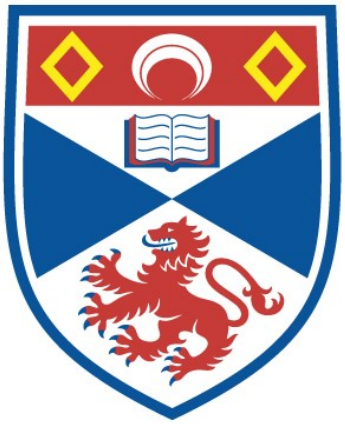
THE NEUROETHOLOGY AND EVOLUTION OF NEST-BUILDING BEHAVIOUR

Zachary Hall

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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The neuroethology and evolution of nest-building behaviour

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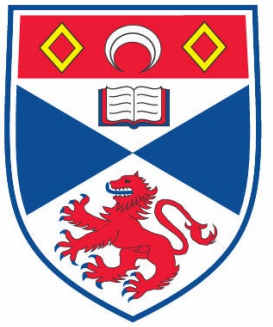

This thesis is submitted in partial fulfilment for the degree of PhD
at the
University of St Andrews

September 2014

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Declaration of publications

The work described in chapter 2 forms the basis of "Hall ZJ, Bertin M, Bailey IE, Meddle SL, Healy SD (2014) Neural correlates of nesting behaviour in zebra finches (*Taeniopygia guttata*). Behaviour Brain Research **264**:26-33."

Chapter 3 will form part of the following manuscript: Hall ZJ, Healy SD, Meddle SL. A role for nonapeptides and dopamine in nest-building behaviour.

The work described in chapter 4 forms the basis of "Hall ZJ, Street SE, Healy SD (2013) The evolution of cerebellum structure correlates with nest complexity. Biology Letters **9**: 20130687.

Chapter 5 will form part of the following manuscript: Hall ZJ, Street SE, Healy SD. Co-evolution of nest structure and location in Old World babblers (Timaliidae).

Declaration of collaboration

I collected all data with the exception of female behavioural data in Chapter 2. These data were collected by the undergraduate student Marion Bertin, under the supervision of me and Dr. Susan Healy. Ida Bailey provided advice regarding the statistical analysis performed in chapter 2. Simone Meddle provided advice regarding immunohistochemical techniques performed in chapters 2 and 3. Finally, Sally Street provided advice regarding the statistical analyses performed in Chapters 4 and 5.

Abstract

A surge of recent work elucidating a role for learning and memory in avian nest-building behaviour has challenged the long-standing assumption that nest building develops under genetic control. Whereas that work has been addressed at describing the cognitive mechanisms underpinning nest-building behaviour, almost nothing is known about either the neurobiological processes controlling nest building or the selection pressures responsible for the diversity in avian nest-building behaviour. Here, I sought to identify both the neural substrates involved in nest-building behaviour and some of those selection pressures. First, I used expression of the immediate early gene product Fos, an indirect marker of neuronal activity, to identify brain regions activated during nest-building behaviour in the brains of nest-building and control zebra finches (*Taeniogypia guttata*). I found that neural circuits involved in motor control, social behaviour, and reward were activated during nest building. Furthermore, I found that subpopulations of neurons that signal using the nonapeptides vasotocin and mesotocin and the neurotransmitter dopamine located within some of these neural circuits were also activated during nest building, suggesting these cell-signalling molecules may be involved in controlling nest-building behaviour. Next, I found that variation in the amount of folding in the cerebellum, a brain structure thought to be involved in manipulative skills, increased with increasing nest structural complexity, suggesting that the cerebellum is also involved in nest building. Finally, using evolutionary statistical models, I found support for the hypothesis that nest-site competition off-ground and increased predation pressure on the ground in Old World babblers (Timaliidae) led to the co-evolution of building domed nests on the ground. Here,

then, I provide the first evidence of potential neural substrates controlling and selection pressures contributing to variation in nest-building behaviour.

Acknowledgements

Firstly, I would like to thank my supervisor, Susan Healy, for her unending help and support throughout my tenure at the University of St. Andrews. Because of Sue's courage and confidence in my abilities, I was able to amass a body of work addressing a topic that some deemed too risky to be central to a PhD thesis. I hope that I will be able to maintain collaborations with such an inspirational researcher and that the work I was able to complete while in St. Andrews recompensed her for at least a fraction of all that she has taught me.

I would also like to thank all of my collaborators, who taught me the techniques that were vital to the integrative approach to studying nest building presented here. Most notably, I would like to thank Simone Meddle for all of her patience and help in improving my lab techniques and Sally Street, who taught me the phylogenetic comparative statistics crucial to my evolutionary work. I would also like to thank Chris Vendetti and Daniel Barker for their help and advice regarding the statistical approaches used in my comparative analyses. For insightful and inspiring discussion about my research, I would like to thank Rob Barton, Dave Shukar, David Sherry, and Scott MacDougall-Shackleton.

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Finally, and most importantly, I would like to thank every bird included in my studies. You were all clever to me, even if you weren't crows or feathered apes or whatever.

Ethical note

All experimental procedures in this thesis were performed with ethical permission from the University of St. Andrews Animal Welfare and Ethics Committee and from the UK Home Office (PPL. 60/3666).

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Chapter 1: Introduction

Of all the constructions made by non-human animals, perhaps none are as widely recognised as the nests built by birds. From the sewing behaviour of the common tailorbird (*Orthotomus sutorius*), which stitches together leaves to form a nest cup later filled with insulating material (Nguembock et al., 2007) through the famous weaving and thatching abilities of weaver birds (Ploceidae; Collias and Collias, 1964) to the unique nest construction of the Horned Coot (*Fulica cornuta*), which deposits upwards of 1 ton of pebbles in bodies of water to form a nesting island before constructing a nest cup (McFarlane, 1975), the daunting diversity in nest-building behaviour has long been celebrated by the likes of Wallace (1867), Tinbergen (1953), and Thorpe (1956). Despite the ongoing accumulation of nest structure descriptions for the majority of extant, known bird species, as seen in the Handbook of Birds of the World book series (for example, del Hoyo et al., 1992), it is then perhaps surprising that so few researchers have sought to elucidate the mechanisms underlying how birds construct nests and why there is such structural diversity in nests across species.

Amongst the handful of studies in which the way birds construct nests has been addressed, research effort has been focused almost entirely on the role of learning and experience in nest building. Historically, nest building was assumed to be an innate behaviour under genetic control and unaffected by experience (Healy et al., 2008). For example, in *Descent of Man*, Charles Darwin stated that, in contrast to human skills, which improve with practice, inexperienced birds will construct nests comparable to those of experienced builders on their first attempt (Darwin, 1882). Experimentally, this view

received early support from studies in which hand-reared birds, deprived of nest material during development and first exposed to nest material as adults, were reported to construct nests resembling those built by experienced builders. For example, hand-reared female canaries (*Serinus canaria*) deprived of nesting material during development constructed species-typical nests upon their first exposure to nest material in adulthood (Hinde and Matthews, 1958). It should be noted, however, that this finding conflicts with earlier, similar experiments in which hand-reared American Robins (*Turdus migratorius)* and Rose-breasted Grosbeaks (*Pheuticus ludovicianus*) failed to construct species-typical nests upon their first exposure to nest material in adulthood (Scott, 1902; 1904).

Soon after Hinde and Matthew's work on canaries, Collias and Collias (1962; 1964), displeased with the limitations of describing the mechanisms underlying nest building as innate, published a series of studies on the nest-building behaviour of African Village weaver birds (*Ploceus cucullatus*) in the wild and captivity. In one of the strongest challenges to a (still-prevalent) genetic-only origin of nest-building behaviour, Collias and Collias (1964) documented the development of weaving abilities in hand-reared and aviary-reared weaver birds, reporting a significant effect of experience with nest material during development on subsequent nest material preferences and construction behaviour. Specifically, hand-reared weaver birds deprived of experience with nest material exhibited weaker preferences for the longer, flexible, green nest material than did experienced weaver birds and were also less able to weave material successfully into the aviary cage and trees. When these naive birds were given experience with nest material and tested again months later, they exhibited material preferences and weaving capabilities similar to those exhibited by birds reared with access to nest material (Collias and Collias, 1962). Although

the studies by Collias and Collias suffer somewhat from a reliance on anecdotal evidence, they provided some of the first evidence that nest-building behaviour cannot be explained purely by genetic, innate origins.

Despite these compelling studies by Collias and Collias, however, it is still common to identify nest-building behaviour as entirely innate, a view that has been used to discount comparisons between nest building and other construction behaviours thought to depend on cognition such as tool manufacture and use (Raby and Clayton, 2009; Seed and Byrne, 2010). The assumption that nest building is innate, however, fails to explain the results of Collias and Collias' work, remains largely untested, and cannot account for apparent phenotypic similarities between nest-building and tool-use behaviour (Hansell, 2005; Healy et al., 2008; Hansell and Ruxton, 2008; Schumaker et al., 2011). Recently, a surge of studies on wild and captive birds has demonstrated a role for learning and experience on subsequent selection of nest material (Muth and Healy, 2011; 2012; Muth et al., 2013), nest location (Mennerat et al., 2009; Hoi et al., 2012), and construction behaviour at the nest (Walsh et al., 2011; Muth and Healy, 2014; Bailey et al., 2014), reigniting the Collias' challenge to the assumed genetic origins of this behaviour.

Although these recent studies have begun identifying the learning processes involved in nest-building behaviour, this body of work addresses only one level of mechanism. Compared to ongoing work on the role of learning and experience in nest building, even less work has addressed the neural mechanisms underlying nest-building behaviour. Similarly, few studies have addressed the evolutionary processes that have lead to the considerable interspecific variation in nest design. The focus of my thesis was, therefore, to establish methodological approaches facilitating research on the

neurobiological substrates underlying and evolutionary influences shaping nest-building behaviour. Using techniques from behavioural neuroscience, I sought to identify neural circuits that were active during the performance of nest-building behaviour. Additionally, by using phylogenetic statistical techniques, I aimed to test whether species differences in brain morphology may relate to variation in nest structure and to identify selection pressures that might influence nest structure and location.

Why study nest building in the brain?

Nest building has the potential to become a powerful behavioural model in the fields of both behavioural and comparative neuroscience. As a model in behavioural neuroscience, nest-building behaviour offers an opportunity to study the neural substrates involved in sequence learning and motor sequencing using a naturally occurring behaviour that has significant fitness consequences. This is firstly because nest-building behaviour can be decomposed into sequences of discrete, organised motor actions. For example, in 1953, Tinbergen observed the nest-building behaviour of long-tailed tits (*Aegithalos caudatus*), which construct domed nests with walls comprised of moss and up to 600 spider egg cocoons. Following construction of most of the dome, long-tailed tits cover the outside of their nests with lichen flakes, which adhere to the spider silk in the nest walls. The birds then create an entrance hole and finish the roof of the nest before finally lining the nest with an estimated 2600 feathers (Thorpe, 1956; Hansell, 2000). Tinbergen's observations led him to decompose nest building by the long-tailed tit into 13 or 14 discrete, highly stereotyped actions that must be organised correctly to produce a viable nest. The correct sequence of building actions required to produce a nest is called the effective sequence, a

term coined by Collias and Collias (1964) while describing the development of nest-building behaviour in Village weaver birds. Whereas the effective sequence of long-tailed tits and weaver birds involves organising many actions over long periods of time, nest building, in its simplest form, involves an effective sequence of nesting material collection and deposition at the nest site.

Current behavioural neuroscience models of sequence learning and motor sequencing include serial reaction time tasks and shaping animals to perform motor sequences using operant conditioning procedures. In serial reaction time tasks, animals are trained to respond to multiple stimuli presented in a sequence. When each stimulus is presented, the animal is required to produce a stimulus-specific response within a limited amount of time to receive a reward. In rodents, for example, an animal must poke its nose through one of five holes when the light above that hole is illuminated to receive a food reward. In the sequence learning condition, five stimuli are presented in the same order each trial, whereas in the control condition, the stimuli are presented in a randomised order each trial (Schwarting, 2009). The animal is assumed to have learned the sequence when the reaction times to stimuli are lower in the sequenced condition compared to stimuli presented in a random order, suggesting the animal has learned to predict the next stimulus in the sequence. Alternatively, other studies use operant conditioning procedures to train animals to press up to five buttons in a specific order, called serial-order tasks. These paradigms have been used to directly compare motor sequence learning between humans, non-human primates, and birds (Scarf and Colombo, 2008). Furthermore, this shaping paradigm has been used to identify neural substrates in the pigeon involved in initiating a memorised sequence of pecks (Helduser and Güntürkün, 2012; Helduser et al., 2013).

One limitation of serial reaction time and serial-order tasks is that both paradigms focus on relatively short action sequences that occur over a few seconds, whereas many of the action sequences that animals perform occur over much longer timespans. Nest building, for example, can occur over hours, days, and even weeks. For example, Red-winged Blackbirds (*Agelaius phoeniceus*) take up to three days to construct cup nests (Holcomb and Twiest, 1968) while the male malleefowl (*Leipoa ocellata*) constructs a large nesting mound over the course of weeks, which he then maintains daily for the majority of the year (Frith, 1959). Comparing the neural substrates involved in nest building to those identified using pre-existing behavioural paradigms will help to increase our understanding of how the brain organises motor sequences across different timescales. Furthermore, both serial reaction time and serial-order tasks rely on immediate and consistent food rewards to change animal behaviour, whereas nest building, alongside many other behaviours performed in the wild, are typically met with no overt, immediate reward. The role of reward contingencies in studies on sequence learning in the lab has only recently been discussed and evidence suggests that such contingencies blur the contributions of learning versus rewards to changes in task performance. For example, in serial reaction time tasks, animals in the sequence learning treatment typically exhibit increased response accuracy over repeated trials (Schwarting, 2009) and, thus, may receive more rewards than controls. This group difference in the amount of reward received can influence task motivation and, in turn, reaction times. By studying nest building, I would be able to test for the involvement of brain regions thought to be involved in motor organisation and sequencing without relying on artificial reward contingencies to change behaviour. Furthermore, in the absence of reward contingencies, I would be able to test whether neural circuits regulating

the motivation and reward associated with ecologically-relevant behaviours such as courtship (O'Connell and Hoffman, 2012) are also involved in reinforcing nest-building behaviour.

In additional to its potential as a model of motor sequencing, I also believe that nest building could become a powerful model in comparative neuroscience. Our understanding of how the brain controls behaviour is often restricted to a few, intensively studied, typically lab-reared animal models. This limitation reduces the cross-species transferability of our knowledge of brain-behaviour relationships and is thought to contribute to the failure of, for example, neuropsychiatric therapeutic interventions first validated on lab animals and subsequently tested in humans (Hall et al., 2014a). By incorporating more species into neurobiological studies, we can produce a more robust understanding of how the brain controls behaviour and generate conclusions that can be transferred across species. One of the biggest, current hindrances for comparative neuroscience is the lack of behavioural and neural data for large samples of species. Although detailed observational descriptions of nest-building behaviour such as that provided by Tinbergen (1953; see above) are relatively rare, descriptions of species-typical nest structure have been collected for the majority of extant bird species and may contain some information about species differences in building behaviour. In conjunction with the availability of nest structure descriptions, databases comprised of neuroanatomical data on multiple bird species are widely accessible and have been used previously to relate brain morphology to species differences in behaviour such as song repertoire size in songbirds (Moore et al., 2011). Although relating brain morphology to species differences in behaviour does not necessarily imply a functional connection between the brain and behaviour, these comparative analyses help identify brain regions of

interest that can be focused on in subsequent functional studies using fewer species. For example, comparative studies on avian neuroanatomy identified significantly larger hippocampal volumes in the brains of bird species that cache and retrieve seeds (Sherry et al., 1989), suggesting that the hippocampus may be involved in learning cache locations. Since that study, evidence from both hippocampal lesions (Sherry and Vaccarino, 1989) and, more recently, impairments of hippocampal adult neurogenesis (Hall et al., 2014b) confirm a functional connection between neurons in the hippocampus and spatial learning of food locations in the black-capped chickadee (*Poecile atricapillus*), a caching species.

How to study nest building in the brain

How patterns of neuronal activity translate into the production of behaviour is a question that has always been at the forefront of neuroscience. A common approach to linking brain and behaviour is to identify brain regions that are active while animals perform behaviour of interest. The popularity of this approach in behavioural neuroscience is evident in the large array of techniques that have been developed to sample activity within the brain. These techniques often differ in the measure of brain activity quantified, the time- and spatial scale across which brain activity is sampled, and the procedures required to prepare an animal for recording brain activity. For example, whereas blood-oxygen-level dependent functional magnetic resonance imaging (BOLD fMRI) measures changes in oxygenated bloodflow occurring 10 seconds after elevated neuronal activity in heavily restrained animals (Ogawa et al., 1990), electrophysiological techniques record individual action potentials instantaneously in small populations of neurons in anesthesised or awake, behaving animals (for example, Hubel and Wiesel, 1962).

Here, I sampled brain activity in nest-building zebra finches (*Taeniopygia guttata*) using immunohistochemistry on sectioned neural tissue to highlight neurons producing an immediate early gene product. As the name suggests, immediate early genes are a group of genes expressed immediately following periods of elevated neuronal activity, specifically the production of action potentials in neurons (Clayton, 2000; but see Kovács [2008] for other factors regulating immediate early gene expression). I focused on the expression of the immediate early gene *c-fos*, which is transcribed and translated to produce the protein product Fos (Morgan and Curran, 1991). Fos protein is the most commonly studied immediately early gene product and has been used to identify patterns of brain activity in most vertebrate taxa, including songbirds (Clayton, 2000). There is a time-dependent profile to the appearance of c-*fos* mRNA such that it accumulates to peak levels roughly 30-60 minutes following a period of elevated neuronal activity. Requiring the additional step of mRNA translation, Fos protein accumulates to peak levels anywhere between 50 to 120 minutes following elevated neuronal activity (Figure 1.1; Clayton, 2000). Neurobiologists exploit the temporal dissociation between neuronal activity and the accumulation of Fos mRNA and protein to indirectly sample levels of brain activity in neural tissue collected up to 120 minutes after an animal performs a behaviour of interest.

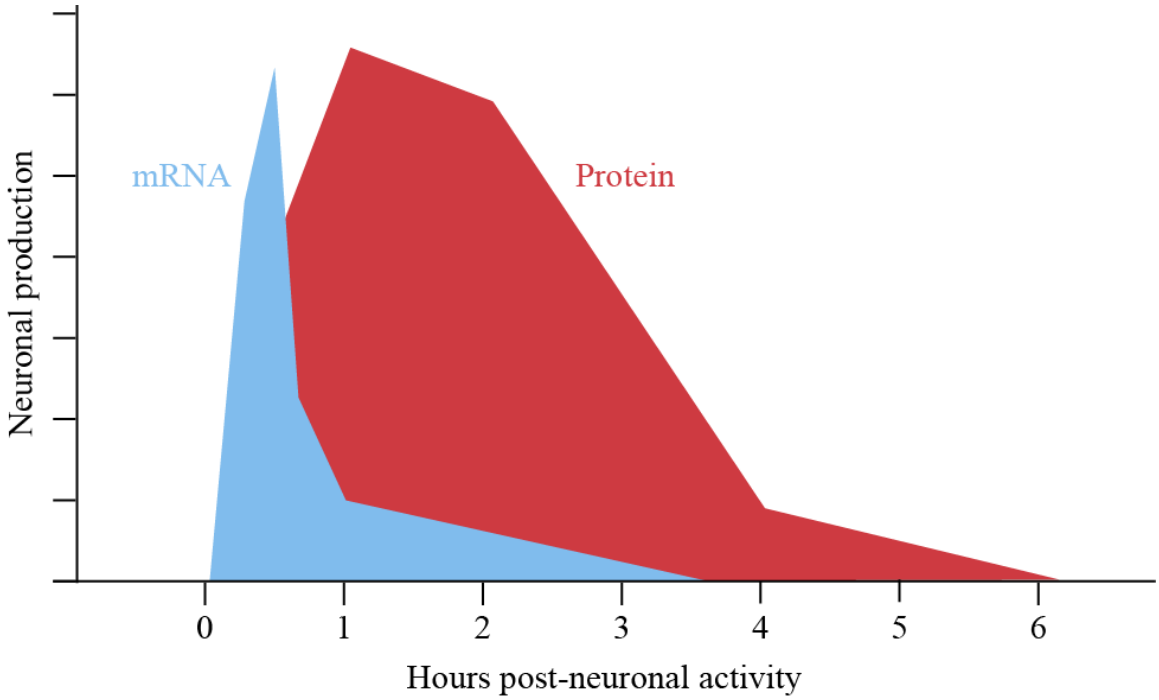

Figure 1.1. The accumulation of *c-fos* mRNA (blue) and protein (red) in neurons following periods of high neuronal activity. 0 hours post-neuronal activity refers to a period of elevated activity and the releasing of action potentials by a neuron. Over the following 30-60 min, *c-fos* mRNA accumulates in the neuron to peak levels. As *c-fos* mRNA is translated, Fos protein accumulates in the neuron to peak levels anywhere between 50-120 min. Figure adapted from Clayton (2000).

By studying immediate early gene expression in neural tissue collected after nest building, I would be able to sample neuronal activity without the need for animal restraint or anaesthetic. Additionally, immunohistochemical labelling of Fos protein provides a "snapshot" of neuronal activity across entire brain sections, allowing me to sample brain activity corresponding to the same period of nest-building behaviour throughout the brain.

Due to the relatively slow accumulation and degradation of *c-fos* mRNA and Fos protein, immediate early gene techniques suffer from reduced temporal acuity in quantifying brain activity. Furthermore, neurons labelled for the production of Fos protein in neural tissue are quantified as "active" or "inactive" based on the intensity of Fos labelling in each neuron, ignoring differences in activity between individual neurons. Despite these limitations, characterising immediate early gene expression patterns is widely and successfully used as a "first step" in identifying candidate brain regions activated during performance of a behaviour. For example, in zebra finches, immediate early gene techniques have been used to identify brain regions exhibiting elevated neuronal activity during birdsong production (Kimpo and Doupe, 1997; Jarvis et al., 1998), song perception (Bailey et al., 2002), and social and agonistic interactions with conspecifics (Goodson, 2005). After candidate brain regions are identified, subsequent studies can focus on these regions and compare neuronal activity to the production of behaviour on a much finer timescale or interfere with neuronal activity in these regions to test for a causal relationship between brain activity and production of behaviour.

In the work presented here, I exploited the temporal delay between neuronal activity and the accumulation of Fos protein to sample neuronal activity in the brains of nest-building zebra finches 90 minutes after nest building began. Although Fos labelling has been used to identify patterns of brain activity across entire brain sections (Sadananda and Bischof 2002; 2006), I chose to focus on sampling neuronal activity in neural circuits that I hypothesised may be involved in nest building based on previous studies on these brain regions.

Anterior and posterior motor pathways

Aside from the song-control system (a group of interconnected brain nuclei involved in producing birdsong: Tramontin and Brenowitz, 2000), the neural substrates involved in motor control in birds were only recently identified. In 2008, Feenders et al. compiled the results of several studies on songbirds, parrots, ring doves, and hummingbirds in which the production of different locomotor behaviours correlated with the expression of immediate early gene mRNA, used as a proxy of neuronal activity. These behaviours included wing-whirring during migratory restlessness in garden warblers (*Sylvia borin*) and hopping in zebra finches. Across these comparisons, and in additional experiments in which birds hopped in a rotating wheel moving at a constant speed, a common set of 11 telencephalic regions exhibited elevated neuronal activity (identified using both *zenk* and *c-fos* immediate early genes) the more locomotor behaviour the birds produced. The authors hypothesised that these 11 regions are organised into two motor pathways, responsible for the production of actions, and two somatosensory pathways, which were known to receive somatosensory input (Feenders et al., 2008). The two motor pathways were named the posterior and anterior motor pathways for their relative location within the telencephalon. The regions within each of these pathways is summarised in Figure 1.2.

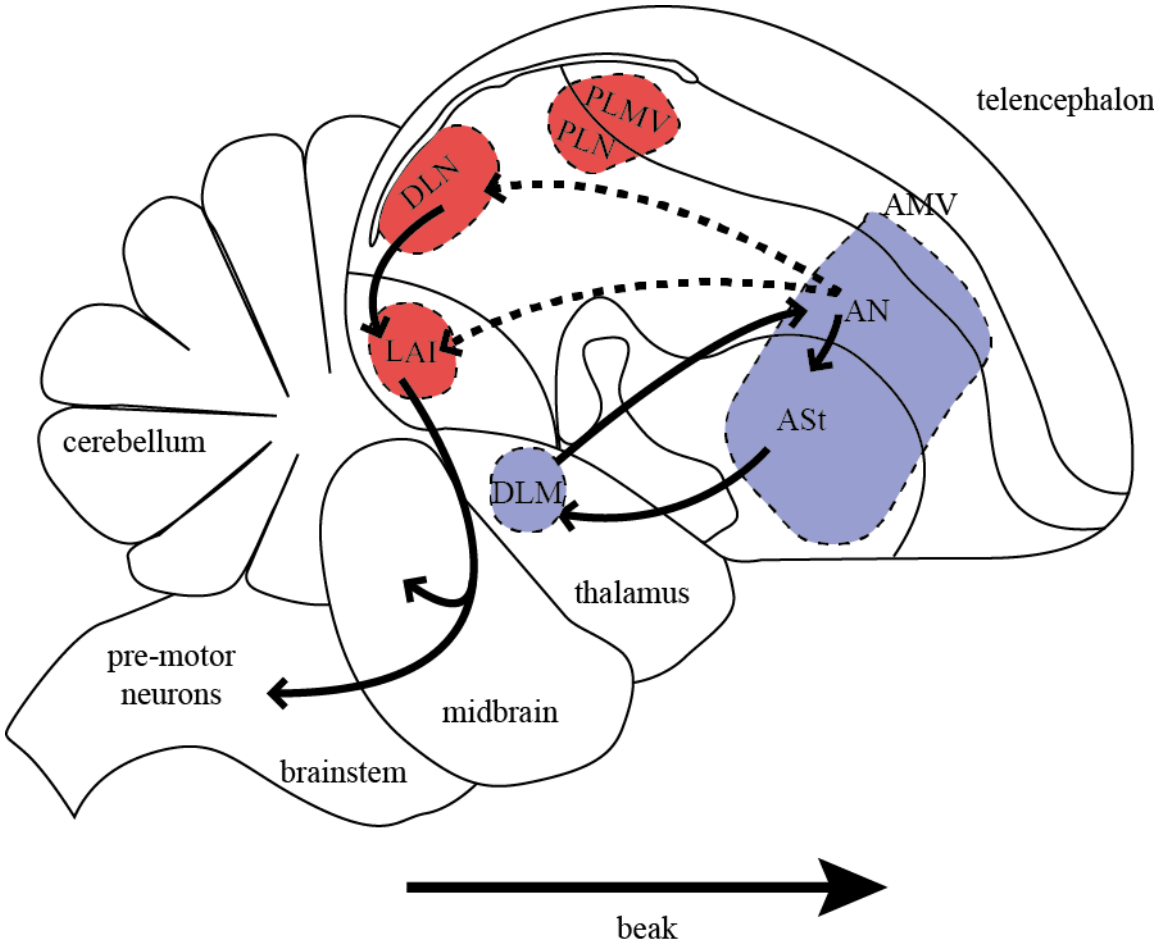

Figure 1.2. The anterior and posterior motor pathways of the avian brain. A sagittal drawing of the zebra finch brain containing the two motor pathways proposed by Feenders et al. (2008). The anterior motor pathway (purple) includes three telencephalic regions—the anterior striatum (ASt), the anterior nidopallium (AN), and the anterior ventral mesopallium (AMV)—and the dorsal magnocellular nucleus of the thalamus (DLM). The posterior motor pathway (red) contains four telencephalic regions: the posterior lateral nidopallium (PLN), posterior lateral ventral mesopallium (PLMV), the dorsolateral nidopallium (DLN), and lateral intermediate arcopallium (LAI). Locations of all regions were adapted from Feenders et al. (2008).

Feenders et al. (2008) noted that, in bird species that learn their songs, both the posterior and anterior motor pathways are located within close proximity to pathways in the song-control system. The authors used functional knowledge about each of these song-learning pathways to suggest functions for the posterior and anterior motor pathway. The posterior motor pathway is located beside the "motor pathway" of the song-control system (consisting mainly of two song nuclei: the robust nucleus of the arcopallium and HVC [used as a proper name]), which sends motor commands to the singing muscle, the syrinx, to produce song (Tramontin and Brenowitz, 2000). Accordingly, Feenders et al. (2008) suggested that the posterior motor pathway sends motor commands out of the telencephalon down into the brainstem and spinal cord to produce movement.

As the anterior motor pathway is located beside the similarly-named "anterior motor pathway" of the song-control system (consisting of three telencephalic song nuclei: Area X in the striatum, magnocellular nucleus of the anterior nidopallium [MAN], and oval nucleus of the mesopallium [MO]), which is involved in the learning and modification of birdsong (Tramontin and Brenowitz, 2000), Feenders et al. (2008) suggested that the anterior motor pathway is involved in the learning, modification, and organisation of actions.

In this thesis, I aimed to determine whether the anterior and posterior motor pathways are involved in controlling the production of nest-building behaviour using Fos protein immunohistochemistry to sample neuronal activity in both pathways. If nest-building behaviour, and specifically the collection and deposition of nesting material, involves motor sequencing, then I expected to see correlations between nest-building behaviour and the number of neurons producing Fos in the anterior striatum, anterior nidopallium, and anterior ventral mesopallium of the anterior motor pathway.

Social behaviour network

Whereas few neurobiological investigations have attempted to identify the neural circuits involved in nest-building behaviour, much work has elucidated the neural substrates involved in courtship behaviour preceding and parental behaviour following, nest building. The majority of these studies have focussed on the social behaviour network, a group of interconnected telencephalic nuclei involved in the production and regulation of social behaviour (Goodson, 2005). Newman (1999) first proposed the existence of a social behaviour network based on previous neurobiological work in mammals. In his review, Newman grouped six brain regions in the limbic system together as a neural system based on reciprocal connectivity between all regions, expression of gonadal hormone receptors in each region, and a common function in mediating affiliative, aggressive, and parental behaviour in mammals. Since then, homologous regions of all six social behaviour network brain regions have been identified in all vertebrate lineages, including fish, reptiles, and birds (Goodson, 2005; O'Connell and Hofmann, 2011). In birds, nuclei in the social behaviour network have been functionally associated with social behaviours including courtship singing and displaying (Heimovics and Riters, 2006), copulation (Balthazart and Surlemont, 1990; Meddle et al., 1999), aggressive interactions (Goodson and Adkins-Regan, 1999) and incubation (Youngren et al., 1989). Because the social behaviour network regulates reproductive behaviour prior to and following nest building I expected that these brain regions might also be involved in controlling nest-building behaviour.

A previous study sampling neuronal activity in the social behaviour network in songbirds included indirect measures of nest building. In 2006, Heimovics and Riters found that captive adult male European starlings (*Sturnus vulgaris*) possessing a nest box

exhibited elevated neuronal activity in several brain regions in the social behaviour network relative to males lacking a nest box. The regions identified in that study included the medial bed nucleus of the stria terminalis, dorsal subdivision (BSTmd), medial bed nucleus of the stria terminalis, ventral subdivision (BSTmv), anterior hypothalamus (AH), medial preoptic area (POM), and ventromedial hypothalamus (VMH). The authors noted that male starlings possessing a nest box also collected and delivered nest material to the nest box, however, as nest-building behaviour was not quantified it is difficult to determine whether the observed changes in neuronal activity were related to nest building specifically and not to other concurrent changes in courtship, territorial, and parental behaviour. In this thesis, I aimed to compare neuronal activity in the social behaviour network with nest-building behaviour in zebra finches with a focus on the nuclei that were observed to be more active during nest possession in starlings (Heimovics and Riters, 2006).

One limitation of quantifying brain activity in the social behaviour network by sampling the number of neurons producing Fos is that all neurons in a given brain region are assumed to serve the same function. Contrary to this assumption, studies on the chemical neuroanatomy of the social behaviour network have demonstrated that several brain regions contain functionally distinct subpopulations of neurons that differ in the type of cellular signal they use to transmit information. Notably, medial divisions of the bed nucleus of the stria terminalis (BST) of the social behaviour network contain at least two, overlapping neuronal subpopulations: vasotocinergic neurons that transmit signals using vasotocin (the avian analog of arginine vasopressin in mammals) and mesotocinergic neurons that transmit signals using mesotocin (the avian analog of oxytocin in mammals; Goodson, 2008). Furthermore, these vasotocin and mesotocin neurons appear to mediate

many of the social behaviours associated with BST function (Goodson, 2008). In this thesis, after identifying regions in the social behaviour network that are activated during nest building, I also tested whether neuronal activity specifically in vasotocinergic and mesotocinergic neuronal subpopulations within these brain regions increased during nest building. By combining Fos protein immunohistochemistry with vasotocin or mesotocin immunohistochemistry, I was able to sample neuronal activity specifically within vasotocinergic and mesotocinergic neurons in the social behaviour network.

Dopaminergic reward system

Alongside studies on the involvement of the social behaviour network in regulating behaviour in birds, similar work has identified the neural substrates that reinforce the performance of social behaviours. A group of interconnected nuclei collectively referred to as the dopaminergic reward system has been extensively studied in the context of controlling the incentive and reward associated with behaviour in both laboratory paradigms and ethological study (Riters, 2011). Much like the social behaviour network, the dopaminergic reward system appears to be functionally and anatomically conserved amongst vertebrates and putative homologs of two of the most commonly studied reward nuclei, the ventral tegmental area and central gray, have been identified in all vertebrate lineages (O'Connell and Hofmann, 2012). O'Connell and Hofmann (2011) have recently proposed that the social behaviour network and dopaminergic reward system be considered a single neural system, called the social-decision making network, based on the deep homology of both the social behaviour network and dopaminergic reward system in vertebrates and extensive reciprocal connectivity between these two circuits. Because the

social-decision making network is a recent hypothetical framework and requires directed studies to justify grouping these two neural circuits, in this thesis I focused on each neural circuit separately.

Functional studies on the dopaminergic reward system show that neuronal activity in this system is related to the speed at which animals approach an environmental stimulus associated with reward and how long the animal engages with that stimulus, suggesting this neural circuit plays a key role in controlling motivational processes (Salamone and Correa, 2012). Changes in neuronal activity in dopaminergic neurons in this circuit predict behavioural changes in reward-based learning tasks, suggesting this neural circuit also plays a role in mediating the effects of reward on reinforcing behaviour (Schultz et al., 1997). Accordingly, dysfunction in the dopaminergic reward system has been associated with addiction disorders (Gardner, 2011). In studies on birds, the dopaminergic reward system also appears to play a role in controlling motivational and reward processes shaping naturally occurring behaviour: the ventral tegmental area is thought to reinforce the production of courtship song (Heimovics and Riters, 2005), copulation (Charlier et al., 2005), affiliation behaviours (Goodson et al., 2009), and pair bonding (Banerjee et al., 2013). Support for the involvement of the dopaminergic reward system in nest-building behaviour comes from evidence that neuronal activity is elevated in the ventral tegmental area in adult male starlings that possessed a nest box compared to males that did not (Heimovics and Riters 2005; 2007). Although this finding suggests a role for the ventral tegmental area in nest building, as in a similar study sampling activity in the social behaviour network described above (Heimovics and Riters, 2006), nest-building behaviour was not quantified and it remains unclear whether increased neuronal activity in the ventral

tegmental area can be attributed to nest-building behaviour or to concurrent changes in reproductive and territorial behaviours. Relative to the ventral tegmental area, much less is known about the function of the central gray in birds. After observing that neuronal activity in the central gray increased the more male zebra finches produced vocalisations directed at conspecifics, however, Goodson et al. (2009) hypothesised that the central gray may be involved in motivational processes controlling social communication. Here, I looked to see whether there was a relationship between neuronal activity in the ventral tegmental area and central gray and nest-building behaviour. If nest building is rewarding, I would expect neuronal activity in dopaminergic reward system nuclei to increase the more birds engage in nest-building behaviour.

As for the social behaviour network, brain regions in the dopaminergic reward system contain subpopulations of neurons characterised for using different cellular signals to transmit information. As the name "dopaminergic reward system" suggests, one such neuronal subpopulation in the ventral tegmental area and central gray uses the neurotransmitter dopamine. Furthermore, as mentioned above, dopaminergic neurons contained in these regions are thought to be central to the dopaminergic reward system's function in reinforcing behaviour. To test whether these neuronal subpopulations are involved in nest building, I compared Fos immunoreactivity in dopaminergic neurons in the ventral tegmental area and central gray with the production of nest-building behaviour.

Hippocampus

As described at the outset, unlike the role that motor or reward pathways may play in the neural underpinnings of nest building, there is an ongoing dispute regarding the role

played by cognition in nest-building behaviour, particularly with regard to comparisons between nest building and other construction behaviours that are thought to involve cognition (Hansell, 2005; Hansell and Ruxton, 2008; Healy et al., 2008). Demonstrating neuronal activation in certain brain regions associated with a behaviour may be useful in this debate as it can potentially inform us of the cognitive/learning processes involved in a behaviour. For example, consistent demonstrations of increased neuronal activity in the hippocampus during spatial cognition tasks in birds (reviewed in Mayer et al., 2012) and mammals (Nakamura et al., 2010; Teather et al., 2005; Guzowski et al., 2001) have suggested these animals share at least a partly homologous neural substrate involved in spatial learning. In addition to spatial learning, the hippocampus is thought to be involved in behavioural sequencing (Remondes and Wilson, 2013) and in regulating the context-specificity of behaviour in both mammals (Behrendt, 2013) and birds, including sexual behaviour (Atoji and Wild, 2006). As nest building might involve one or more of these processes, I compared Fos immunoreactivity in the hippocampus to nest-building behaviour.

Cerebellum

The cerebellum is a brain structure found in all vertebrates and located caudal to the telencephalon. Historically, the cerebellum was thought to serve only motor functions, an assertion supported by connectivity studies, in which it was reported that the cerebellum sent output exclusively to motor and pre-motor regions in the telencephalon, as well as studies connecting cerebellar damage with motor dysfunction including akinesia and rigidity (reviewed in Middleton and Strick, 2000). A surge of hodological studies in the

1990s using a newly-introduced viral-mediated tract tracing protocol, which enabled more extensive tracing of neural tracts across multiple synaptic junctions, however, demonstrated that the cerebellum, in addition to connections with cortical motor regions, was also reciprocally connected with several brain regions thought to be primarily involved in cognitive processing, including prefrontal cortex (Middleton and Strick, 2000). These connectivity studies, in conjunction with ongoing work demonstrating neuronal activity in the cerebellum associated with cognitive tasks, have lead to the current view that the cerebellum is involved not only in motor control, but also in learning, memory, and language processing, at least, in humans (reviewed in Barton, 2012).

In mammals and birds, cerebellar volume and the degree to which the cerebellar cortex is folded (called cerebellar foliation) exhibit tremendous diversity between species (Larsell, 1967). Butler and Hodos (2005) suggested that the expansion of cerebellar cortex, associated with increased cerebellar foliation, increases the neuronal processing capacity of the cerebellar cortex and supports enhanced motor abilities. Although the specific nature of improved motor abilities was not elucidated by Butler and Hodos, positive correlations between cerebellar foliation and tool use in birds (Iwaniuk et al., 2009) and between cerebellar volume and extractive foraging techniques in primates (Barton, 2012) suggest that increasing cerebellar foliation may improve manipulative skill with the beak and hands in birds and primates, respectively. Because nest building likely requires different degrees of manipulative skill to shape, stitch, and weave nest materials into different nest structures, I tested whether cerebellar foliation, as measured using a previously-published list of cerebellar foliation indices (Iwaniuk et al., 2006), relates to variation in species-typical nest structure. To do this, I classified species-typical nest structure based on structural

complexity following the assumption that the nest structure a bird builds is at least partially dictated by the manipulative skill of that species. For example, I predicted that constructing a cup nest, characterised by a nest floor and walls that are shaped by the beak, would require more manipulative skill and a more foliated cerebellum than would building a platform nest, which consists of an un-manipulated pile of collected material.

The evolution of nest structure

Much like the neurobiology of nest building, there has been little work aimed at elucidating the selective forces that have lead to the vast structural diversity in nests among bird species. Previous comparative studies investigating the evolution of nest structure are characterised by a lack of formal statistical tests of evolution and, instead, have described evolutionary patterns by mapping species-typical nest structure onto contemporaneous phylogenies (Winkler and Sheldon, 1993; Eberhard, 1998; Irestedt et al., 2006). In those studies, ancestral nest states and evolutionary transitions were estimated using outgroup comparison, a phylogenetic inference technique that suffers from overestimating the influence of phylogeny and relying on only the species included in the tested phylogeny to reveal the evolutionary history of the whole clade. Furthermore, outgroup comparison cannot account for either the degree of relatedness between species or phylogenetic uncertainty (Pagel and Harvey, 1988).

Despite advances in phylogenetically-informed statistical techniques that overcome the limitations of outgroup comparison (Pagel and Meade, 2006), the application of these tests in studies on the evolution of nest structure have been largely hampered by the lack of accessible phylogeny distributions with detailed information on species relatedness and the

lack of a classification system for the structural complexity of bird nests. Recently, however, Jetz et al. (2012) produced an online, publically accessible database of phylogenies for the largest sample of bird species to date. Usefully, for my purposes, many of these phylogeny estimations are amenable to current techniques in phylogenetic statistical modelling. In conjunction with the classification system I developed to compare cerebellar foliation with species-typical nest structure, I was able to generate phylogenetically-informed statistical models to test evolutionary hypotheses regarding the evolution of nest structure.

In spite of the historic lack of phylogenetic and nest classification data required to investigate the evolutionary origins of nest structure diversity, there are a number of hypotheses regarding the evolutionary pressures influencing nest structure extant in the literature. Notably, Collias (1997) used outgroup comparisons and descriptive statistics to present multiple hypothetical evolutionary routes that he believes have led to the diversity in nest structure seen today. Although Collias' arguments lacked statistical complements to account for the effects of phylogenetic relatedness in his proposal, many of his hypotheses are testable (albeit thus far untested) and supported by ecological work on nest placement and structure. In this thesis, I used phylogenetically-informed statistics to test one of Collias' hypotheses regarding the evolutionary pressures selecting for the construction of domed nests. Specifically, Collias (1997) argued that, from an ancestral state of constructing cup nests in trees, competition for limited nest sites off the ground favoured bird lineages that began constructing nests closer and closer to the ground. The closer a nest is constructed to the ground, however, the greater the risk of predation from ground predators. Collias postulated that birds began constructing enclosed nests to confer

protection from this increased predation risk. Here, I aimed to retest Collias' hypothesis regarding the evolution of domed nests in Old World babblers (Timaliidae) by incorporating phylogenetically-informed analyses to test for co-evolution between nest height and structure, to identify the ancestral state of nests in this clade, and to elucidate the most likely evolutionary transitions between nest heights and structures.

Thesis Aims

In the following chapters, I sought to identify the neural substrates involved in nest-building behaviour in birds and to establish a comparative framework to begin studying the evolutionary pressures that have produced the diversity in nest structures among bird species.

First, I aimed to identify neural circuits exhibiting elevated neuronal activity during the production of nest-building behaviour. To do this, in the work described in Chapter 2 I sampled neuronal activity, indirectly as the number of neurons producing Fos protein, in adult male and female nest-building and control zebra finches. I sampled neuronal activity in neural circuits I hypothesised may be involved in nest building and tested whether neuronal activity in these regions differed between nest-building and control birds. Furthermore, I used stepwise linear regressions to test whether or not any single behaviour explained individual variation in neuronal activity in nest-building finches.

Following the identification of brain regions associated with nest-building behaviour, in the work described in Chapter 3 I sampled neuronal activity in some of these regions again, however, this time I focused on sampling Fos immunoreactivity in neuronal subpopulations located within these brain regions. Specifically, I compared neuronal

activity in mesotocinergic and vasotocinergic neuronal subpopulations in the social behaviour network and dopaminergic neuronal subpopulations in the dopaminergic reward system between nest-building and control birds. Again, I also tested whether any nest-building behaviours explained individual variation in neuronal activity in any of these neuronal subpopulations.

In Chapter 4, I describe my nest classification scheme for species-typical nest structure and how I used this classification system to test whether cerebellar foliation is related to variation in species-typical nest structure, which would suggest that foliation correlates with species differences in manipulative skill with the beak. To do this, I used phylogenetically-informed statistical techniques to compare the degree of cerebellar foliation between species building nests of different structural complexity.

Finally, in Chapter 5 I used my nest structure classification scheme to test the evolutionary hypothesis underlying the evolution of domed nests in Old World babblers as originally proposed by Collias (1997). Specifically, I looked for differences in nest height between cup- and domed-nesting babblers and identified the most likely ancestral state of nest height and structure in Timaliidae and the likely order of transitions in nest height and structure leading the diversity in nest height and structure observed in extant babblers.

Chapter 2: Neural correlates of nest-building behaviour in zebra finches

Introduction

As mentioned in Chapter 1, nest-building behaviour in birds consists of a sequence of actions, which in its simplest form involves the collection and deposition of nest material at the nest-site. For some species this nest-building sequence can be decomposed into just a few actions while for others the construction of nests is more elaborate. For example, arctic terns (*Sterna paradisaea*) nest in unadorned ground scrapes whereas long-tailed tits (*Aegithalos caudatus*) sequence up to 14 motor actions to build a domed nest comprised of moss and spider egg cocoons (Thorpe, 1956). Superficially at least, nest building appears to involve motor actions and sequencing akin to those used in tool manufacture and use (Hansell, 2000; Walsh et al., 2010; 2011; 2013) but to date there is little information regarding the neurobiology of these behaviours in birds.

In this study, I sought to investigate the neural substrates involved in nest-building behaviour in zebra finches. Zebra finches readily build nests in the laboratory (Muth and Healy, 2011; 2012; 2013) using an easily quantified motor sequence of nest material collection and deposition. While the male zebra finch collects and deposits nest material, the female remains within the nest cup and manipulates material to shape a species-typical dome nest (Zann, 1996). As mentioned in Chapter 1, one of the most common ways to implicate brain regions involved in the behaviour of interest is to determine which brain regions are activated whenever this behaviour is performed. As described in Chapter 1, I quantified immunoreactivity for the immediate early gene c-*fos* protein product Fos (Meddle and Follett, 1997, Clayton, 2000) throughout multiple neural circuits that I

predicted may be involved in nest-building behaviour in male and female zebra finches. I did this using birds that did or did not build a nest.

I first quantified Fos immunoreactivity in the anterior motor pathway, which is thought to control motor learning and sequencing (Feenders et al., 2008) and includes the striatum, the input structure of the basal ganglia. The basal ganglia control motor planning and sequencing, are found in all vertebrates (Kuenzel et al., 2011), and are activated during trained tool use in macaque monkeys (Obayashi et al., 2001). By sampling Fos immunroeactivity in the anterior motor pathway, I could test the hypothesis that nest building involves motor sequencing: Fos immunoreactivity in the anterior motor pathway should correlate with the amount of nest-building behaviour exhibited by male zebra finches. I also predicted that Fos immunoreactivity would not differ between nest-building and control birds (birds that were not allowed to build nests) in the posterior motor pathway, a circuit that is involved in the production of motor actions (Feenders et al., 2008; Chapter 1), as both nest-building and control birds could move freely.

In addition to sampling Fos immunoreactivity in these motor pathways, I also quantified Fos immunoreactivity in the social behaviour network, a neural circuit involved in avian courtship and parental behaviour (e.g. Goodson, 2005; Chapter 1). Because nest box possession in male European starlings increases Fos immunoreactivity in several regions in the social behaviour network (Heimovics and Riters, 2006), Fos immunoreactivity specifically in these social behaviour network regions should be greater as a result of nest box possession (the dorsal and ventral subdivisions of the medial bed nucleus of the stria terminalis [BSTmd and BSTmv, respectively], anterior hypothalamus, medial preoptic area, and ventromedial hypothalamus) in nest-building zebra finches than

it is in control birds. Although Heimovics and Riters (2006) noted that starlings that possessed a nest box also built nests, they did not quantify nest-building behaviour and so were unable to test whether Fos immunoreactivity in the social behaviour network was specifically related to nest-building behaviour. By quantifying nest-building behaviour, I could determine whether Fos immunoreactivity in these regions during nest building is associated with nest possession or nest building itself.

Complementary to the social behaviour network, I also quantified Fos immunoreactivity in the dopaminergic reward system, which is involved in reward and motivation of social behaviours including courtship (O'Connell and Hofmann, 2011; Chapter 1). If nest-building behaviour is rewarding, Fos immunoreactivity in this reward pathway should correlate with nest-building behaviour. Furthermore, this correlation should be most conspicuous specifically in the ventral tegmental area and central gray, two regions in the dopaminergic reward system which exhibit elevated neuronal activity following nest box possession in starlings (Heimovics and Riters, 2005; 2007).

Finally, as described in Chapter 1, the avian hippocampus is involved in spatial learning memory and in synthesising multimodal cues to promote context-specific behaviour. If the hippocampus is involved in initiating nest building after zebra finches recognise a reproductive context (Sherry and Hoshooley, 2009; Székely and Krebs, 1996), Fos immunoreactivity in the hippocampus should be elevated in nest-building finches compared to controls.

Methods and materials

Animals

Thirty-two adult zebra finches (n = 16 male, n = 16 female) were bred in captivity at the University of St. Andrews, St. Andrews, Scotland, UK and the University of Glasgow, Glasgow, Scotland, UK. Prior to experimentation, I housed birds in single-sex groups in cages containing 10 to 20 birds with access to finch seed mix and water *ad libitum* but deprived of access to coconut fibre. The room was held on 14L:10D light:dark light cycle (lights on 8:00) with temperatures ranging between 19-27°C and 50-70% humidity. All procedures were performed with ethical permission from the University of St. Andrews Animal Welfare and Ethics Committee and from the UK Home Office (PPL. 60/3666).

Treatment group assignment

I caught zebra finches from group cages, randomly paired birds (one bird of each sex) in wooden/wire mesh cages (44 x 30 x 39 cm), and then moved pairs to a separate room with the same light cycle, temperature, and humidity as the group-housing room. I fitted cages with a wooden nest cup (11 x 13 x 12 cm) and covered the floor with bedding chips. The birds had access to finch seed mix and water *ad libitum*. I paired birds for at least one week before providing them with coconut fibre as nest material. Prior to receiving this nest material, all pairs filled their nest cups with bedding chips at least once and some females laid eggs in these bedding chip nests. I removed all bedding and eggs from nest cups during daily inspection.

At least one week after pairing, at 12:00 (4 hours after lights on) I gave six pairs of birds 7.5 g of coconut fibre each and I inspected cages 24 hours later to identify pairs that

had begun to build in their nest cup. To create an experimental cohort, I randomly assigned a pair of finches that had begun building a fibre nest to each behavioural treatment group (nest-building or control group). I selected only pairs of birds that had begun building a nest to ensure that all of the finches included in this study, both nest-building and control pairs, were motivated and capable of building nests prior to behavioural observation. I removed coconut fibre nests and remaining fibre from the cages of both pairs and also removed the nest cup from the cage of the control pair. I removed the cage bedding chips and lined the cage floor with black plastic to prevent unwanted nest building with bedding. I moved the two pairs of the experimental cohort to a test room where both pairs were visually but not acoustically isolated from each other by a wooden barrier.

Isolation of nest-building behaviour

On the next morning, 1 hour after lights on, I provided the nest-building finch pair with 12 g of coconut fibre and monitored them throughout the day for evidence of nest building. If the nest-building pair began building a nest on the day they received nest material, I scheduled the behavioural observation period for the following morning. If the nest-building pair failed to construct a nest on the first day I provided the material, I replaced the 12 g of coconut fibre the next morning and monitored the nest-building male for the remainder of the day. If a nest-building male failed to deposit any material in the nest cup within two days of material provision, the nest cup and material were removed and a new nest cup and 12 g of coconut fibre were given to the control pair, reversing the treatment assignment of each pair in the cohort. Reversal of treatment conditions occurred

twice and in one case, neither male constructed a nest while in the isolation room. These birds were removed from the study and replaced by a subsequent cohort.

When the lights came on the morning after a nest-building pair began nest building in the test room, I removed unused nest material from this pair's cage but left the nest they had begun building. Both the nest-building and control pairs were left for 30 minutes before I began filming. After 30 minutes, I gave the nest-building pair 9 g of coconut fibre so that the male could resume nest building and I filmed each pair using either a JVC Everio ACVHD (Model no. GZ-HD300AU) or Sony Handycam AVCHD (Model no. HDR-CX115E) camcorder. Nest-building males did not typically resume building immediately so I observed the birds from outside the isolation room via a window until I observed the nest-building male make three consecutive trips with material from the cage floor to the nest, which I considered the initiation of nest building. I recorded the time at which the male began to build.

Behaviour coding

I encoded the birds' behaviour using Noldus Observer (TrackSys Ltd., Nottingham, U.K.) behavioural analysis software. I measured the occurrence of five behaviours that were performed by both nest-building and control finches: *hopping* (a jump between perches, the cage floor, and/or the nest cup), *feeding* (pecks into the ground or cage-mounted feeder), *drinking* (pecks into the cage-mounted water dispenser), *preening* (each preen of the chest, wing, or tail feathers by the beak), and *scratching* (scratch head feathers with foot). In all females, I also recorded *allopreening* (female preens her partner male with her beak). In all males, I assessed singing behaviour in two ways: *song bouts* (number

of song bouts separated by at least 3 seconds) and *time spent singing* (number of seconds a bird spent singing). I measured two nest-building behaviours only in nest-building males: *pick up* (male picked up coconut fibre from the floor of the cage using his beak) and *put down* (male released coconut fibre into the nest cup). In both nest-building males and females, I counted the number of *nest visits* (bird entered the nest cup) and *nest time* (number of seconds the bird spent in nest cup).

Tissue collection

After 90 minutes following the initiation of nest building, I entered the room to confirm visually that material on the floor of the cage was added to the nest. Once confirmed, I sacrificed both the control and nest-building pairs by terminally anaesthetising (0.2 ml Pentobarbitone sodium i.p.; Dolethal, Vétoquinol) birds and then rapidly dissected brains from the skulls. I fixed brains via submersion in 4% paraformaldehyde in phosphate-buffered saline (0.1M, pH = 7.4) for six days and then cryoprotected brains in 20% sucrose in phosphate-buffered saline for 48 hours. I embedded brains embedded in cubes of quail egg yolk, which was subsequently fixed with 4% paraformaldehyde over six days. I sectioned the embedded brains coronally (section thickness = 30 μm) using a freezing microtome and collected sections in three, alternating series (intersection interval = 90 μm) into phosphate-buffered saline.

I repeated all of these procedures until I had observed behaviour of, and collected brains from, eight nest-building pairs and eight control zebra finch pairs. Note: although I will refer to ‘nest-building pairs’ it is the male that is the builder of the nest. The female

may bring material at the end of the process in order to line the nest (Zann, 1996) but the birds in this experiment did not reach that point of nest construction.

Fos immunohistochemistry

I rinsed sections three times in phosphate-buffered saline before incubating them in 0.5% H_2O_2 in phosphate-buffered saline for 30 minutes at room temperature to reduce endogenous peroxidase activity. Following another three phosphate-buffered saline rinses, I incubated sections in 10% Normal Goat Serum (Vector Laboratories) in 0.3% Triton X-100 (Sigma) in 0.1M phosphate-buffered saline (0.3% PBT) for 60 minutes at room temperature. I then removed sections from the blocking serum into the primary Fos antibody (rabbit-anti-Fos antibody diluted 1:1000 in 0.3% PBT, Santa Cruz Biotechnology K-25) and incubated for 21 hours at room temperature. This antibody has previously been validated for use in the zebra finch (see Nordeen et al., 2009). The following day, I rinsed sections three times in 0.1% PBT and incubated sections in biotinylated goat anti-rabbit secondary antibody (diluted 1:250 in 0.3% PBT; Vector Laboratories) for 1 hour at room temperature. After three rinses in 0.1% PBT, I incubated sections at room temperature in ABC Elite avidin-biotin horseradish-peroxidase complex (Vector Laboratories) for 1 hour. Following three rinses in 0.1% PBT I visualised the antibody-avidin-biotin complexes with 0.04% diaminobenzidene solution (Sigma Fast DAB) for 90 seconds and then rinsed sections 4 times with phosphate-buffered saline. I then serially mounted tissue sections on to Polysine microscope slides (VWR), serially dehydrated tissue through alcohol (50 to 100%), cleared tissue in xylene, and cover-slipped slides with DePeX (VWR). I found no immunoreactivity when I omitted the primary Fos antibody.

Quantification of Fos immunoreactivity

In all brain regions, I quantified Fos immunoreactivity by sampling the number of neurons in a given brain region immunoreactive for Fos protein. In males, I quantified the number of nuclei immunoreactive for Fos in HVC (used as a proper name) and the robust nucleus of arcopallium (RA) in the song-control system. I also quantified Fos immunoreactivity in the lateral intermediate arcopallium and dorsal lateral nidopallium of the posterior motor pathway and anterior ventral mesopallium, anterior nidopallium, and anterior striatum of the anterior motor pathway as identified in Feenders et al. (2008). In the social behaviour network, I quantified Fos immunoreactivity in brain regions previously reported to increase immediate early gene expression with nest box possession in starlings: BSTmd, BSTmv, anterior hypothalamus, medial preoptic area, and ventromedial hypothalamus (Heimovics and Riters, 2006; 2007). I also quantified Fos immunoreactivity in the social behaviour network in one other division of the bed nucleus of the stria terminalis (lateral subdivision [BSTl]), four divisions of the septum (ventral caudal subdivision [LScv], lateral ventral caudal subdivision [LScvl], rostral subdivision [LSr], and medial septum), and nucleus taeniae as identified by Goodson (2005) and Heimovics and Riters (2006). Because BSTmd and BSTmv have been found to both increase Fos immunoreactivity with nest box possession but the level of Fos immunoreactivity is differentially influenced by breeding condition in each subdivision (Heimovics and Riters, 2006), I opted to sample these subdivisions separately, unlike a recent study testing for a role of vasotocinergic neuronal subpopulations in BSTm (both BSTmd and BSTmv together) in nest building (Klatt and Goodson, 2013). In the dopaminergic reward system, I

quantified Fos immunoreactivity in the ventral tegmental area and central gray. I quantified Fos immunoreactivity in two regions of the hippocampus (dorsal hippocampus and medial hippocampus). All sampled brain regions are summarised in Figure 2.1.

I located areas of interest in brains using full section architecture and regional anatomy with reference to brain atlases of the canary (Stokes et al., 1974) and zebra finch (Nixdorf-Bergweiler and Bischof, 2007). At each area of interest, I inspected adjacent coronal sections to locate the midpoint of the region in the rostrocaudal axis (Figure 2.1). I took images of each region in both hemispheres and across 3 consecutive coronal sections centred on the rostrocaudal midpoint of the region (intersection interval = 90 μm). For brain regions that are larger in the rostrocaudal plane (anterior striatum and dorsal and medial hippocampus), I took images across 5 evenly-spaced coronal sections centred on the rostrocaudal midpoint of the region with an intersection interval of 270 μm. I captured all images using a Nikon Coolpix E4500 digital camera mounted on a Leitz Diaplan microscope using a 40x objective lens and Leitz Wetzlar 307-148.001 light source.

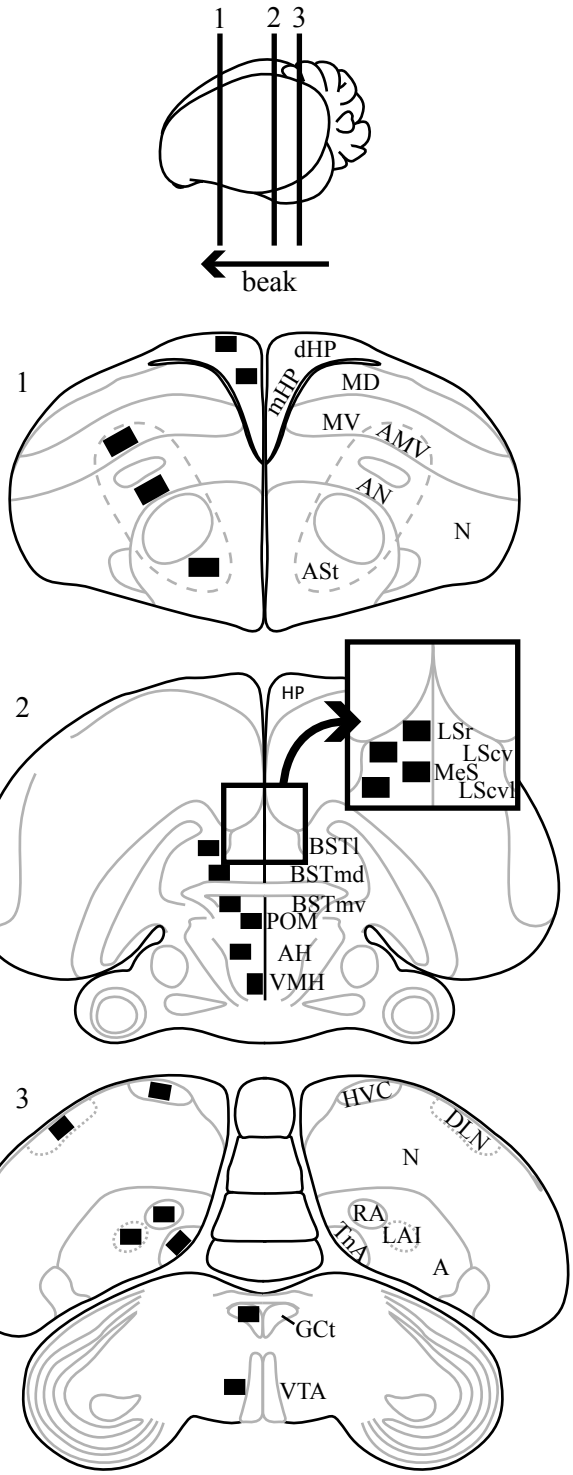
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Figure 2.1. Brain regions quantified for Fos immunoreactivity in the zebra finch brain. Drawing of three coronal brain sections (1-3) and their locations along the sagittal plane (top diagram) depicting all brain regions quantified bilaterally for Fos immunoreactivity in this study. Black squares on the left hemisphere represent sampling squares taken at 40x objective magnification and brain region acronyms are located in the relative position of the sampling square in the right hemisphere. AH = anterior hypothalamus; ASt = anterior striatum; AMV = anterior ventral mesopallium; AN = anterior nidopallium; BSTl = bed nucleus of the stria terminalis, lateral subdivision; BSTmd = medial bed nucleus of the stria terminalis, dorsal subdivision; BSTmv = medial bed nucleus of the stria terminalis, ventral subdivision; dHP = dorsal hippocampus; DLN = dorsolateral nidopallium; GCt = central gray; LAI = lateral intermediate arcopallium; LScv = lateral septum, ventral caudal subdivision; LScvl = lateral septum, lateral ventral caudal subdivision; LSr = lateral septum, rostral subdivision; mHP = medial hippocampus; MS = medial septum; POM = medial preoptic area; RA = robust nucleus of the arcopallium; TnA = nucleus taeniae; VMH = ventromedial hypothalamus; VTA = ventral tegmental area.

During quantification of Fos immunoreactivity, I opened each image in ImageJ software (version 1.45, NIH, Bethesda, MD, USA) and desaturated the image. To isolate Fos nuclei from background staining, I used the *auto levels* function in ImageJ, which saturates a lack of Fos immunoreactivity as white and saturates Fos immunoreactivity as black. Before applying the function to each image, I subtracted 40 units from the *auto levels* adjustment value. This subtraction was necessary because the *auto levels* adjustment value selected by ImageJ saturated both neurons and much of the background, neuropil Fos

immunoreactivity, making neurons indistinguishable from background levels of staining. By subtracting 40 units from the *auto levels* adjustment value, I was able to saturate the more intense Fos immunoreactivity specific to neurons, without saturating the lighter, background neuropil. An experimenter blind to bird treatment confirmed that this subtraction reliably highlighted darkly-stained Fos immunoreactive nuclei from background staining in a set of randomly selected images from multiple birds and brain regions. In the anterior motor pathway regions, I subtracted only 30 units from the *auto levels* value as the same experimenter (blind to bird treatment) found that neuropil staining was notably lighter and better excluded using this modified *levels* manipulation. After applying the *levels* function, I counted the number of highlighted Fos immunoreactive nuclei using the *analyze particles* function in ImageJ. I only counted nuclei if they had a minimum area of 400 pixels^2. An experimenter blind to bird treatment selected this value by measuring the area of the smallest Fos immunoreactive nuclei identified in multiple, randomly-selected regions across birds and brain regions. I summed the number of Fos immunoreactive nuclei in each hemisphere and section to yield a single value of Fos immunoreactivity for each brain region in each bird. I used these total Fos immunoreactive nuclei counts for each brain region in statistical analysis except for HVC because lateralisation in activation in the right hemisphere has been previously reported during short-distance communication with a sexual partner in zebra finches (George et al., 2006). Accordingly, I analysed Fos immunoreactivity in HVC in the left and right hemispheres separately.

Statistical analysis

During the behavioural analysis, I identified one pair of nest-building finches as outliers because the male picked up only small amounts of nest material (<2 SD below the mean for the rest of nest-building males) and the female never interacted with the nest material within the nest cup. As a result I excluded this pair from further statistical analysis.

I performed all statistical analyses using PASW software (version 19.00, SPSS Inc., Chicago, IL, USA). I quantified finch behaviour 80-50 minutes prior to the time at which finches were sacrificed. The delay between this period of behaviour and sacrifice provides sufficient time for the accumulation of Fos protein following neural activation associated with nest-building behaviour (Morgan and Curran, 1991; Chapter 1). All behaviour and Fos data were normally distributed ($p > 0.05$; Shapiro-Wilkes). I compared behaviour and Fos immunoreactivity as dependent variables using GLMs and the independent variables included sex on two levels (male and female) and treatment on two levels (nest-building and control). Because I used these group comparisons to identify differences in Fos immunoreactivity that would be associated with having a nest or not, such as visual perception of the nest, and not Fos immunoreactivity that might be associated with how much nest-building behaviour individual birds exhibited, I treated male and female birds from the same nesting pairs as independent birds. For data on Fos immunoreactivity, I looked specifically for treatment and treatment x sex interaction effects that reflected neuronal activity associated with nest building.

To investigate whether nest-building behaviours explain individual variation in Fos immunoreactivity, I regressed each brain region on all recorded behaviours in nest-building birds as independent predictors of Fos immunoreactivity using multiple linear regression. I ran regression models separately for males and females using a stepwise backwards

elimination procedure that excluded interactions between types of behaviour. Using this statistical approach, I could enter all behaviours measured into my regressional models and identify the behaviour that best predicts Fos immunoreactivity in each brain region compared to all other nest-building and non-nest-building behaviours measured. By using this approach, I can avoid presenting relationships between Fos immunoreactivity and nest-building behaviour that may actually be attributed to concurrent non-nest-building behaviours measured, such as hopping to and from the nest cup. In the song control nuclei (HVC and RA), I entered only singing behaviour (*song bouts* and *time spent singing*) as predictors of Fos immunoreactive nuclei counts in all males (nest-building and control) firstly to test for song-brain correlations as previously reported (Kimpo and Doupe, 1997) and secondly to test whether a relationship between Fos immunoreactivity and birds' behaviour 80-50 minutes prior to sacrifice existed.

Results

Regressional models in which nest-building behaviour significantly explained variation in Fos immunoreactivity in a brain region are summarised in Table 2.1 and Appendix 1.

Table 2.1. Relationships between behaviour and Fos immunoreactivity in brain regions of nest-building adult zebra finches. Correlates were calculated using stepwise linear regression to identify behaviours performed by nest-building zebra finches 80-50 minutes before sacrifice that predicted Fos immunoreactivity in sampled brain regions. When regression models identified more than one behaviour that predicted Fos

immunoreactivity in a single brain region, each behaviour in the model is listed in the order of greatest predictive power. Nest-building behaviours are represented in bold.

Brain Region	Acronym	Sex	Correlated Behaviour(s)	ß	t	*p*
Motor Pathways						
Anterior striatum	ASt	Male	**pick up**	0.808	3.070	0.028
Anterior nidopallium	AN	Male	**pick up**	0.801	6.451	0.003
Anterior nidopallium	AN	Male	time spent singing	0.459	3.696	0.021
Anterior ventral mesopallium	AMV	Male	**pick up**	0.807	3.061	0.028
Social Behaviour Network						
Anterior hypothalamus	AH	Female	**time in nest**	-0.771	-2.711	0.042
Bed nucleus of the stria terminalis, ventromedial subdivision	BSTmv	Female	**time in nest**	1.043	5.399	0.006
Bed nucleus of the stria terminalis, ventromedial subdivision	BSTmv	Female	preening	0.595	3.079	0.037
Medial septum	MS	Male	**put down**	-0.795	-2.928	0.033
Dopaminergic Reward Circuit						
Ventral tegmental area	VTA	Male	**pick up**	0.789	2.870	0.035

Behavioural analyses

Between 80-50 minutes prior to sacrifice, control birds hopped ($F_{1,26} = 22.623$, $p < 0.001$), fed ($F_{1,26} = 9.617$, $p = 0.005$), drank ($F_{1,26} = 7.296$, $p = 0.012$) and preened ($F_{1,26} = 6.049$, $p = 0.021$) more than did nest-building birds. Males scratched more often than did females ($F_{1,26} = 20.362$, $p < 0.001$).

Control females tended to allopreen more than did nest-building females ($t_{13} = 1.991$, $p = 0.087$). Nest-building and control males did not differ significantly in the time they spent singing ($p > 0.05$). In nest-building pairs, males visited the nest cup more often than did females ($t_{12} = 6.128$, $p < 0.001$) but did not spend more time in the nest cup ($p = 0.091$).

Song control system

Time spent singing positively correlated with Fos immunoreactivity in the right HVC (Figure 2.2B; ß = 0.564, $t_{13} = 2.464$, $p = 0.028$) but did not significantly explain variation in Fos immunoreactivity in the left hemisphere HVC in all males. Neither the number of song bouts nor time spent singing significantly explained variation in Fos immunoreactivity in RA in males.

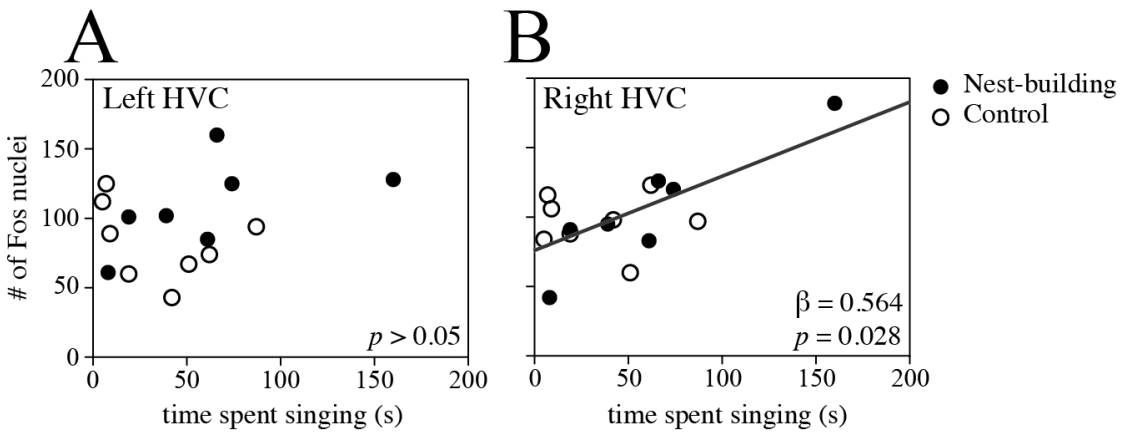


Figure 2.2. Correlations between singing behaviour in adult male zebra finches and Fos immunoreactivity in left (A) and right (B) HVCs. Correlation between the time spent singing (s) 80-50 minutes prior to sacrifice and the number of cells immunoreactive for Fos sampled in the left (A) and right (B) HVC in adult male zebra finches that were either nest building (black circles) or not (white circles). Within each graph, the regression coefficient and *p* value of the model are presented in the bottom right corner. *n* = 15 male finches.

Motor pathways

Fos immunoreactivity in the anterior striatum increased the more males picked up pieces of nest material (Figure 2.3; ß = 0.808; $t_5 = 3.070$; $p = 0.028$). Fos immunoreactivity in the anterior nidopallium increased the more males picked up material (Figure 2.3; ß = 0.801; $t_4 = 6.451$; $p = 0.003$) and the more males spent time singing (ß = 0.459; $t_4 = 3.696$; $p = 0.021$). Fos immunoreactivity in anterior ventral mesopallium increased the more

males picked up material (Figure 2.3; ß = 0.807; $t_5 = 3.061$; $p = 0.028$). None of the behaviours that I measured significantly explained individual variation in Fos immunoreactivity in either of the areas I quantified from the posterior motor pathway, the lateral intermediate arcopallium and dorsal lateral nidopallium.

In nest-building females, neither the number of visits to the nest nor the time spent in the nest significantly explained the variation in Fos immunoreactivity in either the anterior or posterior motor pathway.

I also found no significant difference in Fos immunoreactivity between nest-building and control birds in either the anterior or posterior motor pathway ($p > 0.05$).

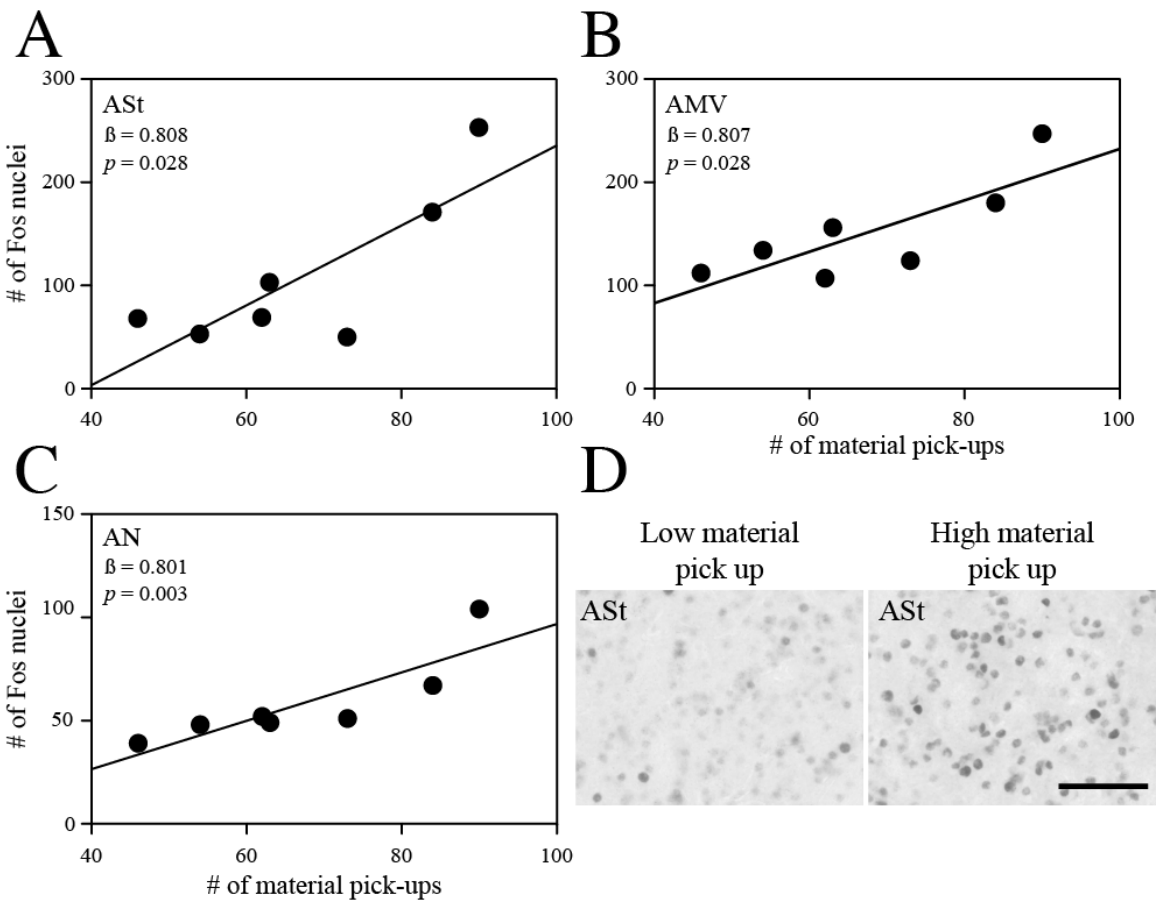


Figure 2.3. Correlations between nest-building behaviours and Fos immunoreactivity in the anterior motor pathway in zebra finches. Correlations between the picking up of nest material and the number of Fos immunoreactive nuclei quantified in the (A) anterior striatum [ASt], (B) anterior ventral mesopallium [AMV], and (C) anterior nidopallium [AN] of the anterior motor pathway in adult male zebra finches. Correlations were derived from stepwise linear regressions. Within each graph, the regression coefficient and *p* value of the model are presented in the top left corner. (D) Micrographs of sampling squares taken in tissue stained to label neurons immunoreactive for Fos in ASt in the right hemisphere of a male finch who picked up the least and a male finch who picked up the most number of times while building a nest. Scale bar represents 50 µm.

Social behaviour network

Fos immunoreactivity in the medial septum decreased the more pieces of material males deposited in the nest cup (ß = -0.795; t_5 = -2.928; p = 0.033). Fos immunoreactivity increased in LScv and decreased in the ventromedial hypothalamus the more time nest-building males spent singing (LScv: ß = 0.928; t_5 = 5.555; p = 0.003; ventromedial hypothalamus: ß = -0.792; t_5 = -2.899; p = 0.034). Fos immunoreactivity in LSr decreased the more nest-building males hopped (ß = -0.778; t_5 = -2.771; p = 0.039) and neither picking up nor depositing nest material significantly explained variation in Fos immunoreactivity in any of the other social behaviour network regions that I quantified.

Fos immunoreactivity in the anterior hypothalamus decreased the more time nest-building females spent in the nest (ß = -0.771; t_5 = -2.711; p = 0.042). Fos immunoreactivity in BSTmv, however, increased the more time these females spent in the nest (Figure 2.4; ß = 1.043; t_4 = 5.399; p = 0.006) and the more time they spent preening (ß = 0.595; t_4 = 3.079; p = 0.037). Fos immunoreactivity in the ventromedial hypothalamus decreased the more nest-building females preened (ß = -0.861; t_5 = -3.790; p = 0.013). Neither the number of times these females visited the nest nor the time these females spent in the nest significantly explained variation in Fos immunoreactivity in any other social behaviour network regions sampled.

Fos immunoreactivity in BSTmd ($F_{1,23}$ = 4.720, p = 0.040) and medial preoptic area ($F_{1,25}$ = 8.095, p = 0.009) was significantly greater in nest-building birds relative to control birds. There was no significant difference in Fos immunoreactivity between nest-building and control birds in any other region sampled (p > 0.05).

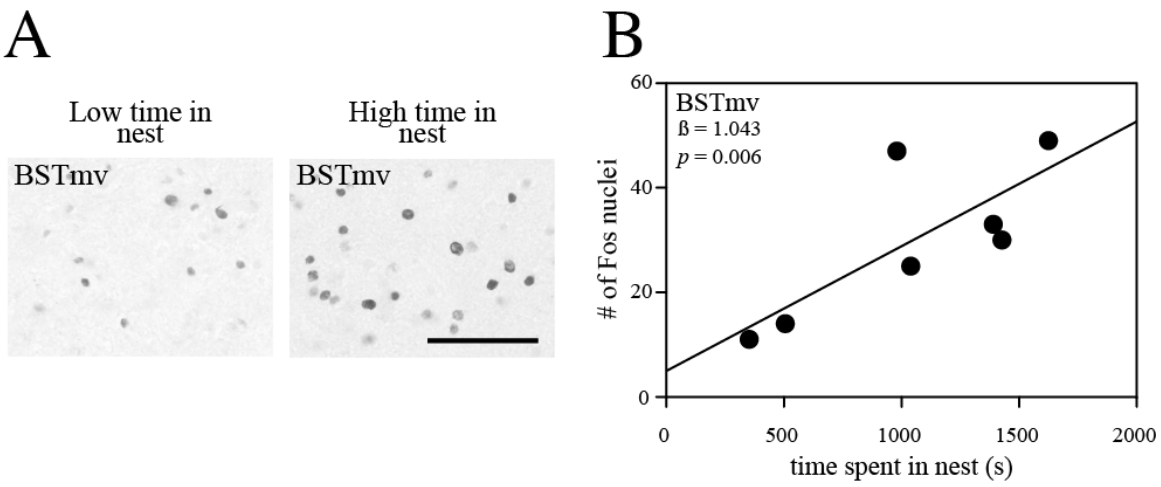


Figure 2.4. Correlations between nest-building behaviours and Fos immunoreactivity in the social behaviour network. (A) Micrographs of sampling squares taken in tissue stained to label neurons immunoreactive for Fos in the medial bed nucleus of the stria terminalis, ventral division (BSTmv) in the right hemisphere of a female finch who spent the most time in her nest and a female finch who spent the least amount of time in her nest. Scale bar represents 50 µm. (B) Correlation between the time a female zebra finch spent in the nest cup and the number of Fos immunoreactive nuclei in BSTmv. Correlation was derived from stepwise linear regressions. Within the graph, the regression coefficient for the behaviour and model *p* value are presented.

Dopaminergic reward system

Fos immunoreactivity in the ventral tegmental area increased the more nest-building males picked up pieces of nest material (Figure 2.5; ß = 0.789; $t_5 = 2.870$; $p = 0.035$).

Conversely, variation in nest-building behaviour did not significantly explain variation in Fos immunoreactivity in the central gray.

In nest-building female finches, neither the number of nest visits nor the time spent in the nest significantly explained variation in Fos immunoreactivity in the ventral tegmental area or central gray.

Fos immunoreactivity in the ventral tegmental area and central gray did not differ between nest-building and control birds ($p > 0.05$).

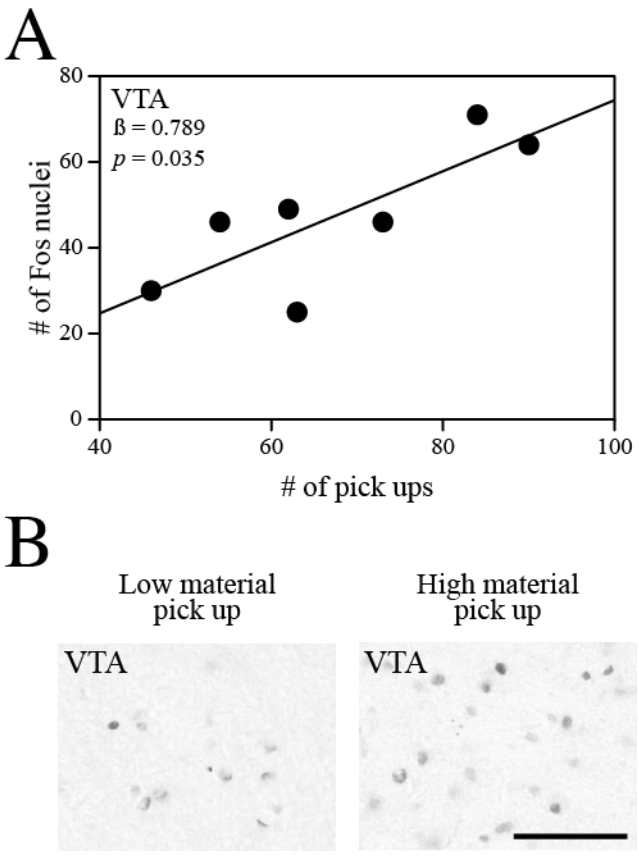


Figure 2.5. Correlations between nest-building behaviours and Fos immunoreactivity in the dopaminergic reward system. (A) Correlation between the picking up of nest

material and the number of Fos immunoreactive nuclei quantified in the ventral tegmental area (VTA) in adult male zebra finches. This correlation was derived from stepwise linear regressions. Within the graph, the regression coefficient for the behaviour and model *p* value are presented. (B) Micrographs of sampling squares taken in tissue stained to label neurons immunoreactive for Fos in the ventral tegmental area in the right hemisphere of a male finch who picked up the most and a male finch who picked up the least amount of nest material while constructing a nest. Scale bar represents 50 μm.

Hippocampus

None of the behaviours that I measured significantly explained individual variation in Fos immunoreactivity in dorsal and medial hippocampus. I also found no significant differences in Fos immunoreactivity in the dorsal and medial hippocampus between nest-building and control birds ($p > 0.05$).

Discussion

In this study I used immediate early gene immunohistochemistry to identify regions of the songbird brain that produce Fos protein during nest building. Based on the assumption that Fos production reflects neuronal activation (Clayton, 2000), these data show Fos immunoreactivity associated with nest-building behaviour (the number of times nest material was picked up by nest-building males or with the time spent in the nest cup by nest-building females) within the anterior motor pathway, social behaviour network, and dopaminergic reward system. To my knowledge, this is the first demonstration of neural

correlates of nest-building behaviour in the anterior motor pathway and dopaminergic reward system.

Prior to discussing my results, an important caveat to address is that this study used a restrictive sample size to test for a relationship between neuronal activity in the brain and behaviour. Because of this small sample size, it is difficult to interpret non-significant results as a demonstration that a specific brain region is not involved in the nest-building behaviours tested here. Compounded with the imprecision of Fos immunohistochemistry as a technique for inferring neuronal activity on a finer timescale (Chapter 1), non-significant results presented in this study and the following chapter (Chapter 3) should not be used to as evidence to preclude a relationship between a given brain region and nest-building behaviour.

Motor pathways

Variation in Fos immunoreactivity throughout the anterior, but not the posterior, motor pathway was explained by the number of times a male finch picked up nest material. Given the involvement of the anterior motor pathway in motor learning and sequencing (Feenders et al., 2008), activation of the anterior motor pathway, and the anterior striatum in particular, during nest building suggests that nest-building behaviour may involve similar motor sequencing and control as has been ascribed to tool use behaviour (which activates the basal ganglia in primates: Obayashi et al., 2001). Fos immunoreactivity in the anterior motor pathway was, however, specifically related to initiation of the sequence of nest-building behaviour (picking up material) but not to the final step in the behavioural sequence that I quantified (depositing material in the nest). This suggests that the anterior

nidopallium in the zebra finch brain (as identified by Feenders et al., 2008) is functionally similar to nidopallium intermedium medialis pars laterale (as identified by Helduser and Güntürkün, 2012), a region in the pigeon brain found in the same location as the anterior nidopallium in zebra finches, which plays a role in executing learned motor sequences.

The number of visits the females partnered to nest-building males made to the nest and time they spent in the nest cup, however, were unrelated to Fos immunoreactivity in the anterior motor pathway. This sex difference suggests that, during nest building, the anterior motor pathway is specifically involved in the collection of nest material and not construction within the nest cup, in which both male and female zebra finches participate (Zann, 1996). The measures of nest-building behaviour in female finches used here, however, were restricted to nest visitation and the time females spend in the nest and may not reflect the degree to which they carry out any construction behaviour while in the nest. Collection of construction behaviour data within the nest by both birds is required to specifically address whether the anterior motor pathway might be involved in female nest-building behaviour.

Social behaviour network

Fos immunoreactivity in the medial preoptic area and BSTmd of nest-building finches was significantly higher compared to control birds. In conjunction with previous reports of increased Fos immunoreactivity in the medial preoptic area and BSTmd during nest box possession in adult male starlings (Heimovics and Riters, 2006), my failure to find correlations between Fos immunoreactivity in the medial preoptic area and BSTmd and nest-building behaviour suggest that this activity is associated with nest possession and not

with nest building itself. It is important to note that there is no obvious control condition to match with a pair of nest-building birds. For example, our control pair were unable to build a nest, but also could not perceive nest material or the nest cup. For this reason, it is possible that group differences in Fos immunoreactivity between nest-building and control birds may reflect group differences not directly associated with nest building but other environmental and behavioural differences between our treatment groups. For this reason, I focus predominantly on my correlational results, which demonstrate a relationship between Fos immunoreactivity and production of a specific, nest-building behaviour.

Although the groups did not differ in Fos immunoreactivity in BSTmv, within nest-building females, Fos immunoreactivity in this region was greater the longer the female spent in the nest. Elevation of Fos immunoreactivity in BSTmv following nest box possession has been attributed to concurrent changes in agonistic behaviour associated with territorial defence of the nest (Heimovics and Riters, 2006). My results in female finches, however, suggest that such changes may be associated with occupation of the nest, a behaviour that is only possible after a nest site has been obtained. Similar to Heimovics and Riters (2006), I found that immediate early gene expression was higher in both BSTmd and BSTmv the more nest-building behaviours birds performed but the specific expression pattern in each subdivision of BSTm differed. These differences in expression patterns dependent on the subdivision of BSTm sampled may explain why there appeared to be no relationship in between nest-building behaviour and activation of vasotocinergic neurons in BSTm in a previous study (Klatt and Goodson, 2013).

As mentioned in Chapter 1, after demonstrating the involvement of BSTmd and BSTmv in nest-building behaviour, I sought to test whether the relationship between

neuronal activity in the social behaviour network and nest-building behaviour existed in specific neuronal subpopulations located in the social behaviour network. Although Klatt and Goodson (2013) have already tested for a relationship between neuronal activity in vasotocinergic and mesotocinergic neuronal subpopulations in the social behaviour network and nest-building behaviour in zebra finches, this study failed to recognise the potential functional division between BSTmd and BSTmv (see above and Chapter 1). In order to test for the potential involvement of vasotocinergic and mesotocinergic neurons in nest-building behaviour while recognising the functional division of BSTm, in Chapter 3 I sampled Fos immunoreactivity within vasotocinergic and mesotocinergic neuronal subpopulations in BSTmd and BSTmv separately and compare these levels of neuronal activity to nest-building behaviour.

Dopaminergic reward system

The more males picked up pieces of nest material the greater the Fos immunoreactivity in the ventral tegmental. As with the increase in Fos immunoreactivity I observed in the BSTmd, it appears that Fos immunoreactivity in the ventral tegmental area is associated with nest building itself rather than with other behavioural changes that occur after a nest site is obtained, which are unrelated to nest building (Heimovics and Riters, 2006). Given the role that the dopaminergic reward system plays in motivating and rewarding behaviour (O'Connell and Hofmann, 2011), I propose that the ventral tegmental area may be involved in rewarding material collection behaviour in male nest-building finches. In Chapter 1, I mentioned that dopaminergic neuronal subpopulations are thought to mediate the reward and motivation functions of the entire dopaminergic reward system.

To test whether these neurons are responsible for the correlation between neuronal activity in the ventral tegmental area and nest-building behaviour, in Chapter 3 I sampled Fos immunoreactivity specifically within this neuronal subpopulation and compared this neuronal activity to nest-building behaviour.

In addition to a potential role in reward, the ventral tegmental may also influence activity in the anterior motor pathway during nest building. In vertebrates, the ventral tegmental area contains dopaminergic projection neurons and, in mammals, these neurons innervate the striatum and provide necessary dopamine to support striatal functions including motor learning and sequencing (Joel and Weiner, 2000; Hikosaka et al., 2008). The possibility that the ventral tegmental area plays a role in influencing activity of the anterior motor pathway is supported by my observation that Fos immunoreactivity was higher in both the ventral tegmental area and anterior striatum the more nest material the males picked up. Further examination of the relationship between Fos immunoreactivity in dopaminergic neuron populations in the ventral tegmental area and nest building is required to test this prediction.

Hippocampus

The absence of a correlation between variation in Fos immunoreactivity in the dorsal and medial hippocampus and nest-building behaviour in male or female finches suggests that the hippocampus does not play a substantial role in nest building, at least in zebra finches.

Singing and HVC

Finally, as has been previously reported Fos immunoreactivity was higher in the HVC the longer the males spent singing. Furthermore, the time a male spent singing explained the variation in Fos expression better than did the number of song bouts (Kimpo and Doupe, 1997; Jarvis et al., 1998).

Conclusion

Here I identified several neural circuits in which neuronal activity, as indicated by production of the immediate early gene c-*fos* protein product Fos (the anterior motor pathway, social behaviour network, and dopaminergic reward system), was correlated with the production of nest-building behaviour in nest-building male zebra finches and their mates. These are the first detailed data to show the neural underpinnings of building behaviour in birds and are, therefore, a major step in determining the role that motor planning and sequencing, and reward and motivation may play in those behaviours.

Chapter 3: A role for nonapeptides and dopamine in nest-building behaviour

Introduction

Understanding the neurobiology of sexual and parental behaviour in vertebrates has long been a focus of neuroendocrine research (e.g. O'Connell and Hoffmann, 2011). In birds, these studies often focus on the production and perception of courtship song (Riters et al., 1998; Heimovics and Riters, 2005; 2006), affiliation (Goodson et al., 2009), copulation (Balthazart and Ball, 2007), and parental care (Youngren et al., 1989). Despite this work on the neurobiology of social behaviour throughout the breeding season, few studies have elucidated the neuroendocrinological systems involved in nest-building behaviour.

The current consensus is that two evolutionarily conserved neural circuits, the social behaviour network and dopaminergic reward system, are important for sexual and parental behaviour in all vertebrate lineages (O'Connell and Hofmann, 2012). Functionally, the social behaviour network is thought to be involved in the production of courtship, sexual, affiliative, and aggressive behaviours, whereas the dopaminergic reward system is thought to be involved in the motivation to perform, and the positive feedback for performing, these social behaviours (O'Connell and Hofmann, 2011). In Chapter 2, I found that neuronal activity in the social behaviour network and dopaminergic reward system increased the more nest-building behaviour male and female zebra finches exhibited, suggesting these neural circuits may also be involved in nest-building behaviour.

Many of the brain regions in the social behaviour network and dopaminergic reward system that I identified as being associated with nest-building behaviour in Chapter 2

contain subpopulations of neurons characterised for using specific signalling molecules to transmit neuronal information to downstream target brain regions (O'Connell and Hofmann, 2012). In zebra finches, these subpopulations include vasotocinergic and mesotocinergic neuronal subpopulations in the medial bed nucleus of the stria terminalis (BSTm) of the social behaviour network, which synthesise and release the nonapeptide hormones vasotocin (the avian analog of arginine vasopressin in mammals) and mesotocin (the avian analog of oxytocin in mammals), respectively. In addition to releasing these nonapeptides, which bind to receptors in sites including the striatum, hypothalamus, and the septum of the social behaviour network (Goodson et al. 2012), these neuronal subpopulations also innervate hypothalamic and social behaviour network targets including the medial preoptic area, which exhibits elevated neuronal activity during nest building (Chapter 2; Goodson et al., 2012). In the dopaminergic reward system, dopaminergic neuron subpopulations in the ventral tegmental area and central gray use the neurotransmitter dopamine to transmit information to dopaminergic receptors in both the striatum and regions in the social behaviour network including BSTm and the septum (Balthazart and Absil, 1997; Kubikova et al., 2010; O'Connell and Hofmann, 2011).

Both the actions of vasotocin, mesotocin, and dopamine released from their respective neuronal subpopulations and neuronal activity within the subpopulations themselves are thought to mediate many of the behavioural functions associated with the social behaviour network and dopaminergic reward system during the breeding season. For example, administering a pharmacological antagonist that blocks the predominant mesotocin receptor in the brain decreased affiliative behaviours associated with pair formation in male and female zebra finches (Pedersen and Tomaszycki, 2012) and neuronal

activity in BSTm vasotocinergic neurons increased in male zebra finches after courting a female (Goodson et al., 2009), suggesting that vasotocinergic and mesotocinergic neuronal subpopulations have a central role in controlling affiliative behaviour. Neuronal activity in dopaminergic neurons in the ventral tegmental area increased the more male zebra finches (Goodson et al., 2009) and European starlings (*Sturnus vulgaris*; Heimovics and Riters, 2005) sang courtship song to female conspecifics and pharmacologically agonising or antagonising dopamine transmission increased and decreased the amount of song produced by male starlings, respectively (Schroeder and Riters, 2006), suggesting that dopaminergic neurons in the ventral tegmental area are involved in the motivation to perform courtship behaviour. Because neuronal activity in the dopaminergic neurons in the ventral tegmental area also increases following the production of reproductive and aggressive behaviour (Bharati and Goodson, 2006), this dopaminergic neuronal subpopulation is thought to serve a general function involved in the motivation to interact with conspecifics (O'Connell and Hofmann, 2011). In the central gray of male zebra finches, however, neuronal activity in dopaminergic neurons increased only after males produced vocalisations directed at conspecifics, leading Goodson et al. (2009) to hypothesise that this neuronal subpopulation is involved in the motivation to communicate vocally.

Following Chapter 2, in which I suggested that brain regions in the social behaviour network and dopaminergic reward system are involved in nest building, here I hypothesised that it may be the vasotocinergic, mesotocinergic, and dopaminergic neuronal subpopulations within these circuits specifically that are involved in nest-building behaviour. To test this hypothesis, I compared nest-building behaviour exhibited by male and female zebra finches with concurrent neuronal activity, as measured indirectly by the

number of neurons producing Fos protein (see Chapter 2), in vasotocinergic and mesotocinergic neuronal subpopulations in subdivisions of BSTm and dopaminergic neuronal subpopulations in the ventral tegmental area and central gray. Because neuronal activity in the ventral subdivision of BSTm (BSTmv) increased the more time female finches spent in the nest (Chapter 2) and systemic administration of a mesotocin receptor blocker reduced the amount of time the female mate of nest-building zebra finch males spent in the nest (Klatt and Goodson, 2013), I predicted that neuronal activity within BSTmv mesotocinergic neurons would increase the more time that the female finches spent in the nest cup. In the dorsal subdivision of BSTm (BSTmd), neuronal activity increased during nest building in both male and female zebra finches (Chapter 2) and, accordingly, I predicted that neuronal activity in vasotocinergic and mesotocinergic neurons in BSTmd would increase during nest building.

In the ventral tegmental area, neuronal activity increased the more male finches picked up nest material (Chapter 2). If picking up nest material is involves dopaminergic neurons, I predicted that neuronal activity in dopaminergic neurons within the ventral tegmental area would also increase the more often male finches picked up nest material. Finally, as wild zebra finch pairs produce “duet-like” song exclusively while in the nest (Elie et al., 2010), I predicted that Fos production in dopaminergic neurons in the central gray would positively correlate with the time a pair of finches spent together in the nest.

Methods and materials

Animals

Thirty-two adult zebra finches (n = 16 male, n = 16 female) were bred in captivity at the University of St. Andrews, St. Andrews, Scotland, UK. All birds were maintained in the same conditions as the experiment in Chapter 2 and all procedures were performed with permission from the University of St. Andrews Animal Welfare and Ethics Committee and the UK Home Office (PPL. 60/3666).

Treatment group assignment

I randomly paired zebra finches and formed experimental cohorts using the same selection procedures as in Chapter 2, however, instead of coconut fibre, in this study I gave birds 15 cm lengths of string (No. 4 Polished Cotton Twine; Rope Source, UK) with which to build their nests. I administered string as a nest material in this study instead of the coconut fibre used in Chapter 2 because string is more easily observed than coconut fibre in videotaped footage of zebra finches building in the lab and finches build more readily and faster using string compared to coconut fibre (Morgan, KV, pers. comm.). After at least a week following pairing, I gave four pairs of birds 50 pieces of string at 12:00 (4 hours after lights on). I inspected cages 24 hours later to identify pairs that had deposited string into their nest cup. As in Chapter 2, to create an experimental cohort, I randomly assigned one pair of finches in which the male had begun building a nest to each behavioural treatment group (nest-building and control group). I selected only finch pairs that had begun building a nest to ensure that all pairs included in this study were motivated and capable of building nests prior to behavioural observation. I removed the string nests and remaining, unused

string from the cages of both selected pairs and also removed the nest cup from the cage of the control pair. I removed the bedding chips from the cages of both pairs, lined the cage floors with black plastic to prevent nest building with bedding chips, and moved the two pairs to a test room, as in Chapter 2. I repeated this selection procedure until I had 8 nest-building and 8 control zebra finch pairs.

Nest building

Once in the test room, the control and nest-building pair were visually but not acoustically isolated from each other by a wooden barrier. To record out-of-nest box behaviour, I positioned a camcorder in front of each pair's cage (Sony Handycam AVCHD, Model no. HDR-CX115E) and to record in-nest box behaviour I suspended a bird-box camera inside each pair's cage (SpyCameraCCTV, Bristol, UK). I left each cohort undisturbed in the test room for 24 hours to habituate.

30 minutes after the lights came on the morning following the habituation, I gave the nest-building pair 250 pieces of string and began filming both pairs. I observed the birds from outside the test room via a window until the male of the nest-building pair made three consecutive trips with nest material from the cage floor to the nest. As in Chapter 2, I recorded these trips as the time at which the male began to build and set the sacrifice time for 90 minutes later. If the male began building immediately after receiving material, I delayed the start of the observation for 15 minutes to avoid sampling Fos production in the brain associated with the bird seeing the experimenter.

Behaviour coding

As in Chapter 2, I encoded the birds' behaviour using Noldus Observer (TrackSys Ltd., Nottingham, U.K.) behavioural analysis software and here I also measured the occurrence of behaviours performed 80-50 minutes prior to sacrifice, a time bin in which Fos production is associated with nest-building behaviour. Briefly, I measured instances of *hopping*, *feeding*, *drinking*, *preening*, *scratching*, and *allopreening* in all birds. In males, I recorded the number of *song bouts* and the *time spent singing*. In nest-building birds, I measured six nest-building behaviours: *pick up*, *put down, tuck* (when the bird picked up a piece of string and tucked the string back into the nest while in the nest cup), *nest visits*, and *nest time*. Unique to this chapter, I also measured *time together* in the nest (the duration both members of a nesting pair spent together in the nest cup [seconds]).

Tissue collection

After 90 minutes following the initiation of nest building, I entered the room to confirm visually that string was deposited in the nest cup. Once confirmed, I terminally anaesthetised (0.2 ml i.p.; Dolethal) both pairs of birds and rapidly dissected their brains from their skulls. I fixed brains via submersion in 4% paraformaldehyde in phosphate-buffered saline (0.1M; pH = 7.4) for six days and then moved the brains into in 20% sucrose in phosphate-buffered saline overnight and then in 30% sucrose in phosphate-buffered saline for another night to cryoprotect them. I removed cerebella from the rest of the brains by cutting and then froze both the cerebella and remaining brain on pulverised dry ice and stored all neural tissue at -80°C before transporting the brains on dry ice to the Roslin Institute, University of Edinburgh, Roslin, UK. I sectioned brains coronally (section thickness = 52 μm) using a freezing microtome and collected sections in four, alternating

series in cryoprotectant and stored the sections at -20°C until immunohistochemical processing.

Double-label immunohistochemistry

Three series of sections were rinsed four times in 0.2% Triton X-100 (Sigma) in 0.1M phosphate buffer (PBT) and once in 0.1M phosphate buffer before being incubated in 0.3% H_2O_2 in phosphate buffer for 15 minutes at room temperature to reduce endogenous peroxidase activity. Following three PBT rinses, sections were incubated in 10% Normal Goat Serum (Vector Laboratories) in PBT for 60 minutes at room temperature. Sections were then moved into the primary Fos antibody (Santa Cruz Biotechnology rabbit polyclonal anti-Fos K-25, sc-253, 1:10,000) in 10% Normal Goat Serum in PBT and incubated for 21 hours at 4°C. The following day, sections were rinsed three times in PBT and incubated in biotinylated goat anti-rabbit secondary antibody (diluted 1:250 in PBT; Vector Laboratories) for 1 hour at room temperature. After another three rinses in PBT, sections were then incubated in avidin-biotin horseradish-peroxidase complex (1:400; Vector Laboratories) in PBT for 1 hour at room temperature. Following four rinses in PBT, one rinse in phosphate buffer, and a brief rinse in 0.1M sodium acetate, tissue was reacted with 0.04% nickel-intensified diaminobenzidene (Sigma) solution for 210 seconds at room temperature to visualise Fos immunoreactivity and then rinsed 5 times with phosphate buffer to stop the reaction.

Immediately after Fos visualisation, I double-labelled each series to visualise tyrosine hydroxylase, vasotocin, or mesotocin. Tyrosine hydroxylase is an enzyme catalysing the rate-limiting step in dopamine synthesis and is used as a marker for

dopaminergic neurons in vertebrate neuroanatomy (e.g. O'Connell and Hofmann, 2012). Briefly, tissue series were rinsed three times in PBT, once in phosphate buffer, and incubated in 0.3% H_20_2 for 15 min. After another three PBT rinses, tissue series were incubated in blocking serum (tyrosine hydroxylase: 10% Normal Horse Serum, Vector; vasotocin and mesotocin: 3% Normal Goat Serum, Vector) in PBT for 60 min at room temperature. Tissue was then moved into a solution containing the appropriate primary antibody (tyrosine hydroxylase: Millipore, MAB5280, 1:1000; vasotocin: rabbit anti-vasotocin: a gift of Dr David A. Gray, University of the Witwatersrand, Johannesburg, South Africa, 1:10,000) and incubated for 60 h at 4°C. The tissue series reacted to visualise mesotocin (primary antibody: Immunostar, 20068, 1:5000) was incubated for 87 hours at 4°C. After three more rinses in PBT, tissue was incubated in a solution containing biotinylated secondary antibody (tyrosine hydroxylase: horse anti-mouse, 1:100, Vector; vasotocin and mesotocin: goat anti-rabbit, 1:100, Vector Laboratories) in PBT for 60 minutes at room temperature. After three rinses in PBT, sections were then incubated in avidin-biotin horseradish-peroxidase complex (1:50; Vector Laboratories) in PBT for 60 min at room temperature. After a final 4 rinses in PBT and a single rinse in phosphate buffer, the second label was visualised by incubating tissue in non-intensified diaminobenzidene at room temperature for different periods of time depending on the tissue series (tyrosine hydroxylase: 110 s; vasotocin: 225 s; mesotocin: 140 s). Tissue was rinsed five times in phosphate buffer to stop the diaminobenzidene reaction. This labelling procedure produced an intensely dark, black Fos labelled nuclei in neurons and a light brown cytoplasmic staining of neurons producing tyrosine hydroxylase, vasotocin, or mesotocin. After double-labelling, all tissue sections were mounted on to 0.5% gelatine-

subbed microscope slides (Thermo), serially dehydrated through alcohol (70 to 99%), cleared in xylene, and cover-slipped with Pertex (VWR).

Quantification of Fos immunoreactivity

I sampled Fos immunoreactivity in neuronal subpopulations characterised by their production of tyrosine hydroxylase, vasotocin, or mesotocin. I located each neuronal subpopulation with reference to full-section architecture (Stokes et al., 1974) and, more specifically, visualisation of tyrosine hydroxylase, vasotocin, and mesotocin. In tyrosine hydroxylase-labelled tissue, I sampled tyrosine hydroxylase-immunoreactive (dopaminergic) subpopulations in the ventral tegmental area in three adjacent sections and central gray in four adjacent sections in each brain. In both vasotocin- and mesotocin-labelled tissue, I sampled vasotocinergic and mesotocinergic subpopulations in BSTmd in three adjacent sections and BSTmv in two adjacent sections in each brain.

In each neuronal subpopulation, I counted the number of neurons producing tyrosine hydroxylase, vasotocin, or mesotocin and the number of double-labelled (tyrosine hydroxylase+Fos, vasotocin+Fos, or mesotocin+Fos) neurons. Although tyrosine hydroxylase+Fos neurons could be counted in the ventral tegmental area visually while using the microscope, single-labelled tyrosine hydroxylase-immunoreactive neuronal subpopulations were too large to be quantified using this method. To count these neurons, I took images of all ventral tegmental area sections using a 20x objective lens and counted the tyrosine hydroxylase-immunoreactive neurons by using ImageJ software (version 1.45, NIH, Bethesda, MD, USA). All neuron counts were made in both hemispheres. To account for differences in tyrosine hydroxylase-immunoreactive, vasotocinergic, and

mesotocinergic neuronal subpopulation sizes between sections and birds, I divided the total number of double-labelled cells by the total number of tyrosine hydroxylase-immunoreactive, vasotocinergic, or mesotocinergic neurons, respectively, in a given brain to quantify Fos immunoreactivity as the percentage of a neuronal subpopulation immunoreactive for Fos.

Statistical analysis

I used PASW software (version 19.00, SPSS Inc., Chicago, IL, USA) for all of my statistical analyses. I compared Fos immunoreactivity in each neuronal subpopulation using GLMs with independent variables including sex on two levels (male and female) and treatment on two levels (nest building and control).

To investigate whether nest-building behaviour explained individual variation in Fos immunoreactivity, I used multiple linear regression including neuronal activity as a dependent variable and all recorded behaviours in nest-building birds as independent predictors, as in Chapter 2. I ran regression models separately for each sex and each vasotocinergic, mesotocinergic, and dopaminergic neuron subpopulation sampled using a stepwise reduction procedure to identify behaviours that significantly explained individual differences in Fos immunoreactivity in these subpopulations.

Results

Full regressional models for all of the significant findings present below are summarised in Appendix 2.

Vasotocinergic neuronal subpopulations

Overall, Fos immunoreactivity in vasotocinergic neuron subpopulations in BSTmd or BSTmv did not differ between nest-building birds and control birds (BSTmd: $p = 0.535$; BSTmv: $p = 0.978$).

Among nest-building males, however, Fos immunoreactivity in vasotocinergic neurons in BSTmd increased the more time a male spent together with his mate in the nest cup (ß = 0.837; $t_6 = 3.748$; $p = 0.010$; Figure 3.1). Additionally, Fos immunoreactivity in vasotocinergic neurons in BSTmv increased the more times males picked up pieces of nest material (ß = 0.784; $t_6 = 3.097$; $p = 0.021$: Figure 3.1). In nesting females, none of the behaviours I measured significantly explained the individual variation in Fos immunoreactivity in vasotocinergic subpopulations in either BSTmd or BSTmv.

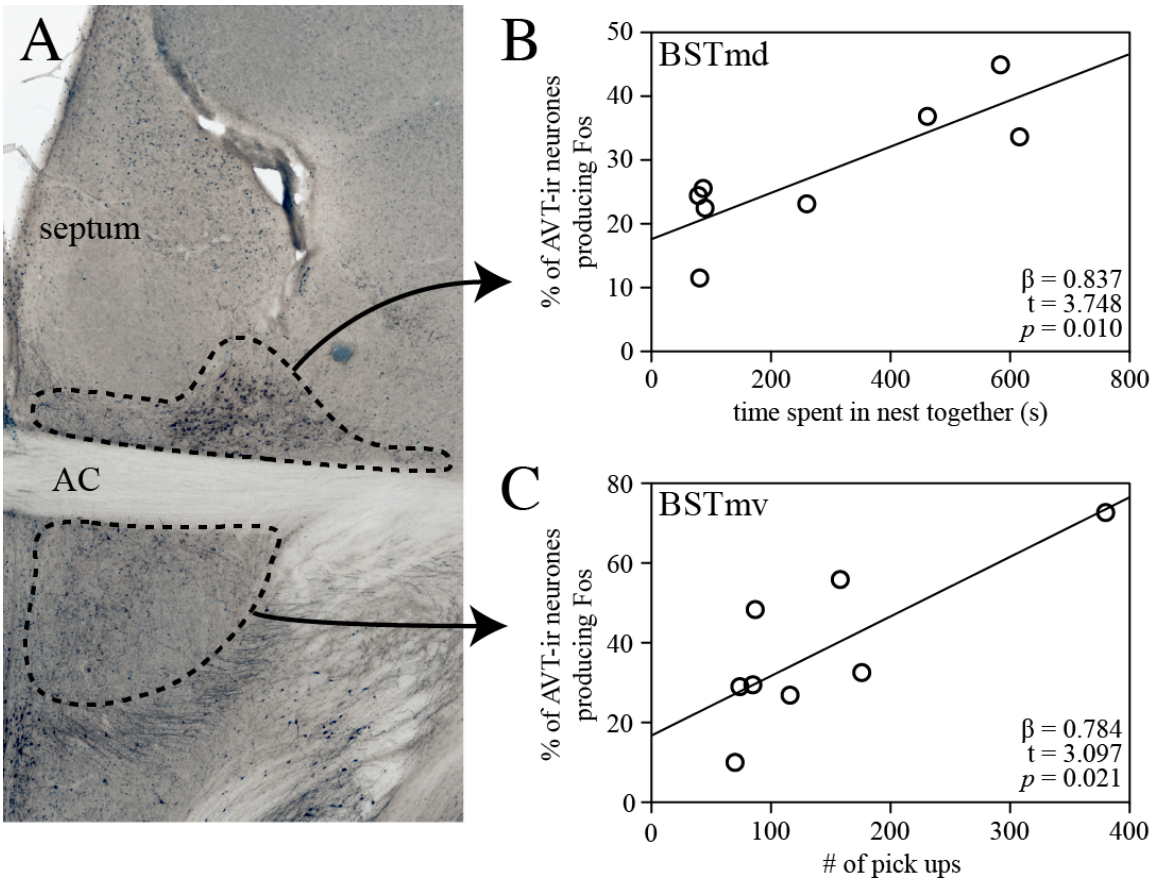


Figure 3.1. (A) A micrograph of medial bed nucleus of the stria terminalis labelled for the production of arginine vasotocin and Fos protein with dotted lines indicating the boundaries of vasotocinergic neuronal subpopulations sampled in this study. (B) Correlation between the time a pair of nest-building zebra finches spent together in the nest and the percentage of arginine vasotocin immunoreactive (AVT-ir) neurons in the medial bed nucleus of the stria terminalis, dorsal subdivision (BSTmd) immunoreactive for Fos in male brains. (C) Correlation between the number of times male nest-building zebra finches picked up pieces of nest material and the percentage of vasotocinergic neurons in the medial bed nucleus of the stria terminalis, ventral subdivision (BSTmv) immunoreactive for Fos in male brains.

Mesotocinergic neuronal subpopulations

Fos immunoreactivity in mesotocinergic neurons in BSTmd, but not BSTmv, tended to be greater in the nest-building birds than in controls (BSTmd: $F_{1,26} = 4.160$, p = 0.052; BSTmv: $p = 0.441$; Figure 3.2).

None of the behaviours that I measured significantly explained individual variation in Fos immunoreactivity in mesotocinergic neurons in either BSTmd or BSTmv.

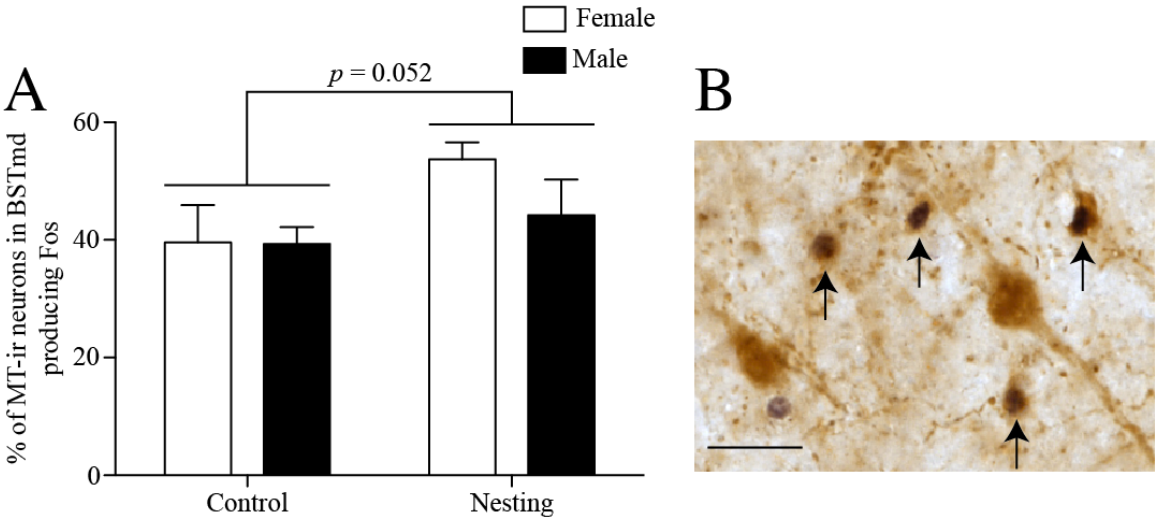


Figure 3.2. (A) Fos immunoreactivity in mesotocin-immunoreactive (MT-ir) neurons in the medial bed nucleus of the stria terminalis, dorsal subdivision (BSTmd) in adult control and nesting zebra finches. Bars represent mean percentage of MT-ir neurons immunoreactive for Fos in BSTmd in female (white bars) and male (black bars) zebra finches of pairs in which the male was or was not constructing a nest ± SEM. (B) A micrograph of neurons immunoreactive for of MT (cytosolic brown stain) and Fos (dark purple nuclear stain). Arrows indicate neurons containing both labels. Scale bar = 20 μm.

Tyrosine hydroxylase-immunoreactive neuronal subpopulations

Overall, Fos immunoreactivity in tyrosine hydroxylase-immunoreactive neurons in either the ventral tegmental area or central gray did not differ between the nest-building and control birds (ventral tegmental area: $p = 0.211$; central gray: $p = 0.794$).

Among nest-building males, however, Fos immunoreactivity in tyrosine hydroxylase-immunoreactive neurons in the central gray increased the more time a male spent with his mate in the nest cup (ß = 0.921; $t_6 = 5.793$; $p = 0.001$; Figure 3.3). Additionally, Fos immunoreactivity in tyrosine hydroxylase-immunoreactive neurons in the ventral tegmental area decreased the more males tucked nest material into the nest (ß = -0.719; $t_6 = -2.531$; $p = 0.045$).

In nesting females, Fos immunoreactivity in tyrosine hydroxylase-immunoreactive neurons in the ventral tegmental area decreased the more a female fed (ß = -0.816; $t_6 = -3.453$; $p = 0.014$). Stepwise linear regression identified no behaviours that significantly explained individual variation in Fos immunoreactivity in tyrosine hydroxylase-immunoreactive neurons in the central gray of female nesting finches.

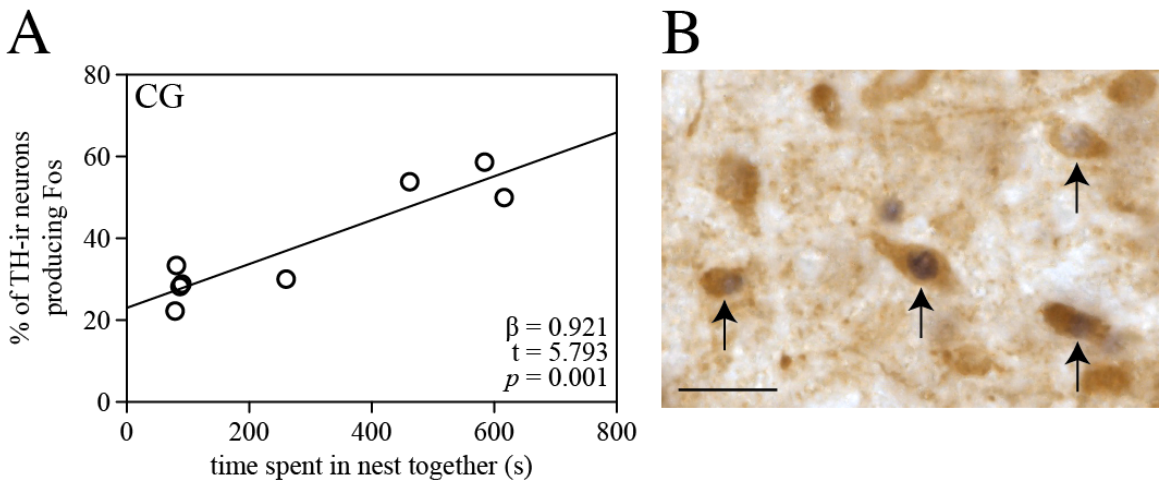


Figure 3.3. (A) Correlation between the time a pair of nest-building zebra finches spent together in the nest cup and Fos immunoreactivity in tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the central gray (CG) of male zebra finches. (B) A micrograph of neurons labelled for TH (cytosolic brown label) and Fos (dark purple nuclear label) immunoreactivity. Arrows indicate neurons containing both labels. Scale bar = 20 μm.

Discussion

I compared neuronal activity in vasotocinergic, mesotocinergic and dopaminergic neuronal subpopulations in the social behaviour network and dopaminergic reward system between male and female zebra finches in which the male of the pair was building a nest or not. In nest-building males, Fos immunoreactivity in vasotocinergic neurons in BSTmd and in dopaminergic neurons in the central gray increased the more time a male spent together with his mate in the nest. Fos immunoreactivity in mesotocinergic neurons in BSTmd was higher in nest-building birds relative to control birds. In BSTmv of nest-building males, however, Fos immunoreactivity in vasotocinergic neurons increased the more a male finch

picked up nest material. Finally, Fos immunoreactivity in dopaminergic neurons in the ventral tegmental area decreased the more a male finch tucked material into the nest. These data provide the first evidence suggesting vasotocinergic and mesotocinergic neuronal subpopulations in the social behaviour network and dopaminergic neuronal subpopulations in the dopaminergic reward system may be involved in controlling nest-building behaviour in zebra finches.

Vasotocinergic and mesotocinergic neuronal subpopulations

Medial bed nucleus of the stria terminalis, dorsal subdivision (BSTmd)

I found that Fos immunoreactivity in mesotocinergic neurons in BSTmd was higher in nest-building finches relative to Fos immunoreactivity in these neurons in control birds (Figure 3.2). These data appear to contradict those from an earlier study in which Fos immunoreactivity in BSTm vasotocinergic and mesotocinergic neurons did not differ between nest-building and control zebra finches (Klatt and Goodson, 2013). As the neurons sampled in that study, however, included subpopulations from both BSTmd and BSTmv as a single measure, coupled with my observation that Fos immunoreactivity in mesotocinergic neurons in BSTmv in nest-building birds did not differ from that of controls, it seems plausible that assessing the activity in neurons across the two subdivisions may have masked a group difference. Aste et al. (1998) originally proposed the division of BSTm into dorsal and ventral subdivisions, BSTmd and BSTmv, respectively, because of the anatomical separation of these two subpopulations by the anterior commissure. Support for such a functional distinction between the two subdivisions comes from two studies, one in which Fos immunoreactivity in both BSTmd and BSTmv increased during nest box

possession in starlings (Heimovics and Riters, 2006) and the other in which Fos immunoreactivity in both subdivisions increased during nest building in zebra finches (Chapter 2). Although neuronal activity in both subdivisions increased following these behaviours, the relationship between neuronal activity and the behaviour observed differed between BSTmd and BSTmv. For example, in Chapter 2, Fos immunoreactivity in BSTmd was higher in nest-building zebra finches relative to that of the non-nesting controls but this increased Fos immunoreactivity did not correlate with any of the nest-building behaviour I quantified. Fos immunoreactivity in BSTmv, however, did not differ between nest-building and control finches, but, within nest-building females, increased specifically with the more time a female spent in the nest cup, suggesting BSTmd may play a role in nest possession or perception, whereas BSTmv is specifically involved in time spent in the nest in female zebra finches (Chapter 2). Here, I also found that neuronal activity in nonapeptidergic neuronal subpopulations in BSTmd and BSTmv exhibited different relationships with nest-building behaviour, supporting the previous assertion that these subdivisions are functionally distinct.

In addition to replicating the increase in BSTmd Fos immunoreactivity in nest-building finches compared to controls that I reported in Chapter 2, here I show that this increase in neuronal activity appears to occur specifically within mesotocinergic neurons. Functionally, because Fos immunoreactivity in mesotocinergic BSTmd neurons was higher in nest-building birds compared to controls but this increased Fos immunoreactivity in this subpopulation did not correlate with any behaviour measured, it seems plausible that the activity in this neuronal subpopulation is related to nest possession or perception of the nest rather than to nest building, as I proposed for BSTmd in Chapter 2.

Also within BSTmd, Fos immunoreactivity in vasotocinergic neurons increased in male nest-building finches the more time he spent together with his mate in their nest, a result that appears at odds with the absence of a relationship between Fos immunoreactivity in vasotocinergic neurons in BSTm and the time spent in the nest in zebra finches (Klatt and Goodson, 2013). Further to the suggestion above regarding differences between sampling BSTmd and BSTmv as a single neuronal subpopulation and sampling them separately, it could be that at least part of the explanation of this discrepancy between studies lies with the behaviours quantified. Both Klatt and Goodson (2013) and I (Chapter 2) measured the amount of time individual birds spent within the nest whereas here I measured the amount of time the pair of finches spent together in the nest. This discrepancy might be particularly important because the social behaviour network is primarily involved in social interactions between conspecifics (Goodson, 2005). For example, in zebra finches, vasotocinergic neurons in BSTm specifically appear to be involved in eliciting affiliative responses to mates (Goodson and Wang, 2006). These results suggest that vasotocinergic neurons in BSTmd of male finches may be involved in affiliative behaviour within the nest during nest building, although more detailed data on the social interactions occurring within the nest are necessary to test this possibility.

Medial bed nucleus of the stria terminalis, ventral subdivision (BSTmv)

Here, I found that Fos immunoreactivity in vasotocinergic neurons in BSTmv increased the more a nest-building male finch picked up nest material, which also appears at odds with the data I reported in Chapter 2. This difference may be explained if the relationship between neuronal activity and picking up nest material is specific to

vasotocinergic neurons in this region and, therefore, may have been masked by Fos immunoreactivity in other BSTmv neuronal subpopulations sampled alongside vasotocinergic neurons in Chapter 2. Functionally, I suggest that vasotocinergic neurons in BSTmv of zebra finches may be involved in picking up nest material. Again, this suggestion contradicts that of Klatt and Goodson (2013), who found no relationship between neuronal activity in BSTm and nest material collection by male zebra finches. As with my results in BSTmd above, I believe this discrepancy in BSTmv may be, in part, explained by differences in the behaviour quantified by Klatt and Goodson (2013) and by myself. Whereas Klatt and Goodson (2013) counted the number of pieces of nest material picked by male finches, in this study, I counted the number of times males picked up nest material. In both this study and Chapter 2, I noticed that male finches often pick up but then drop the same piece of nest material several times and encoding the number of pieces of nest material picked up in lieu of the number of picking up actions, as in Klatt and Goodson (2013), may not reflect nest-building behaviour. By demonstrating that neuronal activity in vasotocinergic neurons in BSTmv increased specifically the more male finches picked up nest material, I suggest that neuronal activity in this subpopulation is involved in the action of collecting nest material and not the number of pieces of nest material collected, as measured in Klatt and Goodson (2013). By manipulating vasotocin signalling using pharmacological agents targeted to BSTmv subpopulations and recording subsequent effects on nest-building behaviour, one could help to determine whether vasotocin from this neuronal subpopulation is involved in picking up actions.

In females, I found no relationship between Fos immunoreactivity in either vasotocinergic or mesotocinergic neuronal subpopulations and nest-building behaviour,

which suggests that the correlation between Fos immunoreactivity in BSTmv and the time a female spent in the nest that I reported in Chapter 2 may be attributed to other neuronal subpopulations located in BSTmv intermingled with the nonapeptidergic subpopulations sampled here, such as the population of neurons expressing receptors for vasoactive intestinal peptide (Goodson et al., 2006). Consistent with this possibility, Klatt and Goodson (2013) found no effect of central infusions of pharmacological antagonists that impair vasotocin and mesotocin signalling on the time female zebra finches spent within the nest. Here, as in Chapter 2, I will reiterate that the lack of a relationship between Fos immunoreactivity in any of the neuronal populations tested here and nest-building behaviour should not be used as evidence to discount a relationship between these neuronal populations and nest-building behaviour because the restricted sample size used in this study may have been too small to have detected this relationship.

Dopaminergic neuronal subpopulations

Ventral tegmental area

In Chapter 2, I found that neuronal activity in the ventral tegmental area increased the more male finches picked up nest material, however, here I saw no change in Fos immunoreactivity in dopaminergic neurons in the ventral tegmental area with regard to the collection of nest material by males, suggesting that dopaminergic neurons in the ventral tegmental area do not play a role in collecting nest material. Instead, here I found a decrease in Fos immunoreactivity in ventral tegmental area dopaminergic neurons the more nest-building male finches tucked material into the nest structure. This may mean that tucking nest material into the nest structure is unrewarding or that the dopaminergic neuron

subpopulation in the ventral tegmental area inhibits tucking behaviour. Such negative relationships between neuronal activity and the production of behaviour have been reported by Goodson et al. (2005), who found neuronal activity throughout the lateral septum negatively correlated with aggressive displays in male song sparrows (*Melospiza melodia*). Pharmacological manipulations could be used to inhibit neuronal activity in ventral tegmental area dopaminergic neurons in order to distinguish between these two possibilities.

Because I did not find an increase in dopaminergic neuronal activity in the ventral tegmental area the more male finches picked up nest material, I believe the relationship between the ventral tegmental area and nest material collection that I reported in Chapter 2 may occur in other, non-dopaminergic neuronal subpopulations in the ventral tegmental area. For example, the ventral tegmental area also contains a neuronal subpopulation that uses the inhibitory neurotransmitter gamma-aminobutryic acid (GABAergic neurons), which also appears to be involved in controlling social behaviours including courtship song production in male zebra finches (Hara et al., 2007; Lynch et al., 2008). Comparing neuronal activity in non-dopaminergic neuronal subpopulations in the ventral tegmental area to nest-building behaviour could test this hypothesis.

Central gray

The increase in Fos immunoreactivity in central gray dopaminergic neurons in male nest-building finches the more time he spent in the nest with his partner supports the proposal that dopaminergic neurons in the central gray play a role in social communication (Goodson et al., 2009). It is possible that this social communication takes the form of duet-

like vocalisations that appear to be performed only within the nest (Elie et al., 2010) but, as yet, we have no data to confirm this possibility.

In this chapter, I provide the first evidence that vasotocinergic, mesotocinergic, and dopaminergic neuronal subpopulations in the social behaviour network and dopaminergic reward system are active when birds are nest building. These brain-behaviour relationships suggest that nest-building behaviour can be classified as a social behaviour regulated by the social behaviour network and dopaminergic reward system.

Chapter 4: The evolution of cerebellum structure and nest complexity

Introduction

In Chapters 2 and 3, I demonstrated the potential involvement of brain regions in nest-building behaviour by comparing neuronal activity in the brain to the production of nest-building behaviour. Although this functional neuroscience approach of comparing brain activity to behaviour is common, it is not the only approach to identify brain regions involved in the behaviour of interest. As described in Chapter 1, identifying correlations between brain morphology and behaviour across species has been used to suggest the function of brain regions. For example, by demonstrating that food-caching bird species have larger hippocampal volumes than do non-caching species, Krebs et al. (1989) and Sherry et al. (1989) both suggested that structural variation in the avian hippocampus was related to variation in its functional capabilities, specifically with regard to spatial learning and memory. In this chapter, therefore, I aimed to test whether morphological variation in the cerebellum was correlated with variation in nest-building behaviour across bird species.

The cerebellum is a caudal brain region found in all vertebrates, which although historically was considered to play a major role in motor control (Ito, 1984), is now known also to be involved in a range of cognitive processes, such as learning, memory, and language in humans (Ito, 1993). Across vertebrates, the morphology of the cerebellum is highly varied in both its volume and foliation (amount of surface folding) across species: amphibians and reptiles have unfolded cerebella while birds and mammals have variably convoluted cerebella (Larsell, 1967; Iwaniuk et al., 2006). Of specific importance to the work I describe in this thesis, increased cerebellar foliation in birds is hypothesised to

increase the density of cerebellar neural circuitry and processing capacity of the cerebellum to enhance motor abilities, specifically manipulative skills (Butler and Hodos, 2005; Iwaniuk et al., 2009). Some support for this suggestion is provided by the positive correlation between cerebellar foliation and tool use in birds (Iwaniuk et al., 2009) and between cerebellum volume and extractive foraging in primates (Barton, 2012) and neural activation (as seen by positron emission tomography) in the cerebellum during tool use in monkeys (Obayashi et al., 2001).

Because nest building in birds also requires some manipulative skills phenotypically similar to those involved in tool use (see Chapter 1) and these skills may vary depending on the structural complexity of the nest built, I hypothesised that the cerebellum may be involved in nest-building behaviour. Here, I examined whether variation in cerebellar foliation index (Iwaniuk et al., 2006) in birds is explained by the variation in the complexity of their species-typical nest structure. I predicted that species that build more structurally complex nests would have higher cerebellar foliation indices than would species that build simpler nests, suggesting the cerebellum is involved in the manipulative skill underlying nest-building behaviour.

Methods and materials

Cerebellar foliation and nest structure

I collected data on cerebellar foliation index, measured as the degree of cerebellar cortex folding compared to a hypothetical unfolded cortex for the same cerebellum size, cerebellum volume, whole brain volume, and body mass from Iwaniuk et al. (2006) for 87 bird species.

I then gathered descriptions of the species-typical nest structure from published studies and texts (Appendix 1). Based on these descriptions, I categorised nest structures as no nest, platform, cup, domed, and excavation nests. Birds that do not excavate or construct a nest but lay eggs directly on a bare substrate or in a nest built by another species were categorised as building no nest. No nest categorisations included birds that build nests in nest boxes and cavities only if they are not described as building any structure within these housings. Birds that construct nests within cavities and nest boxes were classified by the structure they build within these housings. Platform nests are unshaped piles of collected nesting material, including material used to line ground scrapes and depressions. Cup nests have nest walls created during construction by the bird and not by depression of the nest's centre by the weight of the bird and eggs' during incubation. Domed nests have both nest walls and a roof. Finally, excavation nests are tunnels or chambers dug using the beak or feet into a substrate. Unlike Hansell (2005), I did not differentiate between platform nests built in the tree and those on the ground (referred to as "plate" and "bed" nests, respectively, in Hansell, 2005) but I did differentiate between species that excavate nests and those that nest in natural cavities or cavities excavated by other species (both referred to as "cavity nests" in Hansell, 2005). These differences in nest categorisation reflected my focus on the manipulative skill and behaviour required to construct a nest, regardless of nest location or materials used.

I focused on comparing no nest, platform, and cup nest structures because these three nest structures differ in the degree to which material is collected and manipulated during construction: birds building no nest do not collect or manipulate nest material, platform nests require the collection but little manipulation of material while cup nests

require collection and manipulation of nest material to produce walls in the cup structure. Because excavation behaviour involves a distinct set of actions to burrow into a substrate which are difficult to compare to the collection and manipulation of nest material, I excluded species that built excavation nests from further analysis. Furthermore, because only two species (*Acanthiza pusilla* and *Menura novaehollandiae*) in my sample constructed domed nests, I excluded these species from analysis as well as those species without a nest description. After these exclusions, 64 species remained in my analysis. Keywords used to categorise species-typical nest structures compared here are summarised in Table 4.1.

Table 4.1. Terminology in published nest descriptions used to classify species-typical nest structure. In my nest structure classification scheme, I focused on the nest-building behaviour involved in collecting and manipulating nest material as well as manipulating nesting, irrespective of nest location or the materials used.

Nest Structure Classification	Terminology in literature
No nest	No evidence of construction/excavation
	Cavity excavated by other species
	Nestbox
	Tree hollow/hole
	Unlined scrape
	Nest on bare ground
	No nest/no nesting material
	Old stick nest of other species
	Shallow knot-hole
Platform	Platform
	Lined scrape/depression
	Saucer-shaped
	Bed of material
	Pile of material
	Mud nest
Cup	Bowl
	Cup
	Cup-shaped
	Half cup
Domed	Dome
	Ball
	Roofed
Excavation	Burrow
	Digging/Excavating
	Tunnel

Statistical methods and analyses

To account for the statistical non-independence of datasets including multiple species, I analysed data using the phylogenetic generalised least squares (PGLS) approach, which incorporates the phylogenetic relatedness of species into the error term of a regression model (Pagel, 1997). Regression analysis included nest structure as a discrete, independent variable on three levels (no nest, platform, cup) and cerebellar foliation index as a continuous, dependent variable. To account for allometric scaling effects on cerebellar

foliation index, I included cerebellum volume as a covariate. Cerebellum volume was log-transformed to achieve normality (Shapiro-Wilkes test, $p > 0.05$). Although previous cerebellar foliation index analyses included other allometric variables (body size, whole brain volume, and whole brain - cerebellum volume; Iwaniuk et al., 2006), I found that cerebellum volume predicted cerebellar foliation index better than the other allometric measures and after including cerebellum volume as a covariate no other allometric variable explained significant variation in cerebellar foliation index. To test whether nest structure was related specifically to cerebellar foliation, I also tested whether nest structure predicted cerebellar volume using a PGLS with log-transformed whole brain volume and log-transformed body size as allometric co-variates.

In addition to testing the main effect of nest structure on cerebellar foliation, I also made three planned contrasts (no nest vs. platform, no nest vs. cup, and platform vs. cup) by changing which factor level was the reference level in the model. I ran analyses in R (R Development Core Team 2013) using the packages ape (Paradis et al., 2004) and caper (Orme, 2012) and viewed phylogenetic trees in FigTree (Rambaut, 2012) and DensiTree (Bouckaert, 2010).

To account for phylogenetic uncertainty, I ran my PGLS models across a sample of 3000 phylogenies built using a family backbone by Hackett et al. (2008; Jetz et al., 2012) with restricted phylogenetic signal estimation (λ = lower: 0.01-0.1, upper: 0.95-0.99). I used model averaging (following Johnson and Omland, 2004) to estimate average parameters from PGLS regressions across the tree-block, weighted by the probability of the model given each tree. Main effects could not be model-averaged across the tree-block

because they were calculated from comparison of models with and without nest structure using ANOVA. Instead, I present the minimum F and maximum *p* values reported across the tree-block as a conservative means of testing for the main effect across varying phylogenies. Because model comparison requires a fixed λ value in both models, λ was fixed at either 0.85 or 0.95 (values derived from maximum likelihood estimations) when testing for main effects of nest structure on cerebellar foliation. I acquired all bird phylogenies from www.birdtree.org (Jetz et al., 2012). An example phylogeny is presented in Figure 4.1. Finally, because my species sample included two flightless birds (*Rhea americana* and *Struthio camelus*) and flight may also be a behavioural specialisation associated with cerebellar foliation, I reran analyses excluding these two species.

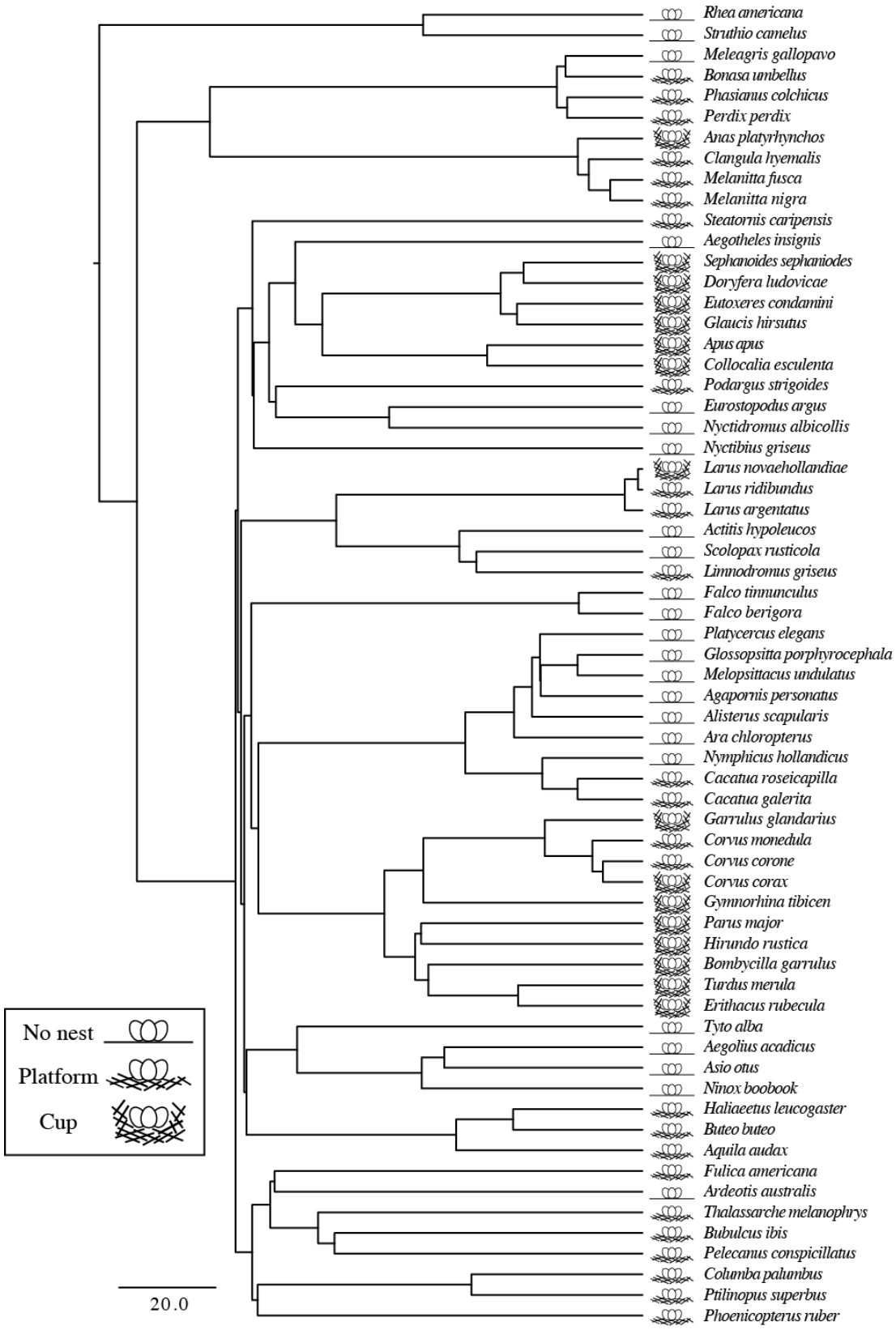

Rhea americana
Struthio camelus
Meleagris gallopavo
Bonasa umbellus
Phasianus colchicus
Perdix perdix
Anas platyrhynchos
Clangula hyemalis
Melanitta fusca
Melanitta nigra
Steatornis caripensis
Aegotheles insignis
Sephanoides sephaniodes
Doryfera ludovicae
Eutoxeres condamini
Glaucis hirsutus
Apus apus
Collocalia esculenta
Podargus strigoides
Eurostopodus argus
Nyctidromus albicollis
Nyctibius griseus
Larus novaehollandiae
Larus ridibundus
Larus argentatus
Actitis hypoleucos
Scolopax rusticola
Limnodromus griseus
Falco tinnunculus
Falco berigora
Platycercus elegans
Glossopsitta porphyrocephala
Melopsittacus undulatus
Agapornis personatus
Alisterus scapularis
Ara chloropterus
Nymphicus hollandicus
Cacatua roseicapilla
Cacatua galerita
Garrulus glandarius
Corvus monedula
Corvus corone
Corvus corax
Gymnorhina tibicen
Parus major
Hirundo rustica
Bombycilla garrulus
Turdus merula
Erithacus rubecula
Tyto alba
Aegolius acadicus
Asio otus
Ninox boobook
Haliaeetus leucogaster
Buteo buteo
Aquila audax
Fulica americana
Ardeotis australis
Thalassarche melanophrys
Bubulcus ibis
Pelecanus conspicillatus
Columba palumbus
Ptilinopus superbus
Phoenicopterus ruber
No nest
Platform
Cup
20.0

Figure 4.1. Sample phylogeny of bird species included in regressional analysis and species-typical nest structure classification. I included species from Iwaniuk et al. (2006) that had a description of the typical nest structure I could classify as no nest, a platform, or cup (using the terminology in Table 1). Branch lengths represent time. Scale bar represents 20 million years (Jetz et al., 2012). Species names taken from Jetz et al. (2012).

Although model averaging and summarising PGLS parameters across a block of phylogenies accounts for phylogenetic uncertainty, this approach cannot account for potential uncertainty in the statistical model. In order to account for both phylogenetic and model uncertainty, I re-ran my main PGLS analyses using Bayesian Markov-Chain Monte Carlo (MCMC) methods in BayesTraits (Version 1; Pagel and Meade, 2006; 2007). I estimated posterior probability distributions for parameters including regression coefficients (β), model R^2, and phylogenetic signal (λ). I report average values for parameters and the percentage of posterior estimates in the predicted direction (% β +ve, following the prediction that cerebellar foliation index should increase when comparing species that build more structurally complex nests to species that build less structurally complex nests). Prior to analysis, I determined that >95% of posterior estimates for regression coefficients above zero would be interpreted as 'strong evidence' for a statistical relationship between variables, as, for example, in Ross et al. (2012). As in the model-averaging analyses, I used cerebellar foliation index as the outcome variable, predicted by log-transformed cerebellar volume and nest structure. I ran MCMC chains for 5,000,000 iterations, sampling every 100 generations. I used uniform prior distributions for regression

coefficients (-100, +100). Mean acceptance rates were between 20-40%, as recommended by Pagel and Meade (2007), and all effective sample sizes were >5,000.

Results

Across 64 species of bird, nest structure was significantly associated with cerebellar foliation index ($F_{1,60} > 3.875$, $p < 0.026$, $R^2 = 0.615$; using $\lambda = 0.85$ = model-averaged estimate from main regression model). This relationship appears specific to cerebellar foliation because nest type did not predict cerebellum volume ($F_{1,60} < 1.686$, $p > 0.194$; $\lambda = 0.95$).

Specific contrasts confirmed my predictions: species that build a platform nest have significantly higher cerebellar foliation indices than do species that do not build nests ($t_{46} = 2.047$, $p = 0.047$), species that build a cup nest have significantly higher cerebellar foliation indices than species that do not build nests ($t_{37} = 3.165$, $p = 0.003$), and species that build a cup nest have significantly higher cerebellar foliation indices than species that build a platform nest ($t_{39} = 2.020$, $p = 0.049$). Altogether, as nests increase in structural complexity (no nest → platform → cup), cerebellar foliation index also increases. Furthermore, my main results were not affected by removing the two flightless species in my sample, in terms of either the main effect of nest structure on cerebellar foliation: ($F_{1,58} > 4.589$, $p < 0.028$, across 3000 trees, $\lambda = 0.85$, using cerebellum volume as a co-variate), or in any of the planned contrasts (all model-averaged $p < 0.05$).

In my re-analysis of the data using the Bayesian MCMC approach, I again found strong evidence for greater cerebellar foliation in species that build cup nests relative to

species that build platform nests (Figure 4.2; average β: 0.24, 100% β +ve), species that build platform nests relative to species that build no nests (Figure 4.2; average β: 0.22, 96% β +ve), and species that build cup nests relative to species that build no nests (Figure 4.2; average β: 0.46, 100% β +ve). The model R^2 was 0.62.

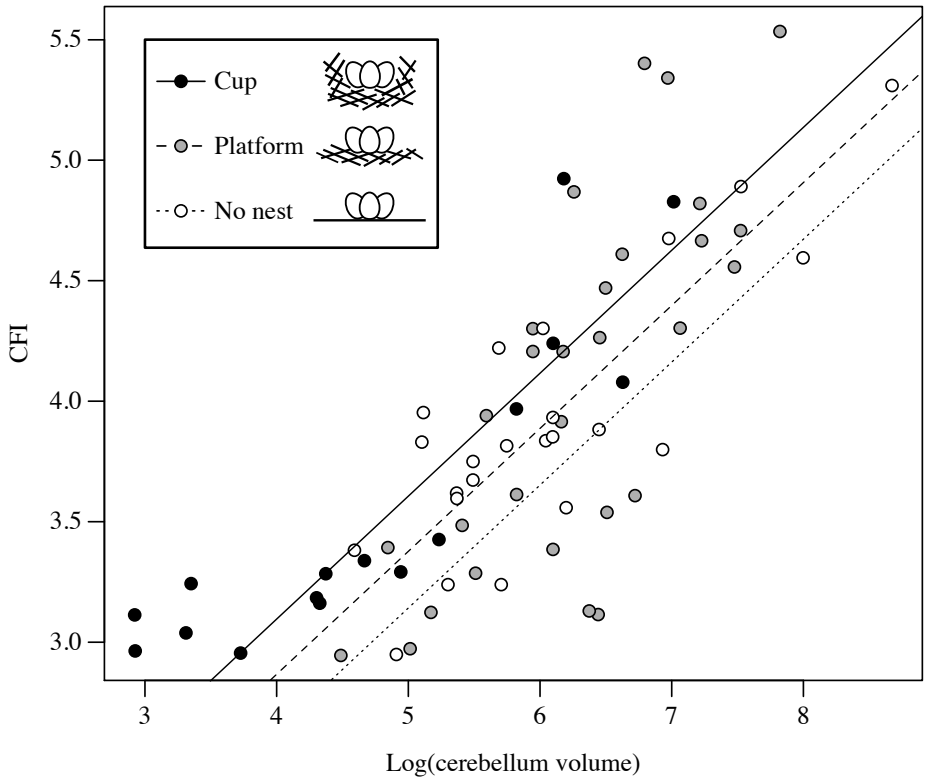


Figure 4.2. Regression lines between log-transformed cerebellum volume and cerebellar foliation index of bird species that build no nest, platform nests, or cup nests. Dots represent log-transformed cerebellum volume and cerebellar foliation index

(CFI) for bird species that build cup (black), platform (gray), and no nest (white). Slopes and intercepts for all three groups were estimated from phylogenetic generalised least squares regression models. For a given cerebellum volume, species that build cup nests have more foliated cerebella than do species that build platform nests and no nest (both $p < 0.05$) and species that build platform nests have more foliated cerebella than species that build no nest ($p < 0.05$).

Discussion

The building of more structurally complex nests is associated with greater cerebellar foliation than it is in birds that build simpler nests. These data support both the hypothesis that increased cerebellar foliation enables enhanced manipulative motor skills (Butler and Hodos, 2005) and that the cerebellum is involved in nest-building behaviour. A relationship between increased cerebellar foliation and 'increasingly sophisticated' behaviours (e.g. agile capture of cephalopod prey in the Tawny nurse shark (*Nebrius ferrugineu*) has also been observed in chondrichthyes (Yopak et al. (2007). Taken together, these data suggest that increasing cerebellar foliation may be a mechanism that is conserved across vertebrates to improve manipulative skill and motor control. In fact, such an increase in foliation may also underpin the positive correlation between cerebellum volume and extractive foraging in primates (Barton, 2012).

Functionally, increased cerebellar foliation is hypothesised to increase the density of Purkinje cells, the predominant neuron in the cerebellar cortex and only source of cerebellar output, which is thereby thought to increase the processing capacity of the cerebellum in birds (Iwaniuk et al., 2009). Although here I suggest that cerebellar foliation

is associated with the manipulative skill required to build nests, other processes involved in nest-building behaviour also supported by the cerebellum, such as motor sequencing and learning, may also explain the correlation between nest structure complexity and cerebellar foliation. By incorporating measures of neuronal activity in the cerebellum into future studies on nest-building behaviour as in Chapters 2 and 3, we can identify which of the processes associated with nest building involve the cerebellum.

My demonstration that cerebellar foliation is positively correlated to nest-building behaviour is based on the same dataset in which cerebellar foliation has been shown to be positively correlated with tool use in birds (Iwaniuk et al., 2006). Although a currently unpopular notion, this parallel between these two construction behaviours suggests that nest building and tool use may involve the same, or similar, neurobiological processes. In Chapter 6, I explore implications of the neurobiological similarities between nest-building behaviour and tool use incorporating not only these morphological cerebellar data but also functional data on neuronal activity during nest building presented in Chapter 2.

In my analyses, I used a much simpler nest classification system relative to those used previously (Hansell, 2005) to examine causes of variation in nest building. For example, I excluded nesting materials, nest attachment to substrates, and nest location from my nest structure classification scheme. By doing so, however, I had a dataset that was amenable to current comparative statistical analytical techniques. The association between variation in cerebellar foliation index and in nest structural complexity that I show here would suggest that this simple classification system may be useful for further investigation of the evolution of nest design. Accordingly, in Chapter 5, I demonstrate that this nest

classification scheme may be more generally useful as it enabled me to investigate the evolutionary history of nest structure and location in Old World babblers (Timaliidae).

In conclusion, I found that variation in cerebellar foliation is positively associated with the complexity of nest structures built by birds. Across all bird species, nest structure varies tremendously, beyond the three nest classifications I tested here (Hansell, 2005). By continuing to identify the neural underpinnings of nest building (as described throughout this thesis), I can take advantage of variation in species-specific behaviour to understand how evolution has shaped the brain to produce unique behaviours and the structural outcomes that result from those behaviours.

Chapter 5: Co-evolution of nest structure with location

Introduction

The tremendous diversity in avian nest structure has long been documented and celebrated. For example, in *The Jungle Book,* Rudyard Kipling (1899) describes nest building by the common tailorbird (*Orthotomus sutorius*), which stitches leaves together to form a deep cup. This diversity in nest structure extends from the simple stick platform of the woodpigeon (*Columba palumbus*) to the intricate woven hanging nest of the Southern masked weaver (*Ploceus velatus*) and it has been suggested that flexible nest-building behaviour, alongside a small body and flight, was one of the key traits that enabled the adaptive radiation of passerines (Collias, 1997). Despite the accumulation of descriptions of nest structure for thousands of bird species (e.g. del Hoyo et al., 2007), together with a flurry of mechanistic studies elucidating the structural properties of nests (Heenan and Seymour, 2011; 2012) and the learning mechanisms associated with nest building (see Chapter 1), there has been little work addressing the evolution of nest structure.

Two major problems have hampered such study. Firstly, the lack of avian phylogenetic information amenable to phylogenetic comparative methods has precluded the use of formal statistical tests of evolutionary hypotheses of nest structure. Instead, past investigations of nest structure evolution superimposed nest traits onto a single phylogeny to describe proposed evolutionary patterns rather than conducting formal phylogenetic analyses (e.g. Winkler and Sheldon, 1993; Eberhard, 1998; Irestedt et al., 2006). Without formal statistical models, however, such studies rely on outgroup comparison to infer ancestral states, which suffers from sampling bias and an inability to incorporate

information on branch lengths and phylogenetic uncertainty (Pagel and Harvey, 1988). The recent availability of posterior probability samples of phylogenetic estimations across the largest sample of birds to date (Jetz et al., 2012) now enables formal statistical analysis incorporating branch length information and phylogenetic uncertainty to address the evolution of nest structure. Secondly, the lack of a standardised nest structure classification scheme has prevented cross-species comparisons. In Chapter 4, I proposed a simple nest categorisation scheme based on structural complexity that can be used for comparative statistical analyses of nest structure.

With these tools and data now available, it is possible to test, for instance, one specific hypothesis regarding the evolution of nest building proposed by Collias (1997): that building domed nests evolved from the building of cup nests by species building nests in trees. Collias specifically suggested that competition for limited nest sites off the ground favoured birds that built their nests nearer to the ground, eventually leading to birds' building nests on the ground. Because open-cup nests built nearer to the ground are thought to be susceptible to greater predation pressure from ground predators than are enclosed, domed nests (Linder and Bollinger, 1995), Collias argued that the shift to ground nesting should, therefore, coincide with the building of an enclosed, domed nest to confer protection against this increased predation risk.

In his original proposal, Collias (1997) supported his hypothesis with data on Old World babblers (Timaliidae) from India, which build either cup or domed nests. Collias reported that the majority of cup-nest building babblers built nests off the ground, whereas the majority of domed-nest building babblers built nests on the ground. This comparison, however, failed to incorporate any information on phylogenetic relatedness of the sampled

species and could not, therefore, formally test either the potential co-evolution of domed-nest building and building on the ground or the ancestral state of and history of evolutionary transitions in nest location and structure in this clade. Here, I investigated the co-evolution of building on the ground and the building of a domed-nest in the Timaliidae, using a large species sample and phylogenetically-informed statistical analyses to elucidate the evolutionary history of nest structure and height in this family. If building a domed nest confers increased protection from predation and that risk increases with increasing proximity to the ground, I would expect domed-nest building species to build their nests closer to the ground than would cup-nest building species. Further, to determine whether ground-nesting co-evolved with the building of a domed nest, I carried out phylogenetic analyses of trait co-evolution, including an ancestral reconstruction and order of evolution analysis to establish the ancestral state of nest structure and location and to test whether subsequent co-evolution was more likely to occur first through changes in nest structure or changes in nest height. Because phenotypic plasticity in nest location within bird species is well-documented (reviewed in Lima, 2009) whereas flexibility in nest structure is less commonly observed, I expected that transitions would be more likely to occur through changes first in nest height rather than nest structure.

Methods and materials

Collection of nest data

I gathered descriptions from previously-published sources of the species-typical nest structure and the lowest height of nests built by 155 species within Timaliidae (del Hoyo et al., 2007). I categorised nest structures as either cup or domed using the nest

classification scheme described in Chapter 4: both cup and domed nests are characterised by a nest floor and surrounding walls created during construction. Domed nests, however, also have a roof. Terminology used to classify nest structure in Timaliidae is summarised in Table 5.1.

Table 5.1. Terminology used in published nest descriptions to classify cup and domed nest structure in Old World Babblers (Timaliidae). I classified nest structures as either cup nests, characterised by the construction of nest walls, or domed nests, characterised by the construction of both nest walls and a partial or full roof from species-typical nest structure descriptions from del Hoyo et al. (2007).

Nest Structure Classification	Terminology in Literature
Cup	Cup
	Cup-shaped
	Basket
	Cradle
	Bowl
Domed	Dome
	Semi-dome
	Oval-shaped
	Dome-shaped
	Ball
	Globe
	Globular structure
	Semi-roofed
	Half-canopy
	Egg-shaped structure
	Roofed

In addition to nest structure, I recorded the lowest height at which nests were built. I used the lowest reported nest height because selection pressure exerted by ground predators should be the greatest at the lowest height at which a nest is built. Whenever nests were described as being placed on the ground, I entered the nest height as 0 m. All nest structure and height data are summarised in the Appendix 2.

Phylogenetic comparative statistical methods

Similar to the second analysis in Chapter 4, I used Bayesian Markov-Chain Monte Carlo (MCMC) methods in order to estimate posterior probability distributions for model parameters across posterior probability distributions of phylogenies (Pagel and Meade, 2006). For all MCMC analyses, I used 3000 phylogenies obtained from a posterior sample in a recent Bayesian phylogeny estimation (Jetz et al., 2012; http://birdtree.org/). I used a version of the phylogenies built only from genetic data and a family 'backbone' provided by a previous phylogenetic estimation (Hackett et al., 2008). I ran all analyses in BayesTraits (Pagel et al., 2004). I excluded species for which I had nest data but that were not included in the phylogenetic sample from Jetz et al. (2012) from further analysis (58 exclusions, final n = 97). A maximum clade credibility phylogeny from the posterior sample of phylogenies is presented in Figure 5.1.

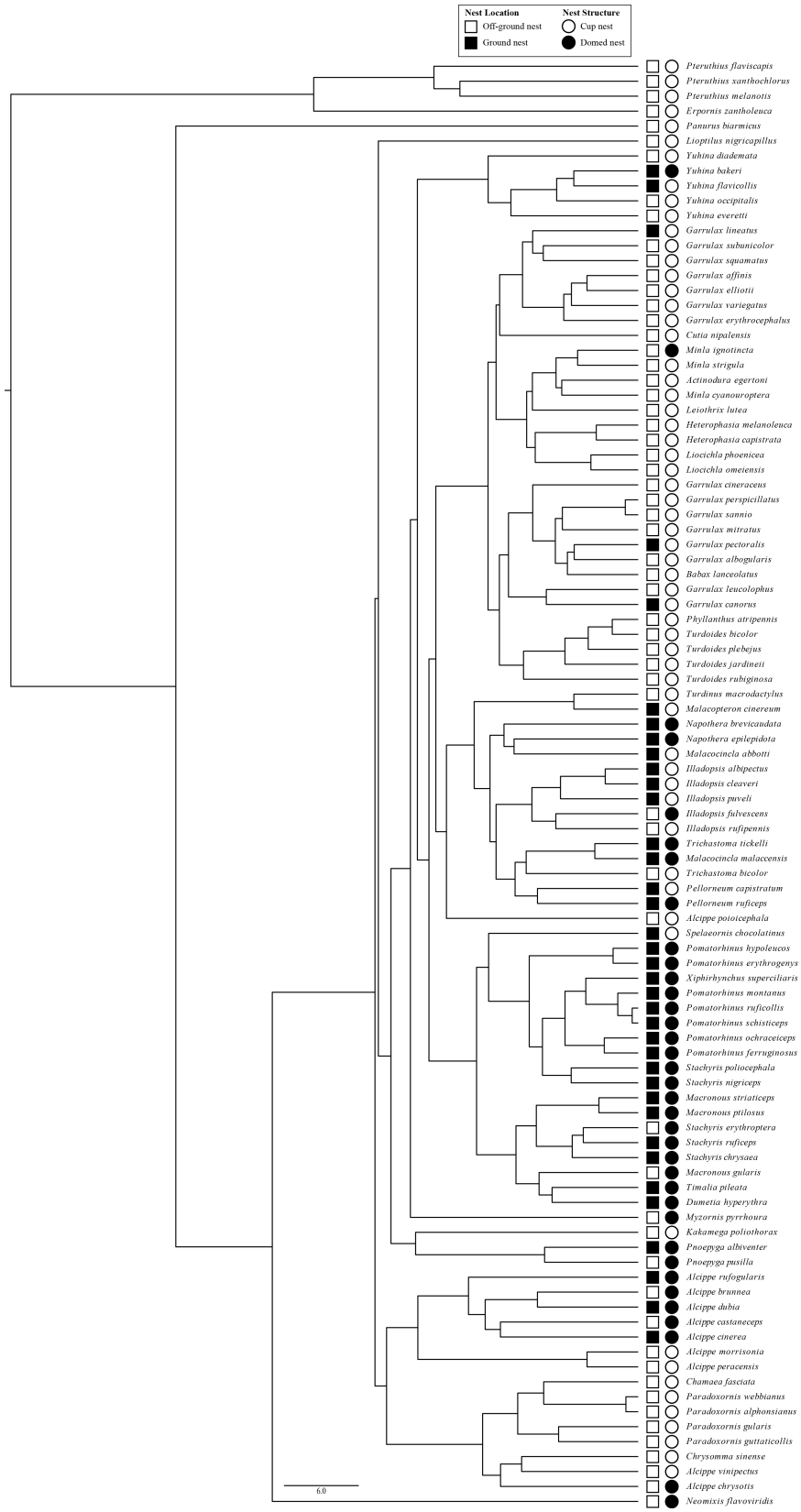

Nest Location
Off-ground nest
Ground nest
Nest Structure
Cup nest
Domed nest
Pteruthius flaviscapis
Pteruthius xanthochlorus
Pteruthius melanotis
Erpornis zantholeuca
Panurus biarmicus
Lioptilus nigricapillus
Yuhina diademata
Yuhina bakeri
Yuhina flavicollis
Yuhina occipitalis
Yuhina everetti
Garrulax lineatus
Garrulax subunicolor
Garrulax squamatus
Garrulax affinis
Garrulax elliotii
Garrulax variegatus
Garrulax erythrocephalus
Cutia nipalensis
Minla ignotincta
Minla strigula
Actinodura egertoni
Minla cyanouroptera
Leiothrix lutea
Heterophasia melanoleuca
Heterophasia capistrata
Liocichla phoenicea
Liocichla omeiensis
Garrulax cineraceus
Garrulax perspicillatus
Garrulax sannio
Garrulax mitratus
Garrulax pectoralis
Garrulax albogularis
Babax lanceolatus
Garrulax leucolophus
Garrulax canorus
Phyllanthus atripennis
Turdoides bicolor
Turdoides plebejus
Turdoides jardineii
Turdoides rubiginosa
Turdinus macrodactylus
Malacopteron cinereum
Napothera brevicaudata
Napothera epilepidota
Malacocincla abbotti
Illadopsis albipectus
Illadopsis cleaveri
Illadopsis puveli
Illadopsis fulvescens
Illadopsis rufipennis
Trichastoma tickelli
Malacocincla malaccensis
Trichastoma bicolor
Pellorneum capistratum
Pellorneum ruficeps
Alcippe poioicephala
Spelaeornis chocolatinus
Pomatorhinus hypoleucos
Pomatorhinus erythrogenys
Xiphirhynchus superciliaris
Pomatorhinus montanus
Pomatorhinus ruficollis
Pomatorhinus schisticeps
Pomatorhinus ochraceiceps
Pomatorhinus ferruginosus
Stachyris poliocephala
Stachyris nigriceps
Macronous striaticeps
Macronous ptilosus
Stachyris erythroptera
Stachyris ruficeps
Stachyris chrysaea
Macronous gularis
Timalia pileata
Dumetia hyperythra
Myzornis pyrrhoura
Kakamega poliothorax
Pnoepyga albiventer
Pnoepyga pusilla
Alcippe rufogularis
Alcippe brunnea
Alcippe dubia
Alcippe castaneceps
Alcippe cinerea
Alcippe morrisonia
Alcippe peracensis
Chamaea fasciata
Paradoxornis webbianus
Paradoxornis alphonsianus
Paradoxornis gularis
Paradoxornis guttaticollis
Chrysomma sinense
Alcippe vinipectus
Alcippe chrysotis
Neomixis flavoviridis
6.0

Figure 5.1. A maximum clade credibility phylogeny of Timaliidae species used in this study. Species-typical nest location (ground or off-ground) and structure (cup or domed) are listed following each species' scientific name. This maximum clade credibility phylogeny was constructed from a Bayesian posterior sample of 3000 phylogenies from Jetz et al. (2012) that had been constructed using genetic data only and a 'backbone' family estimation by Hackett et al. (2008). Scale bar represents 5 mya (Jetz et al., 2012).

Phylogenetic generalised least squares regression

I transformed lowest nest height data using log(x+1) transformation and compared these heights between cup- and domed-nest building species using the phylogenetic generalised least squares regression (PGLS) approach, as in Chapter 4, which incorporates phylogenetic relatedness into the error term of regression models (Grafen, 1989; Pagel, 1997). In this analysis, I included nest structure as an independent factor on two levels ('cup' and 'domed', where cup was the reference level) and nest height as a dependent continuous variable. I used MCMC to estimate posterior probability distributions for regression coefficients (β) and phylogenetic signal (λ; Pagel, 1999). I ran MCMC chains for PGLS analyses for 1 million iterations, sampling every 100 generations, with a 'burn-in' period of 50,000 iterations. I used uniform priors (range -100, 100) for all parameters.

As in Chapter 4, prior to analyses, I specified that where ≥95% of the posterior probability distribution of regression coefficients (β) was in the predicted direction (negative, following the prediction that domed nests are built at lower heights compared to cup nests), I would conclude that there was 'strong evidence' for the predicted relationship

(for example, Ross et al., 2012). I also report the mean λ from the posterior probability distributions.

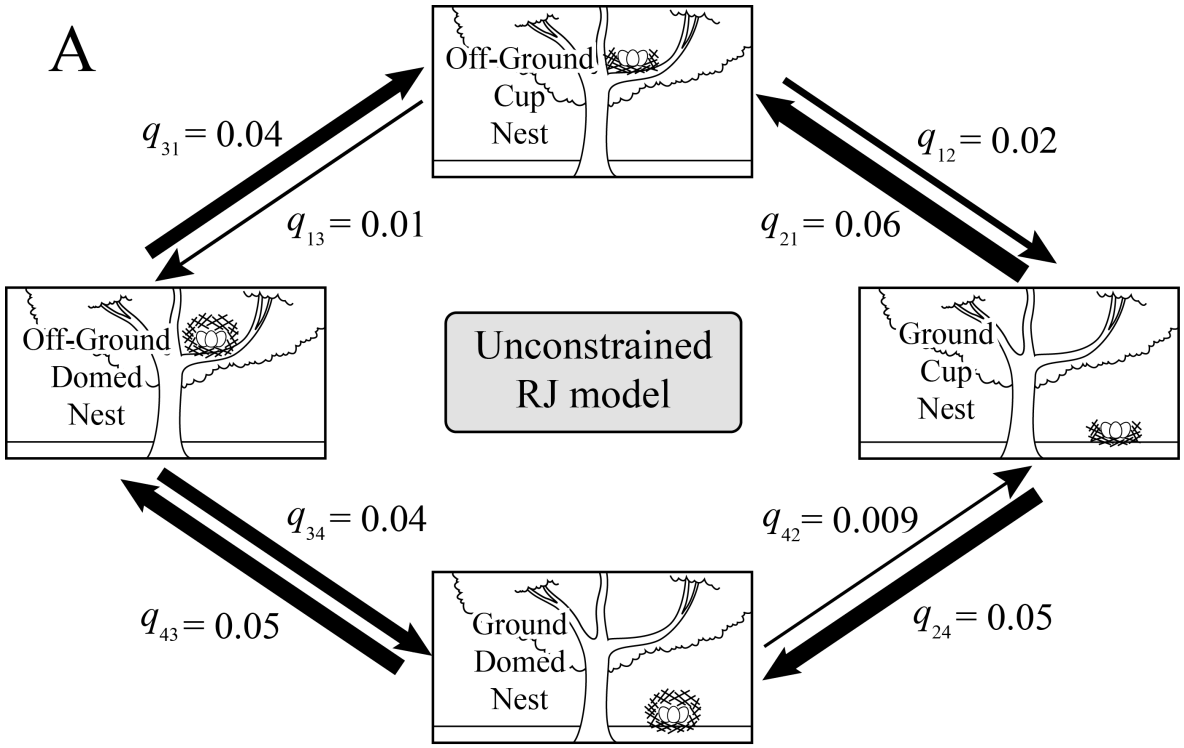
Co-evolution of binary traits

To investigate possible co-evolution of nest height and nest structure, I used Pagel's methods for detecting co-evolution of discrete character traits (Pagel and Meade, 2006). This approach uses continuous-time Markov models to estimate up to 8 transition rates between states of 2 binary traits. I coded nest height as 'ground' where nest height was 0 m, and 'off-ground' where nest height was >0 m. I coded nest structure as before. For these 'discrete' analyses (models depicted in Figure 5.2), I ran chains for 100 million iterations, sampling every 5000 generations, with a 'burn-in' period of 50,000 iterations, using exponential hyper-prior distributions (range 0, 5) for all parameters.

Dependent versus independent evolution

To compare models of dependent versus independent evolution of nest structure and height, I used the reversible-jump MCMC approach, which estimates transition rates whilst simultaneously selecting the best-fitting model of evolutionary change by visiting models in proportion to their posterior probabilities (Pagel and Meade, 2006). In the dependent reversible-jump model (Figure 5.2A), transition rates for each character are permitted to depend on the state of the other character, i.e. it is possible that $q12 \neq q34$, $q13 \neq q24$, $q43 \neq q21$ and $q42 \neq q31$, whereas in the independent reversible-jump model (not shown), transition rates for each character are not permitted to depend on the state of the other character, such that $q12=q34$, $q21=q43$, $q13=q24$ and $q31=q42$.

To investigate specifically the hypothesis that building a domed nest co-evolved with building on the ground, (i.e. *q12<q34, q13<q24, q43<q21,* and *q42<q31*), I also ran a reduced, non-reversible-jump dependent model (Figure 5.2B) in which two transition rates were estimated, one corresponding to state transitions that I predicted would not be favourable (i.e. toward building a cup nest on the ground and building a domed nest off the ground: *q12, q13, q43* and *q42*) and one corresponding to state transitions that I predicted would be favourable (i.e. toward building a cup nest off the ground and building a domed nest on the ground: *q34, q24, q21* and *q31*; Figure 5.2B). I predicted that the former rate would be smaller than the latter rate. I compared this reduced, non-reversible-jump two-rate model to a reduced, non-reversible-jump one-rate model corresponding to independent evolution of the traits (not shown), as well as to the unconstrained dependent reversible-jump model.


A
Off-Ground Cup Nest
Off-Ground Domed Nest
Ground Cup Nest
Ground Domed Nest
Unconstrained RJ model
$q_{31} = 0.04$
$q_{13} = 0.01$
$q_{12} = 0.02$
$q_{21} = 0.06$
$q_{34} = 0.04$
$q_{43} = 0.05$
$q_{42} = 0.009$
$q_{24} = 0.05$

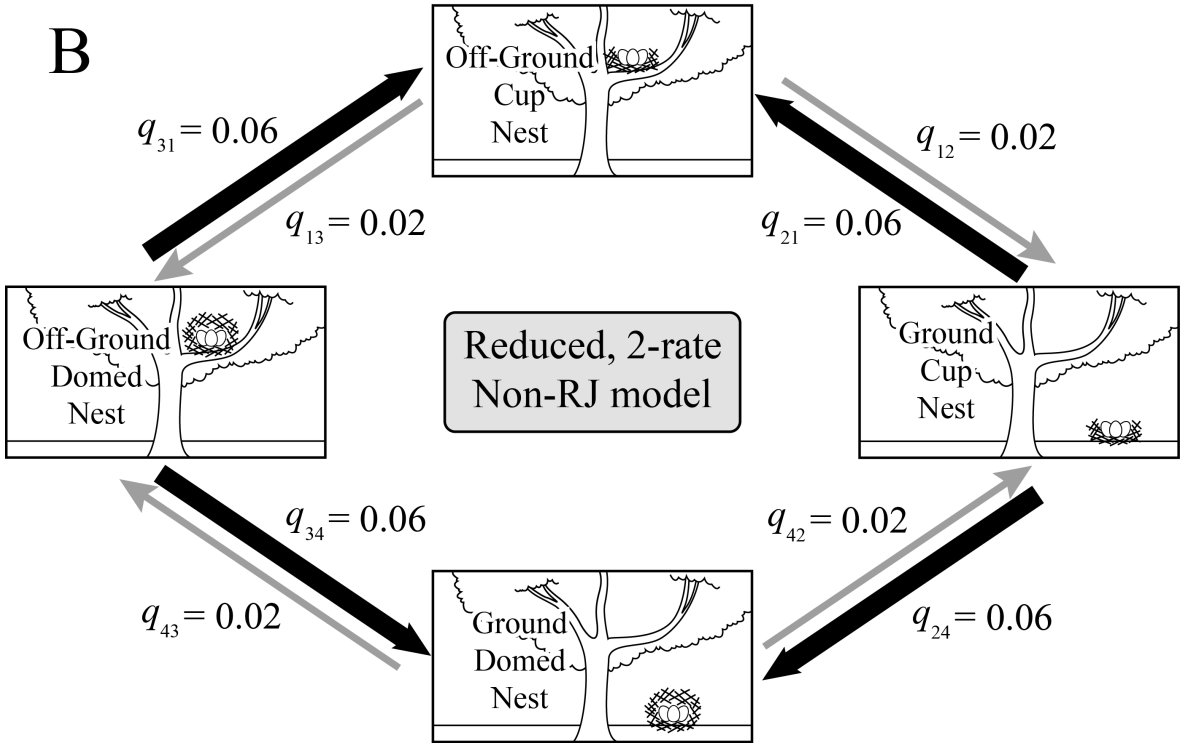

B
Off-Ground Cup Nest
Off-Ground Domed Nest
Ground Cup Nest
Ground Domed Nest
Reduced, 2-rate Non-RJ model
$q_{31} = 0.06$
$q_{13} = 0.02$
$q_{12} = 0.02$
$q_{21} = 0.06$
$q_{34} = 0.06$
$q_{43} = 0.02$
$q_{42} = 0.02$
$q_{24} = 0.06$

Figure 5.2. Two transition rate models used to investigate the co-evolution of nest height and structure in Timaliidae. (A) An unconstrained, dependent reversible-jump (RJ) model used to estimate 8 evolutionary transition rates (*q*) corresponding to all possible transitions between nest height and nest structure state combinations. (B) A reduced, non-RJ dependent model of nest structure and height in which estimated only two transition rates: transitions toward nest states predicted favourable (black arrows; toward off-ground cup nest and ground domed nest; *q34, q24, q21* and *q31*) and transitions away from nest states predicted to be favourable (gray arrows; *q12, q13, q43* and *q42*). Arrow thickness is proportional to likelihood of the associated transition.

Ancestral states

To investigate the most likely ancestral state of nest structure and nest height in the most recent common ancestor, I compared three models in which the most recent common ancestor was fixed as either 1) building a cup nest on the ground, 2) building a domed nest off the ground, or 3) building a domed nest on the ground to a model in which the most recent common ancestor was fixed as the predicted ancestral state (off-ground/cup-nesting). I compared ancestral states models both for the full, dependent reversible-jump model, and for the reduced, non-reversible-jump two-rate dependent model.

Order of evolutionary transitions

I investigated the likely order of evolutionary transitions by testing whether transitions from building a cup nest off the ground to building a domed nest on the ground were more likely to occur through changes in nest height or nest structure (i.e. whether

q12≠q13). I also tested whether transitions from building a domed nest on the ground to building a cup nest off the ground were more likely to occur through changes in nest height or nest structure (i.e. whether *q43≠q42*; Pagel, 1997). I therefore compared reversible-jump dependent models in which the rates of interest were fixed as equal (transitions through nest structure and height being equally likely) to unconstrained reversible-jump dependent models with the prediction that, if the transition rates in nest structure and height differ, the unconstrained models should be supported over the restricted models.

Model diagnostics and comparison

For all analyses, I ran three MCMC chains to ensure that chains converged on similar values. All reported model parameters were averaged across the three chains. I used the program 'Tracer' (Rambaut and Drummond, 2009) for visual examination of chains to ensure convergence and to estimate effective sample size for posterior probability distributions (*E.S.S.)*. No analysis reported an effective sample size below 13,000 for model parameters. I used Bayes Factors (*B.F.*) to compare model fit based on the harmonic means of the model likelihoods where, by convention, a positive value of >2 is taken as 'positive evidence' and 5-10 as 'strong' evidence for the better fitting model (Pagel et al., 2004). I took harmonic means from the final iteration in the MCMC chain.

Results

Nest heights of cup and domed nests

I found strong evidence that species that build domed nests build them closer to the ground than do those species that build cup nests (Figure 5.3; 99% $\beta < 0$, $\lambda = 0.64$, $n = 97$).

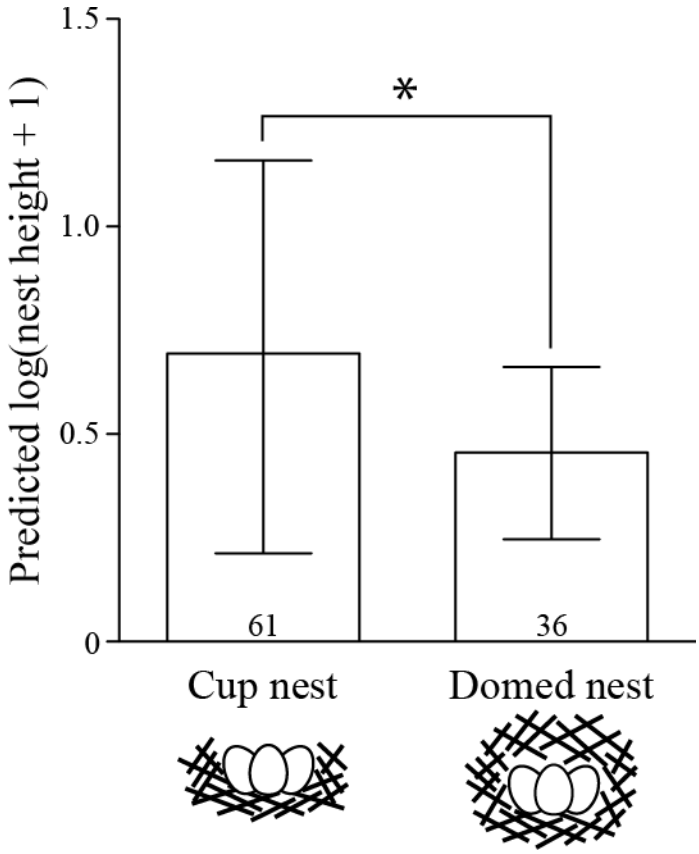


Figure 5.3. Domed-nesting species in Timaliidae build nests at lower heights than cup-nesting relatives. Bars represent average predicted log(x+1)-transformed lowest nest heights of cup and domed nesting species in Timaliidae calculated using phylogenetic least squares regression. Error bars represent 95% confidence interval. *≥95% of the posterior probability distribution of regression coefficients were in the predicted, negative direction (following the prediction that domed-nesting species would construct nests at lower heights than cup-nesting relatives).

Co-evolution of domed- and ground-nesting

I found positive evidence for the unconstrained, dependent reversible-jump model over the unconstrained, independent reversible-jump model (*B.F.* = 4.0, *n* = 97), suggesting

co-evolution of nest structure and nest height. Mean transition rates from in the unconstrained dependent reversible-jump model supported the hypothesis of both co-evolution of building a domed nest on the ground and building a cup nest off the ground, i.e. *q12<q34, q13<q24*, *q43<q21* and *q42<q31* (Figure 5.2A).

The reduced, non-reversible-jump, two-rate model of dependent evolution was strongly favoured over a reduced, non-reversible-jump one-rate model of independent evolution (*B.F.* = 9.0, *n* = 97), further suggesting co-evolution of both building of domed nests with nesting on the ground and the building of cup-nests when nesting off the ground. I also found positive evidence for the reduced, non-reversible-jump, two-rate model over the unconstrained, reversible-jump dependent model (*B.F.* = 4.8). Mean transition rates estimated in the reduced, non-reversible-jump 2-rate model of dependent evolution corresponded to the hypothesis of co-evolution of domed-nests with building on the ground and cup-nests with building off the ground, i.e. *q12<q34, q13<q24*, *q43<q21* and *q42<q31* (Figure 5.2B).

Ancestral states

Under the unconstrained reversible-jump dependent model, the most probable ancestral state was building a cup nest off the ground. I found positive evidence that a cup nest built off the ground was more probable than was a cup nest built on the ground (*B.F.* = 3.28), but I had insufficient evidence to show that a cup nest built off the ground was more probable as the ancestral state than was a domed nest built off the ground (*B.F.* = 1.14). I found strong evidence that a cup nest built off the ground was the more probable ancestral state than was a domed nest built on the ground (*B.F.* = 7.05).

Under the reduced two-rate non-reversible-jump dependent model, the most probable ancestral state was also building a cup nest off the ground. I had insufficient evidence to show that building a cup nest off the ground was more probable than was building a cup nest on the ground (*B.F.* = 1.58), but I found positive evidence that building a cup nest off the ground was a more probable ancestral state than was building a domed nest irrespective of location (on-ground: *B.F.* = 4.72, off-ground: *B.F.* = 2.43).

Order of evolutionary transitions

Transition rates from the unconstrained reversible-jump dependent model (Figure 5.2A) suggest that a change from building a cup nest off the ground to building a domed nest on the ground was more likely to occur through a change in nest height than in nest structure (i.e. $q12 > q13$). Similarly, a change from building a domed nest on the ground to building a cup nest off the ground was more likely to occur through a change in nest height than in nest structure (i.e. $q43 > q42$). Fixing $q12 = q13$, however, did not reduce model fit relative to the unconstrained reversible-jump model (*B.F.* 3.0, in favour of the reduced model), suggesting that changes in nest height and nest structure when building cup nests off the ground are equally likely. Reversible-jump models that fixed $q43 = q42$ did reduce the model fit in comparison to the unconstrained dependent reversible-jump model (*B.F.* 4.2, in favour of the unconstrained model), suggesting that a transition from building a domed nest on the ground to building a cup nest off the ground is more likely to occur through a change in nest height than in nest structure.

Discussion

Using phylogenetic comparative statistical techniques, I found evidence to support the proposed co-evolution of nest height and structure in Old World Babblers (Timaliidae). Together, my analyses showed that those species in this group that build domed nests build their nests at a lower height than do related species that build cup nests and strongly suggest that building a domed nest and nesting on the ground co-evolved as derived traits. Furthermore, although transitions away from building a cup nest off the ground are equally likely to occur through changes in either nest height or nest structure, transitions away from building a domed nest on the ground are more likely to occur through changes in nest height rather than in nest structure. To my knowledge, this is the first demonstration of co-evolution between the structure and location of bird nests.

Using nest height as a continuous variable, I found that domed-nesting babblers construct nests at lower heights than do cup-building relatives. Comparison of dependent models of evolution in which transitions in nest height were permitted to depend on transitions in nest structure were favoured over models in which nest height and structure evolved independently. In restricted models of dependent versus independent evolution, evolutionary transitions towards either building a cup nest off the ground or a domed nest on the ground are more likely than are transitions away from these two nest state combinations. These data support Collias' (1997) original prediction that nest height and structure co-evolve in Timaliidae.

Although my analysis here provides strong support that ground-nesting and building a domed nest co-evolved as derived traits in Timaliidae, my findings can only provide indirect support for these transitions being driven by selective factors including nest-site

competition and predation, as hypothesised by Collias (1997). Future studies should look to obtain direct measurements of nest predation at varying heights in the forest edge environments species in Timaliidae inhabit to provide more direct evidence for a role of predation and nest-site competition in the evolution of nest-building behaviour. Furthermore, other factors, such as protection from weather conditions at different heights in the forest, should be considered and tested in future studies on the evolution of nest structure in Timaliidae. Because this analysis was constructed from Collias' hypothesis and no other current hypotheses have been established to predict the co-evolution described here, I will mainly focus on how our data alongside Collias' hypotheses might suggest a role for predation at specific heights in shaping nest-building behaviour in birds. Outside of species nesting in cavities, a role for nest-site competition has not been measured in species that construct cup and domed nests, but here I suggest Timaliidae may be an interesting system to study whether this competition could influence nest-building behaviour in birds.

In addition to providing support for the co-evolution of nesting on the ground and constructing a domed nest, here I provide some of the first cross-species statistical evidence to support the idea that building a cup nest off the ground or a domed nest on the ground are both more likely to be favoured by selection (hypothesised by Collias [1997] to be attributed to reduced predation pressure) than are either domed nests built off the ground or cup nests built on the ground, at least in the Timaliidae (Collias and Collias, 1984). Also in support of a role for ground predation in influencing nest-building behaviour (as hypothesised by Collias [1997]), ground predation by introduced terrestrial mammals seems to explain the change in nest elevation in the Hawaiian monarch flycatcher (*Oahu elepaio*), which now constructs its open nest 50% higher than was reported in 1995

(Vanderwerf, 2012). A general change in nest height in response to changing predation pressure is not necessarily to be expected, however, as pointed out by Newmark and Stanley (2011): the effect of nest height on nest predation is likely to be species-specific and influenced by the importance of predators operating at different heights in different habitats. In support of selection favouring enclosed nests on the ground, a previous study using artificial nests placed on the ground found that eggs placed in domed nests were less susceptible to predation than were those placed in cup nests (Linder and Bollinger, 1995). Although studies using artificial nests to assess predation rates have been heavily criticised for a lack of external validity (Moore and Robinson, 2004), my results indirectly support the conclusions of Linder and Bollinger (1995), as here I found that selection is likely to favour domed nests over cup nests when building on the ground in Timaliidae.

Both my two different models of dependent evolution (unconstrained reversible-jump and restricted, non-reversible-jump) demonstrated that building a cup nest off the ground is more likely to have been the ancestral state than was building a domed nest on the ground in the Timaliidae. These results support Collias' (1997) prediction that domed-nests and building on the ground co-evolved as derived traits in this family. When I examined the order of evolutionary transitions from cup nests off the ground to the likely derived state of domed nests on the ground, I found that changes in either nest structure or nest height were equally likely, providing support for both of these evolutionary pathways. In contrast, Collias (1997) predicted that transitions from a building a cup nest off the ground state would occur primarily as shifts to ground-nesting to avoid competition for limited nest sites off the ground. The effect of competition for nest sites on nest site selection is well documented in species nesting in natural or excavated cavities

(Brightsmith, 2005). Furthermore, communal defence, territoriality, and an absence of coloniality in Timaliidae (Collar and Robson, 2007) may restrict the number of nesting sites available off the ground. Investigating how competition for nest sites relates to selection for nest sites could help identify the selection pressures involved in the evolutionary transition toward nesting on the ground.

Unlike these transitions to building a cup nest on the ground, Collias (1997) argued that transitions from building a cup nest off the ground to building a domed nest off the ground are unfavourable because nesting off the ground already confers protection from predators and birds should avoid the presumed higher energetic cost of additional construction to create a nest roof (Bailey et al., 2014). Here I found that, from building a cup nest off the ground, transitions to building a domed nest off the ground were equally likely as transitions to building a cup nest on the ground. The evolutionary pathway to a domed nest built off the ground may be a response to increased nest predation: Newmark and Stanley (2011) found that, among nest structures, predation rates were the highest for open and cup nests regardless of nest height in Afrotropical bird communities inhabiting forest edges produced by fragmentation. Alternatively, the transition from cup to domed nest building in off-ground nesting lineages could represent another evolutionary path toward the construction of domed nests proposed by Collias (1997). Specifically, that domed nests may be favoured for those species that construct their nests in the canopy periphery because an enclosed nest could mitigate the effects of increased exposure to aversive weather experienced by nests placed farther away from the tree trunk. Incorporating nest location within off-ground sites could enable statistical tests of this alternative, but equally likely, evolutionary route.

Once birds were building domed nests on the ground, I found that transitions to nesting off the ground were more likely than were transitions to building a cup nest on the ground. This supports my prediction that evolutionary transitions in nest height would be more likely than would changes in nest structure. Previous reports on phenotypic plasticity in nest height (Lima, 2009; Vanderwerf, 2012) also suggest that nest height is more easily changed than is nest structure. Furthermore, transitions from building a domed nest on the ground to building a cup nest on the ground probably increase susceptibility to nest predation due to the abundance of ground predators in forest edge habitats (Söderström et al., 1998), making this transition highly unfavourable. Strong selection pressure against transitions from building domed nests to building cup nests in ground-nesting lineages is also supported by the transitions rates calculated in my unconstrained reversible-jump model (i.e. Figure 5.2A; $q_{42} < q_{43}$).

In sum, here I present the first formal analyses of co-evolution between nest height and structure in Timaliidae. I found that building a domed nest and doing so on the ground is highly likely to have co-evolved in this family as derived traits providing indirect support for suggestions that nest predation and nest site competition are two selective forces that may influence nest structure design and nest site selection.

Chapter 6 – General discussion

During the research in my thesis, I identified a number of neural circuits that may be involved in nest building in zebra finches and I used my own nest structure classification scheme to characterize how the evolution of nest structure relates to brain morphology and the hypothesised influences of nest-site competition and predation. Specifically, I used the expression of the immediate early gene product Fos, an indirect marker of neuronal activity, to identify brain regions exhibiting elevated brain activity during nest-building behaviour. Using phylogenetically-informed statistical techniques, I tested whether variation in cerebellar foliation, hypothesized to play a role in the development of motor control, could be explained by the structural complexity of the species-typical nest built. Also using phylogenetically-informed analysis, I performed the first formal statistical test to investigate the evolution of nest height and structure in Old World babblers (Timaliidae).

Summary of Fos production and nest-building behaviour relationships

By comparing the number of neurons producing the immediate early gene product Fos in different neural circuits to nest-building behaviour exhibited by male and female zebra finches, I identified brain regions that are activated during nest building. I showed that neuronal activity in all three components of the anterior motor pathway, the anterior striatum, the anterior nidopallium, and the anterior ventral mesopallium increased the more male zebra finches picked up nest material (Chapter 2).

In the social behaviour network, neuronal activity in the anterior hypothalamus and medial bed nucleus of the stria terminalis, ventral subdivision (BSTmv) increased the more

female finches spent time in the nest (Chapter 2), however, in BSTmv this relationship does not appear to involve vasotocinergic or mesotocinergic neuronal subpopulations (Chapter 3). Neuronal activity in the medial preoptic area and medial bed nucleus of the stria terminalis, dorsal subdivision (BSTmd) increased in nest-building birds, regardless of sex, compared to controls (Chapter 2) and, in BSTmd, this increased neuronal activity appears to occur specifically in mesotocinergic neurons (Chapter 3). By sampling neuronal activity specifically in vasotocinergic and mesotocinergic neuronal subpopulations in the social behaviour network, I found that, in male finches, neuronal activity in vasotocinergic neurons in BSTmd and BSTmv increased the more time a male spent together with his mate in the nest and the more a male picked up nest material, respectively (Chapter 3).

In the dopaminergic reward system, neuronal activity in the ventral tegmental area increased the more male finches picked up nest material (Chapter 2), however, this relationship did not appear to involve dopaminergic neurons within this brain region (Chapter 3). Instead, neuronal activity in dopaminergic neurons in the ventral tegmental area decreased the more male finches tucked material into the nest structure. Finally, neuronal activity in dopaminergic neurons within the central gray increased in male finches the more time they spent in the nest with their mates (Chapter 3).

In summary, I found evidence suggesting the anterior motor pathway, social behaviour network, and dopaminergic reward system may all be involved in nest-building behaviour. Furthermore, some aspects of nest-building behaviour may involve specifically the vasotocin-mesotocin and dopaminergic neuronal subpopulations contained in the social behaviour network and dopaminergic reward system, respectively. In the following sections of this discussion, I will speculate about how each of these neural circuits may contribute to

the control of nest-building behaviour and, reciprocally, what a role in controlling nest building might tell us about the more general functions of these neural circuits.

The anterior motor pathway and nest building

The pattern of increased neuronal activation in the anterior motor pathway during nest building suggests that the anterior motor pathway controls the initiation of motor sequences. In the original paper in which they described the anterior motor pathway, Feenders et al. (2008) reported elevated activity in this neural circuit following the production of a variety of locomotor behaviours in birds, although the importance of motor sequencing in these behaviours, which included wing-whirring in garden warblers (*Sylvia borin*) and hovering flight in hummingbirds, is difficult to assess. During nest building, however, activity in the anterior motor pathway increased the more male finches exhibited the first step in the nest-building sequence (the collection of material) but was unrelated to the number of times males deposited that material in the nest, the final step in the sequence. Because of this relationship, it seems plausible that the anterior motor pathway would be involved at the beginning of behavioural sequences. In order to test whether this neural circuit is involved in the beginning of motor sequences and not the specific action I quantified (picking up material; Chapter 2), it would be useful to record neuronal activity using electrophysiological techniques in the anterior motor pathway in birds while they perform motor sequences of interest. Using this paradigm both in birds performing sequences comprised of different actions (for example, nest building) as well as birds performing sequences consisting of the same action (for example, a series of pecks to receive a food reward as in Helduser and Güntürkün [2012]), it would be possible to test

whether neuronal activity is always associated with the beginning of a sequence or with the specific actions the sequence contains.

I propose that, in addition to this role in beginning motor sequences, the anterior motor pathway may also be involved in the learning and modification of motor actions, two functions for this neural circuit that were originally proposed by Feenders et al. (2008). Feenders et al. (2008) based their proposal on the evidence that the song-control nuclei that are involved in learning and modifying birdsong are located within close proximity to the brain regions comprising the anterior motor pathway. This proximity was interpreted to suggest that the anterior motor pathway plays a more general role in motor learning than does the nearby song-control system, which is involved exclusively in the motor learning associated with birdsong. We could test whether the anterior motor pathway is involved in motor learning by adapting paradigms previously used to demonstrate the relationship between the song-control system and motor learning involved in birdsong for testing the relationship between the anterior motor pathway and the motor learning involved in nest-building behaviour. For example, the lateral portion of the song nucleus MAN (lMAN), a song nucleus located within close proximity to the anterior nidopallium of the anterior motor pathway, is required to learn how to produce the actions involved in species-typical birdsong in juvenile male zebra finches (Bottjer et al., 1984). If the anterior nidopallium is involved in learning the actions required to build a nest then lesions to the anterior nidopallia in juvenile birds should lead to no improvement in nest-building skills with experience compared to intact controls, but without impairing previously learned motor skills. One behavioural system in which this could be tested is the development of weaving skill by male Village weaver birds who increase the number of pieces of nest material they

can weave successfully into a nest site with weaving experience (Collias and Collias, 1964). In this system, I would predict that anterior nidopallial lesions would prevent birds from improving their ability to successfully weave material into nest-sites compared to controls. Furthermore, such anterior nidopallial lesions should not impair weaving success compared to levels prior to lesioning, suggesting a deficit specifically in the learning of new motor skills and not previously learned skills or motor output. Furthermore, as lMAN is also involved in modifying previously learned birdsong in adulthood (Kao and Brainard, 2005; Kojima and Doupe, 2011), the anterior nidopallium may be involved in modifying nest-building actions in adult birds. If so, then lesions to the anterior nidopallia in adult male zebra finches might then lead to an experienced bird being unable to modify how he picks up and delivers nest material to the nest box and, instead, continue to use nest-building actions expressed prior to lesioning (Muth and Healy 2011). One could also use Helduser and Güntürkün's (2012) paradigm in which a bird is trained to peck five keys in a rewarded order to test if the anterior motor pathway is involved in modifying all behavioural sequences and not just nest building: birds trained to peck a specific sequence of buttons and subsequently given anterior nidopallia lesions should be unable to modify their sequence of pecks in response to changes in the rewarded sequence, for example, by adding a new peck or rearranging the rewarded order of pecks.

Finally, the concerted increase in neuronal activity in all three regions sampled in the anterior motor pathway during nest building provides support for a recent theory regarding the functional organisation of the avian telencephalon compared to mammalian neocortex. Whereas functional divisions in mammalian neocortex consist of a stack of six, abutting layers in which incoming information is received and processed through

connections between the six layers of neocortex, functional divisions in the avian telencephalon are historically considered to be comprised of interconnected, but anatomically isolated nuclei located throughout the brain (termed “nuclear” organisation; Karten, 1997). Because this distinction in brain organisation between mammals and birds has been interpreted as evidence that the neocortex and the avian pallia developed from distinct evolutionary processes (Karten and Shimizu, 1989), avian homologs of mammalian neocortex are rarely proposed, hampering comparative studies with the mammalian neocortex. Via a tract-tracing study in 2010, however, Wang et al. (2010) found that auditory regions abutting one another in the avian brain are heavily interconnected across striatal, nidopallial, and mesopallial brain divisions and resemble the connectivity reported across the six layers of mammalian neocortex. As a result it has been suggested that based on anatomical contiguity of brain regions across major brain divisions and similar molecular profiles during development the anterior striatum, anterior nidopallium, and anterior ventral mesopallium of the anterior motor pathway form a similar functional division akin to that reported in the avian auditory system and in the mammalian neocortex (Chen et al., 2013). Although the arrangement of regions in the anterior motor pathway suggests they may be involved in the same neural processes, data demonstrating that all regions of this pathway are functionally involved in the same types of information processing are crucial to defining them as a functional unit.

The concerted increase in neuronal activity in all three regions of the anterior motor pathway the more male finches picked up nest material is consistent with the notion that all three of these regions are involved in the same functional processes and are likely, therefore, to be interconnected and activated as a functional unit. One could confirm this by using

tract-tracing techniques to visualise connections between these three regions across pallial divisions. If the avian telencephalon and mammalian neocortex exhibit similar functional organisation, we could begin to study potentially homologous regions of the avian brain and mammalian neocortex to understand how the brain controls similar behavioural processes across these distinct taxa.

The social behaviour network and nest building

Whereas the anterior motor pathway appears to be involved in the motor control underlying behaviour, the social behaviour network is thought to be involved in the production of social behaviours including aggression, copulation, and parental care (Goodson, 2005). Prior to the work presented in this thesis, there was no evidence for the involvement of the social behaviour network in nest-building behaviour. Indeed, in the only previous study in which the authors looked for correlations between patterns of neuronal activity in the social behaviour network and nest-building behaviour in birds, they found no evidence for a relationship (Klatt and Goodson, 2013). As discussed in Chapters 2 and 3, it is plausible that those authors failed to find this relationship because, at least in part, they sampled the medial bed nucleus of the stria terminalis (BSTm) as a single brain region. As a result of Klatt and Goodson (2013)'s data, others have assumed that the social behaviour network is not involved in nest-building behaviour but have, instead, suggested that this behaviour may be controlled by the paraventricular nucleus of the hypothalamus (PVN; for example, Kelly and Goodson, 2014). To confirm this lack of relationship, however, the potential parcellation of BSTm into a dorsal and ventral subdivision (BSTmd and BSTmv, respectively) needs to be tested functionally. This could be done by focally lesioning or

administering vasotocin to each BSTm subdivision and testing for subsequent changes in nest-building behaviour. Based on my interpretation of the data from female nesting finches in Chapter 2, lesions to the BSTmv, but not BSTmd, in female finches would lead to these birds spending less time in the nest. Based on my interpretation of the data from male nest-building finches in Chapter 3, using a chronically implanting cannulae to administer vasotocin directly to BSTmd should increase the amount of time a male finch spends with his partner in the nest cup without affecting nest material collection, whereas administering vasotocin to BSTmv should increase the number of times males picked up nest material without influencing the time a male spends in the nest cup with his partner. Given that BSTm is increasingly studied for its role in a whole array of social and breeding behaviours (Goodson, 2005), it would be useful to determine whether functional subdivisions exist in this region sooner rather than later.

Because the social behaviour network appears to be involved in the expression of all breeding behaviours in birds, including courtship, copulation, incubation, territoriality (O'Connell and Hofmann, 2011), and now nest building, I propose that the social behaviour network is involved, at least in part, in coordinating the expression of these behaviours across the breeding season. This coordination could be achieved physiologically through temporal changes in the levels of hormones, nonapeptides, and neurotransmitters released and acting in the social behaviour network. Support for this possibility comes from the demonstration that knocking down mesotocin production in adult zebra finches using antisense mRNA both impairs pair formation and reduces nest occupation behaviour in females (Kelly and Goodson, 2014), suggesting that mesotocin in the brain may be necessary for both pair formation and the subsequent occupation and defence of a nest site

in female zebra finches. Whereas Kelly and Goodson (2014) suggest that this coordinated increase in both affiliative behaviours associated with pair formation and nest occupation occurs due to the actions of mesotocin in PVN, I found evidence that mesotocinergic neurons in BSTmd may be involved in possession of a nest site (Chapter 3). Mesotocin in the brain may, therefore, influence pair formation and nest occupation through its actions in PVN and BSTmd, respectively, in female zebra finches. Such coordinated changes in behaviour caused by the actions of a signalling molecular acting in multiple locations in the brain have been demonstrated in zebrafish (*Danio* rerio), in which widespread release of the neurotransmitter histamine produced changes in aggression, boldness, and exploration in adult fish (Norton et al., 2011). If mesotocinergic neurons in BSTmd are involved nest occupation and mesotocinergic neurons in PVN are involved in pair formation, administering mesotocin to the social behaviour network in female finches with BSTmd lesions should increase affiliation behaviours associated with pair formation without increasing nest occupation exhibited by these birds.

The dopaminergic reward system and nest building

Whereas it seems plausible that the anterior motor pathway and social behaviour network are involved in the motor control and coordination of nest-building behaviour, respectively, the dopaminergic reward system seems to be involved in reinforcing male nest-building behaviour in zebra finches. Specifically, activation of the ventral tegmental area in the dopaminergic reward system seems to reward nest material collection and discourages nest-building behaviour within the nest cup in male zebra finches. One would, then, expect that neuronal activity in the ventral tegmental area and dopaminergic neurons

within this brain region might reflect an individual bird's contributions to nest building in other bird species. For example, in a species in which females collect material and males construct the nest, activity across all neurons in the ventral tegmental area in females should increase the more a female picks up nest material and activity specifically within dopaminergic neurons should decrease the more the female contributes to construction of the nest at the nest site, as found in male zebra finches.

Although compared to the ventral tegmental area, much less is known about the functions of the central gray, my data provide some support for Goodson et al.'s (2009) suggestion that dopaminergic neurons in the central gray are involved in the motivation to communicate vocally with conspecifics. Quantifying as much nest-building behaviour and social behaviour performed by males and females within the nest is crucial for identifying whether or not neuronal activity in the central gray is associated with nest-building actions or, following Goodson et al.'s (2009) hypothesis, vocal interactions between the individuals in a nesting finch pair. Another approach to testing whether this dopaminergic subpopulation is involved in social interaction during nest building would be to sample neuronal activity in a male zebra finch building a nest while exposed to a female in an adjacent cage, where she is unable to enter the nest cup. If central gray dopaminergic neurons are involved in social interactions within the nest during nest building, then neuronal activity in this subpopulation should be both unrelated to any nest-building behaviour exhibited by the lone male and lower than in male finches building a nest with a female partner within the same cage.

Using neurobiology to compare nest building and tool use

In addition to an increasingly large body of work challenging the assumed genetic origins of nest building by identifying a role for learning and experience (see Chapter 1), my work provides new data enabling the comparison of the neurobiology of nest-building and tool use behaviour. Based on the data currently available regarding the neurobiology of nest-building behaviour and tool use, I propose that these two construction behaviours are controlled by the same neurobiological processes and may represent two different elaborations of the same sensory-motor processes (Barton, 2012).

One method for testing whether two behaviours use the same neurobiological processes is to demonstrate that the same brain regions are functionally involved in both behaviours. One brain region involved in both tool use and nest-building behaviour is the cerebellum. In primates, a larger cerebellum appears to have coevolved with the use of extractive foraging techniques (Barton, 2012) and, in birds, a more foliated cerebellum is coincident with tool use (Iwaniuk et al., 2009). Barton has suggested the enlargement of the primate cerebellum enables the learning and execution of increasingly elaborate behavioural sequences, including both tool use and the production and comprehension of language (Barton, 2012). There may be a similar relationship between cerebellar structure and function in birds: the evolution of a more foliated cerebellum may have enabled the learning and execution of increasingly elaborate behavioural sequences including both the manufacture and use of tools (Iwaniuk et al., 2009) and the manipulative abilities and motor sequencing required to construct a more structurally complex nest (Chapter 4).

Although this correlated evolution between the cerebellum, tool use, and nest building suggests that the evolution of a more foliated cerebellum supports these

behaviours, implying the function of a brain region by anatomy alone can be misleading (Healy and Rowe, 2007) and requires complementary functional studies to demonstrate that the cerebellum is active during both tool use and nest-building behaviour. Evidence that the cerebellum is activated during tool use in Japanese monkeys has been shown using positron emission tomography (*Macaca fuscata*; Obayashi et al., 2001) and although this functional imaging technique has recently been adapted for use in crows (Marzluff et al., 2012), current technological limitations prevent this technique from being used in smaller birds such as zebra finches. Using a similar Fos immunohistochemical protocol as in Chapters 2-3 to sample neuronal activity in the cerebellum would, however, address whether cerebellar activity is correlated with the production of nest-building behaviour and tool use in birds, providing some functional support for the involvement of the cerebellum in both behaviours. In addition to the cerebellum, several other regions in the mammalian brain are known to be activated during tool use (Obayashi et al., 2001), however, current debate over the homology of the avian telencephalon and mammalian neocortex complicates comparisons of neocortical brain regions active during tool use. One brain region found in both birds and mammals and activated during tool use is the striatum (Reiner et al., 2004; Obayashi et al., 2001), which I found is also activated during nest building in male zebra finches. With the data currently available, then, at least two brain regions, the cerebellum and striatum, appear to be involved in the production of tool use and nest-building behaviour, suggesting these behaviours use the same neurobiological processes. By mapping patterns of neuronal activity associated with tool use throughout the avian brain, the neurobiological comparison of these two behaviours can extend beyond the cerebellum and striatum to the rest of the brain.

The evolution of nest structure

I developed the first nest classification scheme to adapt pre-existing descriptions of nest structure into data amenable to formal statistical tests of evolution. By demonstrating that this classification scheme can be used to identify neural substrates (Chapter 4) that may be involved in nest structure, I also found evidence that this classification scheme may reflect some aspect of the behaviour underpinning the construction of the nest. Whereas previous studies attempting to identify evolutionary pressures that affect nest building have relied on comparisons between sympatric species, which may ignore other species differences in nest building, or on the use non-formal statistical techniques, such as outgroup comparison, the statistical models I used allowed me to explain variation in nest structure across a large number of species while accounting for species relatedness. The success of this analysis would suggest that this classification scheme and statistical approach could be used to test a number of other theories regarding the evolution of nest building, with the added benefit of using previously compiled descriptions of nest structures, eliminating the need for additional data collection. For example, Winkler and Sheldon (1993) found that the construction of an increasingly enclosed, retort-shaped nest coevolved with higher breeding densities in swifts (Apodidae). The authors hypothesise that constructing a more enclosed nest may lessen the threat of extra-pair fertilisations, a hypothesis that could be tested by investigating potential correlated evolution between nest structure and breeding density in this clade. Additionally, in my own analysis on nest structure, I found evidence suggesting that competition for limited nest-sites and predation are two key evolutionary pressures that have influenced the evolution of nest structure and location. Because Timaliidae is just one radiation of passerines, one would expect to see

nest-site competition and predation pressure influence nest location and structure in other groups of birds. For example, in study sites in Arizona and Arkansas forests, where predation pressure is lowest on the ground (Martin, 1993) one would expect species constructing nests off-ground should be more likely to construct a domed nest to confer protection from the heightened predation pressure. By using such comparative analyses, one might be able to elucidate the variety of evolutionary pressures that may have helped produce the tremendous diversity in nest structure seen today.

In this thesis, I sought to integrate data from behavioural, neural, and evolutionary sources and paradigms to enable a holistic understanding of nest-building behaviour. This approach has led me to not only identify neural substrates involved in nest-building behaviour but also how these neural substrates may specifically contribute to nest building and to identify the evolutionary pressures that may have acted on the brain and behaviour to produce variation in nest structures. For example, extrapolating from my interpretation of the data in Chapters 4 and 5, I could predict that elevated predation pressure on the ground would favour ground-nesting species with more foliated cerebella that may enable the manipulative skills to construct a domed nest and confer protection from this ground predation. By continuing to establish approaches to the neurobiological control of behaviour using both functional neuroscience and comparative studies, we understand not only how the brain controls behaviour but also how these brain-behaviour relationships may vary across species exhibiting behavioural differences.

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Appendix 1. Backwards elimination stepwise regressional models reported in Chapter 2. First, I present the variables entered and excluded by the backwards elimination regression process, followed by the full regressional model selected and the R^2 and R^2_{adj} values.

Models reported for all male finches

Song control system – Right HVC

Behavioural variables entered: **SingTime**

Behavioural variables excluded: SongBouts

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	4579.971	1	4579.971	6.072	0.028
	Residual	9804.962	13	754.228		
	Total	14384.933	14			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		6.142	<0.001
SingTime	0.564	2.464	0.028

$R^2 = 0.318$

$R^2_{adj} = 0.266$

Models reported for nest-building male finches

Anterior motor pathway – Anterior striatum (ASt)

Behavioural variables entered: **PickUp**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	22551.263	1	22551.263	9.427	0.028
	Residual	11960.451	5	2392.090		
	Total	34511.714	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		-1.741	0.142
PickUp	0.808	3.070	0.028

$R^2 = 0.653$

$R^2_{adj} = 0.584$

Anterior motor pathway – Anterior nidopallium (AN)

Behavioural variables entered: **PickUp, SingTime**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SongBouts, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	2650.386	2	1325.193	30.939	0.004
	Residual	171.329	4	42.832		
	Total	2821.714	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		-2.050	0.110
PickUp	0.801	6.451	0.003
SingTime	0.459	3.696	0.021

$R^2 = 0.939$

$R^2_{adj} = 0.909$

Anterior motor pathway – Anterior ventral mesopallium (AMV)

Behavioural variables entered: **PickUp**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	9490.583	1	9490.583	9.369	0.028
	Residual	5065.131	5	1013.026		
	Total	14555.714	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		-0.317	0.764
PickUp	0.807	3.061	0.028

$R^2 = 0.652$

$R^2_{adj} = 0.582$

Social behaviour network – Lateral septum, ventral caudal subdivision (LScv)

Behavioural variables entered: **SingTime**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SongBouts, PickUp, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	6063.135	1	6063.135	30.853	0.003
	Residual	982.579	5	196.516		
	Total	7045.714	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		7.843	0.001
SingTime	0.928	5.555	0.003

$R^2 = 0.861$

$R^2_{adj} = 0.833$

Social behaviour network – Lateral septum, rostral subdivision (LSr)

Behavioural variables entered: **Hop**

Behavioural variables excluded: Feed, Drink, Preen, Scratch, SingTime, SongBouts, PickUp, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	1022.759	1	1022.759	7.677	0.039
	Residual	666.098	5	133.220		
	Total	1688.857	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		11.512	<0.001
Hop	-0.778	-2.771	0.039

$R^2 = 0.606$

$R^2_{adj} = 0.527$

Social behaviour network – Medial septum (MS)

Behavioural variables entered: **PutDowns**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, PickUp, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	5358.035	1	5358.035	8.572	0.033
	Residual	3125.393	5	625.079		
	Total	8483.429	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		5.157	0.004
PutDowns	-0.795	-2.928	0.033

$R^2 = 0.632$

$R^2_{adj} = 0.558$

Social behaviour network – Ventromedial Hypothalamus (VMH)

Behavioural variables entered: **SingTime**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SongBouts, PickUp, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	829.953	1	829.953	8.404	0.034
	Residual	493.761	5	98.752		
	Total	1323.714	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		9.668	<0.001
SingTime	-0.792	-2.899	0.034

$R^2 = 0.627$

$R^2_{adj} = 0.552$

Dopaminergic reward circuit – Ventral Tegmental Area (VTA)

Behavioural variables entered: **PickUp**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	1022.770	1	1022.770	8.239	0.035
	Residual	620.658	5	124.132		
	Total	1643.429	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		-0.042	0.692
PickUp	0.789	2.870	0.035

$R^2 = 0.622$

$R^2_{adj} = 0.547$

Models reported for nest-building female finches

Social behaviour network – Anterior Hypothalamus (AH)

Behavioural variables entered: **NestTime**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, Allopreen, NestVisit

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	445.043	1	445.043	7.352	0.042
	Residual	302.671	5	60.534		
	Total	747.714	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		8.013	<0.001
NestTime	-0.771	-2.711	0.042

$R^2 = 0.595$

$R^2_{adj} = 0.514$

Social behaviour network – Bed nucleus of the stria terminalis, ventromedial subdivision (BSTmv)

Behavioural variables entered: **NestTime**, **Preen**

Behavioural variables excluded: Hop, Feed, Drink, Scratch, Allopreen, NestVisit

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	1146.275	2	573.138	14.831	0.014
	Residual	154.582	4	38.645		
	Total	1300.857	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		-1.417	0.229
NestTime	1.043	5.399	0.006
Preen	0.595	3.079	0.037

$R^2 = 0.600$

$R^2_{adj} = 0.519$

Social behaviour network – Ventromedial Hypothalamus (VMH)

Behavioural variables entered: **Preen**

Behavioural variables excluded: Hop, Feed, Drink, Scratch, Allopreen, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	373.638	1	373.638	14.362	0.013
	Residual	130.076	5	26.015		
	Total	503.714	6			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		17.455	<0.001
Preen	-0.861	-3.790	0.013

$R^2 = 0.742$

$R^2_{adj} = 0.690$

Appendix 2. Backwards elimination stepwise regressional models reported in Chapter 3. As in Appendix 1, first I present the variables entered and excluded by the backwards elimination regression process, followed by the full regressional model selected and the R^2 and R^2_{adj} values. TimeTogether = the time a bird spent in the nest with its mate (s).

Models reported for nest-building male finches

Vasotocinergic Neurons – Bed nucleus of the stria terminalis, mediodorsal subdivision (BSTmd)

Behavioural variables entered: **TimeTogether**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, PickUp, Tucks, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	0.052	1	0.052	14.048	0.010
	Residual	0.022	6	0.004		
	Total	0.074	7			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		5.041	0.002
TimeTogether	0.837	3.748	0.010

$R^2 = 0.701$

$R^2_{adj} = 0.651$

Vasotocinergic Neurons – Bed nucleus of the stria terminalis, medioventral subdivision (BSTmv)

Behavioural variables entered: **PickUp**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, Tucks, PutDowns, NestVisit, NestTime, TimeTogether

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	0.168	1	0.168	9.590	0.021
	Residual	0.105	6	0.017		
	Total	0.272	7			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		1.998	0.093
PickUp	0.784	3.097	0.021

$R^2 = 0.615$

$R^2_{adj} = 0.551$

Dopaminergic Neurons – Central gray

Behavioural variables entered: **TimeTogether**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, PickUp, Tucks, PutDowns, NestVisit, NestTime

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	0.113	1	0.113	33.564	0.001
	Residual	0.020	6	0.003		
	Total	0.133	7			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		6.902	<0.001
TimeTogether	0.921	5.793	0.001

$R^2 = 0.848$

$R^2_{adj} = 0.823$

Dopaminergic Neurons – Ventral Tegmental Area

Ventral tegmental area model in nest-building male finches

Behavioural variables entered: **Tucks**

Behavioural variables excluded: Hop, Feed, Drink, Preen, Scratch, SingTime, SongBouts, PickUp, PutDowns, NestVisit, NestTime, TimeTogether

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	0.006	1	0.006	6.405	0.045
	Residual	0.005	6	0.001		
	Total	0.011	7			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		6.293	0.001
Tucks	-0.719	-2.531	0.045

$R^2 = 0.516$

$R^2_{adj} = 0.436$

Ventral tegmental area model in nest-building female finches

Behavioural variables entered: **Feed**

Behavioural variables excluded: Hop, Drink, Preen, Allopreen, Scratch, Tucks, NestVisit, NestTime, TimeTogether

Model		Sum of Squares	df	Mean Square	F	*p*
1	Regression	0.001	1	0.001	11.923	0.014
	Residual	0.001	6	<0.001		
	Total	0.002	7			

Coefficients

Parameter	Standardized Coefficients	t	*p*
	Beta		
Constant		13.840	<0.001
Feed	-0.816	-3.453	0.014

$R^2 = 0.665$

$R^2_{adj} = 0.609$

Appendix 3. Nest structure classifications, source material, and body and brain measures for all species included in Chapter 4. Nest structure classifications made from descriptions in Book Sources and all body and brain measures were taken from Iwainuk et al. (2006).

Species name	Nest Structure	Book Source	Body size (g)	Brain volume (mm^3)	Brain-Cerebellum volume (mm^3)	Cerebellar Foliation Index
Anas platyrhynchos	Cup	del Hoyo et al., 1992	1111	5440	4683.92	4.0788
Apus apus	Cup	del Hoyo et al., 1999	38	642	535.67	3.3383
Collocalia esculenta	Cup	del Hoyo et al., 1999	5	121	92.49	3.2431
Larus novaehollandiae	Cup	del Hoyo et al., 1996	292	2941	2495.3	4.2401
Bombycilla garrulus	Cup	Anderson, 1915	55.5	1102	961.85	3.2916
Corvus corax	Cup	Soler et al., 1998	1175	14648	13535.2	4.8274
Erithacus rubecula	Cup	Gooders et al., 1982	16.2	592	518.07	3.1841
Garrulus glandarius	Cup	Goodwin, 1951	139	3806	3468.76	3.9679
Gymnorhina tibicen	Cup	Kaplan, 2004	314	5665	5181.73	4.9232
Hirundo rustica	Cup	Snow et al., 1998	19	531	451.71	3.2841
Parus major	Cup	Alabrudzinska et al., 2003	17.5	877	801.25	3.1619
Turdus merula	Cup	Walters, 1994	95	1745	1557.73	3.426
Doryfera ludovicae	Cup	del Hoyo et al., 1999	6	139	111.58	3.0386
Eutoxeres condamini	Cup	del Hoyo et al., 1999	9	257	215.47	2.9549
Glaucis hirsutus	Cup	del Hoyo et al., 1999	123	123	104.35	2.9638
Sephanoides sephaniodes	Cup	del Hoyo et al., 1999	5	134	115.42	3.1133
Aegotheles insignis	No Nest	del Hoyo et al., 1999	2120	1540	1297.6	3.6729
Eurostopodus argus	No Nest	del Hoyo et al., 1999	121	1013	877.48	2.9491
Nyctibius griseus	No Nest	del Hoyo et al., 1999	257	1980	1679.5	3.2389
Nyctidromus albicollis	No Nest	del Hoyo et al., 1999	53	910	709.44	3.2389
Actitis hypoleucos	No Nest	del Hoyo et al., 1996	47	746	647.43	3.3815

Scolopax rusticola	No Nest	del Hoyo et al., 1996; Volume 3	290	2503	2189.8	3.8149
Falco tinnunculus	No Nest	del Hoyo et al., 1994	230	3543	3098.1	3.9325
Falco berigora	No Nest	del Hoyo et al., 1994	562	6032	5400.4	3.8825
Meleagris gallopavo	No Nest	del Hoyo et al., 1994	9839	6781	5757.57	3.7991
Ardeotis australis	No Nest	del Hoyo et al., 1996	4450	10501	9428.8	4.675
Agapornis personatus	No Nest	del Hoyo et al., 1997	52.5	2824	2581.42	3.7498
Alisterus scapularis	No Nest	del Hoyo et al., 1997	160.4	4902	4489.46	4.3019
Ara chloropterus	No Nest	del Hoyo et al., 1997	1430	23497	21641.4	4.8904
Glossopsitta porphyrocephala	No Nest	del Hoyo et al., 1997	37	1855	1690.54	3.8303
Melopsittacus undulatus	No Nest	del Hoyo et al., 1997	43	1487	1320.64	3.9528
Nymphicus hollandicus	No Nest	del Hoyo et al., 1997	92	2161	1946.84	3.6187
Platycercus elegans	No Nest	del Hoyo et al., 1997	129	3628	3333	4.2206
Aegolius acadicus	No Nest	del Hoyo et al., 1999	86	2857	2642.36	3.5963
Asio otus	No Nest	del Hoyo et al., 1999	250	5321	4899.77	3.8359
Ninox boobook	No Nest	del Hoyo et al., 1999	231	6339	5847	3.5581
Tyto alba	No Nest	del Hoyo et al., 1999	290	5857	5412.88	3.852
Rhea americana	No Nest	del Hoyo et al., 1992	25000	19228	16254.11	4.5948
Struthio camelus	No Nest	del Hoyo et al., 1992	90000	39631	33786.69	5.3096
Clangula hyemalis	Platform	del Hoyo et al., 1992	911	4875	4247.59	3.1148
Melanitta nigra	Platform	del Hoyo et al., 1992	1191	5516	4845.15	3.5387
Melanitta fusca	Platform	del Hoyo et al., 1992	1896	7138	6307.88	3.6081
Podargus strigoides	Platform	del Hoyo et al., 1999	387	5759	5313.79	3.385
Steatornis caripensis	Platform	del Hoyo et al., 1999	414	3900	3313.71	3.1297
Larus argentatus	Platform	del Hoyo et al., 1996	1000	4312	3648.2	4.4696
Larus ridibundus	Platform	del Hoyo et al., 1996	250	2714	2239.77	3.9148
Limnodromus griseus	Platform	Harrison, 1978	109	1338	1210.94	3.3926
Bubulcus ibis	Platform	del Hoyo et al., 1992	366	4025	3642.93	4.2061
Columba palumbus	Platform	del Hoyo et al., 1997	450	2315	1977.29	3.6127
Ptilinopus superbus	Platform	del Hoyo et al., 1997	104	1052	901.53	2.9729

Aquila audax	Platform	del Hoyo et al., 1994	3350	15997	14146.55	4.7077
Buteo buteo	Platform	del Hoyo et al., 1994	900	8452	7282.85	4.3031
Haliaeetus leucogaster	Platform	del Hoyo et al., 1994	3004	12541	11164.89	4.6655
Bonasa umbellus	Platform	del Hoyo et al., 1994	650	3136	2867.61	3.9399
Perdix perdix	Platform	del Hoyo et al., 1994	401	1849	1625.66	3.4847
Phasianus colchicus	Platform	del Hoyo et al., 1994	1133	3865	3384.25	4.2058
Fulica americana	Platform	del Hoyo et al., 1996	651	2719	2471.67	3.2863
Corvus monedula	Platform	Wilmore and Wilmore, 1977	200	4593	4210.97	4.3009
Corvus corone	Platform	Wilmore and Wilmore, 1977	537	9382	8628.94	4.6097
Pelecanus conspicillatus	Platform	del Hoyo et al., 1992	5850	24880	23522.25	4.8202
Phoenicopterus ruber	Platform	del Hoyo et al., 1992	3000	10674	8908.31	4.5568
Thalassarche melanophrys	Platform	del Hoyo et al., 1992	3388	14129	11634.59	5.5338
Cacatua roseicapilla	Platform	del Hoyo et al., 1997	355	7456	6934.09	4.8683
Cacatua galerita	Platform	del Hoyo et al., 1997	765	13933	12868.28	5.3408

Appendix 4. Nest structure description, nest structure classification, and minimum nest height (m) for all species in Timaliidae included in Chapter 5.

Scientific Name	Nest description from del Hoyo et al., 1997	Nest Classification	Minimum Nest Height (m)
Actinodura egertoni	largish, rather deep cup	Cup	1
Alcippe brunnea	loose dome or semi-dome with entrance at upper part	Domed	0.1
Alcippe castaneceps	dome	Domed	1
Alcippe chrysotis	very deep cup, sometimes domed or egg shaped with side entrance	Domed	0.4
Alcippe cinerea	deep cup, sometimes domed or semi-domed	Domed	0
Alcippe dubia	loose oval or dome-shaped structure with entrance towards the top	Domed	0
Alcippe morrisonia	very compact, fairly strong cup or hanging basket	Cup	0.2
Alcippe nipalensis	usually neat and compact deep cup, rarely loosely woven and semi transparent	Cup	0.3
Alcippe peracensis	small cup	Cup	1.5
Alcippe poioicephala	roughly built, compact, deep cup, sometimes almost cone shaped	Cup	0.6
Alcippe rufogularis	rather loose dome or semi-dome or cup on large base of leaves, protected by whorl of upward pointing leaves	Domed	0
Alcippe vinipectus	bulky, fairly deep, compact cup	Cup	0.9
Babax lanceolatus	reportedly a loose but well defined open cup	Cup	0.6
Babax waddelli	large, rather rough cup, exterior woven	Cup	1.8
Chamaea fasciata	deep compact cup	Cup	0.3
Chrysomma sinense	small, compact, cone-shaped deep cup	Cup	0.5
Cutia nipalensis	open cup	Cup	3
Dumetia hyperythra	loose or neat dome, with side entrance, sometimes towards the top.	Domed	0
Erpornis zantholeuca	small, deep cradle	Cup	0.5
Garrulax affinis	large but neat cup	Cup	1
Garrulax albogularis	broad, shallow saucer to moderately deep cup	Cup	1
Garrulax austeni	cup	Cup	0
Garrulax caerulatus	reportedly a large, compact, rather shallow to deep cup	Cup	1
Garrulax	reportedly a large cup, outwardly rough but with	Cup	0

canorus	well defined walls		
Garrulax chrysopterus	large, deep, cup	Cup	1.2
Garrulax cineraceus	reportedly a compact but often flimsy cup	Cup	1
Garrulax courtoisi	open cup	Cup	4
Garrulax elliotii	reportedly a fairly crude cup	Cup	0.5
Garrulax erythrocephalus	substantial, rather neat, deep cup	Cup	0.9
Garrulax galbanus	large, roughly made, flattish to deep cup	Cup	0.6
Garrulax gularis	reportedly a bulky, shallow, rather untidy cup	Cup	1
Garrulax leucolophus	large, shallow, rough cup	Cup	1.8
Garrulax lineatus	reportedly an outwardly loose, untidy, thick walled deep cup	Cup	0
Garrulax mitratus	loose cup	Cup	3
Garrulax monileger	broad, often shallow cup	Cup	1
Garrulax pectoralis	large, broad, bulky, rather shallow cup or saucer	Cup	0
Garrulax perspicillatus	large, crude, untidy cup	Cup	1
Garrulax ruficollis	compact, deep cup, untidy externally	Cup	1
Garrulax rufogularis	reportedly a fairly deep cup	Cup	0.6
Garrulax sannio	reportedly fairly compact, thick walled cup	Cup	0.6
Garrulax squamatus	reportedly a bulky, compact, or loose cup	Cup	1.2
Garrulax striatus	broad, usually shallow, strongly made cup	Cup	1
Garrulax subunicolor	cup	Cup	0.6
Garrulax sukatschewi	one nest was a cup	Cup	1.2
Garrulax variegatus	rather compact, sometimes untidy, usually rather shallow cup	Cup	0.15
Garrulax virgatus	reportedly a deep, rather neat, stoutly built cup	Cup	0
Heterophasia annectens	neat and compact cup	Cup	2
Heterophasia capistrata	neat cup, firmly interwoven	Cup	2
Heterophasia melanoleuca	cup	Cup	2.5
Heterophasia picaoides	very deep cup or bag	Cup	6
Illadopsis albipectus	only 1 nest described- a loose shallow cup	Cup	0

Illadopsis cleaveri	large, loose, shallow cup	Cup	0
Illadopsis fulvescens	large, loose, untidy, shallow cup, sometimes with half canopy	Domed	0.5
Illadopsis puveli	one nest was mossy cup, another a loose cup	Cup	0
Illadopsis rufipennis	2 types, a large, loose, deep cup and a rudimentary flat cup	Cup	0.8
Kakamega poliothorax	small, deep cradle	Cup	0.5
Leiothrix lutea	regular or oval cup, of varying depth and solidity	Cup	0.6
Liocichla omeiensis	robust cup with untidy base, completely shielded from above by row of bamboo leaves or placed in bush	Cup	0.3
Liocichla phoenicea	fairly deep, compact cup	Cup	0.6
Lioptilus nigricapillus	simple neat cup	Cup	1
Macronous bornensis	loose rough ball or tangle of material, strongly domed but with large entrance, giving impression of roofed cup	Domed	0
Macronous gularis	ball or rough dome, entrance at front or side (often near top)	Domed	0.3
Macronous kelleyi	untidy globe, slightly flattened in appearance	Domed	3
Macronous ptilosus	small or large loose ball or cup, with often oblong entrance at front or side	Domed	0
Macronous striaticeps	large, quite loose woven ball	Domed	0
Malacocincla abbotti	bulky, open, sometimes deep cup, often scantily lined	Cup	0
Malacocincla cinereiceps	cup	Cup	0
Malacocincla malaccensis	neat cup, sometimes semi roofed with large dead leaves	Domed	0
Malacopteron affine	loose shallow cup	Cup	1
Malacopteron cinereum	neat, fairly flimsy cup	Cup	0
Minla cyanouroptera	fairly small cup	Cup	2
Minla ignotincta	beautiful, small pendant shaped cup or rather deep purse	Domed	1.2
Minla strigula	neat cup	Cup	1.5
Myzornis pyrrhoura	globular structure	Domed	1
Napothera brevicaudata	upright dome with entrance near the top, a semi-dome or deep cup	Domed	0
Napothera epilepidota	dome, semi-dome or cup	Domed	0
Neomixis flavoviridis	an oval ball, with entrance near the top (SOURCE-del Hoyo et al. 2006)	Domed	1
Panurus	a deep cup-shaped structure, nearly always	Cup	0.5

biarmicus	roofed by sheltering vegetation		
Paradoxornis alphonsianus	cup shaped structure	Cup	0.5
Paradoxornis flavirostris	very, neat, compact, deep (rarely shallow) cup	Cup	1
Paradoxornis gularis	beautiful, very neat, compact, cup-shaped struture, sometimes with broad bulging sides	Cup	2
Paradoxornis guttaticollis	very compact and deep cup-shaped structure	Cup	0.9
Paradoxornis heudei	beautiful cup-shaped structure	Cup	1.3
Paradoxornis ruficeps	neat and compact deep cup	Cup	1
Paradoxornis webbianus	neat and fairly stiff, deep cup-shaped structure (rounded or oblong)	Cup	0.3
Pellorneum albiventre	small compact globe or dome, sometimes semi-dome or deep cup	Domed	0
Pellorneum capistratum	outwardly untidy cup	Cup	0
Pellorneum fuscocapillus	loose ball with large lateral entrance, or occasionally a cup	Domed	0
Pellorneum palustre	reportedly ball shaped	Domed	0
Pellorneum ruficeps	large, flimsy ball or dome, entrance at side, or a semi-dome or cup, sheltered by large upward pointing leaf	Domed	0
Phyllanthus atripennis	large, untidy cup	Cup	3
Pnoepyga albiventer	globular structure, entrance two thirds up one side	Domed	0
Pnoepyga formosana	dome or cylinder with entrance hole at one end	Domed	0
Pnoepyga pusilla	small ball of moss, rootlets, bark shreds and leaf skeletons, or a built in structure made of long strands of brilliant green moss, with tiny cup	Domed	0.5
Pomatorhinus erythrogenys	loose dome with broad entrance high up at side, or sometimes open at both ends	Domed	0
Pomatorhinus ferruginosus	oval or bulky cone placed on side, egg-shaped (Hume, A.O., 2004; The Nests and Eggs of Indian Birds, Vol.1)	Domed	0
Pomatorhinus gravivox	untidy dome with side entrance	Domed	0
Pomatorhinus horsfieldii	loose, often large dome, entrance on upper side, or a semi-domed cup	Domed	0.3
Pomatorhinus hypoleucos	large, semi-domed oval, but very open, part forming the roof sometimes flimsier, the cup fairly deep and more solid	Domed	0
Pomatorhinus mcclellandi	loose dome with side entrance	Domed	0
Pomatorhinus montanus	large dome or sheltered cup	Domed	0
Pomatorhinus	oval ball, loosely put together	Domed	0

ochraceiceps			
Pomatorhinus ruficollis	bulky, crude dome with entrance at the side or near top, or a cone on its side	Domed	0
Pomatorhinus schisticeps	large, loose dome, usually on its side, entrance at smaller end or at side	Domed	0
Pomatorhinus swinhoei	dome with side entrance	Domed	0
Pteruthius flaviscapis	loose but strong cradle or shallow cup	Cup	4.6
Pteruthius melanotis	flimsy looking but strong, small cradle	Cup	2
Pteruthius xanthochlorus	flimsy, deep purse or cradle	Cup	1.5
Rhopocichla atriceps	loose dome (also builds cock nests which aren't used for breeding)	Domed	0.6
Rimator malacoptilus	rather loose, untidy globe with entrance near the top	Domed	0
Robsonius sorsogonensis	large ball with large front entrance	Domed	0.6
Spelaeornis caudatus	cup-shaped, resembling earth brown paper mâche or as a dense mass of moss	Cup	0
Spelaeornis chocolatinus	one reported nest, deep cup with long back wall, though not enough to form a roof	Cup	0
Spelaeornis formosus	unauthenticated nest described as a deep, semi domed cup, densely lined	Domed	0
Spelaeornis longicaudatus	rather loose dome, occasionally when natural shelter is afforded it is a deep cup	Domed	0
Spelaeornis oatesi	large domed, sometimes firmly woven oval with entrance near top or side	Domed	0
Spelaeornis reptatus	loose ball	Domed	0
Stachyris chrysaea	dome or ball with entrance near the top	Domed	0
Stachyris erythroptera	loose or quite compact dome with side entrance	Domed	0.4
Stachyris maculata	loose globe or cup	Domed	0.5
Stachyris nigriceps	bulky, often loose cup or dome with wide entrance at front or side, often towards top	Domed	0
Stachyris nigricollis	dome with loose canopy of dry leaves and flat circular base	Domed	0
Stachyris nigrocapitata	a deep cup or cradle	Cup	1.2
Stachyris oglei	large, domed or globular structure with entrance near the bottom	Domed	0
Stachyris poliocephala	rather compact cup or dome covered in dead leaves	Domed	0
Stachyris pyrrhops	fairly deep cup or loose dome	Domed	0.6
Stachyris ruficeps	deep cup, or neat or loose uneven ball, oval or cone, entrance at side, often near the top	Domed	0
Strophocincla	bulky but compact, sometimes externally untidy,	Cup	0

cachinnans	usually deep cup		
Timalia pileata	rough ball, oval or dome with rather large entrance at the side or sometimes a deep cup.	Domed	0
Trichastoma bicolor	small, untidy, open cup	Cup	0.2
Trichastoma celebense	cup	Cup	0.3
Trichastoma rostratum	loose deep cup, roughly lined	Cup	0.4
Trichastoma tickelli	domed, semi-domed or deep cup, scantily or neatly lined ground, base of sapling or low bush or bamboo clump	Domed	0
Turdinus macrodactylus	large cup	Cup	0.4
Turdoides affinis	loose cup	Cup	1.2
Turdoides bicolor	large, rough, fairly deep open bowl	Cup	1.5
Turdoides caudata	neat, compact, rather thick walled, often rather deep cup	Cup	0.6
Turdoides earlei	massive but neat and compact cup (smaller and more compact when placed among reeds)	Cup	0.3
Turdoides fulva	loose deep cup	Cup	1
Turdoides hypoleuca	rough cup	Cup	1.5
Turdoides jardineii	bulky, open bowl	Cup	0.5
Turdoides malcolmi	rather loose but neat cup	Cup	1.2
Turdoides melanops	rough bowl	Cup	1.5
Turdoides nipalensis	deep cup	Cup	0
Turdoides plebejus	large, fairly shallow cup	Cup	0.75
Turdoides rubiginosa	untidy, open cup	Cup	0.3
Turdoides striata	fairly loose, deep or shallow cup	Cup	1.2
Turdoides tenebrosa	fairly deep cup	Cup	1
Xiphirhynchus superciliaris	large globular structure with entrance at one end, or blunt cone on its side with entrance at broad end	Domed	0
Yuhina bakeri	cup-shaped or dome-shaped structure	Domed	0
Yuhina diademata	flimsy almost transparent cup	Cup	0.2
Yuhina everetti	cup	Cup	0.5
Yuhina flavicollis	well made cup	Cup	0
Yuhina occipitalis	one nest was a cup	Cup	4
Yuhina torqueola	compact cup	Cup	0