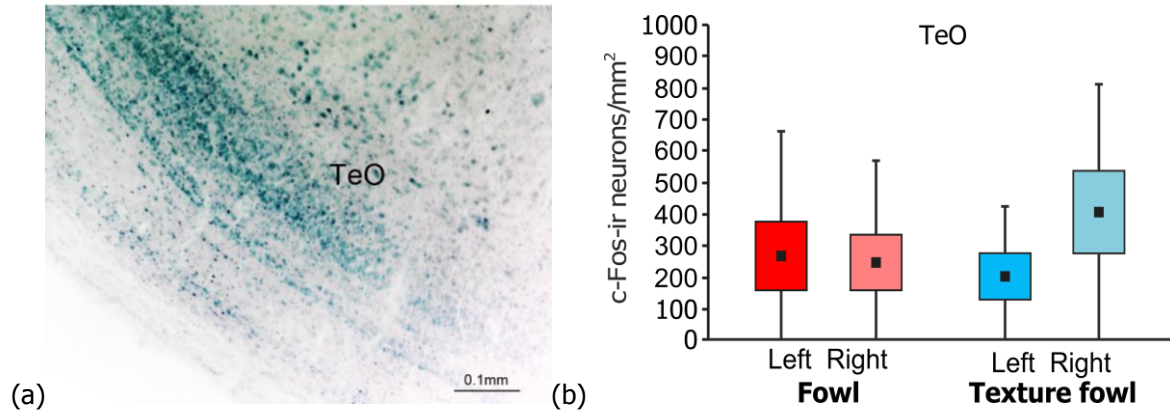


Figure 31: Results in optic tectum.

(a) Photomicrograph of a coronal section through the right TeO of a chick that approached the texture fowl. (b) Estimated c-Fos-ir cell densities in TeO for the two groups for the two hemispheres (red-fowl; blue-texture fowl). Non-significant trend for higher c-Fos-ir cells in the right TeO of the texture fowl group. Graph-plot: mean (black square), S.E.M. (box), S.D. (whiskers). On the Y-axis are represented c-Fos-ir cells per mm².

We performed an additional *post-hoc* baseline activity control experiment. Chicks of this group underwent the same procedure as others that were used for brain activity measurements (lower line in blue, Figure 23), with the only difference that, instead of expressing a choice between the two stimuli, they were exposed to an empty corridor. The IMM of these baseline control chicks contained only a very low number of c-Fos-ir neurons, which was significantly lower than in the other two groups (Figure 29). The measured c-Fos-ir density in the left IMM of the baseline group (mean±S.E.M.: 172.0±41.13 c-Fos-ir neurons/mm²) was three times lower than in the left IMM of the stuffed fowl choice group (mean±S.E.M.: 685.54±124.52 c-Fos-ir neurons/mm²), showing a highly significant difference $t_{(19)}=3.142$; $p=0.005$, $d=1.27$. Also the difference to the left hemisphere of the texture fowl group (mean±S.E.M.: 960.00±161.64 c-Fos-ir neurons/mm²) was highly significant $t_{(15)}=4.468$; $p<0.001$, $d=2.23$. Likewise, the activity in the right IMM of the baseline control group (mean±S.E.M.: 164.67±33.39 c-Fos-ir neurons/mm²) was more than three-times lower than in the right IMM of the stuffed fowl group (mean±S.E.M.: 576.4±136.7 c-Fos-ir neurons/mm²) and revealed a significance level of $t_{(19)}=2.290$; $p=0.03$, $d=1.16$. The activity was more than six-times lower compared to the right IMM of the texture fowl group (mean±S.E.M.: 1259.3±131.7 c-Fos-ir neurons/mm²), also this difference was highly significant $t_{(15)}=7.612$; $p<0.001$, $d=3.81$. The densities of c-Fos-ir

neurons were almost at the same level in the HA of all groups and both hemispheres (Figure 30), showing that differences that were present in the IMM were specific to this brain region.

Discussion

The present study demonstrates differential activation of IMM in chicks that approached a naturalistic visual stimulus (stuffed fowl) compared to those that approached a less naturalistic one (texture fowl). In a spontaneous choice test with visually naïve chicks (first procedure) we were able to obtain a significant preference for the stuffed fowl over a texture fowl, thus successfully replicating the results of Johnson and Horn (Johnson and Horn 1988). In chicks that were exposed to light prior to testing (second procedure), the strength of the preference decreased. In line with our initial hypothesis, comparison of brain activity in these subjects after their choice revealed a differential activation in the IMM. Contrary to our expectations, a significantly higher number of c-Fos-ir neurons was present overall in the IMM of chicks that approached the texture fowl, if compared to chicks that approached the stuffed fowl. However, neuronal activity in IMM of both groups was higher than the baseline condition, where it was nearly absent. This indicates that IMM activity is upregulated by processing of either of the two stimuli. As expected, such differences were not present in the HA, suggesting that the differential activity in IMM was region specific. Contrary to what we predicted, the number of c-Fos-ir neurons was also not significantly different in the TeO, although a slightly higher activity level was present in the right TeO of birds that approached the texture fowl.

IMM in chicks is important for learning and retention of visual properties of an imprinting object (McCabe et al. 1981, 1982; Horn 1998) and it contains neurons that respond to visual features of the imprinting object after training (McCabe and Nicol 1999; Horn et al. 2001 and see Maekawa et al. 2006; Nakamori et al. 2010; Yamaguchi et al. 2011, 2012; Aoki et al. 2015 for other areas involved in imprinting). At the same time, the IMM does not contribute to the emergence of predisposed preferences in chicks (Horn and McCabe 1984). In chicks, lesions to this area abolish learned object preferences, but chicks can still develop predisposition for the stuffed fowl. These results suggested that information about a complex object, which resembled the chicks' own species, is stored in a different manner than information derived from a relatively simple, but very salient, artificial object. In line with this evidence, IMM activity in our experiment differed depending on whether the chicks approached the stuffed fowl or the texture fowl. This indicates that the spatial arrangement

of the otherwise identical local visual features in the two stimuli provides input to IMM, which is different enough to influence its neuronal activity.

Given the very low c-Fos expression in the baseline condition, the higher activity found in the IMM of chicks that approached the texture fowl or the stuffed fowl is likely to reflect increased plasticity in these two groups. Indeed, c-Fos as an IEG product is rapidly activated after an increase of neuronal activity and plays an important role in neuronal plasticity related to learning (Lanahan and Worley 1998; Jones et al. 2001; Guzowski 2002; Fleischmann et al. 2003; Kubik et al. 2007). The higher c-Fos expression in the texture fowl group thus indicates increased plasticity in this group. This can be ascribed to the fact that this group was exposed to a visual object that does not fit to the innate template representing the appearance of the preferred naturalistic object. Encoding the properties of this object can require the storing of additional information for the aspects that do not fit the prewired template. A similar phenomenon has been found in sexual imprinting in zebra finches (Huchzermeyer et al. 2006). In this species, sexual imprinting on conspecifics is facilitated, but cross-species rearing can originate also sexual preferences for non-conspecifics. Rearing by non-conspecifics and subsequent preference for them is associated with higher IEGs expression than preference for conspecifics, in brain areas related to sexual imprinting (Huchzermeyer et al. 2006). In a situation closer to the present study, domestic chicks that show better recognition of their non-naturalistic imprinting object (a red box) have also higher number of Fos-like-ir neurons in the IMM, compared to chicks that have poor recognition performance (McCabe and Horn 1994). Thus, chicks that develop a better representation of the artificial imprinting object, as revealed by their superior recognition performance, have increased expression of plasticity markers in IMM. In addition, electrophysiological recordings of spontaneous activity of IMM neurons in chicks imprinted on a red box or on a stuffed fowl revealed a significant correlation with approach counts during training, but only for individuals imprinted on the red box (Payne and Horn 1984). This evidence reflects increased spontaneous IMM activity after learning of a stimulus to which chicks do not show a predisposition. Finally, it should be noted that the concentration of noradrenaline in the IMM correlates with the strength of filial imprinting for artificial stimuli, but not with the predisposition for hen-like objects (Davies et al. 1985, 1992). Noradrenaline has a crucial role in memory consolidation (see Horn 2004), in line

with our argument that there is a greater need for learning, and thus plasticity, in chicks exposed to artificial stimuli that do not fit the preferred template.

Another interesting finding of the present study is the lateralisation in chicks that approached the texture fowl, with higher activity in the right IMM. Filial imprinting has long been known to be lateralised in the IMM (Horn et al. 1985). Admittedly, our results seem to be at odds with the observation that biochemical and structural changes consequent to imprinting (on artificial objects) are more marked in the left hemisphere (Horn 2004; but see Horn et al. 1973; Johnston and Rogers 1998; for exceptions to this general pattern). However, the lateralisation pattern is more complex than that, with a crucial interaction between the different types of information to be stored and the time course of memory formation. The left and right IMM have been hypothesised to undergo parallel memory consolidation processes with different temporal profiles (see Andrew 1999). However, in the present study we chose to allow chicks only a very brief exposure to the two stimuli (8 minutes), consistent with the duration of the behavioural tests typically used for predispositions. This makes it difficult to “stage” our data with regard to the time course of memory formation in classical imprinting studies, where time is counted after the end of a much longer exposure period. Interestingly, with only 20min of exposure to the imprinting object Bradley and colleagues found transient structural synaptic changes limited to the right hemisphere (Bradley et al. 1981). Also, some behavioural, pharmacological and morphological studies, suggest the right hemisphere has a crucial role in the early stages of imprinting recall, in line with what we found in the present study (Johnston and Rose 2002).

The right hemisphere dominance observed in the present study could be also due to functional specialisation of the right hemisphere to encode some features characterising the texture fowl to a greater degree than the stuffed fowl. Indeed, the two hemispheres have been hypothesised to encode different aspects of the visual stimuli (Vallortigara and Andrew 1991; Vallortigara 1992; Vallortigara and Andrew 1994; Andrew 1999; Andrew and Rogers 2002; Rogers et al. 2013). Right hemisphere functions include the detection of change in a complex stimulus or in its spatial context (Vallortigara and Andrew 1991, 1994; Rogers 1995a; Rosa-Salva et al. 2012b; see also evidence from electrophysiology studies reviewed in Johnston and Rose 2002). Preferential use of the right hemisphere to monitor the environment has also been considered a default condition that is used when there is no

strong reason to involve the left hemisphere. On the contrary, the left hemisphere would be in charge of processing salient cues that identify the category of the object and are used in the guidance of the response (Vallortigara 1999; Andrew and Rogers 2002). It is possible that the scattered placement of the salient local features, scrambled all over the surface of the texture fowl, could make it more difficult to process it in terms of its unnatural configuration of features. This more demanding configuration processing could cause enhanced recruitment of the right hemisphere (Andrew and Rogers 2002; Rosa-Salva et al. 2012a; Rogers et al. 2013). Finally, it should be noted that this adds to the list of lateralisation effects observed in chicks hatched from dark incubated eggs (*e.g.*, Johnston et al. 1995; Deng and Rogers 2002a; Rosa-Salva et al. 2012a). In chicks from dark incubated eggs from very different experimental conditions, we more recently observed a trend for higher levels of c-Fos in the left hemisphere of several brain areas, among them IMM (Mayer et al. 2016a, 2017a; Lorenzi et al. 2017). We hypothesised the presence of a baseline level of activity spontaneously lateralised in favour of the left hemisphere. In the present experiment, the effect of the experimental manipulation could be superimposed on a spontaneously left lateralised IMM. In fact, in both groups the left IMM showed a high density of c-Fos-ir cells. For the discussion of the role of light exposure in lateralisation development (Rogers and Sink 1988; Rogers 1990, 1997, 2008; Rogers and Bolden 1991) and further investigations please see next the Section 2, Study 4.

In contrast to what we observed in the IMM and contrary to our initial expectations, the inner, intermediate and outer layers of the optic tectum (TeO) did not show a significant difference between the two groups, even though we observed a slight, but not significantly higher number of c-Fos-ir neurons in the right hemisphere of chicks that preferred the texture-fowl. Interestingly, lateralised expression of IEGs has been reported also in TeO of zebra finches after sexual imprinting, however the lateralisation occurred independently from the experimental condition and was in favour of the left hemisphere (Lieshoff et al. 2004). The lack of clear differences within the optic tectum might be related to the fact that chicks were exposed to a rich and complex visual scene. Not only both stimuli were visible to the exposed chicks, but also both contained various types of visual information, like movement and visual configurations of different sizes. Other visual textures were also present in the visual environment during the test phase, like those created by the grids at the end of the corridor and the floor and walls of the corridor itself. In the deeper layers of

the optic tectum (Marín et al. 2003; Hellmann et al. 2004), there are different types of neurons that respond to a variety of different visual properties (Wylie et al. 2009; Verhaal and Luksch 2016; see also Rosa-Salva et al. 2015). Given this, we can imagine that such a highly complex visual stimulation should activate multiple neuronal populations in both groups. This could have masked any effect of the only aspect in which the visual scene had any variation (*i.e.* the configuration of the two stimuli).

Despite these partial limitations, our findings represent the first evidence of different neuronal responses to naturalistic and artificial stimuli in naïve chicks. Future investigations could be devoted to confirm and extend these findings to other samples of chicks demonstrating a significant preference for the naturalistic stimulus at the behavioural level.

SECTION 2: Lateralisation

In many experiments using IEGs as neuronal activity marker, we observed in the two brain hemispheres an asymmetrical pattern of activity. Usually, in most of the measured brain areas, the left hemisphere showed a higher number of c-Fos-ir cells, compared to the right hemisphere independently from the experimental conditions. This tendency made us hypothesise the presence of a spontaneous asymmetrical pattern of c-Fos expression in the two hemispheres in visually naïve chicks. Therefore, the following study aimed to investigate if such spontaneous lateralisation pattern can also be observed in chicks without any visual experience. Results from this study could shed some light on our previous results and set the baseline level of c-Fos immunoreactivity for future studies. We also aimed to investigate if light stimulation prior to hatching and after hatching would affect the lateralisation pattern of c-Fos expression. Indeed, it is known that light stimulation within a critical time window during embryo development in the egg can affect behavioural and neuroanatomical lateralisation in chicks (Rogers and Sink 1988; Rogers and Bolden 1991). Surprisingly, our prior observation came however from chicks hatched from dark incubated eggs.

Study 4: Effects of light stimulation on brain lateralisation in developing chicks (*Gallus gallus domesticus*): an immediate early gene study

Abstract

Late embryonic light stimulation during a critical time window before hatching is known to stimulate selectively the left hemisphere of domestic chicks and to influence brain and behavioural asymmetries. At the neuronal level, only few studies showed the presence of lateralisation in the absence of light stimulation of the egg. By using the immediate early gene product c-Fos as neuronal activity marker, we recently observed a general trend for left lateralisation in newly hatched dark incubated chicks. The left hemisphere usually showed more activity than the right one. This asymmetrical activity pattern was observed in different brain areas and in different behavioural conditions, regardless of the experimental manipulations. These observations raised two main questions: (a) Do chicks have a spontaneous left lateralisation of c-Fos expression immediately after birth in the absence of any light stimulation as embryos? (b) Is there any effect of light stimulation on the c-Fos lateralisation pattern? In order to answer these questions, we devised three conditions: a group of chicks was incubated and hatched in complete absence of light (darkness); a second group was light stimulated during the critical window *in ovo* and hatched in darkness; a third group was incubated in darkness and exposed to a homogeneously illuminated featureless environment after hatching. We observed that newly hatched chicks that were incubated and hatched in darkness showed significantly higher density of c-Fos-ir cells in the left hemisphere than in the right. A similar, although less pronounced trend was visible also in the other two groups, suggesting that late embryonic light stimulation is not crucial to elicit this effect. Results from this experiment are important for the interpretation of the results from previous experiments and provide an important basis for future studies using immediate early genes as neuronal activity markers in young chicks. Moreover, this evidence adds to previous knowledge about the presence of a spontaneous left-lateralisation pattern which is independent of embryonic light stimulation.

Introduction

Lateralisation is widely known as a phenomenon apparent in bilateria (animals that have a bilateral symmetrical organisation), which exhibit different types of asymmetries (*i.e.* morphological and functional; Hirokawa et al. 2006; Güntürkün and Ocklenburg 2017). For instance, in humans the left and the right hand serve different functions and have different abilities. Among other forms of lateralisation, brain asymmetries have been widely investigated from both a structural and a functional perspective. In fact, the two hemispheres are hypothesised to encode different aspects of visual stimuli. For example, the right is more involved in the detection of a general configuration of stimuli and in monitoring the spatial configuration of the environment, the left is more devoted to the analysis of salient cues for categorization and for guiding the behavioural output (Vallortigara and Andrew 1994; Andrew 1999; Vallortigara 1999; Andrew and Rogers 2002; Rogers et al. 2013). Evidence from different animal species, ranging from mammals to insects (Rogers and Vallortigara 2008), have been collected to document and understand both the phylogenetic and ontogenetic origins of this phenomenon (Vallortigara and Rogers 2005; Güntürkün and Ocklenburg 2017).

Among vertebrates, domestic chicks have been extensively used to study brain and behavioural lateralisation. Indeed, these animals offer a variety of advantages for the study of visual brain lateralisation and its behavioural correlates. First, as already emerged from the present thesis, chicks are extremely useful animal models in neuroethological research because they are a precocial species, autonomous soon after hatching. This characteristic allows testing them immediately after birth. Second, optic fibres at the optic chiasm almost completely decussate to the contralateral hemisphere (Cowan et al. 1961). Chicks also do not have the corpus callosum, making the visual information coming from one eye mainly staying in the contralateral hemisphere (Zeigler and Bischof 1993). These characteristics provide enormous advantages for the behavioural study of brain lateralisation. In fact, chicks can be tested monocularly (*e.g.* left-eye-system: right eye is occluded and visual information is acquired only from the left eye and mainly processed from the contralateral right hemisphere). Taking advantage of this technique, a huge amount of behavioural evidence on lateralisation was collected (Vallortigara 1992; Deng and Rogers 2002a; Rosa-Salva et al. 2009). For instance, the left-eye-system (right hemisphere) was found to

monitor preferentially the presence of emotional stimuli such as predators, whereas the right-eye-system (left hemisphere) showed a greater involvement in precise discrimination between grains and pebbles (Andrew et al. 1982). Another important advantage clearly emerged from the studies reported in the present work is that chicks' embryonic development occurs *ex utero*. This allows experimental manipulation during the embryonic phase and crucially enables strict control over visual experience pre- and post-hatching. Moreover, asymmetrical light exposure of the egg in a critical window before hatching (embryonic days: E19-E20) is known to be responsible for a structural asymmetry in the visual ascending projections of the thalamofugal pathway to the forebrain (Deng and Rogers 1997), homologue of the mammalian geniculocortical pathway (Shimizu and Karten 1993). This asymmetrical light exposure is triggering some visual lateralised behaviours of both hemispheres in different tasks (Rogers and Sink 1988; Rogers and Bolden 1991). For instance, lateralised chicks, light exposed in a late embryonic stage, aligned their body with the apparent direction of a biological motion display, monitoring it preferentially with the left-eye-system, contrary to chicks from dark incubated eggs that counter aligned and monitored it equally with both eyes (Rugani et al. 2015). Indeed, the late-stage embryo is turned in the egg so that its body occludes the left but not the right eye, which is facing the eggshell and can be selectively stimulated with light (Figure 32). As mentioned above, thanks to the almost complete decussation of the optic fibres, this results in a more pronounced stimulation of the contralateral left hemisphere. Remarkably, the right-turn of the embryo is genetically determined by the Nodal genes, the same genes that determine the location of the internal organs during embryonic development (Schier 2003; Güntürkün and Ocklenburg 2017).

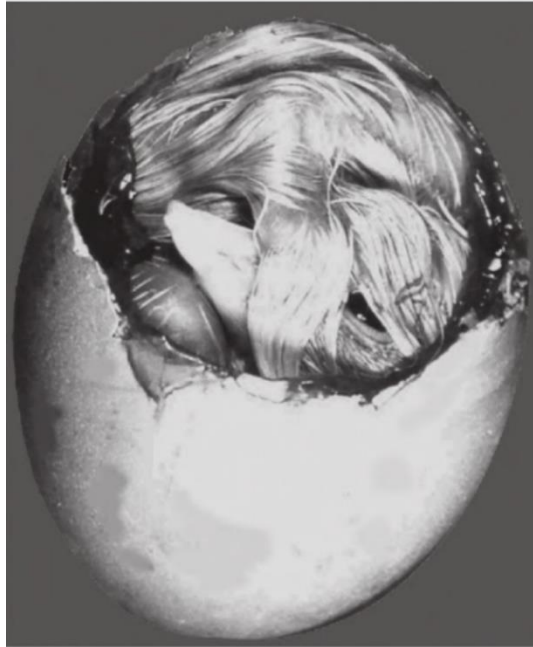


Figure 32: Typical placement of the chick in the egg during the last embryonic days. The right turn. An embryo of chick at day E20. The typical placement of the head in late-stages of embryo development. The head is tilted so that the right eye is always pointing toward the eggshell and the left eye is pointing to the body. This position in the egg is determined by the Nodal cascade genes, which also code for the internal organization of the organs during embryogenesis (image taken from Rogers 1995b).

However, not all forms of brain lateralisation are affected by light stimulation immediately pre-hatching. Indeed, brain and behavioural evidence suggests that chicks may begin their life after hatching with a lateralisation pattern, regardless of the light stimulation during the critical window (Horn and Johnson 1989; Johnston and Rogers 1999; Deng and Rogers 2002b; Andrew et al. 2004). Some authors suggested the presence of another critical period earlier during embryo development (Chiandetti et al. 2013). Here the question arises, do chicks begin their life with a pre-programmed lateralisation pattern, regardless of light stimulation during embryonic development?

In Study 1 and 2 and in other IEGs experiments in our lab we frequently observed a general trend for a left lateralisation pattern without any embryonic light stimulation during the critical window (E19-E20), irrespectively of the different experimental conditions and in different brain areas (Mayer et al. 2016a, b, 2017a; Lorenzi et al. 2017). The most numerous observations come from IEGs studies investigating neural correlates of social predispositions. In Study 2 of the present thesis, we observed in all the areas (septum,

arcopallium and intermediate medial mesopallium) a higher density of c-Fos-ir cells in the left hemisphere in both experimental and control chicks (Lorenzi et al. 2017). This higher density in the left hemisphere could have masked some effects caused by the experimental manipulation (ceiling effect). Indeed, we found significant treatment dependent group differences only in the right septum. A similar trend was observed in the left hemisphere, but the difference was not significant, maybe due to a spontaneous level of activity already present in the left hemisphere of both groups masking the difference related to the independent variable. The same trend was observed in septum and POA (preoptic area) in another experiment investigating social predispositions for motion (Mayer et al. 2017a). In both studies, also arcopallium showed a non-significant trend for higher c-Fos-ir cell density in the left hemisphere of both experimental and control chicks. Moreover, a left lateralisation pattern in IMM (intermediate medial mesopallium) was found significant in the experimental group of Study 2 and there but non-significant in the control group of the same study. In Study 3 IMM of the experimental group showed a trend of left lateralisation and the same trend could be present in the left hemisphere of the control group, but masked by the experimental manipulation effect on this area in that group since the level of activity was very high in both hemispheres (Mayer et al. 2016b). A trend for higher density of c-Fos expression in the left hemisphere regardless of the experimental manipulation was observed also in a study investigating spatial cognition (Mayer et al. 2016a). In the left hippocampus a trend for higher density of c-Fos-ir cells compared to the right hemisphere was apparent in both experimental conditions devised. Intriguingly, this was observed without any embryonic light stimulation during the critical time window (E19-E20). This variety of observations collected in our lab made us hypothesise the presence of an asymmetrical baseline level of c-Fos expression spontaneously more pronounced in the left hemisphere in various brain areas. In order to understand better this set of non-systematic observations and to shed some light on our previous results, we devised three experimental conditions. In one condition, we incubated eggs in the darkness and let them hatch in a complete absence of visual experience. In a second condition, we light stimulated the embryos during the critical time window in the egg and let them hatch in the darkness. In a last condition, we incubated and hatched the eggs in the darkness, but exposed the chicks individually to light in a featureless environment. This was done to provide a symmetrical stimulation to the eyes. Five brain areas of interest were selected based on our previous studies: septum,

preoptic area, arcopallium, intermediate medial mesopallium and hippocampus. We hypothesised to find a trend for left lateralisation in all the three groups for all the five areas, with a more pronounced effect in the light stimulated group prior to hatching and a less pronounced effect in the group exposed to light after hatching.

Materials and Methods

Subjects

Subjects were 29 (12M) domestic chicks (*Gallus gallus domesticus*) divided in three different experimental conditions.

Experimental conditions

Three groups of chicks underwent three different experimental conditions: Condition 1 dark-dark (N=9), Condition 2 light-dark (N=10), Condition 3 dark-light (N=10) (Figure 33). Until embryonic day 18 (E18), eggs were all kept in the exact same dark conditions as for the other studies presented. All chicks hatched in the darkness and after hatching were carefully placed in individual cardboard compartments to distinguish them (11x11x25cm) carefully avoiding any visual stimulation. For Condition 1 (DD: dark-dark) and 3 (DL: dark-light) eggs remained in the darkness until hatching. While chicks from the dark-dark group remained in the dark incubator until perfusion (DD), chicks from the dark-light group were individually extracted from the incubator and placed in a featureless environment for 10min (DL). After the post-natal homogenous light exposure, chicks were placed back in their individual compartments in the dark incubator until perfusion. The featureless environment consisted of a white cylinder (∅23x50cm) illuminated from above (incandescent light bulb 40W) and with the floor covered with sawdust, a cam recorded chick's behaviour hidden from above. For Condition 2 (LD: light-dark) a rectangular translucent plastic panel (36x38cm), with 15 LEDs (270 lm) homogeneously distributed on the surface, was placed behind the roof of the incubator in the morning of day E19 and carefully removed in the evening of day E20 in order to stimulate the eggs during the critical period (for the embryo stimulation procedure please see Rogers and Sink 1988; Rogers and Bolden 1991). Chicks remained in the darkness until perfusion.







	Critical window	After hatch
DD: dark-dark		
LD: light-dark		
DL: dark-light		

Figure 33: Different conditions in Study 4.

Schematic representation of the experimental conditions (DD, LD, DL) and the relative three groups (dark-dark, light-dark, dark-light). In DD chicks were incubated and hatched in the darkness. In LD eggs were light stimulated during the critical window (E19-E20), but chicks hatched in the darkness. In DL incubation and hatching happened in the darkness, but after hatching chicks were individually exposed to light in a featureless environment (10min).

Immunohistochemistry

Please see section Immunohistochemistry in Section 1 of Study 2. For this experiment, after the immunohistochemical staining all sections of both right and left hemispheres were mounted on the microscopic slides with identical orientation, so that the coder could be completely blind while counting.

Analyses

Brains analyses

Based on previous observations in our lab, we selected five different brain regions to estimate hemispherical asymmetries in this study: septum, IMM, arcopallium, Hp and POA.

To estimate labelled cell density in septum up to five sections of each hemisphere were selected by the shape and anatomical landmarks that would correspond to the A(nterior)9.4 to A8.4 (Kuenzel and Masson 1988) and accordingly to our previous studies (Mayer et al. 2017b, a; Study 2: Lorenzi et al. 2017). The septum of each section was parsed into three subdivisions for a more representative counting: dorsal, lateral and medial.

To estimate labelled cell density in the intermediate medial mesopallium (IMM) we relied on our previous studies descriptions of this region (Studies 3 and 2: Mayer et al. 2016b; Lorenzi et al. 2017). Up to five brain slices, from a region where the shape of IMM was corresponding to that observed between A8.6 and A7.6 of Kuenzel and Masson (1988), were selected for the analysis.

To estimate cell densities within the arcopallium and TnA we relied on our previous studies descriptions of these regions (Mayer et al. 2017b, a; Study 2: Lorenzi et al. 2017). Up to three sections of both hemispheres were selected from the region extending from A7.6 to A6.4 in Kuenzel and Masson (1988).

To estimate labelled cell density in hippocampus (Hp) up to five sections of each hemisphere were selected by the shape and anatomical landmarks that would correspond to the A(nterior)7.0 to A6.4 (Kuenzel and Masson 1988) and accordingly to our previous studies (Mayer et al. 2016a). The Hp of each section was parsed into three subdivisions for counting: ventral (HpV), dorso-medial (HpDM) and dorso-lateral (HpDL, Figure 34).

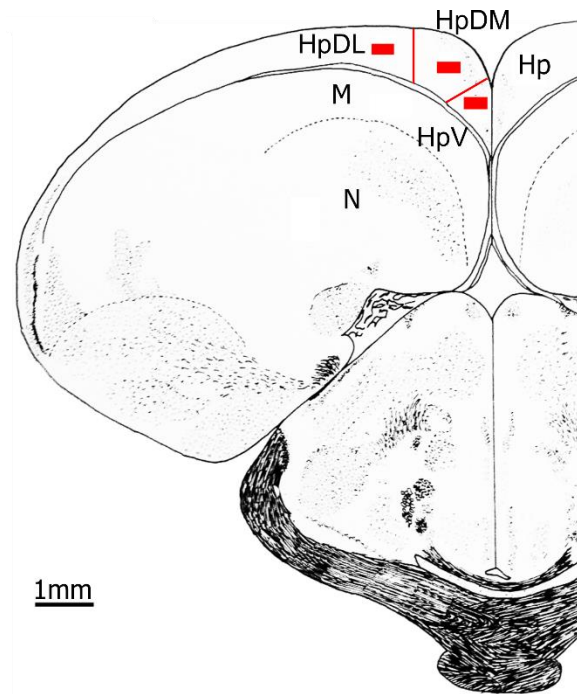


Figure 34: Counting areas in hippocampus.

Schematic drawing of a coronal section through telencephalon showing the Hp and its portioning into dorso-lateral (HpDL), dorso-medial (HpDM) and ventral (HpV, red lines). The red rectangles are in the typical placements for cell counting. Adapted from Kuenzel and Masson atlas (1988). Hp-Hippocampus, M-mesopallium, N-nidopallium.

To estimate labelled cell density in the preoptic area (POA) we relied on our previous studies descriptions of this region (Mayer et al. 2017a; Study 2: Lorenzi et al. 2017). Up to three slices around A8.2 of Kuenzel and Masson (1988) were selected for the analysis.

After completing the cell counts, the mean values derived from the sections were initially calculated for each of the subdivisions independently and cell densities were standardised to 1mm². Cell counts, pooled from the five subdivisions in septum, were further averaged to estimate overall septal activity and the same was done for Hp. Also, the cell counts pooled from the two subdivision in arcopallium and the counting in TnA were averaged to estimate overall activity in this amygdala equivalent. This was done for the two hemispheres separately. The resulting individual bird means were considered overall indicators for the number of c-Fos-ir cells in the regions of interest and were employed for further statistical analysis.

Statistical analyses

The differences in the density of c-Fos-ir cells in the two hemispheres for the five different brain areas was tested by a repeated measurement ANOVA, with a between-subject factor "group" (3 levels: dark-dark, light-dark, dark-light) and a within-subjects factor "area" (5 levels: septum, IMM, arcopallium, POA and Hp). The dependent variable for all the analysis was an index of left lateralisation computed for each area as follows:

$$lateralisation\ index = \frac{Left\ c-Fos-ir\ cells'\ density}{Left\ c-Fos-ir\ cells'\ density + Right\ c-Fos-ir\ cells'\ density}$$

Values of this index range from 0 (maximal density of c-Fos-ir cells in the right hemisphere) to 1 (maximal density of c-Fos-ir cells on the left), with a value of 0.5 identifying a similar density in both hemispheres. For *post-hoc* analyses, one-sample two-tailed *t*-tests were used to compare individually the indices to the expected chance level (0.5).

The differences in the density of c-Fos-ir cells in the two hemispheres for the five different brain areas was also tested by a repeated measurement ANOVA, with a between-subject factor "group" (3 levels: dark-dark, light-dark, dark-light) and a within-subjects factors "area" (5 levels: septum, IMM, arcopallium, POA and Hp) and "hemisphere" (2 levels: left and right). For *post-hoc* analyses, two-tailed paired *t*-tests were used to compare c-Fos-ir densities between the two hemispheres in each condition. Two-tailed independent samples

t-tests were used to compare the overall c-Fos-ir cell density with hemisphere collapsed between the three conditions. For every *t*-test Cohen's *d* was calculated as a measure of effect size with the software G*Power version 3.

For a preliminary analysis, a repeated measurement ANOVA was run to test a gender effect on the lateralisation index in the five different areas measured or any interaction between gender and the experimental conditions. Between-subjects factors used were "group" (3 levels: dark-dark, light-dark, dark-light) and "gender" (2 levels: female, male); within-subjects factor was "area" (5 levels: septum, Hp, IMM, POA, arcopallium).

Results

All 29 brains from the three groups were successfully stained for c-Fos. Not all the subjects provided an intact POA for both hemispheres. In some individuals, this area was damaged and not analysable in at least one hemisphere. However, if the area was intact, the labelled cells were easily distinguishable from the background.

Preliminary analysis confirmed that no significant interaction was present between subjects' gender and the c-Fos-ir cells' density in the five different areas nor with the experimental conditions (gender: $F_{(1,19)}=3.365$, $p=0.082$; area*gender: $F_{(4,76)}=1.000$, $p=0.413$; area*gender*group: $F_{(8,76)}=0.533$, $p=0.829$; gender*group: $F_{(2,19)}=0.099$, $p=0.906$).

The repeated measurement ANOVA on the lateralisation index of septum, IMM, arcopallium, Hp and POA revealed significant main effect of group ($F_{(2,22)}=3.738$, $p=0.040$; see Figure 35). No other significant effects or interactions were found (area: $F_{(4,88)}=0.815$, $p=0.519$; area*group $F_{(8,88)}=0.651$, $p=0.733$). Therefore, since we were not allowed to analyse the five areas separately, we computed the lateralisation index for the three different conditions with all the areas collapsed (for descriptive purposes please find the mean lateralisation indices plotted for each area in the graphs in Figure 36). Comparisons against chance level for the three groups are reported below.

In condition DD the lateralisation index for the group dark-dark was significantly different than chance level, indicating a higher c-Fos-ir cells' density in the left hemisphere (0.5492 ± 0.0126 ; $t_{(8)}=3.903$, $p=0.005$, $d=1.3$; see first column in Figure 35). For condition LD the index of the group light dark was slightly not significantly different from chance level (0.5338 ± 0.01538 ; $t_{(9)}=2.196$, $p=0.056$, $d=0.7$; see second column in Figure 35). For condition DL the index of the group dark-light showed a non-significant trend similar to the group dark-dark (DD) and light-dark (LD) with a tendency for higher c-Fos-ir density in the left hemisphere (0.5430 ± 0.02955 ; $t_{(9)}=1.456$, $p=0.179$, $d=0.46$; see third column in Figure 35).

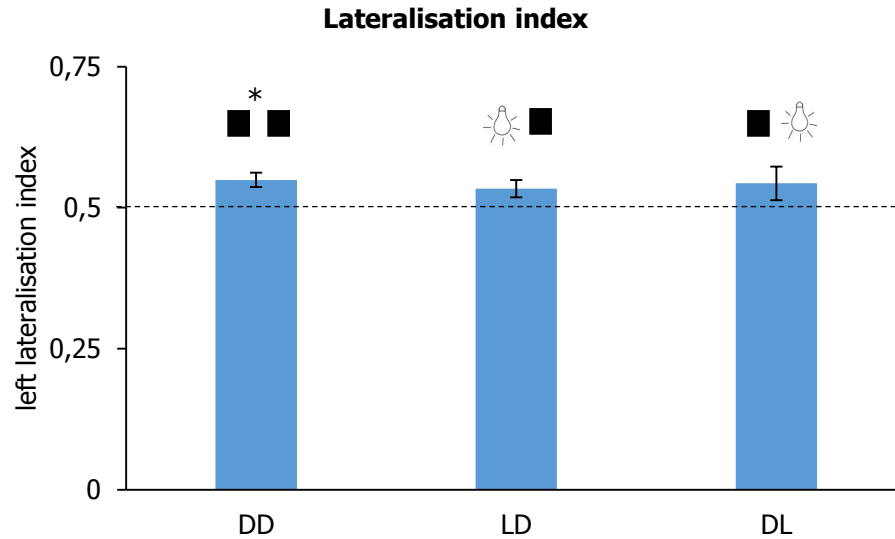


Figure 35: Results in the different conditions.

The three different conditions are reported on the X-axis. In DD the left lateralisation index for group dark-dark is significantly different from chance. In LD and DL the indices for the other two groups light-dark and dark-light show a trend similar to DD, but not significant. Asterisks indicate significance ($\alpha \leq 0.05$), dashed line the chance level (0.5). Error bar indicates S.E.M..

For descriptive reason, we included the plots reporting the left lateralisation indices for each brain area in the different conditions (Figure 36). By looking at the graphs, we can observe that septum had a left lateralisation tendency in all three conditions, whereas IMM was never exhibiting this pattern. POA showed an asymmetry in favour of the left hemisphere in condition dark-dark (DD) and light-dark (LD), but not in the condition dark-light (DL). Hp and arcopallium showed a left asymmetrical pattern in DD and DL condition, although Hp had a less pronounced asymmetry in DL, and no lateralisation was observable in these two areas for LD condition.

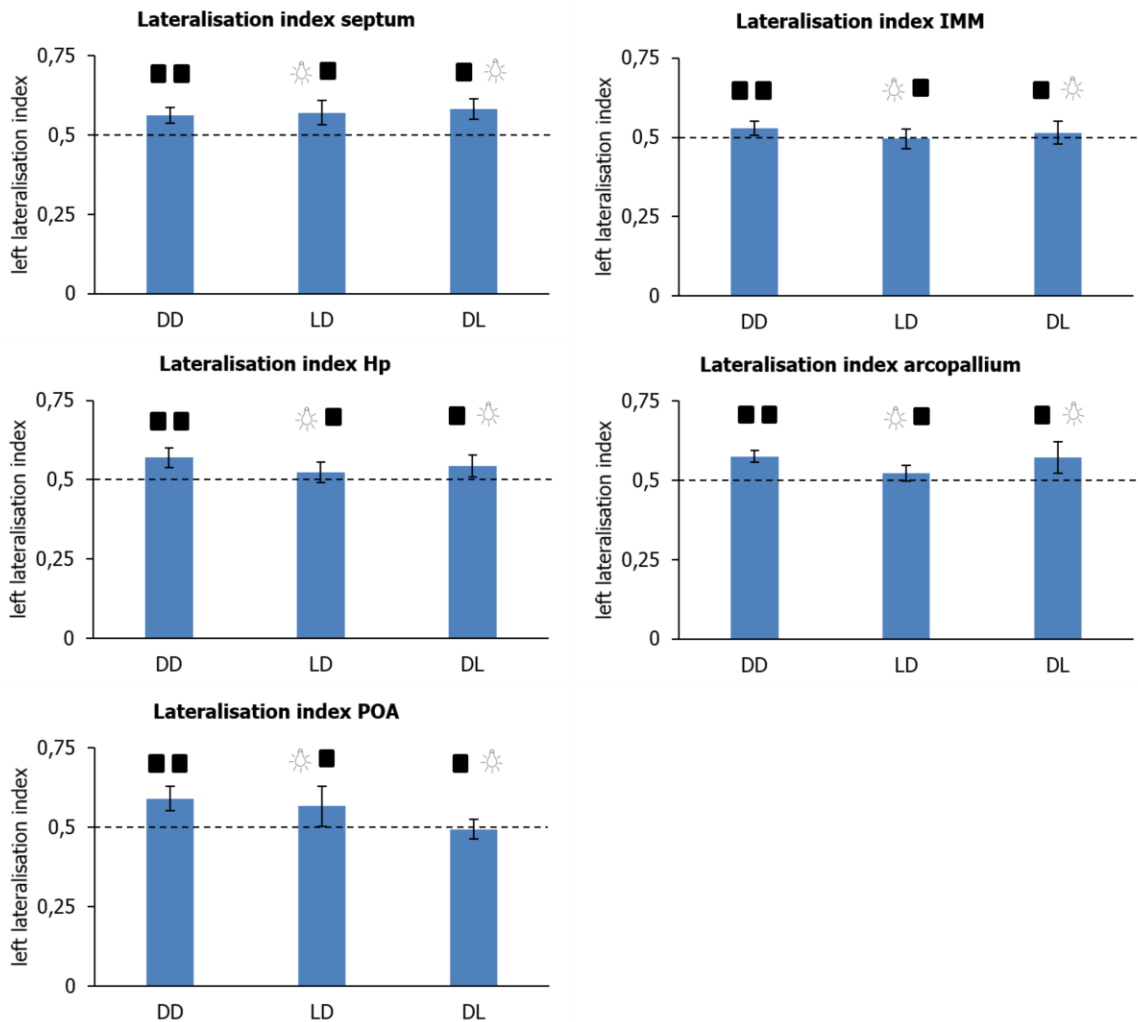


Figure 36: Lateralisation indices in the different areas.

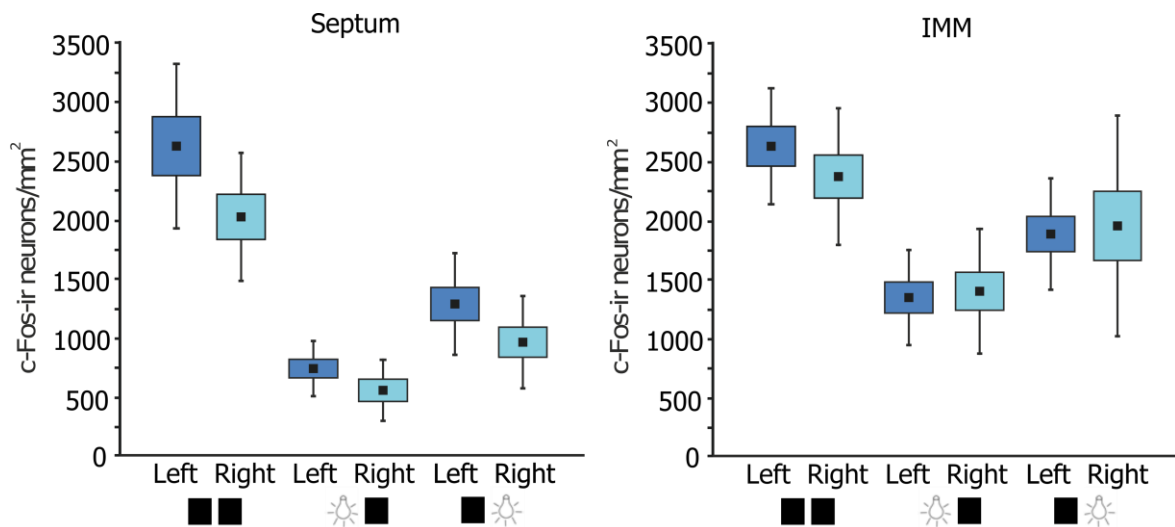
Every plot reports the lateralisation index for the three different conditions for a single brain area. Dashed line indicates the chance level (0.5), error bars indicate S.E.M..

The repeated measurement ANOVA on the c-Fos-ir cell density in septum, IMM, arcopallium, Hp and POA revealed significant main effect of group ($F_{(2,22)}=66.070$, $p<0.001$), hemisphere ($F_{(1,22)}=19.023$, $p<0.001$) and area ($F_{(4,88)}=51.784$, $p<0.001$); a significant interaction of group*hemisphere ($F_{(2,22)}=8.376$, $p=0.002$) and area*group ($F_{(8,88)}=11.601$, $p<0.001$). No other significant interaction was found (area*hemisphere: $F_{(4,88)}=0.913$, $p=0.460$; area*hemisphere*group: $F_{(8,88)}=0.966$, $p=0.468$). Therefore, since we were not allowed to analyse the five areas separately, we computed the mean densities for the three different conditions with all the areas collapsed (for descriptive purposes please find the mean

densities for each area and hemisphere plotted in the graphs in Figure 37). Paired-samples *t*-tests between the two hemispheres for the three groups are reported below.

In condition DD the c-Fos-ir cells' density in the left hemisphere (2249.4 ± 71.8 cells/mm²) was significantly higher than in the right (1854.7 ± 92.2 cells/mm²; $t_{(8)}=3.929$, $p=0.004$, $d=6.47$). For condition LD the c-Fos-ir cell density was slightly significantly higher in the left (1046.78 ± 74.07 cells/mm²) than in the right hemisphere (909.11 ± 53.28 cells/mm²; $t_{(9)}=2.226$, $p=0.053$, $d=2.09$). For condition DL the c-Fos-ir cell density in the left hemisphere (1258.27 ± 76.93 cells/mm²) was not significantly different from the right one (1128.04 ± 133.04 cells/mm²; $t_{(9)}=1.392$, $p=0.197$, $d=0.73$).

For descriptive reason we included the graphs reporting the density of c-Fos-ir cells per mm² in each hemisphere per each area (Figure 37 **Error! Not a valid bookmark self-reference.**).



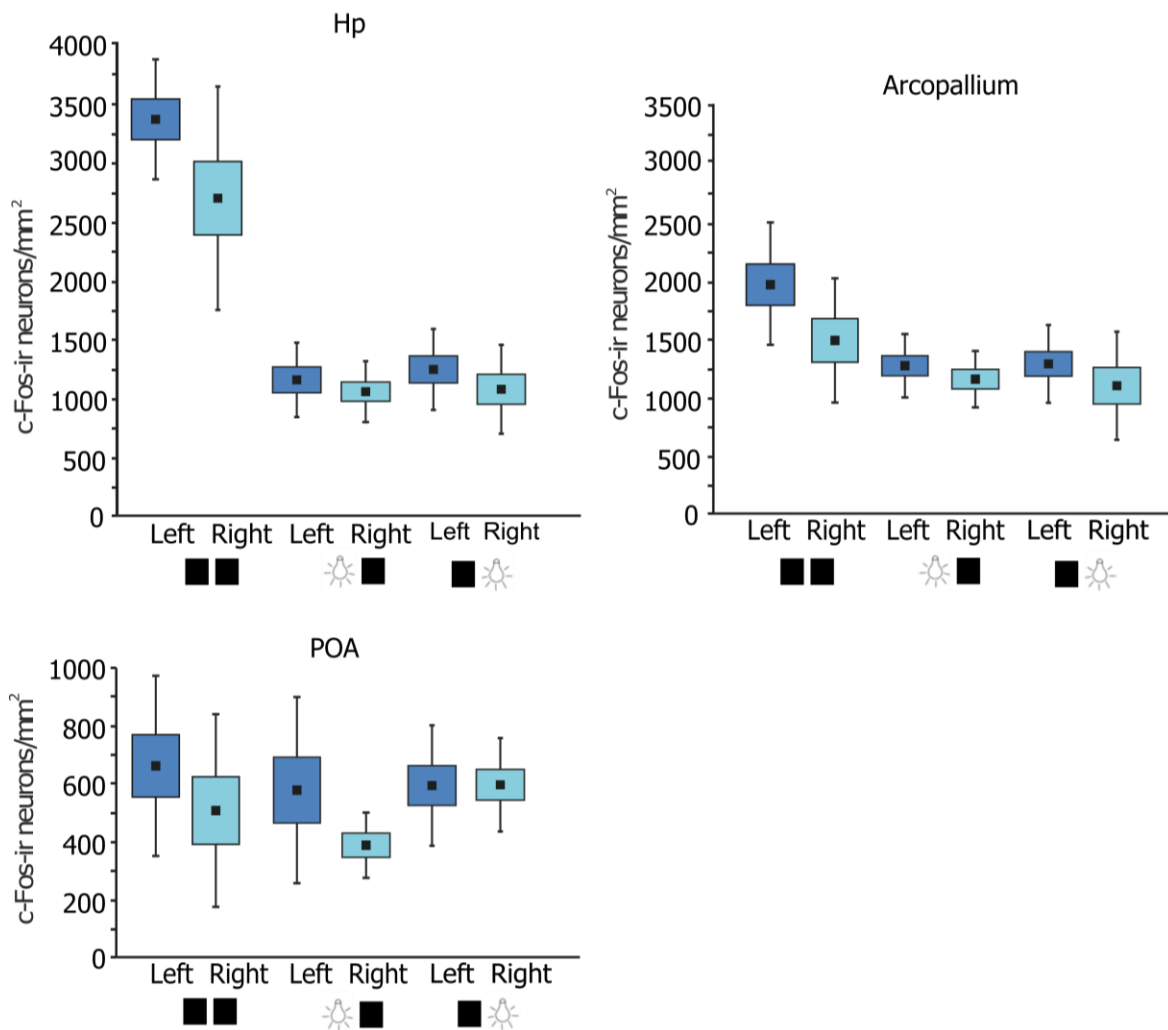
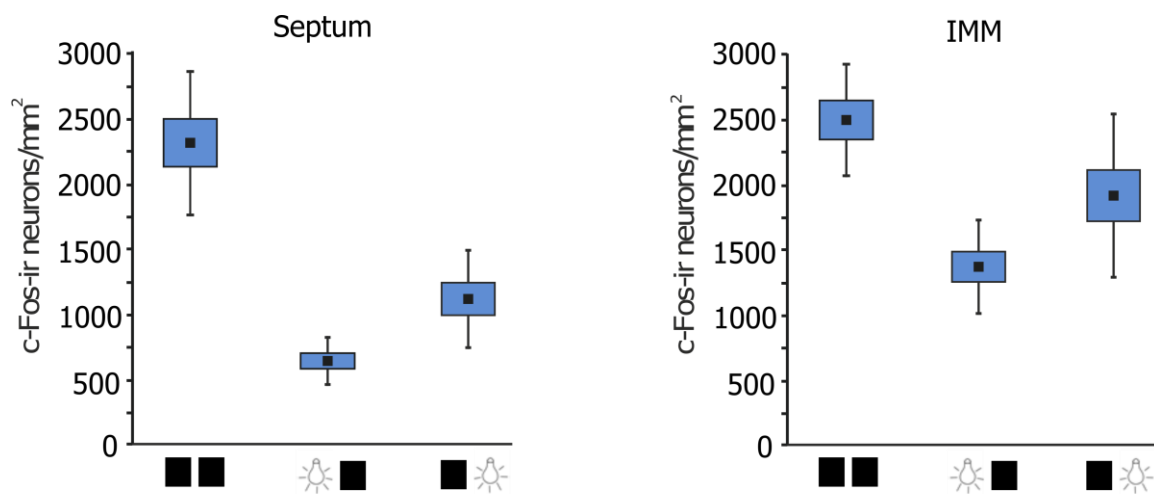


Figure 37: Measured ir-cell density in each area and hemisphere. Plots for the different brain areas report c-Fos-ir cell per mm² on the Y-axis, for the three different conditions (DD, LD, DL) for both hemispheres (left-dark blue and right- light blue) reported on the X-axis. Graph-plot: mean (black square), S.E.M. (box) and S.D. (whiskers).

By looking at the mean densities in Figure 37, we can observe that septum showed in all three conditions a higher density in favour of the left hemisphere. On average septum was showing 23% more c-Fos-ir cells in the left hemisphere than in the right one. IMM was showing no difference in c-Fos-ir cell density between the two hemispheres in none of the conditions. POA showed comparable mean densities in the two hemispheres in condition DD and DL, whereas in condition LD was showing 33% more c-Fos-ir cells in the left than in the right hemisphere. Hp showed 20% more c-Fos-ir cells in the left hemisphere than in

the right one in condition DD, whereas in the other two conditions LD and DL the densities of immunoreactive cells in the two hemispheres were comparable. Eventually, arcopallium showed a similar trend with 25% more immunoreactive cells in the left hemisphere than in the right one of the DD condition and almost comparable densities in the two hemispheres for the other two LD and DL conditions. Overall, these observations confirm previous analyses run with the lateralisation indices. Following analyses will focus on group differences in the level of activation regardless of brain hemispheres.

The significant interaction area*group revealed that some areas differed in some conditions independently from hemisphere. We therefore, computed the total mean density of c-Fos-ir cells with the hemispheres collapsed for each condition. Total densities were compared between groups with independent-samples *t*-tests. A significant higher density of c-Fos-ir cells was found in the DD group compared to the other two groups (DD vs. LD: $t_{(17)}=12.459$, $p<0.001$, $d=5.71$; DD vs. DL: $t_{(17)}=7.108$, $p<0.001$, $d=3.31$). A non-significant trend of higher density was found in the DL condition compared with the LD ($t_{(18)}=-1.9$, $p=0.074$, $d=0.85$). We also compared the overall c-Fos immunoreactivity in the single areas between conditions by collapsing the two hemispheres for each area, mean densities are reported in the plots in Figure 38.



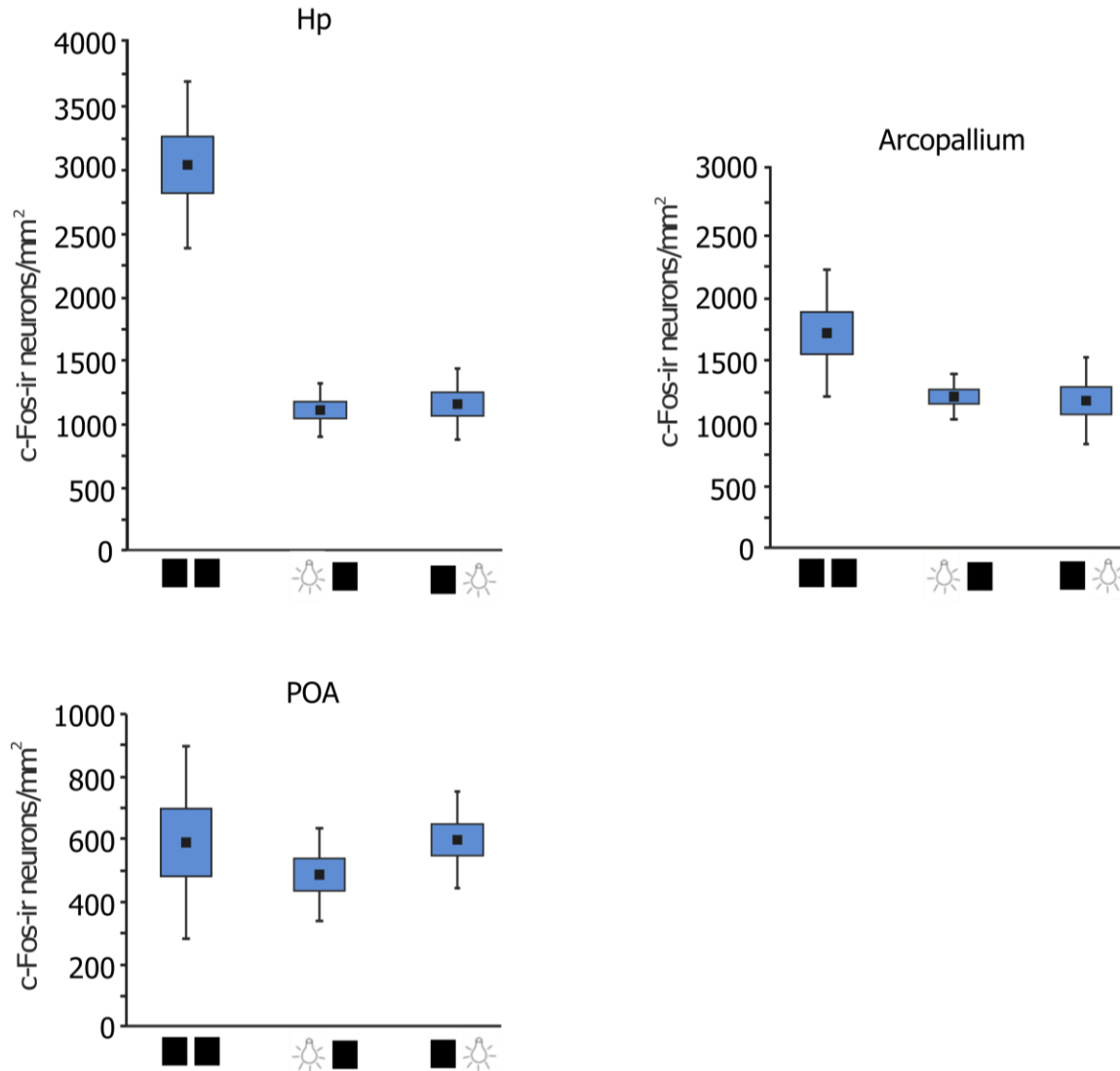


Figure 38: Areas' differences in the total ir-cell density between conditions. Plots report for each area independently the estimated overall c-Fos-ir cell/mm² on the Y-axis with the two hemispheres collapsed for the three different conditions (DD, LD, DL) reported on the X-axis. Graph-plot: mean (black square), S.E.M. (box) and S.D. (whiskers).

Figure 38 shows that in different conditions the range of the absolute number of c-Fos-ir neurons was different in different areas. Septal activity significantly differed between the three conditions: in the DD condition was in the range of 2300 c-Fos-ir cells/mm², whereas in LD condition it was in the range of 600 c-Fos-ir cells/mm² and in the range of 1100 c-Fos-ir cells/mm² in the DL condition (DD vs. LD: $t_{(9.756)}=8.585$, $p<0.001$, $d=4.02$; DD vs. DL: $t_{(17)}=5.557$, $p<0.001$, $d=2.53$; LD vs. DL: $t_{(18)}=-3.492$, $p=0.003$, $d=1.56$). Also IMM activity was significantly different in the three conditions: in the DD condition was in the

range of 2500 c-Fos-ir cells/mm², whereas in the LD condition it was in the range of 1300 c-Fos-ir cells/mm² and in the range of 1900 c-Fos-ir cells/mm² in the DL condition (DD *vs.* LD: $t_{(17)}=6.163$, $p<0.001$, $d=2.82$; DD *vs.* DL: $t_{(17)}=2.293$, $p=0.035$, $d=1.06$; LD *vs.* DL: $t_{(18)}=-2.37$, $p=0.029$, $d=1.06$). In POA no significant differences were found in the range of c-Fos-ir cells measured in the three conditions (DD *vs.* LD: $t_{(14)}=0.851$, $p=0.409$, $d=0.43$; DD *vs.* DL: $t_{(15)}=-0.058$, $p=0.955$, $d=0.03$; LD *vs.* DL: $t_{(15)}=-1.475$, $p=0.161$, $d=0.72$). Eventually, Hp and arcopallium activity was significantly different in DD condition from the other two conditions (Hp: DD *vs.* LD: $t_{(9.525)}=8.348$, $p<0.001$, $d=3.92$; DD *vs.* DL: $t_{(17)}=8.164$, $p<0.001$, $d=3.67$; arcopallium: DD *vs.* LD: $t_{(9.974)}=2.808$, $p=0.019$, $d=1.32$; DD *vs.* DL: $t_{(17)}=2.672$, $p=0.016$, $d=1.21$), but no significant difference was found between LD and DL conditions (Hp: $t_{(18)}=-0.487$, $p=0.632$, $d=0.22$; arcopallium: $t_{(18)}=0.203$, $p=0.841$, $d=0.09$). Activity in the DD condition was in the range of 3000 c-Fos-ir cells/mm² in Hp and 1650 c-Fos-ir cells/mm² in arcopallium, whereas in the LD and DL conditions it was in the range of 1100 c-Fos-ir cells/mm².

Discussion

The results here reported represent preliminary evidence for a spontaneous lateralisation pattern in favour of the left hemisphere in the absence of any light stimulation of the egg and visual experience in newly hatched chicks. In line with our initial hypotheses, chicks showed an overall trend for higher spontaneous c-Fos immunoreactivity in the left hemisphere than in the right in all the experimental conditions. This trend was significant only in the condition dark-dark. As expected in the condition dark-light, where chicks received a symmetrical featureless visual stimulation after hatching, the level of asymmetry between the two hemispheres decreased. Instead, contrary to our expectations the asymmetry in favour of the left hemisphere, even if present, was not significantly different from chance level in the condition light-dark, where eggs were light stimulated during the critical window. According to previous literature (Rogers and Sink 1988; Rogers and Bolden 1991), we expected embryonic light stimulation between E19 and E20 to enhance the existing asymmetry. This could be due to the characteristics of the areas we investigated here. For instance, none of the selected areas is directly involved in visual processing and therefore, it could be less affected by an asymmetrical visual stimulation in egg. Alternatively, it could be due to the characteristics of the neural activity marker we used. In fact, c-Fos is a transcriptional factor related to plastic changes and reaches its peak of expression between 1-2hours after neuronal stimulation. Light stimulation in the egg occurred 48hours before the sacrifice. After the light stimulation, it could be that the neuronal networks reorganise involving expression of c-Fos during the hours following the stimulation. After this reorganisation is terminated, plasticity is decreased and consequently also levels of c-Fos expression are lower. It could also be that light stimulation directly decreased the levels of c-Fos, reducing the spontaneous left lateralisation. In fact, a significant difference in the absolute number of c-Fos-ir neurons was found between the dark-dark group and the other two light stimulated groups, with a higher density of c-Fos-ir cells in the dark-dark group (for an example with mammals on different effects of light on the expression of c-Fos see Shuboni et al. 2015). Noteworthy, in Study 3 chicks from the baseline condition, that were light exposed in a featureless environment after hatching showed also a small number of c-Fos-ir cells in both hemispheres of IMM pointing to a possible interaction between exposure to homogenous light and decrease in c-Fos immunoreactivity at least in some areas (Mayer et al. 2016b).

In general, our results add to previous evidence suggesting that some forms of brain and behavioural lateralisation are not necessarily triggered by light stimulation during a late embryonic stage (Horn and Johnson 1989; Johnston and Rogers 1999; Mascetti and Vallortigara 2001; Andrew et al. 2004; Chiandetti et al. 2013). This lateralisation could be genetically determined involving the Nodal cascade genes, which are also determining the right-turn in the egg, promoting itself an asymmetrical light stimulation to potentially trigger existing asymmetries of functions and structures (Ramsdell and Yost 1998). Alternatively, it could be that there is a second critical window for light stimulation in embryo earlier during development, when visual system is still not functional (*e.g.* E1-E3; Chiandetti et al. 2013). To this regard, it is important to highlight that for this work we never used fresh eggs (just fertilised, at E0), instead we always received eggs from the commercial hatchery around the second week of embryonic development. This could account for an early light embryonic stimulation in all our eggs.

The reason why we originally devised this experiment was to understand better the pattern of left lateralisation in several brain regions observed in other IEGs studies from our lab. Here we confirmed that dark incubated chicks exhibit an asymmetrical level of c-Fos immunoreactivity in favour of the left hemisphere and that this can be considered as a hemispherical baseline level for dark incubated chicks. However, since we did not find a significant interaction of the different areas with the different conditions we are not allowed to draw conclusions about the baseline level of spontaneous activity in the different areas for the different groups. We are well aware that the evidence here reported is still preliminary and that should be interpreted cautiously. Nevertheless, we would like to highlight that septum showed a higher density of c-Fos-ir cells in the left hemisphere in all the three conditions performed suggesting probably a stable left asymmetry in this area. The absence of significant interactions between the single areas measured and the conditions could be due to a lack of statistical power of the test we used to analyse data. Therefore, future studies could try to increase the sample size, and decrease the big variability present between conditions by staining in parallel brains from different conditions for instance. The lack of statistical power of the test could account also for the slightly non-significant trend found in the light-dark condition, and therefore also for this reason increasing the sample size could result in a more clear scenario.

Eventually, the findings of the present Study and the observations from previous IEGs studies could reflect simply a structural difference between the two hemispheres in the total number of neurons they have. It could be that in general the left hemisphere in the brain areas we measured have a higher cell density with respect to the same areas in the right hemisphere, and that this different densities do not directly reflect functional differences. To overcome this potential point of criticism, future studies could estimate the overall cell density in the two hemispheres for different brain areas.

GENERAL DISCUSSION

The aim of the present thesis was to investigate different categories of social predispositions in newly hatched visually naïve chicks from both a behavioural and a neurobiological perspective. Social predispositions are a set of rudimentary knowledge that helps vertebrates to efficiently detect and react to animate entities in their environment. This rudimentary knowledge is of particular relevance for the offspring of precocial species. Immediately after birth, it is extremely important for these younglings to be able to categorise and react to living creatures. For example, our animal model, the domestic chick, a nidifugous species that leaves the nest soon after hatching, demonstrated in previous studies to possess an unlearned preference for both static configuration of features resembling a schematic face (Rosa-Salva et al. 2010, 2012b) and dynamic sequences depicting the movement of a living creature (biological motion point-light displays, Vallortigara et al. 2005). Thanks to comparative neuroethological studies an increasing body of evidence for these social predispositions has been collected in different vertebrate species (*i.e.* Simion et al. 1998; Sugita 2008; Mascalonzi et al. 2013; Nakayasu and Watanabe 2013; Di Giorgio et al. 2016b, 2017; Nunes 2016; Carreira et al. 2017). In order to address our scientific purposes, we conducted four studies.

In the first study, we found a stable and robust preference in visually naïve newly hatched chicks for a simple motion cue related to self-propulsion, an index for an internal energy source to the moving object. In a series of previous experiments, we found a behavioural spontaneous preference to approach a simple moving stimulus that was performing visible changes in speed over an identical one moving at constant velocity. Here we wanted to understand whether chicks' spontaneous preference for speed changes was related to self-propulsion and not to the intrinsic level of variability of the speed changing stimulus. We tested our hypothesis by covering the two single moments of visible speed changes in the preferred stimulus. If chicks were simply attracted by the level of variability present in the speed changing stimulus they would have maintained their preference for it even after covering the two moments of change. In contrast, chicks preference disappeared in this crucial control condition, showing that the preference was driven by the self-induced changes in speed. This adds to previous literature finding self-propulsion as a powerful cue

related to animacy perception (Luo and Baillargeon 2005; Luo et al. 2009; Mascialzoni et al. 2010) and gave rise to similar experiments which found the same predisposition for self-propulsion in days-old human newborns (Di Giorgio et al. 2016b). In a second experiment, we wanted to unveil the role of changes in trajectory. Changes in trajectory had been shown to elicit in adult human observers the perception of animacy (Scholl and Tremoulet 2000). In our previous experiment, the presence of direction change was combined with a change in speed. According to our main result, chicks should have preferred this pattern of motion at least due to the presence of visible speed change. Since this did not happen, we wanted to understand whether the presence of direction change could have had a detrimental effect also on the usually attractive speed change. Therefore, we decided to change the sequence of change in direction and speed by implementing a more natural speed profile for animate creatures inverting their direction. While, the previous pattern (change in direction and sudden acceleration) could have elicited the perception of an inanimate object bouncing against an invisible obstacle. In the second experiment, the stimulus was decreasing its speed stopping before changing direction, and once inverted its motion gradually accelerating in the new direction. Chicks spontaneously preferred this stimulus, demonstrating that direction of change itself has not a detrimental effect on the preference for speed changes. However, this result is not enough to understand whether changes in direction alone are able to trigger spontaneous preference in chicks. Future studies could focus on investigating the role of direction changes alone without speed changes in visually naïve chicks and clarify whether they are enough to elicit social approach. Moreover, in future experiments another interesting aspect that could be investigated is the front-back consistent alignment of the object with its direction of motion.

A potential limitation of the present study could be represented by the poorly naturalistic stimuli used. However, this was necessary on the one hand to exactly target the elementary motion cue subject of the investigation and on the other hand to validate simple stimuli well balanced and controlled for the low-level perceptual properties for the subsequent neural correlates investigation.

In the second study, we took advantage of the stimuli validated in Study 1 to run an IEG experiment targeting the neural structures underlying the predisposed preference for speed changes in chicks. Since we clearly established the preference in our laboratories, we decided to single expose two different groups of visually naïve newly hatched chicks to

either the predisposed stimulus (speed changes) or the control one (constant speed). We visualised brain activity with an immunohistochemical staining of the IEG product c-Fos and estimated neuronal immunoreactivity in four different brain areas, selected from previous literature. Septal, preoptic (POA) and amygdaloid nuclei crucial nodes of the social decision-making network rich in sex steroid receptors (Newman 1999; Gahr 2001; O'Connell and Hofmann 2011; Goodson and Kingsbury 2013) and arcopallium (comprising nucleus Taeniae) supposed avian homologue for the mammalian amygdala (Cheng et al. 1999; Reiner et al. 2004; Jarvis et al. 2005; Yamamoto and Reiner 2005; Yamamoto et al. 2005; Butler et al. 2011) were selected based on findings from previous studies in our lab investigating the first exposure to the alive motion of a conspecific (Mayer et al. 2017b, a). Another area was selected from previous literature on imprinting, intermediate medial mesopallium (IMM, according to the new nomenclature; IMHV, Intermediate medial hyperstriatum ventrale according to the old nomenclature; Reiner et al. 2004; Jarvis et al. 2005). IMM has been shown to be involved in filial imprinting (Horn 1979; Bolhuis 1991; Ambalavanar et al. 1993; McCabe and Horn 1994; Horn 1998, 2004; McCabe 2013). We showed differential activation of septal and preoptic nuclei in response to the predisposed stimulus over the non-predisposed one. The differences were region specific and not reflecting the overall activity of the brains, indeed IMM and arcopallium did not show any difference between the groups. Surprisingly, after only six minutes of exposure to highly artificial stimuli differing only for a subtle elementary feature of animate motion, we found a difference in neural response of visually naïve chicks. Interestingly, we found the difference in the two areas that differentially responded also to the motion of an alive conspecific pointing to the idea that these areas could be indeed part of a neural network for early processing of predisposition for animate motion (Mayer et al. 2017b, a). Intriguingly, recent evidence from teleost fish found an involvement of homologue areas in the perception of similar animacy stimuli, which supports the hypothesis on an ancient phylogenetic origin of this predisposition (Nunes 2016; Carreira et al. 2017). Remarkably, the difference found in septum was significant when comparing the right hemisphere of the experimental group with the right hemisphere of the control group. The c-Fos immunoreactivity in the left hemisphere was high in both groups. A trend for higher density of c-Fos-ir cells in the left hemisphere was found significant in IMM of the experimental group, in line with previous observations that biochemical changes during imprinting are

usually more marked in the left hemisphere (Horn 2004). This was observed in all the areas measured and in both groups, and in previous studies from our group making us hypothesise a baseline level of activity spontaneously lateralised in favour of the left hemisphere. Potentially, a spontaneously higher density in the left hemisphere of septum in both groups *i.e.*, could have masked the effect of the dependent variable also on that hemisphere by a ceiling effect. This adds to the set of observations on lateralisation effects found in chicks from dark incubated eggs (Horn and Johnson 1989; Johnston and Rogers 1999; Mascetti and Vallortigara 2001; Andrew et al. 2004; Mayer et al. 2016a, 2017a), which is surprising because light stimulation during late embryonic stage is known to trigger lateralisation in the chick brain in other tasks (Rogers and Sink 1988; Rogers 1990; Rogers and Bolden 1991; Rogers 1997, 2008).

Unfortunately, due to the intrinsic characteristics of our neural activity marker and of the chosen staining technique in this study we were not allowed to assume that significant higher level of c-Fos-ir neurons in septum and POA were an enhancement caused by the exposure to the predisposed stimulus or a decrement in the control group caused by the non-predisposed stimulus. However, these results show an involvement of these social decision-making areas in differential processing of predisposed and non-predisposed social moving stimuli, future studies will focus on identifying which neurons were stained here with c-Fos (*i.e.* GABAergic or Glutamatergic) replicating the study and using a double-labelling staining technique. This could identify by co-localisation the different populations of neurons c-Fos stained. Moreover, after measuring activation in these brain areas, it would be extremely interesting to inactivate selectively those specific brain regions to see whether their involvement is necessary for the emergence of predispositions. This could be done by selective lesioning techniques targeting precisely the area of interest and not the surrounding or connected one, and afterwards testing the animals for the behavioural preference.

In a third study, we wanted to investigate neural correlates of social predispositions for static configuration of naturalistic features. We referred to a famous set of studies by Gabriel Horn and colleagues investigating imprinting (Bolhuis et al. 1985, 1989; Johnson et al. 1985, 1989; Johnson and Horn 1986, 1988; Davies et al. 1992; Hampton et al. 1995; Bolhuis and Horn 1997; for a review see Horn 2004). They demonstrated a spontaneous preference in dark reared chicks for approaching a stuffed jungle fowl over several other salient objects.

They also observed that the fowl was preferred when the head and neck configuration was visible. In order to investigate the neural correlates of this predisposition we wanted first to replicate the behavioural preference in our laboratory setting. The predisposed stimulus was a stuffed jungle fowl and the non-predisposed one was an identical one cut in small pieces and reassembled scrambled on a box (texture fowl). Low-level perceptual features (*i.e.* luminance, colour, visual complexity) were perfectly balanced between the two stimuli. Since we decided to replicate the behavioural preference in this study, we visualised brain activity in two subsamples of chicks that underwent the behavioural preference test by selecting them for their absolute preference for either one of the two stimuli, the fowl or the texture fowl. For this experiment two brain areas were selected for c-Fos counting based on previous literature. IMM, intermediate medial mesopallium investigated also in Study 2, an area involved in learning the features of the imprinting object (*i.e.* McCabe et al. 1981; Horn 1986, 1990; Bolhuis and Honey 1998) and optic tectum (TeO) the avian homologue of the mammalian superior colliculus (Reiner et al. 2004; Jarvis et al. 2005) theorised to be a candidate area relevant for social predispositions and involved in preferential social orienting (Johnson 2005; Rosa-Salva et al. 2015). A third area was selected as control region, the hyperpallium apicale (HA, hyperstriatum accessorium, old nomenclature) part of the visual Wulst homologue to the mammalian visual cortex. No difference was expected in this region since chicks from both groups were exposed to the same visual environment and stimuli were well balanced. A third group was exposed only to the experimental apparatus, its neural activity was estimated in IMM, and HA to verify the baseline level of activity caused by the simple exposure to the test environment. Behavioural results successfully replicated the spontaneous preference for the fowl. At a neural level, preferential approach toward naturalistic or artificial stimulus resulted in differential plasticity levels in IMM, pointing to an involvement of this area in the perception of static configurations of features. Contrary to our expectations, higher c-Fos-ir density was detected in IMM of the non-predisposed group approaching the texture fowl. Nevertheless, activity in IMM in both groups was higher than in the baseline group, indicating that IMM was involved in processing either of the two stimuli. Moreover, no difference was found in HA suggesting that the effect in IMM was region specific. A possible explanation of the higher density found in IMM of chicks approaching the texture fowl could be that encoding the features of a complex imprinting object not matching the predisposed template (fowl)

could be more demanding in terms of plasticity. Surprisingly, the IMM of the texture fowl group showed significant right lateralisation. This is in contrast with the frequent observation that structural and biochemical changes in IMM consequent to imprinting on artificial objects are more pronounced in the left hemisphere (Horn 2004; for exception to this general trend please see Horn et al. 1973; Johnston and Rogers 1998). Indeed, right IMM of the texture fowl group could be more engaged in the detection of change in the complex stimulus represented by the texture fowl (for hemispherical specialisation in this direction please see Vallortigara and Andrew 1991, 1994; Vallortigara 1992; Rogers 1995a; Johnston and Rose 2002; Rosa-Salva et al. 2012a; Rogers et al. 2013). This complex pattern of lateralisation adds to previous observations in Study 2 and in other IEG experiments stressing the need for future investigation on the level of spontaneous activity in the two hemispheres in dark incubated chick.

Contrary to our expectations, no difference between the two groups was present in TeO. The absence of any effect in this area could be due to the fact that both groups saw a complex visual scene with both the stimuli, itself comprising a variety of visual information. All this complex visual stimulation could have act as confounding factor activating multiple populations in the TeO and masking the effect of the social predisposed configuration. Future studies investigating TeO could focus on providing a more controlled, simple and specific stimulation of the experimental groups of chicks, *e.g.* using a single exposure technique as in Study 2 and providing static simple stimuli such as schematic faces.

In order to understand the lateralisation effects emerged from the two previous studies and other IEG experiments in the lab (Mayer et al. 2016a, b, 2017a; Lorenzi et al. 2017) and to set a baseline activity level for future c-Fos investigations, we devised a fourth study targeting the level of spontaneous c-Fos immunoreactivity present in the two hemispheres. From previous observations a tendency was apparent for higher activity in the left hemisphere regardless of the different experimental manipulations. This tendency was observed in several brain areas: septum, IMM, POA, Hp and arcopallium. Chicks from all these experiments hatched from dark-incubated eggs. It is long known that chicks incubated with light during a late embryonic stage develop functional and structural brain asymmetries mainly in favour of the left ascending projections of the thalamofugal pathway (homologue to the mammalian geniculocortical pathway; Shimizu and Karten 1993) to the forebrain (*e.g.* Rogers and Sink 1988; Rogers and Bolden 1991; Deng and Rogers 1997).

In light of what briefly summarised here, we devised three different conditions. A dark-dark condition, in which chicks received no any visual nor light stimulation, eggs were incubated in the darkness and chicks hatched in the darkness. A light-dark condition, in which eggs were light stimulated in the critical window known to trigger lateralisation, but chicks hatched in the darkness. A dark-light condition, in which eggs were incubated in the darkness, chicks hatched in the darkness and a homogeneous light stimulation was provided individually to chicks in a featureless environment. We showed that in the absence of any light stimulation in the egg and before any visual experience chicks hatch with a spontaneous left lateralisation asymmetry. We partially confirmed what observed in previous experiments finding an overall left lateralisation asymmetry in all the three experimental conditions. However, these preliminary results need to be extended to a larger sample. The reason why we did not find any significant lateralisation in the other two conditions and neither a significant interaction between conditions, area and hemisphere, is probably the lack of statistical power of the test used. This made it hard to draw firm conclusions about these results and to set a baseline level for future c-Fos studies. Indeed, future studies will focus on increasing the sample size while decreasing the variability between the three conditions. Future studies will also focus on the structural differences between the two hemispheres, by targeting the absolute number of neurons in the two hemispheres. Indeed, difference in c-Fos counting between the two hemispheres could simply reflect a numerical structural difference in the population of neurons in the two hemispheres.

In sum, these results adds to the existing knowledge about some kind of brain and behavioural lateralisation not necessarily dependent from late embryonic light exposure (Horn and Johnson 1989; Johnston and Rogers 1999; Mascetti and Vallortigara 2001; Andrew et al. 2004; Chiandetti et al. 2013).

The present thesis adds to previous research emphasising the need for an integrated comparative approach to the nature *vs.* nurture studies in systems neuroscience. The use of different animal models and different techniques to tackle the same research questions in different laboratories, lays the bases for a deeper understanding of the phylogenetic and ontogenetic origins of behaviour and brain.

Compliance with ethical standards

All procedures performed in the present thesis were in accordance with the ethical standards of the University of Trento. Moreover, all applicable European and Italian guidelines for the care and use of animals were followed. All the studies presented here have been approved by the research ethics committee of the University of Trento and by the Italian Ministry of Health.

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