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Study of the cognition
and its neural substrate
in New Caledonian crows

Felipe Salvador Medina Rodríguez

A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor in Philosophy,
The University of Auckland 2013
Dedicated to:

Ukko, Oy, Sampo, Angel, Corbelle, Lulu,
Machine Gun, Pulpo, Joker, Cuba, Ronia,
Quino, Maya, Tzo Tzo, Lazlo, Guille, Djidji,
Samson, Pug, Casper, Enzo, Bess, Junior,
Pepe, Bolt, Caesar, Kingpin, Djinn, Che,
Boxer, Eli, Korben,

and especially to:

Pelé, Batou, Español, Sisu, Tiga, Robin, Egg,
Slevin, and Obo.
Abstract

New Caledonian crows are renowned for their impressive tool culture in the wild and their advanced problem-solving skills in captivity. In toto, this suggests that their complex behaviour is based on flexible cognition. In this thesis, I combine research methods and tools from the fields of experimental and evolutionary psychology and neuroscience to investigate the cognitive abilities and neuroanatomy of the NC crow.

In the first part of this work, I carried out two experiments to show that NC crows’ cognition is indeed flexible, as had been inferred. In the first such study on wild-caught NC crows, I presented birds with a vertical mirror to investigate their mirror-induced responses. I also gave the NC crows a mirror-mediated spatial location task with a horizontal mirror to assess whether they could learn to use the mirror to locate visually displaced objects. The NC crows were able to exploit the correlation between an object’s image in the mirror and its location in the real world, in spite of their continuing agonistic social behaviour in front of vertical mirrors. In the second experiment, I gave NC crows a novel visually-restricted string pulling task to test between two alternative mechanisms behind avian spontaneous string pulling: visual reinforcement-independent insight and visual reinforcement-dependent operant conditioning. This study demonstrated the limitations of using visually-restricted string-pulling tasks to distinguish between insight and operant conditioning. The findings of these experiments support the view that the NC crow’s impressive cognitive abilities extend beyond their tool use.

The second part of this thesis presents the first detailed gross neuroanatomical and cytoarchitectural study of the NC crow’s telencephalon, along with the first brain atlas for this species. This research challenged previous claims that NC crows are a highly encephalised species and that their complex cognition is linked to enlargement of associative regions such as the mesopallium or increased foliation of the cerebellum. I also found no relationship between gross neuroanatomical interhemispheric asymmetries and eye dominance associated with lateralized tool holding. Last but not least, I describe the potentially important discovery of clusters of perineuronal satellite oligodendroglia in the telencephala of healthy NC crows and other passerine birds. In humans, reduced numbers of these clusters is linked to psychiatric disorders and schizophrenia.

I conclude by proposing that the slow development and prolonged parental care observed in NC crows, and the general evolutionary cephalisation process in corvids, have combined to forge a brain-mind capable of extraordinary feats.
Acknowledgements

Many people were instrumental in the completion of this thesis and deserve much more than just the words of appreciation I can fit in these pages.

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Con su corno francés
y su academia sueca
su salsa americana
y sus llaves inglesas
con todos sus misiles
y sus enciclopedias
su guerra de galaxias
y su saña opulenta
con todos sus laureles
el Norte es el que ordena.

Pero aquí abajo, abajo
cerca de las raíces
es donde la memoria
ningún recuerdo omite
y hay quienes se desmueren
y hay quienes se desviven
y así entre todos logran
lo que era un imposible
que todo el mundo sepa
que el Sur,
que el Sur también existe.

Mario Benedetti (1920-2009)
Extract from El Sur también existe
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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Arcopallium</td>
</tr>
<tr>
<td>APH</td>
<td>Area parahippocampalis</td>
</tr>
<tr>
<td>B</td>
<td>Nucleus basalis</td>
</tr>
<tr>
<td>BO</td>
<td>Bulbs olfactorius</td>
</tr>
<tr>
<td>CA</td>
<td>Commissura anterior</td>
</tr>
<tr>
<td>Cb</td>
<td>Cerebellum</td>
</tr>
<tr>
<td>CDL</td>
<td>Area corticoidea dorsolateralis</td>
</tr>
<tr>
<td>CIO</td>
<td>Capsula interna occipitalis</td>
</tr>
<tr>
<td>CO</td>
<td>Chiasma opticum</td>
</tr>
<tr>
<td>DLA</td>
<td>Nucleus dorsolateralis anterior thalami</td>
</tr>
<tr>
<td>DLP</td>
<td>Nucleus dorsolateralis posterior thalami</td>
</tr>
<tr>
<td>DMA</td>
<td>Nucleus dorsomedialis anterior thalami</td>
</tr>
<tr>
<td>DMP</td>
<td>Nucleus dorsomedialis posterior thalami</td>
</tr>
<tr>
<td>DSD</td>
<td>Decussatio supraoptica dorsalis</td>
</tr>
<tr>
<td>DSV</td>
<td>Decussatio supraoptica ventralis</td>
</tr>
<tr>
<td>E</td>
<td>Entopallium</td>
</tr>
<tr>
<td>FA</td>
<td>Tractus fronto-arcopallialis</td>
</tr>
<tr>
<td>FPL</td>
<td>Fasciculus prosencephali lateralis</td>
</tr>
<tr>
<td>FRL</td>
<td>Formatio reticularis mesencephali pars lateralis</td>
</tr>
<tr>
<td>FRM</td>
<td>Formatio reticularis mesencephali pars medialis</td>
</tr>
<tr>
<td>GCt</td>
<td>Substantia grisea centralis</td>
</tr>
<tr>
<td>GLd</td>
<td>Nucleus geniculatus lateralis pars dorsalis</td>
</tr>
<tr>
<td>GLv</td>
<td>Nucleus geniculatus lateralis pars ventralis</td>
</tr>
<tr>
<td>GP</td>
<td>Globus pallidus</td>
</tr>
<tr>
<td>HA</td>
<td>Hyperpallium apicale</td>
</tr>
<tr>
<td>HD</td>
<td>Hyperpallium densocellulare</td>
</tr>
<tr>
<td>Hip</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>HM</td>
<td>Nucleus habenularis medialis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>HVC</td>
<td>High vocal centre</td>
</tr>
<tr>
<td>ICo</td>
<td>Nucleus intercollicularis</td>
</tr>
<tr>
<td>Imc</td>
<td>Nucleus isthmi pars magnocellularis</td>
</tr>
<tr>
<td>Imp</td>
<td>Nucleus isthmi pars parvocellularis</td>
</tr>
<tr>
<td>IO</td>
<td>Nucleus isthmo-opticus</td>
</tr>
<tr>
<td>L</td>
<td>Area L pallii (also Field L)</td>
</tr>
<tr>
<td>LA</td>
<td>Nucleus laminaris</td>
</tr>
<tr>
<td>LM</td>
<td>Nucleus lentiformis mesencephali</td>
</tr>
<tr>
<td>LSt</td>
<td>Striatum laterale</td>
</tr>
<tr>
<td>M</td>
<td>Mesopallium</td>
</tr>
<tr>
<td>MAN</td>
<td>Nucleus magnocellularis nidopallii anterioris</td>
</tr>
<tr>
<td>MLd</td>
<td>Nucleus mesencephalicus lateralis pars dorsalis</td>
</tr>
<tr>
<td>MSt</td>
<td>Striatum mediale</td>
</tr>
<tr>
<td>MVL</td>
<td>Mesopallium ventro-lateralis</td>
</tr>
<tr>
<td>N</td>
<td>Nidopallium</td>
</tr>
<tr>
<td>NIII</td>
<td>Nervus oculomotorius</td>
</tr>
<tr>
<td>NC</td>
<td>Nidopallium caudale</td>
</tr>
<tr>
<td>OM</td>
<td>Tractus occipito-mesencephalicus</td>
</tr>
<tr>
<td>Ov</td>
<td>Nucleus ovoidalis</td>
</tr>
<tr>
<td>PT</td>
<td>Nucleus pretectalis</td>
</tr>
<tr>
<td>RA</td>
<td>Nucleus robustus arcopallialis</td>
</tr>
<tr>
<td>Rt</td>
<td>Nucleus rotundus</td>
</tr>
<tr>
<td>Ru</td>
<td>Nucleus ruber</td>
</tr>
<tr>
<td>SAC</td>
<td>Stratum album centrale</td>
</tr>
<tr>
<td>SGP</td>
<td>Substantia grisea et fibrosa periventricularis</td>
</tr>
<tr>
<td>SL</td>
<td>Nucleus septalis lateralis</td>
</tr>
<tr>
<td>SM</td>
<td>Nucleus septalis medialis</td>
</tr>
<tr>
<td>SNCc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SpL</td>
<td>Nucleus spiriformis lateralis</td>
</tr>
<tr>
<td>SpM</td>
<td>Nucleus spiriformis medialis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TeO</td>
<td>Tectum opticum</td>
</tr>
<tr>
<td>TnA</td>
<td>Nucleus taeniae amygdalae</td>
</tr>
<tr>
<td>TrO</td>
<td>Tractus opticus</td>
</tr>
<tr>
<td>TSM</td>
<td>Tractus septopallio-mesencephalicus</td>
</tr>
<tr>
<td>VeM</td>
<td>Nucleus vestibularis medialis</td>
</tr>
<tr>
<td>X</td>
<td>Area X</td>
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Chapter 1

Introduction

Cognition has many definitions. Here cognition refers to the mechanisms by which animals behave as if they had acquired, processed, stored, and acted on information from the environment\(^1\). This view recognises both the external and internal domains involved in the study of cognition, to which we (as observers/experimenters) sometimes may have simultaneous access. The external domain comprises what researchers usually distinguish as the animal’s interactions with the environment and the history (ontogeny) of such interactions. What is considered as the internal domain will depend on the particular focus of study or on the researcher’s background. For instance, to psychologists this is the domain of the mind and of mental processes. To neuroscientists, it may be something as radically different as the concerted changes in internal structure and activity state of the cells in the animal’s neural network at the time at which the observations/measurements were made. Both views generate different levels of explanation of the behaviours under study and have proved useful in the fields of psychology and neuroscience.

In thus defining cognition, I have specified the distinctions between (1) what we can observe and measure (i.e. behaviour, information from the environment), (2) what we can conceptually propose as generative explanation (i.e. mechanisms, acquisition, processing, storage), and (3) what behaviour fits best with the working hypothesis (i.e. to act on). These are often disregarded but crucial distinctions (Maturana & Varela 1987; Cronwell & Panksepp 2011). From a neurocognitive–biological viewpoint (see below), the use of words such as ‘information’ and ‘environment’ has a distinct communicational value but not a mechanistic explanatory one. The reason for this is that the nervous system does not work with information nor does it perceive the environment as we, the observers, do. Instead, the nervous system

\(^1\) Modified from Shettleworth (p. 4, 2010a).
operates with changes of structure (e.g. synaptic plasticity) and changes in activity state (e.g. electric membrane potential). This is why I have purposefully specified that the studied animals behave as if they knew something about the environment. In saying so, I have made explicit that the effectiveness or adequacy of the studied behaviours is entirely context-dependent, i.e. it is defined by the questions we (as researchers) make and the hypotheses we use to answer them (Maturana & Varela 1987).

Psychology meets neuroscience

Historically, the study of cognition in vertebrates has focused on animal models with allometrically\(^2\) large brains, such as primates, elephants, cetaceans, psittacids and corvids, because they all seem to exhibit what is usually called complex cognition\(^3\) (Schusterman 1986; Pepperberg 1991; Barton & Harvey 2000; Povinelli 2003; Emery & Clayton 2004a, 2004b, 2005; Emery 2006; Shoshani 2006; Connor 2007; Dunbar & Shultz 2007; Marino et al. 2007; Hart et al. 2008; Sol et al. 2008; Byrne et al. 2009). Recently, however, researchers have started to challenge this top-down view of cognition\(^4\) and the usefulness of continuing to treat certain animal behaviours as cognitively more complex has been called into question. The reason behind this shift is twofold. Firstly, more often than not, separating cognition into dichotomist subcategories, such as simple and complex, or associative and nonassociative, ignores abundant evidence that shows that complex behaviours can emerge from simple processes (von Bayern et al. 2009; de Waal & Ferrari 2010; Hemelrijk & Bolhuis 2011; Shettleworth 2010a, 2010b;

\(^2\) Originally, allometry was coined to indicate the rate of growth of a body structure (i.e. usually expressed in terms of size) with respect of that of the whole body or another standardised body part or structure (Huxley & Teissier 1936; Huxley et al. 1940). Today however, its use has broaden to include all biological scaling relationships such as the ones that exist between physiological and ecological traits as well as the morphological traits of living beings. Therefore, in its modern sense allometry “describes how biological traits or processes scale with one another” (Singleton 2010).

\(^3\) Primate-like complex cognition has been proposed to involve a “tool kit” consisting of: (1) causal reasoning (about both the physical world and mental states of conspecifics), (2) imagination, (3) behavioural flexibility, and (4) prospection (i.e. imagining possible future events) (Emery & Clayton 2004a).

\(^4\) In other words, to envisage cognition with an arbitrary human-animal divide, with humans on the top and the rest of the animals below.
Heyes 2012). Thus, the misuse of conceptual dichotomies has led to confusion and to unconstructive debates (see Cromwell & Panksepp 2011).

Secondly, a bottom-up approach to cognition is more in line with evolutionary biology and neuroscience\(^5\) (de Waal & Ferrari 2010; Shettleworth 2010b; but see also Bolhuis & Wynne 2009 and Hemelrijk & Bolhuis 2011). It has been recently shown that complex *human-like* capacities, such as future planning, reciprocal altruism, theory of mind, imitation and numerical representation can be effectively broken down into behavioural components that are present in many nonhuman species (e.g. Brosnan & de Waal 2002; Rizzolatti & Craighero 2004; Schacter et al. 2007; Call & Tomasello 2008; Pastalkova et al. 2008; Prather et al. 2008; Ferrari et al. 2009; Nieder & Dehaene 2009; Raby & Clayton 2009). Thus, in challenging the outdated fixation with complex cognition, it has been argued that priority should be given to efforts to document and understand common cross-species components of behavioural processes at the neural level (de Waal & Ferrari 2010).

The notion that the brain is the organ of the mind\(^6\) is not new. In the 19\(^{th}\) century a French physiologist, Pierre Flourens, said: “All sensory and volitional faculties exist in the cerebral hemispheres and must be regarded as occupying concurrently the same seat in these structures.” (p. 264, Gregory 1987). Today’s researchers who study comparative cognition agree with this view\(^7\) (e.g. Heyes & Huber 2000; Watanabe 2004), which is based in the implicit hypothesis

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\(^5\) Especially if one embraces the notion that the neural mechanisms of behaviour are indeed ancient, and that the same mechanism in the brain (e.g. a mirror neuron system) is responsible for complex behaviours such as bird song learning and cultural acquisition of tool use in primates (de Waal & Ferrari 2010).

\(^6\) This is the case at least in chordates.

\(^7\) In other words, modern researchers sustain that cognition in vertebrates is linked to the functioning brain. This view is held, regardless of how they study the development of brain function and its organisation. That is, whether cognitive neuroscientists believe it is helpful to reduce cognitive functions to the activity of a specific, specialised cerebral locus or to describe them as the result of the activity of several, integrated cerebral loci (see Hebb 1949; Puccetti & Dykes 1987; Sarter et al. 1996; Bach-y-Rita 2005).
that all mental processes are cerebral processes\(^8\) (Bunge 2002). Thus, when using the biological approach to animal cognition, one should explicitly acknowledge that when we speak of mental or cognitive abilities of individuals, we are referring to underlying processes produced by the activity of each individual’s nervous system\(^9\).

However, this does not mean that psychology (or cognition for that matter) should be reduced to neurophysiology. Far from it, psychology contributes with its own conceptual framework, hypotheses and methods of study to the field of animal cognition. Also, the activity of nervous systems is coupled\(^10\) with the environment. Importantly, this allows changes of state in the neural network that are triggered by environmental stimuli to happen (many of which can be controlled, altered or designed by the enthusiastic experimenter). The nervous system is also coupled with other systems present in the body (e.g. digestive, immune, endocrine), which further adds serious resistance to any attempt to reduce cognition to the physiology of nerve cells.

*Explaining behaviour in neural terms*

From the point of view of physiological psychology and cognitive neuroscience, behaviour\(^11\) can be explained in terms of activity of the nervous system\(^12\) (Hebb 1949; Thompson 1975, Kandel et al. 2000; Gazzaniga et al. 2009), which is not to say that behaviour does not exist without nerve cells or that behaviour is exclusively associated with the nervous system. Rather,

\(^8\) However, complex *vertebrate-like* brains are not strictly needed (see Chittka & Niven 2009). A more modern proposal of this hypothesis would state that *all animal cognitive processes emerge from operations of the nervous system*.

\(^9\) By doing so, we bypass the problematic mind-brain dualism that still permeates psychology (see Miresco & Kirmayer 2006).

\(^10\) *Couple* in this context means to connect electrochemically.

\(^11\) Behaviour as changes of posture or position of an organism that an observer describes as movements or actions in relation to a certain environment (*sensu* Maturana & Varela 1987).

\(^12\) This deterministic approach is also in accordance with model-dependent realism and the construction of mechanistic explanatory models and the search of general laws in science (Hawkins & Mlodinow 2010).
what nerve cells do well is to couple points from the sensory surfaces with points in the motor surfaces, and thus they “... expand the realm of possible behaviours by endowing the organism with a tremendously versatile and plastic structure.” (p. 92, Maturana & Varela 1987). Indeed, in vertebrates early multimodal (e.g. sensorimotor) experience (which is mediated by interneurons, sensory and motor neurons) helps to hone coordination in a way that is instrumental for developing the rich vertebrate behavioural repertoire\textsuperscript{13}, as revealed by classical experiments (e.g. Sperry 1956; Held & Hein 1963). Also, the enormous effect of the nervous system in behavioural versatility and plasticity lies in the microscopic structural dynamics of the neural network, especially the astronomical number\textsuperscript{14} of electrical and chemical synapses nerve cells can form among themselves. The total number of synapses and their distribution is in constant change (Maturana et al. 1984; Cooper 2005; Ben Achour & Pascual 2010), in a process of plasticity that is not only fundamental during early development, but that continues to play a major role in learning and in recovery from injury throughout an animal’s lifespan (Wiesel & Hubel 1965; Bach-y-Rita et al. 1969; Merzenich et al 1983; Bach-y-Rita 1990; Buonomano & Merzenich 1998; Pascual-Leone et al. 2005).

Also, the biological approach to the study of animal behaviour assumes that cognition is best understood in the context of evolution (Shettleworth 2010a). This means that the evolution of the brain is also linked to the evolution of cognition. This is well illustrated by the phenomenon of cephalisation which occurred during the ontogenetic history of cephalopods (octopuses, squid and cuttlefish), insects and vertebrates\textsuperscript{15}. Reported evidence of convergent evolution of cognitive abilities in these taxa, such as associative learning, observational learning, spatial

\textsuperscript{13} Its relevance in the construction of behaviour and cognition is further shown by research in neuroconstructivism and embodied cognition (e.g. Garbarini & Adenzato 2004; Smith & Gasser 2005; Sirois et al. 2008).

\textsuperscript{14} One estimation sets the number of synapses in the human neocortex alone in $1.4 \cdot 10^{14}$ (Drachman 2005).

\textsuperscript{15} In fact, two major transformations of the nervous system in the history of living organisms are thought to have triggered the radiation of the Chordate phylum: (1) the assembly and organisation of nervous cells into a single elongated compartment (i.e. the notochord), and (2) the concentration of a greater volume of nervous cells in the (cephalic) anterior portion of the body (i.e. cephalisation) (Maturana et al. 1984).
memory, tool-use and tool manufacture, is already at hand and continues to grow (e.g. Fiorito & Scotto 1992; Menzel & Giurfa 2006; Reznikova 2007; Dornhaus & Nigel 2008; Hochner 2008; Mather 2008; Bentley-Condit & Smith 2009; Chittka & Niven 2009; Edelman & Seth 2009; Ikeda 2009; Avarguès-Weber et al. 2012; Wystrach & Graham 2012). Importantly, by the process of cephalisation the nervous system adds thousands of millions of nerve cells between the afferent (sensory) and efferent (motor) neurons. This phenomenon increases the complexity of the nervous system circuitry and, consequently, increases the complexity and variety of animal behaviour (Hebb 1949; Maturana et al. 1984).

Enter an extraordinary tool manufacturer: the New Caledonian crow

As mentioned above, the behaviour of allometrically large brained vertebrates such as corvids has always captured the attention of animal cognition researchers (e.g. Balda et al. 1996; Heinrich 1999; Emery & Clayton 2004a; Lefebvre et al. 2004; Marzolf & Angell 2005, 2012; Cnotka et al. 2008a; Mehlhorn et al. 2010a). One species in particular, the New Caledonian crow, *Corvus moneduloides* (hereafter NC crow), stands out among corvids because of its complex tool culture and extraordinary tool manufacturing skills (Hunt 1996; Hunt et al. 2001; Weir et al. 2002; Hunt & Gray 2003; Weir & Kacelnik 2006; Hunt & Gray 2007; Holzhaider et al. 2008, 2010a, 2010b). To date, NC crows are the only nonhuman species capable of crafting hooked tools (Hunt 1996; Hunt & Gray 2004; Shumaker et al. 2011). Recent evidence also indicates that NC crows can use tools in novel ways (Taylor et al. 2010a, 2011, 2012a; Wimpenny et al. 2009, 2011).

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16 Corvidae, Corvoidea, Oscines, Passeriformes.

17 Fine three-dimensional sculpting of tools (i.e. tool crafting by imposing form to raw material) was unknown outside humans until NC crows were observed to sculpt hooks on their tool twigs (Hunt 1996; Hunt & Gray 2004; see also Weir et al. 2002). Hook sculpting represents the last of the three steps NC crows carry out during tool manufacture (i.e. [1] the selection of raw material, [2] trimming of side twig and removal of leaves, and [3] sculpting of the hook), and may well constitute the largest part of the entire process (see Hunt & Gray 2004). This type of dedicated sculpting by NC crows suggest that such complex manipulations are executed towards a predefined goal (i.e. to manufacture hooked tools) and further supports the view that this species may possess a rudimentary understanding of folk physics (Weir et al. 2002; Hunt & Gray 2004; but see also Hunt et al. 2006; Taylor et al. 2007, 2011, 2012a; Wimpenny et al. 2009, 2011).

Although extremely proficient tool-users, NC crows are also exceptional in solving cognitively demanding tasks using tools unlike those that they use in the wild (von Bayern et al. 2009; Taylor et al. 2011). This new evidence suggests that the NC crows’ underlying cognition is flexible and at the same time domain-general and domain-specific (Chiappe & MacDonald 2005; Shettleworth 2010a). This thesis has two main aims: (1) to build on previously published experimental work (Taylor et al. 2009, 2010b) by presenting NC crows with new tests of cognition, and (2) to investigate the neural underpinnings of NC crows’ complex behaviour. To accomplish these aims, I first carried out cognitive experiments with temporarily captive NC crows held in an outdoor aviary on the island of Maré, New Caledonia. These individuals were tested with novel versions of classical non-tool related tasks (see below). I then studied the brains of nine NC crows from Maré, focusing on their gross neuroanatomy and telencephalic cytoarchitecture. In doing so, I attempted to verify previous findings on bird brain–behaviour relationships (see below). I also discovered a cellular feature in NC crows and other passerine birds that could be important for understanding the evolution of complex behaviour in vertebrates generally, not just in birds.

Thesis description

The first part of this thesis deals with experiments that were designed to test whether NC crows are capable of solving problems that do not involve tools, at least not those that crows habitually use. The NC crows on Maré manufacture tools out of twigs and the barbed edges of Pandanus spp. bush leaves to extract invertebrates living in the tropical forest (Hunt 1996, Hunt & Gray 2003; Holzhaider et al. 2010a). I carried out experiments to investigate if the cognitive abilities of NC crows were indeed flexible, as had been inferred from this species’ extraordinary
tool-use and apparent ability to reliably transmit tool skills between generations via social learning (Holzhaider et al. 2010b).

In Chapter 2 I investigated NC crows’ responses to mirrors and tested whether they could learn to use mirrors to find hidden objects in novel locations. Studies of the mirror ‘mark’ test have usually been used to assess self-recognition in nonhumans and humans (Gallup 1970, Berenthal et al. 1978; Anderson 1983; Swartz 1997). In nonhuman primates, success in the ‘mark’ test is generally thought to indicate the presence of self-awareness (Bard et al. 2006; de Waal et al. 2005), in spite of evidence to the contrary (Lewis & Brooks-Gunn 1979; Johnson 1983; Mitchell 1993a, 1997a; Heyes 1994, 1996). Animals that do not conclusively pass the ‘mark’ test or cannot be easily tested with mirrors do, however, show other interesting mirror-induced responses such as mirror-triggered search, mirror-mediated spatial location and mirror-guided reaching task (Gallup 1970; Povinelli 1989; Pepperberg et al. 1995; de Waal et al. 2005). The study of such responses holds considerable unexploited potential for the comparative analysis of cognitive abilities necessary to perceive and process information made available by mirrors.

The study was designed to fill an important gap revealed by two previous mirror studies on corvids that reported contrasting findings. While Japanese jungle crows (Corvus macrorhynchos) continuously responded socially to their image when confronted with mirrors, Eurasian magpies (Pica pica) were reported to show self-contingent\(^\text{18}\) responses and to pass the ‘mark’ test for self-recognition (Kusayama et al. 2000; Prior et al. 2008). Because I suspected that the apparent contradictory nature of corvid mirror-induced responses could be partially explained by the different way mirrors were introduced to the birds in the above mentioned studies, the experiments were carefully designed to allow NC crows to quickly develop

\(^{18}\) Self-contingency is the ability to match sensory feedback received during a movement with internal motor and sensory representations. In mirror studies, self-contingent behaviour consists of reiterative animal movements performed in front of the mirror as if testing the relationship between its actions and the reflection of those actions (Prior et al. 2008). An example of contingent behaviour in magpies is available online in the Video S3 in the link: http://www.plosbiology.org/article/info:doi/10.1371/journal.pbio.0060202#s4.
responses beyond the default social ones produced by the sudden introduction of a vertical mirror into their daily routine. Thus, I wanted to determine whether the crows, when tested individually, would develop self-contingent behaviour in a relatively short period of time. Self-contingency had been described in the magpies that passed the ‘mark’ test, during their early mirror exposure, but not in the jungle crows. I also gave a mirror-mediated spatial location task with a horizontal mirror to four of our crows (two experienced and two naïve) in order to assess whether they could learn to use the mirror to locate hidden food. The crows’ behaviour would indicate whether they are able to exploit the correlation between an object’s mirror reflection and its location in the real world, or if they could develop an understanding of how mirrors represent objects in the environment, regardless of their behaviour in front of a vertical mirror. These findings attracted international media interest\textsuperscript{19}.

In Chapter 3 I studied NC crows’ string pulling behaviour. In a previous study (Taylor et al. 2010b), the findings had suggested that spontaneous string pulling was based on a sensorimotor reinforcement cycle (i.e. operant conditioning) rather than ‘insight’, as claimed by many researchers (Bierens de Haan 1933; Fischel 1936; Thorpe 1943, 1945; Heinrich 1995; Pepperberg 2004; Werdenich & Huber 2006). Therefore, I investigated string pulling in both the standard test (i.e. meat cubes attached to a perch via a length of string) and a novel visually-restricted version (i.e. an opaque disc was attached near the end of the string just above the meat cube) in order to test whether the crows’ spontaneous string pulling behaviour depended on visual feedback. Insight allows animals to build mental scenarios about how to solve novel problems before choosing an appropriate course of action (\textit{sensu} Heinrich 1995). Therefore, if our crows relied on insight to pull up the string, it was expected their performance would not be affected by the presence of an occluding disc.

\textsuperscript{19} BBC Nature article “Crows use mirrors to find food”, available in http://www.bbc.co.uk/nature/14897544. September 20, 2011.
The second part of the thesis involves an extensive neuroanatomical study of the NC crow brain with a special emphasis on the description of the crows’ telencephalon (i.e. major part of the forebrain). Importantly, I present the first NC crow brain atlas for future reference and investigated the gross and microscopic morphological aspects of the crows’ telencephalon, which are described in detail for the first time.

In Chapter 4, I analyse how the allometric study of brain–behaviour relationships can be useful in explaining the evolution of cognition and tool-use. I attempted to verify previously reported findings and inferences of evolutionary relationship and causality between brain measures and proxies to cognition (Cnotka et al. 2008a; Mehlhorn et al. 2010a; Iwaniuk et al. 2009). The brain measures under study were total brain mass (Cnotka et al. 2008a), the volume of the mesopallial component of the telencephalon (Mehlhorn et al. 2010a) and the degree of foliation of the cerebellum (Iwaniuk et al. 2009).

In Section 1, I first analysed our subjects’ brain and body weight data to verify previous claims of relatively higher encephalization rates in NC crows (Cnotka et al. 2008a). The records of captive weight data of nine crows led me to further test the reliability of capture body weight as a predictor of brain size in comparative log brain/body allometry studies. I also discussed the appropriateness of the allometric regression analysis and statistical methods used in Cnotka et al. (2008a) to estimate species encephalisation indices.

In Section 2, I tested whether lateralised tool holding, or tool handedness (sensu Rutledge & Hunt 2004), in six captive NC crows was correlated with potential volumetric asymmetries between brain hemispheres. The NC crows’ preference to manipulate tools exclusively with the proximal end on either the right or left side of their heads may be indicative of asymmetries between left and right sides of the avian equivalent of the mammalian ‘neocortex’. This volumetric study focused on the following brain regions: total telencephalon, mesopallium (part of the telencephalon), visual nucleus rotundus and auditory nucleus ovoidalis (both major relay nuclei in the thalamus). Nucleus ovoidalis was included as a control for a general sensory lateralisation of the brain. Also, I attempted to verify
previous findings that claimed NC crows have relatively larger mesopallia than other passerine species (Mehlhorn et al. 2010a). For this purpose, I allometrically reanalysed the volumetric estimates of the mesopallium against the volume of the rest of the telencephalon (known as telencephalon rest).

In the final section (Section 3), I tested a derived hypothesis based on the work of Iwaniuk et al. (2009), which suggested that the presence of tool-use in avian families was positively correlated with increasingly complex cerebellar folia. Fractal analysis was used to estimate the complexity of the cerebellar Purkinje cell layer in 37 avian species. Given the complexity of the NC crow’s tool skills, I expected their cerebellar complexity to stand out from that of 18 other reported tool-using bird families20.

In Chapter 5, I investigated the cytoarchitecture of the NC crow’s telencephalon and provided the first description of cell morphology in the central nervous system. The aim was to describe any distinctive microscopic features of the NC crow’s brain that might play a role in this species’ complex cognitive abilities. In the NC crow’s pallium I discovered clusters of nervous cells possibly functionally similar to satellite neuroglia clusters in the human central nervous system. We also detected these cellular structures in four other species of Passeriformes (Japanese jungle crow; Australian magpie Gymnorhina tibicen; Indian mynah, Acridotheres tristis; and zebra finch, Taeniopygia guttata) but not in a non-passerine (Gruiformes: New Zealand pukeko, Porphyrio porphyrio).

In the Discussion I summarise the findings and discuss how they can help elucidate the mechanisms behind the evolution of cognition, complex tool-related behaviour and advanced problem-solving skills. I also suggest new lines of experimental research by combining comparative psychology with neuroscience, such as the study of the nervous activity of the brain during behavioural tasks in awake, unrestrained animals.

20 We used data from 37 species to produce a comparative scale of avian cerebellar complexity. However, only 19 out of the 37 species shared phylogenetic closeness with reported tool-using species (i.e. they belonged to the same taxonomic family). The family tool-use level of the remaining 18 species was unknown. Therefore, we could only rigorously test the cerebellar complexity–tool-use relationship with the 19 species with reported tool-using family relatives.
PART I

THE MIND
Chapter 2

New Caledonian crows’ responses to mirrors

Introduction

Mirror-image stimulation (MIS) has become a standard test in comparative animal psychology ever since Gallup (1970) conducted the first objective ‘mark’ test for mirror self-recognition. In theory, during the mark test animals able to recognise their own self in their mirror-image should touch marks (e.g. coloured dots) on their bodies that are only visible in a mirror. In this article, we use the term mirror self-recognition (MSR) to define the objective behaviour an animal performs when passing such mark tests (i.e. animals that touch the mark in front of a mirror have MSR). Originally however, this test was specifically designed to test the long-held view in primatology that chimpanzees, Pan troglodytes, “...realised that their behaviour was the source of the behaviour seen in the mirror...” (Gallup & Povinelli 1993). In spite of the ongoing debate on whether animals that show MSR possess human-like self-awareness (Heyes 1994, 1995, 1996; Mitchell 1993a, 1993b, 1995, 1997a; Gallup & Povinelli 1993; Gallup et al. 1995; Swartz 1997; Bard et al. 2006), mirror-induced responses in animals continue to be reported. Such reports range from animals that continuously exhibit species-specific social behaviours to those that spontaneously engage in self-exploratory behaviour in front of mirrors (Pepperberg et al. 1995; Reiss & Marino 2001; Gallup et al. 2002; de Waal et al. 2005; Reznikova 2007).

Until recently, animals other than the great apes were thought to view mirror-images only as conspecifics (Gallup 1970; Kusayama et al. 2000). Non-primates now reported to pass the mark test are one bottlenose dolphin, Tursiops truncates, (Reiss & Marino 2001), one Asian elephant, Elephas maximus, (Plotnik et al. 2006) and two Eurasian magpies, Pica pica, (Prior et al. 2008).

Animals that cannot be tested or do not conclusively pass the mark test nevertheless show other interesting, but less controversial, intermediate mirror-induced responses (Gallup 1970; Povinelli 1989; Pepperberg et al. 1995; de Waal et al 2005). For example, mirror-triggered search is a basic task in which animals in the presence of mirrors search for hidden food (visible in the mirror) that is placed in fixed, familiar places (Menzel et al. 1985; Anderson 1986; Povinelli 1989). As food is always hidden in the same location, subjects may use the mirror only as a cue to start searching rather than to obtain information of the food's precise whereabouts (Povinelli 1989; Pepperberg et al. 1995). In contrast, in the mirror-mediated object discrimination task (Menzel et al. 1985; Pepperberg et al. 1995) subjects are required to look at mirror-images of hidden objects that are either aversive or rewarding. They then must consistently choose to move towards them or move away from them. Animals can do this by exploiting the correlation between an object and its reflection, but they do not need to understand that the object is being represented in the mirror or use the mirror to continuously guide their actions (Pepperberg et al. 1995).

The mirror-mediated spatial location task requires more sophisticated cognition (Menzel et al. 1985; Anderson 1986; Povinelli 1989; Pepperberg et al. 1995). Subjects must use mirrors without recourse to trial and error to find a reward that is hidden in one of several novel locations. To do so, they must understand the implicit correspondence between an object’s location in the mirror and its location outside the mirror. That is, understanding that the object reflected in the mirror is in the real world, in the exact same location as shown in the mirror. However, Povinelli (1989) has argued that animals capable of understanding the duality between mirrored objects and the environment may not necessarily perceive the duality between their own body movements and the image of those body movements in the mirror. In other words, an animal may understand that objects reflected in a mirror have the exact same spatial location in the environment (i.e. understanding mirror duality or mirror correspondence), but it may not understand that the reflected object and the real object are one and the same.
The more elaborate mirror-guided reaching task (Menzel et al. 1985; Anderson 1986) has been designed to test if animals understand how mirrors can be used in synchrony with their own body movements. While animals that possess self-recognition should pass this test without training, others incapable of self-recognition may do so only after intensive, sequential training (Povinelli 1989). This training would involve an animal continuously monitoring the correspondence between its body parts and the reward object when both are visible only in the mirror (see Itakura 1987).

The heterogeneous taxonomic origin of animals reported to pass the ‘mark test’ suggests, if rigorously confirmed, that this type of mirror use is an exceptional example of convergent cognitive evolution between primate and non-primate animals (Reiss & Marino 2001; Plotnik et al. 2006; Prior et al. 2008). As mirror responses in children are well documented and provide cues to their developmental stages (Amsterdam 1972; Berthenthal & Fischer 1978; Anderson 1983; Asendorpf & Bauonnière 1993; Asendorpf et al. 1996), most animal studies have focused exclusively on mirror-triggered behaviour with potential self-awareness implications. However, they continue to ignore two non-trivial issues: (1) passing the mark test does not imply self-recognition, and (2) MSR does not imply self-awareness (sensu Gallup 1987) (Mitchell 1993a; Swartz 1997). Children able to recognise themselves in a mirror or a video recording should only touch the mark when they see it on their face. Instead, children often wipe nonexistent marks off their noses when they see another person with a mark on her/his nose (Lewis & Brooks-Gunn 1979; Johnson 1983). This behaviour casts doubts on self-awareness in very young children who pass the mark test. Also, self-awareness is not the only proposed explanation behind MSR. For example, Mitchell (1993a, 1993b, 1995, 1997a, 1997b, 2002) has proposed that MSR may also initially develop without self-recognition via kinaesthetic-visual matching (i.e. the ability to match the visual experiences of our body movements with the proprioceptive representation of our body) (for a complete list of theoretical explanations of MSR, see Bard et al. 2006).
Regardless of the ambiguous nature of animal MSR, studies often fail to recognize that MIS holds a broader unexploited potential for cross-species comparison of cognitive abilities, especially those related to the perception and processing of mirror information (Pepperberg et al. 1995). Thus, researchers may overlook the more basic aspects and levels of mirror-contingent behaviour if they hold an all-or-nothing view of self-awareness (Swartz 1997; Reznikova 2007).

The two mirror studies that have been conducted with corvids are of particular interest regarding MIS in avian species. In agreement with other published studies (Pickering & Duverge 1992 with lesser flamingos, *Phoeniconais minor*; Pepperberg et al. 1995 with African grey parrots, *Psittacus erithacus*), four captive jungle crows, *Corvus macrorhynchos*, viewed their mirror-image as a conspecific and showed no self-contingent behaviour during 150 mins of mirror exposure (Kusayama et al. 2000). In contrast, three hand-raised Eurasian magpies showed self-contingent behaviour: two after 150 mins of open mirror exposure and one during the mark test (Prior et al. 2008). Two of the Eurasian magpies were also reported to pass the mark test after 250 mins of cumulative exposure to mirrors. Methodological issues in the above-mentioned studies could partly explain the differences in performance between jungle crows and the Eurasian magpies for two reasons. First, while the jungle crows were immediately tested in a small confined area (90 cm × 90 cm), the Eurasian magpies were tested only after 150 mins of open mirror exposure in a 4 m × 4 m room. The following 100 mins of testing were carried out in a cage with open compartments (60 cm × 100 cm) which gave them free access to a mirror. Second, the jungle crows could not explore behind the mirror as it was up against a wall or floor. The Eurasian magpies could explore freely behind the mirror during the initial MIS but not during the mark tests. Thus the methodology used by Kusayama et al. (2000) may have restricted the jungle crows to developing social mirror-responses and prevented the appearance of other, perhaps more interesting, mirror-contingent behaviours.
New Caledonian crows, *Corvus moneduloides*, have remarkable tool skills in the wild (Hunt 1996, Hunt & Gray 2003, 2004). Wild-caught NC crows also show problem solving skills in captivity that rival those of primates (Weir et al. 2001; Taylor et al. 2007, 2009, 2010a; Wimpenny et al. 2009). Here, we tested wild-caught NC crows for their responses to mirrors in two ways. We first gave the crows MIS using a vertical mirror in a large cage where birds could look behind the mirror. We predicted that NC crows would engage in similar social (aggressive) displays described in other avian studies when first confronted with their mirror-image. However, with ample opportunity to explore both sides of the mirror and to freely search for the ‘mirrored crow’ in the cage, we expected that their social responses would extinguish over time.

Exposure to both reflective and non-reflective vertical mirror surfaces should lead to increasingly more mirror-directed exploratory and self-contingent behaviour (Pepperberg et al. 1995; Prior et al. 2008). We then gave the crows a mirror-mediated spatial location task to see if they could use visual feedback in a horizontal mirror to locate hidden food. As tool-using NC crows search for larvae cached in dead wood (Hunt 2000), we expected them to rapidly learn to use the mirror as a tool to locate a hidden food reward. Birds were first trained to extract hidden food in a 2-box apparatus. They were then tested on a more difficult 4-box apparatus where only one compartment was baited. We predicted that if NC crows were using the mirror to locate food they would consistently search for it only in the baited compartment. However, if they usually first searched in any of the unbaited compartments it would suggest that they were instead using strategies such as mirror-triggered search.
EXPERIMENT 1: VERTICAL MIRROR-IMAGE STIMULATION

Methods

Subjects

We carried out the experiment with 10 New Caledonian crows captured on the island of Maré, New Caledonia, in August/October 2007. We captured the birds using a 8 m x 4 m 'whoosh net’ obtained from SpiderTech Bird Nets, Helsinki, Finland. Based on predictable associations between age and mouth colour, we aged the crows by mouth colour. Adults were over 2 years old and juveniles were under 2 years old. Female and male distinction was based on bill morphology and body size. All the crows were housed in a 5-cage outdoor aviary situated in primary forest inland from the coast. The cages were 3 m high and at least 4 m x 2 m in area. All cages were provided with ample perching space, branches and feeding logs. We held up to three crows at a time in a single cage. The crows were left to get accustomed to the aviary and human presence for three days before any experimental procedures began. The crows were fed a main meal in the evening consisting of soaked dog biscuits, bread or rice and occasionally raw egg. Papaya and clean drinking and bathing water were available throughout the day. Crows were given a half ration of food ca. 15 hours prior to their participation in the experiment. Their bodyweights were closely monitored to maintain them at or above capture weight. All birds were captured before breeding season (November-January) and kept in the aviary for up to 5 months. All ten crows were presumed to be naïve to mirrors before the experiment began.

Experimental procedure

Experiments were conducted in a 4 m x 5 m x 3 m high cage. In the top corner of the cage was a small observation cage 0.5 m wide x 0.5 m high x 2 m long. The observation cage had a single perch in front of an observation window (25 cm x 25 cm). A vertical mirror (50 cm high x 40 cm wide x 6 mm thick) was fixed to a wooden base and positioned on top of a 1 m high ×
70 cm wide table in the middle of the large cage. Crows could easily access the table from either the perches or the ground. A test crow was first moved into the observation cage and remained there for 2 minutes, with free access to the observation window from which they could see the back of the mirror in the large cage. After 2 minutes had elapsed the crow was moved into the large cage.

Crows were given a single 10-minute long mirror session per day, and up to a total of six daily sessions. To control for effects due to exposure to a novel setup (i.e. the mirror on the table), we (1) reversed the mirror after each session and (2) assigned the crows pseudo-randomly into two experimental groups (N = 5 per group). When reversed, the back of the mirror faced the crows instead of the reflective side. The mirror’s reflective surface at the back was covered with a cardboard to prevent crows from seeing their image in it if they inspected behind the mirror. Each group had an equal mix of crows of different ages (Table 2.1). Group 1 started with the reflective surface (R) of the mirror, and Group 2 started with the non-reflective surface (nR). At the end of the experiment each crow had been exposed to 3 reflective and 3 non-reflective vertical mirror sessions as follows:

(a) Group 1: R₁, nR₂, R₃, nR₄, R₅, nR₆

(b) Group 2: nR₁, R₂, nR₃, R₄, nR₅, R₆

Table 2.1 Study crows and their assigned groups for Experiment 1

<table>
<thead>
<tr>
<th>Group 1</th>
<th></th>
<th></th>
<th>Group 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td>Sex</td>
<td>Age</td>
<td>Subject</td>
<td>Sex</td>
<td>Age</td>
</tr>
<tr>
<td>Español</td>
<td>male</td>
<td>adult</td>
<td>Robin</td>
<td>male</td>
<td>adult</td>
</tr>
<tr>
<td>Angel</td>
<td>female</td>
<td>juvenile</td>
<td>Egg</td>
<td>female</td>
<td>juvenile</td>
</tr>
<tr>
<td>Boxer</td>
<td>male</td>
<td>juvenile</td>
<td>Slevin</td>
<td>male</td>
<td>juvenile</td>
</tr>
<tr>
<td>Sisu</td>
<td>female</td>
<td>juvenile</td>
<td>Ronia</td>
<td>female</td>
<td>juvenile</td>
</tr>
<tr>
<td>Cuba</td>
<td>male</td>
<td>juvenile</td>
<td>Tiga</td>
<td>male</td>
<td>juvenile</td>
</tr>
</tbody>
</table>

An adult is > 2 years old; a juvenile is < 2 years old
In each session the front of the mirror was baited four times with small meat cubes (ca. 1 cm³) to ensure crow interaction with the reflective or the non-reflective mirror surface. One minute after a session had started, the experimenter entered the cage and left one meat cube on the table in front of the mirror (ca. 5 cm from the ledge) and immediately left the cage. One and a half minutes later, the experimenter repeated this procedure. Again, one and a half minutes later the experimenter re-entered the cage and after leaving six meat cubes in front of the mirror (five along the mirror ledge and one on the table) left the cage. Finally, two and a half minutes later the experimenter entered the cage and left five meat cubes in front of the mirror (along the mirror ledge). The experimenter then left the cage and the session was terminated once 10 minutes had elapsed. Thus, each crow was offered a total of 13 meat cubes per session. We increased the number of meat cubes over the session to try and ensure a standard period of time in front of the mirror across individuals. Crows left the table as soon as the cage door was opened, and did not return until the experimenter had left the cage. Therefore they could have not seen the experimenter in the mirror. However, crows could see the camera lens reflected in the mirror when standing in front of it. Mirrors were thoroughly cleaned before each session to preclude possible responses to cues (olfactory, gustatory and/or visual).

Data analysis

All trials were recorded on video camera through a small hole in the opaque walls of the experimental cage. A total of 10 hours of video footage was analysed by F.S.M.R.² and the total frequency of nine mirror-responses listed in Table 2 was scored (see Movie 2.1). Similar responses have been described for children (Amsterdam 1972, Brooks-Gunn & Lewis 1984), chimpanzees (Lin et al. 1992), gibbons (Suddendorf & Collie-Baker 2009), macaques (Straumann & Anderson 1991), marmosets (Eglash & Snowdon 1983), talapoins (Posada &

² Felipe Salvador Medina Rodriguez, author of this doctoral thesis.
Table 2.2 Mirror-responses and definition

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Social responses</strong></td>
<td></td>
</tr>
<tr>
<td>Vocalization</td>
<td>Bird makes a ‘caw’ call, sometimes accompanied by subtle wing flapping</td>
</tr>
<tr>
<td>Rapid head movement (RHM)</td>
<td>Bird does a series of six or more quick head movements in front of mirror, generally with stretched neck and accompanied by short series of non-‘caw’ calls</td>
</tr>
<tr>
<td>Tail-up</td>
<td>Acute tail lifting, usually after a sudden opening of wings</td>
</tr>
<tr>
<td>Attack</td>
<td>Bird jumps at mirror image, usually with claws up in frontal position</td>
</tr>
<tr>
<td><strong>Exploratory responses</strong></td>
<td></td>
</tr>
<tr>
<td>Peck</td>
<td>Bird pecks at the surface of mirror or mirror image (not associated with attack)</td>
</tr>
<tr>
<td>Peekaboo</td>
<td>Bird stares at mirror-image and then quickly moves its head out of view and then back, within 3 seconds</td>
</tr>
<tr>
<td><strong>Search responses</strong></td>
<td></td>
</tr>
<tr>
<td>Look under table (LUT)</td>
<td>While at the edge of the table, bird orients its head towards the ground, usually bending the whole upper body (including turning of the eye towards ground)</td>
</tr>
<tr>
<td>Look behind mirror (LBM)</td>
<td>Bird moves from front area to the back area of the table or while perching on the mirror top ledge, turns its head or body from facing towards the front to towards the back of the mirror</td>
</tr>
<tr>
<td><strong>Other responses</strong></td>
<td></td>
</tr>
<tr>
<td>Startle</td>
<td>Sudden wing flapping usually followed by a backwards jump or by flight, or any other sudden aversive reaction to the mirror image (i.e. leave table before eating bait)</td>
</tr>
</tbody>
</table>

1 The table is divided by an imaginary line into ‘front’ and ‘back of the mirror’

Collel 2005), elephants (Povinelli 1989; Plotnik et al. 2006), dolphins (Marino et al. 1994), pigs (Broom et al. 2009), parrots (Pepperberg et al. 1995), chickadees (Censky & Ficken 1982), finches and parakeets (Gallup & Capper 1970), Eurasian magpies (Prior et al. 2008) and jungle crows (Kusayama et al. 2000). We did not witness any self-contingent (i.e. ‘contingent’ behaviour) (Prior et al. 2008) or self-directed (Bard et al. 2006) behaviours during the MIS sessions.

An independent scorer coded half of the reflective mirror sessions using only behavioural descriptions from Table 2.2. Scorer consistency was measured with Spearman rank correlation tests (Martin & Bateson 1986). To detect any trend or habituation to vertical MIS, we used non-parametric Friedman’s ANOVA. We carried out non-parametric Mann-Whitney U-tests with
SPSS v15.0. We calculated Wilcoxon signed-ranks tests manually because of low sample sizes. The alpha level for the above tests was set at 0.05, unless otherwise indicated.

**Results**

We scored a total of 1246 and 110 behavioural responses during reflective (R) and non-reflective (nR) mirror sessions, respectively (Tables 2.3 & 2.4). Scores between observers were highly correlated (two-tailed Spearman correlation tests: vocalization: $r_s = 1.0$; rapid head movements: $r_s = 0.923$; tail-up: $r_s = 0.949$; attack: $r_s = 1.0$; peck: $r_s = 0.986$; peekaboo: $r_s = 0.935$; look under table: $r_s = 0.931$; look behind mirror: $r_s = 0.997$; startle: $r_s = 0.949$; all $P < 0.001$).

We found no significant difference in both the R and nR conditions between groups in the frequency of the behaviour responses in Table 2.2 (Mann-Whitney U-tests: all $P$-values > 0.05). Therefore, we combined the data for each condition across groups 1 and 2.

With exception of the search response ‘look under table’, crows made fewer responses to the mirror when it was reversed (Wilcoxon signed-ranks tests; rapid head movement: $T = 0, N = 9, P = 0.004$; tail-up: $T = 0, N = 10, P = 0.002$; attack: $T = 0, N = 6, P = 0.031$; peck: $T = 0, N = 6, P = 0.002$; look behind mirror: $T = 8, N = 10, P = 0.049$; startle: $T = 0, N = 10, P = 0.002$; look under table: $T = 15.5, N = 8, P = 0.74$) (Fig. 2.1). We were unable to test for significant differences in ‘vocalization’ and ‘peekaboo’ frequencies across mirror conditions due to low sample size ($N = 3, N = 5$, respectively). However, three birds made a total of 69 vocalizations during R sessions and only 7 during nR sessions. Also, five birds made 61 peekaboos exclusively during R sessions.

The crows’ social, exploratory, search and startle behaviours did not extinguish completely over time, as was predicted (Fig. 2.2). However, the frequency of the social responses across all crows tended to decrease over the 10 trials in the R sessions and the frequency of the
<table>
<thead>
<tr>
<th></th>
<th>Español</th>
<th>Angel</th>
<th>Boxer</th>
<th>Sisu</th>
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</tr>
<tr>
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</tr>
</tbody>
</table>

<sup>a</sup> Rapid head movement  
<sup>b</sup> Look under table  
<sup>c</sup> Look behind mirror

<table>
<thead>
<tr>
<th></th>
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<th>Slevin</th>
<th>Ronia</th>
<th>Tiga</th>
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<td>2</td>
<td>0</td>
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<tr>
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<td>0</td>
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<td>0</td>
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<td><strong>Other</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Startle</td>
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<td>20</td>
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<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rapid head movement  
<sup>b</sup> Look under table  
<sup>c</sup> Look behind mirror

Exploratory responses tended to increase, but these trends were not significant (Friedman’s ANOVA tests; social responses: $\chi^2 = 1.895$, $P = 0.42$; exploratory responses: $\chi^2 = 0.176$, $P = 0.95$; search responses: $\chi^2 = 1.947$, $P = 0.41$; startle: $\chi^2 = 1.600$, $P = 0.48$).
Figure 2.1 Comparison of responses to vertical mirror stimulation. Mean frequencies (group mean ± s.e.m, $N = 10$) are shown. RHM: rapid head movement; LUT: look under table; LBM: look behind mirror. Black bars represent data from reflective mirror surface (R) sessions and white bars show data from non-reflective mirror surface (nR) sessions. Significant differences are indicated by * $P < 0.05$ and *** $P < 0.005$ (Wilcoxon signed-rank test).

Birds approached the baited table as soon as the experimenter left the cage. With few exceptions, a crow immediately viewed its reflection as a threat and reacted agonistically (rapid head movement, tail-up, attack). As a consequence, birds often perched on the top mirror ledge or ended up close to the mirror side (usually after an attack). In both cases, crows had free access to the back of the mirror and frequently examined it. Birds also searched behind the mirror right after their mirror-image disappeared from view. Such exploratory responses increased in frequency across mirror sessions (Fig. 2.3, Tables 2.5 & 2.6).

On several occasions, a crow flew off the table as soon as it saw its mirror-image (startle responses), but they usually returned to the table a few seconds later. Crows also initially ate the meat in between or after making social responses to their mirror reflection. As mirror sessions continued, they started social interactions only after all or most of the meat cubes had been eaten.
Table 2.5 Responses to vertical reflective (R) mirror sessions: Group 1

<table>
<thead>
<tr>
<th>Responses</th>
<th>Español</th>
<th>Angel</th>
<th>Boxer</th>
<th>Sisu</th>
<th>Cuba</th>
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<tbody>
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<td>R5</td>
<td>R1</td>
<td>R3</td>
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<td>1</td>
<td>21</td>
<td>12</td>
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<td>8</td>
<td>38</td>
<td>27</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td>Peekaboo</td>
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<td>0</td>
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<td>38</td>
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</tr>
</tbody>
</table>

\textsuperscript{a} Rapid head movement \textsuperscript{b} Look under table \textsuperscript{c} Look behind mirror

Table 2.6 Responses to vertical reflective (R) mirror sessions: Group 2

<table>
<thead>
<tr>
<th>Responses</th>
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<th>Egg</th>
<th>Slevin</th>
<th>Ronia</th>
<th>Tiga</th>
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</thead>
<tbody>
<tr>
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<td>R4</td>
<td>R6</td>
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<td>R4</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Tail-up</td>
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<td>5</td>
<td>8</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Attack</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Exploratory</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peck</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Peekaboo</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Search</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
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<td>6</td>
<td>10</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Rapid head movement \textsuperscript{b} Look under table \textsuperscript{c} Look behind mirror

Important individual differences in mirror-responses were observed during R sessions (Tables 2.5 & 2.6). For example, only three crows (Español, Cuba and Sisu) from Group 1 made ‘vocalizations’ during the entire experiment. During their first reflective (R\textsubscript{1}) mirror session they made submissive begging-like calls in front of their mirror reflection. Only two of
Figure 2.2 Frequency of behavioural categories during vertical mirror stimulation. Mean frequencies (group mean ± s.e.m, N = 10) are shown. Social responses include vocalization, rapid head movement, tail-up and attack. Exploratory responses include peck and peekaboo. Search responses include look under table and look behind mirror. Startle responses only include startle. Black bars represent data from first reflective mirror surface (R) sessions; gray bars, data from second R sessions; and white bars, data from third R sessions.

these three crows (Español and Cuba) continued to vocalise in subsequent reflective mirror sessions. Crows other than Español made no ‘vocalizations’ during reversed mirror sessions (Tables 2.3 & 2.4). Prior to the mirror experiment we had never observed Español’s surprising begging-like behaviour in mature male NC crows.

Discussion

Our study provides the first description of the initial reaction to a vertical mirror by captured corvids that had become independent in the wild. When first confronted with the mirror NC crows treated their mirror-image as a conspecific. Their agonistic responses to their mirror-image (e.g. rapid head movements, tail-up and attack) continued over the experimental sessions, with no significant decrease in frequency over time. However, the crows also engaged in search (look behind the mirror) and mirror-directed exploratory behaviour (peck and peekaboo) during reflective sessions. Therefore, after 30 mins of exposure to a vertical mirror the NC crows that
we tested did not show any of the self-contingent responses recently found in Eurasian magpies (Prior et al. 2008). As in the jungle crow study (Kuyasama et al. 2000), our NC crows were probably not given enough mirror exposure to elicit any self-contingent behaviour. We could not give the crows extended exposure to a vertical mirror because of the limited time that we had use of the birds in the aviary.

Responses during MIS were significantly more frequent during reflective (R) than non-reflective (nR) sessions (Fig. 2.1). Therefore, other than ‘look under the table’, all responses were triggered by the reflection of ‘another bird’ in the mirror. The equally frequent ‘look under the table’ behaviour observed across mirror sessions can be explained if birds expected to find food when visiting the experimental table. Birds often search for objects of interest or food in the sand of cages, especially when they have just been rewarded with a small number of meat blocks. In the absence of the ‘other bird’ after they had eaten the meat bait on the table, crows immediately searched for food under the table. Thus, ‘look behind mirror’ appeared to be the only valid mirror-induced search behaviour.

Only three birds made ‘vocalizations’ during mirror sessions. We expected ‘vocalizations’ to be more evenly distributed across groups and individuals, given that begging in the wild is common in juveniles begging for food from adults and adult females begging for food from their partners in courtship behaviour. We were surprised that the dominant male adult among the study crows, Español, made submissive juvenile-like begging calls in front of his own mirror-image. Other than Cuba, juveniles in this study made no ‘vocalizations’ in front of the reflective mirror (see Tables 2.3 & 2.4). One possible explanation for the lack of begging by juveniles is that they might have recognised another juvenile in the mirror and therefore did not beg to it. Most crows (adults and juveniles) did perform other forms of social displays (i.e. agonistic), though. Therefore, Español’s begging might have been a submissive response to recognising a high-ranking adult male crow in the mirror. He also was the only adult to attack
the mirror, doing so twice. Nevertheless, we have never observed such begging when free-
living dominant males are in close proximity at feeding sites.

We found no evidence that NC crows habituated to their mirror-images over time. Other than
for Egg and Slevin, agonistic social behaviours (tail-up, rapid head movement and attack) by
crows restarted on the next session (see Tables 2.5 & 2.6). Given that Egg and Slevin made
almost no mirror-directed exploratory behaviours in the R sessions (Slevin made one peekaboo
in R₃ session), they still appeared to have no understanding of mirror properties or showed any
self-contingent behaviour in front of the mirror. Chimpanzees and orang-utans, *Pongo
pigmiaeus*, show a rapid extinction of social responses and initiate self-directed behaviour after 3
days of MIS (when given 8 and 5 hours of mirror exposure per day, respectively) (Gallup 1970;
Suarez and Gallup 1981). Eurasian magpies also show transient social behaviours during the
first 150 min of mirror exposure, then start showing contingent behaviour in the following 100
min of exposure. Some gorillas, *Gorilla gorilla*, and orang-utans have become intensely curious
about mirrors and even grown very attached to them, resisting attempts by experimenters to
remove the mirrors from their cages (see Gallup 1968). Although three out of five captive
Eurasian magpies preferred compartments with mirrors (Prior et al. 2008), the wild-caught NC
crows in our study did not spontaneously approach the mirror or stay in its proximity unless it
was baited with food. However, this may have been because we placed the vertical mirror on a
low table and wild-caught crows prefer to perch higher up in their cages.

Despite these differences, our study crows appeared to actively search for their mirror-image
in a very similar way to that described in primates and children. They repeatedly searched
behind the mirror during reflective mirror sessions (Fig. 2.1), and this often happened
immediately after they lost sight of their mirror-image (e.g. when they moved away from the
mirror during social displays). It was interesting that three juvenile NC crows in Experiment 1
(Angel, Sisu and Tiga) reacted to their mirror-image in a way similar to how primate infants
react by repeatedly performing ‘peekaboo’ behaviours (see Tables 2.5 & 2.6). Children and
orang-utan infants start experimenting with mirror movement synchronism at two years of age (Robert 1986). Two-year-old children also actively seek their mirror-image when they lose sight of it (Kleeman 1973; Modarressi & Kenny 1977), at about the time they recognise themselves in mirrors (Amsterdam 1972). They also search behind the mirror (Dixon 1957), probably in an attempt to re-establish contact with their mirror-image. Two African Grey parrots have also been reported to search behind mirrors, the juvenile (Kyaaro) doing more so than the adult (Alo) (Pepperberg et al. 1995). Similarly, monkeys (Anderson 1984) and apes (Suddendorf & Collie-Baker 2009) reach behind the mirror while in front of it as if to touch the monkey in the mirror.

**EXPERIMENT 2: MIRROR-MEDIATED SPATIAL LOCATION TASK**

*Methods*

*Subjects*

We used mirror-experienced birds Español and Sisu from Experiment 1 and two new crows (Pelé and Obo) kept in captive conditions as per Experiment 1. Pelé was a male adult and Obo a female juvenile. Both Pelé and Obo were also presumed to be naïve to mirrors at the start of Experiment 2.

We only had four crows available for Experiment 2 at the time we carried it out. We could not use birds from Experiment 1 other than Español and Sisu because they had been given mirror-mediated search experience for an unrelated experiment (Taylor et al. 2010b).

*Experimental procedure*

Experiments were conducted in a 4 m long × 2 m wide × 3.0 m high cage. Crows could not see into this cage from the other aviary cages. A mirror (50 high cm × 40 cm wide × 6 mm thick) was laid (horizontally) on top of a 1.5 m high × 60 cm wide table at one side of the cage. As in Experiment 1, mirrors were thoroughly cleaned before each session.
**Habituation**

Subjects were each given one 10-minute long habituation session with a horizontal mirror (same dimensions as in Experiment 1) on the experimental table prior to training. This involved placing 10 target meat cubes (ca. 1 cm$^3$) on the horizontal mirror at the start of the session. The crows either quickly ate all the bait while they were standing over the mirror or took them away to eat elsewhere in the cage. The following day we installed a 70 cm long perch 11 cm above the horizontal mirror.

We then gave birds one session where they had to retrieve a minimum of 10 target meat cubes from the perch and from the mirror surface. The experimenter entered the cage and baited the apparatus 4 times. Birds were thus offered 1, 1, 6, and 5 meat cubes on each baiting event, respectively. A total of 11 meat cubes were left on the perch and 2 meat cubes on the mirror surface (1 cm from the mirror edge on the back side of the table). All sessions terminated before 10 mins had elapsed. All crows showed some level of caution when approaching the mirror surface. Their horizontal reflection caused initial startle responses that quickly extinguished. Only one crow (Pelé) did not approach the mirror surface after a startle, when it failed to retrieve the meat cubes there.

We then hung meat from the perch on a 2 cm long piece of cotton string. We gave birds 3 opportunities to retrieve a meat cube. All subjects readily bent their bodies over the perch to retrieve the cubes. As in the previous session, the crows only stayed perching over the mirror for up to a few seconds while they retrieved the meat. In some cases, when a crow would not approach the horizontal mirror or the new perch we placed smaller pieces of meat on top of the perch. We did not keep a record of how many of these extra small pieces of meat a crow received before it had taken the three meat cubes on hanging string.

Next we introduced a wooden 2-box choice apparatus (32 cm long × 10 cm high × 9 cm deep) into the cage. It was placed on the horizontal mirror under the perch. The front of the apparatus consisted of two separated, open compartments (15 cm long × 9 cm high × 8 cm deep).
deep) (Fig. 2.3a). Each compartment had a 3 cm deep piece of occluding board at the top front of the compartment that could be used to gradually obstruct a crow’s view of the meat baits (by manipulating the length of the string, see below) (Fig. 2.3c). A crow could at all times, clearly see the meat bait reflected in the horizontal mirror. When the food was hidden behind the occluding board a crow had to distinctly bend its head below the board to see it directly. Furthermore, crows could only see their heads in the mirror while searching for food inside the boxes because their bodies were completely occluded by the reflection of the apparatus. The aim of this habituation phase was to get birds comfortably extracting hidden meat bait before it moved onto testing with the training phase.

The experimenter entered the experimental cage and baited one of the two compartments at random. To do this, a 1 cm piece of string with a meat bait on one end was attached to the roof in the centre of the compartment and just behind the occluding board. A crow was then allowed to retrieve the meat. To encourage birds to locate meat hanging behind the occluding board, we first hung it on a longer piece of string so they could see it without the need to bend their heads into a compartment. The four crows were given a total of 10 habituation trials. However, each bird had a different habituation experience. We had to modify the procedure to suit each bird’s level of interest and cooperation. Pelé only extracted meat twice over the 10 habituation trials.

Training

After habituation the four crows were given 3 blocks of 10 trials each with the 2-box apparatus. The aim of the training was to (1) to make crows equally proficient at extracting meat hidden behind the occluding board before testing, and (2) allow us to determine valid criteria for scoring trials in the 4-box condition. As with the habituation phase, the experimenter entered the cage and baited one of the two compartments at random (counterbalanced, see Fig. 2.4). With the exception of Pelé, all crows were given trials with completely occluded meat cubes from the start (Fig. 2.4). Sessions were terminated once 10 meat cubes had been retrieved or 10
Figure 2.3 Mirror-mediated spatial location task setup and apparatus. a The 2-box training apparatus b The 4-box test apparatus c Cross-section of a baited compartment. The black arrow points to the occluding board. The red square indicates the meat bait hanging from a piece of string. The light blue rectangle represents the mirror.

minutes had elapsed. As before, we sometimes placed a meat cube on the middle of the perch before leaving the cage if a bird was reluctant to come down to the perch.

Reversed mirror control

After the training three of the four crows were transferred to the 4-box spatial location task. The best performing bird (Obo) in training was given a 10 min reversed-mirror control before moving to the 4-box apparatus. In the control, we reversed the horizontal mirror so that the
Figure 2.4 Trial-by-trial performance in the 2-box training task. A row of squares in each block indicates one trial. The two squares in a row that make up a trial represent the 2 compartments from left to right as seen in Fig. 2.3a.

The control was divided into two trials of 5 mins each separated by 1 min. One compartment was baited behind the occluding board in each trial, the right compartment in trial 1 and the left one in trial 2. In the first trial, if Obo did not extract the occluded bait by 2 mins we placed visible bait on the apparatus every minute for 3
mins to keep her interested in it. The first two baits were left on the middle of the perch. The third and final bait was left on the mirror surface, in front of the separation between both compartments. In the second trial we did not use any visible meat bait.

**Mirror-mediated spatial location task**

We used a wooden 4-box apparatus (43 cm long × 10 cm high × 9 cm deep) that only differed from 2-box apparatus used in training in the number and size of compartments (Fig. 2.3b). Three crows (Español, Sisu and Obo) were given daily sessions of 10 trials. Due to bad weather conditions, Pelé was tested on the same day, with sessions 3 hours apart. In each trial, the experimenter baited pseudo-randomly one of the compartments when outside the experimental cage (out of the test crow’s view) and made sure that the meat cube could not be seen from the front. The apparatus was then placed in the experimental cage between the mirror and perch as with the 2-box apparatus. The experimenter then left the cage and the crow was allowed to retrieve the bait. We did not use any extra meat bait during the testing. A 10-trial session was terminated once 10 meat cubes had been retrieved. At the end of a session, all four compartments had been baited at least 2 times and no compartment was baited in two consecutive trials.

**Data analysis**

All trials were recorded on video camera through a small hole in the tarpaulin covering the walls of the experimental cage. We scored mirror-mediated search in a trial if a crow’s behaviour met two criteria: (1) it inserted its head *only* into the baited compartment; and (2) it did not lower an eye below the bottom edge of the 3 cm deep occluding board along the top of an unbaited compartment (or the unseen line of the bottom of the board that projected out either side of the apparatus). Sometimes when a crow clearly moved to insert its head into a baited compartment it was manipulated in such a way that it violated criterion 2 by partially moving in front of an adjacent non-baited compartment. This behaviour always occurred when a crow was
not standing directly above the baited compartment. We still classified these trials as successful if the baited compartment was the first one a crow searched in ($N = 5$ trials; Pelé: B2 trial 1; Obo: B2 trial 10; Sisu B3 trial 4; Español: B2 trial 7 and B3 trial 7). We recorded in which compartment(s) in unsuccessful trials a crow violated criterion 2.

We did not statistically test the crows' performance in the 2-box training phase because the compartments were baited in view of the test birds and meat was also sometimes placed on the perch. To test if crows successfully solved the 4-box task, we tested each block of 10 trials for significance using the binomial test ($\alpha = 0.05$); a crow successfully solved the task if they had 6 or more correct trials out of 10 (two-tailed test: $P = 0.039$; exact binomial probability: $P = 0.016$). We terminated testing immediately after a crow met the significance criterion in a block of 10 trials. For each trial, we recorded the approximate position on the perch that a crow initially flew to before it started to inspect the apparatus. We also recorded the time that it took the crow to retrieve the hidden bait from the time it landed on the perch (i.e. latency to food retrieval).

After the present mirror study, the 12 crows used in Experiments 1 and 2 participated in further aviary experiments. The research reported in this paper was approved by the University of Auckland Animal Ethics Committee and complies with the laws of New Caledonia.

**Results**

**Training**

All four crows readily extracted the occluded meat from the 2-box apparatus at the end of training. As Pelé never extracted the occluded bait without another piece of meat on the mirror surface in his first block (see Fig. 2.4), we only used data from the last 2 blocks for each crow when analysing success rates and latencies to food retrieval. Over the last two blocks (B2 and B3) crows chose to first inspect the baited compartment in 50 of the 79 trials. On the remaining 80th trial (B2 trial 10, Fig. 2.4), the crow (Obo) did not visit the apparatus because it was
probably satiated. Sisu and Pelé had 10 out of 20 successful trials in the last 2 blocks, Español 12 out of 20, and Obo 18 out of 19. In 2 of her 19 trials, Obo choose the previously baited compartment. Sisu did so on 9 of the 20 trials, Pelé 8 out of his 20 trials and Español 4 out of his 20 trials.

We found no evidence that crows had improved their extraction times from block 2 to block 3 (Wilcoxon signed-ranks tests; Español: \( T = 22, N = 10, P = 0.63 \); Sisu: \( T = 24, N = 10, P = 0.77 \); Pelé: \( T = 13, N = 10, P = 0.16 \); Obo: \( T = 22, N = 10, P = 0.63 \). However, Obo and Español were faster at getting the meat after landing on the perch (mean ±s.e.m, 3.99 ±0.03 s and 4.02 ±0.06 s, respectively, \( N = 20 \) trials) compared to Sisu and Pelé (6.77 ±0.10 s and 7.60 ±0.09 s, respectively, \( N = 20 \) trials).

**Reversed mirror control**

Obo had no obvious neophobic response to the reversed mirror in the first session. As soon as the experimenter left the cage on the first trial, Obo came down and sat on the far end of the perch and scanned the mirror as she usually did during her training trials. After scanning the mirror Obo left the apparatus without inspecting any compartment (clip 1 Movie 2.2). The experimenter then baited the perch and left. Obo came back to the apparatus immediately and ate the extra bait. She slightly lowered her head and scanned the mirror directly below the baited compartment, but again left the apparatus (clip 2 Movie 2.2). The perch was baited again. This time after scanning the mirror directly below the unbaited compartment, Obo inspected the compartment then left the apparatus (clip 3 Movie 2.2). The last bait was placed on the non-reflective side of the mirror. Obo returned to the perch and reached down and got the bait on the back of the mirror. After taking the meat on the mirror she immediately extracted the meat behind the occluding board (clip 4 Movie 2.2).
Figure 2.5 The performance of crows in the mirror-mediated spatial location task. Pelé and Obo reached criterion in session 2, and Español and Sisu did so in session 3. The y-axis shows the number of correct trials per session and the x-axis shows the session number.

In the second session, Obo immediately flew down and scanned the mirror from the far end of the perch. She then moved along the perch until she was positioned directly over the wall that separated both compartments. Obo scanned the mirror again then left the apparatus. She did not return again to the apparatus for the remainder of the session (clip 5 Movie 2.2).

**Mirror-mediated spatial location task**

All four crows successfully located hidden meat baits by using the bait’s reflection in the horizontal mirror (clip 1 Movie 2.3). Obo and Pelé reached criterion in their second block (Obo scored 10/10 and Pelé 6/10) (Figs. 2.5 & 2.6). Both Sisu and Español succeeded in their third block (both scored 6/10). In unsuccessful trials across all blocks (Español: \( N = 17 \); Sisu: \( N = 16 \); Pelé: \( N = 9 \); Obo: \( N = 5 \)), the crows either searched in the compartment that they had last found meat and/or inserted their heads into at least one other empty compartment before choosing the correct one (clip 2 Movie 2.3).
Figure 2.6 Trial-by-trial performance in the mirror-mediated spatial location task. A row of squares in each block indicates one trial. The four squares in a row that make up a trial represent the 4 compartments from left to right as seen in Figure 2.3b. The black arrows represent a crow's approximate landing position.
On 18 of the 47 unsuccessful trials a crow first potentially inspected a compartment non-adjacent to the baited one before it found the food (Español: \( N = 7 \); Sisu: \( N = 7 \); Pelé: \( N = 4 \)) (Fig. 2.6). On these occasions the crows either (1) inspected the nearest compartment to their landing position on the perch, or (2) inspected the previously baited compartment. Sisu appeared to use the first strategy by default at the start of every block. However, by trial 4 in block 3 she had almost completely switched to using the mirror (Fig. 2.6). On the other hand, Español used both the above strategies in unsuccessful trials (see his blocks in Fig. 2.5). Pelé used the second strategy in five of nine unsuccessful trials (Fig. 2.6). The number of trials (\( N = 14 \)) in which the crows initially searched the compartment where they last found meat tended to decrease over time (Table 2.6).

**Table 2.6 Number of searches in the last baited compartment**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Session 1</th>
<th>Session 2</th>
<th>Session 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sisu</td>
<td>1 / 10 trials</td>
<td>1 / 10 trials</td>
<td>2 / 10 trials</td>
</tr>
<tr>
<td>Español</td>
<td>4 / 10 trials</td>
<td>1 / 10 trials</td>
<td>0 / 10 trials</td>
</tr>
<tr>
<td>Pelé</td>
<td>2 / 10 trials</td>
<td>3 / 10 trials</td>
<td>-</td>
</tr>
<tr>
<td>Obo</td>
<td>0 / 10 trials</td>
<td>0 / 10 trials</td>
<td>-</td>
</tr>
</tbody>
</table>

The crows other than Español usually landed on the far left end of the perch (when viewing the apparatus from the front as in Fig. 2.3b) then moved along it to inspect the apparatus. On only six out of their 70 trials did these crows land partially or completely on top of one compartment (Sisu, \( N = 5/30 \); Pelé, \( N = 1/20 \)). On three of Sisu’s five trials she inspected the compartment directly below her (Fig. 2.6). All the six trials were unsuccessful ones. In contrast, Español inspected the compartment directly below his landing position on 17 out of his 30 trials. Five of those 17 trials were successful (B1 trials 3 and 5, B2 trials 4 and 8, B3 trial 10).

The time crows took to retrieve the meat after landing on the perch was significantly shorter on successful trials (Español: 1.64 s, 1.08–3.04; Sisu: medium 1.36 s, range 1.04–3.84; Obo:
1.92 s, 1.12–3.2; Pelé: 2.24 s, 1.64–6.0) than on unsuccessful trials (Sisu: 3.24 s, 1.4–7.96; Español: 2.56 s, 1.4–6.0; Obo: 3.56 s, 1.84–6.4; Pelé: 6.6 s, 2.4–18.28) (Mann-Whitney U-test; Español: \( U = 37.5, N_1 = 11, N_2 = 19, P = 0.003 \); Sisu: \( U = 21.5, N_1 = 13, N_2 = 17, P < 0.001 \); Obo: \( U = 12.5, N_1 = 14, N_2 = 6, P = 0.012 \); Pelé: \( U = 13, N_1 = N_2 = 10, P = 0.004 \)).

**Discussion**

All four wild-caught NC crows successfully learnt to use a horizontal mirror to locate hidden food in the 4-box spatial location task, doing so in either 20 or 30 trials. The four crows did not seem to be using olfaction to find the meat baits. First, Obo’s poor performance in the reversed mirror control suggested that she at least was not using olfaction to find hidden baits. Without a meat bait on the mirror surface Obo’s behaviour gave no indication that she knew one of the compartments was baited. Second, if crows were using olfaction and not the mirror to locate meat we would have expected little difference in latency to food retrieval between trials. However, Obo, Pelé and Sisu took significantly less time to retrieve the meat in successful trials compared to unsuccessful ones. Last, if crows depended on olfaction alone we would also have expected them to consistently search the previously baited compartment on unsuccessful trials where meat had recently been hanging. Obo, Pelé and Sisu had 30 unsuccessful trials in total. On only 10 of those 30 trials did these crows search in the previously baited compartment (5 trials each for Pelé and Sisu, see Fig. 2.6). As the meat bait was never in direct contact with the walls of a compartment but hung from string, any residual odour once the bait was removed should have been very weak compared to that coming from the baited compartment.

Our study crows may have had some experience with horizontal reflections of themselves in the wild (e.g. when drinking from pools of rain water). Therefore, it was possible that they had used this past experience to solve the spatial location task. However, given that only one of the four crows solved the 2-box training phase in the last two blocks this possibility seems highly unlikely.
Although Español met the statistical criterion for solving the problem, he may not have used the mirror to locate food in five of his 13 successful trials. In these five trials, he found the bait in the compartment directly below his landing position, which he immediately inspected after landing (see Fig. 2.6). On the remaining eight successful trials he did not look in the compartment directly below his landing position. On 11 of 17 unsuccessful trials, Español immediately looked in the compartment directly below his landing position. Similar behaviour was reported for one of two African Grey parrots (Kyaaro, juvenile) in a study by Pepperberg et al. (1995) which used a 3-box apparatus. Kyaaro appeared to have used the mirror to locate hidden pasta in 44 out of 60 trials. However, the authors argue that Kyaaro developed a position preference and inspected the compartments in consecutive order, starting with the same compartment on one side of the apparatus (Pepperberg et al. 1995).

It was interesting that the two crows which had no vertical mirror experience (Obo and Pelé) solved the 4-box spatial location task faster than Sisu and Español who had had experience with vertical mirrors in Experiment 1. In fact, Obo’s performance was by far the most impressive. In the 2-box training phase she chose correctly 9 out of 10 times in each of her 3 blocks, and she scored a perfect 10 out of 10 in the final block of the 4-box task.

Obo’s poor performance in the reversed mirror control (Movie 2.2) suggests that she had used the mirror in the training and the second block of the 4-box condition to decide which compartment to inspect first. However, her performance in the last six trials in the first block of the 4-box task was surprisingly very poor (unsuccessful in trials 5-8 and 10; Fig. 2.6). We conservatively scored trials 5-8 and 10 as unsuccessful, but it was unclear whether Obo had first inspected a non-baited compartment. It was possible that she had lowered her head in front of an unbaited compartment (violation of criterion 2) in the process of moving it to extract the meat in the baited one. Such a false violation of criterion 2 could occur because of two related factors: a bird not standing directly over the meat and the relatively narrower compartments of the 4-box apparatus.
How were Obo and Pelé using the mirror to solve the 4-box task? It seems unlikely that Obo and Pelé relied on mirror-triggered search because their performance met our criterion for significance in block 2. That is, they went directly to the baited compartment more times than would have been predicted by chance. It also seems unlikely that they had developed a full understanding of mirror correspondence before starting the 4-box task because both birds made errors in their first block of trials. Povinelli (p. 129, 1989) argued that an animal capable of mirror-mediated spatial location must “...understand the correspondence between the object's relative position in a mirror and the real world counterpart to the position.” He also speculated animals could use mirrors to first estimate and move towards the approximate location of the food. Once close enough, they would then use other proprioceptive means to find the exact location of the food (Povinelli 1989).

In our view, this type of mirror-use would not require the mental representation that the food reflected in the mirror was the same as that in the real world behind the occluding board. Rather, it would only require learning to expect to find food (visible in the mirror) when moving towards the food's mirror-image. Therefore, Obo and Pelé were more likely to have been exploiting the correlation between the location of objects and their mirror-images. In other words, they learnt to associate the mirror-image of the meat with finding the bait in the compartment at that location. However, we cannot exclude the possibility that crows like Obo could use this correlation to heuristically develop over time an understanding of mirror correspondence based on mental representations of visually displaced objects.

Conclusion

Our study is the first to investigate the mirror-induced behaviour of captured corvids that had become independent in the wild. In agreement with the jungle crow study (Kusayama et al. 2000), NC crows did not habituate to vertical mirrors. Instead, they consistently engaged in agonistic behaviour in response to their mirror reflection. However, unlike the jungle crows
which could not look behind the mirror, NC crows made exploratory behaviours similar to those seen in primates. Our study suggests that mirror-directed behaviour and exploration in birds as well as primates is facilitated by free access to the back of the mirror.

We also found that NC crows could successfully use a mirror to locate food, probably by exploiting the correlation between the location of objects and their mirror-images. Not only did all four crows reach criterion within 3 sessions (30 trials), the crows without vertical mirror experience (Obo and Pelé) did so in fewer trials than the two crows with experience. These findings suggest that neither self-contingent responses to mirror reflections nor prolonged exposure to mirrors is necessary for some species to exploit the basic correspondence between mirrored objects and their location in the real world.

The performance of three crows (Español, Pelé and Sisu) in the training and test conditions indicated that they had to learn by experience how to exploit the correspondence between the mirror image and the actual location of the meat. In contrast to these three crows, Obo’s performance suggested that she had learnt the correspondence during the very first trial of training. Individual differences in mirror-use performance might reflect each individual's cognitive abilities (e.g. brain capacity to process visual and spatial information) and/or the amount of experience with mirrors. Obo’s relatively error-free performance tends to suggest that the speed at which NC crows learn to use mirrors depends on their cognitive abilities rather than the amount of mirror experience given. She was also the fastest of three crows in solving the trap-tube problem (Taylor et al. 2009). Therefore, Obo’s performance with mirrors may be associated with a superior ability to solve visuospatial problems. Additional studies will help determine if there are any significant age or sex effects on mirror-use in NC crows.

Previous mirror studies have not been designed to provide cross-species data in birds (Pepperberg et al. 1995; Kusayama et al. 2000; Prior et al. 2008). Also, most avian mirror studies have exclusively focused on using mirrors and the mark test to look for evidence of complex social cognition (for a complete list of species that have failed the mark test, see
Pepperberg et al. 1995). Rigorous cross-species comparisons about birds’ understanding of how mirrors work will require careful attention to the design of mirror tasks. For example, the use of large or vertical mirrors may trigger social responses that inhibit learning about how mirrors work. The design of mirror-guided reaching tasks also needs to take into account the absence of manipulatory limbs. Birds have yet to be tested in mirror-guided reaching tasks, although one design involving string-pulling has been proposed (Pepperberg et al. 1995). In an unrelated visually restricted string-pulling study, the string-pulling efficiency of naïve NC crows increased when visual feedback was available in a nearby mirror (Taylor et al. 2010b). This suggests that with the appropriate experimental design NC crows might be able to learn how to use mirrors in synchrony with their body movements.

Along with the African grey parrot study (Pepperberg et al. 1995), our findings show that birds are suitable for investigating a range of cognitive abilities using mirrors. Although we do not know if NC crows can pass the mark test after extended mirror exposure, their ability to succeed at a mirror-mediated spatial location task demonstrates that Corvus species are capable of primate-like processing of mirror information. NC crows are an appropriate model because they react to mirrors and can use them to find visually displaced objects. More importantly, they help to fill the large gap in mirror studies on corvids, a group of birds considered to be the primate equivalents of the avian world (Emery & Clayton 2004a).
Chapter 3

Is string pulling really based on insight?¹

Introduction

A major issue in the study of cognition is whether animals are capable of insightful behaviour. Köhler (1925) claimed that chimpanzees used this ability to obtain out-of-reach food in his classic problem-solving studies, which included tasks such as the stacking of boxes to reach a hanging banana. The string-pulling paradigm has been used to investigate insightful behaviour in birds (e.g. Heinrich 1995). In this task, a bird is presented with a length of string usually hung vertically from a perch with a small food reward on the end of it. To obtain the reward the bird must pull up the string. To our knowledge, successful string pulling has been documented in 23 out of 32 avian species tested (Supplementary Table, p. 66). Insight has often been claimed to explain the behaviour of birds in these studies when they spontaneously execute a sequence of pulling and stepping actions to obtain the reward (Bierens de Haan 1933; Fischel 1936; Thorpe 1943, 1945; Heinrich 1995; Pepperberg 2004; Werdenich & Huber 2006; see Supplementary Table). However, alternative explanations based on innate feeding behaviour, conditioning and trial and error learning have also been proposed to explain string pulling performances in birds (Hertz 1926; Altevogt 1953; Vince 1956, 1958, 1961; Seibt & Wickler 2006; see Supplementary Table).

Heinrich (1995) reported that naïve hand-raised common ravens, Corvus corax, solved the standard string-pulling problem using insight. Heinrich (2000) considered that an animal which used insight was, “...conscious, capable of building mental scenarios, so that alternative choices or motor patterns are expressed or suppressed depending on their probable outcome, either before or after such outcome has been experienced.” Heinrich (1995) also reported that one raven showed evidence of understanding the ‘connectivity’ (sensu Piaget 1954; Hauser 1997; ¹ Collaborative work manuscript. Authors: Medina, F. S., Taylor, A. H., Hunt, G. R. & Gray, R. D.)
Povinelli 2000) between the meat and the string. Based on his findings, Heinrich (p. 1002, 1995) stated that “…‘seeing into the situation’ before executing the behaviour appears to be the most-parsimonious explanation to account for the results.”

In a recent study with New Caledonian crows, *Corvus moneduloides*, Taylor et al. (2010b) proposed that insight did not underlie the crows’ spontaneous string-pulling. Instead, the authors suggested that the crows’ rapid success was based on operant conditioning via a perceptual-motor feedback cycle. The initial pull and then step on the string brings the attached meat closer to the animal, which reinforces a bird’s actions so it continues pulling and stepping until the meat is close enough to reach. They also suggested that this feedback cycle is scaffolded by NC crows’ use of their feet and bill in natural foraging situations and an ability to integrate sensory information rapidly due to their relatively large associative forebrain areas (Mehlhorn et al. 2010).

In the Taylor et al. (2010b) experiment, the crows had to pull up the string through a small hole in the middle of a horizontal wooden platform. The design prevented a crow seeing the meat on the end of the string when the bird moved away from the hole in the process of pulling the string up. It also prevented the crows from accurately assessing if a pull-step results in the food moving closer to the crow. This is because the crows could only assess distance to the food from a head-on angle, rather than from as side angle as in standard string pulling. When the crows’ view of the meat was obstructed in this way their performance was significantly worse than their performance in standard string-pulling. However, when another group of four naïve crows\(^2\) had the opportunity to obtain visual information about the movement of the meat in an adjacent mirror two of them solved the task in a manner similar to the experienced crows. The crows subsequently failed various crossed-string discrimination tests. Taylor et al. (2010b) concluded that the crows solved standard string pulling without an understanding of the causal

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\(^2\) Called the 'mirror group' (p. 2, Taylor et al. 2010b).
relation between the string and the meat (i.e. connectivity). Instead, a perceptual-motor feedback cycle appeared sufficient to explain their spontaneous string pulling.

Although the results of Taylor et al. (2010) are intriguing, the experimental design they used had several possible methodological problems: (1) crows lost sight of relevant elements of the task (most of the string and the ground) when they moved away from the hole in the process of pulling up the string, thus making the task more cognitively demanding than standard string pulling, (2) since it was not possible to reliably track their eye movements, the two successful crows in the mirror group might have succeeded without the aid of a mirror, and (3) the sample sizes of the three treatment groups were low ($N = 4$).

To address these methodological issues we carried out a simpler visually-restricted string-pulling experiment. We fitted an opaque plastic disc just above the meat on the end of the vertical string hung from the perch (opaque condition). A crow pulling up the string could therefore still see the string and the ground but not the meat. We used a transparent plastic disc on the string as a control (clear condition). We first made sure that all the crows were experienced at standard string pulling before being tested on disc string pulling. This was because even an insightful bird may not initially understand the physical properties of the novel string material, therefore some learning about how to handle it would be expected.

We investigated the role of visual feedback in string-pulling by testing between two mutually exclusive hypotheses:

(1) **Spontaneous string-pulling depends on direct visual reinforcement**

A coherent sequence of pull-step actions can only be executed when subjects directly perceive the positive effects of their actions on the position of the reward.

(2) **Spontaneous string-pulling does not depend on direct visual reinforcement**

A coherent sequence of pull-step actions can be mentally simulated (*sensu* Heinrich 2000) and then successfully executed independent of visual feedback.
If spontaneous string pulling by NC crows depends on direct visual reinforcement, their performance in the opaque disc condition should be considerably worse than their performance in both standard string pulling and the clear disc condition where they could clearly see the meat while pulling up the string. We also expected the clear group’s performance to differ little between standard and disc string pulling. In contrast, if insight was behind NC crows’ string-pulling performances, we expected birds to solve the opaque disc condition in a manner that was little different to their most recent (proficient) performance in standard string pulling. This is because an insightful bird presented with the opaque disc condition would know that the meat is connected to the string (and it has seen nothing to suggest that the meat has been knocked off) and be experienced at the pull-step technique. In other words, when a crow approached the string with the opaque disc and the meat disappeared from view, it would be able to compensate for the lack of direct visual reinforcement by calling on past experience and using its causal knowledge about connectivity to solve the problem. Therefore, the experimental design allowed us to test between the predictions of the ‘insight’ and ‘visual reinforcement’ hypotheses.

Methods

Subjects

We conducted the experiment with 19 wild New Caledonian crows on the island of Maré, New Caledonia, in November/December 2007 and August/November 2008. Birds were captured using a whoosh net (www.spidertech.fi/whoosh.php) at sites next to local gardens frequented by crows. A unique combination of coloured leg bands was fitted on each crow at the time of capture. Each crow was also treated for parasites with Ivomec applied externally on the skin at the back of their neck. Based on observations of banded birds in the field we aged the crows by mouth colour. Adults were over 2 years old and had entirely or almost entirely black mouths. Juveniles were under 2 years old with mouth colour that ranged from distinctly red (birds at least ca. 8 months old) to predominantly black. All subjects were housed in a 5-
caged outdoor aviary close to the capture locations. They were held in 4 m × 3.2 m × 3.3 m high
cages with a maximum of 10 crows in the aviary at any one time. The aviary floor was covered
with a layer of sand which was cleaned of droppings and food remains daily. All cages were
provided with ample perching space, dead branches and feeding logs. The crows were left to get
accustomed to the aviary and human presence for three days before any experimental
procedures began. Four to six perches were installed in the experimental and holding cages.
During captivity each crow was fed a main meal in the evening that generally consisted of 40
ml of dry cat/dog biscuits soaked in water, bread or rice and raw egg. As well, crows had access
to clean drinking water and papaya throughout the day. Crows were given a half ration of food
ca. 15 hours prior to their participation in the experiment. Crows’ bodyweights were closely
monitored by at least weekly weighing, in association with a feeding regime designed to keep
the weights within 10% of capture weights. We usually weighed crows on electronic scales
without handling the birds. All birds were captured before the breeding season (November-
January) and kept in the aviary for up to 5 months as per Ethical Approval R602 (University of
Auckland Animal Ethics Committee). After testing, they were released in a healthy state at their
site of capture.

Twelve crows (Batou, Djidji, Korben, Maya, Obo, Tzo Tzo, Samson, Lazlo, Caspar, Pug,
Djinn and Sisu) were naïve to string pulling before the experiment began. Seven crows
(Español, Robin, Egg, Ronia, Slevin, Tiga and Pelé) had participated in a string-pulling
experiment one week before being used for our study (Taylor et al. 2010b). In that experiment
they had all carried out standard string pulling and/or problems where they had to discriminate
between two strings hung from a perch. In addition, six (excluding Pelé) of these seven crows
participated in experiments with visually-restricted string pulling.
**Procedure**

All 19 experimental crows were given 10 standard string-pulling trials just prior to testing; the 12 crows not used in Taylor et al. (2010b) did not receive any prior habituation to the string. Crows were given at most 10 trials per day.

String was hung from a 3 cm diameter × 2 m long wooden perch positioned 1.8 m above the ground. On each trial a small meat cube (less than 1 cm³) was tied onto the distal end of a 45 cm long white nylon string (2-3 mm of diameter) attached to the perch; the distance between meat and the perch was ca. 40 cm. Trials ended when a crow pulled up the meat or 10 minutes elapsed.

Two crows (Djidji and Maya) failed to develop successful string pulling after 100 minutes of exposure to a baited string. They were then given five demonstrations of standard string pulling by an experienced bird (Tzo Tzo). After these demonstrations, both crows were given a further 10 standard string-pulling trials.

![Figure 3.1](image_url) **Figure 3.1** Disc string-pulling task setup. A clear or an opaque disc was attached to a 45 cm long string 40 cm from the perch. A small meat reward is tied to the end of the string just below the disc.
### Table 3.1  Study crows and their assigned groups

<table>
<thead>
<tr>
<th>Opaque disc group</th>
<th>Clear disc group</th>
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<tr>
<td>Batou (^j)</td>
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<td>Djidji (^j)</td>
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<td>Korben (^a)</td>
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<td>Obo (^j)</td>
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<td>Tzo Tzo (^a)</td>
<td>Tiga (^j, e)</td>
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<td>Robin (^a, e)</td>
<td>Pelé (^a, e)</td>
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<td>Ronia (^j, e)</td>
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<td>Slevin (^j, e)</td>
<td>Sisu (^j, n)</td>
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<td>Español (^a, e, n)</td>
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\(^a\) Adult over 2 years old  \(^j\) Juvenile less than 2 years old  
\(^e\) Participated in Taylor et al.’s (2010b) string pulling study  
\(^n\) Excluded from disc string pulling because of a neophobic reaction to the disc.

The 19 crows were assigned pseudo-randomly to the two experimental groups: nine in the opaque disc group and 10 in the clear disc group (Table 3.1). Each group had a mix of crows with or without previous experience of visually-restricted string pulling and of different ages.

All subjects were given 10 string-pulling trials with a disc attached without prior habituation to the disc. Crows were given at most 10 trials per day. We used clear 2 mm thick plastic discs 5.5 cm in diameter. The position of a disc was 5 cm from the end of the string and 40 cm from the perch (Fig. 3.1). One disc was made opaque by placing white masking tape on both sides of it. A test crow entered the experimental cage when the unbaited apparatus was in position. Trials began when the experimenter left the cage after tying a small meat reward underneath the disc. All crows were perched at least 2 m away from the apparatus when the experimenter moved out of the cage. This meant that they easily had a direct line of sight to the (fresh) meat tied under both the clear and opaque discs before initially approaching the test perch (we calculated that a crow perching at the same height as the test perch had to be at least 1.2 m away to see the meat under the disc). Conversely, when a crow was directly over the string on the test perch it could
only see the meat under the clear disc, not the opaque disc. Trials ended when the crow pulled up the disc or 10 minutes elapsed.

Following string pulling with the discs, the crows in both the clear and opaque groups were each given three trials to assess whether they were attending to the presence of meat on the end of the string. The previous procedure with the disc was used except that a 2 cm long length of string was tied under the disc instead of meat.

An independent neophobia test was carried out in July 2010 with both the clear and opaque discs, using seven NC crows naïve to disc string pulling. The crows were tested individually and given 10 meat cubes placed on top of a disc. Birds were left alone for 2 minutes before the experimenter entered the cage and left the baited disc on a table. A trial started when the experimenter left the cage and ended when a crow took the last meat cube from the disc (which all crows did within 10 minutes). Three of the seven crows (Bess, Junior and Pepe) were given the clear disc and the remaining four (Bolt, Caesar, Guille and Kingpin) the opaque disc.

Data analysis

All trials were recorded on video camera through a small hole in the tarpaulin covering the walls of the experimental cage. We analysed the video footage and scored six variables associated with string-pulling performance for each trial: frequency of pulling attempts; frequency of pull-step sequences, time to solution, frequency of release errors, frequency of bend-looks and the number of times the test crow moved more than 40 cm from directly over the string after it first moved to that location. A pulling attempt was coded whenever a crow first tried to pull or yank the string upwards or sideways regardless if the pull or yank was followed by a step or a release error. A pull-step sequence consisted of three consecutive actions: (1) pulling up the string with the bill, (2) stepping on and holding the string, and (3) releasing the string from the bill. When several of these sequences were carried out consecutively a crow usually obtained the meat on the end of the string or at least could touch
the disc. A successful trial was when the crow either obtained the meat (standard string pulling) or touched the disc with its bill (disc string pulling). In disc string pulling a touch indicated success rather than meat retrieval because some crows had to learn how to retrieve the meat under the disc once they had pulled it up to the perch. **Time to solution** was defined as the cumulative time crows spent directly interacting with the string, until they succeeded in getting the bait or touching the disc. If a crow took more than one trial time was accumulated across trials. A **release error** occurred in two ways: (1) when a crow completely released the string right after pulling or yanking at it, without obtaining the meat or touching the disc and the string returned to its original vertical position hanging from the perch, and (2) when a crow made a partial release. A partial release occurred after a successful pull-step when a crow was attempting to pull the string up again and released it from the bill but not the foot. A **bend-looking** occurred when a crow lowered its head by bending its upper body and neck so that the eyes were at perch level. A crow that made a bend-looking at a distance of more than 20 cms along the perch away from the string was able to visually confirm that the meat was still attached to string under the disc (we confirmed this by video analysis, establishing a direct line of sight between a crow’s eyes and the meat under the disc). We finally recorded the number of times (visits) a crow moved more than the string’s length (40 cm) away from where the string was tied to the perch. Continued unsuccessful visits to the location on the perch where the string was tied by the opaque group across all trials would suggest that the crows had object permanence difficulties.

It was important to control for individual variation in standard string-pulling performance before birds transferred to disc string pulling. To do this we used Kendall’s non-parametric test for monotonic trend (Bradley 1968; Kendall 1975) to detect similarity in the standard string-pulling performance of the 12 naïve crows. We calculated Kendall’s test manually. Kendall’s test required equal sample sizes for each comparison, therefore we could only look for a trend in the data over the first eight successful trials for each naïve crow. We could not analyse 10
successful trials because we only gave crows 10 trials and some birds did not succeed in every trial.

Non-parametric Mann-Whitney $U$-tests were carried out with SPSS v15.0. Wilcoxon signed-ranks test results were calculated manually because of low sample sizes. We carried out the $G$-test of independence and the Fisher exact test of independence manually. All tests were one-tailed unless otherwise indicated. We modified the critical alpha level using Bonferroni’s correction to account for multiple pair-wise tests where appropriate. The adjusted alpha level for these tests was $P = 0.017$.

We excluded two crows (Español and Sisu) from the analyses of disc string pulling because they had a neophobic reaction to the disc. Sisu flew away from disc as soon as it came close to her and Español repeatedly yanked, pecked and ripped at the string to cut it and get the meat.

For the neophobia tests, we recorded (1) the time it took a crow to start feeding from the disc ($total$ $approach$ $time$), and (2) the time it took to eat all 10 meat cubes ($total$ $feeding$ $time$).

**Results**

**Standard string-pulling**

Crows had three distinctive behaviours in standard string pulling: (1) consecutive pull-steps while either stationary or moving sideways on the perch, (2) after the first step they created a loop of string under the feet and subsequent pull-steps were possible by pulling only the loop without further stepping (‘shackle bolt’ technique in Thorpe 1963), and (3) loosely holding the string in the bill then turning around and/or moving sideways along the perch to raise the meat without pull-stepping (first described by Taylor et al. 2010b; see Movie 3.1).

Nine of the 12 naïve crows were successful at the standard string pulling task on their first trial (Fig. 3.2). However, two crows (Tzo Tzo and Caspar) produced a sudden, complete and error-free string pulling performance on the first attempt. Tzo Tzo was the only crow to produce
Figure 3.2 Standard string pulling trials for the 12 naïve crows. The coloured cells indicate the level of performance per trial. Blue: successful in first attempt. Green: successful after first attempt. Yellow: interacted with string without success. Red: did not interact with string

There was little difference in the performance of the 12 naïve crows in standard string pulling, and they improved their performance over the first eight trials (Fig. 3.3). Their solution times decreased (Kendall’s monotonic trend test $S = -84$, $N = 12$, $P = 0.001$) (Fig. 3.3a), as did the frequency of their release errors ($S = -80$, $N = 12$, $P = 0.002$) (Fig. 3.3c). The frequency of their pull-step sequences did not vary across the trials ($S = 5$, $N = 12$, $P = 0.571$) (Fig. 3.3b), indicating that four pull-step sequences were sufficient to pull up the 40 cms long piece of string and get the meat. In an exceptional trial, Sam made 51 release errors in trial 4, which accounts for the spike in that trial in Figs. 3a and 3c.

In standard string-pulling trials 5 to 8 we found no significant difference in performance between naïve crows and those used in a previous experiment (excluding Sisu and Español who
Figure 3.3 Standard string pulling performance (group mean ± s.e.m) across successful trials. a Time to solution b Pull-step sequences c Release errors. Sample size is shown above the x-axis for each trial. The vertical line between successful trials 8 and 9 indicates the cut off (N = 12) for inclusion in Kendall’s monotonic trend test (P-values for the tests are shown).
Figure 3.4 Performance in disc string-pulling trials and a trial-by-trial description of bend-look behaviour (numbers within cells). For example, the bend-look data in trial 2 for Ronia (4/3(1)) first indicates the number of bend-looks occurring on the string-pulling perch more than 20 cms away from the string (4), then the number occurring within 20 cms (3), and finally the number occurring within 20 cm during a pull-step sequence (1). The coloured cell key is the same as in Figure 3.2. Crows that participated in Taylor et al. (2010b).

were later neophobic to the disc), indicating that proficient string pulling by all the crows was reached by trial 8 (data for trial 8, two-tailed Mann-Whitney U-test: \( U = 19.5, \ N_1 = 11, \ N_2 = 6, \ P = 0.186 \) for time to solution; \( U = 25, \ N_1 = 11, \ N_2 = 6, \ P = 0.442 \) for pull-step sequences; \( U = 28.5, \ N_1 = 11, \ N_2 = 6, \ P = 0.734 \) for release errors). Crows in the opaque and clear groups also had the same level of standard string-pulling performance at trial 8 (two-tailed: \( U = 19.5, \ N_1 = 9, \ N_2 = 8, \ P = 0.117 \) for time to solution; \( U = 20, \ N_1 = 9, \ N_2 = 8, \ P = 0.126 \) for pull-step sequences; \( U = 27, \ N_1 = 9, \ N_2 = 8, \ P = 0.241 \) for release errors) (Fig. 3.4). Therefore, all crows were similarly proficient in standard string pulling before they progressed to disc string pulling.

<table>
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<tr>
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E Crows that participated in Taylor et al. (2010b).
Disc string pulling

Seven of the eight crows in the clear group solved the task on their first trial, with four of these seven birds doing so on their first attempt. In distinct contrast, only two of the nine crows in the opaque group solved the problem in the first trial, and they did so after their first attempt (Fig. 3.4, Movie 3.2). Up to their first successful solution of the problem (the data were cumulative when crows took more than one trial), the clear group made significantly fewer pull-step sequences, release errors and took less time to pull up the disc compared to the opaque group (clear group medians; pull-steps: $Mdn = 4, N = 8$; release errors: $Mdn = 0.5, N = 8$; time: $Mdn = 11$ s, $N = 8$; opaque group medians; pull-steps: $Mdn = 9, N = 9$; release errors: $Mdn = 8$, $N = 9$; time: $Mdn = 46$ s, $N = 9$) ($U = 10.5, N_1 = 8, N_2 = 9, P = 0.006$; $U = 2, N_1 = 8, N_2 = 9, P < 0.001$; $U = 6, N_1 = 8, N_2 = 9, P = 0.001$; respectively) (Figs 3.5a-c). Crows from the clear group also made significantly fewer pulling attempts, total visits and visits with pulling attempts (clear group medians; pulling attempts: $Mdn = 1.5, N = 8$; total visits: $Mdn = 1, N = 8$; visits with pulling attempts: $Mdn = 1, N = 8$; opaque group medians; pulling attempts: $Mdn = 8$, $N = 9$; total visits: $Mdn = 8, N = 9$; visits with pulling attempts: $Mdn = 5, N = 9$) ($U = 3, N_1 = 8, N_2 = 9, P < 0.001$; $U = 2.5, N_1 = 8, N_2 = 9, P < 0.001$; $U = 2.5, N_1 = 8, N_2 = 9, P < 0.001$; respectively) (Figs 3.5d-f).

Crows from the opaque group appeared confused after their first pull-step and then released the string. They then usually either moved along the perch to try and view the meat under the disc or flew to another perch nearby. Once they had seen the meat from a distance they again tried to pull up the string (Figs. 3.5f & 3.6a). The opaque group made a total of 113 bend-looks (mean ± s.e.m, $1.256 ± 0.282$ times per trial, range 1–41), 195 total visits ($2.167 ± 0.206$ times per trial, range 13–37) and 118 visits with pulling attempts ($1.311 ± 0.108$ times per trial, range 7–22) in the 10 transfer trials. The clear group only made a total of 27 bend-looks ($0.338 ±$
Figure 3.5 Comparison of performance between standard and disc string pulling. **a** Time to solution **b** Pull-step sequences **c** Release errors **d** Pulling attempts **e** Total visits **f** Visits in which pulling was attempted. The data for standard string pulling is from trial 8, and for disc string pulling is up to and including the first successful trial (group mean ± s.e.m., $N = 17$). Significant differences are indicated by $P$-values (Mann-Whitney U-test).
0.096 times per trial, range 0–10), 119 total visits (1.488 ± 0.163 times per trial, range 10–27) and 99 visits with pulling attempts (1.238 ± 0.107 times per trial, range 8–19) in the 10 transfer trials.

The opaque group made significantly more bend-looks (Mdn = 2.5, N = 8) than the clear group (Mdn = 8, N = 9) (U = 16, N₁ = 8, N₂ = 9, P = 0.027), however it was only during transfer trial 1 that the bend-look frequency was significantly different between groups (U = 15.5, N₁ = 8, N₂ = 9, P = 0.029). The opaque group also made relatively more visits without pulling attempts compared to the clear group (G-test of independence: G = 18.84, N = 314, P < 0.001).

Interestingly, while the total number of bend-looks quickly decreased across trials for the opaque group (Fig. 3.6b), relatively few of them occurred during pull-step sequences (opaque group: 9 out of 113 (7.96%); clear group: 2 out of 27 (7.41%)) (Fig. 3.4). Other than Obo, Ronia and Slevin (from the opaque group) and Pelé (from the clear group), no crows stopped their pull-step sequence to bend and look under the disc. Ronia and Slevin did so during their first successful disc string-pulling trial (one and two times, respectively). Birds in the clear group possibly may have had to bend and look under the disc to see the meat if the sunlight was
reflected off the clear disc and impaired direct visual feedback. Within 20 cm bend-looks with
the opaque disc were ineffective as the disc occluded direct viewing of the meat. When bend-
looks that occurred within 20 cm of the string were excluded, the total number decreased to 75
and 10 for the opaque and clear group, respectively.

The performance of crows in the opaque group differed between standard (data from trial 8)
and disc string pulling (cumulative data until the first successful solution) (Wilcoxon signed-
ranks tests; time to solution: $T = 0, N = 9, P = 0.004$; number of pull-step sequences: $T = 1.5, N = 9, P = 0.008$; release errors: $T = 0, N = 9, P = 0.004$; pulling attempts: $T = 0, N = 9, P = 0.004$;
total visits: $T = 0, N = 9, P = 0.004$; visits with pulling attempts: $T = 0, N = 9, P = 0.004$) (Fig.
3.5). In contrast, the performance of crows in the clear group did not differ between standard
(data from trial 8) and disc string pulling (cumulative data until the first successful solution)
time to solution: $T = 10.5, N = 7, P = 0.578$; number of pull-step sequences: $T = 13.5, N = 7, P = 0.938$; release errors: $T = 6.5, N = 5, P = 0.813$; pulling attempts: $T = 6.5, N = 5, P = 0.813$; total visits: $T = 1.5, N = 3, P = 0.500$; visits with pulling attempts: $T = 3, N = 3, P = 1.000$) (Fig.
3.5).

We also found no visible effect on performance due to previous experience with a different
visually-restricted string-pulling test (Taylor et al. 2010b) of any of the six experienced crows
that were equally allocated to both the clear and opaque groups (Fig. 3.4). In the sham trials,
two of the eight crows (Pelé and Lazlo) from the clear group pulled up the unbaited string (in 4
of their combined 6 trials), as did five of the nine (Ronja, Djidji, Korben, Maya and Tzo Tzo)
from the opaque group (in 11 of their combined 15 trials). The group difference was not
significant ($U = 19, N_1 = N_2 = 8, P = 0.086$).

Neophobia tests

The seven crows readily approached their respective disc and took all the food from it soon
after the experimenter left the cage. They did not show any obvious aversion or startle reactions
to either the opaque or clear disc. There was no association between either total approach time or total feeding time and disc type (Fisher exact test for independence; approach time: \(N = 7, P = 1.000\); feeding time: \(N = 7, P = 1.000\)).

**Discussion**

The success rate of the 12 naïve NC crows in standard string pulling was very similar the reported insightful performances of African grey parrots, *Psittacus erithacus*, keas, *Nestor notabilis*, and ravens. Nine of the 12 the NC crows solved the problem on the first trial, compared to two of four African grey parrots (Pepperberg 2004), six of seven keas (Werdenich & Huber 2006) and five of six ravens (Heinrich & Bugnyar 2005). However, the NC crows’ performance in disc string pulling was not compatible with the predictions of the insight hypothesis. The clear group’s performance over multiple measures did not differ between standard and disc string pulling. For example, seven of the eight crows solved the problem on the first trial. In contrast, the performance of crows in the opaque group did differ across these same measures. Crucially, only two of the nine crows solved the task on their first trial. Up to their first success, the birds made more release errors and visits to the apparatus in the disc condition than in standard string pulling, and consequently took longer to get the meat and made more pulling attempts and pull-steps (Fig. 3.5). In the first five trials, the nine crows in the opaque group had four successful disc-pulling trials that were error-free (10% of trials when an attempt to pull the string was made), whereas the eight crows in the clear group had 19 such trials (49%) (Fig. 3.4). These combined results indicate that NC crows’ spontaneous string pulling depends on *on-line* visual feedback rather than *off-line* mentally simulated actions (i.e. insight).

Furthermore, the crows in the opaque group also differed from those in the clear group in often interrupting disc string pulling. These interruptions led to release errors that were followed either by (1) movement away from the apparatus (e.g. flight to a nearby perch) or (2)
distinctive bending behaviour in the proximity of the apparatus (Figs 3.5e-f & 3.6b). Both of these actions allowed the crows to directly view the meat under the disc, after which they again attempted to pull up the string (Fig. 3.6a). It was usually only after several failed pulling attempts that the crows in the opaque group succeeded for the first time (Fig. 3.5d). Their interrupted behaviour before first solution was not consistent with mental planning or the simulation of actions predicted by the insight hypothesis. Moreover, it shows that they had not planned string pulling actions off-line, before their first pull in any of their previous string-pulling trials.

However, in spite of their inefficient string pulling in early trials, the birds in the opaque group progressively made fewer visits, pulling attempts and bend-looks over the 10 trials. This improvement in their disc string pulling proficiency cannot be explained by a decrease in frequency of the bending behaviour. This is because few bend-looks (7.96%) occurred during pull-step sequences and therefore did not account for the higher release error rate observed in the opaque group. Also, as the difference in the frequency of bending behaviour between groups was significant only in transfer trial 1, and in the opaque group it decreased relatively quickly across transfer trials, bending behaviour alone does not explain the poor performance of the opaque group (Fig. 3.5).

Motivational problems due to uncertainty about the presence of the meat below disc are also an unlikely explanation for the crows’ poorer performance with the opaque disc. The fact that the opaque group crows were able to initiate pull-step sequences (Fig. 3.5b) not only suggests that they were well motivated, but also that they were trying to retrieve the out-of-sight meat under the disc. Moreover, the mental simulation of actions predicted by the insight hypothesis can only occur if there is a constant internal psychological motivation to run such simulation prior to the initial pull-step. Such motivation should become stronger after the first successful execution of the simulation. Since the mentally modelled solution remains the same across standard and disc conditions (i.e. no new actions are needed), crows should not have had
problems accessing such simulation and carrying out disc string pulling in a similar manner to standard string pulling.

There was no obvious neophobia to either the clear or opaque disc by the crows given disc string pulling. The clear group’s performance remained constant in both standard and clear disc string pulling. This suggested that, other than Sisu, the birds in the clear group did not have a neophobic reaction to the disc in spite of it being reflective. All birds except Español in the opaque group displayed similar non-neophobic behaviour towards the opaque disc. This non-neophobic behaviour by both groups was consistent with the lack of neophobia to the discs by the seven naïve crows tested in 2010.

A crucial methodological aspect of our disc string pulling experiment is that it controlled for trial and error learning in the development of motor mastery because all crows were similarly proficient at standard string pulling before the first trial. Consequently, the failure of the opaque group to retain the same levels of performance when transferring from standard to disc string pulling cannot be explained by individual differences in motor coordination abilities. Again, the failure to retain the same level of motor mastery when confronted with the opaque disc is inconsistent with suggested insightful problem-solving mechanisms (e.g. ‘mental pondering of possible solutions’: Köhler 1925; ‘means-end apprehension’: Thorpe 1943, Heinrich and Bugynar 2005; ‘connectivity’: Hauser 1997, Povinelli 2000; ‘mental scenario building’: Heinrich 2000). Overall, our findings provide important support for the idea that string pulling is based on operant conditioning via a perceptual-motor feedback cycle (Taylor et al. 2010b).

Previous authors have suggested that the transfer of the string from the bill to the feet is probably scaffolded by natural foraging behaviour (Altevogt 1953; Thorpe 1943; Seibt and Wickler 2006; Taylor et al. 2010b). However, not all birds that use their feet while feeding succeed in string pulling tasks, including Corvus species such as common ravens (see Supplementary Table). Corvids and psittacids, though, show an extraordinarily rare tendency to handle objects when feeding and playing (Lockie 1956; Clark 1973; Hunt 1996; Hunt et al.
2002; Friedman and Davis 1938; Smith 1971; Harris 1989; Diamond and Bond 1991; Pepperberg and Shive 2001; Borsari and Ottoni 2005; Sazima 2008). They also have uncommonly larger pallial brain regions which are considered homologous to the mammalian neocortex (Rehkämper and Zilles 1991; Rehkämper et al. 1991; Medina and Reiner 2000; Lefebvre et al. 2002; Emery and Clayton 2004; Reiner et al. 2004; The Avian Brain Nomenclature Consortium 2005; Schuck-Paim et al. 2008; Mehlhorn et al. 2010). These behavioural and neuroanatomical features could underlie the impressive string pulling performances in corvids and parrots by supporting higher online integration capabilities than in other avian taxa (Taylor et al. 2010b).
**Supplementary Table**  Summary of published reports on string pulling by birds

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Common name</th>
<th>Details of subjects</th>
<th>Success level</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hertz 1926</td>
<td><em>Corvus monedula</em></td>
<td>jackdaw</td>
<td>1 wild caught fledging</td>
<td>S³</td>
<td>innate feeding</td>
</tr>
<tr>
<td></td>
<td><em>Corvus corone</em> f</td>
<td>carrion crow</td>
<td>1 wild caught fledging</td>
<td>F</td>
<td>behaviour</td>
</tr>
<tr>
<td>Teyrovsky 1930</td>
<td><em>Sylvia borin</em></td>
<td>garden warbler</td>
<td>1 captive male au</td>
<td>F³</td>
<td>ideational behaviour</td>
</tr>
<tr>
<td>Bierens de Haan</td>
<td><em>Carduelis carduelis</em> f</td>
<td>goldfinch</td>
<td>3 naïve au</td>
<td>2S⁴, 1F</td>
<td>primary problem</td>
</tr>
<tr>
<td>1933</td>
<td><em>Fringilla coelebs</em> f</td>
<td>chaffinch</td>
<td>2 birds au</td>
<td>F⁵</td>
<td>understanding</td>
</tr>
<tr>
<td></td>
<td><em>Carduelis spinus</em> f</td>
<td>siskin</td>
<td>1 adult pair au</td>
<td>2F</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Carduelis chloris</em> f</td>
<td>greenfinch</td>
<td>2 birds au</td>
<td>2F</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Carduelis cannabina</em> f</td>
<td>linnet</td>
<td>1 adult female, 1 male juvenile au</td>
<td>2F</td>
<td></td>
</tr>
<tr>
<td>Fischel 1936</td>
<td><em>Ara ararauna</em> f</td>
<td>blue-and-yellow macaw</td>
<td>2 birds au</td>
<td>2S⁶</td>
<td>insight</td>
</tr>
<tr>
<td></td>
<td><em>Amazona amazonica</em></td>
<td>orange-winged Amazon parrot</td>
<td>1 bird au</td>
<td>1S⁶</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Carduelis spinus</em> f</td>
<td>siskin</td>
<td>1 captive bred</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Melopsittacus undulatus</em></td>
<td>budgerigar</td>
<td>1 female, 1 male au</td>
<td>2F</td>
<td></td>
</tr>
<tr>
<td>Herter 1940</td>
<td><em>Parus caeruleus</em> f</td>
<td>blue tit</td>
<td>2 wild caught male adults</td>
<td>S⁷</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td><em>Parus niger</em> f</td>
<td>black tit</td>
<td>1 wild caught male</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Sitta europaea</em> f</td>
<td>Eurasian nuthatch</td>
<td>1 wild caught</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Thorpe 1943</td>
<td><em>Parus major</em> f</td>
<td>great tit</td>
<td>1 wild caught</td>
<td>S⁵</td>
<td>insight</td>
</tr>
</tbody>
</table>

a anecdotal observation, usually from feeding tables, f tested previously, au origin/sex/age unclear, f reported to use feet while feeding
### Supplementary Table continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Common name</th>
<th>Details of subjects</th>
<th>Success level $^1$</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thorpe 1945</td>
<td><em>Parus caeruleus</em> $^{1,f}$</td>
<td>blue tit</td>
<td>1 bird, unclear</td>
<td>S$^5$</td>
<td>insight</td>
</tr>
<tr>
<td></td>
<td><em>Pyrrhula spp.</em> $^f$</td>
<td>bullfinch</td>
<td>hand-reared, number unclear</td>
<td>F$^a$</td>
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</tr>
<tr>
<td></td>
<td><em>Carduelis spinus</em> $^{1,f}$</td>
<td>siskin</td>
<td>wild caught, number unclear</td>
<td>1S, rest F$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Passer domesticus</em> $^f$</td>
<td>house sparrow</td>
<td>1 wild male</td>
<td>S$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Carduelis flammea</em> $^f$</td>
<td>redpoll</td>
<td>1 wild caught</td>
<td>F$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Fringilla coelebs</em> $^{1,f}$</td>
<td>chaffinch</td>
<td>unclear</td>
<td>F$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Carduelis chloris</em> $^{1,f}$</td>
<td>greenfinch</td>
<td>1 bird $^{ou}$</td>
<td>S$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Erithacus rubecula</em></td>
<td>robin</td>
<td>unclear</td>
<td>F$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Corvus frugilegus</em> $^f$</td>
<td>rook</td>
<td>unclear</td>
<td>S$^a$</td>
<td></td>
</tr>
<tr>
<td>Altevogt 1953</td>
<td><em>Parus caeruleus</em> $^{1,f}$</td>
<td>blue tit</td>
<td>1 tame bird, 9 juveniles $^{ou}$</td>
<td>all S$^8$</td>
<td>innate behaviour</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(tactile-proprioceptive stimulus situation)</td>
</tr>
<tr>
<td>Vince 1956</td>
<td><em>Parus major</em> $^{1,f}$</td>
<td>great tit</td>
<td>12 wild caught adults</td>
<td>1S, 11F$^a$</td>
<td>classical conditioning</td>
</tr>
<tr>
<td>Vince 1958</td>
<td><em>Fringilla coelebs</em> $^{1,f}$</td>
<td>chaffinch</td>
<td>6 wild caught adults, 4F</td>
<td></td>
<td>classical conditioning</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 hand-reared adults</td>
<td>4F</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 hand-reared juveniles, 6F</td>
<td></td>
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</tr>
<tr>
<td>Vince 1958, 1961</td>
<td><em>Chloris chloris</em> $^{1,f}$</td>
<td>greenfinch</td>
<td>6 wild caught adults, 5F</td>
<td></td>
<td>classical conditioning, trial and error learning</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 aviary-reared juveniles, 1S, 4F</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 hand-reared juveniles, 2S, 5F</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ anecdotal observation, usually from feeding tables, $^1$ tested previously, $^{ou}$ origin/sex/age unclear, $^f$ reported to use feet while feeding
<table>
<thead>
<tr>
<th>Study</th>
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<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vince 1958, 1961</td>
<td><em>Serinus spp.</em>)</td>
<td>domestic canary</td>
<td>6 adults&lt;sup&gt;ou&lt;/sup&gt;</td>
<td>6F</td>
<td>classical conditioning,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 juveniles&lt;sup&gt;ou&lt;/sup&gt;</td>
<td>6S&lt;sup&gt;10&lt;/sup&gt;</td>
<td>trial and error learning</td>
</tr>
<tr>
<td>Dickinson 1969</td>
<td><em>Parus bicolor.</em>)</td>
<td>tufted titmouse</td>
<td>1 bird&lt;sup&gt;ou&lt;/sup&gt;</td>
<td>1S&lt;sup&gt;a,11&lt;/sup&gt;</td>
<td>none</td>
</tr>
<tr>
<td>Dücker &amp; Rensch 1977</td>
<td><em>Melopsittacus undulatus</em>)</td>
<td>budgerigar</td>
<td>3 birds&lt;sup&gt;ou&lt;/sup&gt;</td>
<td>3S&lt;sup&gt;12&lt;/sup&gt;</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td><em>Acridotheres tristis</em></td>
<td></td>
<td>1 bird&lt;sup&gt;ou&lt;/sup&gt;</td>
<td>S&lt;sup&gt;13&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Corvus monedula</em>)</td>
<td></td>
<td>1 bird&lt;sup&gt;ou&lt;/sup&gt;</td>
<td>S&lt;sup&gt;14&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Heinrich 1995</td>
<td><em>Corvus corax.</em>)</td>
<td>common raven</td>
<td>5 hand-reared adults,</td>
<td>1S, 4F&lt;sup&gt;15&lt;/sup&gt;</td>
<td>insight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 hand-reared juveniles</td>
<td>4F&lt;sup&gt;16&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 wild caught;</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 birds in the wild;</td>
<td>all F</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>14 wild caught</td>
<td>6S, 8F</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>13 wild caught</td>
<td>5S, 8F</td>
<td></td>
</tr>
<tr>
<td>Funk 2002</td>
<td><em>Cyanoramphus auriceps</em></td>
<td>yellow-crowned parakeet or kakariki</td>
<td>6 parent-raised juveniles</td>
<td>S&lt;sup&gt;17&lt;/sup&gt;</td>
<td>none</td>
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<tr>
<td>Pepperberg 2004</td>
<td><em>Psittacus erithacus.</em>)</td>
<td>African grey parrot</td>
<td>3 captive bred adult</td>
<td>2F&lt;sup&gt;18&lt;/sup&gt;, 1S</td>
<td>insight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 hand-reared adult</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Heinrich &amp; Bugynar 2005</td>
<td><em>Corvus corax.</em>)</td>
<td>common raven</td>
<td>12 hand-reared juveniles</td>
<td>5S, 1F&lt;sup&gt;19&lt;/sup&gt;</td>
<td>means-ends comprehension</td>
</tr>
</tbody>
</table>

<sup>a</sup> anecdotal observation, usually from feeding tables,  <sup>1</sup> tested previously,  <sup>ou</sup> origin/sex/age unclear,  <sup>f</sup> reported to use feet while feeding
(Supplementary Table continued)

<table>
<thead>
<tr>
<th>Study</th>
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<th>Details of subjects</th>
<th>Success level</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seibt &amp; Wickler 2006</td>
<td><em>Carduelis carduelis</em> f, t</td>
<td>goldfinch</td>
<td>52 aviary-reared subadults(^{20})</td>
<td>25S(^{21}), 27F</td>
<td>innate behaviour, trial and error, and social learning</td>
</tr>
<tr>
<td></td>
<td><em>Carduelis spinus</em> f, t</td>
<td>siskin</td>
<td>29 aviary-reared subadults(^{22})</td>
<td>21S(^{23}), 8F</td>
<td></td>
</tr>
<tr>
<td>Werdenich &amp; Huber 2006</td>
<td><em>Nestor notabilis</em> f</td>
<td>kea</td>
<td>2 females, 5 males(^{oa})</td>
<td>6S, 1F(^{24})</td>
<td>insight learning</td>
</tr>
<tr>
<td>Schuck-Paim et al. 2009</td>
<td><em>Anodorhynchus</em></td>
<td>Hyacinth macaw</td>
<td>1 hand-reared mated pair</td>
<td>2S(^{24})</td>
<td>goal-directed means-end understanding</td>
</tr>
<tr>
<td></td>
<td><em>Hiacynthinus</em> f</td>
<td></td>
<td>1 wild caught mated pair</td>
<td>2S(^{25})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Anodorhynchus leari</em></td>
<td>Lear's macaw</td>
<td>1 wild caught mated pair</td>
<td>2S(^{26})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Amazona aestiva</em> f</td>
<td>blue-fronted Amazon parrot</td>
<td>2 hand-reared females</td>
<td>2S(^{27})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Guaruba guarouba</em> f</td>
<td>Golden Conure</td>
<td>1 wild caught adult pair</td>
<td>2F(^{28})</td>
<td></td>
</tr>
<tr>
<td>Magat &amp; Brown 2009</td>
<td><em>Nymphicus hollandicus</em></td>
<td>cockatiel</td>
<td>5 captive-reared adults</td>
<td>5F</td>
<td>enhanced cognition</td>
</tr>
<tr>
<td></td>
<td><em>Melopsittacus undulatus</em> t</td>
<td>budgerigar</td>
<td>5 captive-reared adults</td>
<td>5F</td>
<td>theory</td>
</tr>
<tr>
<td></td>
<td><em>Eolophus roseicapilla</em> f</td>
<td>galah</td>
<td>5 captive-reared adults</td>
<td>S(^{29})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Callocephalon fimbriatum</em> f</td>
<td>gang-gang cockatoo</td>
<td>5 captive-reared adults</td>
<td>S(^{30})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Calyptorhyncus banksii</em> f</td>
<td>red-tailed black cockatoo</td>
<td>5 captive-reared adults</td>
<td>S(^{31})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Cacatua scapularis</em> f</td>
<td>sulphur-crested cockatoo</td>
<td>5 captive-reared adults</td>
<td>S(^{32})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Alisterus scapularis</em> f</td>
<td>Australian king parrot</td>
<td>5 captive-reared adults</td>
<td>S(^{33})</td>
<td></td>
</tr>
<tr>
<td>Taylor et al. 2010b</td>
<td><em>Polystelis swainsonii</em> f</td>
<td>superb parrot</td>
<td>5 captive-reared adults</td>
<td>S(^{34})</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Corvus moneduloides</em> f</td>
<td>New Caledonian crow</td>
<td>7 adults, 5 subadults, wild caught</td>
<td>4S(^{34})</td>
<td>operant conditioning via perceptual-motor feedback</td>
</tr>
</tbody>
</table>

\(^{a}\) anecdotal observation, usually from feeding tables, \(^{t}\) tested previously, \(^{oa}\) origin/sex/age unclear, \(^{f}\) reported to use feet while feeding
whether tested birds succeeded (S) or failed (F) in the standard string pulling problem.
failed with strings, succeeded with wide stripes (>5mm).
failed also after shortening the string, succeeded only with horizontal string.
setup consisted of a stringed wagon on a ramp (at a 45 degrees angle) next to cage that was filled with water or food; one subject showed flawless performance, the other succeeded through trial and error.
1 subject died, the other never stepped over the string.
flawless performance, string was 1m long.
string was progressively lengthened.
non-baited strings were pulled up.
successful subject exhibited flawless performance in first trial, 4 subjects that failed succeeded after training with short strings.
4 of the juveniles were given wire instead of string, thus string pulling was not possible for these subjects.
bird was observed pulling up a caterpillar hanging from a ca. 18-inch long silken thread.
fastest subject pulled up a 15-cm long string after 5 trials.
pulled up a 15 cm long string after 6 trials.
flawless performance at first trial.
3 more hand-reared adults succeeded after observing a demonstrator raven.
3 juveniles succeeded 20 months later, when adults.
4 subjects succeeded in first session, 5 more needed one or two extra sessions.
2 language-trained parrots did not pull up the string but instead asked the experimenters to give them the bait.
only 6 raven juveniles were given the standard string pulling test, the other 6 failed in a counterintuitive task.
12 subjects were deprived of branches during their ontogeny prior to testing.
results suggested early experience with branches has a positive effect (but it is not necessary) on later string-pulling behaviour; also, 13 unsuccessful goldfinches subsequently succeeded after demonstrations by an experienced bird.
2 subjects were deprived of branches during their ontogeny prior to testing.
3 unsuccessful siskins subsequently succeeded after demonstrations by an experienced bird.
unsuccessful juvenile succeeded 1 month later after three 10 min sessions.
tested in pairs, birds worked simultaneously to pull up the string.
26 tested in pairs, female participation was probably prevented by dominant males.
tested in pairs, subjects took turns at pulling up the string.
ever interacted with the string.
no subjects solved the problem at first exposure; success rate reported as 66 ± 5.1% across 10 trials, with 50-cm long string.
2 subjects solved the problem at first exposure; success rate was 88 ± 5.8%.
1 subject solved the problem at first exposure; success rate was 86 ± 5.8%.
1 subject solved the problem at first exposure; success rate was 88 ± 4.9%.
1 subject solved the problem at first exposure; success rate was 82 ± 5.8%.
no subjects solved the problem at first exposure; success rate was 62 ± 3.7%.
3 crows succeeded in first trial and 1 in the second trial (only these 4 crows were given the standard string pulling problem before a visually-restricted task); 4 experienced and 1 naïve crow solved a visually-restricted task in 1-6 trials, 2 other naïve crows solved a visually-restricted task when a mirror could provide visual feedback.
PART II

THE BRAIN
Chapter 4

Brain–behaviour relationships: what do they actually tell us about the evolution of cognition and tool-use?

Introduction

The study of brain–behaviour relationships originated from the early concern of neuropsychology with the identification of cerebral structures that subserve human cognitive functions (Godefroy et al. 1998). The notion of the brain being related to cognition is far older, though. Aristotle, put forward the idea that humans, in proportion to their size, were endowed with the largest brain of all animals (Aristotle 350 BC). Along with the idea of *scala naturae*, also from Aristotle, this idea permeated throughout the history of Western philosophy and science until the modern misconceptions of brain evolution of the past century (Hodos & Campbell 1969; Jerison 1977; Deacon 1990; Campell & Hodos 1991). While such anthropocentric views have lost scientific support, with the morphing of the old field of comparative cognition into the new field of animal cognition (Healy et al. 2009; van Horik & Emery 2011), brain–behaviour relationships have gained renewed interest because they supposedly serve to link the evolution of the brain (Barton & Harvey 2000; Clark et al. 2001; de Winter & Oxnard 2001; Iwaniuk et al. 2004; see Iwaniuk & Hurd 2005 for a full list of vertebrate studies) with the emergence of *human-like* cognition among vertebrates (Heyes & Huber 2000; Becoff et al. 2002; Watanabe 2004; Reznikova 2007; Shettleworth 2010).

Studies often compare areas of the brain that have been traditionally associated with cognition (e.g. the mammalian neocortex) with the species’ body mass (e.g. Rehkämper & Zilles 1991), or more recently, with the total brain volume (e.g. Barton & Harvey 2000; Iwaniuk & Hurd 2005). The resulting ‘relative brain size’ or ‘brain fraction’ is then plotted against other cognition proxies such as, group size (Dunbar & Shultz 2007), sociality (Burish et al 2004; Shultz & Dunbar 2007), behavioural innovation (Timmermanns et al. 2000; Lefebvre
et al. 2004) and tool-use (Lefebvre et al. 2002), in search for strong correlates that may be proposed as drivers of the evolution of the vertebrate brain architecture. Unfortunately, the ongoing pouring of work of brain–behaviour correlates (Marino 2002; Burish et al. 2004; Emery & Clayton 2004a; Emery 2006; Dunbar & Shultz 2007; Marino et al. 2007; Cnotka et al. 2008a; Iwaniuk et al. 2009; Mehlhorn et al. 2010a; Shultz & Dunbar 2010a, b) does little to further increase our understanding of brain evolution or its role in the emergence of extraordinary cognition. For this body of evidence to be useful (i.e. in leading to the formulation of mechanistic explanations behind cognition), it should form the basis of exploratory experimental analyses that may lead to confirmation that the alleged brain parts really do participate in generating the behaviour in question (Healy & Rowe 2007). Certainly, a different approach to the analysis of brain–behaviour relationships is much needed and birds, in particular, may represent a rare opportunity to help elucidate the precise nature of such findings in terms of the evolution of the vertebrate brain and its internal architecture.

It has been recently recognised (Reiner et al. 2004) that the avian cerebral nomenclature had been based on flawed assumptions of homologies to mammals. Instead, the mammalian and avian brains share deep homologies that have led to a major re-description of the avian brain to emphasize the neocortex-like cognitive functions of the avian pallium (Jarvis et al. 2005). At the same time, recent findings suggest that birds have evolved cognitive abilities that rival, and in some cases exceed those of primates (Pepperberg 1999; Emery & Clayton 2004b; Emery 2004). Indeed, a wide range of complex behaviour has been reported in corvids that includes the manufacture sophisticated tools (Hunt 1996), episodic-like memory (Clayton & Dickinson 1998), planning for the future (Raby et al. 2007), tactical deception (Heinrich & Pepper 1998; Bugnyar & Kotschach 2002, 2004), making complex social inferences (Bond et al. 2003; Paz-y-Mino et al. 2004), metatool use (Taylor et al. 2007), causal reasoning (Taylor et al. 2009) and context dependent tool-use (Taylor et al. 2012b). But how different exactly is the avian brain from the mammalian brain? While a definitive answer is still a work in progress, the most
obvious differences are related to the cytoarchitectonic organisation of the forebrain (i.e. the telencephalon), and particularly the pallium. Basically, the cells in the bird telencephalon cluster together to form several distinct pallial regions or nuclei, laid around the striatum and on top of each other like irregular sheaths, whereas in the mammalian telencephalon cells are arranged in six layers or laminae, giving rise to a typically folded neocortex\(^1\) (Karten 1991; Puelles et al. 2000; Jarvis et al. 2005).

Of the avian pallial regions, several have been found to render strong brain–behaviour correlates so far. For instance, a comparatively enlarged mesopallium has been found to best indicate feeding innovation rate\(^2\) among bird taxa (Timmermans et al. 2000). However, the nidopallium has been found to best predict bird true tool-use (Lefebvre et al. 2002). Noticeably, while both studies (Timmermans et al. 2000; Lefebvre et al. 2002) support the notion of co-evolution of large brains and complex cognitive processes in birds\(^3\), they do reach different conclusions depending on whether they focus on the total frequency of feeding innovation reports (Timmermans et al. 2000) or, more specifically, on the reported degree of tool-use (i.e. emphasis on categories like *borderline* and *true* tool-use) (Lefebvre et al. 2002). Both studies agree, though, that the anatomical and functional evidence of the integrative nature of the nidopallium (which receives primary and secondary multimodal sensory projections) favours its potential role over that of the mesopallium (considered a higher order, tertiary area) in both the cognitive and sensorimotor aspects of tool-use. Other studies support the view that the

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\(^1\) In spite of this apparent dissimilitude between the physical organisation of the avian telencephalon and the layers of the mammalian neocortex, the fundamental telencephalic circuitry of auditory and visual pathways has been shown to be common to all amniotes (Wild et al. 1993; Karten 1997). Furthermore, a recent study by Wang et al. (2010) has demonstrated the existence of comparable columnar functional modules in the laminated auditory telencephalon of the chick brain (*Gallus gallus*). Together, these findings challenge the long-held view in which the laminated nature and circuitry of mammalian neocortex are thought to be unique in the Vertebrate clade.

\(^2\) Also known as feeding flexibility rate, the feeding innovation rate is operationally defined as the frequency of *innovations* collated from the short notes sections of ornithology journals; an *innovation* is defined as a food type or foraging technique stated to be ‘noteworthy’, ‘unusual’, ‘unknown’, ‘rare’, ‘opportunistic’, ‘interesting’, ‘adaptable’, ‘strange’, ‘not noted before’, ‘not recorded’, or ‘not mentioned’ (see Lefebvre 1997).

\(^3\) An observation that is generally used to imply that larger neural substrates should favour greater information processing abilities and thus favour *human-like* cognition (i.e. the bigger the brain the better; however, for a critique see Chittka & Niven 2009).
caudolateral portion of the nidopallium plays a central role in juvenile and adult learning (Mogensen & Divac 1993; Braun et al. 1999), and even that this region is homologous to the mammalian prefrontal cortex (Timmermans et al. 2000; Güntürkün 2005; Emery 2006). Finally, Iwaniuk et al. (2009) have recently suggested that tool-using birds have significantly more complex cerebellar cortices (i.e. cerebella with increased number of folds) but not a larger cerebellum than non-tool-using birds, further suggesting that the integrative role of the cerebellum may have been crucial in the evolution of cognitive processes.

After the discovery that the New Caledonian crow, *Corvus moneduloides*, manufactured complex tools (Hunt 1996) the species entered the field of comparative cognition as another model for investigating the evolution of cognition. To date, NC crows are the only nonhuman species capable of crafting hooked tools4 (Hunt 1996; Hunt & Gray 2004; but see also Weir et al. 2002). Although great tool-users, it remained unknown until recently that NC crows were also exceptional in solving cognitively demanding tasks not involving stick or stick-like tools (von Bayern et al 2009; Taylor et al. 2011). Furthermore, the NC crow behavioural performance in the trap-tube problem (Taylor et al. 2009) and the mirror-mediated spatial location task suggested an enhanced capacity to solve visuospatial problems (Medina et al. 2011; Chapter 2). Also, it was recently stated that “... NC crows’ exceptional skills are associated with one of the highest encephalisation values in avian species studied so far.”(Cnotka et al. 2008a, p 243).

However, the finding that NC crows possess a higher than expected degree of encephalisation5 should be weighed with caution because statistical analyses were carried out with merged data.

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4 See footnote 17, in p. 6 Chapter 1.

5 Brain mass and body mass correlate allometrically (Jerison 1977). This allows us to compare brains from different species on a logarithmic scale. However, since its inception this type of comparative analysis was based on fallacies of evolutionary progression (i.e. *scala naturae*). Also, this type of analysis considers that the slope of the log brain/body trend as a ‘mental equivalence’ axis despite differences in body size. Any residual deviations above or below this trend line are presumed to be the correlates of relative increase or decrease in encephalisation with respect to this baseline (Deacon 1990). Also, studies of degree of encephalisation more often than not choose to ignore species-typical body mass variations due to diet, metabolism, maturity or sex. Different axes of ‘mental equivalence’ or intelligence can be created for the same dataset when individuals are categorised according to these variables.
datasets from different sources and because there was still not a comprehensive character-based phylogeny of *Corvus* species (but see recent studies: Jønsson et al. 2012; Haring et al. 2012), and thus regressions were carried without phylogenetic corrections\(^6\).

It is the aim of our present study to provide a detailed account of the neuroanatomical features of the NC crow’s brain, building on previously published work (Cnotka et al. 2008a; Mehlhorn et al. 2010a). We present here an atlas for future studies. We also measured the variation of the log brain mass–log body mass relationship due to body mass fluctuation that occur in captivity using encephalisation indices (Jerison 1977; Cnotka et al. 2008a) and brain size residuals (Lefebvre et al. 2002), and discuss the reliability of encephalisation degree as a proxy for cognition in comparative studies.

Next, we investigated whether the naturally occurring handedness in NC crows’ tool behaviour (Rutledge & Hunt 2004; Weir et al. 2004) was correlated with individual, interhemispheric volumetric asymmetries in the telencephalon and in the nucleus rotundus of the thalamic visual pathway (Karten & Hodos 1970; Reiner et al. 2005; Shimizu et al. 2010). This behavioural lateralisation (tool handedness) may be a result of the visual lateralisation that starts early in the development of the embryo inside the egg (Kuo 1932; Rogers 1982; Güntürkün 2002; Wichman et al. 2009). Alternatively, experience with tools (and therefore, tool handedness) may induce volumetric brain asymmetries in a similar way as navigational experience does in homing pigeons, *Columbia livia* (see Cnotka et al. 2008b and Mehlhorn et al. 2010b)\(^7\). Therefore, our working hypothesis was that the side of the bill on which a NC crow

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\(^6\) Also, improper use of statistical tests by wrongly assuming that 21 individuals from 5 different species (seven European carrion crows, two Eurasian magpies, three European jays, five NC crows and four domestic sparrows) may be treated as independent data points (i.e. \(N = 21\) was used, instead of \(N = 5\) using the species means) and that parametric tests may be more suitable than non-parametric tests with such low sample size.

\(^7\) Cnotka et al. (2008b) measured the size of 14 different brains structures/subdivisions in 20 homing pigeons. That study revealed that in individuals with navigational experience (\(N = 10\)) the hippocampal volume (relative to the telencephalon) was 11.2% larger than that of non-experienced individuals (\(N = 10\)). The authors drew the conclusion that navigational “... experience is a precondition to full hippocampal development.” (p. 233, Cnotka et al. 2008b). In the Mehlhorn et al. (2010b) study, the volumetric analysis of the brains of 14 homing pigeons revealed that in experienced subjects (\(N = 7\)) the right mesopallium is significantly larger than the left one, whereas in non-experienced individuals (\(N = 7\)) the trend is inversed (i.e. left mesopallium is larger than the right one). In
chooses to hold its tool is a direct function of the position of the tip of the tool with respect to
the crow’s potential dominant eye (i.e. tools are held so that crows can survey the tip with their
preferred eye). To control for overall sensory (brain) lateralisation, we also measured the
volume of nucleus ovoidalis of the auditory pathway (Karten 1968; Reiner et al. 2005; Wild et
al. 2010) which was not expected to show volumetric lateralisation associated with tool-use. We
thus investigated if tool handedness did co-occur with an increase in size of the ipsilateral
nucleus rotundus which receives indirect input, predominantly, from the contralateral eye retina
(via optic tectum).

In addition, we used our telencephalic volumetric data to re-examine previously published
findings on relative size of the mesopallium of the NC crow’s brain (Mehlhorn et al. 2010a). Last,
we investigated if different measures of cerebellar complexity yield the same results as
those of Iwaniuk et al. (2009) who studied the ‘cerebellar–tool-use’ relationship. The authors
reported that complexity of tool-use in avian taxa positively correlated with cerebellar
complexity. Given the complexity of the NC crow’s tool skills, we expected that the complexity
of its cerebellum would stand out amongst bird species.

SECTION 1: GENERAL DESCRIPTION AND BRIEF NEUROANATOMICAL STUDY
OF THE NC BRAIN

Study scope

We give a detailed description of the NC crow brain by presenting the first brain atlas for the
species. Also, we analyse the effects of using different samples of the same geographical
population and the appropriateness of Model I regression analysis in comparative study of brain
encephalisation. Finally, we present evidence that challenges the view that single measurements

addition, this study reported that subjects with navigational experience had significant volumetric asymmetries in
the following brain nuclei: hyperpallium apicale (left was larger), nidopallium (right was larger), hippocampus (left
was larger) and optic tectum (right was larger) (all in relation to the telencephalon). In contrast, non-experienced
subjects only exhibited a larger left hyperpallium apicale.
of body mass yield reliable encephalisation indices and we discuss the limitations of using body mass-based encephalisation degree as a proxy for cognition in comparative studies.

**Methods**

**Subjects**

Nine New Caledonian crows (*Corvus moneduloides*) captured on the island of Maré, New Caledonia, in August/October 2007 were used for this study (Table 4.1). We captured the birds using a ‘whoosh net’ (obtained from SpiderTech Bird Nets, Helsinki, Finland) and aged by mouth colour. Crows over 2 years old were classified as adults and those under 2 years old as juveniles (see Table 4.1). Female and male distinction was based on bill morphology and bodyweight on the day of capture (Kenward et al. 2004).

**Table 4.1 Study crows details**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Tool preference</th>
<th>N of trials</th>
<th>Body mass$^b$ (g)</th>
<th>Brain mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batou</td>
<td>male</td>
<td>juvenile</td>
<td>58.3% pandanus</td>
<td>24</td>
<td>335</td>
<td>7.426</td>
</tr>
<tr>
<td>Español</td>
<td>male</td>
<td>adult</td>
<td>100% stick</td>
<td>24</td>
<td>320</td>
<td>7.474</td>
</tr>
<tr>
<td>Sisu</td>
<td>female</td>
<td>juvenile</td>
<td>100% stick</td>
<td>24</td>
<td>270</td>
<td>6.211</td>
</tr>
<tr>
<td>Robin</td>
<td>male</td>
<td>adult</td>
<td>62.5% stick</td>
<td>24</td>
<td>310</td>
<td>6.901</td>
</tr>
<tr>
<td>Pelé</td>
<td>male</td>
<td>adult</td>
<td>100% stick</td>
<td>24</td>
<td>309</td>
<td>7.581</td>
</tr>
<tr>
<td>Egg</td>
<td>female</td>
<td>juvenile</td>
<td>91.7% stick</td>
<td>24</td>
<td>292</td>
<td>6.407</td>
</tr>
<tr>
<td>Tiga</td>
<td>male</td>
<td>juvenile</td>
<td>77.8% pandanus</td>
<td>27</td>
<td>357</td>
<td>8.073</td>
</tr>
<tr>
<td>Obo</td>
<td>female</td>
<td>juvenile</td>
<td>96.2% stick</td>
<td>26</td>
<td>288</td>
<td>8.024</td>
</tr>
<tr>
<td>Slevin</td>
<td>male</td>
<td>juvenile</td>
<td>55.6% stick</td>
<td>27</td>
<td>323</td>
<td>7.210</td>
</tr>
</tbody>
</table>

$^a$An adult is > 2 years old; a juvenile is < 2 years old  
$^b$Body mass on perfusion day

All the crows were housed in a 5-cage outdoor aviary situated in primary forest inland from the coast. The cages were 3 m high and at least 4 m × 2 m in area. All cages were provided with ample perching space, branches and feeding logs. The crows were left to get accustomed to the aviary and human presence for three days before any experimental procedures began. The crows were fed a main meal in the evening consisting of soaked dog/cat biscuits (40+ mL of dry
biscuits left on water for 15 min), bread or rice and occasionally raw egg. Papaya and clean drinking and bathing water were available throughout the day.

Their bodyweights were closely monitored during captivity to maintain them at or above capture weight (an extract of the bodyweight records is in Table 4.2). This was done by weighing them on an electronic scales at least 2 times per week, once they landed on a perch set up on the scales. All the crows were captured before breeding season (November-January) and kept in the aviary until they were perfused approximately 5 months later. During the captivity period all subjects participated in daily experimental work for which they were regularly rewarded with small (~ 1 cm³) blocks of raw meat. The amount of meat given per day varied according to the experiment in which a crow participated (see Taylor et al. 2009, 2010b; Medina et al. 2011).

The present research was approved by the University of Auckland Animal Ethics Committee (Approval R602) and complies with the laws of New Caledonia.

Animal euthanasia

One crow was perfused per day. In the morning of each day, a crow was caught and put into a clean, dark cloth bag and its bodyweight was immediately recorded with an electronic scale (Table 4.2). A crow was then injected with a lethal dose of pentobarbital (30 mg/kg, intramuscular) (Ludders, 2008) and held calmly in a dark bag by the experimenter until cardiac arrest. Immediately after cardiac arrest the crow was transcardially-perfused by gravity with 0.5 L of 0.9% saline followed by 0.5 L of 4% formaldehyde solution (obtained from Mesachimie, Nouméa, New Caledonia). The crow’s brain was then extracted from the skull and immersed in 4% formaldehyde for safe transportation and importation into New Zealand.

Tissue preparation

For all specimens, the brain was re-immersed and fixed in 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB) at arrival in May 2008 (10 days after perfusion) to the Anatomy Laboratory in New Zealand (Department of Anatomy with Radiology, Faculty of Health
Table 4.2 Extract of the body mass (g) records per individual during captivity

<table>
<thead>
<tr>
<th>Year</th>
<th>Date</th>
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<th>Batou</th>
<th>Obo</th>
<th>Egg</th>
<th>Slevin</th>
<th>Robin</th>
<th>Tiga</th>
<th>Sisu</th>
<th>Español</th>
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<td>+2.79</td>
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<td>±2.95</td>
</tr>
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<td></td>
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<td>323</td>
<td>310</td>
<td>357</td>
<td>270</td>
<td>320</td>
</tr>
</tbody>
</table>

and Medical Science, University of Auckland). Also, each brain was weighed (before re-immersion) to the nearest milligram (Table 4.1) and left for storage in a separate container with
The remaining eight brains were processed in pairs between May 2010 and May 2011 by F.S.M.R.\textsuperscript{9} One pair of brains was fully processed before the processing of the next pair began.

First, brains were sectioned mid-sagittally and both hemispheres were cryoprotected in 30% sucrose in PBS for 4 to 7 days (until they sank twice in fresh sucrose solution). Brain halves were then placed in a solution of 15% gelatine with 30% sucrose (cryoprotective gelatine solution) at 40° C for one hour. The hemispheres were then placed in a custom-made mould so that fiducial points could be made in the gelatine for later alignment of tissue sections. The mould consisted of a custom made plastic box with a removable base. The base had small holes drilled in a 3 mm grid pattern, on top of which a perfectly flat 5 mm thick layer of cryoprotective gelatine solution was left to set for 10 min prior to hemisphere placement. This layer would later allow collection of the whole brain tissue in the freezing microtome. The brains were placed on top of this cryoprotective gelatine base with the midline facing down and left to set for 3 min at 6° C. Then, seven to ten sewing pins were inserted into the holes that had been drilled in the base of the mould so that they surrounded the brain hemisphere. A different cryoprotective gelatine solution containing a teaspoon of black fabric dye (to darken the solution) was then poured over the brain. This coloured solution had been previously prepared and kept in liquid state just above room temperature. Once poured, it was left to set first at 6° C in a freezer and then at -4° C, for a total time of 15 min.

The resulting cryoprotective gelatine block (containing the brain) was then removed from the mould, trimmed and placed, along with the pins, into 4% PFA 30% sucrose overnight. The pins were then removed and the block was sectioned on a sliding freezing microtome at 50 μm thickness in the sagittal plane. Sections were collected in 0.01% sodium azide PBS solution. For each hemisphere every third section was mounted serially onto gelatin chrome-alum coated

\textsuperscript{8} Dr. Jeremy Richard Corfield.

\textsuperscript{9} Felipe Salvador Medina Rodriguez, author of this doctoral thesis.
slides, stained with haematoxylin neutral-red, neutral-red or cresyl violet (see Appendix A),
dehydrated and coverslipped with DPX mounting medium (Scharlau) from xylene. The
remaining sections were set apart in two series, one for storage and the other for calcium-
binding proteins calbindin (mouse monoclonal anti-calbindin D28k; Swant) and parvalbumin
(goat anti-parvalbumin; Swant).

Sections and fiduciary points in the surrounding dyed gelatine were imaged using a Leica
M205 FA stereomicroscope with a mounted Leica DFC 500 digital camera, and the images
subsequently merged and flattened in Photoshop CS3 Extended (Adobe) and then loaded into
AMIRA 5.2.0 (Visage Imaging) for alignment. The aligned image stacks were then labelled
using CorelDRAW X5 for the production of a complete brain atlas for the species. Brain
regions were identified using boundary lines that could be recognised from the histological
staining in the image stack. Boundaries were carefully identified with the aid of several avian
brain atlases (Karten & Hodos, 1967; Kuenzel & Masson, 1988; Izawa & Watanabe, 2007) and
named according to the Consortium Nomenclature (Reiner et al. 2004; Jarvis et al. 2005).

**Encephalisation estimates**

The degree of encephalisation was plotted according to Jerison (1977) (i.e. log₁₀ transformed
brain and body mass data). A standarised major axis (SMA) Model II linear regression was
performed using PASW Statistics 18.0.2 using  \( y = a + b \cdot x \) as the model equation and  \( l = (y - pred)^2 / |b| \) as the loss function, where  \( a \) is the intersect,  \( b \) is the slope and  \( pred \) is the predicted
\( y \) value from the model equation (i.e.  \( a + b \cdot x \))(Ludbrook 2010, 2012). The resulting intercept
and slope values were then contrasted with SMA regressions performed with SMATR ver 2.0
Computed slope and intercept values from both programs were identical. SMATR also returned
95% confidence intervals (CIs) for every model equation. PASW Statistics 18.0.2 calculated
brain residuals by subtracting actual log brain mass values and predicted log brain mass values from the model equation.

Brain allometric encephalisation indices (E) were also calculated. This was done by dividing the actual mass of the brain (i.e. not log data) by the inversely transformed predicted brain mass value from the SMA regression (as per Cnotka et al. 2008a). Logarithmic brain/body mass and brain residual plots were done using Systat SigmaPlot 11.2. For the reanalysis of the original Rehkämper et al. (1991) study data, SigmaPlot 11.2 was also used to calculate Wald 95% confidence intervals (CIs) which were drawn around the mean of brain residuals. In addition, we used the free-trial version of Systat 13.00.05 to calculate Wald 95% CIs for the slope (b). The CIs were also calculated by hand from the returned asymptotic standard errors, as described by Ludbrook (2012). We compared SMATR’s, SigmaPlot’s, Systat’s and by-hand values for 95% CIs for the model equation’s slope (b). Methods were found to yield identical\(^{10}\) results to the nearest centesimal.

**Data analysis**

Two-tailed non-parametric Wilcoxon signed-ranks test were calculated manually because of low sample sizes. Results were then contrasted with an automated JavaScript version of the Wilcoxon signed-rank test available on Dr. Richard Lowry’s (Vassar College) website for Statistical Computation in http://vassarstats.net/. Computed results were identical to those calculated by hand. Unpublished individual volumetric data for previously studied five NC crows (courtesy of Dr. Julia Mehlhorn) and published volumetric data for three other passerine species (sparrows: *Passer domesticus*, European jays: *Garrulus glandarius*, European carrion crow: *Corvus corone*) (Rehkämper et al. 1991; Mehlhorn et al. 2010a) were used in addition to the data in the present study.  

\(^{10}\) The formula can be accessed in http://www.sigmaplot.com/products/sigmaplot/productuses/prod-uses46.php. It is also available in the supplemental information in Ludbrook (2012). We used \( t_{0.05,2} = 4.303 \) from Student’s t-distribution tables as the \( t_{a,P} \) value (\( a \) or \( P = 0.05 \), two-tailed test).
Results

*Atlas of the NC crow brain*

From a total of 150 sagittal sections from the right brain hemisphere of the adult male Slevin, stained with cresyl violet (see Appendix A), 14 representative slices were stacked together to produce the first brain atlas for NC crows (Fig. 4.1). For every plate in Fig. 4.1, there is a twin labelled image identifying the mayor nuclei in the telencephalon, thalamus and midbrain. The large majority of the nuclei located in the hindbrain were left unidentified, as more studies (i.e. tracing and immunohistochemical) are needed for a positive identification. An Abbreviation list for the brain regions can be found at the start of this thesis (pp. vii-ix).

*Effect of captive body mass fluctuation on encephalisation indices and brain size residuals*

Analysis of the aviary body mass record extract for the December 2007 - April 2008 period (Table 4.2) showed that body mass during captivity for the nine crows fluctuated, in most cases considerably (Pelé: $Mdn = 309$ g, $R = 287-335$, $N = 19$; Batou: $Mdn = 324$ g, $R = 308-353$, $N = 23$; Obo: $Mdn = 284$ g, $R = 272-310$, $N = 23$; Egg: $Mdn = 292.5$ g, $R = 280-323$, $N = 24$; Slevin: $Mdn = 320$ g, $R = 298-348$, $N = 24$; Robin: $Mdn = 314$ g, $R = 285-328$, $N = 23$; Tiga: $Mdn = 336$ g, $R = 310-358$, $N = 23$; Sisu: $Mdn = 268.5$ g, $R = 255-281$, $N = 18$; Español: $Mdn = 327.5$ g, $R = 290-352$, $N = 24$). On average, the female crows’ body mass was below 300 g with a differential of up to 43 g (Egg) between the minimum and maximum recorded body masses. Male crows’ average body mass was above 300 g with a differential of up to 62g (Español) between minimum and maximum recorded body masses (Table 4.2). These acute fluctuations of body mass had a noticeable effect on individual encephalisation indices (Table 4.3) and on brain residuals calculated from SMA regression lines for the day of capture, perfusion day and for the mean body mass during captivity (Fig. 4.2). This is especially well illustrated by the case of Tiga (see in Fig. 4.2).
Figure 4.1 Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.

102
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Figure 4.1 (continued) Brain atlas of the New Caledonian crow. Images correspond to midsagittal sections the right brain hemisphere of Slevin (adult male). Rostral direction is left, ventral is down. Scalebar: 2 mm.
Table 4.3 Effect of body mass fluctuation on allometric encephalisation indices (E)

<table>
<thead>
<tr>
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<th>E_perfusion</th>
<th>E_mean captivity</th>
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<td>1.054</td>
<td>1.047</td>
</tr>
<tr>
<td>Español</td>
<td>adult</td>
<td>0.937</td>
<td>1.001</td>
<td>0.954</td>
</tr>
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<td>0.941</td>
</tr>
<tr>
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<td>1.205</td>
<td>1.208</td>
</tr>
<tr>
<td>Tiga</td>
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<td>juvenile</td>
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</tbody>
</table>

We also determined the variation per month for the group’s mean body mass during captivity (Fig. 4.3a). In spite of monthly group mean differences being small between December 2007 (307.9 ±2.520 g, N = 52), January 2008 (303.58 ±3.112 g, N = 45) and April 2008 (313.9 ±4.592 g, N = 26) with respect to the average group body mass for the whole captivity period (309.6 ±1.625 g, N = 201) (mean group body mass ±s.e.m and total number of mass estimates per period, respectively; data from Table 4.2), the crows’ individual mean body mass for these three months were significantly different from their respective average body mass during the whole captivity period (Wilcoxon signed-rank test; December: T = 45, P < 0.01; January: T = 41, P < 0.02; April: T = 37, P < 0.05; all N₁ = N₂ = 9). To better visualise the effect of mean group body mass variation per month, we re-plotted the ‘mean during captivity’ log brain/body mass SMA regression line from Figure 4.2 and added the monthly mean group body mass values (Fig. 4.3b).

Although the sample size was too low to statistically test for an effect based on sex or maturity (as shown by Table 4.1), we did not observe in our data any such potential effect on encephalisation indices or brain residuals. However, one female juvenile (Obo) and one male
Figure 4.2 Impact of body mass fluctuation on the individual degree of encephalization and brain size residuals. a Logarithmic plots for brain/body mass (g) relationship from records from the day of capture, the perfusion day and mean during captivity for all study crows (Table 4.2). Dashed SMA regression lines are shown for each dataset with their respective slope (b) and intercept (a) values (see Methods) b Brain residuals calculated from regression lines in a. Black triangles indicate positive residuals and white inverted triangles, negative residuals. Asterisks serve to highlight Tiga’s body mass fluctuation.

adult (Pelé) were found to deviate consistently from the log brain/body trend lines (Fig. 4.2) and held the highest encephalisation indices observed in captivity (Table 4.3).
Figure 4.3 Impact of the monthly variation of mean group body mass on the degree of encephalisation for NC crows. a Mean group (±s.e.m) body mass for study crows over captivity period (capture day, per month, all 5 months together and perfusion day). P-values indicate the significance level for individual (not mean group) differences detected with Wilcoxon signed-ranked test (see text) b Logarithmic variation of the mean group body mass(g) in relation to SMA regression line for mean body mass during the whole captivity period shown in Fig. 4.2.

Discussion

With regard to the degree of encephalisation of NC crows, we found that the body mass fluctuations (which may depend on any of countless internal and external variables, such as stress, metabolism, body hydration, humidity and temperature, and human experimental manipulation) obscured any attempt to determine encephalisation based on captive bodyweight. We did not find a solid, unchanging trend in individual body mass in spite of birds being fed daily with an abundant and balanced diet (see Methods). In retrospect, it would have proved useful to monitor how individual body mass varied according to variables such as time and frequency of feeding, amount of food eaten, etc. Interestingly, our best performing crow Obo (female, juvenile) from previous behavioural studies (Taylor et al. 2009; Medina et al. 2011) consistently had a high encephalisation index and brain residual in every log brain/body analysis we carried out (see Table 4.3 and Fig. 4.2). She was closely followed by Pelé, who also quickly solved the mirror-mediated spatial location task (Medina et al. 2011). In contrast, Sisu
and Español who also participated in the mirror study but took more time to solve the mirror-mediated task than Obo and Pelé, had lower encephalisation indices (Table 4.3) and were usually found closer to the ‘isoponderal’ cephalic lines (Fig. 4.2).

Studies often ignore the effect of body mass fluctuations on encephalisation indices and brain residuals from allometric brain/body mass relationships altogether. It is generally assumed that by using a mean body mass per species the effects of body mass fluctuation will somehow cancel out within and across species. However, as the present monthly analysis shows, mean species body mass does not follow a stable pattern under constant captive feeding conditions nor does it remain stable or increases/decreases at a constant rate (see December-February period in Fig. 4.3a). Group means also obscure important, significant individual mean differences. For instance, while the crows’ March and April group means are almost identical, only the April individual means are significantly different from the individual means for the whole captivity period (see Fig. 4.3a). The NC crows’ encephalisation degree and individual encephalisation indices change according to whether we choose to plot March mean group values or the April ones (Fig. 4.4).

These findings suggest that we should treat comparative encephalisation analyses with caution, especially when they are based on brain residuals extracted from common regression lines built from large collection of mixed datasets (typically gathered from literature, e.g. Lefebvre et al. 2002 and Cnotka et al. 2008a) unless of course, that they give a detailed account of the naturally occurring body mass fluctuation for each species in the comparison and that they interpret results under the light of such variation (see also Healy & Rowe 2007). Unfortunately, compile-studies usually lack any mention of this source of error. Instead, it is often assumed that the best way to counteract or thwart any methodological errors associated with compiling a huge dataset (or even merging two different sources of brain and body mass data) is to use confidence intervals (CIs) around the Model I regression line (typically 95%
**Figure 4.4** Monthly variation of log transformed mean group and individual body mass (g) data. Black squares, white circles and black triangles indicate March, April and whole captivity period individual body mass values, respectively. Black arrows show a few examples of the direction of individual body mass shift between the two time periods. Grey and black diamonds show the month’s and whole captivity mean body masses, respectively. Red dashed lines indicate SMA regressions for March and April, and the blue solid line for the whole captivity period.
CIs). If any given species’ brain residuals, calculated from the model equation, are then found above three standard deviations (Yan & Su 2009) from the predicted isoponderal line but also located beyond the 95% CI line, they are safely interpreted to represent significant outliers to the allometric trend11.

For example, in case of NC crows it has been stated that this species possesses “extraordinarily large brains...” (Cnotka et al. 2008a, p. 242) which are “... characterized by a relatively large mesopallium, striatopallidal complex, septum and tegmentum” (Mehlhorn et al. 2010a, p. 63) when compared to other passerines (results based on parametric t-test analyses). The validity of these findings is suspect for two reasons. First, the inadequate use of Model I regression for the allometric analysis12. Model II regression is a more appropriate method because it does not assume x (body mass) values to be fixed by the experimental design but rather allows both x and y (brain mass) values to vary freely (Ludbrook 2010). Unfortunately, the misuse of Model I regression (i.e. standard least squares method) analysis is still a common practice in scientific research (see Ludbrook 2012) in spite of many attempts to point this out in the past century (see in Berkson 1950 and Mandansky 1959) and the introduction of Model II regressions by Brace in the late 70s (Brace 1977). As long as allometric studies intend to provide meaningful brain data (especially in close-related species) with respect to the slope of the regression (isoponderal) line, they must account for potential variation in both body and brain mass. Most studies base their analyses in capture bodyweights and the mass of perfused brains without first demonstrating that they have controlled for variation of body mass related to aspects such maturity, seasonal effects (i.e. type of diet and food quantity available in the wild

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11 Several other methods have also been described to detect outliers (e.g. Tukey 1977; Healy 1979; Schwertman et al. 2004).

12 In the Cnotka et al. (2008) study, there is no mention of the regression being a Model II type. Mehlhorn et al. (2010) also do not specify whether the type of regression they use is Model II. They cited Warton et al.’s (2006) article on bivariat line-fitting methods for allometry, but the paper describes major axis regressions as well as other types of linear fitting methods.
Figure 4.5 Reanalysis of the degree of encephalization of NC crows with respect to three other passerine birds.  

(a) Logarithmic brain/body mass (mg) plot for four species of passerine birds using partial data (see text) from Cnotka et al. (2008a) (original from Rehkämper et al. 1991) (N = 19 individuals, including 5 NC crows) b Residuals calculated from the SMA regression line in a.  

(c) Logarithmic brain/body mass plot for four species of passerine birds using combined data from this and the Rehkämper et al. (1991) study (total of 23 individuals, including 9 NC crows) d Residuals calculated from the SMA regression line in c. Black dashed SMA regression line, slope (b) and intercept (a) values are shown. Red dashed lines indicate 95% confidence intervals for the slope, calculated using SMATR. Blue dashed lines indicate 95% CIs for the residuals, calculated using SigmaPlot 11.2. Black triangles indicate positive residuals and white inverted triangles, negative residuals.

at the time of capture), body hydration levels (which impinges also in the final mass of the brain) or the digestive system contents. The studies by Cnotka et al. (2008a) and Mehlhorn et al. (2010a) are no exception as they did not take into account such potential effects on bodyweight in the wild. Because we could not determine by which precise linear-fitting method we could
observe the alleged brain enlargement reported by Cnotka et al. (2008a), we reanalysed the previous crows’ \((N = 5)\) and our crows’ \((N = 9)\) mean capture bodyweight and mean brain mass data with data from Rehkämper et al. (1991), which included means for three European jays \((Garrulus glandarius)\), seven European carrion crows \((Corvus corone)\) and four domestic sparrows \((Passer domesticus)\). These were the original data used to run the allometric regressions in Cnotka et al. (2008a) (with the exception of two Eurasian magpies , \(Pica pica^{13}\)) and Mehlhorn et al. (2010a), which we wished to repeat. However, in replicating the analysis we did not treat each individual as an independent data point. Such treatment of the data would bring bias into the analysis because independent data points contribute equally to the construction of the best-fitting regression line. In Cnotka et al. (2008a), the over-represented species (the two \(Corvus\) species, \(N = 7\) and \(N = 5\) for carrion and NC crows, respectively) contributed more to the regression than the under-represented species (sparrows, magpies and jays, \(N = 4\), \(N = 3\), \(N = 2\), respectively). Instead, we ran the analysis with each species’ mean capture weights. As a result, both the degree of encephalisation and the brain residuals for NC crows failed deviate significantly from the model equation (i.e. they remained within the 95% CIs) (Fig. 4.5). Importantly, this was regardless of whether the NC crow mean body and brain mass data came from the previous five crows (Cnotka et al. 2008a) or our nine crows (see Fig. 4.5).

Secondly, in the present study we describe the fluctuating nature of body mass for nine captive NC crows. Although we have no evidence to support that such fluctuations also happen in the wild, neither is there any evidence to support the opposite (i.e. that birds maintain their body mass constant throughout the year, despite potential seasonal effects on their diet). We can safely sustain, however, that individual fluctuations occur despite of our captive crows having regular access to fresh water, fresh fruit and balanced food throughout the day. Nevertheless, if we were

\[^{13}\text{We failed to find this data in the cited literature. We only found data for one Eurasian magpie in Rehkämper et al. (1991). We deemed this sample size too small to include into our analyses.}\]
Table 4.4 Body and brain mass (g) for the total New Caledonian crows studied so far.

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**Means**

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**Log_{10} transformed**

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<td>all combined</td>
<td>2.452</td>
<td>0.867</td>
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*a bodyweights from capture day  
*b data courtesy of Dr. Julia Mehlhorn  
*c present study crows

to allometrically determine the degree of encephalisation for NC crows with respect to other bird taxa based on capture weights, our results would vary according to the moment at which birds were weighed (as illustrated by Fig. 4.3b). In Cnotka et al. (2008a) crows were perfused one week after capture. Our weight data shows important individual mass fluctuations, even over short weekly periods (Table 4.2), which in turn suggests that using capture weight together with delayed perfusion brain mass may produce over/underestimated encephalisation values.

Furthermore, the previously reported capture body and brain mass for five NC crows were 277.25 ±29.87 g (mean ± s.d.) and 7.56 ±0.77 g, respectively (Cnotka et al. 2008a, p. 242; Mehlhorn et al. 2010a, p. 66). In our study, they were 307.56 ±23.64 g and 7.26 ±0.65 g for
nine crows. Up to a point, the observed differences may reflect dissimilar methodologies (i.e. that we used different scaling instruments or that our brain mass data were collected later than in Cnotka et al. 2008a), however, these potential sources of bias are usually ignored when merging brain datasets from literature (as it was in Cnotka et al. 2008a, when they used data from Rehkämper et al. 1991). Thus, if we were to compare the resulting encephalisation indices we would undoubtedly find that they would have decreased according to the body mass increment as occurred to the log_{10} transformed data (see Table 4.4). This was regardless of the fact that the mean capture bodyweight and mean brain mass were not statistically different between the Cnotka et al. (2008a) and our study (two-tailed Mann Whitney U-test; body mass: \( U = 15; \) brain mass: \( U = 18; N_1 = 5, N_2 = 9; \) ns).

However, it would be unfair not to remark that the results of any brain study are always determined by the collected sample size. The previous NC crow studies were based on a sample of five subjects. Close observation of the brain data in Table 4.4 allows direct comparisons between the samples. For instance, while the Cnotka et al. (2008a) study only had one crow brain below 7 g of weight (subject 1033) we had three (Egg, Robin and Sisu). Also, the proportion of relatively larger brains (i.e. over 8 g of weight) in their sample was higher than in ours (two out of five against two out of nine crows, respectively). These examples serve to illustrate the fact that it is unrealistic to assume that low samples can accurately represent an entire species’ population.

There are several reasons why the data in Cnotka et al. (2008a) should be viewed with caution: (1) they treated individuals as independent data points; (2) they assumed that singular measurement of bodyweight (i.e. capture weight) is a reliable estimator for comparative allometry; and (3) they used Model I regression and parametric testing for detecting significant differences in relative brain size. It is instrumental to critically examine, before making inferences about comparative brain–behaviour relationships (e.g. causality, evolutionary trends, etc), whether the chosen estimators are statistically sound (i.e. unbiased, consistent, efficient
and robust). In addition, we demonstrated that when the Cnotka et al. (2008a) body/brain data is analysed with Model II regression, the NC crow brain does not deviate significantly from the regression line.

As there is no simple way to evaluate across several different studies the equivalence of body mass measurements and their error (e.g. the error of measuring instruments and the error associated to each method of measurement) or their intrinsic variation, it seems reasonable also to be cautious about the conclusions reached from encephalisation indices derived from allometric compile-studies that use body mass as a scaling factor and whether these indices can be used as proxies for cognition. Our study consistently demonstrated (by the use of several different approaches) that body size is neither a good estimator for predicting brain size, nor a good scaling variable. Therefore, previous claims by Cnotka et al. (2008a) that NC crows are a comparatively highly encephalised species need to be revised in light of the current analysis.

SECTION 2: HANDEDNESS, BRAIN ASYMMETRIES IN THE NC CROW AND REANALYSIS OF THE EVIDENCE SUGGESTIVE OF NC CROWS HAVING AN ENLARGED MESOPALLIUM

Study scope

In vertebrates, the left brain hemisphere controls visually guided object manipulation (Andrew & Rogers 2002; Rogers 2002). In altricial birds, this functional lateralisation of the brain is related to the visual lateralisation that starts early in the development of the embryo inside the egg (Deng & Rogers 2002; Rogers 2008). Recently, four wild NC crows have been reported to exhibit strong tool laterality or handedness by Rutledge and Hunt (2004). That study suggested that in crows, one brain hemisphere might initially have a slight advantage for tool manipulation, perhaps from embryonic light exposure, and that this asymmetry might later increase through experience. Here we studied the brains of six crows with documented
lateralised tool behaviour during captivity (three right and three left-handed crows) and analysed whether their strong tool holding preference was correlated with volumetric asymmetries in the telencephalon, mesopallium, and nucleus rotundus. Our findings led us to further compare the volumetric estimates of the mesopallium of three crows with previously reported values, which had been used to suggest that (when compared to three other Passeriformes) NC crows have comparatively enlarged mesopallia\(^1\) (Mehlhorn et al. 2010a).

**Methods**

**Subjects**

Six of the nine birds described in Table 4.1 were used for this study. Brain and body mass records for the six crows was also described in Table 4.1 and Table 4.2.

The present research was approved by the University of Auckland Animal Ethics Committee (Approval R602) and complies with the laws of New Caledonia.

**Behavioural data**

The crows’ individual tool behaviour was closely observed and video recorded for posterior analysis by two experimenters (A.H.T.\(^1\) and F.S.M.R.). Recordings were always made from outside the cage through a hole in the cage wire (at 2-3 m of distance from the crows). Written notes were also kept to complete a behavioural record for each crow, as we were not always able to capture every behavioural event on tape.

Both written notes and video recordings were used to calculate a tool preference percentage for the nine crows across all *tool choice* trials (see Table 4.1). A *tool choice* trial consisted of a

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\(^1\) Since the mesopallium receives no direct projection from sensory organs it has been described as a “true associative forebrain area” by Mehlhorn et al. (2010a). Evidence from molecular expression studies in psittacids (Jarvis & Mello 2000) and songbirds (Jarvis & Nottebohm 1997) indicates that the anteroventral and the caudal portions of the mesopallium are active during vocalization learning (see also Feenders et al. 2008). In addition, several studies performed with domestic chicks (*Gallus gallus domesticus*) have revealed that the intermediate medial mesopallium is critical for visual imprinting and passive-avoidance learning (e.g. Cipolla-Neto et al. 1982; see also Horn 1980, 2004, and Rose 2000). Finally, ablation the medial mesopallium in adults pigeons (*Columba livia*) result in impaired performance in a colour-discrimination learning task (Chaves & Hodos 1997).

\(^1\) Dr. Alex Harwood Taylor.
task in which a crow was presented with a pair of stick and pandanus tools along with a 80 cm long wooden log, baited with meat blocks in drilled holes (each 10 cm deep). We scored a stick preference when a crow used the stick tool to extract meat from the wooden log, and a pandanus preference when it used the pandanus tool instead.

When possible, we also recorded the side of the bill against which crows held their tools (or handedness\(^{16}\); see Rutledge & Hunt 2004) during captive tool behaviour (Table 4.5). We only used data on videotape to determine the crows’ handedness. All study crows showed a 100% preference to either right or left handedness. When crows held their tools straight in the beak it was impossible to detect any obvious trend of laterality. Therefore, such trials were not used in this study.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Handedness</th>
<th>N of trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obo</td>
<td>left</td>
<td>14</td>
</tr>
<tr>
<td>Robin</td>
<td>right</td>
<td>35</td>
</tr>
<tr>
<td>Tiga</td>
<td>left</td>
<td>27</td>
</tr>
<tr>
<td>Español</td>
<td>left</td>
<td>12</td>
</tr>
<tr>
<td>Egg</td>
<td>right</td>
<td>22</td>
</tr>
<tr>
<td>Sisu</td>
<td>right</td>
<td>18</td>
</tr>
</tbody>
</table>

*Table 4.5* Tool-use handedness for study crows in Section 2

Animal euthanasia and tissue preparation

A detailed description is given in the Methods section of Section 1.

Brain region surface labelling in AMIRA

Individual images of every third brain section for each of the six birds were loaded into AMIRA (Fig. 4.6) from the main menu. An Alignslice module was attached to the data and

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\(^{16}\) Handedness is determined by the side of the bill in which the tool’s proximal end is held during tool-use. NC crows hold their tools either straight inside their bills or slightly angled, with the proximal end leaned against one side of the skull. The distal end of the tool is what is usually referred as the probing tip, while the proximal end is the tip closest to the head. Therefore, if a crow always held the tool with the proximal end set adjacent to the right side of the skull it was a right handed crow.
Figure 4.6 Labelling of brain regions in AMIRA. Purple area: mesopallium; green area: rest of telencephalon; yellow area: nucleus rotundus; light blue area: nucleus ovoidalis; white arrowheads: fiducial points for section alignment; white circle: AMIRA brush tool. Notice that at the base of the forebrain an imaginary straight line drawn from the posterior to the anterior commissure separates the diencephalon from the telencephalon.

each section was aligned according to the fiducial points. A LabelField module was then attached to the newly aligned image stack and new materials were created to correspond with each brain region that was to be labelled. Each brain region was segmented out from each image using the Brush tool and assigned its corresponding material. Two telencephalic regions and two diencephalic nuclei were identified using boundary lines that could be recognised from the histological staining in the AMIRA image stack. Boundaries were carefully identified with the aid of several avian brain atlases (Karten & Hodos, 1967; Kuenzel & Masson, 1988; Izawa & Watanabe 2007) and named according to the Consortium Nomenclature (Reiner et al. 2004; Jarvis et al. 2005).

The four areas that were labelled were the mesopallium, the telencephalon surrounding the mesopallium (containing nidopallium, arcopallium, entopallium, basal ganglia, nucleus
basorostralis, and hyperpallium), nucleus rotundus from the visual thalamus and nucleus ovoidalis from the auditory thalamus. The mesopallium included both the dorsal and ventral subdivisions. The nidopallium included all of the nidopallial subregions (e.g. L-field) except for nucleus basorostralis, entopallium and arcopallium. The hyperpallium included hyperpallium densocellulare, hyperpallium apicale, and interstitial hyperpallium apicale. As the diencephalon-telencephalon boundary becomes undistinguishable towards the brain midsection, an imaginary straight line was drawn between the anterior and posterior commissures in order to standardise the measurement of the telencephalic volume across individuals (as shown in Fig. 4.6).

**Stereological volumetric reconstruction**

Labelled image stacks exported from AMIRA for each brain region were converted into .tif files (greyscale, 8-bit) using Adobe Photoshop and imported into ImageJ 1.45s freeware (http://rsbweb.nih.gov/ij/). Histograms for each brain region were produced using the *Analyze* module. These in turn gave the total count of black pixels (corresponding to the area labelled in AMIRA) for each brain region.

The exact same process was repeated with an image of a 1 mm microscopic scale bar, which had been taken right after the brain section images on the Leica microscope. Ten measurements of scale bar length were done (using the AMIRA program) in both horizontal and vertical positions to control for any image distortions. The total length of the 1 mm scale bar calculated was 59 pixels (no error in 20 measurements). This number was then used to calculate the conversion rate: $59^2 \text{ pixels} = 3481 \text{ pixels} = 1 \text{ mm}^2$ to convert total count of black pixels in the image stack to total regional surface area. Finally, the surface area was multiplied by three (because we used every third section) and then by the thickness of each sections (i.e. 50 μm) to render total brain region volume. By using this particular method every labelled pixel in AMIRA was converted into mm$^3$ of brain region. No additional image processing (e.g.
thresholding) was thus used during the whole procedure from labelling to the final volumetric calculation.

Because we accidentally destroyed one brain section (Obo’s left hemisphere) in the process of mounting the sections over subbed slides, we replaced the missing volumetric estimates with mean values calculated from the telencephalic and mesopallial component volumes of the previous and next sections. Thus, the replaced values were 8.053 mm$^3$ and 0.8138 mm$^3$ for the telencephalon and mesopallium, respectively. They represent 1.08% and 0.62%, respectively, of the total volumes estimated for Obo’s left hemisphere’s telencephalon and mesopallium.

**Margin of human error in labelling**

A control image was obtained from the World Wide Web\(^\text{17}\) and used to estimate the error of drawing in AMIRA. As with the brain sections, a region of interest was defined within the image (i.e. an object with clear boundaries in the image) and this region was drawn ten times to produce the same number of greyscale histograms in ImageJ. The total pixel count for the surface area of the drawn control object estimated was 3183.5 ±11.156 pixels (mean ±s.e.m). We then divided the obtained mean by its s.e.m to determine the percentage of intrinsic human error in the process of drawing object boundaries in AMIRA. The error was exactly 0.35% ($N=10$).

**Brain fraction estimates**

We utilised the same SMA regression analysis as described in the Methods section of Section 1. Regressions were ran on log$_{10}$ transformed volumetric data. The mesopallial component was plotted against the ‘telencephalon rest’ volume (i.e. total telencephalon volume

\(^{17}\) This was done by running a search with the words *wacom tablet* in Google Images on February 2012. We chose an image with a grip pen set on top of a graphic tablet. The grip pen was used as the control image. A copy of the original image used has been uploaded for future references and can be accessed through via the following link http://img137.imageshack.us/img137/7872/illustratordrawingtut13.jpg
- mesopallium volume) to prevent Deacon’s part/whole fallacy\(^{18}\) (Deacon, 1990). As in Section 1, PASW Statistics 18.0.2 calculated brain fraction residuals by subtracting actual log brain region volumes and predicted log brain region volumes from the model equation. SigmaPlot 11.2 was used to plot the data and to calculate 95% confidence intervals which were drawn around the mean of brain residuals. We also used the free-trial version of Systat 13.00.05 to calculate 95% CIs for the slope \((b)\) from the returned asymptotic standard errors, as described by Ludbrook (2012).

**Data analysis**

Left and right brain hemispheres were labelled as dominant or contralateral according to the observed handedness in tool-use. Since virtually the entire avian retinal projection obliquely crosses from one side to the other (Cowan et al. 1961; Hart et al. 2000), the dominant hemisphere was assigned to the brain half that receives input from the eye directly above the distal end of the tool, during captive tool behaviour (i.e. lateralised extractions on videotape) (see Table 4.5). In other words, the hemisphere that receives the nerve fibres from the eye that could potentially be responsible for the crucial sensory input required for precise sensorimotor coordination during tool-use, would be dominant in relation to the contralateral hemisphere.

We chose to measure the size of nucleus rotundus and nucleus ovoidalis because (1) they serve as proxies for any potential asymmetries in two of the major sensory pathways: visual and auditory, respectively, (2) their boundaries are easily identified in sagittal sections (unlike the optic tectum, for example) and (3) their relatively small size.

We ran the two-tailed non-parametric Wilcoxon signed-rank test in the automated JavaScript version of the test available in http://vassarstats.net/ (see Methods in Section 1). Unpublished individual volumetric data for previously studied five NC crows (courtesy of Dr. Julia

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\(^{18}\) Since the adult brain represents only a small fraction of the total bodyweight and this brain/body size fraction decreases with increasing body size, it is possible that in comparative analysis of brain/body mass for its bias to be unevenly distributed across species. In other words, the brain/body fraction bias would be more significant in smaller species (e.g. sparrows) than in larger ones (e.g. ravens) (see also pp.209-216, in Deacon 1990). We further discuss the potential bias due to this effect on p. 129 of this Section.
Mehlhorn) and published volumetric data for three other passerine species (sparrows: *Passer domesticus*, European jays: *Garrulus glandarius*, European carrion crow: *Corvus corone*) (Mehlhorn et al. 2010a, using data from Rehkämper et al. 1991) was used in addition to the present study data.

**Results**

A total of 603 brain sections were imaged and used to produce volume estimates for the telencephalon and the mesopallium from both hemispheres for Obo (199), Robin (193) and Tiga (212). Summarised data are shown in Tables 4.6 and 4.7. In addition to these 603 images, Table 4.7 includes another 119 sections for Español (43), Sisu (39) and Egg (37). Brain hemisphere data are shown using dominant and contralateral categories according to video data on individual tool-use laterality during captivity (Table 4.3) (see Methods).

Reconstructed telencephalic and mesopallial volumes were very dissimilar between study subjects, but in perfect accordance to differences observed in brain mass (Table 4.1). For the first 3 subjects (Obo, Robin and Tiga) we found no consistent, observable pattern for volumetric asymmetries between the telencephalic and mesopallial halves (i.e. regions per hemisphere) of the NC crow brain (Fig. 4.7). This observation was supported by the fact that when we assigned a 100% volume to the dominant side, the percentage that represents volumetric difference in the contralateral side did not reach a level of 10%. More precisely, asymmetry estimates for three crows were not greater than 4% for the telencephalon (Fig. 4.7b) and 7% for the mesopallium (Fig. 4.7d). Unfortunately, we could not run any statistical analysis due to low sample size (*N* = 3).

We found a consistent asymmetry pattern in the visual pathway of the first three crows (Fig. 4.8a). The size of nucleus rotundus on the dominant side was consistently larger than on the non-dominant side (Fig. 4.8b). Furthermore, this asymmetry pattern was absent in the (control)

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19 Labelled images are available in the Electronic Supplementary Material for this Chapter.
Table 4.6 Volumetric estimates (mm$^3$) of the telencephalon and its mesopallial component in three NC crows

<table>
<thead>
<tr>
<th>Subject</th>
<th>Telencephalon</th>
<th></th>
<th></th>
<th></th>
<th>Mesopallium</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dominant</td>
<td>contralateral</td>
<td>total</td>
<td>dominant</td>
<td>contralateral</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obo</td>
<td>2231.20</td>
<td>2281.36</td>
<td>4512.56</td>
<td>391.82</td>
<td>398.55</td>
<td>790.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robin</td>
<td>1893.14</td>
<td>1948.47</td>
<td>3841.61</td>
<td>303.72</td>
<td>323.77</td>
<td>627.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiga</td>
<td>2666.57</td>
<td>2576.06</td>
<td>5242.63</td>
<td>443.53</td>
<td>437.49</td>
<td>881.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.7 Volumetric estimates (mm$^3$) of the nucleus rotundus and nucleus ovoidalis in six NC crows

<table>
<thead>
<tr>
<th>Subject</th>
<th>Nucleus rotundus</th>
<th></th>
<th></th>
<th></th>
<th>Nucleus ovoidalis</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dominant</td>
<td>contralateral</td>
<td>total</td>
<td>dominant</td>
<td>contralateral</td>
<td>total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obo</td>
<td>3.932</td>
<td>3.853</td>
<td>7.785</td>
<td>0.3665</td>
<td>0.3740</td>
<td>0.7405</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robin</td>
<td>4.542</td>
<td>4.322</td>
<td>8.864</td>
<td>0.3094</td>
<td>0.3266</td>
<td>0.6359</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiga</td>
<td>4.467</td>
<td>4.306</td>
<td>8.773</td>
<td>0.2815</td>
<td>0.2716</td>
<td>0.5531</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Español</td>
<td>4.914</td>
<td>5.934</td>
<td>10.849</td>
<td>0.3179</td>
<td>0.3637</td>
<td>0.6816</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sisu</td>
<td>4.269</td>
<td>4.534</td>
<td>8.802</td>
<td>0.2566</td>
<td>0.3266</td>
<td>0.5832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>4.129</td>
<td>3.898</td>
<td>8.027</td>
<td>0.2996</td>
<td>0.3112</td>
<td>0.6108</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Yet this enlargement of dominant rotundus became non-dominant side (Fig. 4.8b). Furthermore, this asymmetry pattern was absent in the non-dominant side (Fig. 4.8b). Furthermore, this asymmetry pattern was absent in the (control) auditory pathway (Fig. 4.8c-d). Yet this enlargement of dominant rotundus became unclear after we increased the sample size by adding the volumetric estimates of three more crows (Español, Sisu and Egg) (see Table 4.7). Nevertheless, the dominant rotundus component was slightly larger in four out of six crows (see Fig. 4.9a). In addition, we could now observe a clear asymmetry in nucleus ovoidalis with a larger contralateral component for the auditory pathway (Fig. 4.9b). Neither of these potential deviations from symmetry was significant when tested bidirectionally (nucleus rotundus: $T = -1$; nucleus ovoidalis: $T = -17$; $N = 6$; ns).
Figure 4.7 Forebrain region volume estimates per hemisphere for our study crows. a Absolute telencephalon volume b Percent telencephalon volume c Absolute mesopallium volume d Percent mesopallium volume. White symbols: left handed crows. Black symbols: right handed crow.

However, the observed difference in the contralateral nucleus ovoidalis was so obvious and proportionally consistent (five out six crows exhibited it) that if we had run a directional (i.e. 1-tail) statistical analysis instead of a bidirectional (2-tailed) one we would have found it to be significant ($T = -17$, $N = 6$, $P = 0.047$).

When we compared the estimated volumes for the brain regions of our first three subjects (Table 4.6) with those from the Mehlhorn et al. (2010a) study, we found them very dissimilar. While our mean volumetric estimate for total telencephalon was $4532.27 \pm 495.483$ mm$^3$, the previous study reported $5558.55 \pm 664.442$ mm$^3$ (Mehlhorn et al. 2010a, p. 66). Hence, compared to our data, the previous reported value is 22.64% larger. The difference is even bigger when we compared the mesopallium estimates. Our mean mesopallial volume was
Figure 4.8 Thalamic sensory nuclei volume estimates per hemisphere for our first three study crows. a Absolute volume of nucleus rotundus b Percent volume of nucleus rotundus c Absolute volume of nucleus ovoidalis d Percent volume of nucleus ovoidalis. White symbols: left handed crows. Black symbols: right handed crow.

Figure 4.9 Thalamic sensory nuclei volume estimates per hemisphere for our increased study group. a Percent volume of nucleus rotundus b Percent volume of nucleus ovoidalis. White symbols: left handed crows. Black symbols: right handed crows.

766.29 ±90.836 mm³ while the previous reported value was 1158.72 ±121.010 mm³, an increment of 51.21% with respect to our values. Therefore, when plotted together, our transformed log brain data appears to fall below the other five crows from the Mehlhorn et al. (2010a) study (Fig.4.10a-b).
Figure 4.10 Mesopallial brain fraction analysis of the NC crow telencephalon. a Logarithmic brain fraction (mm$^3$) plot for $N = 8$ crows (previous and present study data combined). b Brain fraction residuals calculated from the SMA regression line in a. c Logarithmic brain fraction (mm$^3$) plot for $N = 3$ crows (present study data only). d Brain fraction residuals calculated from the SMA regression line in c. Black dashed SMA regression line, slope ($b$) and intercept ($a$) values are shown (see Methods section of Section 1). Blue dashed lines indicate 95% CIs for the residuals, calculated by SigmaPlot 11.2 (see Methods). Black symbols indicate positive residuals and white inverted symbols, negative residuals. Circles: data from previous studies (courtesy of Dr. Julia Mehlhorn), triangles: present study data.

Plots for our three study crows and corresponding SMA regressions were also generated for visualization purposes (Fig. 4.10c-d). This was done due to our interest in seeing if our best performing crow (Obo) on the mirror and trap tube tests (Taylor et al. 2009; Medina et al. 2011) deviated positively from the mesopallial fraction trend as she did for the log brain/body mass.
Figure 4.11 Comparison of Model I (OLS) and Model II (SMA) regression analysis. a Logarithmic mesopallial fraction (mm$^3$) plot with OLS regression analysis for $N = 8$ crows (combined data) b Logarithmic mesopallial fraction plot (mm$^3$) with SMA regression analysis for $N = 8$ crows (combined data). Black dashed SMA regression line and green solid OLS regression line, slope ($b$) and intercept ($a$) values are shown. Green dashed lines indicate 95% CIs for the OLS slope, calculated by SigmaPlot 11.2. Red dashed lines indicate 95% CIs for the SMA slope, calculated by SMATR. Black symbols indicate positive residuals and white inverted symbols, negative residuals. Circles: data from previous studies (courtesy of Dr. Julia Mehlhorn), triangles: present study data.

trend (as shown in Section 1, see Figs. 4.2 & 4.4). Obo had the largest mesopallial component of our study group, despite having only the second heaviest brain (see Table 4.1).

Interestingly, when we ran a Model I OLS regression analysis on the combined data two outliers (subjects 1033 and Tiga) were detected beyond the 95% CIs for the residuals and slope (Figs. 4.10b & 4.11a), although, we could not detect any outliers when running a SMA regression analysis on the same data (Fig. 4.11b). Because of the large disparity found between our study crows and the crows from the previous studies (Cnotka et al. 2008a; Mehlhorn et al. 2010a) but also because of the studies’ apparent misuse of Model I regression analyses to determine the positive deviation of the mesopallial component of NC crow brain, we reanalysed the original data used in the Mehlhorn et al. (2010a) study (i.e. Rehkämper et al 1991) (see Discussion section of Section 1 for a similar argument with

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20 Ordinary least squares (see Ludbrook 2010, 2012).
Figure 4.12 Logarithmic plot of mesopallium volume (mm$^3$) versus mean body mass (g) for four passerine species.

a Reanalysis of the Mehlhorn et al. (2010a, using Rehkämper et al. 1991) study data using species means ($N = 16$ individuals, including 5 NC crows) b Residuals calculated from the SMA regression line in a c Analysis of the mesopallial volumetric and body mass combined data from Mehlhorn et al. (2010a, using Rehkämper et al. 1991) and present study using species means ($N = 13$ individuals; including 3 NC crows) d Residuals calculated from the SMA regression line in c. Black dashed SMA regression line, slope ($b$) and intercept ($a$) values are shown. Red dashed lines indicate 95% CIs for the slope, calculated by SMATR. Blue dashed lines indicate 95% CIs for the residuals, calculated by SigmaPlot 11.2. Black symbols indicate positive residuals and white inverted symbols, negative residuals. Circles: data from Mehlhorn et al. (2010a, using Rehkämper et al. 1991), triangles: combined data (see text).

regard to the supposedly high encephalisation degree reported in Cnotka et al. 2008a). Unlike the previous studies, we did not treat every individual as an independent data point. Such treatment of the
Figure 4.13 Mesopallial brain fraction analysis of the telencephalon of four species of passerine birds. a Logarithmic mesopallium/telencephalon rest (mm\(^3\)) plot using data showing data from the Mehlhorn et al. (2010a) study using \(N = 4\) species (including 5 NC crows; total \(N = 16\) subjects) (see text) b Residuals calculated from the SMA regression line in a c Reanalysis using combined data from the Mehlhorn et al. (2010a) and present study using \(N = 4\) species (including 3 NC crows; total \(N = 13\) subjects) d Residuals calculated from the SMA regression line in c. Black dashed SMA regression line, slope (\(b\)) and intercept (\(a\)) values are shown. Red dashed lines indicate 95% confidence intervals calculated by SigmaPlot 11.2 (see Methods). Black symbols indicate positive residuals and white inverted symbols, negative residuals. Circles: data from Mehlhorn et al. 2010a, triangles: combined data.

data always brings bias into the analysis (see pp. 108-116, this Chapter). Instead, we used species species means and directly compared how much they deviated from the allometric trends obtained via SMA regression analyses (Fig. 4.12). In doing so, we contrasted these results (Fig. 4.12) with an analysis of the mesopallial component against the telencephalon rest (Fig. 4.13) in
order to evaluate any potential part/whole fallacy effect due to the asymmetric distribution of biased relationships between brain and body mass across species (Deacon 1990). Since the bodyweights of European carrion crows, NC crows and European jays are roughly 17, 10 and 5 times larger, respectively, and the brain weights are only 10, 7 and 4 times larger than that of the common sparrow, it is possible that a significant bias may distort the results when the body part comprises a relatively large fraction of the whole body (i.e. in the sparrow, the brain comprises 3.64% of total body mass while in the carrion crow it only comprises 2.12% of total body mass) (see also Deacon 1990).

We found no evidence of a potentially significant positive deviation for the size of the NC crow mesopallium with respect to the other three passerine species, when using SMA regression analysis on mesopallial volume vs. body mass (Figs. 4.12a-b) or mesopallial volume vs. telencephalon rest volume plots (Fig. 4.13a-b). Furthermore, when we replaced the NC crow data from the Mehlhorn et al. (2010a, using Rehkämper et al. 1991) study with data from Obo, Robin and Tiga, the deviation changed its direction consistently and the residuals became negative with respect to the allometric slope (Figs. 4.12c & 4.13c). Importantly, regardless of the different variables or the origin of the data sets used, brain residuals invariably remained within the 95% CIs (Figs. 4.12 & 4.13).

Discussion

Our detailed analysis of the mesopallial and telencephalic volumes in three NC crows did not reveal a consistent pattern of volumetric asymmetry between hemispheres for these higher function areas of the avian brain. Neither did we find a significant correlation between behavioural laterality (i.e. tool handedness) and the size of the mesopallium and total telencephalon in what we designated as the visually ‘dominant’ half of the brain (Fig. 4.7). We do not mean to say, however, that there are no other types of laterised brain asymmetries in the NC crow telencephalon (i.e. asymmetry in the number of projections or functional
asymmetry between cerebral hemispheres). Future morphological studies may well reveal that the NC crow brain is indeed asymmetrically lateralised (see below). Brain asymmetries have been studied experimentally to an extent in other avian and non-avian vertebrate models (e.g. Nottebohm 1971; Nottebohm et al. 1976; Rogers & Anson 1979; Denenberg et al. 1981; Güntürkün & Hoferichter 1985) and are described at length elsewhere (for a summary, see Rogers & Andrew 2002).

Our volumetric analysis of two of the sensory thalamic nuclei located in the diencephalon led us to less straightforward interpretations. This was due to the initial detection of a trend in nucleus rotundus that was absent in nucleus ovoidalis (Fig. 4.8), based on the preliminary analysis of Obo, Robin and Tiga. This was first thought to be suggestive of an increase in size of nucleus rotundus in the dominant hemisphere associated with tool laterality. However, the trend disappeared in nucleus rotundus when we increased sample size (now N = 6). Instead, a new trend appeared in nucleus ovoidalis, suggesting that this auditory component is consistently lateralised with respect to tool-use (especially for right-handed crows) (Fig. 4.9b). This was illustrated with a ‘hypothetical’ directional (1-tail) statistical test. These results challenged our initial assumption over the adequacy of using nucleus ovoidalis as a control for any potential, tool-use related lateralisation in the visual pathway. If we would have found any strong evidence suggestive of lateralisation in the nucleus rotundus, the trend in nucleus ovoidalis would have prevented us from appropriately control for overall sensory brain lateralisation. This was not the case though. There is also an important aspect of using secondary relay nuclei of the sensory pathways to estimate (nucleus rotundus) and to control (nucleus ovoidalis) for lateralisation, which we have ignored so far. In fact, both the nucleus ovoidalis and nucleus rotundus receive bilateral projections from nucleus mesencephalicus lateralis pars dorsalis (MLd) (Karten 1967) and optic tectum (Benowitz & Karten 1976), respectively. However, because both the MLd and the optic tectum project primarily to the ipsilateral ovoidalis (Karten 1967) and the ipsilateral rotundus (Benowitz & Karten 1976), it was unlikely that the lesser
bilateral projection would hinder the quantification and, therefore, the detection of a major volumetric asymmetry in the ascending sensory pathways. At any rate, selection of another sensory nuclei to control for a general sensory lateralisation in the forebrain and midbrain would have produced similar concerns.

Possibly, a volumetric study of the optic tectum (primary recipient of retinal projections in the tectofugal pathway) or the assumed homologue of the dorsal lateral geniculate nucleus (dLGN) (primary recipient of retinal projections in the thalamofugal pathway) may reveal that there is lateralisation upstream, in the visual pathway. We were unable to accurately estimate the volumes of the primary visual recipient nuclei because our tissue material was not sectioned to optimise the detection of the optic tectum or the dLGN boundaries. This also applies to the boundaries of the MLd nucleus of the auditory pathway, which would have been a more suitable control for general sensory brain lateralisation. Brains that are sectioned coronally produce the ideal tissue material for this purpose. Future studies may measure the sizes of these nuclei with more accuracy, but also measure the growth of the projections of the visual pathways during development or estimate synaptic density of the retinal projection into these nuclei (via retrograde neurotracing), in order to detect asymmetries at a cellular level. Future research may also indicate whether tool laterality in NC crows is determined by another sensory modality (e.g. tactile feedback), and whether it correlates with particular aspects of that modality (e.g. uneven distribution and density of mechanoreceptors in the bill skin).

In addition, our volumetric estimates for mesopallium and total telencephalon were, unexpectedly, much lower than the ones reported by Mehlhorn et al. (2010a). Regrettably, in the absence of a clear description of the methodology used to estimate brain region volumes from sections (Mehlhorn et al. 2010a) we are forced to make assumptions (see below) in order

21 The NC crow tectorotundal projection may still be otherwise asymmetric. In pigeons, for instance, “... the number of neurons projecting contralaterally from the right tectum to the left rotundus are about twice in number than vice versa...” (Güntürkün et al. 1998).

22 It is also possible that there are lateralised asymmetries at a cytomorphological level as revealed for neurons in the optic tectum (Güntürkün 1997) and nucleus rotundus (Manns & Güntürkün 1999) in pigeons Columba livia.
to try explain the differences we found between our crows and the five previous crows in Mehlhorn et al. (2010a). Initially, we expected some disparity due to differences in methodology. In the previous study, brains sections were cut along the coronal plane while in our study brains were sectioned along the sagittal plane. Accurate brain region boundary detection may be more or less difficult depending on the plane of section. This is of course, because brain nuclei seldom have a spherical form (for 3D reconstructions of brain regions for several species, see Corfield 2009). Therefore, it is possible that some error originates from the tediously long process of reconnoitring the boundaries and drawing them by hand (a process that has not yet been automated). More importantly, our reconstructed data per individual come from pixel histograms of roughly 200 sections, which represented exactly a third of each brain. Also, we digitally drew the region boundaries in AMIRA with a level of precision (thanks to the Zoom function in AMIRA) that is not possible when delineating boundaries on enlarged paper prints (as described in Rehkämper et al. 1991) or when using camera lucida to project images of slides over drawing paper (a technique we also considered using before we began this study).

Unfortunately, no published record exists to indicate how many sections per brain were used to estimate brain region volumes in the previous study or whether these were drawn by hand using digital images or enlarged prints (see Mehlhorn et al. 2010a). However, the authors do mention that they included previously reported brain data by Rehkämper et al. (1991) of three passerine species (sparrows, European jays and carrion crows) into their regression analyses. Rehkämper et al. (1991) state that “… at least 65 sections of each brain…” (p. 126) were used to reconstruct total volumes but no indication is given as to how representative was the sample of 65 sections for each brain (or if they even used the same proportion of total sections per brain per species). If Mehlhorn et al. (2010a) chose the same methodological approach for estimation, then it well may be the case that our estimators are closer to the real brain region volumes and that previous reports overestimated the brain region volumes (Rehkämper et al. 1991; Mehlhorn et al. 2010a).
Such overestimation could occur by the use of volumes from ‘representative’ sections that are too far apart from each other to accurately reconstruct the whole volume. In our study, our 50 μm thick sections were only 100 μm apart from each other and together they represented up to 10 mm of continuous (if laid one after the other) tissue per brain. Instead, if we had used 65 sections of 20 μm each (Rehkämper et al. 1991, p. 126) our sample would have only represented 1.3 mm of continuous tissue per brain. Incorrect boundary detection could have also been partly responsible of the inconsistencies found, especially when an appropriate brain atlas is not available. In the future, studies should share high-resolution images in the supplementary information in order to help detect incongruities before stacking or compiling large datasets for comparative study analysis. Following this line of action, we have chosen to add these data (original images, colour-labelled and binary images per region) into the Electronic Supplementary Material for this Chapter, for future reference.

Since we could not exactly determine which estimator was closer to the true volumetric values, we re-ran two separate SMA regression analyses with the Mehlhorn et al. (2010, using Rehkämper et al. 1991) and our own data. Because mixing different datasets is a standard practice in comparative studies, we chose to repeat this practice only to illustrate the potential risk of misinterpreting strong brain–behaviour relationships. When reanalysing the Mehlhorn et al. (2010a) data and, by extension the Rehkämper et al. (1991) data, we found that the mesopallium of NC crows positively deviated, albeit not significantly so, from the log mesopallium/body mass trend (Fig. 4.13a-b). However, as we have shown in Section 1, single body mass measurements are not reliable predictors for allometric comparisons and, therefore, they should be used with caution or their use should be abandoned altogether. When we ran the analysis on data controlled for Deacon’s part/whole fallacies (Deacon 1990) (i.e. log mesopallium/telencephalon rest plot) the initial positive deviation decreased noticeably (Fig. 4.13). Most importantly, the NC crow relative mesopallial size did not deviate significantly
from the regression lines regardless of whether we used Mehlhorn et al. (2010a) or our study data.

In addition to these analyses based on species means, we ran non-parametric non-directional tests with the actual and predicted mesopallial volumes from the 4-species trend lines in Figs. 4.12a and 4.13a and found that differences were also not significant, regardless of whether the slope was calculated from body mass or from telencephalon rest values ($T = 13$ and $T = 15$, respectively; $N = 5$; $ns$). Furthermore, when analyses were based on our data, the NC crows’ mesopallium deviated negatively from the trend lines (Figs. 4.12c & 4.13c). In sum, our analysis of the telencephalon and mesopallium of the NC crow brain did not reveal anything ‘special’ about their allometric relationships. On the contrary, our present findings cast doubt over the previous interpretations of NC crows having comparatively enlarged mesopallia (Mehlhorn et al. 2010a), and, do not substantiate the view that “... evolutionary increase of cognitive skills goes along with a volume increase of associative forebrain structures [Rehkämper et al., 1991].” (Mehlhorn et al. 2010a, p. 69). However, we do acknowledge the need for a full scale analysis that should include the volumetric estimates of all the other telencephalic nuclei (i.e. nidopallium, hyperpallium, etc.) extracted from a larger sample size, to fully reject the interpretations in the Mehlhorn et al. (2010a) study.

SECTION 3: CEREBELLAR COMPLEXITY AND CEREBELLAR–TOOL-USE RELATIONSHIP

Study scope

Recently, Iwaniuk et al. (2009) suggested that the morphology of the cerebellum, a brain structure largely ignored by comparative study of animal cognition, is related to tool-use in birds (Iwaniuk et al. 2009). The study reported that tool-using species have significantly more complex cerebella, but not larger cerebella, than non–tool-using species and was described as
“... the first demonstration of an empirical relationship between the folding of a neural structure and a cognitive behaviour...” providing “... critical insight into the neural substrate of tool use...” (p. 1). By using the comparative approach, Iwaniuk et al. (2009) described patterns of cerebellar–tool-use evolution and raised the question to whether these evolutionary trends could also be found when carrying out more restricted comparisons. They specifically mentioned the NC crow (for which the neural data was still unknown) as a good candidate to test this hypothesis. Here, we provide another comparative study of the relationship of cerebellar complexity and tool-use. We carried these comparisons with two different estimators for cerebellar complexity and used a similar approach to that of Iwaniuk et al. (2009) to test whether these estimators were indeed related to tool-use.

**Methods**

**Specimens**

We included images of most midsagittal sections from slides that belong to different laboratory collections and sources. Initially, we collected images from two NC crows (Batou and Slevin, see Table 4.1), two common mynahs (*Acridotheres tristis*), two zebra finches (*Taeniopygia guttata*), two Australian magpies (*Gymnorhina tibicen*) (all Passeriformes), one pukeko (*Porphyrio porphyrio*, Gruiformes), one budgerigar (*Melopsittacus undulatus*, Psittaciformes) and three Japanese jungle crows (*Corvus macrorhyncos*, Passeriformes). The mynah, Australian magpie and pukeko were perfused post-mortem in the wild by J.R.C\(^{23}\) between 2005 and 2008, in the North Island of New Zealand. He also sectioned, stained and mounted the tissue using the same methods described in Section 2. We were also granted access to the zebra finch (stained with cresyl violet) and the budgerigar (stained with calbindin-DAB) slides, courtesy of Dr. Priscilla Logerot and Dr. Fabiana Kubke (from the University of Auckland), respectively. Images of the cerebella of three Japanese jungle crows (stained with

\(^{23}\) Dr. Jeremy Richard Corfield.
cresyl violet) were sent to us electronically, courtesy of Dr. Ei-Ichi Izawa (from Keio University). Regrettably, we were later forced to drop these from our analyses due to incomplete state of the Purkinje cell layer (see below). We also discarded the Australian magpie and budgerigar material in favour of the images available in the Iwaniuk et al. (2006a, b) studies. This was done because their images were more medial than ours and thus, best represented the maximal cerebellar foliation for those species (see below).

We also included sagittal images of the cresyl violet stained cerebella of one chick (Gallus gallus, Galliformes) and one barn owl (Tyto alba, Strigiformes) available on the website http://brainmaps.org (last accessed on June 2012). In addition to these, we used representative greyscale or binary images of 31 species from the literature (Iwaniuk et al. 2006a, b). They included members of the following avian taxa: Anseriformes (mallard, Anas platyrhyncos), Charadriiformes (short-billed dowitcher, Limnodromus griseus; silver gull, Larus novahollandiae), Coraciiformes (belted kingfisher, Ceryle alcyon; laughing kookaburra, Dacelo novaguineae), Columbiformes (brush bronzewing, Phaps elegans; peaceful dove, Geopelia placida; superb fruit-dove, Ptilinopus superbus), Falconiformes (brown falcon, Falco berigora), Accipitriformes (brown goshawk, Accipiter fasciatus; wedge-tailed eagle, Aquila audax; white-bellied sea eagle, Haliaeetus leucogaster), Galliformes (ruffed grouse, Bonasa umbellus), Gruidae (American coot, Fulica americana), Otidae (Australian bustard, Ardeotis australis), Passeriformes (Australian magpie; brown thornbill, Acanthiza pusilla; Gouldian finch, Erythrura gouldiae; little raven, Corvus mellori; superb lyrebird, Menura novaehollandiae), Pelecaniformes (Australian pelican, Pelecanus conspicillatus; cattle egret, Bulbucus ibis), Procellariiformes (black-browed albatross, Diomedea melanophris; short-tailed shearwater, Puffinus tenuirostris), Psittaciformes (Australian king parrot, Alisterus scapularis; budgerigar; cockatiel, Nymphicus hollandicus; galah, Cacatua roseicapilla; sulphur-crested cockatoo, Cacatua galerita), Sphenisciformes (little penguin, Eudyptula minor) and
Strigiformes (northern saw-whet owl, *Aegolius acadicus*). Together, they represent a sample size of 37 avian species.

Cerebellar images used in this study had to satisfy two criteria. Every image (1) had to represent the most midsagittal section possible for the cerebellum of the species, and (2) it had to show a perfectly continuous Purkinje cell layer. Chosen images are available in the Electronic Supplementary Material for this Chapter.

*Cerebellar complexity estimation*

To measure the degree of foliation of the cerebellum, we first produced a set of binary images from the actual cerebellar images. This was done by separately loading each species’ image of the cerebellum into Adobe Photoshop to create a background layer on top of which we created a blank layer, which in turn, was used to draw the outline of the Purkinje cell layer (Fig. 4.14) (using Photoshop’s Pencil Tool, set at 1 pixel of thickness) as shown in Fig. 4.15. The new outline layer was then transformed into a binary image in ImageJ, and a histogram was produced to calculate the total length of pixels of the outline. We then measured the length of the hull or envelope using the FracLac v2.524 fractal analysis module for ImageJ (via the Standard Box Count method). The hull calculated by FracLac is the smallest containing convex hull drawn around any given binary outline/image (see Fig. 4.15b for a graphic example).

We also used FracLac to calculate the mean box-counting dimension (mean $D_B$) and the minimum cover box-counting dimension (minC $D_B$) of the drawn outline of the Purkinje cell layer. For this, we modified the Standard Box Count default setup by incrementing the number of grid positions from four to eight and by setting the minimum box size to two pixels. We left the default maximum box size of 45% of the total image size.

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Figure 4.14 Purkinje cells in the cerebellum of the New Caledonian crow (*Corvus moneduloides*). Microphotograph of a sagittal section with parvalbumin positive-DAB stain. Arrowheads indicate the soma of a Purkinje cell. Black line shows the boundary between the granule cell layer (G) and the molecular cell layer (M) of the cerebellum.

Figure 4.15 Different approaches to the estimation of cerebellar complexity. Midsagittal cerebellar section of the Peaceful dove (*Geopelia placida*) extracted from Iwaniuk et al. 2006a (frontal direction is to the right). a White dashed line indicates the extent of the Purkinje cell layer on all folia (in Roman numbers) and the black line shows the envelope measurement of the Purkinje cell layer according to Iwaniuk et al. (2006a, 2009) b Black line indicates the length of the Purkinje cell layer (drawn from the original image of the same photo in a) and the red line corresponds to the minimum convex hull around the Purkinje cell layer, calculated by FracLac in ImageJ according to our study.
The box-counting dimension is a measure of complexity based on fractal analysis\(^{25}\). It is calculated from the ratio of increasing detail with increasing scale. Basically, it involves placing several grids of decreasing box size over an image until (in this case) the box is two pixels long. The number of boxes that contain black (or non-empty) pixels is counted for every grid. Fraclac then calculates D\(_B\) as the negative limit of the ratio of the log of the number of boxes at a certain scale over the log of that scale. In other words, D\(_B\) is the slope of the regression line for the log box size/box count plot. Both mean D\(_B\) and the minC D\(_B\) are calculated according to the number of grids applied over the cerebellar image. The mean D\(_B\) is the most common box-counting fractal dimension and it is calculated from all the scans done at different grid positions. The minC D\(_B\) is generated from the most efficient grid covering, which means that for each box size only the lowest box count (required to cover the outline) is selected from all of the grid positions tried. In our analysis, we used eight different initial, fixed positions for grids for the nonoverlapping box count. This meant that each box in a grid was the same size and that the grid did not change position nor diminished its calibre until all boxes had been checked.

Unlike Iwaniuk et al. (2006a, 2009), we avoided using cerebellum foliation indices (CFIs) because the degree of cerebellar foliation is significantly affected by allometry (Iwaniuk et al. 2006a). Also, we could not find a precise instructions as to how CFIs are calculated, which would have allowed a direct replication of the work in the Iwaniuk et al. (2009) article. Described by Iwaniuk et al. (2006a), the CFI corresponds to the total length of the Purkinje cell layer divided by the length of the envelope. The envelope was defined as “... the surface of the Purkinje cell layer without counting the depth of the folia...” (Iwaniuk et al. 2006, p. 49). Unfortunately, they do not give further details as to how exactly this length was measured or how the estimate was standardized across folia within and across species. However, it appears

\(^{25}\) Fractal analysis is a standard approach for morphometric analyses of biological structures (e.g. Smith & Bejar, 1994; Ryugo et al. 1997; Jelinek & Fernandez 1998; Kubke & Carr 2000; Bernard et al. 2001; Milošević & Ristanović 2006; Di Ieva et al. 2007, 2011; Bizzarri et al. 2011; D'Anselmi et al. 2011). See Smith et al. (1996) for a review on the use of fractal analysis.
Figure 4.16 Comparison between estimators for cerebellar complexity. Two cerebellar Purkinje cell layer outlines: a (in blue) and c (in black) are shown flanking their containing envelope b (in red). Hypothetical cerebellum a has the exact same CFI value (6.2805) as the NC crow (Batou) cerebellum shown in c, but they differ in box counting dimension values. The mean $D_B$ and minC $D_B$ values for cerebellum a are 1.5197 and 1.5803, respectively. In contrast, the mean $D_B$ and minC $D_B$ values for cerebellum c are 1.4838 and 1.534, respectively. Hypothetical cerebellum a was drawn from c, within the envelope boundaries and with the exact same pixel length as in c. The open envelope has been produced by erasing the segment connecting both ends of the red line (the complete outline corresponds to the convex hull calculated by FracLac).

by their Fig. 1 (Iwaniuk et al. 2006a, p. 49) (shown in Fig. 4.14a), that the envelope was measured by hand instead of using a normalised approach like minimum containing polygon or convex hull. Also, when we attempted to estimate CFI using an alternative definition (i.e. the length of the Purkinje cell layer outline divided by the length of the convex hull that contains that outline) we found that box counting dimension $D_B$ allowed us to measure differences in structural complexity when CFI did not (as illustrated by Fig. 4.16). Combined, the fact that we could not replicate the CFI estimation done by Iwaniuk et al. (2009) and the fact that $D_B$ could detect differences that CFI could not, demonstrated that $D_B$ was a more reliable estimator for cerebellar complexity than CFI.

Data analysis

In order to test the relationship between cerebellar complexity and tool-use we separated the 37 species into mutually exclusive categories according to their phylogenetic links with tool-
use. Lefebvre et al. (1997) have developed a literature-based approach to study comparatively the capacity of a given species to perform a cognitively demanding behaviour. Ten years ago, they applied the same approach to study avian tool-use (Lefebvre et al. 2002). In that study, they surveyed 39 true and 86 borderline cases of tool-use in 104 species and concluded that true tool-users were more encephalised than (borderline) prototool-users and that the size of the nidopallium was the best predictor for true tool-use per taxon (Lefebvre et al. 2002). Since then, two more cases of true tool-use have been reported (hyacinth macaws: Anodorhynchus hyacinthinus, Psittaciformes, in Borsari & Ottoni 2005; one Australian magpie, in McCormick 2007). In spite of the anecdotal (i.e. unconfirmed) nature of the tool-use report of one Australian magpie, we chose to treat this species as a true tool-using one because the reported behaviour fits the description of stick-tool probing.

Because Iwaniuk et al. (2009) also described that the two tool-use categories described by Lefebvre et al. (2002) correlated with cerebellar complexity, we first decided to order our whole sample by increasing values of mean $D_B$ and minC $D_B$. Then we checked, using the above mentioned articles (Lefebvre et al. 2002; Borsari & Ottoni 2005; McCormick 2007), whether tool-use reports of any kind existed for members of the species family (listed in Table 4.8). Species that had tool-using family members were classified according to the level of tool-use reported (either prototool-use or true tool-use). Species with no reported tool-using family members were classified as unknown (see Table 4.8). Only three species out of the 37 (8.11%) were directly reported to be tool-using species (NC crows, Australian magpies and sulphur-crested cockatoos; all reported true tool-users).

Following the example set by previous studies, we then treated the reported tool-use level as a scalar variable, with true tool-use at the higher end and prototool-use at the lower end of the

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26 The distinction between true tool-use and borderline or prototool-use was first defined by Beck (1980). It has been recently revised by St Amant & Horton (2008), Bentley-Condit & Smith (2009) and Seed & Byrne (2010).
Table 4.8 Species classification according to the reported level of tool-use (RLTU) of family members

<table>
<thead>
<tr>
<th>Taxa</th>
<th>(Superfamily) Family [Subfamily]</th>
<th>Species</th>
<th>RLTU&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
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<td>(Anatoidea) Anatidea</td>
<td>mallard</td>
<td>unknown</td>
</tr>
<tr>
<td>Charadriiformes</td>
<td>Scolopacidae</td>
<td>short-billed dowitcher</td>
<td>TTU&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Laridae</td>
<td>silver gull</td>
<td>PTU</td>
</tr>
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<td>Coraciiformes</td>
<td>Cerylidae</td>
<td>belted kingfisher</td>
<td>PTU</td>
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<td>Halcyonidae</td>
<td>laughing kookaburra</td>
<td>PTU</td>
</tr>
<tr>
<td>Columbiformes</td>
<td>Columbidae</td>
<td>brush bronzewing</td>
<td>unknown</td>
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<td>Columbidae</td>
<td>superb fruit-dove</td>
<td>unknown</td>
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<td>TTU</td>
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<td>Accipitridae</td>
<td>wedge-tailed eagle</td>
<td>TTU</td>
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<td>Accipitridae</td>
<td>white-bellied sea eagle</td>
<td>TTU</td>
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<td>Australian magpie</td>
<td>TTU*</td>
</tr>
<tr>
<td></td>
<td>(Corvoidea) Corvida</td>
<td>little raven</td>
<td>TTU</td>
</tr>
<tr>
<td></td>
<td>(Corvoidea) Corvida</td>
<td>New Caledonian crow</td>
<td>TTU*</td>
</tr>
<tr>
<td></td>
<td>(Muscipapoidea) Sturnida</td>
<td>common mynah</td>
<td>PTU</td>
</tr>
<tr>
<td></td>
<td>(Meliphagoidea) Acanthizidae</td>
<td>brown thornbill</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td>(Passeroidea) Passeridae [Estrildidae]</td>
<td>Gouldian finch</td>
<td>PTU</td>
</tr>
<tr>
<td></td>
<td>(Passeroidea) Passeridae [Estrildidae]</td>
<td>zebra finch</td>
<td>PTU</td>
</tr>
<tr>
<td></td>
<td>(Menuroidea) Menuridae</td>
<td>superb lyrebird</td>
<td>unknown</td>
</tr>
<tr>
<td>Pelecaniformes</td>
<td>Ardeidae</td>
<td>cattle egret</td>
<td>PTU</td>
</tr>
<tr>
<td></td>
<td>Pelecanidae</td>
<td>Australian pelican</td>
<td>unknown</td>
</tr>
<tr>
<td>Procellariiformes</td>
<td>Diomedeida</td>
<td>black-browed albatross</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td>Procellariida</td>
<td>short-tailed shearwater</td>
<td>unknown</td>
</tr>
<tr>
<td>Psittaciformes</td>
<td>(Psittacoidea) Psittaculida [Psittaculinae]</td>
<td>Australian King parrot</td>
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</tr>
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<td></td>
<td>(Psittacoidea) Psittaculida [Loriinae]</td>
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<td>unknown</td>
</tr>
<tr>
<td></td>
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<td>cockatiel</td>
<td>TTU</td>
</tr>
<tr>
<td></td>
<td>(Cacatuoidae) Cacatuidae [Cacatinae]</td>
<td>galah</td>
<td>TTU</td>
</tr>
<tr>
<td></td>
<td>(Cacatuoidae) Cacatuidae [Cacatinae]</td>
<td>sulphur-crested cockatoo</td>
<td>TTU*</td>
</tr>
<tr>
<td>Sphenisciformes</td>
<td>Spheniscidae</td>
<td>little penguin</td>
<td>unknown</td>
</tr>
<tr>
<td>Strigiformes</td>
<td>Tytonidae</td>
<td>barn owl</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td>Strigidae</td>
<td>Northern saw-whet owl</td>
<td>unknown</td>
</tr>
</tbody>
</table>

<sup>a</sup> reported level of tool-use  <sup>b</sup> true tool-use  <sup>c</sup> prototool-use  * target species is also the reported species
scale (see Lefebvre et al. 2002 and Iwaniuk et al. 2009, for arguments as to why true tool-use is supposed to be cognitively more demanding than prototool-use) in order to carry out our analysis of cerebellar-tool-use relationship. We analysed the quartile distribution of the species and their respective reported level of tool-use. After ordering the 37 species by increasing mean D_B and minC D_B, we discarded those whose reported tool-use level was unknown (N = 18) because we cannot assume that absence of tool-use reports is equivalent to absence of tool behaviour (unlike with presence of tool-use reports).

**Results**

A comparison of the rankings for the 19 species with reported tool-using family relatives (Tables 4.9 & 4.10) revealed that species closely related to true tool-users (with the exception of the short-billed dowitcher and cockatiel) clustered in the 3rd and 4th quartiles (Q_3 and Q_4, respectively) whereas species closely related to prototool-users (except for the Australian pelican) were found throughout the first three quartiles.

Interestingly, the cerebellum of NC crows did not appear to be comparatively more complex than the cerebella of other reported true tool-users (Australian magpie and sulphur-crested cockatoo) or other species with tool-using family members (two eagles, brown goshawk, little raven and galah). This was regardless of which estimator was used to produce the ranking. Invariably, NC crows were situated in Q_3, with the 8th most complex cerebellum of the list with values of 1.4808 for mean D_B and 1.5337 for minC D_B.

Differences in ranking position were dependent on whether we used mean D_B or minC D_B to describe the complexity of the cerebellar Purkinje cell layer. This was in spite of both estimators having a high correlation coefficient when using 8 grid positions ($R^2 = 0.972$, OLS regression, $R^2 = 0.962$, SMA regression) (see Fig. 4.17). However, mean D_B values depend on the amount of grid positions laid over the image, whereas minC D_B should not vary, granted
Table 4.9 Quartile distribution of species with tool-using family relatives according to mean $D_B$

<table>
<thead>
<tr>
<th>Rank</th>
<th>PTU-level family relatives</th>
<th>mean $D_B$</th>
<th>TTU-level family relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td></td>
<td>1.3948</td>
<td>short-billed dowitcher</td>
</tr>
<tr>
<td>18</td>
<td>Gouldian finch</td>
<td>1.3988</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>zebra finch</td>
<td>1.4136</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>American coot</td>
<td>1.4195</td>
<td>Q₁</td>
</tr>
<tr>
<td>15</td>
<td>laughing kookaburra</td>
<td>1.4228</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>belted kingfish</td>
<td>1.4300</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>common mynah</td>
<td>1.4302</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>pukeko</td>
<td>1.4480</td>
<td>Q₂</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>1.4526</td>
<td>cockatiel</td>
</tr>
<tr>
<td>10</td>
<td>silver gull</td>
<td>1.4533</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>cattle egret</td>
<td>1.4643</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>1.4808</em> NC crow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1.4825</td>
<td>brown goshawk</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>1.4933</td>
<td>wedge-tailed eagle</td>
</tr>
<tr>
<td>5</td>
<td><em>1.4986</em></td>
<td></td>
<td>Australian magpie</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1.5020</td>
<td>galah</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.5028</td>
<td>white-bellied sea eagle</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1.5184</td>
<td>little raven</td>
</tr>
<tr>
<td>1</td>
<td><em>1.5209</em></td>
<td></td>
<td>sulphur-crested cockatoo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
* species reported as true tool-user

Table 4.10 Quartile distribution of species with tool-using family relatives according to minimum cover $D_B$

<table>
<thead>
<tr>
<th>Rank</th>
<th>PTU-level family relatives</th>
<th>minC $D_B$</th>
<th>TTU-level family relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td></td>
<td>1.4311</td>
<td>short-billed dowitcher</td>
</tr>
<tr>
<td>18</td>
<td>Gouldian finch</td>
<td>1.4485</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>American coot</td>
<td>1.4555</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>laughing kookaburra</td>
<td>1.4670</td>
<td>Q₁</td>
</tr>
<tr>
<td>15</td>
<td>zebra finch</td>
<td>1.4793</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>common mynah</td>
<td>1.4870</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>belted kingfish</td>
<td>1.4886</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>pukeko</td>
<td>1.5171</td>
<td>Q₂</td>
</tr>
<tr>
<td>11</td>
<td>silver gull</td>
<td>1.5198</td>
<td>cockatiel</td>
</tr>
<tr>
<td>10</td>
<td>cattle egret</td>
<td>1.5223</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>1.5249</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>1.5337</em> NC crow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1.5378</td>
<td>brown goshawk</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>1.5539</td>
<td>wedge-tailed eagle</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.5548</td>
<td>galah</td>
</tr>
<tr>
<td>4</td>
<td><em>1.5587</em> Australian magpie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.5605</td>
<td>white-bellied sea eagle</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1.5717</td>
<td>little raven</td>
</tr>
<tr>
<td>1</td>
<td><em>1.5799</em> sulphur-crested cockatoo</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* species reported as true tool-user
Figure 4.17 Linear correlation between both types of $D_B$ estimates for the 37 species in this study. Black dashed OLS regression line, slope ($b$) and intercept ($a$) values are shown. SMA regression analysis yields similar results (data not shown), with model equation: $y = 0.013 + 1.030 \cdot x$ and $R^2 = 0.962$. Non-filled symbols indicate species with no reported tool-using family members (unknown). Half filled symbols indicate species with reported protoutil-using family members (PTU-level). Filled symbols indicate species with reported true tool-using family members (TTU-level). Arrows show the only 2 species reported as true tool-users along with the potential true tool-user, Australian magpie (see Methods).

that we provide for an optimal minimum number of grid positions for its calculation. For instance, when we recalculated the box-counting dimensions for the NC crow’s (Batou) Purkinje cell layer with 100 grid positions, the mean $D_B$ decreased from 1.4808 to 1.4716 but $\text{minC } D_B$ remained at 1.5337. This confirmed that our initial minimum of 8 grid positions were adequate for detecting the most efficient covering box dimension for the Purkinje cell layer outlines.


**Discussion**

Our analysis based on the fractal box-counting dimension of the folding of the Purkinje cell layer showed that species with tool-using family relatives tend to cluster together forming two main groups: *prototool-user relatives* distributed across $Q_1-Q_3$ and *true tool-user relatives* distributed (with exception of the short-billed dowitcher) across $Q_3-Q_4$. In spite of the fact that this result is in accordance with the results produced by Iwaniuk et al. (2009) (i.e. that *prototool-user relatives* have comparatively lower values of cerebellar complexity estimators than *true tool-user relatives*), we do not share their interpretations of the body of data (i.e. that cerebellar complexity correlates positively with *tool-use* in birds). Fractal dimension analysis of the complexity of the most sagittal section of Purkinje cell layer does not substantiate the view that the level of foliation of the cerebellum is more closely related with *true tools* than with *prototools*, nor the view that *true tool-use* and *cerebellar complexity* are evolutionary correlated.

When we decided to treat the reported tool-use level as a scalar variable, with *true tool-use* at the higher end and *prototool-use* at the lower end of the scale, we accepted the underlying assumption that differences exist in the cognitive demands of tool-use. More precisely, as stated by Iwaniuk et al. (2009, p. 156): “The main distinction between the two types of tools is that true tool-use involves the fine manipulation of an implement, including more intricate movements and coordination of the eye and beak (e.g., use of probe tools). In contrast, the use of a prototool, such as a stone anvil to break open a snail, involves a much more repetitive and stereotyped series of movements. Birds that use true tools correspondingly have a relatively larger brain than birds that use prototools (Lefebvre et al. 2002).” They also suggested that their comparatively higher values of CFI of corvids and psittacids “... may not only reflect their ability to use tools, but also the motor, sensory, and cognitive demands of tool manufacture”. This statement was based on their observation that, other than a handful members of corvids and psittacids, most species do not manufacture their tools (they pick them from the environment instead) (Iwaniuk et al. 2009, p. 156). Therefore, by following this reasoning we expected to find NC crows to exhibit the highest
cerebellar complexity of our group sample because of their extraordinary ability to manufacture a diverse and versatile tool kit (Hunt 1996). To date, no reports exist of psittacids manufacturing such variety of complex tools. The few reports of tool manufacture in the wild by parrots (i.e. the use of leaf bits during nut cracking to supposedly prevent nuts from falling off from the mandibles) are revised in Borsari & Ottoni (2005, p. 48-49), along with the description of the use of wooden pieces (shredded from perches) as wedges to open indaia nuts (Attalea dubia).

Surprisingly, the Purkinje cell layer complexity of NC crows (minC D_B = 1.5337, rank 8) did not reflect their ability to produce an ample variety of tools. Instead, NC crows ranked very close to species with prototoo-tool-user relatives (e.g. cattle egret and silver gull), preceded by the cockatiel (Table 4.10). Furthermore, to our knowledge none of the species with a more complex cerebellum than that of the NC crow has been reported to manufacture tools.

In addition, the lowest minC D_B value we report here (1.4311, rank 19) belonged to the short-billed dowitcher, related to the true tool-user bristle-thighed curlew (Numenius tahitiensis, Scolopacidae) which has been described by Marks and Hall (1992) to repeatedly throw hard objects (e.g. coral pieces, stones) onto abandoned eggs in order to break them open to allow feeding from their contents. A similar behaviour has been described (Aumann 1990) for the black-breasted buzzard (Hamirostra melanosternon, Accipitridae), a family relative to the brown goshawk (minC D_B = 1.5378, rank 13), the wedge-tailed eagle (minC D_B = 1.5539, rank 14) and the white-bellied sea eagle (minC D_B = 1.5605, rank 17). Importantly, the observed disparity in the ranks for the related species for this type of true tool behaviour strongly suggest that the complexity of the cerebellar foliation does not correlate well with (nor reliably predicts) tool-use. If it did, we would have expected ‘egg crackers’ to cluster together in our quartile distribution Tables 4.9 and 4.10.

Is it possible then, to reliably uncover evolutionary trends in cerebellar-tool-use relationships by analysing the cerebellar complexity of tool-using related bird taxa? In our view, it is not possible because of several reasons. Firstly, because of lack of suitable data for the analyses. Only
3 out of 37 (8.11%) species in our study have been reported to use tools. Similarly, only 12 out of 91 (13.19%) species in the Iwaniuk et al. (2009) dataset (from Iwaniuk et al. 2006a, p. 47-48) have been reported to use tools. With these data, Iwaniuk et al. (2009) attempted to empirically demonstrate that there exists a relationship between cerebellar foliation and tool-use. In order to this, they made use of tool-use data compiled by Lefebvre et al. (2002) to assign tool-use levels to the families of the species with no reported tool-use behaviour (86.81% of total cases) in their data set, in a similar way we did in this study. Furthermore, when inferring evolutionary trends for the cerebellar-tool-use relationship Iwaniuk et al. (2009) treated the absence of tool-use reports in the literature as absence of the behaviour itself. In contrast, we carried out our analyses without that assumption, since reports hardly constitute an unbiased way of collecting behavioural data (as they generally describe behaviour from a subjective point of view) nor they reliably represent all bird taxa (e.g. the behaviour of nocturnal birds is rarely reported compared to that of diurnal birds). However, our decision to ignore the species with unknown-level of tool-use when interpreting our results did not free us from the uncertainty involved in combining different sources to produce the data necessary for testing our initial hypotheses. In sum, the error associated with the way previous data (from different sources) was combined to produce new cognitively meaningful variables for the cerebellar-tool-use analysis, undermines any attempt to grant the complexity of cerebella the power to reliably predict evolution of true tool-use.

Secondly, in order to increase their sample from 48 to 91 species depicted in the literature Iwaniuk et al. (2006a) had to justify their use of midsagittal measures as an estimate of the CFI of the entire cerebellum. This was done by testing “... for a significant relationship between the CFI of the entire cerebellum and the CFI of the midsagittal section of all of [their] specimens.”, and they found that “... midsagittal CFI significantly correlated with the volume CFI (n = 31; P < 0.01; r = 0.93).” (Iwaniuk et al. 2006a, p. 49). From our point of view, a significant correlation between midsagittal CFI and volume CFI only justifies the interchangeable use of one estimator over the other within their study specimens. No method can reliably establish that the same
correlation existed for the other 60 cases Iwaniuk et al. (2009) included into their analysis without direct testing, therefore, they made inappropriate use of statistics in treating their samples as equivalent. This inadequate treatment of data obscures any attempt to directly evaluate whether midsagittal estimators of cerebellar complexity are truly representative of the complexity of the entire cerebellum.

In addition, Iwaniuk et al. (2006a) does not describe how they dealt with the naturally occurring discontinuity of the Purkinje cell layer (as illustrated by Fig. 4.18) when they measured the volume CFI. Since this discontinuity does affect the length of envelope it would have produced underestimated values of CFI in the lateral portions of the cerebellum. Finally, Iwaniuk et al. (2006a, 2009) admitted that midsagittal CFI was not an allometrically independent variable. Birds with large cerebella relative to the rest of the brain and a large body mass were found to have high midsagittal CFIs in Iwaniuk et al. (2006a). Therefore, they had to use a measure of relative-CFI (which was 95.5% free of the variation due to allometric effects) to carry out their analyses (Iwaniuk et al. 2009). Upon closer inspection of their methodology, it appears Iwaniuk et al. (2006a, 2009) were not dealing with problematic effects of allometry. Rather, they failed to notice than when they interchanged volume CFI (an estimator that represents cerebellar complexity three-dimensionally) for midsagittal CFI (an estimator that represents cerebellar complexity two-dimensionally), the estimator lost its power to account for the variation from the one missing dimension. In other words, while volume CFI accounts for the complexity of the Purkinje cell layer in all the directions of the cerebellum (i.e. the rostrocaudal, dorsoventral and left-right axes), the midsagittal CFI only does so for the rostrocaudal and dorsoventral axes. Therefore, it may well be possible Iwaniuk et al. (2009) were dealing with variation that originated from the missing cerebellar data from the left-right axis instead of allometric size effects.
Figure 4.18 Discontinuity of the Purkinje cell layer in the cerebellum. Microphotograph of a lateral sagittal section of the cerebellum of the NC crow, calbindin positive-DAB stain. Dark brown bodies indicate Purkinje cells (shown at higher magnification in Fig. 4.14). Arrowheads indicate the places where the Purkinje cells are missing.

Figure 4.19 Logarithmic plot of the minC_D_B and cerebellar hull perimeter (or envelope length) for the 37 species in this study. The independence of minC_D_B values from the size of the cerebellum is clearly shown by the dispersed pattern of the data points. Half filled symbols indicate species with reported protoutil-using family members (PTU-level). Filled symbols indicate species with reported true tool-using family members (TTU-level). Arrows show the only 3 species reported as true tool-users.
In contrast, our log-log plot for the fractal dimension of the midsagittal Purkinje cell layer and the perimeter of its hull (calculated by FracLac) for the 37 species (Fig. 4.19) revealed that minC $D_B$, as an estimator for cerebellar complexity, was free from an effect due to the size of the cerebellum (estimated with the Purkinje cell layer envelope or containing convex hull). Together, our results challenge the view that there is an appropriate body of data to test whether there exists a strong correlation between the degree of cerebellar foliation (i.e. complexity) and the level of tool-use among bird families that contain tool-users. In order to test for this rigorously, the field must first produce more data on the neural substrates thought to play a major role in cognition. More importantly, data must become available for the species involved in tool-use and not their family members. The practice of producing large quantities of data using one group of individuals who do not necessarily exhibit the behaviour under study, to then infer evolutionary causal relationships between that behaviour and some brain region on another group of individuals, contradicts decades of research and presents methodological difficulties that are impossible to ignore.

We are not suggesting that the cerebellum is not involved in tool-use. Several lines of evidence are viewed as suggestive of the potential role of the cerebellum in the evolution of tool-use (see Iwaniuk et al. 2009, p. 152), however, what our study clearly shows is that we are still far from being able to demonstrate that the folding of the cerebellum has driven the evolution of tool-use in birds. Our analysis suggests that true tool-use may have appeared in several families regardless of their cerebellar complexity (for an avian phylogeny with reference to tool-use and cerebellar complexity, see Fig. 4.20 in the Electronic Supplementary Material). This view is supported by the fact that not all true tool-using bird families have more convoluted cerebellar cortices than prototool-using families (e.g. the Scolopacidae, represented by the short-billed dowitcher in this study), and that the cerebellar cortex of the NC crow, perhaps the most canny and resourceful avian tool manufacturer, is not more complex than the
cortices of the Australian magpie and the sulphur-crested cockatoo who have been reported to use tools but not to manufacture them.

**Conclusions**

Throughout this study we have revealed several methodological limitations of the comparative approach in the study of brain-behaviour relationships in the field of animal cognition. Especially, that the practice of compiling large data sets from the literature to test for and infer evolutionary trends and causal relationships, does not necessarily augment our understanding of the function or evolution of brain areas and the behaviour they are supposed to produce or to be associated with. Instead, due to inappropriate statistical handling of the data, they usually lead to misinterpretations about how brain structures (that carry out many functions) are linked to complex behaviour (such as problem solving, tool manufacture and use, etc.). As pointed out previously by Healy and Rowe (2007), authors continue to fail to justify why, other than because they want to increase the amount of data, they combine data produced by different sources without clearly demonstrating that this is appropriate. We have shown that two different studies (i.e. Cnotka et al. 2008a and our study) based on two different samples extracted from the same population of NC crows (from the island of Maré, New Caledonia) produce dissimilar brain encephalisation values and volumetric estimates for the telencephalon and the mesopallium, and hence, leading to contradictory interpretations. This was mainly due to differences in methodological approach. Correlations between brain and body size, used to calculate encephalisation estimates, are confounded by the sample size and inappropriate use of Model I regressions. Brain volume correlates are confounded by the method chosen to estimate region volumes (boundary detection and the selection of a representative number of sections to produce whole volume estimates) and inappropriate use of Model I regressions.

Also, we have pointed out methodological inconsistencies in the treatment of cerebellar complexity data produced to test whether the degree of foliation of the cerebellum has driven the
evolution of tool-use in birds (Iwaniuk et al. 2009). Furthermore, our reanalysis of the cerebellar data (based on fractal dimension) revealed that the complexity of the cerebellar cortex does not correlate well with the presence of true tool-use behaviour in avian families, nor it does predict the development of complex tool behaviour among reported true tool-users. On the contrary, our results suggest that the evolution of NC crow tool behaviour did not require an increase of the folding of the cerebellum.

We are not suggesting, however, that comparative allometric analyse should be completely discarded. Rather, when cautiously interpreted, they both help (1) to point out those possibly interesting aspects of brain architecture that require more attention and study (e.g. Rehkämper & Zilles 1991), and (2) narrow the breadth of hypotheses to be more rigorously tested. Also, by the means of other methods (like brain fraction analysis) it is possible to analyse comparatively brain evolution and animal cognition trends without incurring well-known numerical fallacies (see Deacon 1990).

With regard to our limited volumetric study of brain asymmetries, our findings failed to support our starting hypothesis of tool handedness having a volumetric, neuroanatomical basis. NC crows do not seem to hold their tools in a way that suggests a direct relationship between the position of the working/probing tip of the tool and the position of the potentially dominant eye. In the wild, NC crows converge both eyes forward when probing holes in search of insect larvae that are hidden deep in dead wood (personal observations from footage from the NHK TV documentary on NC crows filmed in 2009, available upon request). Possibly, NC crows like the American crow (Corvus brachyrhyncos) may achieve a 66° binocularity angle when fully converging the eyes forward (Fernández-Juricic et al. 2010). If so, NC crows may not need to hold the tool’s working tip directly under the dominant eye in order to optimise motor control during tool behaviour. Further research will help elucidate whether NC crows must keep both eyes constantly converged forward during tool-use, whether monocular testing affects their tool-
use performance, and importantly, whether lateralisation of visual brain structures can be found elsewhere in the visual pathway (i.e. in the optic tectum and dLGN).

Importantly, our failure to find evidence to support our initial hypothesis of lateralised tool behaviour being visually determined, does not imply NC crows do not manifest other visually lateralised behaviour. The Australian magpie, another member of the Corvoidea superfamily, has been shown in the wild to preferably use the right eye (left hemisphere) when approaching a potential predator and the left eye (right hemisphere) when withdrawing from it (Koboroff et al. 2008). Future studies may determine whether captive NC crows show similar visually lateralised behaviour when encountering novel (potentially threatening) objects. If confirmed, it would offer an interesting opportunity to study whether in NC crows lateralisation confers any cognitive advantage as suggested by other avian studies (see in Rogers et al. 2004).

For instance, Australian parrots with a strong left-foot preference have been shown to outperform less strongly lateralised parrots in standard string-pulling (Magat & Brown 2009). In the case of tool handedness, however, the performance of our study crows in past experiments does not support this view. In our mirror study, left-handed Obo and right-handed Pelé ($N = 15$ trials) outperformed left-handed Español and right-handed Sisu ($N = 18$ trials) on the mirror-mediated search task (Medina et al. 2011; Chapter 2). In the trap-tube test, left-handed Obo, Tiga and Slevin ($N = 17$ trials) outperformed both left-handed Español and Batou ($N = 14$ trials) and right-handed Egg (Taylor et al. 2009). Finally, in a mirror-mediated visually-restricted version of the string-pulling task, left-handed Slevin outperformed left-handed Tiga and right-handed Egg (Taylor et al. 2010b). Combined, these findings suggest at least two new complementary hypotheses. First, that neither right- nor left-handedness confers a consistent manipulatory and/or cognitive advantage over the other. Second, that behavioural tool laterality only confers an advantage when tasks involve carrying out the complex manipulatory actions associated with tool-use (as is similarly the case with footedness in string-pulling by parrots, Magat & Brown 2009).
Collectively, our body of data strongly challenges previous views that the complex tool-use and problem-solving skills of the NC crows can be linked with or be explained by the macroscopic aspects of their brain or the relative size of some of their brain structures. Previous evidence for comparatively enlarged brains and mesopallia (Cnotka et al. 2008a; Mehlhorn et al. 2010a) can be disputed on the basis of: (1) lack of evidence that demonstrates that capture bodyweight is a reliable predictor for brain size and that it is safe to assume, when merging large datasets, that conditions in which each specimen was captured were equivalent across studies (i.e. time of the year, maturity and sex of the individuals, seasonal body mass and hydration levels, etc); (2) inappropriate use of parametric statistics and Model I regression analysis; and (3) inadequate treatment of individual species data as equally-contributing (independent) data points in constructing the allometric regression line.

Most importantly, when the comparative brain data is analysed with Model II regression, we draw the same conclusion regardless of whether regressions were carried out with data either from previous studies or our present study. That is, despite the differences in methodology between these studies NC crows do not possess significantly larger encephalisation indices or relatively larger mesopallia when compared to other passerines. Our reanalysis of the apparently strong cerebellar complexity-tool-use relationship (Iwaniuk et al. 2009) also revealed to not hold true when complexity was measured through the more suitable fractal analysis.

In sum, our results provide further evidence of the inadequacy of using correlations as a proxy to causation, to suggest that cognition emerges from large and/or complex brain regions. Correlations per se cannot replace experimental techniques in demonstrating causal relationships between brain structures and cognitively interesting behaviour. As long as we continue to mine the same species sample data to produce new analyses of brain–behaviour correlates without further checking their power to represent the whole species population nor testing the hypotheses in new species samples, we will not settle the issue of how the concerted nervous activity of the brain forges the wonders of behaviour.
Chapter 5

Perineuronal satellite neuroglia in the telencephalon of New Caledonian crows and other Passeriformes: Evidence of satellite glial cells in the central nervous system of healthy birds?

Introduction

Nearly ten years ago the Avian Brain Nomenclature Consortium recognised that the avian pallium and the mammalian neocortex were homologous (Reiner et al. 2004; Jarvis et al. 2005). Although highly contentious (Puelles 2001), this view nevertheless changed the way we look at the neural substrate behind avian cognition. Around that time, Emery and Clayton (2004) suggested that the many cognitive abilities of corvids were evidence of convergent evolution of complex cognition between these birds and primates. Previously, corvids had been reported to deviate positively from regression lines in comparative brain-behaviour studies (e.g. Lefebvre et al. 1997; Timmermans et al. 2000; Emery 2004; Lefebvre et al. 2002, 2004), and two independent research teams had confirmed that wild and captive New Caledonian crows, Corvus moneduloides, could craft/shape hook tools, demonstrating corvids could rival nonhuman primates in tool-related cognitive skills (Hunt 1996; Weir et al. 2002; Hunt & Gray 2004). Also, before the proposal of convergent evolution of intelligence between corvids and primates, animal cognition researchers had sought to explain the extraordinary cognition (e.g. feeding innovation, tool-use) found in birds in terms of their brain architecture (Timmermans et al. 2000; Lefebvre et al. 2002).

Since 2004, studies focused on corvids have attempted to determine whether the emergence of the advanced cognitive skills present in this group (i.e. complex tool-use, insight, causal reasoning, folk physics, etc.) can be explained by potentially enlarged brain areas that could

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1 Until then, it was thought that the avian telencephalon consisted of overgrown basal ganglia (Reiner et al. 2004).
support or produce such behaviours (Emery & Clayton 2005; Emery 2006; Cnotka et al. 2008a; Mehlhorn et al. 2010a). Two major candidates are suggested to best predict innovative and flexible behaviour in birds: the nidopallium and the mesopallium brain nuclei (Timmermans et al. 2000; Lefebvre et al. 2002, 2004; Emery & Clayton 2004, 2005). In relation to our study subjects, it has been reported that “... the brains of NC crows were characterized by a relatively large mesopallium, striatopallidal complex, septum and tegmentum... ” (Mehlhorn et al. 2010a, p. 63) when compared to carrion crows, *Corvus corone* (*N* = 5), or to the combined group of sparrows, *Passer domesticus*, European jays, *Garrulus glandarius*, and carrion crows (*N* = 4, 2 and 5, respectively) (Mehlhorn et al. 2010a, but see criticisms of this study in Chapter 4).

Our examination of the findings in Mehlhorn et al. (2010a) does not substantiate the view that the extraordinary cognitive abilities of NC crows emerged from, or are supported by, comparatively enlarged brain structures (Cnotka et al. 2008a; Mehlhorn et al. 2010a). We reached similar conclusions when comparatively analysing the degree of foliation of the cerebellum (or cerebellar complexity) of the NC crow (see Chapter 4). Instead, we found that NC crows do not possess comparatively enlarged mesopallia nor do they possess comparatively more complex cerebella, which suggests that the extraordinary behaviour of this species may not be simply explained in terms of their gross neuroanatomy. If the evolution of advanced cognition does not require an enlargement of telencephalic brain areas (as in the case of NC crows with respect to other corvids and passerines), then how can we explain the emergence of innovative behaviour and the development of advanced problem-solving skills in birds?

In order to answer this question, we will shift our comparative approach from the macrostructural aspects of the telencephalon to the microstructural characteristics, because of the limitations associated with the former method. The analysis of brain-behaviour relationships

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2 Therefore, it is assumed that the brains of animals that show advanced cognition must share something in common (e.g. be larger than expected when compared to other animals) or at least reflect (somehow) the power to compute and produce extraordinary behaviour.
based on comparative allometry generally involves the use of inadequate proxies to cognition\(^3\) (e.g. brain-body size, brain region-body size) and the reutilisation of the same body of data published by Portmann (1946, 1947) (e.g. Iwaniuk et al. 2006a, 2006b; Cnotka et al. 2008a; Mehlhorn et al. 2010a) with little consideration as to the statistical appropriateness of carrying out new analyses by merging few new samples with old data that ignores variations between different samples of a given population (e.g. the use of data from Rehkämper et al. 1991 in Mehlhorn et al. 2010a)\(^4\).

Here, we initiate another line of research by briefly describing the cytoarchitecture of some telencephalic areas of interest in the NC crow brain, which we have studied with the use of classic histology staining techniques (described in Appendix A). In doing so, we attempt to shed light on some of the characteristics of the cytoarchitecture of the NC crow brain that may play a role in this species’ extraordinarily advanced cognitive abilities.

**Methods**

**Specimens**

Brains of nine NC crows (see Table 4.1 in p. 85 for details), two Indian mynah (*Acridotheres tristis*), two Australian magpies (*Gymnorhina tibicen*), one pukeko (*Porphyrio porphyrio*), one Japanese jungle crow (*Corvus macrorhyncos*) and two zebra finches (*Taeniopygia guttata*) were used in this study (other than the NC crows, subjects were of unknown age). The mynahs, Australian magpies and pukeko were perfused post-mortem after being collected in the wild by J.R.C\(^5\) between 2005 and 2008 in the North Island of New Zealand. He also sectioned, stained and mounted the tissue using the same methods described in Section 1, Chapter 4. The brain sections (35 μm thick, cresyl violet staining) of two zebra finches were provided by Dr. Priscilla Logerot (University of Auckland). High-resolution whole images of brain sections (50 μm

\(^3\) More appropriate analyses are based on brain region-brain rest as described by Deacon (1990).

\(^4\) Which we have discussed at length in Chapter 4.

\(^5\) Dr. Jeremy Richard Corfield.
thick, cresyl violet staining) of one Japanese jungle crow were provided by Dr. Ei-Ichi Izawa (Keio University).

We have already described how the brain tissue was ultimately obtained and prepared from nine NC crows in the Methods section in Chapter 4. Therefore, in the following section we only give a detailed description of the immunocytochemistry we performed on the tissue (Appendix B describes how to prepare solutions and buffers).

**Calcium-binding protein immunocytochemistry**

Antibodies for calbindin (mouse monoclonal anti-calbindin D28k; Swant, Cat. no. 300) and parvalbumin (goat anti-parvalbumin; Swant, Cat. no. PVG-214), biotin (donkey anti-rabbit; Jackson ImmunoResearch, Cat. no. 711065152), and streptavidin, horseradish peroxidase (HRP) conjugate (Invitrogen, Cat. no. S911) were used in the incubation process along with normal horse serum (Abacus, Cat. no. 008-000-021). Primary antibodies calbindin and parvalbumin were diluted to 1:5000 and secondary and tertiary antibodies biotin and streptavidin (respectively) were diluted to 1:1000 in a 2% normal horse serum 0.4% Triton X-100 PBS solution.

Floating brain sections left in 0.01% sodium azide PBS solution were collected and washed thrice in PBS (for 5 min each wash) before left in bleaching solution for 10 min. Then, sections were washed thrice in PBS prior to the initial 24 h of incubation in primary antibody, left on shaker at room temperature. The next day, we started the first bout of incubation by washing the sections thrice in PBS and leaving them for 1 h in secondary antibody. Next, we washed the sections thrice in PBS and left them for 1 h in streptavidin. We then repeated this bout of incubation (PBS wash, 1 h in biotin, PBS wash, 1 h streptavidin) to strengthen the reaction product.

Sections were finally washed thrice in PBS prior to diaminobenzidine (DAB) peroxide staining reaction (sections were left floating freely in DAB peroxide reaction medium for 90 s).
Figure 5.1 Sagittal section of the New Caledonian crow stained with cresyl violet. A arcopallium APH area parahippocampalis Cb cerebellum CO chiasma opticum DMA nucleus dorsomedialis anterior thalami GP globus pallidus (paleostriatum) Hip hippocampus HA hyperpallium apicale HD hyperpallium densocellularare M mesopallium MST striatum mediale N nidopallium NC nidopallium caudale TrO tractus opticum.

The reacted tissue was washed again in PBS, mounted on gelatine chrome-alum coated slides, left to dry and finally dehydrated and coverslipped with DPX mounting medium (Scharlau) from xylene.

Tissue analysis

Microphotographs of the tissue were taken from different regions of the telencephalon (labelled as in Fig. 5.1), using a Nikon Digital Sight DS- 5MC camera attached to an Eclipse 80i Nikon microscope and then loaded to Adobe Photoshop CS3 for cropping and figure
production. Length measurements for major soma axis and cell processes for NC crows were done with ImageJ 1.45s.

Cell morphology and classification

We identified the cell classes according to standard cell morphology descriptions found in textbooks (e.g. Nieuwenhuys et al. 2008), review (Nieuwenhuys 1994) and research articles (Fortune & Margoliash 1992; Tömböl et al. 2000; Srivastava et al. 2009).

Results

General description of the cellular morphology in the NC crow

We identified several types of calcium-binding immunopositive neurons in the telencephalon of the NC crow brain (Fig. 5.2). In most cases, the calbindin antibody produced a more complete view of the neuronal processes than parvalbumin (e.g. cells shown in HA 1 and Pc, Fig. 5.2). Parvalbumin typically was less selective and produced a tissue that yields images with a higher level of neuropil background staining. However, at least in two cases we used parvalbumin and not calbindin to identify cells, as was the case with the area corticoidea dorsolateralis (CDL*, Fig. 5.2) and the striatum mediale (MSt 1*, Fig. 5.2). Probably due to the technical quality and unequal penetration of the formaldehyde during perfusion, it was not possible for the antibodies to react with the cells in the entopallium to the same degree achieved for cell populations within the other forebrain nuclei (see E and E*, in Fig. 5.2).

The following cell classes were identified in NC crow telencephalon: fusiform bipolar neurons, pyramidal-like neurons, and round, ovoid and angular multipolar neurons. Few fusiform bipolar neurons were found in the dorsal portion of the hyperpallium densocellulare with processes running parallel to the nucleus boundaries (HD 1, Fig. 5.2), and in the striatum mediale with processes aligned in horizontal direction (rostro-caudal axis) (data not shown). Pyramid-like neurons were located in the hyperpallium apicale (HA 2), hyperpallium...
Figure 5.2 Morphology of calcium-binding immunopositive neuron populations in the major telencephalic nuclei of the New Caledonian crow brain. Neuron classes: $rM$ round multipolar, $aM$ angular multipolar, $P$ pyramidal-like, $fB$ fusiform bipolar. Calbindin staining. **HA** hyperpallium apicale **HD** hyperpallium densocellulare **M** mesopallium. Scalebar 20 $\mu$m.
Figure 5.2 (continued) Morphology of calcium-binding immunopositive neuron populations in the major telencephalic nuclei of the New Caledonian crow brain. Neuron classes: $aM$ angular multipolar, $oM$ ovoid multipolar, $P$ pyramidal-like. Calbindin and (*) parvalbumin staining. $M$ mesopallium $N$ nidopallium $NC$ nidopallium caudale $Ap$ arcopallium $MSt$ striatum mediale. Scalebar 20 $\mu$m.
Figure 5.2 (continued) Morphology of calcium-binding immunopositive neuron populations in the major telencephalic nuclei of the New Caledonian crow brain. Neuron classes: $aM$ angular multipolar, $oM$ ovoid multipolar, $Pc$ Purkinje cells. Calbindin and (*) parvalbumin staining. E entopallium CDL area corticoidea dorsolateralis Nb cerebellum. Scalebar 20 μm.

densocellulare (HD 2) and mesopallium (M 1 and M 3) (Fig. 5.2). Multipolar neurons were much more ubiquitous. Round multipolar cells were identified only in the hyperpallium apicale (HA 1) and striatum mediale (data not shown), while ovoid multipolar cells were mostly located in the nidopallium (N), striatum mediale (MSt 1 and MSt 2) and area corticoidea dorsolateralis (CDL*) (Fig. 5.2). Angular multipolar neurons were located in the hyperpallium apicale (HA 1), hyperpallium densocellulare (HD), mesopallium (M 2 and M 3), nidopallium caudale (NC), striatum mediale (MSt 1) and arcopallium (Ap) (Fig. 5.2).
Unfortunately, our limited tissue preparation prevented us from carrying out a detailed, rigorous classification of dendritic trees and axons\(^6\). As the observed length of neuron processes will depend on the thickness of the slice and the relative position of the somas in the slice we could not base our classification on this variable. The same is true for a classification based on the number of processes projecting from the somas or the projections’ orientations since the detection of cytoarchitectonic regularities is affected by the plane of section used in the analysis. For instance, while the (hypothetical) \(W\) type cells would be detected in sagittal sections because their projections form a large dendritic sphere around the soma, we would fail to detect \(Q\) type cells because their projections form columns that are oriented in the left-right axis. In order to detect the hypothetical \(Q\) type cell regularities, we would have to include both coronal and horizontal sections in our analysis.

\textit{Description of a novel cell structure in the telencephalon of NC crows}

When inspecting the NC crow tissue stained for perikarya, we found conspicuous groups of cells located in the mesopallium (Fig. 5.3). More precisely, they consist in clusters of cells with one or two larger cells with long projections (neurons) in the centre, surrounded tightly by four or more compact cells with no visible, or very short, projections, which we classified as perineuronal (satellite) neuroglia. We initially saw satellite neuroglia in cresyl violet stained tissue (Figs. 5.3 & 5.4a), then confirmed their presence with three other stains (haematoxylin, neutral-red and haematoxylin neutral-red) in multiple sections from different individuals (Fig. 5.4b-d). This allowed us to eliminate the possibility that the cell clusters were a histological artifact. Notably, these structures were not visible in the immunocytochemical preparations.

A well differentiated haematoxylin stain followed by a rapid neutral-red stain (see Appendix A) provided the clearest delineation of the different cell types (Fig. 5.5a) and their distribution.

\(^6\) Tissue stained with Golgi technique would prove more appropriate (e.g. Tömböl et al. 2000; Srivastava et al. 2009).
Figure 5.3 Microphotograph of cell clusters in the mesopallium of the NC crow telencephalon (cresyl violet). Black arrowheads indicate cell clusters and orange arrowheads indicate neurons. Magnification: 400x. Scalebar: 20 μm.

Figure 5.4 Microphotographs of cell clusters in the mesopallium of the NC crow telencephalon. a Cresyl violet staining (magnification 400x) b Carazzi’s modified haematoxylin (strong) staining (magnification 400x) c Neutral red staining and DAB to reveal nuclear IEG cJun expression (magnification 400x) d Haematoxylin-neutral red dual staining (magnification 600x). Black arrows: cell clusters. Orange arrows: neurons with their projections. White arrows: possible neuroglia with no visible projections. Scalebar: 20 μm.
Figure 5.5 Microphotographs of cells in the telencephalon of the NC crow (haematoxylin-neutral red). a Image of large neuron with its dendritic projections and small neuroglia in the hyperpallium apicale b Image of a cell cluster in the mesopallium with a labelled zoomed inset showing the visible contour of the central neuron (black dashed line) and 14 surrounding neuroglia (white dashed line). Orange arrowheads indicate neurons and white arrowheads indicate neuroglia. Magnification: 600x. Scalebar: 20 μm.

Figure 5.6 Microphotograph of a cell cluster in the hyperpallium of the NC crow (light haematoxylin stain). A large neuron (N) with its dendritic projections (visible contour indicated by dashed black line), a large round nucleus(solid black circle) with a darkly stained nucleolus (n) in its centre, and nine surrounding small neuroglia are shown in the zoomed inset (white dashed lines). Orange arrowheads indicate neurons and white arrowheads indicate neuroglia. Magnification: 600x. Scalebar: 20 μm.
in the cluster (Fig. 5.5b). Based on our perikarya stains, neurons were distinguished from neuroglia by the presence of dark, coarsely stained Nissl substance (granular substance in the endoplasmic reticulum) in the cytoplasm, a large nucleus with a distinct central nucleolus, and lightly stained proximal segments of dendritic processes (Fig. 5.5a). In contrast, glial cells contained much less endoplasmic reticulum and perinuclear cytoplasm, which gives them a compact round or oval form with darker stained nuclei and often with multiple aggregates of condensed chromatin (as shown in Fig. 5.6). Similar criteria have been previously used to distinguish neuroglia from neurons (e.g. Sherwood et al. 2006).

Gross distribution of cell clusters in the telencephalon of Passeriformes

Once we had successful criteria to identify these structures in the NC crow tissue we carried out a careful microscopy survey in the other telencephalic areas in this and four other passerine species (Japanese jungle crows, Australian magpie, Indian mynah, zebra finch) as well as in a non-passerine (the pukeko, order Gruiformes) using cresyl violet stained tissue (Figs. 5.7-5.13). Far from having a simple, homogeneous pattern of distribution (i.e. presence or absence) across the whole telencephalon, we found that the number of cell clusters and their size varied according to the brain region under study.

In the striatum mediale, the high cell density prevented unambiguous identification of clusters in most species (Fig. 5.7). In both Corvus species, however, we identified a few clusters. With the exception of the nidopallium caudale in the NC crows (Fig. 5.9a), cell clusters in the pallial subdivisions of the passerine telencephalae were easily recognised because in these regions cells are more sparsely distributed (Figs. 5.8-5.13). The clusters appear most conspicuous in hyperpallium densocellulare (Fig. 5.11) and the mesopallium (Fig. 5.10) of the five passerines, where the number of perineuronal satellite neuroglia appeared to be higher.

Clusters of cells were more difficult to identify in the pukeko tissue due to its poor quality. Nevertheless, we recognised a handful of clusters that appeared to be mainly composed of large
Figure 5.7 Microphotographs of the striatum mediale of six avian species. a NC crow b Japanese jungle crow c Australian magpie d Indian mynah e zebra finch f pukeko. Magnification: 100x. Scalebar: 100 μm.
Figure 5.8 Microphotographs of the nidopallium of six avian species. 

- a NC crow
- b Japanese jungle crow
- c Australian magpie
- d Indian mynah
- e zebra finch
- f pukeko.

Magnification: 100x. Scalebar: 100 μm.
Figure 5.9 Microphotographs of the nidopallium caudale of six avian species. a NC crow b Japanese jungle crow c Australian magpie d Indian mynah e zebra finch f pukeko. Magnification: 100x. Scalebar: 100 μm.
Figure 5.10 Microphotographs of the mesopallium of six avian species. 

a NC crow  
b Japanese jungle crow  
c Australian magpie  
d Indian mynah  
e zebra finch  
f pukeko. Magnification: 100x. Scalebar: 100 μm.
Figure 5.11 Microphotographs of the hyperpallium densocellulare of six avian species. a NC crow b Japanese jungle crow c Australian magpie d Indian mynah e zebra finch f pukeko. Magnification: 100x. Scalebar: 100 μm.
Figure 5.12 Microphotographs of the hyperpallium apicale of six avian species. a NC crow b Japanese jungle crow c Australian magpie d Indian mynah e zebra finch f pukeko. Magnification: 100x. Scalebar: 100 μm.
Figure 5.13 Microphotographs of the hippocampus of six avian species. a NC crow b Japanese jungle crow c Australian magpie d Indian mynah e zebra finch f pukeko. Magnification: 100x. Scalebar: 100 μm.
neurons (e.g. Fig. 5.7f). Neuron clusters were also detected in the NC crow nidopallium caudale (Fig. 5.9a) and have been described elsewhere (Fortune & Margoliash 1992). Finally, cell clusters appeared absent in the hippocampus (Fig. 5.13), area parahippocampalis, area corticoidea dorsolateralis, arcopallium (data shown only for the NC crow) (Fig. 5.14a-c). As we described in the previous section, the entopallium was especially difficult to study under microscope possibly due to low penetration of the fixative (Fig. 5.14d).

**Discussion**

Our microscopy survey based on antibodies for calcium-binding proteins revealed that the NC crow telencephalon has the typical neuronal classes described previously in other avian species (Fortune & Margoliash 1992; Tömböl et al. 2000; Srivastava et al. 2009) (Fig. 5.2). In contrast, our survey based on perikarya stains revealed, in at least five passerine species (including the NC crow), the presence of clusters of cells that had not been described before in the avian brain literature (Figs. 5.10 & 5.11). These clusters resemble a specific subtype of perineuronal satellitosis described previously in humans and rodents (see below).

Satellite glia were first described in the dorsal root ganglia by Ramón y Cajal in healthy peripheral nervous tissue of humans, cats and rams (Ramón y Cajal 1910) and in the 1930s the term perineuronal satellitosis (PS) was coined to describe neurons closely surrounded by multiple neuroglia in the central nervous system (see Vijayan et al. 1993). Today, most neuropathology textbooks (e.g. Oehmichen et al. 2006; Haberland 2007; Perry & Brat 2010; Tonn et al. 2010) teach students to recognise PS in the process of diagnosis of common pathologies affecting human nervous tissue, which may explain the lack of reports of their existence by avian brain researchers (for evidence of PS in both ill and healthy rodents, see Ludwin 1984; Krinke et al. 2000; Szuchet et al. 2011). To our knowledge, in one earlier study involving zebra finch brains PS clusters were thought to indicate the presence of a
Figure 5.14 Microphotographs of the telencephalic regions with no cell clusters in the NC crow. a area parahippocampalis, magnification 400x b area corticoidea dorsolateralis, magnification 400x c arcopallium, magnification 400x d entopallium, magnification 200x. Scalebar: 50 μm.

neuropathology in the study subjects (Dr. Martin Wild, University of Auckland, personal communication).

However, a potentially different kind of PS has been described in healthy tissue in different regions of the human brain such as the cerebral cortex, hippocampus, basal ganglia and thalamus. The satellite neuroglia present in this type of PS are oligodendrocytes (Brownson 1956; Vijayan et al. 1993; van Landeghem et al. 2007; Vostrikov et al. 2007; Kim & Webster 2010, 2011; Takasaki et al. 2010). Importantly, one recent study has identified that gray matter perineuronal oligodendrocytes (pN-OLG), in both rats and humans, are of the non-myelinating
phenotype (Szuchet et al. 2011). In mice, evidence also suggests that these pN-OLG in the somatosensory cortex support neuronal survival, differentiation and function, and protect against neuronal apoptosis but do not play a major role in the plasmalemmal uptake of extracellular glutamate neurotransmitter (Takasaki et al. 2010). In contrast, evidence from one human study (van Landeghem et al. 2007) shows that pN-OLG in the neocortex and hippocampus express specific (PACAP) neuropeptides which are known to (1) both promote proliferation and retard maturation and myelogenesis in oligodendrocyte progenitors (Lee et al. 2001; Lelievre et al. 2006), and (2) modulate neuronal synaptic strength (Kondo et al. 1997). Human pN-OLG also express region-specific glutamate transporters, which suggests they might help clear extracellular glutamate and thus prevent delayed neuronal and glial death in hypoxia-sensitive regions following transient global human ischemia (van Landeghem et al. 2007).

Perineuronal satellitosis, therefore, is associated either with normal or pathological nervous tissue depending on the functions performed by the type of neuroglia (i.e. NG2-type macroglia, astrocytes, microglia or myelinating and non-myelinating oligodendrocytes) occupying the perineuronal space (Ludwin 1984; van Landeghem et al. 2007; Yokota et al. 2008; Faber-Zuschratter et al. 2009; Takasaki et al. 2010; Szuchet et al. 2011). Upon closer inspection of the NC crow tissue (Figs. 5.5 & 5.6), the visible dense chromatin aggregates of our satellite cell nuclei suggests that the cells correspond to dark oligodendrocytes described by Mori and Leblond (1970). An independent neuropathologist also identified these cells as oligodendrocyte-like after inspecting our microphotographs (Dr. Arie Perry, University of California, San Francisco, personal communications). Future immunohistochemical studies will, however, determine the exact lineage of the neuroglia we have discovered in our passerine study birds.

Interestingly, recent findings have linked human schizophrenia and other psychiatric disorders with reduction in number of pN-OLG in the prefrontal cortex (Vostrikov et al. 2007; Kim & Webster 2010, 2011). This suggests that pN-OLG may play an instrumental role in the
homeostasis and normal activity of the nervous system, which involves cognitive functions (see Szuchet et al. 2011). It seems highly unlikely that the selective presence of perineuronal satellite neuroglia in the telencephala of the brains that we studied was associated with developed neuropathologies. This is because the brains we studied came from birds in different geographical locations and they showed no obvious sign of illness before being perfused.

Intriguingly, the largest clusters appeared to be present in the densocellular subregion of the hyperpallia (HD) and the mesopallia (M) of the two Corvus species, Australian magpie, Indian mynah and zebra finch (Figs. 5.10 & 5.11). Recent studies with domestic chicks (Gallus gallus domesticus) have revealed that both brain subdivisions (HD and M) are part of the neural circuit that is critical for learning during audiovisual imprinting (Bradley et al. 1985; Nakamori et al. 2010; Town & McCabe 2011; Yamaguchi et al. 2011; see also footnote 14 in p. 117, Chapter 4). This circuit has also been described in pigeons (Columba livia) (Atoji & Wild 2012). Interestingly, there is an increase in glutamate release in the intermediate medial mesopallium during visual imprinting in chicks (Gruss & Braun 1996; Tsukada et al. 1999; Meredith et al. 2004). In addition, blockage of mesopallial glutamate NMDA receptors impairs imprinting behaviour in chicks (McCabe et al. 1992; Bock & Braun 1999). The role played by the mesopallium in learning is not limited, however, to audiovisual imprinting behaviour nor to chicks. For instance, ablation of the left intermediate medial mesopallium leads to impaired passive-avoidance learning in chicks (Patterson et al. 1990; Patterson & Rose 1992). Also, the mesopallium engages in colour-discrimination in both chicks (Patterson & Rose 1992) and adult pigeons (Chaves & Hodos 1997). Together, these findings suggest that the structural organisation for imprinting in the mesopallium is maintained in adults and that new functions other than imprinting are incorporated (Atoji & Wild 2012).

We could not determine unambiguously whether perineuronal satellite neuroglia were present in the pukeko brain tissue, but if present it would be unlikely that they are as ubiquitous as they are in passerine species. Also, when closely inspecting whole high-resolution sagittal
images of perikarya stained brains of the barn owl (*Tyto alba*, Strigiformes) and the chick (*Gallus gallus*, Galliformes), available in the http://brainmaps.org/ public domain, we failed to find evidence of neuron-glia clusters in these species. Together, our findings indicate that PS is both region- and taxon-specific, suggesting that their presence in healthy passerines and humans (as shown by Brownson 1956; Vijayan et al. 1993; van Landeghem et al. 2007; Vostrikov et al. 2007) is the result of convergent evolution. Future comparative quantification studies are needed to confirm whether in birds PS is an exclusive characteristic of Passeriformes and if it is correlated with the emergence of complex tool behaviour and/or advanced problem-solving skills in this group.

According to the neuronal doctrine, neuroglia only play a role in the maintenance and support of neurons (Somjen 1988; Privat et al. 1995). However, many functional aspects of neuroglial cells remain unknown (Barres 2008). They actively control the neuronal extracellular space (Simard & Nedergaard 2004; Haydon et al. 2009) and regulate synaptic transmission (Perea et al. 2009). Neuroglia also transmit calcium-wave signals over long distances via gap-junctions (Bennett et al. 2003; Scemes & Giaume 2006) and participate in the development of the nervous system and synapsis formation (Barres 2008). They play a role in plasticity and map formation associated with the sensory neocortex (Min & Nevian 2012; Rossi 2012), and together with microglia participate in pathologically-induced inflammation and pain responses (Giaume et al. 2007; Milligan & Watkins 2009). Glial cells in the cerebellum are also required for fine motor coordination (Saab et al. 2012). Furthermore, recent evidence shows that both neurons and neuroglia arise from the radial glia lineage present early in development and also during adult neurogenesis (Malatesta et al. 2000, 2003; Alvarez-Buylla & Garcia-Verdugo 2002; Miyata et al. 2001; Noctor et al. 2001, 2002; Morest & Silver 2003; Anthony et al. 2004; Rowitch & Kriegstein 2010).

Verkhratsky (2010, p. 1) pointed out that because the neuronal web is embedded into a glial syncytium it gives rise to a sophisticated neuronal–glial network in which “... both types of
neural cells [work] in concert, ensuring amplification of brain computational power.”. While we may choose to model the nervous activity of the brain based exclusively on neuronal networks (because thus it is easier to study), “... every aspect of the neuronal function is controlled by neuroglia and every neuronal signal is instantly perceived by neuroglia.” (Verkhratsky 2009, p. 119). As long as the mechanisms responsible for the functioning of the neuronal-glial circuitry are unknown, the possibility remains that the complex animal behaviour we endeavour to understand does not emerge exclusively via the operation of neurons (see Verkhratsky 2006).

In sum, our findings raise the question of whether, in Passeriformes, the observed neuron-glial clusters play a significant role in normal brain functioning. It seems unlikely that the apparent increase of perineuronal (satellite) glia in the areas of the avian telencephalon involved in different types of multisensory learning (i.e. hyperpallium densocellulare and mesopallium), has no effect on the electrochemical activity carried out by those brain areas. Importantly, our discovery opens the way for animal experimentation, which will prove critical if the fields of animal cognition and neuroscience are to understand the role of neuroglia in the origin and evolution of cognition in vertebrates.
Chapter 6

Conclusion

Throughout human history the ancient natural philosophers and, more recently, scientists have sought to understand the nature of behaviour and cognition. Although history has been most fertile with the different philosophical approaches to the study of behaviour and cognition, scientists today seek mechanistic explanations\(^1\) (Bunge 2010). The task ahead, therefore, is both vast and intricate. A crucial task for the comparative fields of modern biology, neuroscience and psychology has been to define common parameters and methods that help create a reliable body of knowledge about the evolution of animal cognition. Far from finished, it constitutes an ongoing quest that gives rise to constructive debate and, sometimes, important paradigm shifts (e.g. Jarvis et al. 2005). For the construction of a truly comparative field, it is therefore necessary that we make use of an ample variety of study models. Animals become prospective study models when scientists discover some unique external or internal aspect of their lifestyle. Whether it is by their successful dispersion pattern and ability to thrive in many different habitats (e.g. urban invasion by mice and crows), the accumulation of reports of some rare or unusual behaviour that makes use of natural resources in a unique, novel way (e.g. the opening of milk bottles by blue tits in England or the washing of potatoes by Japanese snow macaques in the island of Koshima) or the development of complex neural networks (e.g. the avian song system or the primate visual system), some animals stand out in the taxon to which they belong (Lefebvre et al. 2004).

The New Caledonian crow is one exceptional example of such study models. Its impressive tool manufacturing and using skills are in many ways similar to the tool skills of nonhuman primates. For example, both chimpanzees and NC crows are capable of metatool use (Köhler 1925; Taylor et al. 2007), show lateralised tool behaviour in the wild and in captivity (Hopkins et

\(^1\) Mechanistic explanations are conceptual systems capable of generating the phenomena they wish to explain, and as such, must generate predictions and stipulate the precise conditions under which those predictions can be observed (Varela & Maturana 1972; Bunge 1984; Maturana et al. 1984).
al. 2004; Lonsdorf & Hopkins 2005; Rutledge & Hunt 2004; Weir et al. 2004) and appear to be able to transmit tool traditions via social learning (Whiten et al. 2009; Holzhaider et al. 2010a, b). Furthermore, the geographical distribution of the three tool designs that the crows make from *Pandanus* spp. leaves, which consist of both simple and complex designs, suggests *human-like* cumulative technological evolution (Hunt & Gray 2003). Also, NC crows are the only nonhuman species known to manufacture hook tools (Hunt 1996; Shumaker et al. 2011).

Considerable work has been conducted on the extent of NC crows’ cognitive abilities in tasks that involve stick or stick-like tools (Weir et al. 2002; Weir & Kacelnik 2006; Holzhaider et al. 2008; Taylor et al. 2009, 2010a, 2012; Wimpenny et al. 2009, 2011). Investigation of the degree of flexibility of NC crows’ cognition, especially related to their non-tool problem solving skills, has been limited (von Bayern et al. 2009; Taylor et al. 2010b, 2011). Finally, there are only two reports examining the neuroanatomy of this species (Cnotka et al. 2008; Mehlhorn et al. 2010). The general aim of my thesis was to investigate further the cognitive flexibility of the NC crow and its neural substrate. In this conclusion, I briefly summarize the findings of my investigation and discuss their significance to the field of comparative study of the evolution of animal cognition.

*Cognition in NC crows is flexible*

Tool-use in animals, and especially birds, is usually associated with food procurement (Bentley-Condit & Smith 2010; Shumaker et al. 2011). Rather than being an expression of flexible cognition, this suggests that NC crows’ tool-use may represent a behavioural adaptation for a specific problem: food extraction and retrieval. However, it was recently shown that NC crows could use tools to explore potentially threatening novel objects even though they had not been previously known to use tools in non-food-related contexts (Wimpenny et al. 2011; Taylor et al. 2012). This suggested that tool-use in NC crows was associated with flexible cognitive abilities.
The results of my mirror study in Chapter 2 further suggest that NC crow cognitive abilities are flexible and extend beyond tool-related problems. Despite the fact that their mirror-induced social displays did not decrease over time in the vertical mirror condition, the crows used a horizontal mirror to locate hidden food. Their behaviour in the mirror-mediated spatial location task importantly demonstrated that NC crows could rapidly learn to exploit the correspondence between the mirror image of a hidden object and its location in the real world, regardless of the amount of previous experience with mirrors. Furthermore, one crow (Obo) appeared to have exploited this mirror correspondence from the first training trial, which suggested NC crows may understand that mirrors serve to represent objects in the environment.

The evidence of context-dependent tool-use (Wimpenny et al. 2011; Taylor et al. 2012) suggests that NC crows possess multimodal spatial representations of their bodies (i.e. internal body schemas², see Povinelli et al. 2010). Body schemata can be extended (remapped) to cover external objects such as tools³ (Holmes et al. 2004; Bonifazi et al. 2007). Also, tool-use has been shown in primates (human and nonhuman) to extend the neural representation of the visuotactile space immediately surrounding their bodies (also known as peripersonal space) (Iriki et al. 1996; Farné & Ládavas 2000; Maravita et al. 2002; Cardinalli et al. 2009). After critical tactile object manipulation with tools, the macaque (Macaca fuscata) monkeys’ visual receptive fields expand to cover the space that can be reached with tools, thus effectively expanding the perihand visual space (Iriki et al. 2001; Maravita et al. 2003; Maravita & Iriki 2004).

Perhaps experience with mirrors and, more crucially, experience in mirror-mediated tasks such as the four-box apparatus in Chapter 2 may crossmodally⁴ facilitate the modification of NC

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² Iriki (p. 661, 2006) defines body schemas as “knowledge or information about the configuration, posture, location and movement of the body in space, together with assessments of the anatomical and functional relationships of different body parts.”

³ In humans only the tips of actively manipulated tools in extrapersonal space are incorporated into the brain’s visuotactile representations of the body and of peripersonal space (Holmes et al. 2004).

⁴ In other words, by integration of multiple modes such as visual, tactile, proprioceptive and motor modes.
crows’ existing visuotactile space\(^5\). This could be accomplished by a rapid expansion of the crows’ visual receptive fields to cover the otherwise hidden space right below the perch (i.e. the interior of the four boxes) to facilitate mirror-mediated ‘food retrieval’ action execution\(^6\). Under this scenario, mirrors would constitute an unorthodox type of tool (i.e. mirrors were not physically connected nor manipulated by the animal) since they would enable crows to act upon distant and hidden objects in a way that is otherwise impossible. This view is supported by the fact that Obo failed to locate food when the mirror was reversed. Unlike probing tools, which would extend the peribill space (as primate tools extend the perihand space), mirrors would extend the visible space around the entire body (much like mirrors do in cars). The novelty of such visual stimuli and their potentially confusing crossmodal (i.e. visual-proprioceptive) effects may partially explain why extended exposure and meaningful (i.e. self-contingent) experiences are required for coherent multisensory integration and effective motor execution in front of mirrors (e.g. the passing of the mark-test). The individual differences in the performance of our study crow in the mirror-mediated object location task may therefore reflect each subject’s neural capacity (or plasticity) to build/reorganise new mirror-induced visuospatial body schema and to maintain them over time.

**Spontaneous string pulling**

In Chapter 3, I investigated spontaneous string-pulling behaviour in NC crows and the role visual feedback may play in the correct execution of a series of pulls and steps required to bring the meat within reaching distance. By presenting a visually-restricted version of the problem to NC crows, I attempted to test between two alternative mechanisms that explain spontaneous string pulling: visual reinforcement-independent *insight* and visual reinforcement-dependent *operant conditioning*. After receiving 10 trials in the standard string pulling task, the crows were given a modified version of the apparatus. We fitted a plastic disc just above the meat on the end

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\(^5\) Which undoubtedly plays a major role in avian self preening behaviour.

\(^6\) The expansion of receptive visual fields in monkeys takes only minutes after intentional tool-use (see Maravita & Iriki 2004). In humans, changes in perihand space representation during tool-use takes seconds (Farné & Ládavas 2000; Holmes et al. 2007).
of the vertical string. The disc was opaque for one group and transparent for the other. Reviewers of this experiment pointed out two methodological limitations of the experimental design, which challenged the interpretations of the data in Chapter 3.

Firstly, study crows were not habituated to discs. Without habituation, the opaque group’s poorer performance might be explained by neophobia to the moving opaque disc. As the opaque disc came closer, neophobic birds could have been afraid to complete the necessary pull-step sequences, thus explaining their higher rate of release errors, visits and pulling attempts. The 2010 neophobia control revealed that when stationary, clear and opaque discs were not perceived as threatening objects and crows fed from them without showing neophobic responses. This control was dismissed because “neophobia is heavily dependent on personality and on rearing and experimental history” (anonymous reviewer), and since it was conducted with birds that were not involved in the disc string pulling task the control could not rule out neophobia in the test crows. I intentionally prevented our study crows from forming any associations (i.e. presence of reward, direct manipulatory experience) with discs prior testing. If the test crows were allowed to manipulate discs before testing, they may have pulled up the discs to explore or play with a known object in a new configuration, as keas did under the same experimental design (Dr. Gyula Gadjon, personal communication). If they were allowed to feed next to discs before testing, they may have pulled up the discs because they had previously been presented simultaneously with food. Therefore, a suitable habituation control would be one that presented birds with approaching opaque and clear non-disc objects in the absence of any reward.

The second methodological limitation was related to motivation. By visually occluding the view of the meat at the end of the string, crows might have lost their motivation to attempt to pull up the string or lost motivation to complete the string pulling process. Such an energetic cost-benefit argument is valid. Failure or poor performance in any visually-restricted task by animals can always be due to a sudden loss of motivation.
In sum, these two limitations revealed that the design of the string pulling experiment was methodologically flawed. In other words, by restricting the view of the meat at the end of the string I could not test between mechanisms such as insight and operant conditioning. Future research may help determine which mechanism is behind spontaneous string pulling in birds. To that aim, horizontal string pulling tasks may prove more useful than vertical tasks such as the counter-intuitive string-pulling\(^7\) described in Heinrich & Bugnyar (2005). Failure of naïve but not experienced ravens to pull down the string was interpreted by the authors as absence of counter-intuitive means-end understanding. In contrast, the performance of experienced ravens was suggestive of transfer of previously learnt skills from the standard to the novel string pulling task (Heinrich & Bugnyar 2005). An alternative explanation, based on the perceptual-motor feedback cycle hypothesis proposed by Taylor et al. (2010b), for the naïve ravens’ performance is ‘the need of split attention’. Naïve ravens had to look up at the string in order to pull it down, which prevented their view of the meat moving up as a direct consequence of pulling down the string. Therefore, positive perceptual feedback was removed by the ravens’ actions\(^8\). Experienced ravens, having previously mastered the necessary pull-step-release motor actions, could focus their attention exclusively on the approaching meat while string pulling (Dr. Alex Taylor, personal communication).

Horizontal string pulling tasks (Fig. 6.1a) can be more easily designed to study different aspects of this paradigm (as described by Povinelli 2000). Modifications can be done \textit{a posteriori} by manipulating string length and setup (Figs. 6.1b-d). For instance, it is possible to test if animals understand connectivity or rely on path continuity when pulling strings by coiling two separate strings (Fig. 6.1c) but making it look like the reward is attached to the end of the string closest to

\(^7\) In this task, the string was looped up through the cage mesh wire and then down again so the meat hung outside the cage, at the same distance as in the standard version, set below the perch where ravens had to pull down on the string in order to move the meat up (see Heinrich & Bugnyar 2005).

\(^8\) Also, it could be argued that by having to reduce view of the meat, ravens lost motivation to complete the series of necessary pulls and steps.

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Figure 6.1 Horizontal string pulling tasks (designs developed in collaboration with Dr. Alex Taylor). a Standard horizontal string pulling problem. A block of meat (red square) is attached to a string (white line) and left on top of a table (black surface). A mesh wire (in gray) prevents subjects from reaching the meat unless they pull the length of string. b Standard delayed reinforcement task. c Two different connectivity/path continuity tasks with two lengths of string. Yellow arrowhead indicates where the string is cut in one task. Green arrowhead shows where strings overlap in the other task. d Delayed reinforcement task with a hidden loop of string. The view of the table from above is shown in d1, and the cross section of the experimental setup in d2 (with a black string). e Counterintuitive string pulling task. A hidden pulley system (brown circles) pulls initially the meat block away from the test subject.

the animal (Povinelli 2000). Also, horizontal string pulling performance is not confounded by the lack of bill-feet coordination, as it can be in the vertical task. Species that have little need in the wild to hold items in their feet are disadvantaged in the vertical string pulling task. They may well understand how to solve the problem, but may need much practice before the correct assembly of the necessary motor actions. Their behaviour, therefore, may appear clumsy in spite of being insightful. Finally, in a horizontal setup it is possible to test whether birds need visual
reinforcement to continuously pull strings. The string can be arranged so that it loops under a table through a small hole, and different loop configurations can be used to test subjects in counterintuitive and standard string pulling tasks (Figs. 6.1d-e).

Gross neuroanatomy of an extraordinary tool-user

In Chapter 4 I carried out a study on brain–behaviour relationships with NC crows. Also, I attempted to replicate previous neuroanatomical findings in the NC crow (Cnotka et al. 2008; Mehlhorn et al. 2010). My findings challenged the view that NC crows are a highly encephalised species and that they possess a relatively larger mesopallial brain component. The apparent contradiction between my findings and those from previous studies was found to be the result of inappropriate allometric regression and statistical methods used in Cnotka et al. (2008) and Mehlhorn et al. (2010), rather than differences in brain tissue treatment methods between my investigation and previous research. This I demonstrated by comparatively analysing both sets of brain data with Model II regression. As a result, I drew the same conclusions regardless of the data set used to produce the allometric regressions. In addition, I tested whether the presence of tool-use in bird families was positively correlated with their cerebellar complexity (i.e. the degree of foliation of the cerebellar Purkinje cell layer), an hypothesis proposed recently by Iwaniuk et al. (2009). My findings did not support the cerebellar complexity-tool-use relationship reported by Iwaniuk et al. (2009) when I measured complexity via the more suitable fractal analysis.

With regard to brain asymmetries and behavioural lateralisation in NC crows, my investigation suggests that the crows’ unidirectional tool holding is not visually determined (at least not in terms of eye dominance or gross neuroanatomical interhemispheric asymmetries). An analysis of the performance of my study crows in several cognitively demanding tasks (Taylor et al. 2009, 2010b; Chapter 4), led me to propose that neither right- nor left-handedness for tool-use provides a consistent manipulatory and/or cognitive advantage over the other. This hypothesis, if
confirmed, may explain why handedness in NC crows does not appear to occur at the population level (Rutledge & Hunt 2004; Weir et al. 2004).

Taken together, my findings do not substantiate the view that NC crows have relatively larger brains than the brains of other Passeriformes and corvids (Cnotka et al. 2008). They also suggest that the evolution of sophisticated cognitive skills in the NC crow is not associated with a significant increase in the volume of associative forebrain structures (Mehlhorn et al. 2010) or increased cerebellar foliation (Iwaniuk et al. 2009). In sum, I provide further evidence of the inadequacy of using correlations as a proxy to causation, to suggest that cognition emerges from enlarged and/or more complex brain regions. Last but not least, I produced the first brain atlas for the NC crow for future reference. The atlas is still being finalised and the complete digital version will include over 150 sagittal brain sections and a more detailed description of the brainstem nuclei.

*Brain cytoarchitecture*

In Chapter 5, I provide the first morphological description of calcium-binding protein immunopositive neuronal populations in the NC crow telencephalon. Also, for the first time I report the presence of perineuronal satellitosis-like neuroglia-neuron clusters in the telencephala of five Passeriformes species (i.e. NC crow, Japanese jungle crow, Australian magpie, Indian mynah and zebra finch). A microscopic survey of the tissue of these species suggested that the largest clusters were located in the hyperpallium apicale and mesopallium brain regions. When inspecting (similarly stained) brain sections from one Gruiform (pukeko), one Strigiform (barn owl) and one Galliform (chick), I failed to find evidence of the neuroglia-neuron clusters that I observed in the passerine species. This led me to the conclusion that perineuronal satellitosis in Aves is both brain region- and taxon-specific.

As indicated by the chromatin masses visible inside the nuclei of these satellite neuroglia, the glial cells in these agglomerates appear to correspond to dark perineuronal oligodendroglia (pN-
OLG) (Mori & Leblond 1970; Dr. Arie Perry, personal communications). The phenotype of these pN-OLG was recently identified in rats and humans as non-myelinating (Szuchet et al. 2011). Evidence suggests that pN-OLG in mouse gray matter support neuronal survival, differentiation and function, and protect against neuronal apoptosis (Takasaki et al. 2010). In humans, they also appear to actively maintain the potential for myelogenesis by both promoting the proliferation of oligodendrocyte progenitor cells and retarding their maturation (Lee et al. 2001; Lelievre et al. 2006; van Landeghem et al. 2007). They also express neuropeptides and region-specific glutamate transporters, which modulate neuronal synaptic strength (Kondo et al. 1997), and participate in the extracellular glutamate balance (van Landeghem et al. 2007), respectively. Intriguingly, recent studies have linked reduction in number of pN-OLG in the prefrontal cortex with human schizophrenia and other psychiatric disorders (Vostrikov et al. 2007; Kim & Webster 2010, 2011). Therefore, pN-OLG may play a decisive role in the homeostasis and normal activity of the human nervous system. Together, these findings suggest that the presence of pN-OLG in healthy human and avian gray matter (as shown by Brownson 1956; Vijayan et al. 1993; van Landeghem et al. 2007; Vostrikov et al. 2007; this thesis) is the result of convergent evolution. Future comparative studies will help determine whether pN-OLG are only present in Passeriformes and if avian perineuronal satellitosis is correlated with the evolution of complex behaviour and advanced problem-solving skills in this group.

The discovery of pN-OLG in the zebra finch is of great importance because for many reasons this species (rather than NC crows) has the potential to become a robust animal model in which to study the function of pN-OLG in healthy and diseased brains. For instance, a stereotaxic atlas of the zebra finch has been developed recently (Nixdorf-Bergweiler & Bischof 2007). In vivo intracellular physiology and recording from awake behaving adult animals can readily be performed in this species (Lewicki 1996; Fee 2000; Mooney 2000; Sturdy et al. 2003). Ex vivo electrophysiology (on slice preparation) can be readily performed in adult brain tissue (Kubke et al. 2005; Roberts et al. 2007, 2008). Also, a genome for the zebra finch is now available (Warren et al. 2010), which means
pN-OLG can be studied molecularly as well as electrophysiologically. Finally, zebra finches are much more available for avian brain researchers and they can be bred in captivity. Therefore, future studies focused on avian pN-OLG could be carried out simultaneously by several different laboratories throughout the globe.

Tracing studies in the jungle crow brain could also prove useful as little is known of the corvid brain interconnections. However, general descriptions of the avian somatosensory and motor control brain systems are already available (see Necker 2000; Dubbeldam 2000). The trace study of these systems will set the basis for future brain research in avian tool-use (see below). As with zebra finches, jungle crows are a much more widespread corvid species than the endemic NC crow (dos Anjos et al. 2009). In addition, it is the only corvid species for which a stereotaxic brain atlas has been published (Izawa & Watanabe 2007), and intensive brain and behavioural research is being carried on these crows in Japan (e.g. Izawa et al. 2005; Rahman et al. 2006; Tsukahara et al. 2006, 2008, 2009; Izawa & Watanabe 2008, 2011; Yokosuka et al 2009; Kondo et al. 2010, 2012; Kondoh et al. 2011; Nishikawa et al. 2011).

**Future neuroethological studies with NC crows**

The discovery of mirror neurons in a songbird (swamp sparrow, *Melospiza georgiana*) has opened the possibility of the existence of an underlying mirror-neuron mechanism for avian tool-use (Prather et al. 2008). In macaque monkeys, a subtype of such visuomotor neurons called *toolResponding mirror neurons* discharge when the animal observes actions performed with tools that are executed by human experimenters (Ferrari et al. 2005; Iriki 2006). As I described earlier, tools become assimilated in the animal body schema, and in humans they are perceived as externalised hands (Iriki 2006). Based on these findings, I propose that tools in NC crows become part of their body schema.

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9 Neurons located in the macaque monkey’s premotor cortex and areas around the intraparietal sulcus that discharge during both the observation and execution of a goal-directed motor action, thus *mirroring* the actions of self and others (Rizzolatti & Craighero 2004; Iriki 2006). An equivalent mirror system are also been described in humans (Ferrari et al. 2009; Heyes 2010), however, no direct single-unit neuronal discharges have been recorded as yet.
internal bill schema (which most likely is located in, or is linked to, the telencephalic nucleus basalis) (see Necker 2000; Dubbeldam 1984). Interestingly, monkey mirror neurons also respond differentially\(^{10}\) to motor actions performed in different regions of space (i.e. peripersonal and extrapersonal), which suggests that the proximity to motor action demonstrators affects the population of neurons recruited in action understanding and execution (see Caggiano et al. 2009). I will return to this neural aspect of tool-use, which may play a role in the development of specific tool-use techniques in NC crows, in the next section.

The way towards future electrophysiological studies with free moving, fully awake tool-using NC crows will have to be patiently paved. Miniature microdrive multichannel recording systems (Fee & Leonardo 2001) are being developed and tested in unrestrained small animals such as swamp sparrows and Bengalese finches (Prather et al. 2008) and mice and rats (Fan et al. 2011). In contrast to tethered device designs (e.g. Fee & Leonardo 2001; Prather et al. 2008), Fan et al. (2011) have recently tested a 5 m-range\(^{11}\) wireless recording system that can be staged in the head of the animal (allowing them to behave with unprecedented freedom) and have demonstrated that the recorded signals are comparable to those from tethered recording. Undoubtedly, in the next few years we will witness even more dramatic technological advances that will help us understand the neural basis of avian tool-use behaviour, if successfully applied in neurophysiological research with NC crows. To that aim, efforts should be directed to construct larger facilities in New Caledonia where brain and behavioural research may be conducted in unison, or alternatively, to attempt breeding them in captivity elsewhere. In the meantime, advances could be made by testing miniature recording devices in unrestrained jungle crows.

\(^{10}\) Caggiano et al. (2009) suggested that space-sensitive mirror neurons encode space both metrically and operationally. This means that mirror neurons discharge in function with fixed distances related to the monkey’s body position (the meaningful units of distance being the length of the monkey’s arms), and that mirror neurons change their internal operations according to the possibilities that the monkey will act.

\(^{11}\) Another, more heavier wireless recording device, has been reported to possess an effective range of 60 m (Szuts et al. 2011).
It is instrumental for the construction of a truly comparative understanding of the evolution of animal cognition and the emergence of complex avian tool manufacture and use, to research the neural underpinnings of social transmission of behaviour in birds. NC crows represent the best animal model for this purpose, and unlike nonhuman primates, their tool culture may have been shaped by human-like cumulative technological evolution (Hunt & Gray 2003; Holzhaider et al. 2011).

**Conclusion**

In the Introduction I described how during the evolutionary history of organisms, cephalisation crucially endowed species with the potential for developing a larger behavioural repertoire by increasing the complexity of the neural network. As more interneurons became part of the brain machinery, the more sophisticated was the neural network (Northcutt 2002). The exponential increment in the total number of electrochemical synapses provided organisms (and especially vertebrates) with arguably the most complex structure in the known universe. As a direct consequence, the possibilities for developing increasingly complex behaviour multiplied in animals. Interestingly, corvids have brains that are relatively comparable to the size of the chimpanzee brain. Also, corvids share other features in common with primates such as large telencephala, slow development and prolonged parental care (Lefevbre et al. 2004; van Horik & Emery 2011). These features possibly play a role in developing complex behaviour and flexible cognition in NC crows (a view I explain below).

As viewed by Hebb (1949), the development of the capacity to learn in vertebrates is marked by the relative inefficiency of early learning. As individuals mature, their learning skills appear to hone up to a point in which learning takes little effort and time. This is a crucial question for neuroethologists, because slow development and extended parental care co-occur in large-brained vertebrates prone to develop complex tool-use (e.g. chimpanzees, elephants, dolphins, NC crows, and humans) (Ross 2001; Hart et al. 2008; Noren 2008; Holzhaider 2010). The spontaneous firing
of the large assembly of neurons located in the associative pallial regions is especially important in any neuropsychological theory, and must be taken into account when attempting to explain cognition in neural terms.

The social structure of NC crows was recently studied and described in detail by Dr. Jennifer Holzhaider (Holzhaider 2010; Holzhaider et al. 2010a, 2010b, 2011). She and her colleagues reported that NC crow juveniles usually live closely with their parents up until the next breeding season and sometimes longer (Holzhaider et al. 2011). In this period, NC crow juveniles learn to manipulate tools (first, the ones left by their parents, and later, their own) and to perfect their tool manufacture techniques (Holzhaider et al. 2010a). A neural Hebbian perspective to learning (see Cooper 2005) describes mechanistically how slow (extended) cumulative learning is possible within the self-organising and intricate vertebrate brain pallium. As animals evolved larger brains, the ratio between the so-called associative and sensory pallia increased (Hebb 1949). This meant a greater variability in the spontaneous nervous activity of multimodal association areas, especially during development. Since early perceptual learning involves the establishment of control over the activity of associative brain areas by sensory events (as illustrated by Sperry 1956; Held & Hein 1963), the larger the associative pallium the slower the establishment of such a control must be and the less rigid and more complex its final form (Hebb 1949).

Once mature, the adult animal continues to profit from the organised neural network in events that lead to learning. At this stage, both environmental stimuli and motor action sequences already evoke well established trains of pallial activity, most of which are very familiar and have a number of other previously integrated associations. This means that learning is not anymore an association between totally unrelated processes (Hebb 1949). Instead, learning amounts to the synaptic strengthening of existing connections which in turn leads to a more efficient conduction of impulses.

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12 Importantly, as animals mature the day-to-day sensory events become more volitional in character.

13 For example, bill-foot coordination which develops step-by-step in natural feeding behaviour, encompasses many visuotactile and visuomotor activity patterns that involve bill, neck, leg and feet muscles (all of which most probably are crucial for spontaneous string pulling).
(Cooper 2005). At this point, it is important to consider that if pN-OLG are shown to participate in avian perineuronal glutamate homeostasis, then their density and distribution in the pallial telencephalon may play a major, unprecedented role in synaptic metaplasticity\textsuperscript{14} and, therefore, in cognition (Abraham & Bear 1996; Lee et al. 2010).

Finally, I propose that the early tool-related experiences that NC crow juveniles are exposed to by their parents provide this species with critical moment-to-moment behavioural contingencies that allow such recapitulative Hebbian associative learning to happen in every generation. Also, I speculate that avian mirror neurons, homologue to those that encode peripersonal and extrapersonal space in monkeys, might play a central role in the transmission of complex tool-use in this species (an hypothesis that may well be tested through neurophysiological experiments in the future).

In this integrative view, the slow development and prolonged parental care observed in NC crows (Holzhaider et al. 2010; Hunt et al. 2012) and the brain cephalisation process that corvids experienced during vertebrate evolution, have worked in concert to produce a brain-mind capable of extraordinary feats\textsuperscript{15}. The evolution of flexible cognition in birds is thus linked to the evolution of complexity and plasticity of the avian nervous system.

\textsuperscript{14}A higher form of synaptic plasticity which can be modulated by previous synaptic activity. In other words, prior synaptic activation/modulation may produce a long-lasting trace that effectively affects subsequent induction of synaptic plasticity (Abraham & Bear 1996).

\textsuperscript{15}A similar view has recently been proposed to explain the covariation between brain size and behavioural complexity in primates (Charvet & Finlay 2012). The authors suggest that extended “... developmental time, and not simply the number of neurons...” may mediate behavioural complexity in large-brained primates. Most importantly, they state: “[The] extended learning period may enable these large [primate] infants to better develop the categorization skills to differentiate individuals and their motivations, learn elaborate methods of food processing, or learn the unique characteristics and affordances of foraging sites, depending on what the social and natural ecology presents.” (p. 84, Charvet & Finlay 2012).

Likewise, during the extended period of parental care exhibited by NC crows, juveniles are invariably closely exposed to several tolerant adults (family and nonfamily) (Holzhaider et al. 2011). This gives juveniles ample opportunity to observe and learn different behavioural repertoires, which in turn may prove critical not only for the development of tool-related skills (i.e. specific techniques for their manufacture and use) but may also represent an invaluable source of rich stimuli (social, technical, etc.) for a large, developing brain. Thus, being part of a social network may have a strong effect in forcing highly flexible cognition during development.

For instance, by observing a handful of adults, a juvenile crow may be exposed to (1) more than a handful of possible solutions to a single problem (e.g. getting out a live Cerambycidae larva from a hole in a log) or (2) several situations (or contexts) in which one object (e.g. a tool) may be used to the holder’s advantage. In any case, a socially rich environment may help prevent the formation of fixed, stereotyped behaviours in the NC crow adult-stage. However, such an effect is conceivable only if there is an extensive overlap between the periods of development of the NC crow brain and the exposure to complex behavioural repertoires.
Appendix A: Histology

Staining methods

Neutral-red nuclear staining method

Staining procedure

1. Dehydrate and bring sections to xylene.
2. Dewax sections in xylenes I-III for 2 min each.
3. Quickly bring sections to tap water.
4. Stain in neutral-red for 2-4 min.
5. Briefly rinse in warm tap water.
6. Check tissue differentiation. Differentiate using 95% ethanol.
7. Dehydrate and bring sections to xylene.
8. Clear in xylenes I-III for 2 min each.
9. Coverslip with mounting medium and let dry.

Carazzi’s modified haematoxylin staining method

Staining procedure

1. Dehydrate and bring sections to xylene.
2. Dewax sections in xylenes I-III for 2 min each.
3. Quickly bring sections to tap water.
4. Stain in haematoxylin solution for 30-40 min.
5. Blue in running, warm tap water¹ for 5 min.
6. Differentiate in acid alcohol² for 2-5 s.

Haematoxylin neutral-red nuclear staining method (with Carazzi’s haematoxylin)

Staining procedure

7. Dehydrate and bring sections to xylene.
8. Dewax sections in xylenes I-III for 2 min each.
9. Quickly bring sections to tap water.
10. Stain in haematoxylin solution for 30-40 min.
11. Blue in running, warm tap water for 5 min.
12. Check tissue differentiation. Differentiate using 95% ethanol.
13. Stain in neutral-red for 2 min.
14. Briefly rinse in warm tap water.
15. Check tissue differentiation. Differentiate using 95% ethanol.
16. Dehydrate and bring sections to xylene.
17. Clear in xylenes I-III for 2 min, each.
18. Coverslip with mounting medium and let dry.

¹ Use a large water container or bucket, leave the slides in the bottom and then open the tap. This will prevent direct water blasting of the mounted sections.

² 1% hydrochloric acid (HCl) in 70% ethanol.
Fast cresyl violet staining method

Staining procedure

1. Dehydrate and bring sections to xylene.
2. Dewax sections in xylenes I-III for 2 min, each.
3. Quickly bring sections to tap water.
4. Stain in fast cresyl violet solution for 2 min.
5. Briefly rinse in warm tap water.
6. Check tissue differentiation. Differentiate using 95% ethanol.
7. Dehydrate and bring sections to xylene.
8. Clear in xylenes I-III for 2 min, each.
9. Coverslip with mounting medium and let dry.

Reagents

Acetate buffer 0.037 M solution (pH 4.8):

<table>
<thead>
<tr>
<th>Sodium acetate anhydrous</th>
<th>153 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glacial acetic acid</td>
<td>60 μL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Preparation:

1. Mix the sodium acetate anhydrous and the glacial acetic acid with distilled water.
2. Check pH.

Neutral-red stain solution:

<table>
<thead>
<tr>
<th>Neutral-red</th>
<th>5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>500 mL</td>
</tr>
<tr>
<td>Acetate buffer 0.037 M (pH 4.8)</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Preparation:

1. Dissolve 5 g of neutral red in 500 mL of distilled water.
2. Add 20 mL acetate buffer.
3. Filter before use.
Carazzi's Haematoxylin solution:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin</td>
<td>5 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100 mL</td>
</tr>
<tr>
<td>Potassium alum</td>
<td>25 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>400 mL</td>
</tr>
<tr>
<td>Potassium or Sodium Iodate</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>

Glacial acetic acid or hydrochloric acid\(^5\) 20 mL/L or 5 mL/L, respectively.

Preparation:

1. Mix 5 g of haematoxylin with 100 mL of glycerol.
2. Dissolve the potassium alum in 390 mL of distilled water\(^6\) (keep the other 10 mL).
3. Mix by adding the alum solution in small volumes to the haematoxylin solution, shaking well between each addition.
4. Dissolve the K- or Na-iodate in the remaining 10 mL of distilled water\(^7\), and then add it to the mixture.
5. Shake (stir) well. The solution is ready for use and will keep for 4-6 months.

Fast cresyl violet stock solution:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast cresyl violet</td>
<td>5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 mL</td>
</tr>
</tbody>
</table>

Preparation:

1. Dissolve 5 g of fast cresyl violet in 500 mL of distilled water.
2. Shake (stir) well. The solution is ready for use and will keep until depletion.

Fast cresyl violet working solution:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution</td>
<td>60 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>540 mL</td>
</tr>
<tr>
<td>10% acetic acid</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

Preparation:

1. Mix the stock solution with the distilled water.
2. While stirring, add the acetic acid slowly but continuously.
3. Filter before and after use. This solution deteriorates after a few days.

---

\(^3\) Aluminium potassium sulphate.

\(^4\) Double (50g) or triple (75g) concentration of mordant usually increases selectivity for nuclei.

\(^5\) Add any one of these when a lower final pH level is needed. This will increase selectivity to nuclei.

\(^6\) This may take several hours. Do not heat the solution.

\(^7\) Use very little (if any) heat.
Appendix B: Solutions and buffers for immunocytochemistry

Normal saline solution:

<table>
<thead>
<tr>
<th>Sodium chloride</th>
<th>4.5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water to make volume to</td>
<td>500 mL</td>
</tr>
</tbody>
</table>

Sodium phosphate buffer (PB) (pH 7.4) 0.4 M stock solution\(^8\):

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 46 \text{ g} \\
\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} & \quad 10.49 \text{ g} \\
\text{Distilled water to make volume to} & \quad 1 \text{ L} \\
\end{align*}
\]

OR

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 46 \text{ g} \\
\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O} & \quad 11.86 \text{ g} \\
\text{Distilled water to make volume to} & \quad 1 \text{ L} \\
\end{align*}
\]

Fixative 4% paraformaldehyde in 0.1 M sodium phosphate buffer solution:

<table>
<thead>
<tr>
<th>Paraformaldehyde (high grade) (PFA)</th>
<th>40 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 M stock PB solution</td>
<td>250 mL</td>
</tr>
<tr>
<td>Distilled water to make volume to</td>
<td>1 L</td>
</tr>
</tbody>
</table>

Preparation:

1. Heat 600 mL of distilled water to 50 °C under fume hood.
2. Suspend PFA in the hot water and leave stirring for 1-2 hours.
3. Regularly check that the temperature does not reach 55 °C.
4. If not dissolved after 2 hours, add 1M NaOH drop-wise until dissolution.
5. Add 250 mL of stock PB solution and complete with distilled water to make volume to 1 L.
6. Filter before storage.

Sodium hydroxide 1 M solution:

<table>
<thead>
<tr>
<th>Sodium hydroxide</th>
<th>4 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H2O to make volume to</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

Phosphate buffered saline (PBS) (pH 7.4) 1 M stock solution\(^9\):

<table>
<thead>
<tr>
<th>KCl (add first)</th>
<th>5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH(_2)PO(_4)</td>
<td>5 g</td>
</tr>
<tr>
<td>Na(_2)HPO(_4)(^{10})</td>
<td>28.75 g</td>
</tr>
<tr>
<td>NaCl (add last)</td>
<td>200 g</td>
</tr>
<tr>
<td>Distilled H2O to make volume to</td>
<td>2.5 L</td>
</tr>
</tbody>
</table>

---

\(^8\) Dilute to 0.1 M for use (i.e. 250 mL diluted to 1 L). Filter and check final pH before use.

\(^9\) Dilute to 0.1 M for use (i.e. 100 mL to 1 L). Filter and check final pH before use.

\(^{10}\) Alternatively, use 36.05 g of Na\(_2\)HPO\(_4\) · 2 H\(_2\)O for a total volume of 2.5 L.
Phosphate buffered saline 0.4% Triton X-100 solution:

Triton X-100 (Sigma) 4 mL
PBS to make volume to 1 L

Bleaching solution (with 1% hydrogen peroxide):

Methanol 50 mL
30% hydrogen peroxide 3 mL
Distilled water to make volume to 100 mL

30% sucrose PBS (pH 7.4) solution:

Sucrose (high grade) 30 g
PBS 100 mL

Diaminobenzidine (DAB) peroxide reaction medium:

DAB (Sigma) 25 mg (or 1 mL aliquot of DAB stock)
PBS 97 mL
30% hydrogen peroxide 15 µL
0.5% Cobalt Chloride\(^{11}\) 3 mL

DAB stock solution:

DAB (Sigma) 1 g
Distilled water 40 mL

Preparation:

1. Prepare a completely dark glass container for this solution\(^{12}\).
2. Dissolve 1 g of DAB in 40 mL of distilled water.
3. Leave stirring for 2-3 hours.
4. Make 1 mL aliquots (final concentration 25 mg/mL) and freeze until needed.
5. Use 1 mL aliquot instead of measuring 25 mg of dry DAB.

\(^{11}\) Add only if a black reaction product is needed. Otherwise, the reaction product will have a brown colour.

\(^{12}\) DAB is photosensitive and should always be kept in the freezer, in a dark container.
References


Aumann, T. 1990. Use of stones by the black-breasted buzzard Hamirostra melanosternon to gain access to egg contents for food. Emu, 90, 141–144.


Electronic Supplementary Material

A data DVD is included in the folder bound at the back of this thesis. It contains the following folders:

**Electronic thesis**: a copy of this thesis in .pdf format.

**Audiovisual material**: 

Chapter 2: New Caledonian crows’ responses to mirrors

*Movie 2.1 Mirror-responses*

This video shows the different responses of crows in front of vertical mirrors as described in Table 2.2 (p. 21).

*Movie 2.2 Reversed Mirror Control*

This video shows Obo’s performance during the 10 min reversed-mirror control, before she was transferred to the 4-box apparatus.

**Clip 1**: Obo’s initial responses (trial 1). Obo lands on the left end of the perch, looks shortly towards at the mirror and then leaves.

**Clip 2**: Obo’s responses after first bait on perch (trial 1). Obo lands on the middle of the perch and eats a block of meat. She then looks at the reversed mirror surface for a while and leaves.

**Clip 3**: Obo’s responses after second bait on perch (trial 1). Obo lands on the left end of the perch, looks at the mirror, moves next to the bait at the middle of the perch, eats the block of meat and lowers her head on top of the left compartment. Next, she turns on the perch and leaves.

**Clip 4**: Obo’s responses after final bait on the mirror (trial 1). Obo lands on the left end of the perch, moves to the middle of the perch and lowers her head to reach the visible block of meat on the reversed mirror surface (between the two compartments. She eats the bait and lowers her head again to scan both compartments. She then reaches for the bait on the right compartment and eats it.

**Clip 5**: Obo’s responses in the absence of visible baits (trial 2). Obo lands on the left end of the perch and looks at the mirror, she moves then to the middle of the perch and looks again down at the mirror. Obo stays on the perch for a while without obvious looks at the mirror and then leaves.
Movie 2.3 Mirror-mediated spatial location performance

This video shows one successful trial (Obo) and one unsuccessful trial (Pelé) in the 4-box apparatus. Success was scored in accordance to the two criteria described in p. 34.

Clip 1: Successful trial Obo (trial 1 block 1). Obo lands on the left end of the perch, looks at the mirror and then moves to the section of the perch right on top of the third (baited) compartment from the left. She lowers her head to reach the block of meat and eats it.

Clip 2: Unsuccessful trial Pelé (trial 6 block 1). Pelé lands on the left end of the perch, moves to the section of the perch right on top of the first (unbaited) compartment from the left. He lowers his head and scans the empty compartment. He then moves to the third (unbaited) compartment from the left and repeats his earlier behaviour. Finally, he moves until he is on top of the fourth and final (baited) compartment, reaches for the block of meat and eats it.

Chapter 3: Is string pulling based on insight?

Movie 3.1 (S1) Techniques used to solve the standard string pulling problem

This video shows the crows’ different string pulling techniques described in p. 54.

Clip 1: Technique: Consecutive pull and steps while stationary at the perch (Tiga trial 2, Español trial 9). Tiga pulls up the whole string by using first his right foot and then his left foot, to step on the string. Español pulls up the string using his left foot only, to step on the string. Both crows stay stationary while pulling up the string.

Clip 2: Technique: Consecutive pull and steps while moving along the perch (Robin trial 9). Robin pulls up the string, steps on it with his right foot and then moves along the perch to the left in the next pull-step sequence, always using his right foot to secure the pulled-up length of string. He moves once again to the left before reaching the meat.

Clip 3: Technique: Using a foot as ‘shackle bolt’ the bird pulls the loop without further steps (Maya trial 1). Maya has stepped on the first pulled-up length of string using her right foot. Next she pulls the string loop under her foot until the meat is at reach. In order to do this, she keeps hold of the string loop with her right foot.
Clip 4: Technique: Moving along the perch while loosely holding the string in the bill (Slevin trial 10). After getting hold of the string with the bill, Slevin moves his head and body to the left while loosely holding the string. The quickly reaches the meat without ever having to step on the string.

Movie 3.2 (S2) First attempts in clear and opaque disc string pulling
This video shows the initial performance of crows in the clear and opaque disc tasks.

Clip 1: Clear disc (Caspar trial 1). Caspar pulls up the string while stationary at the perch using both feet to step on the string. He takes the clear disc and holds it under his left foot, and attempts to reach the meat through the plastic disc.

Clip 2: Opaque disc (Robin trial 1). Robin makes his initial pull-step with the right foot, but then releases the string and looks down at the opaque disc. He then pulls up the string again and releases it without stepping on it. Next, he looks down at the disc, moves along the perch and looks down a final time. He then takes off.

Chapter 4: Brain–behaviour relationships: what do they actually tell us about advanced cognition and tool use?

Figure 4.20
A diagram of the avian phylogeny with reference to tool-use and cerebellar complexity. Due to the large size of the figure, two legends have been inserted in the lower left and upper right corners. The phylogeny was based on data from the Tree of Life Web Project site: tolweb.org/tree (last accessed in July 2012).

Labelled brain section images
These image stacks correspond to each crow’s brain sections, with brain regions identified and coloured in AMIRA 5.2.0, as described in pp. 111-112.

Midsagittal cerebellar images
Cerebellar most midsagittal images of the cerebella of one Galliform (chick), one Gruiform (pukeko), two Passeriform (Indian mynah and NC crow) and one Strigiform (barn owl) bird species. These cerebella satisfied our criteria for fractal analysis (as described in pp. 130-131). Also, discarded images (see pp. 129-130) of the cerebella of two Passeriform (Australian magpie and Japanese jungle crow) and one Psittaciform (budgerigar) species.