The ecology and anatomy of scent

in the critically endangered kakapo (*Strigops habroptilus*)

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Hoki, the kakapo - Photo by Dr. Luis Ortiz Catedral

Kakapo chicks born in 2008;©Photo by Chris Birmingham

ABSTRACT

The focus of the research presented here is the analysis of feather scent emitted by a parrot, the kakapo (*Strigops habroptilus*) and the kakapo's ability to perceive scent by studying the anatomy of its brain and the olfactory bulb. In addition, behavioural research was conducted to determine the capability of the kakapo's closest relatives, the kea (*Nestor notabilis*) and kaka *(N. meridionalis*) to detect scents and to distinguish between different concentrations of scents.

The strong odour of the kakapo is one of the many unique characteristics of this critically endangered parrot, but its sense of smell has never been described in detail. The kakapo is the largest parrot worldwide, it is nocturnal and flightless. Kakapo are herbivorous and it is the only parrot with a lek breeding system. Males defend several display arenas during the breeding season and continuously produce low frequency booming calls. Females come from afar and appraise different males and choose one with which they want to mate. As in all lek mating systems some males make major contributions to the gene-pool of the next generation while others make little or no contribution. Currently it is not known what the female's choice is based on and why some male kakapo are 'favoured' over others. However, it has been observed that favoured males appear to emit a stronger odour than less attractive ones (pers. comm. Kakapo Recovery Team). This study is the first to compare the chemical composition of the kakapo's scent in relation to season, age and sex. It is also the first study to uncover the kakapo's ability to smell by conducting a comparative examination of the anatomy and histology of the brain and the olfactory bulb.

In spite of its endangered status, the kakapo is a good model in which to study olfaction, as the birds are closely monitored by the Department of Conservation, New Zealand. The birds undergo regular health checks and transmitter changes, allowing access to a large proportion of the population at once and for which their individual history is known. The study of olfaction in kakapo is important as it can contribute to the growing field of avian olfaction, and by elucidating the kakapo's potential for olfaction conservation managers will be able to make better decisions in their attempt to save this species. The research approach adopted in this dissertation includes the analysis of feather samples from individuals of different sex and age as well as from different seasons using gas chromatography-mass spectrometry. The opportunity to examine the brain as well as the eyes of a kakapo that died at Auckland Zoo, Auckland, New Zealand, allowed a comparative study of the brain, the olfactory bulb and the visual centres (of both the thalomofulgal and the tectogucal pathways) with other Australasian parrots. Additionally, behavioural experiments with kea and kaka, the closest relatives

of the kakapo, give insight into two of New Zealand endemic parrot's and their ability to distinguish between different scents and scents of different concentrations.

The findings from this research provide evidence that kakapo distinguish themselves by having one of the largest olfactory bulbs measured in parrots and the highest number of mitral cells, responsible for the transmission of an olfactory neural signal into a behavioural response, counted in any species to date. They also have a strong odour, whose chemical composition shows sexual, age-dependent and seasonal distinction. Furthermore, the study found that kea and kaka are both able to distinguish between different scents and different concentrations of scents.

The main conclusions drawn from this study are that kakapo appear to be equipped with a functional olfactory bulb, able to sense olfactory information, but also communicate information that is likely to be of social importance using their plumage scent. In conclusion, this dissertation provides the foundation for future research, in particular to examine the role of the scent in the social life in kakapo, and it provides fundamental insight into the olfactory and visual sensory abilities of the New Zealand endemic kakapo.

Rebecca Wu

REFLECTION AND ACKNOWLEDGEMENTS

I guess my mother Heidemarie would have been fascinated by the many stories I can tell about kakapo, a bird so curious it seems to originate from a different world. She had an eye for detail and taught my sister and myself to look at things with the eye of an eagle and to appreciate them. I started collecting feathers when I was seven years old. I had a big cardboard box with Indian red patterns on its side, which held all the treasured feathers found on many excursions to various west European woods and the Swiss Alps. It was greatly improved by my early voluntary work in the zoo, which meant that I had access to the most exotic feathers. They were all stored in this box, and I was welcomed with a nice wooden, earthy smell whenever I opened the box.

Little did I know at that time how important feathers would become later in my life. I still collect them, but not only for pleasure anymore. Before that though, my journey took me to Africa - inspired by my grandfather who had worked as a medical doctor in Ifakara, Tanzania, in his early days and who wanted to see this amazing continent for one last time. He invited the whole family to a memorable trip to Zambia, where my wish to work in Africa was born. This wish was fulfilled when I conducted by MSc in Tim Clutton-Brock's unique meerkat research project in the Kalahari and worked on aspects of the rich variation of marking behaviour in the social mongoose, the meerkat, (*Suricata suricatta*). I was familiarised in a vivid way with the many ways scent and marks can be transferred in animals. Work in the Botswana Wild Dog Research Project on the African wild dog (*Lycaon pictus*) to develop a biological method to repel wild dogs from farmland taught me the conservational aspect scent can have. A call to New Zealand to work on olfaction in the Northern brown kiwi (*Apteryx mantelli*) heralded a new and fascinating period in my life. Hitherto, little was known about olfaction in birds and the offer to work on the Northern brown kiwi was all the more tempting since I had to deal with a bird whose behaviour, in many aspects, is more reminiscent of a mammal than a bird. Preliminary behavioural experiments undertaken on free ranging birds indicated the use of body scent in the social life of kiwi and a pilot analysis of the chemical compounds in body scent using gas chromatography-mass *spectrometry* revealed the presence of aromatic oils and alcohols that could account for the strong smell of this bird (Castro *et al*. 2010). Periods of waiting for permits were filled with work on rats, with the aim to trial and use the scent of conspecifics to attract and lure them. In a land where food is plentiful, the attraction to mate might be stronger and serve as a good option to lure and catch rats while presenting a low risk to other wildlife. All this work on diverse animals using scent and olfaction was a good introduction for my PhD on another endangered and strong smelling bird, the kakapo (*Strigops habroptilus*), surely the most bizarre and controversial, but rewarding, animal I have worked on so far.

All these ventures to far away destinations were supported by my father Hans-Otto, who in return got to see all these exciting places. I am indebted with deep thanks and gratitude to my father for having supported all along his daughter's unusual, extensive, and not always easy, travel destinations in many ways. I am not quite sure how I came to land so far way, but I think we can blame the kakapo.

I am greatly thankful to my sister Alena Sonja for being all ears to all sorts of issues that come along in the course of a PhD. I would like to thank her for proofreading and commenting on some aspects of my work. Let alone the countless care parcels of food she and my father supplied, primarily fine Swiss and Belgian chocolate, as well as the supply of Italian coffee and other specialities, bringing home a little closer. I think we kept MAF quite busy over the last few years.

Deepest and sincere thanks go to my partner David Vincent, who supported me in the best possible way. He encouraged me in the first place to return to New Zealand in order to study New Zealand's smelly avian fauna, which I am more than thankful for. I would also like to thank David for proofreading some of my work. Coming from a very different background, his knowledge in economy brought me a different perspective to my life. I am also thankful for that reason.

Apart from all the personal support that has been given to me by my partner and my family, this thesis could not have been completed without the continuous and never failing support of my supervisor Assoc. Prof. Dr. Dianne Brunton (Director of the Ecology and Conservation Group, Institute of Natural Sciences, Albany Campus, Massey University, New Zealand). Dianne has all the aspects that a student wishes for in a supervisor, and I am thankful for her openness to new research projects, for sharing the vision and supporting and refining ideas and helping to bring them to their successful completion. Dianne makes time for her students even if she has no time, and lives the Kiwi 'can do' attitude to the fullest. Without her input and help, this project would not have been possible.

The second person in my academic field I am indebted to is Dr. Ron Moorhouse (Scientist, Kakapo Recovery Programme, Invercargill, New Zealand). Not only am I thankful for his support and help with pursuing an initially unusual project idea and helping with the permits, but also for being a marvellous source of knowledge and providing me with deep insights into the wondrous world of the kakapo.

My co-supervisor Dr. Weihong Ji knew Ron Moorhouse personally and not only enabled me to get to know Ron from a different perspective, that is as a marvellous Scottish pipe player, but made the first contact possible. I am grateful for Dr. Weihong Ji's support and help.

There were several large groups with which I worked on my exciting subject. One of the most remarkable groups, however, was my 'GC-MS group', filled with people that were just as much in love with the subject as I am, and always eager to know more – no matter how late at night it was. So it was more than natural to share knowledge and to keep each other up to date with the newest information available. A vibrant, high wired exchange of knowledge took place between my co-supervisors Prof. Tom Goodwin (Hendrix College, Conway, Arkansas, USA) and Assoc. Prof. Dr. Julie Hagelin (Institute of Arctic Biology, University of *Alaska,* Fairbanks, Alaska, USA) and my collaborators Prof. Dave Greenwood (The School of Biological Sciences, Auckland University, Auckland, New Zealand) and Dr. Andrew Fidler (Cawthron Institute, Nelson, New Zealand). I would like to thank Dave for his tremendous help with the gas chromatographic work and evaluation of the GC-MS output, thank you for putting endless hours into this work, but it has all been worthwhile! I would like to thank Julie and Andrew as well as Pat Holland (Cawthron Institute, Nelson, New Zealand) at this point for allowing me to continue their exciting journey in olfaction in kakapo. Their initial results opened the way for this thesis and helped it to success. I think we all were highly inspired and carried away by Tom's unforgettable humour. It has been great pleasure to work with you people and Tom's visits to New Zealand (Figure 1) are just as vivid as our (Tom, Dave, Julie and myself) trip to the Chemical Signals in *Vertebrates* Conference in *Berlin,* Germany, August, 2011. Thank you for being there, and I truly hope, we will continue our adventure in the world of scent in kakapo.

Figure 1 Dianne (left), Tom (middle) and Andrew (right) on a work lunch meeting that we enjoyed close to Massey University, Albany, New Zealand.

Insight, knowledge and an understanding of not only working with brains but getting a flair for understanding their anatomy, were all facilitated with a lot of patience and understanding by Dr. Jeremy Corfield (Department of Neuroscience, *University of Lethbridge, Lethbridge*, Alberta*, Canada*). Without his unlimited help and time, I would never have come to the point of anatomical understanding that I gained in my work. I am deeply indebted to profound and sincere thanks for all the advice, help and understanding Jeremy has given me.

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'Nothing is by chance'

Eileen Caddy

THESIS STRUCTURE AND FORMAT

This thesis is written as a series of seven interrelated chapters, two of which (Chapters Five and Six) have been published in part in peer reviewed journals (Corfield *et al*. 2011; Gsell *et al*. 2012). ***Chapter One: scent and olfaction in birds; kakapo (Strigops habroptilus) as a model species*** introduces the main subject of my thesis by discussing the current knowledge on olfactory signalling in birds and reviewing the literature on the use of olfaction in birds. The particular case of the kakapo is reviewed by addressing its history and evolution, as well as its behavioural characteristics and the current conservation status. Additionally, it is described why the kakapo makes an excellent model in which to study olfaction in birds. At the end of this first chapter, a brief summary of the specific aims of this thesis is presented.

Chapters Two to Six are data chapters and can be divided into four sections. The first section, Chapter Two, deals with the characteristics of the scents emitted. The second section, Chapter Three and Four, deals with the anatomical specialization for scent perception, the brain and the olfactory bulb, in particular. The third section, Chapter Five, looks at the visual system in kakapo, in order to evaluate the importance of olfactory versus visual cues. The fourth section, Chapter Six, combines both aspects of emitting and perceiving scent and looks at whether the kakapo closest relatives, the kea (*Nestor notabilis)* and the kaka (*N. meridionalis)* possess olfactory abilities by using behavioural experiments. Chapter Seven consolidates all findings.

Chapter Two: The chemical analysis of kakapo (Strigops habroptilus) feather scent.

The strong, sweet smell of the kakapo has been described on many occasions, yet it is not known what role it plays. Regular health checks and transmitter changes in the remaining kakapo population on Codfish Island, New Zealand, provided me with the opportunity to obtain feather samples from different kakapo individuals, of different age and sex and collected at different seasons. This allowed me to conduct a complete analysis of the chemical composition of the feather odour in kakapo, encompassing age related, sexual and seasonal factors. Equipped with that data, I was able to assess what information kakapo can convey through its body odour. While Chapter Two discusses the qualities of the sweet smell of the kakapo and examines what type of information the kakapo is able to convey with the smell of its plumage, Chapters Three and Four address the ability of kakapo to receive and process olfactory information.

Chapter Three: A comparison of brain stuctures of the nocturnal kakapo (Strigops habroptilus) and the diurnal sulphur-crested cockatoo (Cacatua galerita) with special emphasis on the olfactory bulb and the optic lobe : The rare opportunity to obtain the brain of an old, male kakapo that had died at Auckland Zoo, presented the unique opportunity to look at the general brain anatomy of the kakapo and to compare it with that of the diurnal sulphur-crested cockatoo.

Chapter Four: Anatomy and histology of the olfactory bulb of the kakapo (Strigops habroptilus) in comparison to other Australasian parrots: Thanks to collaborators in Australia and Canada, I was able to examine and compare the detailed anatomy of the olfactory bulb of the kakapo and nine Australasian parrots of different behavioural ecology and size. These were the Australian king parrot (*Alisterus scapularis*), the cockatiel (*Nymphicus hollandicus*), the crimson rosella (*Platycercus elegans*), the Eastern ground parrot (*Pezoporus wallicus*), the Eastern rosella (*Platycercus eximius*), the galah (*Cacatua roseicapilla*), the rainbow lorikeet (*Trichoglossus haematodus*), the red-rumped parrot (*Psephotus haematonotus*) and the sulphur-crested cockatoo (*Cacatua galerita*). A detailed and comparative study of the anatomy and histology of the olfactory bulb in the kakapo, allowed me to address questions such as whether the kakapo has an acute sense of smell.

Chapter Five: Anatomy and histology of the visual system of the kakapo (Strigops habroptilus) in comparison to other birds: An environment is always perceived through a variety of senses, although some sensory systems are more developed than others. Therefore, the general findings regarding the olfactory system of different bird species were compared to the development and the character of the visual systems, with particular reference to the specific situation in the kakapo.
The visual system was assessed in two ways because retinal information is conveyed over two major pathways: the thalamofugal pathway and the tectofugal pathway. In order to assess visual abilities, it is therefore important to examine the retina and specific brain compartments. For that reason, I describe the retina of the kakapo, while comparing it to the retina of other typical diurnal birds (the domestic chicken, *Gallus gallus*, and the rock pigeon, *Columba livia*), and nocturnal birds (the barn owl, *Tyto alba*, and the predominantly nocturnal morepork, *Ninox novaeseelandiae*). Additionally, I compared four visual brain centres (the entopallium, the nucleus rotundus, the tectum opticum and the Wulst) among nine different parrots. I contrasted the visual brain centres in the kakapo brain with those of the parrots used in the comparison made in the olfactory bulb. Only the Eastern ground parrot had to be replaced with a sample from the kea (*Nestor notabilis*), as the preservation quality of the brains did not always allow me to use them for all examinations. Parts of this work have been published in a peer-reviewed journal (Corfield *et al*. 2011 and Appendix A, Figure A1). Dr.

Jeremy Corfield gained first authorship in this paper due to his connections to Dr. Andrew Iwaniuk and for making this paper possible. Laboratory work has been equally conducted by myself and Jeremy Corfield, data analysis for the paper was mainly conducted by Dr. Jeremy Corfield and Dr. Andrew Iwaniuk, while write up was predominantly done by myself, Dr. Jeremy Corfield and Dr. Andrew Iwaniuk. The data presented in Chapter Five differs from what is presented in the paper, because I conducted my calculations with a different set of parrot species.

Chapter Six: Olfactory sensitivity in kea and kaka: In order to evaluate whether the sense of smell and the action of scenting play any role in the ecology of the Nestoridae, I conducted scent experiments with the kakapo's closest relatives, the kea (*Nestor notabilis)* and the kaka (*N. meridionalis)* at Auckland Zoo. The experiments tested whether kea and kaka possess olfactory abilities and whether they are able to discern different scents and different concentrations of scents. The work presented in this chapter has been published in a peer-reviewed journal (Gsell *et al*. 2012 and Appendix B, Figure B1). I conducted all the experiments, the statistical analysis and the write-up, while my co-authors provided useful input.

Chapter Seven: Conclusions and outlook: The last chapter summarises all information and puts it into context. The relevance of my findings is discussed and research directions are suggested.

Appendixes A&B: Statement of contribution for a publication

Appendixes C-H: present supporting information to Chapter Two.

References: All references are listed at the end of the thesis to minimise repetition. All literature cited is consistent with the format used for the scientific journal: *Proceedings of the Royal Society, Sciences B*. For a list of title word abbreviations, see:
http://www.csa.com/ids70/serials_source_list.php?db=biolclust-set-c.

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CHAPTER ONE

SCENT AND OLFACTION IN BIRDS; KAKAPO (*STRIGOPS HABROPTILUS*) AS A MODEL SPECIES

Drawing by Dylan van Winkel (2011)

The online version of the Encyclopaedia Britannica (2012) notes on olfactory abilities in birds:

"Their sense of smell is not highly developed"

1.1 Overview

Olfactory sensitivity in birds has been a neglected topic for a long time, yet a wide variety of birds have now been documented to use olfactory cues in diverse ways: to express information about their identity and health, to broadcast their sexual availability and to find specific locations of resources, such as homing grounds and food (Roper 1999; Hagelin & Jones 2007).

I have chosen the kakapo (*Strigops habroptilus*), a nocturnal, flightless, and lek breeding parrot, endemic to New Zealand, as the focus of my studies because they are reported to have a strong, long lasting and sweet smell associated with their feathers. Thus, the kakapo is an excellent model to examine odour related activities such as emission and detection of scent.

Although the broader context of this investigation is to understand the nature of the information being transmitted and the social and behavioural relevance of scent transmission in kakapo, the research presented in this thesis has focussed on the anatomical specialisations of kakapo for scent perception and the characteristics of the scents emitted. Therefore, this work examines: 1) the nature and characteristics of the body odour as the scent carrying medium, 2) the anatomy of the olfactory bulb as an organ that receives volatile signals and processes them by transmitting them in the form of neural signals to higher brain areas, and 3) behavioural scent experiments in the kakapo's closest relatives (kea and kaka), which are relevant to kakapo. This indirect approach to the role of scent in kakapo behaviour was deemed appropriate given the kakapo's critically endangered status and the highly restricted access to the islands they inhabit. Access to the brain of a deceased kakapo and regularly sampled feathers of free-living birds provided the opportunity to comprehensively describe the olfactory sense in kakapo. This study is unique in that the olfactory bulb of the kakapo is examined for the first time. It is also the first study to examine the volatile scent of males, females and chicks throughout all seasons.

In this introductory chapter, I discuss the current knowledge on olfactory signalling in birds and review the literature on the use of olfaction in birds. The particular case of the kakapo is reviewed by addressing its history and evolution, as well as its behavioural characteristics and the current conservation status. I discuss why kakapo are a good model for understanding the role of scent production and olfaction.

1.2 Signals

Animals produce a great variety of signals with a multitude of functions. Signals are acts or traits that have evolved to elicit a response in the behaviour of other organisms. They are effective because the receiver's response has altered and evolved alongside the sender's signal (Endler 1992; Maynard Smith & Harper 2003). They can serve to attract an individual's attention, such as elaborate traits in a male's courtship displays or the brightly coloured mouths of chicks begging for food (Kilner 1997). This usually implies that the sender and receiver mutually benefit from creating and reading the signal (Endler & Basolo 1998). Peacock (*Pavo cristatus*) hens for example prefer males with higher numbers of eye-spots in their tail feathers (trains) over males with less elaborate trains. They will also have larger clutches when they have mated with such males compared to males with less elaborated trains (Petrie *et al.* 1991; Petrie & Williams 1993). Trains are hypothesised to have evolved due to female mate choice, but also to act as an 'honest signal' indicating the male's genetic and health constitution to the female. Other traits driven by sexual selection are, for example, the complex bower displays of male satin bowerbirds (*Ptilonorhynchus violaceus*) (Morell 2004), as well as their complex songs, dances and colouration of their plumage (Doucet & Montgomerie 2003). These all fuction to attract females, attain copulations, and maximise reproductive success. Signals can also serve to warn an individual of looming danger; for instance, by listening to warning calls of conspecifics or in some cases the calls of other species (Fallow & Magrath 2010). Galahs (*Cacatua roseicapilla*) for example respond to the sentinel warning calls of sulphur crested cockatoos (*Cacatua galerita*) (Juniper & Parr 1998). However, signals can also be dishonest, as when a bird expresses a danger call where there is none just to secure more food for itself (Maynard Smith & Harper 2003). Apart from visual (bright coloured plumage and displays) and acoustic signals, there is mounting evidence for the importance of olfactory signals in birds.

1.3 Olfactory signals

Although the role of chemical signals in social interactions and behaviour has been studied in invertebrates and a large number of vertebrate species (Darwin 1871; Ralls 1971; Gorman 1990; Wyatt 2003), there has only recently been interest in examining the possibility of birds using chemical signals for communication (Roper 1999; Hagelin & Jones 2007; Rajchard 2007). The use of scent in this sizeable group of animals has largely been ignored possibly because birds have very good eye sight, produce loud and complex songs and display a large repertoire of stereotypical social behaviours, making the role of olfaction appear to be minor in comparison. However, birds are capable of both producing and sensing odours. Some have a comparatively large olfactory bulb (Bang & Cobb 1968;

Northcutt 2011) and other anatomical structures associated with the production and detection of scent (Bang 1960; Bang 1966; Bang & Cobb 1968; Bang 1971; Wenzel *et al.* 1984; Bang & Wenzel 1985).

In the last three decades, scent and olfaction have been shown to have many different functions in birds (Roper 1999; Hagelin & Jones 2007), and scent is therefore an important tool of communication. It has many applications in social behaviours, for example in the location of specific places such as homing- and feeding grounds Nevitt *et al.* 1995, Benhamou 2003b) and to protect an individual from parasitism (Mennerat *et al.* 2009a) and even predation by use of deterrent scents (Münch 2002; Burger *et al.* 2004).

1.4 Social scents

The use of scent in sexual, partner and chick recognition has been established in a number of species, primarily in colonial seabirds. Antarctic prions (*Pachyptila desolata*), for example, live in colonies and are socially monogamous. They spend up to two weeks foraging at sea between incubation shift. When they return at night, they need to be able to find their own nest site among hundreds of other, similar looking nests (Bonadonna *et al.* 2003). Researchers have concluded that the prions recognise their site by the body odour of their partner, whose smell has likely integrated with the nest material (Bonadonna *et al.* 2003b). A similar situation has been described in other colonial breeding seabirds known for their strongly smelling rock caves. Olfactory preference for own burrows over conspecific nest sites has been experimentally shown in blue petrels (*Halobaena caerula*) (Bonadonna & Bretagnolle 2002; Bonadonna *et al*. 2003b; Bonadonna & Nevitt 2004; Bonadonna *et al.* 2004; Bonadonna *et al.* 2009) and other, mainly nocturnal seabirds, such as the snow petrel (*Pagodroma nivea*), the Leach's storm-petrel (*Oceanodroma leucorhoa*) (Grubb 1973; Bonadonna *et al.* 2003a) and Wilson's petrel (*Oceanites oceanus*) (Jouventin *et al.* 2007). The ability of birds to discriminate specific scents is supported in a study by Whittaker *et al*. (2009). She found that female dark-eyed juncos (*Junco hyemalis*) significantly shorten their incubation bout lengths when confronted with hetero- and conspecifics preen gland secretions as opposed to their own preen gland secretion or a control.

Chicks are also able to recognise their own nest site. While this ability is associated with self-odour recognition and / or the recognition of the smell of their parents, it is especially important for chicks in colonial breeding bird species to recognise their own nest site when they get close to fledging and begin to explore areas close to the nest site. Examples are the chicks of the colonial breeding British storm-petrels (*Hydrobates pelagicus*) and the black-footed albatrosses (*Phoebastria nigripes*); chicks

of both species are able to identify their nest by scent within a few days of hatching (Minguez 1997). Chicks of the European storm-petrel (*Hydrobates pelagicus*) prefer bedding with their own body odour to the odour of a conspecifics or a neutral control (De Leon *et al.* 2003). Similarly, domestic chicken chicks (*Gallus gallus*) prefer the smell from the litter of their rearing cage to that from an unfamiliar cage (Jones & Gentle 1985; Burne & Rogers 1996). Goslings from two geese species, *Anser anser* and *Branta canadensis*, given a choice, prefer the nesting material of their own rearing boxes (Würdinger 1982). Furthermore, a colony-breeding songbird, the zebra finch (*Taeniopygia guttata*), shows olfactory nest site preference (Caspers & Krause 2011). Self-odour recognition has been associated with the presence of an individual olfactory signature, which not only broadcasts the compatibility and quality of a potential mate but may also act as chemical kin label (Bonadonna *et al.* 2007; Celerier *et al.* 2011).

An important aspect of social olfactory signals is the volatility and seasonality of cues. Female mallards (*Anas platyrhynchos*) for example release seasonal scent volatiles communicating their sexual status and males assess these on a regular basis (Balthazart & Schoffeniels 1979; Kolattukudy & Rogers 1987). Crested auklets (*Aethia cristatella*) have been reported to perform a special courtship display where they are likely to transmit their seasonal and strong tangerine-like odour for appraisal and mate choice (Hagelin *et al.* 2003). Antarctic prions appear to prefer their partner's scent over other conspecifics and their own scent, suggesting that scent-recognition in this species may be associated with individual recognition and mate-choice (Mardon & Bonadonna 2009). In mallards, crested auklets, European starlings (*Sturnus vulgaris*) and the dark-eyed junco, the responses to odours have been shown to be seasonal (Kolattukudy *et al.* 1987; Clark & Smeraski 1990; Hagelin *et al*. 2003; Soini *et al.* 2007), suggesting an altering sensitivity to and occurrence of odours during the breeding season and the possibility of scent being used as a signal of quality in mate-choice.

The findings above are reinforced by studies showing differences in the chemistry of preen oils (produced by the uropygial gland) at individual, sexual and population levels in two different populations of the dark-eyed junco (Whittaker *et al.* 2010). Furthermore, female dark-eyed juncos also discriminate between the scents of individuals of different body size. They preferred, contrary to what would have been expected, the smell of smaller sized individuals over the scent of larger individuals with more elaborate ornaments, and it is suggested that the female's preference may be for smaller and less aggressive males (Whittaker *et al.* 2011). However, in juncos, female preference appears to be complex and may include traits other than scent and size. Hill *et al.* (1999) found that female juncos prefer males with specific and static ornaments on their plumage. Nonetheless, Whittaker *et al's*. (2010) comparison between the two junco populations supports the hypothesis that preen oils are

likely to have a genetic basis and are different for the two populations, and that these oils provide information relevant to mate recognition and choice. Age related and seasonal differences were also found in the chemical composition of preen oil from the uropygial gland in grey catbirds (*Dumetella carolinensis*) (Shaw *et al.* 2011), indicative of the underlying chemical differences in uropygial gland secretions, preen oils, and their ability to function as an informative signal.

1.5 Spatial movements

Apart from finding nest sites, olfactory cues are also used to locate homing grounds and to find food. Homing experiments have shown pigeons *Columba livia*) use air-born scents or so called 'air maps' when migrating (Walcott 1974; Papi 1991; Guilford *et al.* 1998). Pigeons can derive the direction from long-range gradients of traces of air gases. These gas ratios remain stable even under varying weather conditions (Wallraff 2003). Swifts (*Apus apus*) (Fiaschi *et al.* 1974), European starlings (Wallraff & Hund 1982) and various seabirds such as black-footed albatrosses (Bonadonna *et al.* 2005), blue petrels (Benhamou *et al.* 2003a) and Antarctic prions (Bonadonna & Bretagnolle 2002; Bonadonna *et al*. 2003a) all use air scent traces to find both their breeding and wintering grounds.

Much of the current understanding of avian olfaction comes from the study of foraging, particularly for pelagic seabirds such as many species within the Procellariiformes (tube-nosed birds). Olfaction can be the best option to find food patches in open water, where sensory inputs other than scent are scarce. Pelagic birds such as petrels, albatrosses and penguins find their food using olfactory cues (Culik 2002; Benhamou *et al.* 2003b). In a field experiment, Nevitt (Nevitt 1999; Nevitt 2000; Nevitt *et al.* 2004) evaluated the time it took tube-nosed birds to respond to krill-scented vegetable oil and found that there were big differences in response times between bird species. Verheyden and Jouventin (1994) found that these differences depended on external components such as wind direction rather than the olfactory skills of the birds. The ability to recognise certain olfactory cues appears to already be present in seabird chicks. Using maze tests, Bonnadonna *et al*. (2006) showed that blue petrel chicks were able to detect and orientate towards a foraging cue, in this case dimethyl sulphide, which is used by adults to find their prey, without ever having experienced this odour before. Turkey vultures (*Cathartes aura*) are a well known example of a bird with outstanding olfactory abilities. These vultures can find carcasses by olfactory cues alone (Houston 1986). Mäntylä *et al*. (2004) found that willow warblers (*Phylloscopus trochilius*) were attracted by volatile compounds produced by sand-fly damaged tree branches of the mountain birch (*Betula pubescens* ssp.), demonstrating a bird responding to a plant signal. The first test of the function of the olfactory system in birds was carried out by Michelsen (1959), who showed in a controlled experiment that pi-

geons were able to learn food discrimination using odours as cues. The first report on parrots using olfaction came from Roper (2003), who showed that yellow-backed chattering lories (*Lorius garrulous flavopalliatus*), which feed on nectar, flowers and fruits, are able to discern different odour cues presented in different dispensers. Buitron and Nüchterlein (1985) and later Harriman and Berger (1986) experimentally demonstrated in magpies (*Pica pica*) and common ravens (*Corvus corax*) the ability of these birds to find food under conditions where scent was the sole cue.

In New Zealand, Haast tokoeka (*Apteryx australis*) and the Northern brown kiwi (*A. mantelli*) have both been shown to discriminate between different containers of food (Wenzel 1968; Wenzel 1971b) and concentrations of odours (Jenkins 2001). The Northern brown kiwi has a distinct and pungent smell (Castro *et al.* 2010). In the only experimental trial conducted to date in kakapo, Hagelin (2004) found that kakapo can discriminate between boxes with food and boxes without food using olfaction alone.

1.6 Scent as defence

Defensive odours that deter ectoparasites and potential predators can either be self-produced or attained from external sources. An example of a bird that attains deterring smells is the North American burrowing owl (*Athene cunicularia*). This species actively places horse and cow dung in front of their ground burrow entrances. This tactic attracts beetles, which are eaten by the owls, but also seems to camouflage their nests from potential predators (Martin 1973; Levey *et al.* 2004). Using a similar strategy, the common waxbill (*Estrilda astrild*), a small African finch, is reported to accumulate carnivore scat in, on and around their nests, which may act as an olfactory deterrent (Schuetz 2005). Blue tits (*Parus caeruleus*) cover their nests with fragrant herbs and flowers (Lambrechts *et al.* 2000; Petit *et al.* 2002), which seems to camouflage the smell of the nest and the chicks. However, Lafuma *et al.* (2001) argue that the fragrance could also protect the birds from blood-sucking insects, and it has been shown that the smell from some herbs can reduce the bacteria load and diversity on chicks (Mennerat *et al.* 2009a; Mennerat *et al.* 2009b).

Birds not only use environmental scents as deterrents but they can also produce defensive scents themselves. One group of birds that produce deterring odours are the hoopoes (*Upupa epops* and *Phoeniculus purpureus*). During the breeding season, hoopoes produce peculiar foul smelling excrements, which they discharge at invaders to deter them from their nest (Münch 2002; Burger *et al.* 2004). Shovelers (*A. clypeata*) and eiderducks (*Somateria mollissima*) protect their nests by defecating over their eggs when disturbed by an intruder. Laboratory experiments demonstrated that rats

and ferrets, their main predators, were deterred by even small quantities of faeces from breeding birds, interestingly enough though not by faeces of non-breeding birds (Swennen 1968).

Three species of the colourful passerine genus *Pitohui* (*P. dichrous, P. kirhocephalus* and *P. ferrugi-nueus*) endemic to the forests of the New Guinea sub-region, store high levels of steroidal alkaloids in their skin and feathers. The repulsive smelling and highly poisonous secretion consists of varied concentrations of homobatrachotoxin, a substance otherwise only found in the neotropical poison dart-frogs of the genus *Phyllobates* (Dendrobatidae). Pitohuis attain the poison from their diet and they are resistant to the toxin. The toxin protects the birds from ectoparasites and more importantly from predation, by deterring the potential predators (Dumbacher *et al.* 1992; Wrangham 1992; Glendinning 1993; Dumbacher *et al.* 2009). Unlike their sister taxa, four subspecies of hooded pitohui (*P. dichrous*) do not release the poisonous secretion described above. However, they mimic their sister taxa not only in colouration, but also in the sour body smell the birds effuse (Dumbacher *et al*. 1992).

A different approach to predator defence is exhibited by migrating sandpipers (Scolopacidae). On their way to their breeding grounds, they replace the lighter and more volatile monoesters with heavier and less volatile diesters. Birds have already paired up in their home grounds, and it is likely that the monoesters served as a signal of quality during mate choice. Nesting on the ground though, it is likely that the birds shifted to a less detectable volatile as a means of predator avoidance. Indeed, dogs were not able to pick up the scent of the nests, indicating that the sandpiper's change in odour is deemed to camouflage the nest's odour (Reneerkens *et al.* 2002; Reneerkens *et al.* 2005).

1.7 Chemotaxonomy

The chemical composition of the uropygial gland secretion has a species' specific signature and can be used as a taxonomic tool. This relationship has been demonstrated by J. Jacob for a range of species, such as corvids (Jacob & Grimmer 1973), woodpeckers (Jacob & Poltz 1974b), owls (Jacob & Poltz 1974a), geese (Jacob & Glaser 1975), tinamous and kiwis (Jacob & Hörschelmann 1985) as well as for some passerines (Jacob & Grimmer 1975) and the budgerigar (Jacob & Poltz 1974c). For example, based on behavioural and vocal characteristics, the Hume's ground jay (*Pseudopodoces humilis*) was placed in the parids, however, genetic analysis as well as the gas chromatographic analysis of the uropygial gland secretions classified the birds clearly as corvids (Gebauer *et al.* 2004).

1.8 Detection of scent: the olfactory region of the brain

Olfactory information is processed by the olfactory bulb, which is located in the frontal lobe of the brain (Andres 2008). The olfactory bulb receives volatile information and transforms it into a neural response (Crosby & Schnitzlein 1982). Some early anatomists noticed the large differences in size of the olfactory bulb in birds (Bang 1960). Analysing the relative size of the olfactory bulb was long ago used to classify and assess whether birds possess olfactory abilities (Edinger 1903; Hill 1905; Strong 1911). Comparative studies of the size of the olfactory bulb have been conducted by Strong (1911) and later more comprehensively by Bang and Cobb (1968), who compiled a comprehensive list of 108 birds and the relative size of their olfactory bulbs. Birds with comparatively larger olfactory bulbs were assumed to have a more acute sense of smell (Bang & Cobb 1968). While the functional organisation of the olfactory bulb is conserved throughout all vertebrates (Rose 1914; Andres 2008; Su *et al.* 2009), Nieuwenhuys (1966) divided the main avian olfactory bulb into seven layers, which neurophysiologically analyse the input from the olfactory epithelium and correspond to the functionality and organisation of the layers found in vertebrates. In particular, the number of mitral cells in the mitral cell layer, which builds the port between incoming and outgoing neural signals and projects them to higher brain areas (Nickell & Shipley 1992), indicates the importance of olfaction in an organism (Wenzel & Meisami 1987). Spatial and temporal differences in the number of mitral cells occur, and it has been shown that their numbers can change according to the exposure to and use of scent, as well as with age. The higher the exposure, the greater the number of mitral cells, but this diminishes with age (Hinds & McNelly 1977; Meisami & Noushinfar 1986; Bhatnagar *et al.* 1987).

1.9 Production of scent

As diverse as scent signals are, so are the ways in which they are produced and dispersed. Olfactory signals are distributed through faeces (e.g. ducks and hoopoes) (Swennen 1968; Clark & Wobeser 1997; Jones & Roper 1997), via stomach oils with potential deterring modalities (e.g. seabirds) (Rosenheim & Webster 1927), and through a range of scent producing glands (e.g. uropygial gland, anal glands and sebaceous glands) (Jacob & Ziswiler 1982; Menon & Menon 2000; Rajchard 2010).
Body-odour has frequently been associated with feather preening, where birds spread uropygial gland secretions over the plumage during preening that conditions and waterproofs their feathers (Giraudeau *et al.* 2010). Uropygial gland secretions may help waterproof the feathers due to their hydrophobic qualities. In addition, antibacterial properties of the uropygial gland secretions and / or the action of other bacteria present in the preen glands may act against feather-degrading bacteria (Shawkey *et al.* 2003; Ruiz-Rodriguez *et al.* 2009). Finally, symbiotic bacteria that are housed either

in the uropygial gland or on the feathers have also been suggested to produce or alter the bird's body odour (Shawkey *et al*. 2003).

The many examples presented above show that olfactory signals are produced and used in many different ways in birds. They are used in a vast range of social and behavioural scenarios. Nevertheless, our knowledge of the role and importance of scent and scenting is limited. The opportunity arose to study the olfactory behaviour in a rare parrot, the kakapo. Despite the kakapo's endangered status it is an ideal model for studying olfaction, because all individuals are monitored very closely by New Zealand's Department of Conservation. Hence, for each individual, extensive life history data are available and the birds are handled on a regular basis for health checks and transmitter changes. These circumstances enabled me to receive feather samples from a range of individuals of different age and sex and at different seasons.

1.10 The Kakapo: a parrot unlike any other

The kakapo is a New Zealand endemic parrot and is the heaviest parrot worldwide; it cannot fly and is nocturnal. It is the only lek-breeding parrot worldwide and while males perform conspicuous booming calls and flamboyant dances at close quarters, females come from as far as seven kilometres to choose a mate (Ballance 2010). Both sexes have a strong, sweet smell, which has been described in numerous accounts. A poem by Sonja Yelich even paid tribute to the unusual smell of kakapo: "The kakapo is the smell of some honey, papaya and the inside of an old clarinet case which is spitty. Have a go kakapo, sniff yourself" (Priestley 2008). Maori hunters, for example, knew that kakapo had to be hunted from 'down-wind' suggesting that their sense of smell is acute (Best 1925; Butler 1989; Tipa 2006). Hagelin (2004) described kakapo using olfaction in their search for food. She also measured the external size of an olfactory bulb following the method of Bang and Cobb (1968). She found it to be nearly as large as in kiwi, another endemic, nocturnal and flightless bird that has been reported to use smell extensively (Wenzel 1968; Hagelin 2004; Castro *et al*. 2010). In spite of all these accounts, the role of olfaction in kakapo and the chemical composition of its unique smell have never been looked at in detail.

1.11 History, taxonomy, appearance, flightlessness and nocturnality of kakapo

Kakapo belong to the world's oldest parrot family (Wright *et al.* 2008). Much controversy has occurred over the years as whether to place the genera *Strigops* (*S. habroptilus*) and *Nestor* (kea, *N. meridionalis* and kaka, *N. notabilis)* independently from each other or to unite them within the fam-

ily of Nestoridae. New molecular data and analyses, though, place the two subfamilies Strigopinae and Nestorinae in a single sister clade. Hence, within the avian order Psittaciformes *the* kakapo is considered to have formed alongsid*e* the two sister subfamilies kea and kaka*. T*ogether these species form a distinct parrot family: the Nestoridae (De Kloet & De Kloet 2005; Astuti *et al.* 2006; Tokita *et al.* 2007; Wright *et al*. 2008; Gill *et al.* 2010; Mayr 2010). This family is likely to have diverged from that of all other parrots during the Cretaceous some 80 million years ago, coinciding with New Zealand's geographical separation and isolation from Gondwanaland.

Understanding the geological history of New Zealand is important since it has contributed to a unique fauna. Isolated from the rest of the world for more than 80 million years and in the absence of mammalian predators, New Zealand's avifauna is characterised by having more flightless birds than any other place in the world (Wilson 2004). Many New Zealand birds distinguish themselves through peculiar mammal-like habits. Each bird for example filled ecological niches typically taken by mammals elsewhere: moa and kakapo as 'forest browsers', takahe as 'grass eaters', kiwi, as the `honorary mammal' and wrens as ground 'insect eaters'. Hence, it is not surprising that New Zealand sets itself apart by having a bird fauna with a high level of endemism'. 133 birds species occur only in New Zealand, with 87% of all terrestrial birds and 44% of all breeding seabirds being endemic (Wilson 2004). Many New Zealand birds are remarkable for having a number of unique characteristics. Gigantism (or dwarfism as in kiwi (Apterygidae)) is one of them; a feature observed in many island habitats, where constraints related to predation and / or competition, for instance, are removed (Lomolino 2005). Kakapo are a clear example of gigantism and are the largest parrot species in the world.

Although their dimorphism is not overtly obvious, female kakapo are smaller with an adult bodyweight of 2-2.5 kg compared to males which can reach 3.5 to 4 kg (Elliott *et al.* 2001; Eason *et al.* 2006). Female's heads are slimmer and proportionally longer than males. The female's beak is slightly narrower than the male's, and they have smaller nares. The female's plumage is moss green with slightly brownish and yellowish spots, giving them a vaguely more yellowish colouration than males. Their feet are also slender compared to males and they have a comparatively longer tail (Higgins 1999). The most reliable way to physically sex birds regardless of their age is through the colouration of the four outermost primary feathers on their wings. While in males the lower side of the four outermost primary feathers is mottled with a vague water-mark pattern to the tips, in females there are no such marks (Powlesland *et al.* 2006). Both sexes have a facial disc orientated like owls, which earned them the moniker "owl parrot". Their cryptically coloured plumage perfectly

merges with the foliage so that they are hard to spot. Kakapo remain motionless to avoid being detected when approached (Morris 1977; Powlesland *et al*. 2006). Due to the long absence of mammalian predators many New Zealand bird species, including kakapo, have not developed a typical fear response (Brown *et al.* 1999, Laundré *et al.* 2010), but instead freeze when exposed to a potential danger (Morris 1977; Whitwell 2009).

Even though kakapo are well camouflaged, they are considered to have become nocturnal due to predation from the air (Williams 1956; Wilson 2004). This idea is supported by the plumage colouration of the closely related kea, known for its bright, orange red underwings, which are only visible from below when the wings are extended, while from the top this bird is perfectly well camouflaged. Historically avian predators would have included the Haast eagle (*Harpagornis moorei*) and the Eyles harrier (*Circus eylesi*) (Holdaway & Worthy 1997), both of which are extinct today. It can be assumed that nocturnality in kakapo appeared after the split from kea and kaka, which both are diurnal but can also be active at night (pers. comm. R. Hitchmough and D. Brunton).

Kakapo still possess large wings but they cannot fly and Douglas Adams & Carwardine (1990) noted: "that not only has the kakapo forgotten how to fly, but it has also forgotten that it has forgotten how to fly", denoting the fact that birds climb trees and attempt to flap to the ground. Flightlessness is a feature kakapo share with more than half of New Zealand's terrestrial avifauna (extinct and extant species). Flightlessness evolved several times independently among different lineages (Cooper *et al.* 1992). While the ratites evolved from flightless ancestors (Cooper *et al*. 1992), all other flightless birds have lost their ability to fly after their ancestors colonised New Zealand (Wilson 2004). Due to the lack of mammalian predators many birds in New Zealand could afford to lose the power of flight (McNab 1994). For kakapo the loss of flight enabled this species to develop a larger body mass, which in thermodynamic terms is far more cost-effective. A large body size is also associated with a herbivorous diet and the need to spend more time foraging in order to meet their nutritional needs (Raubenheimer & Simpson 1999 and 2006).

Fossil fevidence and early observations of kakapo suggest they were abundant on the three main islands of New Zealand and inhabited a variety of vegetation types from sea level to alpine regions. They were considered most common in rimu *(Dacrydium cupressinum)* and beech *(Nothofagus* sp.*)* forests in areas of high rainfall (Worthy & Holdaway 2002; Wood 2006). Kakapo were not exclusively forest-dwelling since historic accounts often associate kakapo with grasslands (Henry 1903; Worthy

& Holdaway 2002). Kakapo are currently confined to a few offshore islands and the species has only survived due to enormous conservation efforts.

Kakapo are exclusively herbivorous and feed on bark, twigs, leaves, seedlings, nectar, fruit, ferns, rhizomes and fungi (Haast 1864; Potts 1873; Henry 1895; Pascoe 1957; Best 1984; Butler 2006). Like all parrots, kakapo use their feet to hold food. Their strong feet are also used to excavate subterranean and nutritious bulbs, tubers, corms and rhizomes (Best 1984). Females, due to their smaller size and lighter weight, are more agile and thus are thought to benefit by reaching the outer branches of trees that hold most of the fruit (Henry 1903; Powlesland *et al*. 2006). Using their powerful beak and short but strong tongue, kakapo generally squeeze vegetable material against the ridged upper palate allowing the plant juices to be extracted. All other food is finely ground with the help of the lower mandible and large, keratinised regions on the tongue. Unlike kea and kaka, the kakapo's tongue is not adapted for collecting nectar (Kirk *et al.* 1993).

Kakapo are solitary year round and occupy large home ranges (Reischek 1890). Studies on home range size in kakapo have found considerable variation (Burt 1943; Best 1984). Male home range size varies between 26 and 50 ha and can include their 'track and bowl' mate attraction system. Females have home ranges approximately 10 - 20 ha larger than those of males. Female home ranges are located between male ranges but in general some distance from the 'track and bowl' systems (Moorhouse 1985; Cockrem 1989; Farrimond *et al.* 2006a). There is a correlation between breeding success and the size and quality of female home ranges; additionally, quality home ranges tend to include abundant rimu forest (Whitehead 2007; Whitehead *et al.* 2012).

1.12 Lek-Breeding

Lek mating strategies are characterised by a number of unique properties. Males display on a long-established arena that has no obvious resources to offer to females except for the males themselves. The arena represents only a small fraction of the habitat used by females. Females visit different display arenas and choose a male with which to mate (Bradbury 1981). Males contribute no parental care. Given the absence of paternal care, females should choose a male that guarantees them either sons that are more attractive to future females or offspring that have higher survival rates (Avery 1984). Quantifying these benefits is often difficult because many environmental and inherent parameters can contribute to the fitness of an individual. An often cited study by Partridge (1980) found that female fruit flies can influence the fitness of their offspring by their choice of a mate.

In lek breeders da number of traits have been shown to signal that a male is a 'good' choice of mate. Signals such as colourful plumage, long tail feathers and complex displays, all of which are costly to produce may be 'honest' signals of male quality. Males of the lekking great snipe (*Gallinago media*), whose display arenas were in a more central location, and who displayed more intensely for example, gained more matings (Höglund & Lundberg 1987). *Escherichia coli* infected males in the lekking houbara bustard (*Chlamydotis undulata undulata*) have reduced courtship displays and low semen quality compared to non-infected control birds (Chargé *et al.* 2010). Therefore, the male's phenotypic traits can inform females about the genetic quality of their bearers. In the lekking fallow deer (*Dama dama*), for example, antler size as well as vocalisations and scent marking reflect the social status and dominance of the male and therefore act as an honest signal of phenotypic quality (Ciuti & Apollonio 2011).

Lek breeding is a relatively rare mating strategy in mammals and birds (Avery 1984) and the kakapo is the only lek breeding parrot in the world. Male kakapo exhibit an 'exploded' lek system with widely distributed leks, comprised of a series of shallow burrows. Males excavate one or more shallow bowls of 30 to 60 cm diameter, which are interconnected with 30 to 60 cm wide tracks. Bowl systems can spread over 50 m, and track systems between neighbouring males are 15 m to several hundreds of metres apart (Merton *et al.* 1984). Bowls are preferably excavated against overhanging banks, rock faces or trees, as these create a sound wall to reflect their low frequency booming calls (Merton *et al*. 1984). These bowls or display ''arenas'' are vigorously defended and males can become very aggressive towards potential competitors. They meticulously clear their arena of twigs and debris while standing in the middle of their bowl and bite off any vegetation within reach (Reischek 1890; Westerskov 1981; Powlesland 1992). They also clip any vegetation bordering the tracks. Placing twigs into the bowl is therefore a good method to test whether a breeding bowl is currently in use.

In breeding years, males continuously call from nightfall until the early hours of the morning and display with flamboyant dances that involve flapping their wings and performing characteristic side to side rocking movements (Merton *et al*. 1984). The low frequency calls (<100 Hz) occur in sequences of 20-50 calls at a time, each in one to two second intervals, and can be heard from distances of up to five km (Merton *et al*. 1984). At close range, distances up to 50 m, the calls can only be felt as tremors (Merton *et al*. 1984). Some males call up to 8000 times per night (R. Moorhouse pers. comm.). Females come from far afield and closely approach booming males, choosing one or

more to mate with. To date no correlation has been found between booming rates and mate-choice (pers. comm. G. Harper).

Females raise their chicks alone and lay up to three eggs in a burrow made in a hollow tree trunk. The incubation time is 30 days and chicks are altricial when they hatch. They are fed with a nutritious fluid made by the female. Breeding only occurs in years where rimu fruit is abundant, as chicks require this high calcium palatable food for growth and development (Raubenheimer & Simpson 2006). Chicks fledge around 80 days and continue to be fed by their mother for up to six months (Farrimond *et al.* 2006b).

1.13 Conservation, a species at the brink of extinction

New Zealand's avifauna is a striking feature of its biodiversity; it represents the evolution of unique species over a long period of isolation without mammalian predators. New Zealand's bird life has dramatically declined due to human settlement and the introduction of mammalian predators (Caughley 1989; Holdaway 1989; Verheyden & Jouventin 1994). Introduced mammals, such as, stoats (*Mustela erminea*), weasel (*M. nivalis*) and ferrets (*M. furo*), cats (*Felis catus*), dogs (*Canis lupus familiaris*), three species of rats (*Rattus rattus*; *R. Norvegicus* and *R. exulans*) and mice (*Mus musculus*), largely rely on olfactory cues to find their prey (Hughes *et al.* 2010). A total of 197 native terrestrial, coastal and oceanic bird species have been identified in New Zealand. Forty-nine terrestrial birds (47%) have become extinct since human colonisation and in particular since the introduction of predatory mammals 700 years ago (Elliott *et al*. 2001; Wilson 2004). Kakapo was abundant across the three largest islands of New Zealand. Its numbers have rapidly and catastrophically declined as a result of land management and the introduction of mammalian species (Ballance 2010). The population dropped as low as 50 birds, and this triggered the formation of the National Kakapo Team in 1995 and the implementation of a Kakapo Recovery Plan with the aim to systematically increase kakapo numbers (Clout 2006). Since then the species has been successfully managed and the population increased (Merton 2006; Ballance 2010). Today (2012), the kakapo population has reached 124 individuals.

1.14 A perfume like no other and how an idea became a thesis

The strong smell of the kakapo has likely been a key element by which introduced mammalian predators have located the birds; scent has contributed to this species' dramatic decline. Nonethe-

less, the kakapo's strong odour is likely to play a significant role in social interactions, providing complex and critical information in a behavioural context.

To find out how scent can work as a signal it is necessary to quantify the olfactory abilities of a bird and the structures associated with olfactory detection and production. A unique opportunity to examine the brain of a kakapo, an approximately 100 year old male (Lee), enabled me to perform an in-depth analysis of the brain and olfactory bulb to understand olfactory detection in the kakapo. The strong smell of the kakapo appears to be primarily associated with its body odour. Hence, to address the question of scent production, I examined the chemical composition of kakapo feather scent using gas chromatography-mass spectrometry. Sampling feathers was the least invasive method to study olfaction in kakapo. Uncontaminated feather samples could be taken while birds underwent their half yearly health checks and transmitter changes. Although kakapo faecal samples also have a distinctive odour (Powlesland 1988; Cockrem 1989), using scats for the analysis was logistically not possible given the uncertainty of their collection and the many environmental variables that could alter and affect the scent. This study aims to contribute to the growing field of olfaction in birds and more importantly to provide insights into the behaviour of kakapo; findings that may prove useful to conservation managers and decision makers involved in conserving this enigmatic species.

1.15 Thesis outline and structure

The thesis included the following approaches to assess kakapo olfactory abilities:

1) Gas chromatographic analysis of the feather odour in kakapo of different sex and age groups and during different seasons.
2) A comparison of selected sections through the brains of the nocturnal kakapo (*Strigops habroptilus*) and the diurnal sulphur-crested cockatoo (*Cacatua galerita*).
3) Anatomy and histology of the olfactory bulb of the kakapo (*Strigops habroptilus*) in comparison to other Australasian parrots.
4) Assessment of the visual system in the kakapo (*Strigops habroptilus*) in comparison with other birds.
5) Behavioural assessment of the olfactory abilities of the kakapo's closest relatives, the kea (*Nestor notabilis*) and the kaka (*N. meridionalis*).

Chapter Two examines the quality of emitted signals and includes results on the chemical composition of kakapo feather volatiles and how that varies with age, sex and season. The next three chap-

ters examine the brain of the kakapo and compare it with other Australasian parrots in order to assess the importance of olfaction by examining the olfactory bulb and contrasting it with the visual system. Specificially, ***Chapter Three*** assesses the overall differences of the nocturnal kakapo's brain anatomy in comparison to a diurnal and phylogenetically different parrot, the sulphur crested cockatoo. ***Chapter Four*** examines the olfactory bulb of the kakapo and contrasts it with nine Australasian parrots to assess the speciality of the kakapos' olfactory bulb. Next, ***Chapter Five*** compares the visual system of kakapo with other diurnal and nocturnal birds and discusses the importance of vision in the nocturnal kakapo***.*** This chapter has been published in part in PLoS One. The results presented in Chapter Four however, examine a different set of species and therefore the chapter presented here is a greatly modified version of the published work. ***Chapter Six*** is dedicated to the olfactory abilities of the kakapo's closest relatives, the kea and kaka and has been published in IBIS. ***Chapter Seven*** synthesises all of the findings and discusses the nature of kakapo olfaction and ideas for future work. Each of the results chapters (Two-Six) are written as independent papers and some repetition inevitably occurs.

CHAPTER TWO

CHAPTER TWO: THE ROLE OF BREEDING CONDITION, SEASON, SEX AND AGE ON THE CHEMISTRY OF KAKAPO (*STRIGOPS HABROPTILUS*) FEATHER SCENT

Rebecca Wu

2.1 Abstract

The kakapo (*Strigops habroptilus*) is known for its sweet, strong smell. Females have been reported by the Kakapo Recovery Team to favour stronger smelling males over weaker smelling individuals, but little is known about the significance of olfactory cues in kakapo. Olfaction is widely distributed in birds, with many roles, such as individual-, nest- and partner-recognition, navigation, sexual advertisement and mate choice. I examined the role of feather scent in kakapo by means of gas chromatography-mass spectrometry analysis. The feathers of 67 individuals of different sex, age and seasons revealed strong differences in their chemical composition. Feather samples of booming males had a significantly greater diversity and individual variation of chemical components compared to feather samples of non-booming males. Feather samples collected from non-booming males during the breeding season also had a significantly greater individual variation of chemical components compared to feather samples of males collected during the non-breeding season. Females, in contrast, maintained high levels of individual variation of chemical compounds on their body odour year round. Greater variation of chemical components present in booming males and the seasonal pattern to male odour is consistent with the use of scent as a sexually intrasexual selected signal. The chemically diverse, year-round odour of females is harder to explain, but may relate to competition for high quality territories or diet differences. Quantification of kakapo odour chemistry and behavioural experiments aiming at understanding the social function of olfaction in kakapo are promising future steps in identifying the role of odour in kakapo behaviour.

2.2 Introduction

The strong and sweet smell of the kakapo is likely to be a key element by which introduced mammalian predators locate these birds (Hughes *et al*. 2010) and has significantly contributed to this species' dramatic decline (Holdaway 1989; Elliott *et al*. 2001). However, the odour may also provide the birds with the ability to communicate information that is of social and behavioural relevance. The idea that kakapo convey information through their body odour seems plausible, since their vision is poor, and their retina bears elements of both diurnal and nocturnal birds (Chapter Four and Corfield *et al*. 2011).

The kakapo is a critically endangered, flightless and nocturnal, lek-breeding parrot, endemic to New Zealand (Merton *et al*. 1984; Powlesland *et al*. 2006; IUCN 2011). It is the largest parrot in the world and an obligate herbivore. Males inherit and compete for display 'bowls' at elevated places. These places serve

to amplify their booming calls. The low frequency calls (<100Hz) occur in sequences of 20-50 calls at a time and can be heard from nightfall until the early hours of the morning (Merton *et al*. 1984). The booming calls can travel as far away as five kilometres (Merton *et al*. 1984). Females approach the males from afar, and are welcomed with complex displays at close quarters. After visiting one or more males the female will choose a male to mate with (Powlesland 1992; Powlesland *et al*. 2006). As in all lek mating systems some males possess beneficial attributes, reflecting superior genetic qualities, and make major contributions to the gene pool of the next generation. Others, who lack these attributes, make little or no contribution (Höglund & Lundberg 1987). It is currently not known why some male kakapo are 'favoured' over others, although it has been observed that favoured males appear to emit a stronger smell than less attractive ones (pers. comm. Kakapo Recovery Team).

Contrary to commonly held beliefs, a growing body of literature shows that environmental and self-produced olfactory cues have a significant function in avian ecology (Roper 1999; Hagelin & Jones 2007), and that birds are capable of both sensing and producing odours. For example, they show strong anatomical structures associated with the production and detection of scent (Bang 1960; Bang & Cobb 1968; Bang 1971). Birds use scent and olfaction in navigation (Benhamou *et al*. 2003b; Benvenuti & Ranvaud 2004), deterrence of potential predators (Burger *et al*. 2004), antimicrobial defence (Hagelin & Jones 2007), nest site recognition (Bonadonna & Bretagnolle 2002; Bonadonna *et al*. 2003a; Bonadonna *et al*. 2003b), partner recognition (Bonadonna & Nevitt 2004), individual recognition (Bonadonna *et al*. 2007) and mate choice (Hagelin *et al*. 2003; Hagelin 2007) (for more information, see Chapter One). Moreover, nocturnal birds, like the kakapo, may require a better sense of smell than day-active birds to compensate for limited vision during the hours of darkness (Healy & Guilford 1990). Kakapo have been shown to actively use olfactory cues to find food (Hagelin 2004). Maori traditions are consistent with the kakapo's sense of smell. Hunters approached birds from downwind, as wary birds would otherwise become alert of the hunter's presence (Butler 1989; Best 2005; Tipa 2006). Additionally, kakapo have been shown to have a large, functionally intact olfactory bulb (Chapter Three and Hagelin 2003), as well as a high number of functionally intact olfactory receptor genes (Steiger *et al.* 2009a). The chemistry of feather scent has been studied in a pilot-project by Drs. Andrew Fidler and Patrick Holland (Cawthron Institute), who detected a range of chemical volatiles using gas chromatography-mass spectrometry (pers. comm. A. Fidler). However, they were not able to detect any significant quantitative or qualitative differences between genders in their small sample (n=14 birds).

Gas chromatography–mass spectrometry (GC-MS) is a widely used analytical method to qualitatively and quantitatively analyse the nature of chemical molecules present in a sample (Grob & Barry 2004). These molecules separate in the chromatography column due to differences in their chemical properties. Different chemicals are retained by the column and then elute at different times (i.e.: retention times). In the second part of the reaction, the mass spectrometer breaks the components into ionised classes and separates these based on their mass to charge ratio (Grob & Barry 2004; McMaster 2008). GC-MS has been used extensively in insect and insect-plant interactions (Kessler & Baldwin 2001) and in determining socially and behaviourally relevant molecules in mammalian behaviour (Lin *et al.* 2005 Goodwin *et al.* 2006; Scordato *et al.* 2007 Charpentier *et al.* 2008). Gas chromatographic analysis has also been widely applied in avian olfaction (Campagna *et al.* 2012). For example, the chemical composition of the uropygial gland secretion has a species-specific signature and can be used as a taxonomic tool. This has not only been demonstrated in the budgerigar (*Melopsittacus undulatus)* by Jacob & Poltz (1974c), for example, but has also found direct application in avian systematic; genetic and gas chromatographically analysis of uropygial gland secretions have been used to reclassify the Hume's ground jay (*Pseudopodoces humilis*) as corvids (Gebauer *et al.* 2004). Furthermore, gas chromatography has been used to determine seasonal shifts in the odour of secretions of the uropygial gland in red knots (*Calidris canutus*) (Piersma et al. 1999; Sinninghe Damsté et al. 2000; Reneerkens et al. 2007), rock doves (*Columba livia*) (Montalti et al. 2005) and a range of passerines (Haribal et al. 2005; Zhang et al. 2009). Gas chromatographic analysis allows refining the search for differences in the qualitative nature of molecules determining sexual and individual differences of uropygial and preen gland secretions (e.g. Antarctic seabirds, Bonadonna *et al.* 2003; Bonadonna & Nevitt 2004; Bonadonna *et al.* 2007; spotless starlings (*Sturnus unicolor*, Amo *et al.* 2012; chickens *Gallus gallus* Karlsson *et al.* 2010, and budgerigars (Zhang *et al.* 2010). Female budgerigars were able to distinguish between the sexes and a bioassay test was performed using synthetic mixtures of uropygial gland secretions. Zhang *et al.* were able to show that specific alkanols created a female attractant. However, the work has been critiqued by Mardon *et al.* (2011), because their choice of chemical components for the bioassay was unfounded.

Here I examine the body odour of 67 kakapo from Codfish Island, one of two remaining populations on New Zealand and half of the current population of 124 birds. My goals were to examine feather chromatographic signatures for evidence of sexual, age related and seasonal differences in the chemical diversity between male and female birds year round. Despite its endangered status, the kakapo is an excellent model to study bird olfaction because the population, including their reproductive status and

movement of all individuals, is closely monitored by staff and volunteers of the Department of Conservation, New Zealand. Insight into the functions of the kakapo's strong smell should help conservation managers and decision makers in their effort to save this enigmatic species.

2.3 Methods

2.3.1 Permit

The permit to hold and analyse kakapo feathers was issued through the Department of Conservation (Te Papa Atawhai) and the Te Rūnanga o Ngāi Tahu; permit number: NM-21496-DOA

2.3.2 *Sampling of feathers*

Feathers from around the neck region were collected by members of the Kakapo Recovery Team on Codfish Island between 2007 and 2010. Birds are caught for health screening and transmitter change twice a year, offering a good opportunity to sample feathers in the least biologically disruptive way. Gloves were worn during collection to ensure samples were not contaminated. Feather samples were cut with scissors, which were cleaned with ethanol between sample bouts. Feathers were then individually placed into a 10 ml amber headspace vial (Agilent Technologies, Germany), closed with a PTFE silicone cap (Part No. 5188-2759, Agilent, USA) and stored in a -80 °C freezer until they were analysed. Feather samples collected by Dr. J. Hagelin in 2006, and stored as indicated above, complemented the set.

The feather set used in the feather scent analysis consisted of 67 feather samples (n = 50 males, n = 17 females). There were also 16 replications, i.e. birds that have been sampled multiple times (n = 14 males, n = 2 females), but in different seasons and years (see Table 2.1 for details and Table G1 in Appendix G, for a detailed list of all birds used). The analysis took place at Auckland University and was conducted by Dr. D. Greenwood and Dr. Dung Nguyen (Centre for Genomics & Proteomics, School of Biological Sciences, Auckland University, Auckland, New Zealand) using headspace chromatography-mass spectrometry (GC-MS).

2.3.3 GC-MS analysis of feather sets

The GC-MS system consisted of an Agilent 6890 Gas Chromatograph coupled with a 5975C inert Mass Spectrometry Detector (Santa Clara, CA, USA) and a CTC CombiPAL Autosampler (CTC analytics AG,

Zwingen, Switzerland). Separation of the analytes was performed on a Zebron ZB-1701 GC-capillary column (30 m × 250 µm (id) × 0.15 µm (film thickness) with 5 m guard column, Phenomenex). Helium was used as carrier gas at a constant flow rate of 1 ml/minute. The headspace vial was placed on a CTC CombiPAL sampler tray before being transferred to a CTC Agitator for incubation at 70 °C for 5 minutes before injection. After that, 1 ml of the headspace was taken by SPE syringe: 2.5 ml SYR H LT CTC (Part number NS620023; Target Brand, HPLC & GC Glass Syringes for Chromatography, Automated and manual precision injections, Rockwood, TN 37854, www.nationalscientific.com) set at 80 °C, and was transferred to the GC injection port for injection under pulse splitless mode and separation of analytes. The oven temperature program was initially set up at 40 °C and held for 5 minutes, then raised to 150 °C at 10 °C/minute, followed by an increase at 20 °C/minute to 240 °C and held for 6 minutes. The mass spectrometer operated in electron impact (EI) mode at 70 eV. The temperature of the transfer line was set at 250 °C. The temperatures of the MS source and quadrupole were 230 °C and 150 °C, respectively.

2.3.4 Data analysis

The data were directly imported from the Agilent 6890 Gas Chromatograph as Agilent .dat files (Table C1, Appendix C). The area + RT times and peak heights were copied into an Excel file and alignments were made (i.e. manually). Only peaks and retention times that had a height above 700 nanoAmps were included into the analysis. An example of the evaluation of peaks is discussed in Appendix C, Figures C1-3. Therefore, data were based on the retention times of the chemical components (the characteristic time a particular chemical constitute needs to pass through the system (from the column inlet to the detector) under set conditions (Sparkman *et al.* 2011)).

All 67 birds were included into the analysis, since replicate samples were from different seasons and/or years. Data were standardised according to internal inbuilt control standards and different exploratory tools, such as Principal coordinate analysis (PCO) (see 2.3.5), tests of homogeneity of dispersion (PERMDISP) (see 2.3.6), permutational ANOVA and MANOVA; PERMANOVA (see 2.3.7), and canonical analysis of principal coordinate (CAP) (see 2.3.8) were applied to analyse the chemical composition of feather odour volatile phenotypes according to age, sex and breeding season. All statistical tests were conducted using the PRIMER v6 (*PRIMER-E Ltd, Lutton, UK*) computer program (Clarke & Gorley 2006) with the PERMANOVA+ add-on package (Anderson *et al.* 2008). All graphs were done using SigmaPlot12 (Systat Sofware Inc. San José, CA, US).

Table 2.1 Birds divided by categories used in the feather scent analysis.

		Traits	Number of feather samples
		Total	**67**
General traits		adults	61
		Juveniles	6
		Males	50
		Females	17
		adult males	47
		adult females	14
		juvenile males	3
		juvenile females	3
Breeding	season	breeding season	45
		non-breeding breeding season	22
Booming	status	booming males	26
		non-booming males	10
		male booming status unknown	11
Sexual activity		sexually active males (feathers collected during the breeding season)	41
		sexually inactive males (feathers collected in the non-breeding season)	6
		sexually active females (feathers collected during the breeding season)	14
		sexual inactive females (feathers collected in the non-breeding season)	10
replicates		replicate males	14
		replicate females	2

2.3.5 Principal coordinate analysis (PCO)

Principal coordinate analysis (PCO) is a multidimensional scaling method, based on a (symmetric) resemblance matrix (Gower 1966). All samples are projected onto Euclidean axes using a matrix of interpoint dissimilarities, but in the space of the dissimilarity measure chosen (Anderson *et al*. 2008). For all calculations, I chose the Bray Curtis resemblance matrix as it has a range of desirable criteria: for example, its integrity against the inclusion or exclusion of a chemical component that is absent in any two birds, a condition that many other coefficients do not share (Anderson *et al*. 2008). The PCO allows to ordinate the chemical volatiles (based on the retention times obtained by the gas chromatography-mass spectrometry) found in the feather scent alongside the Euclidean space and to reduce the dimensionality of the data to observable patterns and structures according to different parameters (sex/age/season). A PCO was produced to compare booming versus non-booming birds and for sex and age related effects during the breeding and non-breeding season. A PCO of feather scent from samples of four individuals collected during the breeding and the non-breeding season allowed the investigation of individual changes in the chemical composition of feather odour between seasons.

2.3.6 Test of homogeneity of dispersion (PERMDISP)

I also tested the data for homogeneity of multivariate dispersions (dispersion effect) within groups using (PERMDISP) (Anderson 2006). The test compares the within-group multivariate distances from the observations, in this case the chemical volatiles in the multidimensional room, to their group specific centroid. This allows conclusions to be drawn about the variation or spread of the chemical components that are present in a group (such as breeding males versus non-breeding males). A greater variability, however, can be caused by differences in diversity (the number of components), or it could also be caused by differences in the identity of the components. The calculations are done on the basis of any distance or dissimilarity measure, such as Bray Curtis and using permutations.

2.3.7 Permutational ANOVA and MANOVA: PERMANOVA

Due to the visual separation between sex, age and season related effects in the PCO, the extent to which the 'Location' (location effect) of the chemical composition in feather samples differed between different groups was analysed using PERMANOVA (Anderson 2001). Location is about where the group of sample points is centred. If both groups have the same centre, then (on average) their compositions are not different (even though one group might be more variable in composition than another, and therefore show a dispersion effect). If, on the other hand, the centres of the groups are in different places (in

the multivariate space – so different locations), then this means that the chemical compositions (on average) differ from one another. Therefore, PERMDISP tests for differences in the variation (dispersion or spread) of the two groups. PERMANOVA tests for differences in the average condition (centre or location) of the two groups. Instead of F values PERMANOVA usws Pseudo F values because the F ratio does not have a known distribution under a true null hypothesis.

2.3.8 Canonical analysis of principal coordinates (CAP)

Canonical analysis of principal coordinates (CAP) is used to find axes through the multivariate cloud of (in this case) chemical components to discriminate between groups that were not obvious in the PCO. But it can also help to predict group membership and allocate new data points, based on their resemblance with earlier observations to already existing groups (Anderson & Robinson 2003; Anderson & Willis 2003). This feature was used to assign males whose booming status was not known to groups of booming males or non-booming males (see 2.4.7).

2.3.9 Procedure

Data were tested for normality and a Bray Curtis resemblance matrix was fitted. Subsequently, data were logtransformed and analysed for homogeneity of multivariate dispersion (PERMDISP) to test the average dissimilarities of the chemical components from the central location of their group (Anderson *et al*. 2008). This test was followed by a non-parametric multivariate analysis of variance (PERMANOVA) to test whether the groups, i.e. clouds of chemical components, differed in their location (Anderson *et al*. 2008). The number of permutations used for both calculations was 9999. Differences between the feather odour of booming and non-booming males were evaluated. Feather samples collected during the breeding season (November to March) and the non-breeding season (April to October) were tested for sexual differences between the chemical components of feather odour in males (regardless of their booming status) and females. Additionally feather samples across and between adult males and females during the breeding and non-breeding season were compared. Finally, age related differences in the body odour across and between juvenile birds (up to six years old) and adult birds (older than six years) were compared with each other. Note that the interpretation of PERMANOVA is subjective when PERMDISP is significant, in that the differences could occur due to differences in dispersion and possibly location as well. In such cases, the visual output of the principal coordinate analysis is particularly helpful (Anderson *et al*. 2008) in interpreting the result.

2.4 Results

Chromatograms from the Agilent 6890 Gas Chromatograph showed strong individual, sexual and seasonal differences (Figure 2.1).

2.4.1 Chromatograms

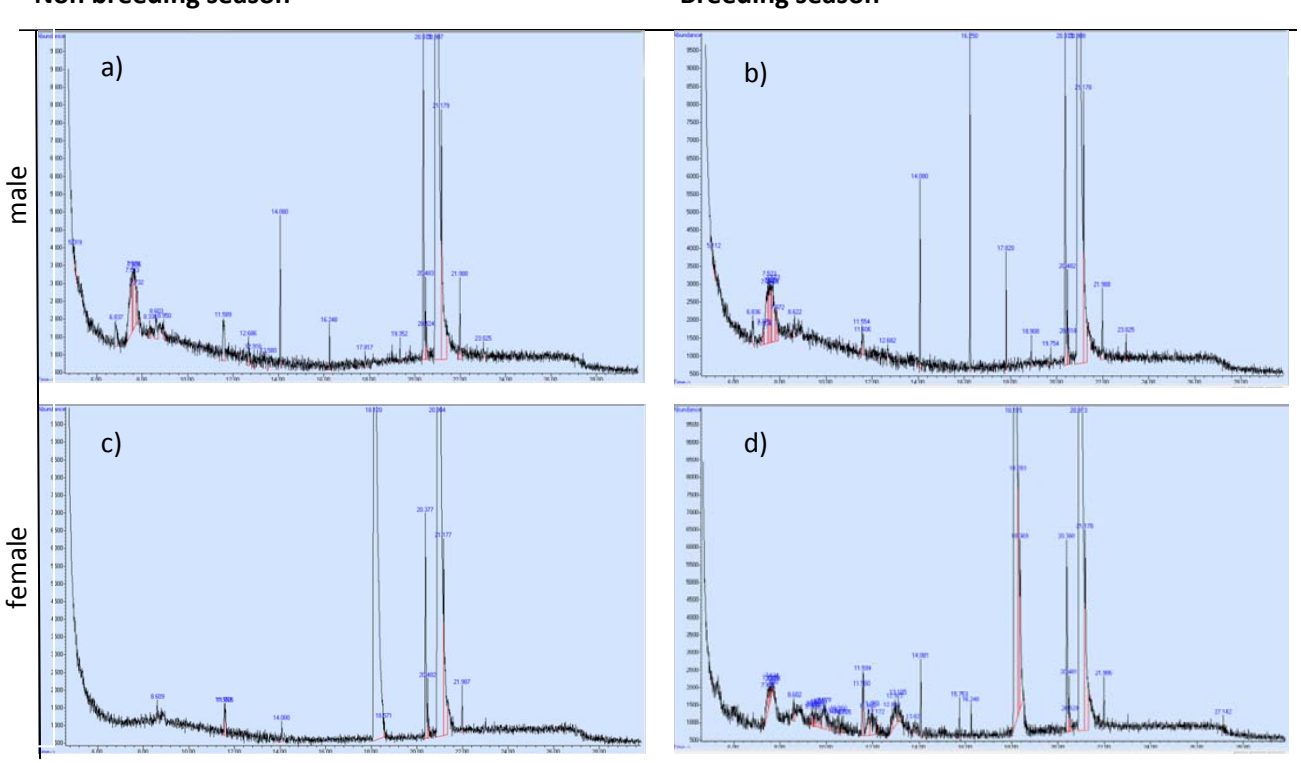


Figure 2.1 Chromatograms of a male and a female kakapo during the breeding and non-breeding season: a) Male kakapo 'Blade' during the non-breeding season; b) 'Blade' during the breeding season; c) female kakapo 'Flossie' during the non-breeding season; and d) 'Flossie' during the breeding season. Runtime (which is a reference to the Retention Time) of the experiment is indicated on the x-axis, and the Abundance is represented on the y-axis. The chromatograms during the breeding season show a higher number of sharp peaks compared to chromatograms during the non-breeding season. An assembly of all chromatograms can be found in the accompanying CD, Appendix H, CD1.

2.4.2 Same sex comparisons

2.4.2.1 Booming versus non-booming males

Comparisons between feather samples from booming males and non-booming males collected during the breeding season (between November and March) showed significant variability in their chemical composition and, taking into account the PCO (Figure 2.2), (on average) in their chemical composition (Table 2.2). Males whose booming status was not known were not included in this analysis. A representation with individual measurements between booming and non-booming males is presented in Appendix D, Figure D1.

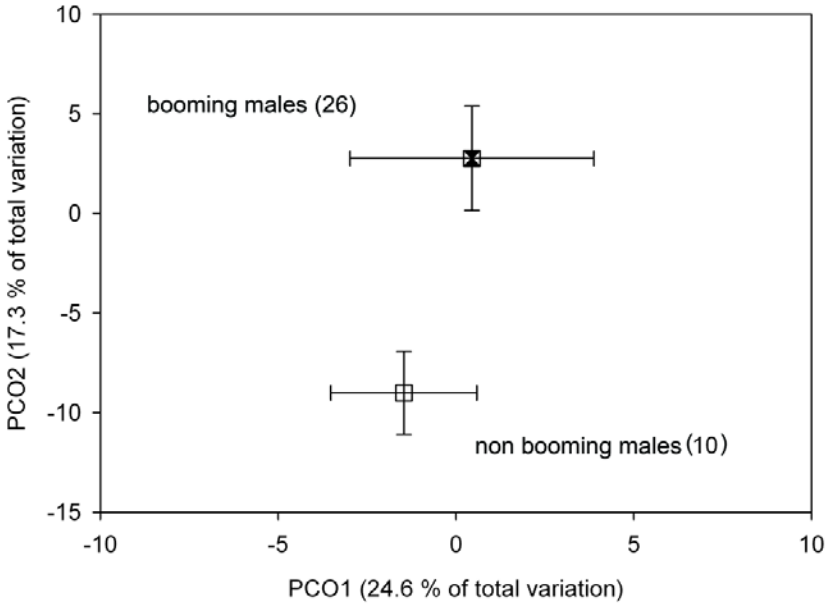


Figure 2.2 Principal coordinate analysis (PCO) of the chemical composition of feather scent from booming versus non-booming males. Feather samples were collected during the breeding season (November to March). Numbers of individuals are indicated in brackets and standard error bars indicate the significantly larger dispersion of chemical components in the feather scent of booming males. The small sample size of non-booming males contributes to exceedingly large error bars.

2.4.2.2 Males – breeding season versus non breeding season

The chemical composition of the odour of feather samples from adult males collected during the breeding season (regardless of their booming status) showed significant differences in the dispersion or variability of chemical components compared to adult males during the non-breeding season. Apart from the strong dispersion effect, there might also be a location effect, as the two groups significantly dif-

fered in their multivariate location from feather samples collected from males during the breeding and non-breeding season, indicating a significantly different composition of chemical components in the two groups (Figure 2.3a and Table 2.2).

2.4.2.3 Females – breeding season versus non breeding season

In females, the chemical composition of the feather odour from samples collected during the breeding season did not significantly differ from feather samples collected outside the breeding season in the multivariate dispersion of the chemical components among two groups (during breeding season versus non-breeding season). However, the two groups did significantly differ from each other in their multivariate location, indicating that females during the breeding season have a significantly different composition of chemical components in their feather scent compared to females in the non-breeding season. (Figure 2.3b and Table 2.2).

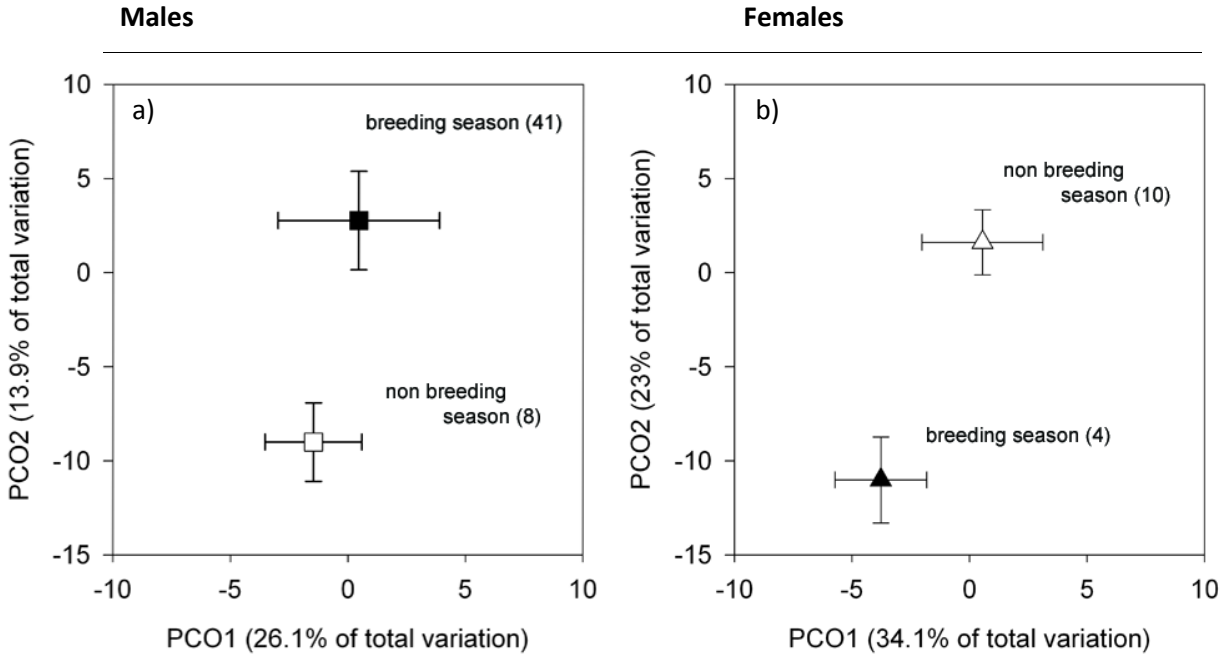


Figure 2.3 Principal coordinate analysis (PCO) of the chemical composition of feathers scent from a) males and b) females during the breeding and non-breeding season. (Number of individuals are in brackets). Small sample sizes contribute to comparatively large standard error bars in males during the non-breeding season. Individual representations of measurements are presented in Appendix E, Figure E1A&B.

Tabel 2.2 Summary of: comparisons within sex and groups. Sample size (N); mean and standard error (SE) for each group comparison; Measures: dispersion effect (testing data for homogeneity of multivariate dispersions (PERMDISP)) and location effect (analysing, where the location of group of sample points is centred (PREMANOVA)); F values: F; Pseudo F values (under PERMANOVA the F ratio does not have a known distribution under a true null hypothesis) and P values.

Comparisons	**Sex**	**Measure**	**N**	**Mean ± SE**	**df**	**F**	**Pseudo F**	**P value: <**
Within sex comparisons								
booming versus	males	**dispersion**	26	45.69 ± 2.75	1,34	21.15		**0.0004**
non-booming males			10	23.84 ± 2.62				
		location			1,34		2.47	**0.021**
breeding versus		**dispersion**	41	42.62 ± 2.42	1,20	10.09		**0.007**
non-breeding season			6	22.08 ± 2.93				
		location			1,45		2.02	**0.05**
	females	dispersion	4	46.86 ± 6.96	1,12	3.19		0.19
			10	32.25 ± 4.35				
		location			1,12		3.58	**0.01**
Group comparisons								
breeding season	males	dispersion	41	42.61 ± 2.42	1,43	0.28		0.65
	females		4	46.86 ± 6.96				
		location			1,43		2.47	**0.024**
non-breeding season	males	dispersion	6	22.08 ± 2.93	1,14	2.77		0.26
	females		10	32.26 ± 4.35				
		location			1,14		0.78	0.63
breeding season	juveniles	dispersion	6	43.03 ± 4.74	6,45	5.22		0.06
	adults		45	44.30 ± 2.30				
		location			1,49		3.10	**0.009**
non-breeding season		dispersion	6	43.03 ± 4.74	1,20	5.23		0.06
			16	29.15 ± 3.26				
		location			1,20		6.99	**0.0007**

2.4.3 Group comparisons

2.4.3.1 Sexual differences within and outside the breeding season

During the breeding season, the multivariate dispersion of the chemical compounds present in the feather odour of adult females and males (booming and non-booming) did not significantly differ when the data were examined between the two genders. However, there was a significant difference in the multivariate location between the two groups, indicating that the chemical composition of the body odour of males and females significantly differed from each other.

During the non-breeding season, there were no significant differences between the odour of adult females and males, neither in the multivariate dispersion among the groups, nor in the multivariate location of these two groups, indicating that the two groups were similar in both their dispersion and the composition of chemical components (Figure 2.4b and Table 2.2).

2.4.3.2 Age related differences within and outside the breeding season

During the breeding season, there were no significant differences in the variation between the odour of juvenile birds (of either sex) versus adult birds (including booming and non-booming males) when the data were examined by the two age groups. However, in spite of the pooled samples, juvenile birds had a significantly different composition of chemical components compared to adult birds (Figure 2.4c and Table 2.2).

During the non-breeding season, there were no significant differences in the dispersion of chemical components between juveniles and adults. However, there were significant differences between the the chemical composition between the two groups (Figure 2.4d and Table 2.2).

Breeding season **Non-breeding season**

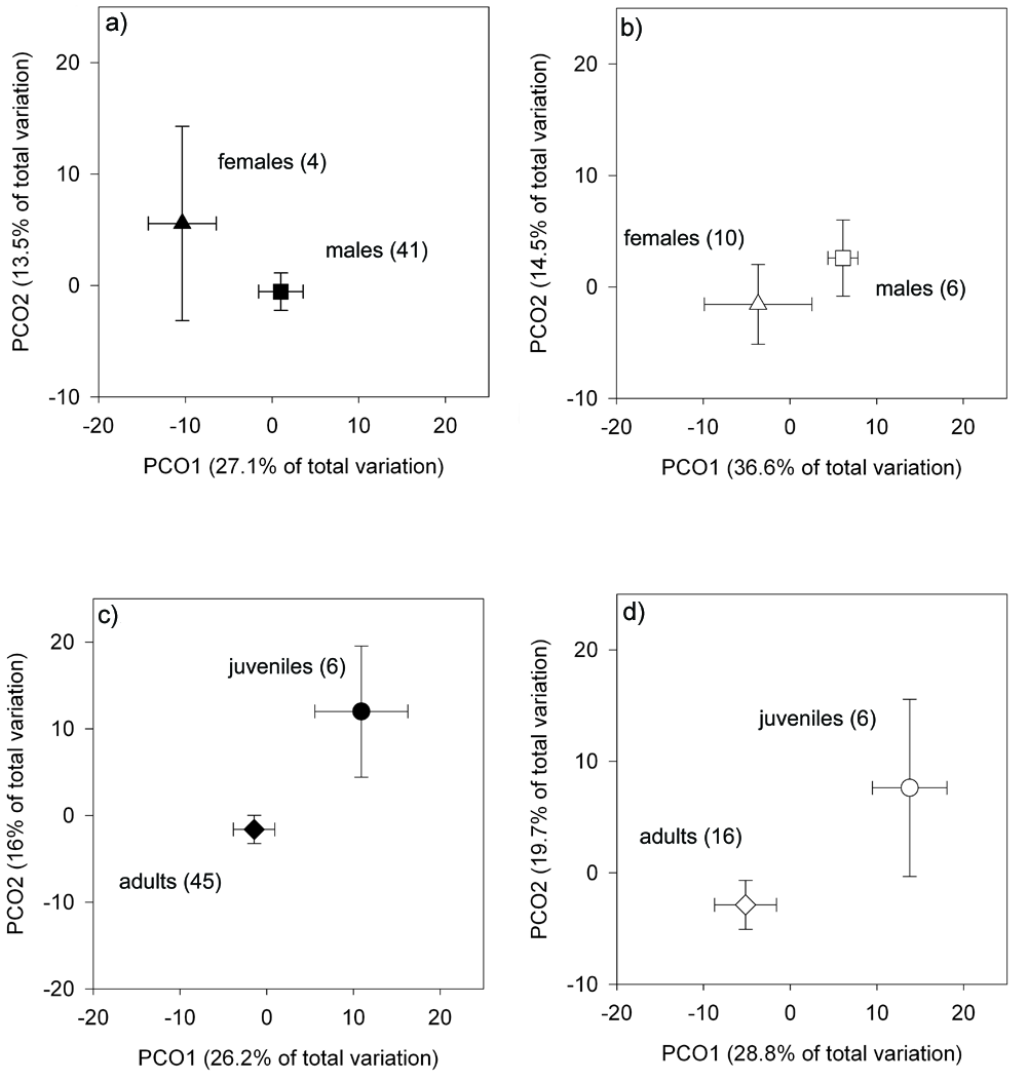


Figure 2.4 Principal coordinate analysis (PCO) of the chemical composition of feathers scent from adult males versus females (Figure 2.4a and b) and adult birds versus juvenile birds (Figure 2.4c and d). Small sample sizes (in brackets) contribute to large standard error bars in females during the breeding season and juveniles in both seasons. Individual representations of measurements are presented in Appendix F, Figures F1-4.

2.4.4 Males of unknown booming status

Males whose booming status was not known at the time were not included in the comparison above (see 2.4.2.1). However, the chemical composition of the feather scent of these males could be examined in the comparative analysis between males during the breeding season versus males outside the breeding season (see 2.4.2.2). By using a canonical analysis of principal coordinates (CAP), not only is it possible to find axes that separate groups in the multivariate space, but also to allocate samples of unknown affiliation to their corresponding axes (see 2.3.9). A CAP analysis for booming males showed that booming males are clearly separated from non-booming males, with a large canonical correlation for both axes: (δ^{1}_{2} = 69.23) and (δ^{2}_{2} = 80), respectively. Samples of booming males were assigned to the first canonical axis with (69.2%, 18/26) and samples of non-booming males were assigned to the second canonical axis with (80%, 8/10). Based on these two canonical axes, 72.2% (8/10) of samples of unknown affiliation could be assigned as booming males (Figure 2.5).

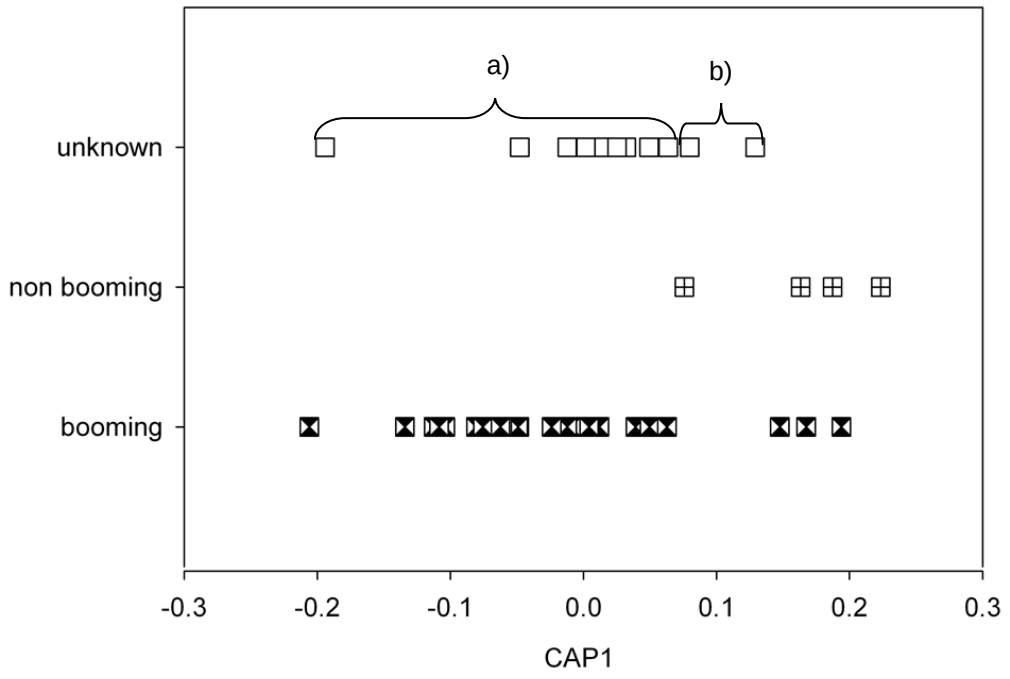


Figure 2.5 Canonical analysis of principal coordinates (CAP) separating booming males (⊠) from non-booming males (⊞) in the multivariate space. With this model, CAP successfully (72.2%) allocated males of unknown booming status (□) into groups of a) booming males (8/10) and b) non-booming males (2/10).

The chemical compositions of feather scent in four birds that have been sampled in both the breeding and non-breeding season are illustrated in a PCO (Figure 2.6).

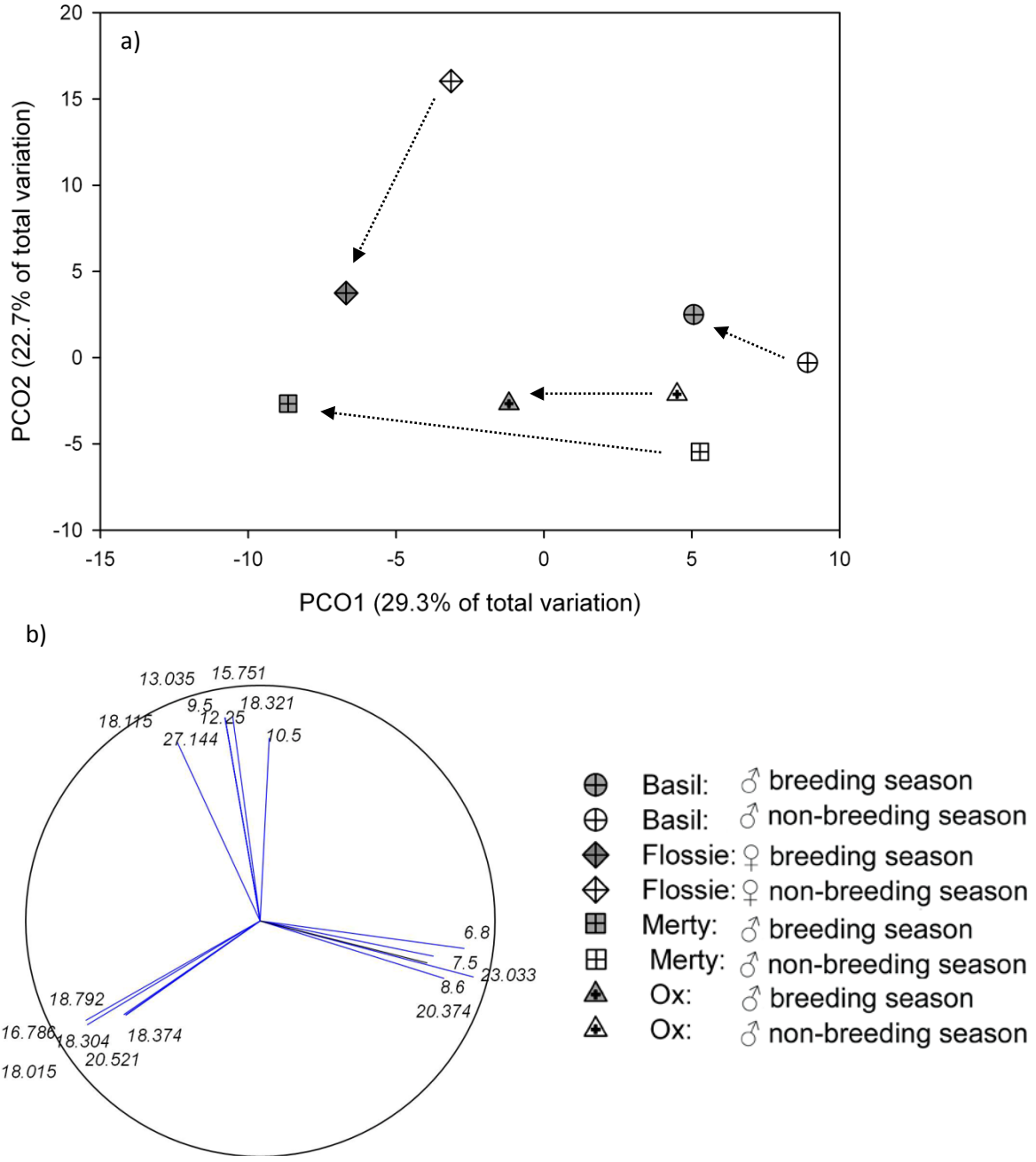


Figure 2.6 a) Principal coordinate analysis of the chemical composition of feather scent in four birds that have been sampled during the breeding and non-breeding season. Arrows indicate the change of the chemical composition from feather samples taken outside the breeding season to that of feather samples taken during the breeding season. **b)** Correlation (0.7) of the chemical components with the two PCO axes. The numbers indicate the retention times of the chemical components in the samples.

2.5 Discussion

Plumage odour of booming male kakapo has a significantly greater diversity and individual variation of chemical components than non-booming males. They also have a significantly different composition of chemical components in their feather scent compared to non-booming males. Plumage odour from males during the breeding season, regardless of their booming status, on the other hand, also has a greater diversity of chemical components compared to plumage odour of males during the non-breeding season. Additionally, the feather scent from males during the breeding season shows an overall significantly different composition of chemical components compared to males during the non-breeding season. The diversity of chemical components observed between the two groups could in both cases be caused by the quantity of particular chemicals and the presence of different chemicals.

The patterns observed are consistent with the idea that kakapo may be capable of detecting chemical differences in plumage odour and could potentially use scent as a social chemical signal within inter- and/or intrasexual behaviours. Lekking ochre-bellied flycatchers (*Mionectes oleagineus*), for example, were found to use song in male-male interactions, as provisionally muted males were more likely to temporarily lose their territories to competitors compared to control birds. Additionally, females assess potential mates by male song (Westcott 1992). While there are numerous examples in mammals (Gosling & McKay 1990; Gosling & Roberts 2001; Scordato *et al.* 2007), scent has never been described as a means of intra- sexual signal in birds, even though it has been suggested (Amo *et al.* 2012). Spotless starlings, for example, are able to discriminate the sex of conspecifics by using olfactory cues and the analysis of the chemical composition of the uropygial gland secretions using gas chromatography–mass spectrometry revealed differences between sexes, ages and the reproductive status of the birds. Intersexual olfactory recognition, however, has been found in a number of seabirds (Bonadonna & Nevitt 2004; Celerier *et al*. 2011) and has even been linked to individual recognition (Bonadonna *et al*. 2007).

Kakapo are lek breeders (Merton *et al*. 1984) and in breeding years males vigorously compete for suitable booming places, which they defend in aggressive fights that often end in injuries (Cockrem 1989). Apart from the competition for the best booming site, males also compete for females by performing long ranging booming calls and display with flamboyant dances, flap their wings and perform characteristic side to side rocking movements (Merton *et al*. 1984). Females come from afar to assess different booming sites, and choose a sexual partner. Although booming appears to be a significant investment of time and energy for males, no correlations between booming calls and mate

choice have been found (G. Harper pers. comm.). Additionally, the kakapo's eyesight is poor (Chapter Four and Corfield *et al*. 2011), which means that their ability to use specific plumage patterns as ornaments, as in birds that use visual traits for communication and camouflage (Gluckman & Cardoso 2010), is limited and suggests it is unlikely that the kakapo's mottled feather coat is used as a visual signal for mate choice. This leaves the males, apart from their booming calls, with few options to signal their qualities to the females. In contrast, and as the sole care provider for the young, females would want to assess specific signals expressed by the male that can give them information about the genetic qualities of the male and help them in their decision (Emlen & Oring 1977). Peahens (*Pavo cristatus*) for example prefer males with larger and more elaborated trains and will lay more eggs for them (Petrie *et al*. 1991; Petrie & Williams 1993). Trains are hypothesised to have evolved due to female sexual selection, but also to act as an 'honest signal' indicating the male's genetic and health constitution to the female. It is currently not known, what kind of signal females assess in kakapo males. However, a higher variety or number in chemical components present on the body odour of booming males could indicate the use of olfactory cues in sexual advertisement in a similar fashion as it has been suggested for crested auklets (*Aethia cristatella*) (Hagelin *et al*. 2003), that use scent for sexual appraisal. An excellent sense of smell (Chapter Three and Hagelin 2004; Steiger *et al*. 2009a) supports this hypothesis, as kakapo possibly would be able to discern qualitative and quantitative differences in the smell. The high individual variation in the body scent of qualitatively better males suggests the presence of an olfactory platform for female to choose from.

However, in order for a scent to be a reliable sign of quality its production would have to bear a cost. Costs and benefits of having a smell can incur by producing a scent (production cost) and/or by emitting a scent that simultaneously makes it more vulnerable to predation by revealing the senders' location. Red knots (*Calidris canutus*), for example, undergo a seasonal change from more volatile monoesters of lower molecular weight, to the less volatile diesters of higher molecular weight when migrating from their wintering grounds to their breeding grounds in the high Arctic (Piersma *et al.* 1999). While the diesters are likely to serve the birds for sexual advertisement and mate choice in their home grounds, the less volatile monoesters have been shown to serve as an antipredatory measure and are less likely to give away the location of the birds' nests (Reneerkens *et al*. 2002; Reneerkens *et al.* 2006). In an experiment with captive birds, Reneerkens *et al*. (2007) showed that birds with limited food supply would not undergo an olfactory change, indicating that the production of diesters bears a cost, but which is offset by the benefits of having a lowered risk of losing their clutch. As New Zealand originally did not have mammalian predators (Wilson 2004), kakapo presumably were able to use olfaction as a tool of communication throughout all seasons. Therefore, it

can be hypothesised that some chemicals could be expensive to produce, and not all males are able to do so.

Seasonal variation in the chemical composition of volatiles emitted as in the example of the red knots (Reneerkens *et al*. 2005) has been found in a number of studies and has generally been linked to sexual advertisement. The crested auklet (*Aethia cristatella*), for example, has a range of short-chained and highly volatile aldehydes, alcohols and acids, accompanied by a strong citrus-like scent during the breeding season (Douglas *et al.* 2001; Hagelin *et al*. 2003; Hagelin 2007). Likewise, dark-eyed juncos (*Junco hyemalis*) emit greater amounts of linear alcohols in their preen oils during the breeding season compared to birds in the non-breeding season and possibly thereby express their reproductive readiness (Soini *et al*. 2007).

Scent in kakapo has strong seasonal shifts (Figures 2.2-2.3) in addition to sex-based differences (Figure 2.4 Table 2.2) in the chemical components of males and females examined during the breeding and non-breeding season. Male feather scent had both a greater variety or number of chemical components as well as shifts in their composition than females which seem to have high levels of individual variation in chemical signal all year round. Nonetheless, the females' plumage odour has a different composition of chemical components during the breeding season compared to the non-breeding season. Female kakapo raise their chicks without the help of the males. Prior to the breeding season, females compete for good home-ranges, providing a rich source of food at close range (Whitehead *et al*. 2012). They also compete for males and have been observed to mate guard selected males (pers. comm. R. Moorhouse). Females may guard to ensure that they get enough sperm from their male of choice to fertilise their eggs. But they cannot be sure whether their male has mated before their arrival. By guarding their preferred male for a few days, they can make sure that their male does not mate with anyone else until they mate with her. This phenomenon has also been observed and described in lek breeding peahens (*Pavo cristatus*) (Petrie *et al.* 1992). Females therefore compete all year round, either for better home ranges during the non-breeding season or for males during the breeding season. Kakapo feathers could thereby serve as 'scent-marks', since the typical kakapo smell remains on them for years (pers. obs.). Female scent might also be important for the chicks. As they spend a long time (up to three months) at the burrow (Cockrem 1989; Farrimond *et al*. 2006b), the chicks need to learn where their home is just as the mother may bond with the chicks by recognising their smell. This idea is supported by juveniles having a significantly different composition of chemical components in their body scent compared to adult birds (Figure 2.4 and Table 2.2). After fledging, females care for their young for up to seven months or longer and may

continue to feed them (Cockrem 1989). Nest recognition has been shown in a number of species, such as zebra finch (*Taeniopygia guttata*) fledglings and the chicks of the British storm-petrel (*Hydrobates pelagicus*) and the leach storm-petrel (*Oceanodroma leucorhoa*) (Minguez 1997; O'Dwyer *et al.* 2008; Caspers & Krause 2010). There is a strong indication from my data that juvenile kakapo have a different chemical profile compared to adult birds, which could signify that olfactory cues might play a role in individual and chick recognition.

It is likely that male kakapo have multiple indicators of 'quality' to attract females: vocalisations to draw females from long distances and olfactory signals at close range. Olfactory signals may be extenuated by the vibrant male dance displays that occur when females approach. Furthermore, I propose that females could assess the males' individual variation in body scent and therefore perhaps a male's genetic 'quality' at close range by using scent. Olfactory signals may perhaps even inform females about the male's abilities to compete against other males, or their health condition, much in the same fashion as is known for mammals (Rich & Hurst 1998; Hurst & Rich 1999; Zala 2004).

While this research is the first to suggest a social relevance for kakapo's plumage scent and has shown significant differences in the variation of feather volatiles associated with sex, season and age, the exact identification of the chemical volatiles still needs to be determined. As the comparative shift of profiles of four individual birds during the breeding and non-breeding season has shown, specific chemical components may be indicative of the seasonal change that seems to occur in the plumage scent of the birds (Figure 2.6a). However, as the mix of volatiles of low and long retention times in this example shows (Figure 2.6b), it is not yet possible to identify these components; an important next step.

CHAPTER THREE

A COMPARISON OF BRAIN STUCTURES OF THE NOCTURNAL KAKAPO (*STRIGOPS HABROPTILUS*) AND THE DIURNAL SULPHUR-CRESTED COCKATOO (*CACATUA GALERITA*) WITH SPECIAL EMPHASIS ON THE OLFACTORY BULB AND THE OPTIC LOBE

Rebecca Wu

3.1. Abstract

A brain atlas was created based on selected sections through the brain of two phylogenetically and behaviourally different parrots, the diurnal sulphur-crested cockatoo (*Cacatua galerita*) and the nocturnal kakapo (*Strigops habroptilus*). By comparing selected sections from the brains of these two species, distinct differences between some brain regions were noted and the implications of these are discussed. The kakapo has a relatively large olfactory bulb, whereas the sulphur-crested cockatoo has a tiny olfactory bulb but a prominent optic lobe. The kakapo's optic tectum (or lobe) is displaced caudally with respect to the 'temporal' pole, and it is comparatively small. These observations indicate that the kakapo has a well-developed olfactory sense but only limited or very different visual capabilities than are usual for diurnal birds. The sulphur-crested cockatoo, in contrast, appears to have a restricted olfactory sense, but a well-developed tectofugal pathway. Indeed, behavioural traits support the hypothesis that the kakapo uses olfactory cues in its daily life, but, owing to its nocturnal lifestyle, may not depend as much on its visual system. In contrast, the sulphur-crested cockatoo seems primarily to rely on auditory and visual cues.

3.2 Introduction

Ecological, behavioural and phylogenetic differences are all reflected in the morphology and anatomy of the brain (Lefebvre *et al.* 1998; Lefebvre *et al.* 2002; Lefebvre *et al.* 2004; Gonzalez-Voyer & Kolm 2010; Maklakov *et al.* 2011). Hence, the brain is a good reference not only in order to study the evolutionary, behavioural and phylogenetic differences within and between species. It also helps us to identify any specialisations in the sensory and cognitive abilities of different species. Various studies have confirmed that an increase in size of a particular part of the brain reflects an enhancement of its function (Mace *et al.* 1980; Mace *et al.* 1981). This implies that larger brains ultimately are the result of greater specialisation. A larger size of certain compartments of the brain therefore reflects their increased functional importance (Jerison 1973; Healy & Rowe 2007). Species with larger brains also have been shown to express greater behavioural flexibility. Reptiles and birds with larger brains, for example, adapt more successfully to novel environments (Sol *et al.* 2005a; Amiel *et al.* 2011). Similarly, temperate Palaearctic birds with larger brains, relative to their body size, and a higher tendency for innovative behaviours to overcome resource-poor seasons, tend to be resident. Less behaviourally flexible species, however, with smaller brains relative to their body size, tend to be migratory (Sol *et al.* 2005b). In addition, birds with larger song repertoires have been shown to have significantly larger song control nuclei (Devoogd *et al.* 1993). Other studies have correlated differences in the size of certain brain regions across species with differences in their behavioural and eco-

logical traits (Healy & Guilford 1990; Iwaniuk *et al.* 2004; Iwaniuk *et al.* 2007; Corfield *et al.* 2008; Iwaniuk *et al.* 2010). Iwaniuk & Hurd (2005), for example, studied the volume of nine brain regions in 67 birds and grouped them according to their similarities in locomotion, behaviour, cognition and prey capture. Tool use in animals can be positively correlated with the enlargement of specific telencephalic regions, such as the nidopallium and the mesopallium (Lefebvre *et al*. 2002; Emery & Clayton 2004; Mehlhorn *et al.* 2010).

Much, therefore, can be gained by examining the size and structure of the brain. The best overview of the structure and architecture of a specific brain can be achieved by creating a comparative atlas of the brain, which not only allows me a comparison of the structure and location of certain brain regions or compartments, but also allows me to draw (tentative) conclusions and to make predictions about the general importance of certain senses reflected in the brain. Therefore, I created an atlas for the nocturnal kakapo and another for the diurnal sulphur-crested cockatoo. By comparing two parrot species of similar physical size but seemingly different behaviour, I was able to comment on some aspects of their sensory system and possibly provide some insight as how the kakapo has adapted to its unique environment. The description will mainly focus on the olfactory and the visual system.

The olfactory lobe or bulb is located at the frontal pole of the forebrain and as is responsible for the reception and perception of olfactory volatiles and cues. The anatomy and size of the olfactory bulb can vary considerable in birds (Cobb 1960; Bang & Cobb 1968). The olfactory bulb is composed of seven to nine layers (Nieuwenhuys 1967), whose function will be discussed in Chapter Four.

Visual information is processed in the brain via two major pathways: the thalamofugal pathway and the tectofugal pathway (Shimizu & Karten 1993; Shimizu & Bowers 1999). In the thalamofugal pathway information from the retina projects to a structure in the dorsal nidopallium called the visual Wulst, a visible protuberance on the dorsal hemisphere of the brain comparable to the primary visual cortex of mammals (Karten *et al.* 1973). In nocturnal species, such as owls (Iwaniuk & Hurd 2005; Iwaniuk & Wylie 2006; Iwaniuk *et al.* 2008), the Wulst is rather pronounced and plays a key role in global stereoscopic vision (Pettigrew 1978; Pettigrew 1986; Wagner & Frost 1993; Nieder & Wagner 2000; Nieder & Wagner 2001; Iwaniuk & Wylie 2006), although its role in modulating stereopsis in other taxa has been debated (Iwaniuk *et al*. 2008; Martin 2009). In barn owls (*Tyto alba*), the Wulst has been described as forming a distinctive physical protuberance dedicated to the representation of the contralateral claw and might in the owl's predatory behaviour (Wild *et al.* 2008).

The tectofugal pathway is regarded as the primary pathway in other bird species (Shimizu & Karten 1993; Bischof & Watanabe 1997). Retinal information projects to the tectum (TeO) (Mpodozis *et al.* 1996), which sends a projection to the nucleus rotundus (nRt), a large, round nucleus that transmits motion and colour based information from the optic tectum to the telencephalon (T) (Laverghetta & Shimizu 2003). The optic tectum in turn, projects to the entopallium (E) of the telencephalon (T) (Benowitz & Karten 1976; Husband & Shimizu 2001; Krützfeldt & Wild 2004).

The optic tectum is made up of 15 different layers (Ramon y Cajal 1911). The most peripheral or exterior layer of the optic tectum, the stratum opticum (SO), is composed of the axons of retinal ganglion cells (optic tract fibres), which enter the tectum ventrally and extend dorsocaudally around the tectal bulge to terminate in the outermost seven layers. The next stratum, the stratum griseum et fibrosum superficiale (SGFS) is the thickest layer of the optic tectum in birds and consists of alternating fibrous and cellular layers (Huber & Crosby 1933; Hamdi & Whitteridge 1954). It receives various optic and non-optic impulses and the degree to which its layers are differentiated is directly correlated with the volume and importance of optic and non-optic impulses (Butler & Hodos 2005). The next stratum, the stratum griseum centrale (SGC), consists of neurons that possess both long dendrites that extend to the periphery of the tectum and axons that project to the nucleus rotundus, as well as shorter dendrites directed towards the ventricle that receive information from the periventricular system (Huber & Crosby 1933; Butler & Hodos 2005). The latter is present in all vertebrates in varying degree and is regarded as phylogenetically very old (Huber & Crosby 1933). Adjacent to the SGC is the stratum album centrale (SAC). This bears major efferent pathways from the tectum to lower centres of the optic tectum (Huber & Crosby 1929). The nucleus dorsolateralis anterior thalami, pars lateralis (DLL), and the nucleus dorsolateralis anterior thalami, pars medialis (DLM), form a shell around the dorsal aspect of nucleus rotundus (Butler & Hodos 2005). The nucleus isthmi pars parvocellularis (Ipc) forms strict, reciprocal connections with the optic tectum (Wang *et al*. 2004; Maczko *et al*. 2006; Wang *et al*. 2006). The nucleus isthmi pars magnocellularis (Imc) and pars parvocellularis (Ipc) both influence the receptive field structure of neurons in the optic tectum (Wang *et al*. 2004). The pretectal nucleus pretectalis (PT) is significant for its reciprocal connections with the optic tectum, but lacks any direct retinal input.

While this is a descriptive approach, the size of some of the structures discussed here will be compared in following chapters: the olfactory bulb in Chapter Four and the visual centres of both, the thalamofugal as well as the tectofugal pathway in Chapter Five. I will use a comparative approach and regression analyses using a range of Australasian parrots. This will enable me to further examine

the functional implications associated with the differences in the size of these brain compartments noted here.

3.3 Methods

3.3.1 *Specimens and permits*

Kakapo (*Strigops habroptilus*): A male kakapo's head (brain, eyes and tongue) was received within eight hours post-mortem from a bird that died from a crop infection at Auckland Zoo (Auckland, New Zealand) in October 2008 (Permit: SO-24483-RES, Figure 3.1a).

Sulphur-crested cockatoo (*Cacatua galerita*): A male sulphur-crested cockatoo, was shot in a culling programme in the Waitakere Ranges (Auckland, New Zealand) in October 2010, and was received within four hours post-mortem (Permit was received as a discretionary consent under the Auckland Regional Council Parks Management Plan) (Figure 3.1b).

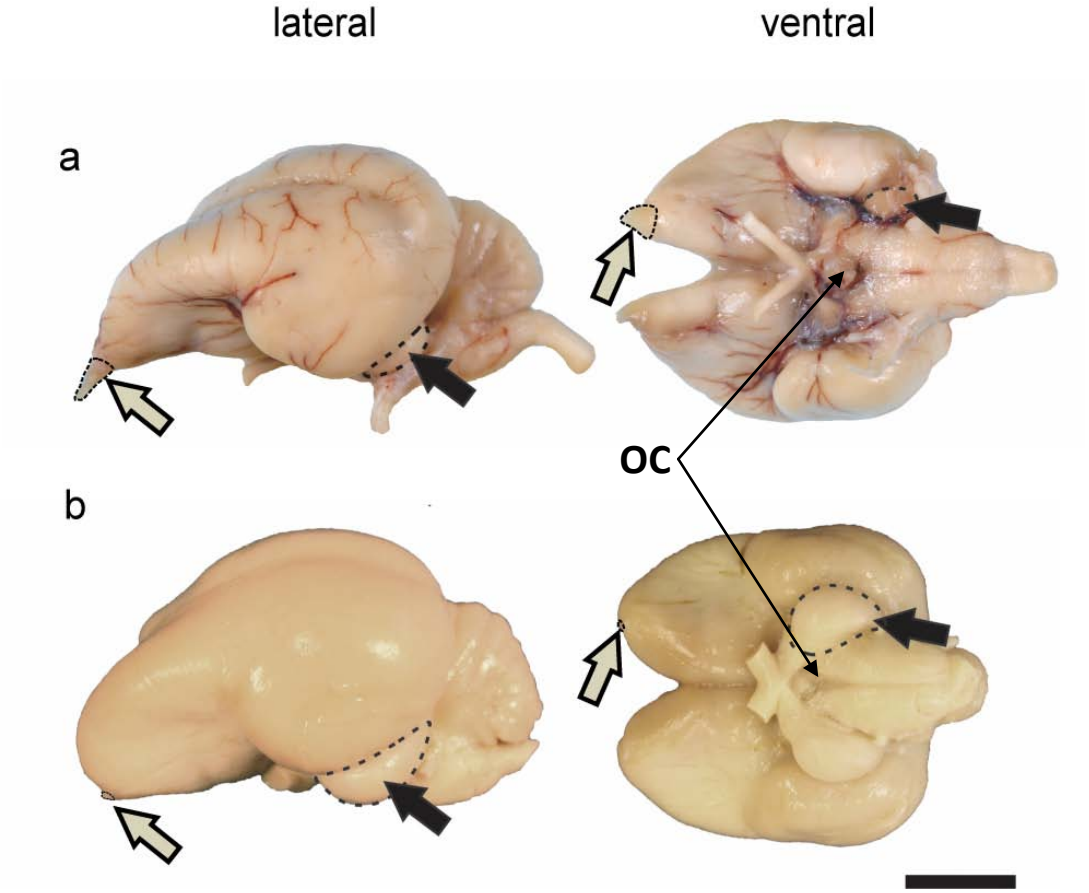


Figure 3.1 a) Brains of kakapo (*Strigops habroptilus*) and **b)** sulphur-crested cockatoo (*Cacatua galerita*). The dotted lines indicated by the open arrow outline the left olfactory bulb in both species. The dotted lines indicated by the black arrow outline the left optic lobe in both species. The optic chiasm (OC) is much smaller in kakapo compared to the sulphur-crested cockatoo. Scale bar = 10 mm. Photos by Nick Duggan (Manager, AMRF Medical Sciences Learning Centre – *Whakaaro Pai,*

Department of Anatomy with Radiology, The University of Auckland, Auckland, New Zealand) using a Nikon D2Xs and a 105mm f/2.8D AF Micro-Nikkor lens.

3.3.1 Preparation of brains and sectioning

The brains of the kakapo and the sulphur-crested cockatoo were dissected and the brain and eyes (for Chapter Five) were immersion-fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) until they had hardened (four weeks). Both specimens were blocked mid-sagittally with a razor blade and the right hemisphere of each brain was cryoprotected in 30% sucrose in 0.01 M PBS, which was renewed every other day. Once the penetration of sucrose was complete, the brain tissue sank to the bottom of the container. With the large brains of kakapo and sulphur crested cockatoo this process took two and a half weeks. Subsequently, the brains were embedded in a solution of 15% gelatine with 30% sucrose at 45°C for one hour and then placed into a prepared mould to allow them to cool and harden at 4°C. Pins were positioned around the brain and into the hardening gelatine block to create fiduciary points, that later could be used to correctly orientate the brain sections (see Chapter Four, section 4.3.6 and Figure 4.2). The hardened block was immersed in paraformaldehyde overnight, the pins were removed and the block was serially sectioned in the sagittal plane on a sliding freezing stage microtome at a thickness of 45 µm. The sections were subsequently collected in PBS, mounted onto subbed slides, stained with cresyl violet, dehydrated and cover slipped with DePeX (Serva GmbH) from xylene.

3.3.2 Specific methods in creating brain atlases

I created an atlas based on 15 kakapo and 14 sulphur-crested cockatoo sagittal sections (out of a total of 162 sections in kakapo and 154 in cockatoo) stained for histology. The sections were selected to include all major brain structures. I created line diagrams to illustrate and label the different regions using CorelDRAWGraphicSuiteX5© (Corel, Ottawa, ON, Canada) and Adobe Illustrator CS4 (Adobe Systems complex, San José, CA, US). I concentrated on the identification of seven telencephalic regions and delineated their boundaries on the basis of the cresyl violet stained sections and guided by several bird brain atlases (Huber & Crosby 1929; Karten & Hodos 1967; Nieuwenhuys 1967; Showers 1982; Wenzel 1987; Künzel & Masson 1988; Halasz 1990; Matochik *et al.* 1991; Butler & Hodos 2005; Atoji & Wild 2006; Nixdorf-Bergweiler. B & Bischof.H 2007; Puelles *et al.* 2007; Brauth *et al.* 2010), while following to the *Consortium Nomenclature* (Reiner *et al.* 2004; Jarvis *et al.* 2005).

3.3.4 ***Areas identified on the atlas***

The areas identified encompass the telencephalon consisting of the hyperpallium (H), the mesopallium (M), the nidopallium (N), the entopallium (E), the arcopallium (A), the basal ganglia or the dor-

sal and ventral striatopallidal complex (SPC) (Smeets *et al.* 2000), the nucleus basorostralis (Bas) and the olfactory bulb (OB). In the budgerigar the nucleus basorostralis (Bas) contains a distorted, but somatotopic precise representation of the bill, other oral components and the body (Wild *et al.* 1997; Butler & Hodos 2005). The olfactory bulb (OB) is responsible for processing olfactory information (see Chapter Four). The nidopallium includes all the nidopallial sub-regions except for the nucleus basorostralis, the entopallium (E) and the arcopallium (A). The mesopallium includes both the dorsal (Md) and ventral (Mv) subdivisions. The hyperpallium (H) or Wulst, contains the hyperpallium apicale (HA), the hyperpallium intercalatum (HI) and the hyperpallium densocellulare (HD) (Butler & Hodos 2005).

Different brainstem structures known to process visual information were identified in the brainstem and are indicated where possible in both kakapo and sulphur-crested cockatoo. These are the optic tectum (TeO) and the main layers associated with it: the stratum opticum (SO), the stratum griseum et fibrosum superficiale (SGFS), the stratum griseum centrale (SGC), the stratum album centrale (SAC). Additionally, the nucleus isthmi pars parvocellularis (Ipc) and pars magnocellularis (Imc) were indicated.

Auditory information is initially processed by the basilar papilla and then by the cochlear nuclei: nucleus angularis (responsible for the intensity) and the nucleus magnocellularis (time difference channel) (Boord & Rasmussen 1963; Karten 1967; Takahashi & Konishi 1988; Butler & Hodos 2005). In the midbrain, auditory information is received and processed by the nucleus mesencephalicus lateralis, pars dorsalis (MLd) (Karten 1968; Correia *et al.* 1982; Conlee & Parks 1986; Krützfeldt & Wild 2005). From there the information is transferred to the nucleus ovoidalis (Ov), which is as the name refers to of ovoid shape, and distributed into the auditory Area L. pallii (Karten 1968; Butler & Hodos 2005). The nucleus reticularis pontis caudalis, pars gigantocellularis (RPgc) belongs to the reticular formation, a little understood part of the brainstem, the descending part of which is responsible for rhythmic motor patterns such as respiration, flying and chewing, as well as the voluntary movement of the head and body. The ascending part is responsible for processes such as sleep and dreaming as well as attention (Butler & Hodos 2005).

Tactile information from the head is conveyed to the nucleus sensorius principalis nervi trigemini (PrV). PrV receives information through the tactile sense organs in the bill via the trigeminal nerve and in some species also from the tongue via the glossopharyngeal and hypoglossal nerves (Dubbeldam 1990; Wild 1990; Wild & Zeigler 1996). The PrV then transmits information to the telencephalic sensory end-station, the nucleus basorostralis (Wallenberg 1903; Dubbeldam *et al.* 1981; Wild 1985).

From there a multisynaptic circuit traverses the brain to the arcopallium and then descends to terminate on the jaw premotor nuclei (Berkhoudt *et al.* 1981; Dubbeldam et al. 1981; Wild *et al.* 1985; Dubbeldam & Visser 1987; Wild & Farabaugh 1996; Wild & Krützfeldt 2012).

3.4 Results

External examination of the two brains showed that the brain of the kakapo was large and elongated towards its rostral pole, whereas the brain of the sulphur-crested cockatoo was more shaped like a square (Figure 3.1a & b). The following description is divided into brain areas.

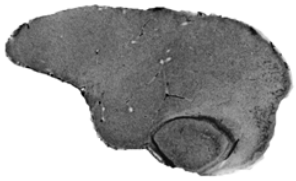
3.4.1. Brainstem

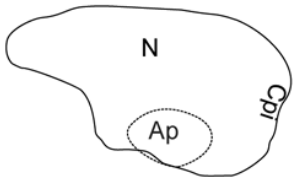
Externally, the optic lobe of the kakapo is miniscule in comparison to that of other parrots (A. Iwaniuk, *pers. comm.*). Because of its small size, it appears to be displaced further caudally than in most other avian brains. In addition, the optic nerve seems to be significantly reduced in diameter (Fig 3.1a). Internally, the visual centres of optic tectum, nucleus rotundus and entopallium seem to be significantly reduced in comparison to the visual centres of the sulphur-crested cockatoo. The PrV seems prominent in kakapo, but its examination and analysis is beyond the scope of this thesis. Externally as well as internally, the visual centres of the sulphur-crested cockatoo do not appear atypical in size and shape (Figures 3.1b & 3.2 f-h). The visual centres will be examined in Chapter Five.

3.4.2. Telencephalon

Externally the kakapo and the sulphur-crested cockatoo have a pedunculated olfactory bulb at the frontal pole of their forebrain (Figure 3.1). The olfactory bulb of the kakapo is strikingly larger compared to that of the sulphur-crested cockatoo and emphasises the apparent elongation of the kakapo's brain (Figures 3.1a & 3.3j-o). The olfactory bulb of the sulphur-crested cockatoo, in contrast, is tiny and hardly visible (Figures 3.1b & 3.2h), which is also reflected in the fact that it extends only over a small number of histological sections compared to the case of the kakapo. The exact volume of the olfactory bulbs and their layers will be discussed in Chapter Four. The atlas also reveals that the Wulst (Figure 3.2e-o) seems to be enlarged in kakapo, whereas it is smaller in size in the sulphur-crested cockatoo (Figure 3.3f-n). The Wulst will be further discussed in Chapter Four.

a)





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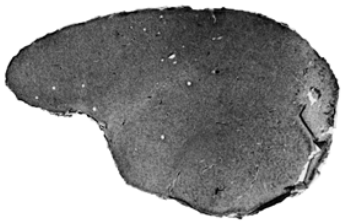

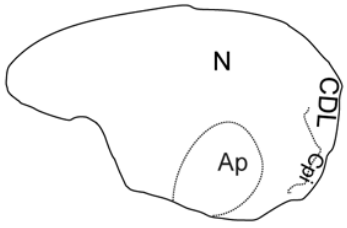

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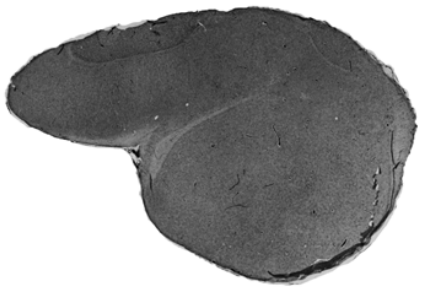

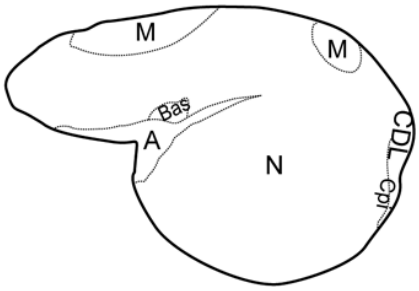

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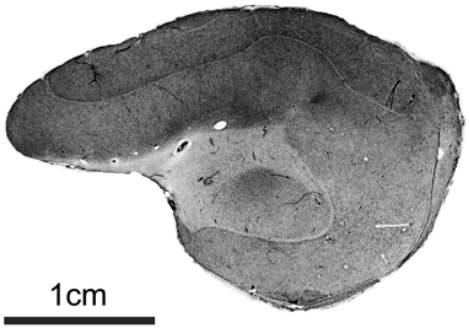

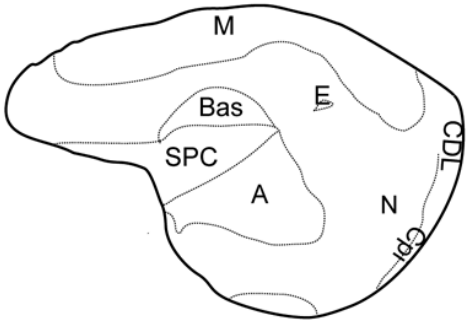

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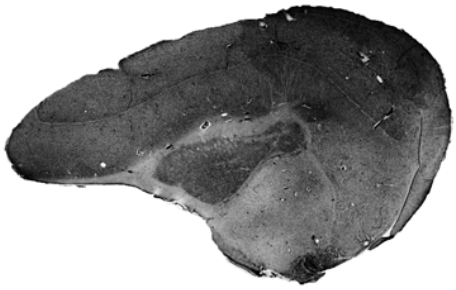

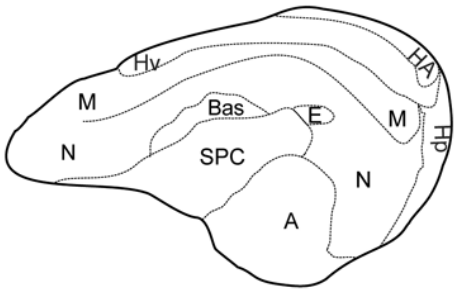
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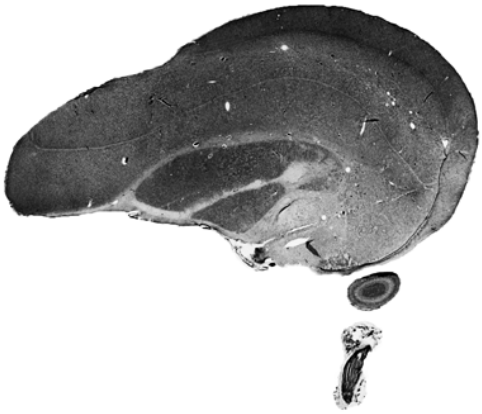

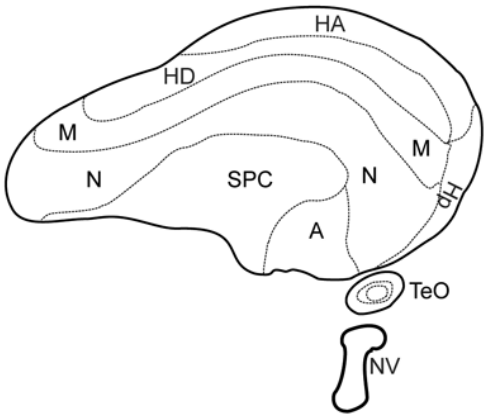
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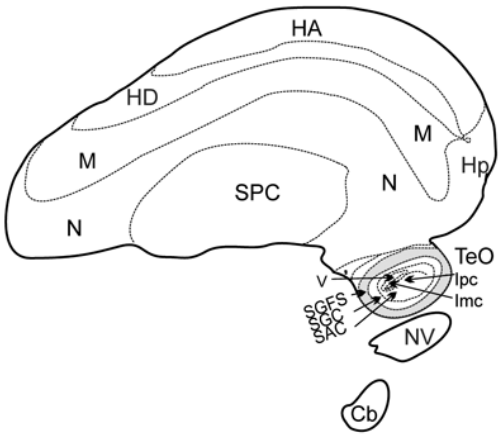
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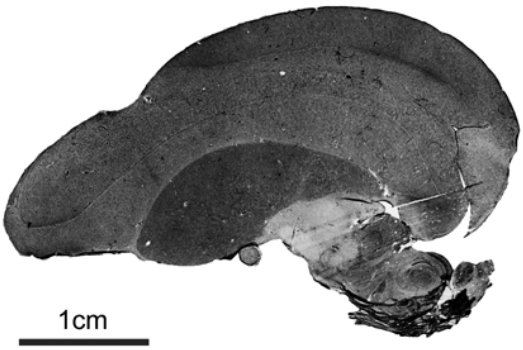
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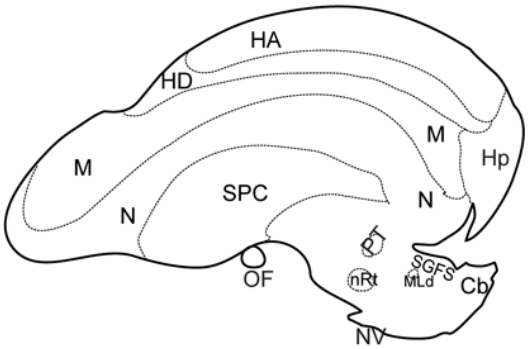
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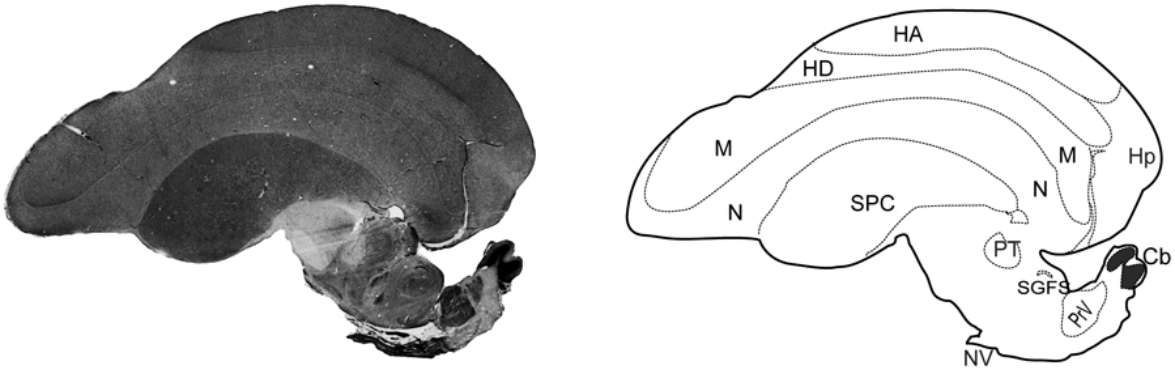
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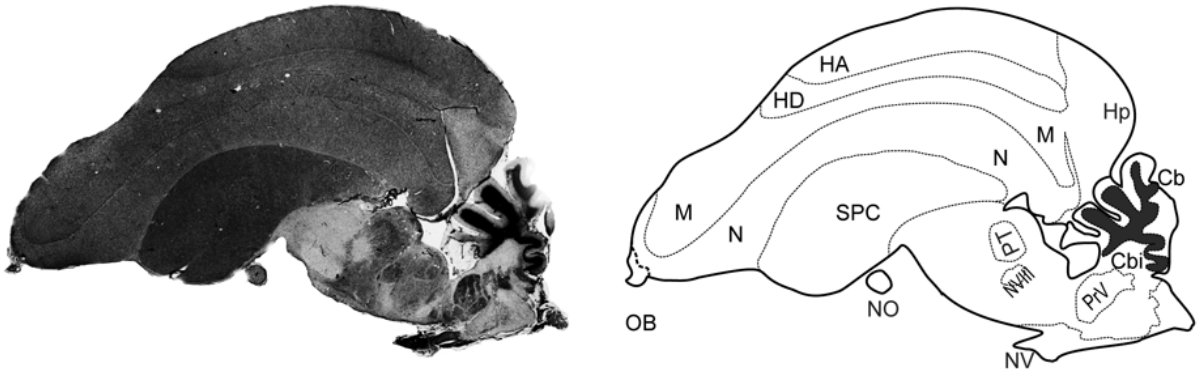
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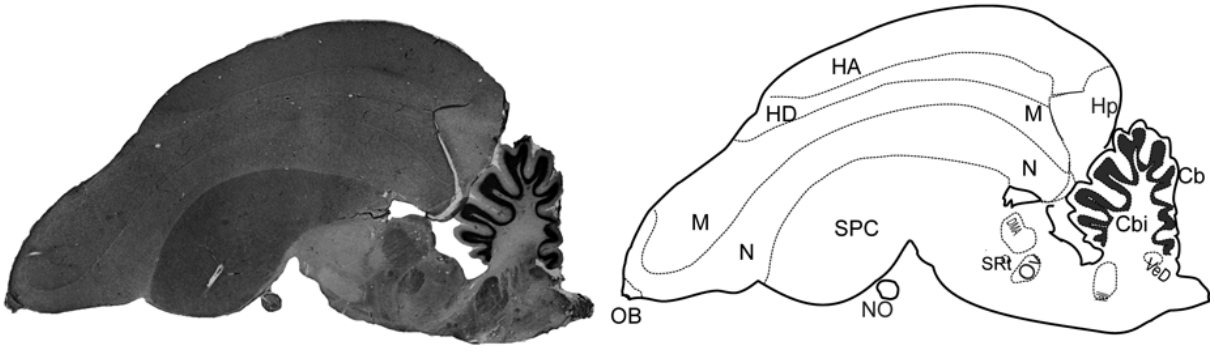
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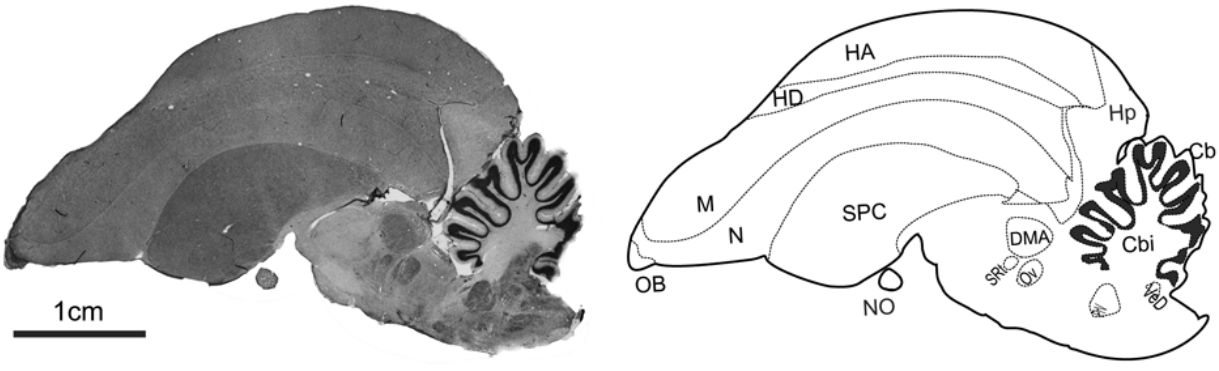
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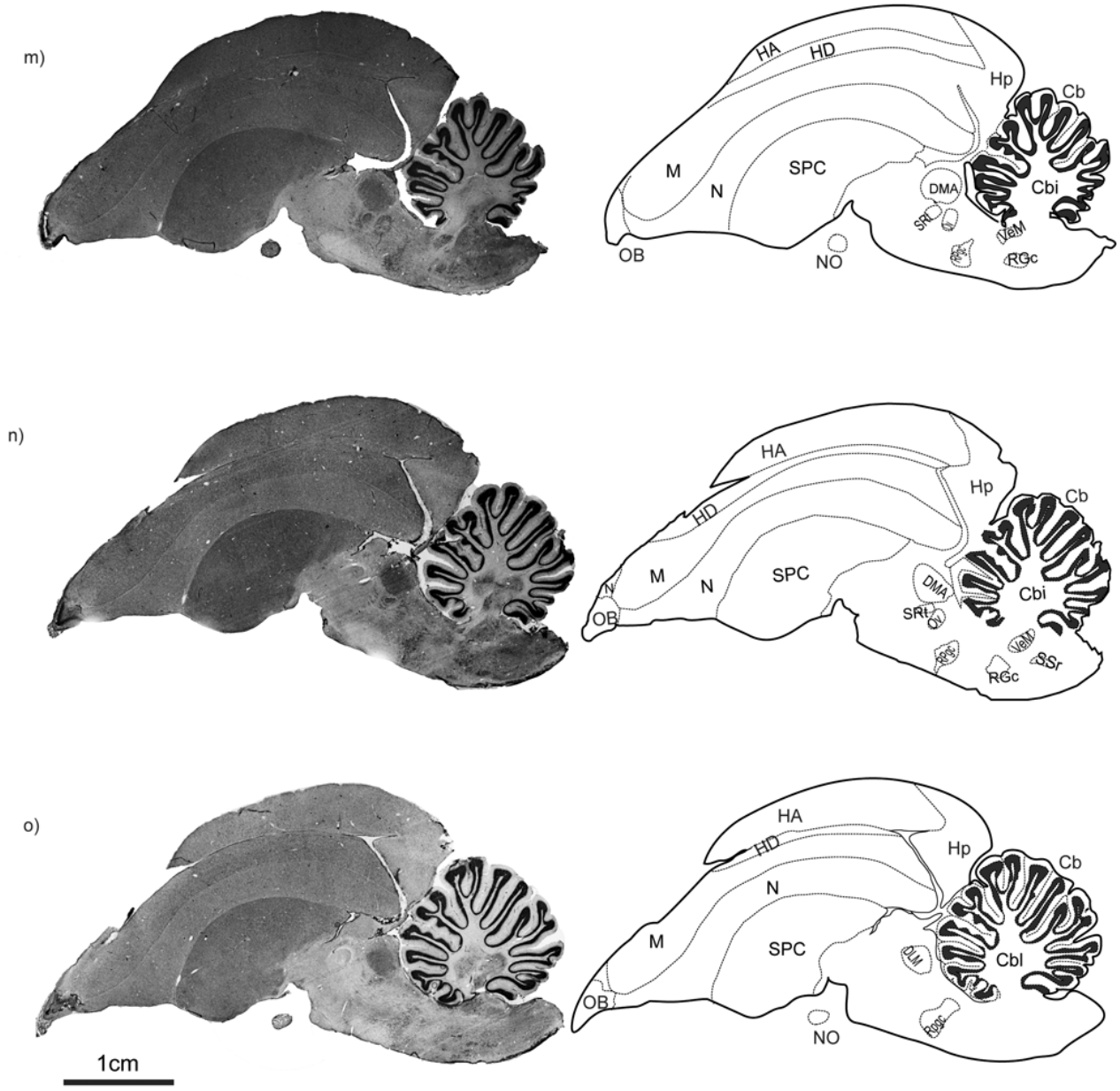
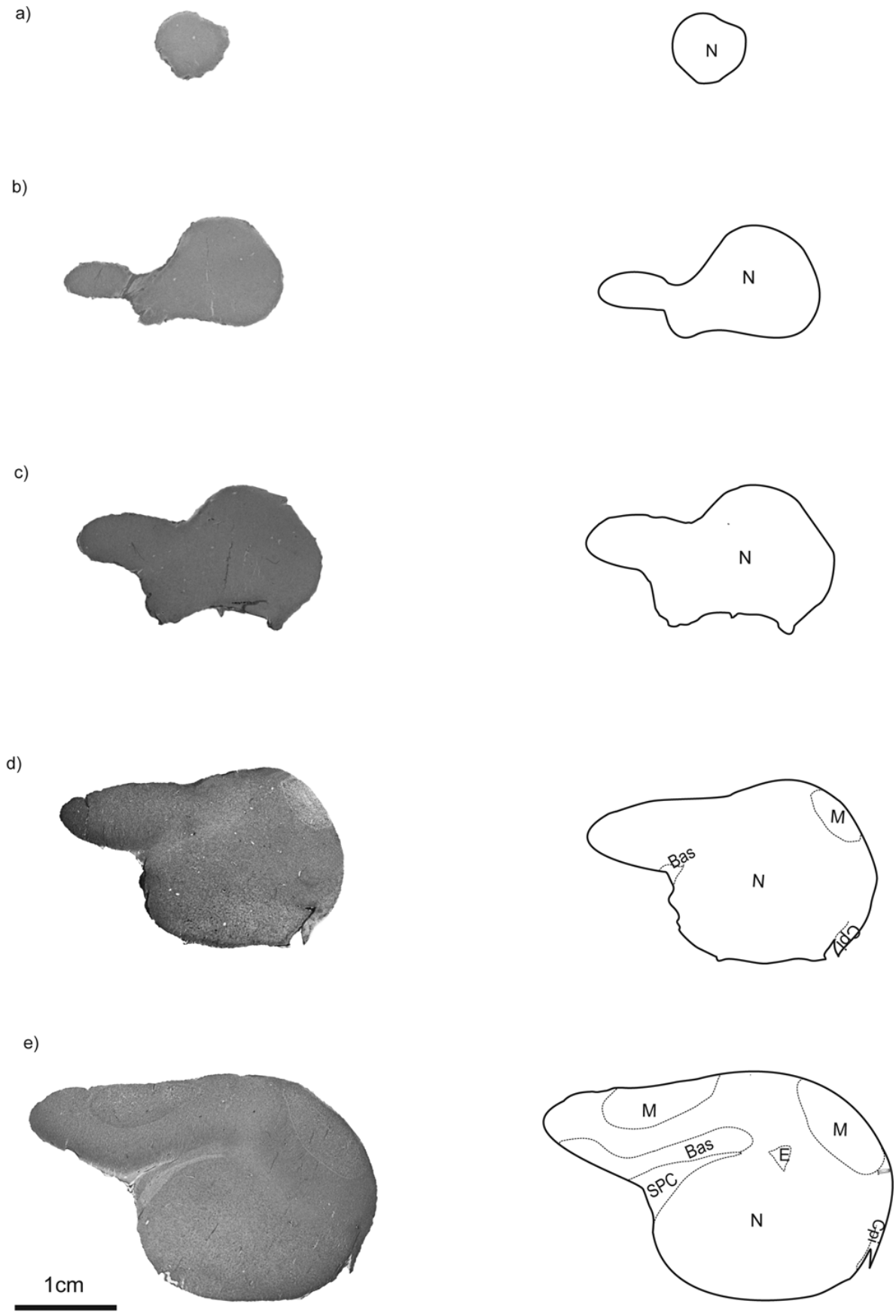


Figure 3.2 a-l Brain atlas of the kakapo based on 15 images revealing the main brain areas of interest. Note the extensive olfactory bulb (j-o). Visual centres are visible in: Entopallium (E) (e); Optic tectum (TeO) (f-g); Nucleus rotundus (nRt) (h) and Wulst, i.e. Hyperpallium apicale (HA) (e-o) and Hyperpallium dorsale (HD) (f-n). See Table 3.1 for definitions of abbreviations used.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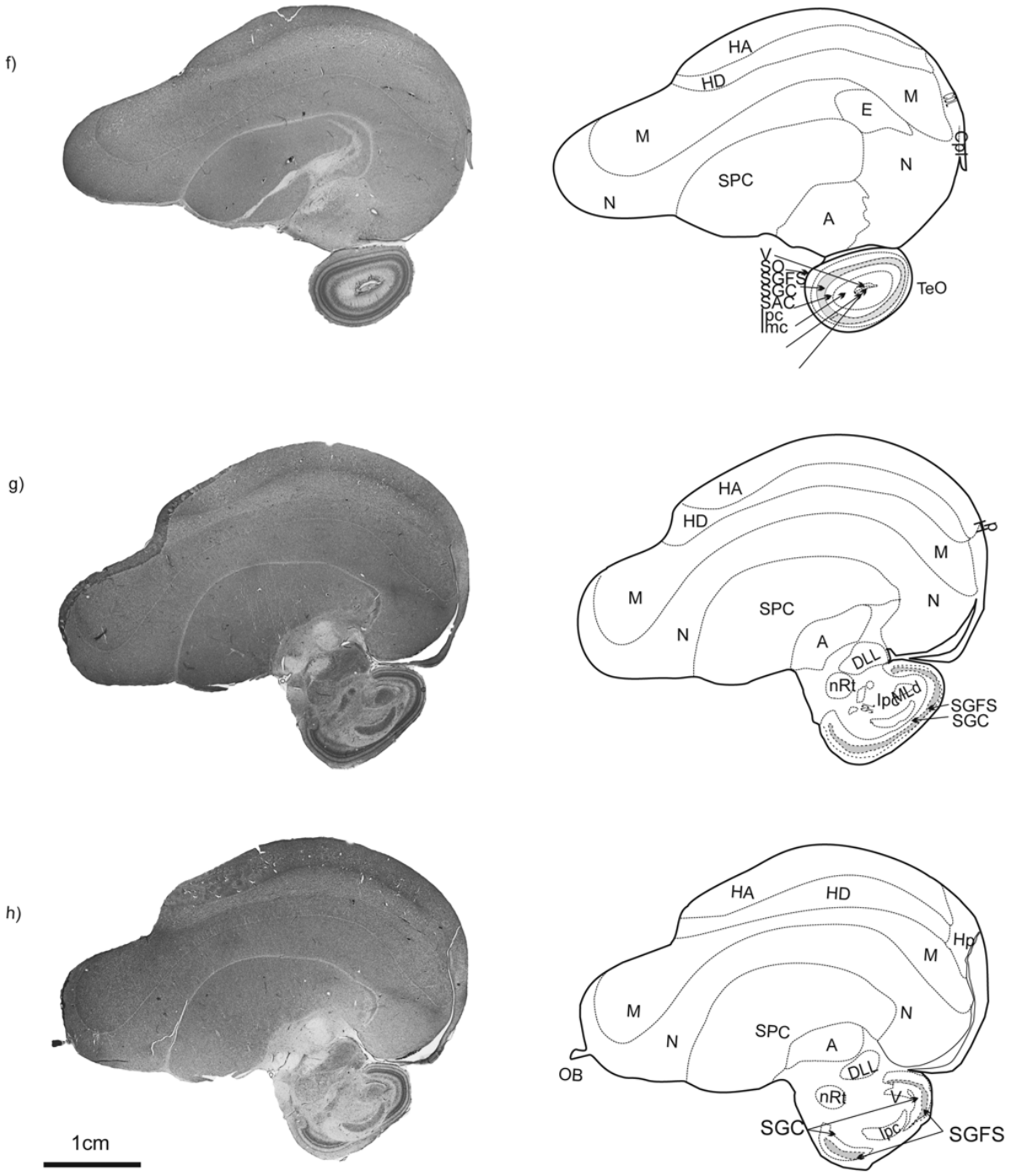
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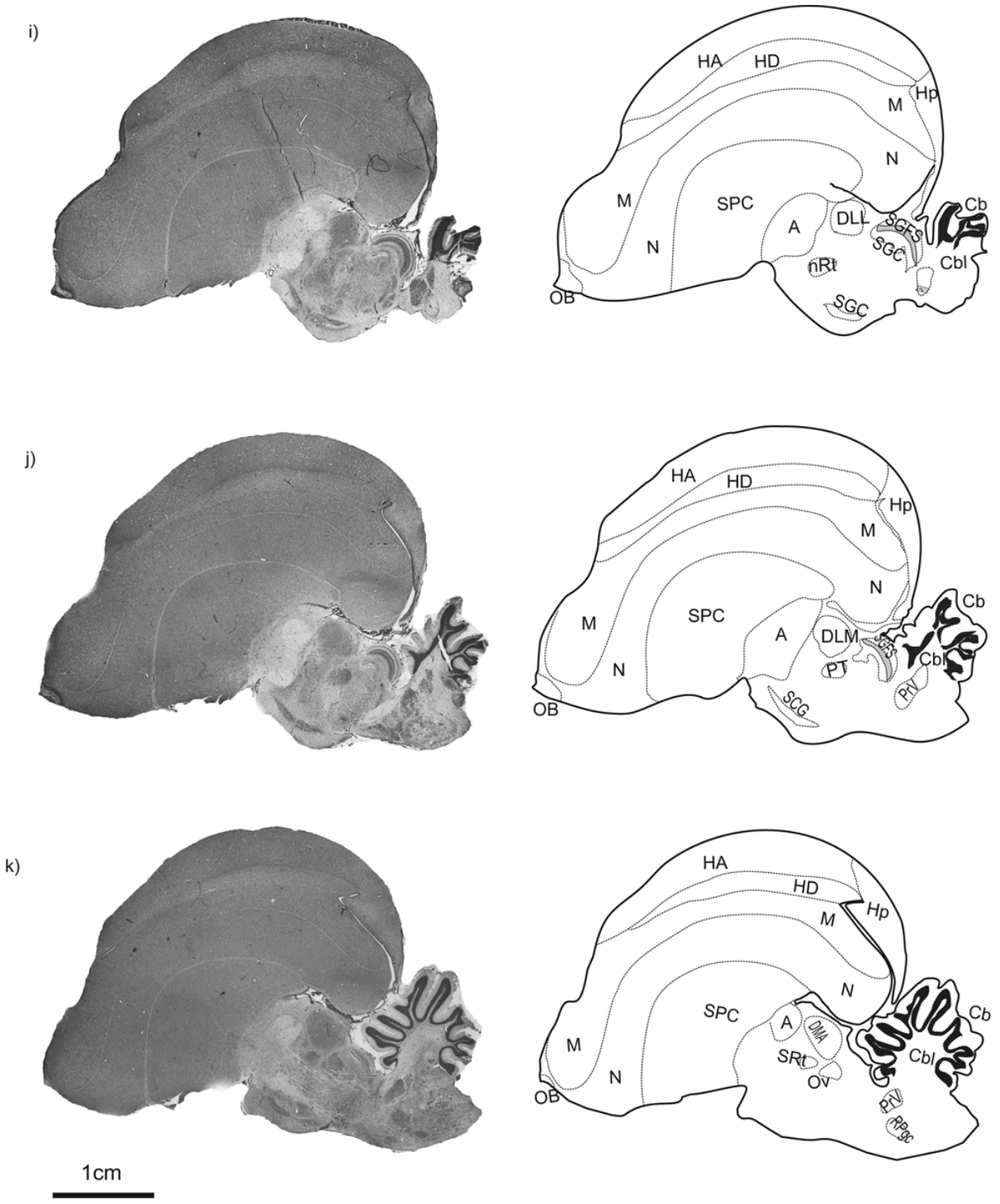
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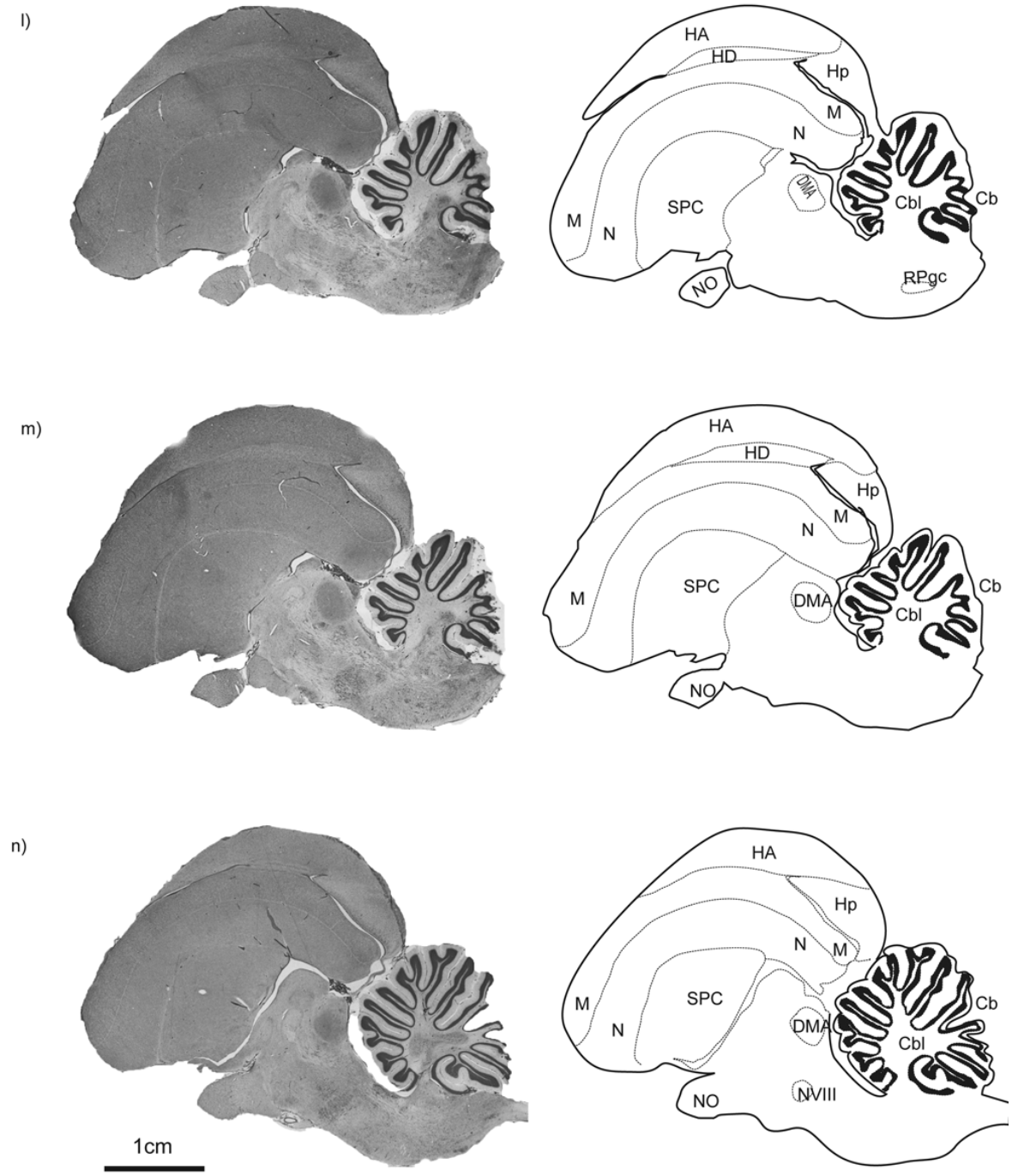


Figure 3.3 a-o Brain atlas of the sulphur-crested cockatoo based on 15 images revealing the main brain areas of interest. Note the small olfactory bulb (h-k). Visual centres are visible in: Entopallium (E) (e-f); Optic tectum (TeO) (f-g); nucleus rotundus (nRt) (g&h) and Wulst (W) i.e. Hyperpallium apicale (HA) (f-n) and Hyperpallium dorsale (HD) (h-m). See Tab 3.1 for abbreviations used.

3.5 Discussion:

The brains of the nocturnal kakapo and the diurnal sulphur-crested cockatoo were compared by the visual examination of selected sections in which major brain regions could be identified. Based on this visual and descriptive approach, two main features are apparent: a comparatively large olfactory bulb in the kakapo and a comparatively small, barely visible olfactory bulb in the sulphur-crested cockatoo. The sulphur-crested cockatoo had a large optic lobe while kakapo had a very small and apparently reduced visual centre

Indeed, there are no reports of sulphur-crested cockatoos using olfaction or olfactory cues. Instead of using preen oils, they have been reported to create a fine white powder to waterproof themselves (Forshaw 2006). The birds also have no noticeable smell, whereas kakapo emit a very strong, sweet and long-lasting smell. The kakapo is nocturnal, its eyesight has been described as poor, and it has a retina that has features of both diurnal and nocturnal birds (Chapter Five and Corfield *et al*. 2011). It is not surprising, then, that in kakapo the optic tectum was found to be greatly reduced in size and displaced caudally in the midbrain. The optic nerves appeared particularly thin and the optic chiasm was conspicuously small. A completely different picture emerged in the sulphur-crested cockatoo. Optic chiasm and nerves were large and firm and the optic tectum pronounced. Sulphur-crested cockatoo are diurnal and volant. They live in open areas and timbered habitats (Higgins 1999; Forshaw 2006) and are of conspicuous appearance due to their white feather-coat. They like to assemble in groups at dusk, and their white appearance may help them in locating their conspecifics. The kakapo instead has a moss-green, brownish feather-coat, which easily integrates with the surrounding vegetation. The kakapo used to be diurnal and changed its activity pattern 20 million years ago (Wright *et al.* 2008), presumably due to predation pressure by the extinct Haast eagle (*Harpagornis moorei*) and the Eyles harrier (*Circus eylesi*) (See Chapter One and Holdaway & Worthy 1997; Wilson 2004). In the absence of mammalian predators, there was no need to maintain the expensive flight apparatus, and kakapo lost the ability to fly (See Chapter One and Wilson *et al.* 2007).

Both species are vocal. Male kakapo are known for their low frequency, long-range booming calls, but these are only used by males and during the mating season (Merton *et al*. 1984). Sulphur-crested cockatoo, in contrast, are known to call vigorously at dawn and dusk at their roosting sites (Forshaw 2002). Their calls can easily travel far and may help birds finding their way back to their mutual roost sites. They also have a sentinel warning system while feeding and roosting, with one or several lookouts that raise the alarm if danger looms (Forshaw 2002). The highly visible feather-coat and vocal character might indicate that sulphur-crested cockatoo depend more on visual and audi-

tory cues. The behavioural traits of kakapo, in turn, indicate that they may depend more on auditory and olfactory cues. In an environment where the main danger originally arose from avian predators, communication using olfactory cues might have been favourable. In comparison, communication using vocal means might be more beneficial in a mammalian dominated world, as olfaction is known to be used by mammals as a means of communication in a wide variety of functions. For example, territorial marking between families, hierarchical marking within families and sexual advertisement (Roper *et al.* 1993; Gsell 2002; Hurst & Beynon 2004), including in finding their prey (Hughes *et al*. 2010).

While the descriptive brain atlases of the two species indicate that in the kakapo olfaction might be more important, while in the sulphur-crested cockatoo it is the visual system, an in-depth analysis is needed to confirm this hypothesis. Chapter Four uses classical histology to examine the size of the olfactory bulb in the kakapo and the sulphur-crested cockatoo, as well as in a range of other Australasian parrots. Furthermore, I will describe the layers associated with the olfactory bulb and analyse their volumetric size. Finally, I will analyse the characteristics of the mitral cell layer, which is known to transfer chemical signals to other parts of the brain. Mitral cells are, therefore needed to trigger a behavioural or physiological response to an olfactory signal. A comparative analysis of the number of mitral cells present in kakapo and other parrots will thus be useful for understanding the size and functionality of the olfactory bulbs with the importance for olfaction in kakapo.

Future studies may want to examine other aspects revealed in the two brain atlases. Volumetric, comparative analysis could disclose more about the specification of some areas. For example, it may be worthwhile to examine in future the principal sensory trigeminal nucleus (PrV). This structure seems to be particularly prominent in kakapo and is known to receive information from the beak and the tongue via the glossopharyngeal and hypoglossal nerves (Dubbeldam 1990; Wild & Farabaugh 1996). Kakapo have a short, wide and thick tongue densely populated with numerous taste buds (Kirk *et al*. 1993). A study to investigate the properties of the taste buds on the tongue of the kakapo could prove worthwhile.

Table 3.1 Abbreviations used in the two brain atlases

Abbreviation	Definition	Abbreviation	Definition
A	Arcopallium	**Mv**	mesopallium ventrale
Ap	*archistriatum posterior*	**N**	nidopallium
Bas	nucleus basorostralis	**NO**	nervus opticus
BP	basilar papilla	**NV**	nervus trigeminus
Br	Brainstem	**nRt**	nucleus rotundus
Cb	Cerebellum	**N VIII**	nervus octavus, pars cochlearis
Cbi	nucleus cerebellaris internus	**OB**	olfactory bulb
CDL	area corticoidea dorsolateralis	**OF**	olfactory fila
Cpi	pyriform cortex	**ONL**	olfactory fila
DLL	nucleus dorsolateralis anterior thalami, pars lateralis	**Oc**	Optic chiasm
DLM	nucleus dorsolateralis anterior thalami, pars medialis	**Ov**	nucleus ovoidalis
DMA	nucleus dorsomedialis anterior thalami	**PL**	periventricular layer
E	Entopallium	**PrV**	nucleus sensorius principalis nervi trigemini
Ep	Ependyma	**PT**	nucleus pretectalis
EPL	external plexiform layer	**RPgc**	nucleus reticularis pontis caudalis, pars gigantocellularis
GCL	granule cell layer	**SAC**	stratum album centrale
GL	glomerular layer	**SGC**	stratum griseum centrale
H	hyperpallium	**SGFS**	stratum griseum et fibrosum superficiale
HA	hyperpallium accessorium	**SPC**	striatopallidal complex
HD	hyperstriatum densocellulare	**SPL**	nucleus spiriformis lateralis
HI	hyperpallium intercalatum	**SRt**	nucleus subrotundus
Hp	hippocampus	**SSp**	nucleus supraspinalis
Imc	nucleus isthmi pars magnocellularis	**SO**	stratum opticum
Ipc	nucleus isthmi, pars parvocellularis	**T**	telencephalon
IPL	inner plexiform layer	**TeO**	tectum opticum
MCL	mitral cell layer	**V**	ventricle
MLd	nucleus mesencephalicus lateralis, pars dorsalis	**VeD**	nucleus vestibularis descendens
M	mesopallium	**VeM**	nucleus vestibularis medialis
Md	mesopallium dorsale		

CHAPTER FOUR

ANATOMY AND HISTOLOGY OF THE OLFACTORY BULB OF THE KAKAPO (*STRIGOPS HABROPTILUS*) IN COMPARISON TO OTHER AUSTRALASIAN PARROTS

Kakapo skeleton from Andreas Reischek and stored at the Natural History Museum in Vienna, Austria. Picture by A. Gsell

4.1 Abstract

One unusual feature of the kakapo (*Strigops habroptilus*), a New Zealand endemic parrot is its strong body-odour. However, little is known about the kakapo's ability to sense odours. To make the best decisions to conserve this critically endangered species, a better understanding of its sensory abilities is imperative. I dissected the olfactory bulb of an old male kakapo specimen and compared the volume against the volume of the telencephalon, the brainstem and the whole brain in a number of Australasian parrots. The volumes of the olfactory bulb layers were regressed against the olfactory bulb volume and the number of mitral cells in the mitral cell layer was compared across the species used. The kakapo's olfactory bulb volume was significantly larger compared to the ofactory bulb volume of all other compared parrots. Larger brained birds, such as the kakapo, are distinctive in having both a main- and an associated olfactory bulb, the later consisting of ependymal and ventricular layers. Smaller brained birds, in contrast, lack those two layers and therefore have only a main olfactory bulb, consisting of seven different layers. Ependym and ventricle were deeply embedded into the periventricular layer in the kakapo, which was significantly larger compared to those of other parrots. The other layer that was significantly larger in the kakapo compared to that of any other bird was the mitral cell layer. The kakapo was unusual in having not only a significantly larger mitral cell layer volume compared to that of other parrots, but also in having an unparalleled number of mitral cells compared to any other species (avian and mammalian) studied to date. The results suggest that the nocturnal, lek-breeding kakapo might rely on olfaction in a much greater extent than so far suggested.

4.2 Introduction

The use of scent in social interactions and behaviour has been studied in invertebrates and a large number of vertebrate species (Darwin 1871; Ralls 1971; Gorman 1990; Wyatt 2003; Müller-Schwarze 2006; Kaupp 2010), but rarely in birds. As birds generally display a large repertoire of visual and aural social behaviours, the potential use of smell was assumed unimportant. However, birds are capable of both producing and sensing odours. Anatomical examinations of the olfactory apparatus were among the first attempts to assess whether birds possess olfactory abilities (Edinger 1903; Hill 1905; Strong 1911). Based on the size of the avian olfactory bulb, Strong (1911) concluded that the sense of smell in birds should be considered functional.

Bang and Cobb (1968) compared the size of the olfactory bulb in 108 bird species by calculating the ratio of the greatest diameter (no matter in what axis) of the olfactory bulb to the greatest diameter

(no matter in what axis) of the corresponding cerebral hemisphere (Figure 4.1). Olfactory acuity, the ability to discriminate between different smells, is thereby based on the assumption that an increase in size of an organ reflects its increased importance in function. This correlation has been exemplified by numerous studies (Jerison 1973; Mace *et al*. 1980; Healy & Rowe 2007). In vertebrates, for example, the size of the olfactory bulb has long been suggested to be correlated with olfactory abilities (Edinger 1908). The number of mitral cells seems to correlate positively with olfactory acuity (Mackay-Sim & Royet 2006). Equally, the number of odour receptors (Mori *et al.* 1999) is positively correlated with the number of olfactory receptor genes (Steiger *et al.* 2008).

Bang and Cobbs'(1968) extensive study pioneered what has been reinforced by many studies on avian olfaction in the last decade. Olfaction seems to play an important role in birds and is elemental to a wide variety of tasks such as homing, individual, partner and nest recognition, mate attraction and the search for food (Nevitt 1999; Bonadonna & Bretagnolle 2002; Hagelin *et al*. 2003; Bonadonna & Nevitt 2004; Bonadonna *et al*. 2007; Soini *et al*. 2007). One group in Bang and Cobb's analysis had particularly small olfactory bulbs: the Psittacidae (Figure 4.1).

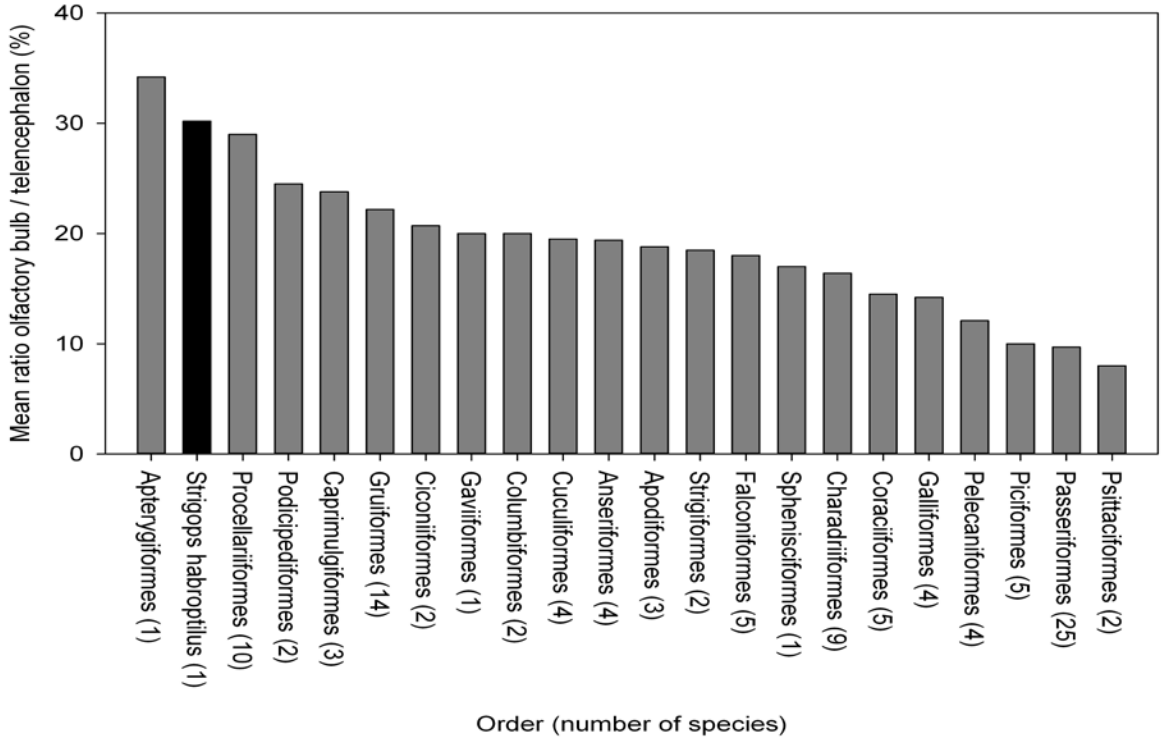


Figure 4.1 Relative size of the olfactory bulb in 108 species of birds. The graph is plotted from data of Bang and Cobb (1968) and integrates data from Hagelin (2004) in black. The mean ratio of olfactory bulb / telencephalon is derived from the greatest diameter (no matter in what axis) of one olfactory bulb divided by the greatest diameter (no matter in what axis) of the corresponding cerebral hemi-

sphere. Mean ratios for each order have been used. The number of species used is indicated in brackets.

Nonetheless, olfactory ability in parrots was established by Roper (2003) who found that yellow-backed chattering lories (*Lorius garrulus flavopalliatus*) can distinguish between different fruit odours. Hagelin (2004) later reported that the kakapo (*Strigops habroptilus*) also has olfactory abilities, which it uses to find food. The same method as used by Cobb and Bang (1968) was employed by Hagelin to measure the olfactory bulb ratio of a kakapo (Hagelin 2004). Unlike the two Psittacidae (budgeriar (*Melopsittacus undulatus*) and rose-ringed parakeet (*Psittaculak rameri*)) examined by Bang & Cobb (1968), Hagelin's kakapo stood out by having a very large olfactory bulb (Figure 4.1), second in size to that of the brown kiwi (*Apteryx australis*). Kiwi are a New Zealand endemic species known to use olfactory cues (Wenzel 1971b; Jenkins 2001; Corfield 2009; Castro *et al*. 2010). Hagelin's result inspired me to examine this relationship further.

4.2.1 A unique opportunity

When a kakapo died from a crop infection at Auckland Zoo (Auckland, New Zealand) in October 2008, a unique opportunity arose to examine the histology of the brain and the olfactory bulb in detail. The kakapo is the largest and heaviest parrot worldwide; an obligate herbivore, flightless, nocturnal (Powlesland *et al*. 2006) and the only parrot that exhibits a lek breeding system (Merton *et al*. 1984). The kakapo is also distinguished by another unusual feature in that it gives off a strong body-odour (Butler 1989; Eason *et al.* 2005). Its odour makes it highly vulnerable to introduced mammalian predators, such as stoats (*Mustela erminea*), weasel (*M. nivalis*) and ferrets (*M. furo*), cats (*Felis catus*), dogs (*Canis lupus familiaris*), three species of rats (*Rattus rattus*; *R. Norvegicus* and *R. exulans*) and mice (*Mus musculus*) (Atkinson 1985; Atkinson 2006; Towns *et al.* 2006; Jones *et al.* 2008; Clavero *et al.* 2009), which find their prey mainly by using olfactory cues. But the same mechanism also has been used in favour of the kakapo. In the effort to conserve this critically endangered species, kakapo-scent trained search dogs were used to locate the few remaining wild birds (Hill & Hill 1987; Ballance 2007). The kakapo also actively uses its sense of smell (Hagelin 2004). Even though olfaction is likely to play an important component in the daily behaviour of kakapo, hardly anything is known about the olfactory or any other sensory abilities of kakapo. In order to make the best decisions when conserving this enigmatic species a better understanding of the sensory abilities is imperative.

4.2.2 The olfactory bulb, its layers and their functionality

The main olfactory bulb of all vertebrates is composed of multiple, and in birds arch-shaped, layers, the cytoarchitecture and functional organisation of which are conserved throughout all vertebrates (Rose 1914; Andres 2008; Su *et al*. 2009). Nieuwenhuys (1966) divided the main avian olfactory bulb into seven layers, which make up the so-called proper olfactory bulb, a region that neurophysiologically analyses the input from the olfactory epithelium. From surface to deep these are: olfactory nerve fila (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), granule cell layer (GCL) and periventricular layer (PL). The size and structure of each layer is specific to each taxon and species according to its ecological and behavioural needs and possibly also its phylogeny (Wenzel 1987; Andres 2008). Birds with smaller olfactory bulbs also tend to have less distinct layers with some layers missing and others merged (Crosby & Schnitzlein 1982; Wenzel 1987). The associated olfactory bulb consists of the associated vegetative cells and is characterised by the ventricle and the associated ependym (McLean & Shipley 1992). Although not involved in olfactory processing as such, these layers are involved in tissue functions, which are important for the basic biological needs of the olfactory bulb, such as providing new granule cells in the early life of the animal and other vegetative functions (Meisami & Bhatnagar 1998).

Functionally, external odour molecules are sampled through breathing or actively "sniffing" (Hagelin *et al*. 2003) and enter the nasal cavity via the nares. The transformation from an external signal to a neural response starts when these molecules encounter the olfactory sensory neurons embedded in the olfactory epithelium lining the nasal cavity (Gomez & Celii 2008). Unlike other neurons these receptor neurons have a high turnover rate and are replaced at a constant pace, presumably to guarantee full functionality at all times (Graziadei & Monti Graziadei 1978; Vollrath *et al.* 1985). The neural coding of odour quality is thereby modulated by either a number of olfactory receptor cells, each responsible for particular properties of the odorous molecules (Buck & Axel 1991), or receptor cells might be responsive to a number of different odours (Nickell & Shipley 1992). A combination of both is likely and the intensity of an odour is meanwhile evoked by the number of receptors activated (Su *et al*. 2009). Firing rates and odour responsiveness found in avian neural receptor cells are comparable to those found in reptiles and mammals and suggest a well developed olfactory system in birds (McKeegan 2002). Likewise, the high numbers of olfactory receptor cells found in birds (Steiger *et al*. 2008; Steiger *et al.* 2009b; Steiger *et al.* 2010) are important for a fully functional olfactory system in birds.

The most superficial layer of the olfactory bulb is the olfactory nerve layer (ONL). This layer is surrounded by axons of the olfactory sensory neurons and fed with unidirectional information (Vollrath

et al. 1985; McLean & Shipley 1992). The olfactory filaments lead the information onto characteristic, spherical neuropils (Pinching & Powell 1971), the glomeruli, which make up the glomerular layer (GL). This cell-poor region sits between the olfactory fila and the external plexiform layer (EPL). Of ovoid shape, they are enclosed by a thin layer of neurons and glia cells (Kratskin 1995; Kosaka *et al.* 1998). Each glomerulus receives input from a single or a few olfactory receptor neurons (Shepherd 1991). This unidirectional connection and the physical arrangement of the glomeruli create the platform of an odorant receptor map (Uchida *et al.* 2000; Mori 2003; Mori *et al.* 2006). The glomeruli are also interconnected between each other and with the more dorsally situated mitral cells. Using a circuit of incoming and outgoing information the periglomerular cells are at the centre of information exchange and responsible for the creation of a topographic odour map while assessing quality and intensity of olfactory information (McLean & Shipley 1992; Su *et al*. 2009). The external plexiform layer (EPL) and internal plexiform layer (IPL) are both thin layers with only a low density of cell bodies. The EPL is adjacent to the glomerular layer and the mitral cell layer (MCL). It has a large number of dendrites, which originate in the mitral and the more dorsally situated granule cell layer. The densely populated cell bodies of the granule layer are distinguished by clusters of thick somata, that are the cell bodies of the neuron (McLean & Shipley 1992). In some cases neurons of the granule cell layer (GCL) fluently merge with the periventricular layer (PL). Each cell in the granule cell layer gives rise to short central dendrites and single, long apical dendrites. Long dendrites travel through the mitral cell body layer and terminate at the external plexiform layer, where they fuse with lateral dendrites of mitral and tufted cells. The dendrites receive synaptic input from mitral and tufted cells and create synaptic outputs through reciprocal dendrodendritic synapses (Kratskin 1995; Shepherd 1998). While in fish, amphibians and reptiles, the mitral cells connect with only one or a few glomeruli, in birds they send dendrites to more than eight glomeruli (Allison 1953).

As the main input and output port of the olfactory bulb, the mitral cells in the mitral cell layer essentially are responsible for all reactive responses to olfactory cues (Nieuwenhuys 1966). They project signals, by communicating through the usual electrochemical process of signal conduction via their axons (Banich & Compton 2011), from the olfactory bulb to higher brain compartments (Nickell & Shipley 1992). They are also the biggest neurons in the olfactory bulb, although of non-uniform size and shape. Usually the mitral cell layer appears in well-defined lines of one or two layers but sometimes, this layer also infiltrates neighbouring layers (Crosby & Schnitzlein 1982; Halasz 1990; McLean & Shipley 1992). Apart from their interconnection with the glomerular layer and the external plexiform layer within the olfactory bulb, efferent axons of the mitral cell layer leaving the olfactory bulb target three higher brain regions in vertebrates: the piriform cortex, the hippocampus (HP) and the

amygdala (in mammals) and the nucleus taeniae in birds. There is considerable behavioural evidence for the involvement of olfaction in homing, such as in pigeons, swifts and petrels (See Chapter One and Benvenuti *et al.* 1973; Baldaccini *et al.* 1974; Fiaschi *et al.* 1974; Papi 1991; Benvenuti *et al.* 1993; Benvenuti & Ranvaud 2004; Bonadonna *et al*. 2004; Gagliardo *et al.* 2011). Ablation experiments of the piriform cortex in pigeons showed a lessened ability to navigate through unfamiliar areas, suggesting that the piriform cortex must play a role in homing using olfactory cues (Papi & Casini 1990; Gagliardo *et al.* 2000).

Mitral axons also project into the hippocampus (HP) in pigeons (Atoji & Wild 2006), which has been shown to be involved in spatial memory tasks (Broadbent *et al.* 2004). Nocturnal Leach storm petrels (*Oceanodroma leucorhoa*) for example had larger hippocampi volumes when nesting in forest, compared to birds nesting on flat meadows. Presumably, birds living in the forest had larger spatial demands associated with returning to their nest sites at night in the darker, navigationally more demanding forests (Abbott *et al.* 1999). Leach's and other nocturnal petrels are known to use olfactory cues to home (Grubb 1974; Bonadonna & Bretagnolle 2002), hence a correlation between homing and the involvement of the hippocampus is likely. Food storing birds also have been found to have a relatively large hippocampus and it is likely, that olfaction plays a part in the proper relocation of hidden food items (Buitron & Nuechterlein 1985; Harriman & Berger 1986; Krebs *et al.* 1989; Shettleworth 2003). The nucleus taenia is located close to the striatopallidal complex (SPC) and deemed to function much in the same way as the amygdala in mammals (Reiner & Karten 1985; Cheng *et al.* 1999). The amygdala processes, among other things, associative learning based on olfactory cues (Schoenbaum *et al.* 1998).

Owing to these large and comprehensive connections between the mitral cell layer and higher brain areas, it can be assumed that the relative volume of the mitral cell layer as well as the density of cells with which it is populated, can provide information about the overall importance of the olfactory system in birds. Larger bulbs for example should have larger numbers of mitral cells. Or if they had a comparatively lower number of mitral cells, they should have comparatively larger cells that are able to process more information at the same time. Here and for the first time, I uncover the histological organisation of the brain (see Chapter Three) and the olfactory bulb of the kakapo and discuss its delineation against the forebrain. In a comparative approach, I analyse the overall size of the olfactory bulb of the kakapo and examine the different layers encompassing the olfactory bulb, particularly the mitral cell layer.

4.3 Methods

4.3.1 Specimens

The choice of parrots included in this study (Table 4.1) was based on the accessibility of suitable specimens having an intact olfactory bulb, and was particularly restricted by having to obtain the necessary permits. The brains needed to be processed and preserved within six to eight hours post-mortem, which also limited the choice of species. Additionally, the body size of the parrots, their lifestyle and phylogeny were taken into account when choosing specimens, which often led to compromises. For example, although the brain of a kea (*Nestor notabilis*) was received and would have made the perfect choice, owing to its phylogenetic relatedness to kakapo (Wright *et al*. 2008), it lacked an intact olfactory bulb. However, it was still used for the comparison of the visual system (see Chapter Five).

4.3.2 Permits

Permits for kakapo and the sulphur crested cockatoo are discussed in Chapter Three (3.3.1).

Kea (*Nestor notabilis*): A male kea's brain was obtained from the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University, Palmerston North, New Zealand in February 2009. The bird was euthanised after having sustained serious leg injuries. (Permit via Institute of Veterinary, Animal and Biomedical Sciences, Accession No.: 42724.)

Ground parrot (*Pezoporus wallicus*): A ground parrot of unknown sex was provided by Walter Boles (Australian Museum, Sydney, Australia). The specimen was found as road kill, fixed in 10% paraformaldehyde and preserved in 70% ethanol. (Transaction according to CITES resolution Conf. 11.15 (Rev.CoP12) Number: BIRD201045.)

Other parrots used: Photo micrographs of the brains of the Australian king parrot (*Alisterus scapularis*); the galah (*Cacatua roseicapilla*); the red-rumped parrot (*Psephotus haematonotus*); the Crimson rosella (*Platycercus elegans*); the cockatiel (*Nymphicus hollandicus*) and the rainbow lorikeet (*Trichoglossus haematodus*) were provided by Prof. Andrew Iwaniuk (Lethbridge University, Canada). Dr. Jeremy Corfield (Lethbridge University, Canada) ensured that the specimens were processed in the same way as mine. Since a release of the specimens was not allowed, I received photomicrographs of sagittal and coronal sections of the brains and high-resolution photo micrographs of the right olfactory bulbs. Additionally, I received slides and photo micrographs from three specimens

of Eastern rosella (*Platycercus eximius*), originally processed by Dr. J. Corfield in the laboratory of the Department of Anatomy with Radiology, School of Medical Sciences, Auckland University, Auckland, New Zealand. All materials were obtained according to institutional and governmental guidelines.

Table 4.1 List of birds used and some of their main traits

Name	Scientific name	Body size	Emit smell	Use scent	Diurnal	Crepus-cular	Noctur-nal	Endemic	References
kakapo	*Strigops habropti-lus*	large	yes	yes	-	-	yes	yes	(Best 1925; Butler 1989; Powlesland *et al*. 2006; Tipa 2006; Corfield *et al*. 2011)
kea	*Nestor notabilis*	large	-	yes	Yes	yes	yes	yes	(Gsell *et al*. 2012)
sulphur-crested cockatoo	*Cacatua galerita*	large	-	unkn.	Yes	yes	-	-	(Juniper & Parr 1998; Higgins 1999)
Australian king parrot	*Alisterus scapularis*	large	-	unkn.	Yes	-	-	-	(Juniper & Parr 1998; Higgins 1999)
galah	*Cacatua roseicapilla*	medium	yes	unkn.	Yes	yes	-	-	(Juniper & Parr 1998; Higgins 1999; Forshaw 2002)
Eastern ground parrot	*Pezoporus wallicus*	medium	yes	unkn.	Yes	-	-	-	(McFarland 1991a; McFarland 1991b; McFarland 1991c)
red-rumped parrot	*Psephotus haematonotus*	medium	-	unkn.	Yes	-	-	-	(Juniper & Parr 1998; Higgins 1999)
crimson rosella	*Platycercus elegans*	medium	-	unkn.	Yes	-	-	-	(Juniper & Parr 1998; Higgins 1999)
Eastern rosella	*Platycercus eximius*	medium	yes	unkn.	Yes	-	-	-	(Juniper & Parr 1998; Higgins 1999)
cockatiel	*Nymphicus hollandicus*	small	-	unkn.	Yes	-	-	-	(Juniper & Parr 1998; Higgins 1999)
rainbow lorikeet	*Trichoglossus haematodus*	small	-	unkn.	Yes	-	-	-	(Juniper & Parr 1998; Higgins 1999)

4.3.3 Preparation of brains and sectioning

All specimens were dissected and the brain (kakapo, kea, ground parrot and cockatoo) and eyes (kakapo, cockatoo) were immersion-fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) until they had hardened (two weeks for the ground parrot and four weeks for the kakapo, kea and cockatoo). The brain of the kea was dissected and immersion-fixed by IVABS before being sent to me for analysis. It had a badly damaged olfactory bulb, hindbrain and cerebellum so that its examination was limited to the optic lobe and telencephalon (see Chapter Five). To compare brain anatomy, all specimens were processed in a similar way. All of them were blocked mid-sagittally with a razor blade and the right hemisphere of each brain was cryoprotected in 30% sucrose in 0.01 M PBS, which was renewed daily. Once the penetration of sucrose was complete the brain tissue sank to the bottom of the container. With the large brains of kakapo, kea and the cockatoo this process took two and a half weeks; the brain of the ground parrot needed one week. Subsequently the brains were embedded in a solution of 15% gelatine with 30% sucrose at 45°C for one hour and then placed into a prepared mould to allow them to cool and harden at 4°C. Pins were positioned around the brain and into the hardening gelatine block to create fiduciary points (Figure 4.2a), that later could be used to correctly orientate the brain sections (see section 4.3.7). The hardened block was immersed in paraformaldehyde overnight, the pins were removed and the block was serially sectioned in the sagittal plane on a sliding freezing stage microtome at a thickness of 50 µm (kea and ground parrot) and 45 µm (kakapo and cockatoo). The sections were subsequently collected in PBS, mounted onto subbed slides, stained with cresyl violet, dehydrated and cover slipped with DePeX (Serva GmbH) from xylene. For sections used, see Table 4.2.

4.3.4 Shrinkage factors

Shrinkage factors were calculated by comparing brain volumes prior to processing with brain volumes calculated by measuring serial sections on the slides. The areas of entire coronal and sagittal sections were measured throughout the brain and multiplied by section thickness and the sampling interval. The difference between this measurement and the original brain volume yielded a shrinkage factor, which was subsequently applied to all of my measurements (as in Boire 1989; Rehkamper *et al.* 1991; Ebinger *et al.* 1992; Ebinger 1995; Iwaniuk & Hurd 2005; Iwaniuk & Wylie 2007).

Table 4.2 Overview of parrots used, including scientific name, the plane the brain was sectioned (sagittally or coronally), section thickness, which sections were used for analysis, the brain weight (Wt) in grams as well as indication (Photo only) where photomicrographs only were available. The order shown in this table relates to the average brain size of the birds in grams, with the largest birds at the top.

Name	Plane sectioned	Section thickness		Section used		Brain Wt (g)	Photo only
kakapo	sagittal	45	µm	Every	2nd	15.3	no
kea	sagittal	45	µm	Every	2nd	13.87	no
sulphur-crested cockatoo	sagittal	45	µm	Every	2nd	11.3	no
Australian king parrot	coronal	40	µm	Every	2nd	4.56	yes
galah	coronal	40	µm	Every	4th	7.72	yes
ground parrot	sagittal	40	µm	Every		2.6	no
red-rumped parrot	sagittal	40	µm	Every	2nd	1.86	yes
crimson rosella	sagittal	40	µm	Every	4th	4.09	yes
Eastern rosella (1)	sagittal	50	µm	Every	2nd	2.71	no
Eastern rosella (2)	sagittal	50	µm	Every	2nd	2.60	no
Eastern rosella (3)	sagittal	50	µm	Every	2nd	2.99	no
cockatiel	sagittal	40	µm	Every	2nd	2.41	yes
rainbow lorikeet	sagittal	40	µm	Every	4th	3.45	no

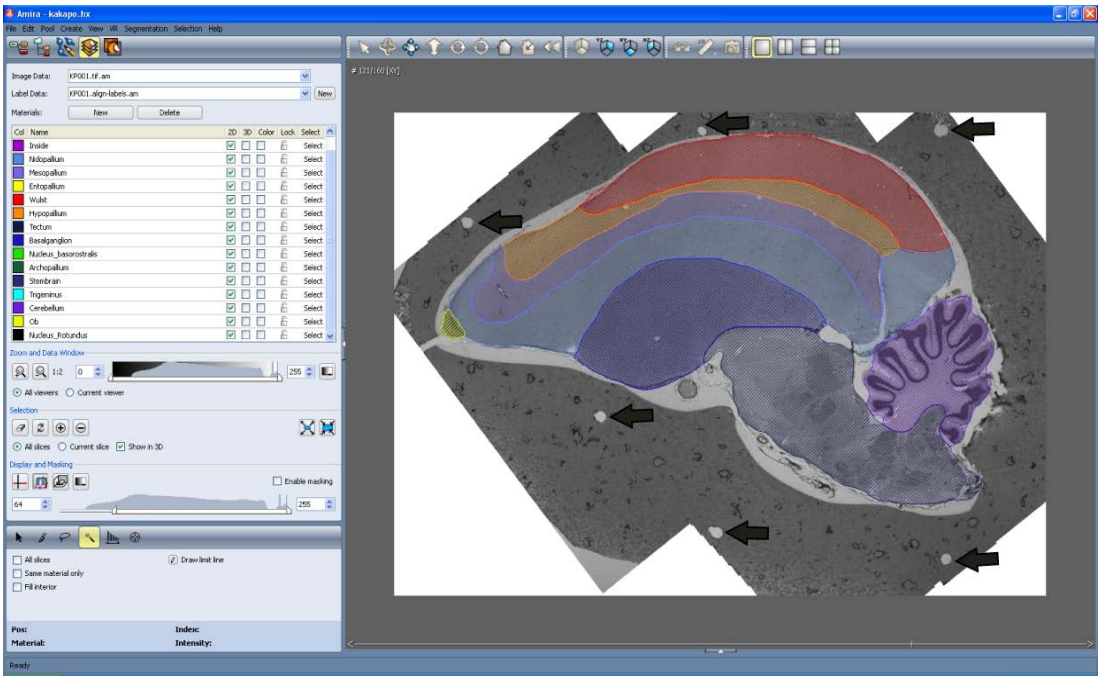
4.3.5 Creation of photo-micrographs

For the brain areas, brain sections from kakapo, sulphur-crested cockatoo, Eastern ground parrot and Eastern rosella, including a scale bar (as reference), were imaged using a Leica stereomicroscope. For the olfactory bulb and its layers, high-resolution photo-micrographs of the olfactory bulb, including a scale bar (as reference), were obtained from kakapo, sulphur-crested cockatoo and Eastern rosella using a Leica DC 500 camera and a 2x objective and 10x ocular lens.

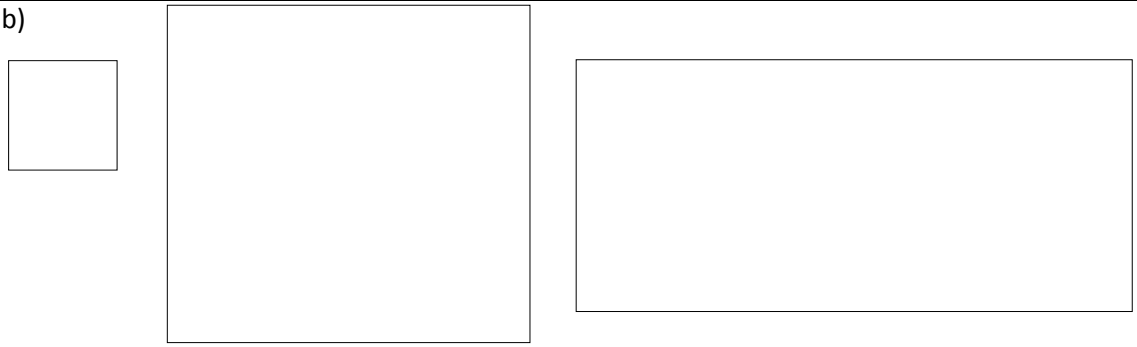
4.3.6 Modelling of brain compartments in Amira

These pictures as well as the brain and the olfactory bulb photo-micrographs provided by Dr. A. Iwaniuk and Dr. J. Corfield (see section 4.3.2 and Table 4.1), were assembled with Adobe Photoshop CS4 (Adobe Systems complex, San José, CA, US). Subsequently the pictures and their corresponding scale bar images were loaded into Amira (v 5.2, Mercury Computer Systems, San Diego, CA, US) for alignment and modelling. The imported pictures were aligned according to their fiduciary points, so that each picture corresponded to the orientation of the previous one. Each brain compartment was then individually labelled for each picture loaded in Amira (as in Figure 4.2a) using a different colour code provided by the programme menu. The mesopallium for example, was labelled throughout all images with the same colour and the nidopallium was labelled with a different colour. Amira recognises objects of the same colour code and from different pictures as a single object, which is the basis for the volumetric calculation of the brain compartments.

a)



b)



ob	basalganglia	Wulst

Figure 4.2 3D modelling using Amira for volume calculations. **a)** Screenshot of the user interface of Amira used to create 3D models of the brains. The panel on the left contains the material and colour codes objects and the tools that are used to segment and select different regions to be modelled. The panel on the right shows an example of a brain section and how different regions were labelled. The arrows indicate the fiduciary points, which were used to align the sections. **b)** Three examples of the pictures that were exported from Amira to imageJ for the volumetric analysis.

4.3.7 Delineation of brain compartments

I identified the following compartments: the total brain volume, the telencephalon and the brain-stem (Chapter Four), the entopallium (E), the nucleus rotundus (nRt) and the optic tectum (TeO) (see Chapter Five). The total brain volume encompassed all regions of the brain, except the optic- (NO)

and trigeminal (NV) nerves. The telencephalon included the hippocampus, mesopallium, nidopallium, archopallium, stratium and area corticoidea dorsolateralis (CDL). The brainstem, as defined here, included the hindbrain, midbrain and thalamus. In terms of the olfactory bulb, I defined its delineation or border against the forebrain (nidopallium and mesopallium), and I identified and labelled nine olfactory bulb layers. These were the olfactory fila (ONL), the glomerular layer (GL), the external plexiform layer (EPL), the inner plexiform layer (IPL), the mitral cell layer (MCL), the granule cell layer (GCL), the periventricular layer (PL), the ependym (EP) and the ventricle (V), a fluid filled space. Where layers merged (such as IPL and MCL in the red-rumped parrot and MCL and EPL in the Eastern rosella), equal parts were assigned to either side. The identification of the different brain compartments was guided by a number of bird brain atlases and publications (Huber & Crosby 1929; Karten & Hodos 1967; Nieuwenhuys 1967; Showers 1982; Wenzel 1987; Künzel & Masson 1988; Halasz 1990; Matochik *et al*. 1991; Butler & Hodos 2005; Atoji & Wild 2006; Nixdorf-Bergweiler. B & Bischof.H 2007; Puelles *et al*. 2007; Brauth *et al*. 2010) while holding to the *Consortium Nomenclature* (Reiner *et al*. 2004; Jarvis *et al*. 2005).

4.3.8 Modelling of brain compartments

The outlines of each brain region obtained in Amira (Figure 4.2a) were exported as a series of TIFF files. The labelled brain regions were thereby filled in black against a white background (Figure 4.2b), allowing me to export a particular brain region as a compact stack of files or sections. These TIFF stacks were then used for volumetric estimates of each region using ImageJ (National Institutes of Health, US, http://rsb.info.nih.gov/ij/). Each image was then analysed to obtain the cross-sectional area of the brain region. The cross-sectional areas were added for each brain region and then multiplied by the section thickness and the number of sections (as in Table 4.2) between stacks, resulting in the total volume of that particular area (Table 4.3).

4.3.9 Data analysis of brain compartments

To account for allometric effects on brain region volume and to avoid Deacon's whole part fallacy (Deacon 1990), all measurements were examined relative to multiple scaling variables. The data were $\log_{10}$ transformed prior to all analyses and the volume of each brain region was compared with brain volume minus the volume of the region of interest. For example, the olfactory bulb volume was compared to the brain volume minus that of the olfactory bulb. Regarding the different scaling variables the olfactory bulb volume (Chapter Three) and the volumes of the entopallium, nucleus rotundus, optic tectum and Wulst (Chapter Four) were contrasted against (1) total brain volume (not including the region of interest), (2) telencephalon volume (not including the region of interest), and

(3) brainstem (not including the region of interest). To determine whether the kakapo brain differed from the brains of other parrots, I performed a box and whiskers plot in SigmaPlot12 (Systat Sofware Inc. San José, CA, US) and least squares linear regressions with fitted confidence intervals (also in SigmaPlot12) using each of the dependent variables against the scaling variables outlined above.

4.3.10 Analysis of the olfactory bulb layer size

To determine whether the olfactory bulb layers of the kakapo brain differed in size from that of other parrots, all layer measurements were examined relative to the whole volume of the olfactory bulb. The volume of each layer was compared with the olfactory bulb volume minus the volume of the layer of interest. For example, the volume of the mitral cell layer was compared with the volume of the olfactory bulb minus the volume of the mitral cell layer. Box-and whiskers plots were performed for each relevant olfactory layer.

4.3.11 Creation of line diagrams of the olfactory bulb

Photo-micrographs (as indicated in section 4.3.5) of the central part of the olfactory bulb of each species were chosen. Line diagrams were created with CorelDRAWGraphicSuiteX5© (Corel, Ottawa, ON, Canada) and Adobe Illustrator CS4 (Adobe Systems complex, San José, CA, US) (see Figs.3.6 and 3.7a-h). Volumetric calculations and line diagrams were not created for the olfactory bulbs of the Eastern ground parrot (*Pezoporus wallicus*) or the cockatiel (*Nymphicus hollandicus*), since the poor quality of the tissue and the tiny olfactory bulb, respectively, did not allow for different layers to be distinguished.

4.3.12 Mitral cell counts

High-resolution photo-micrographs of the olfactory bulb of eight different parrot species and obtained as indicated in section 4.4.1 were loaded into Adobe Photoshop CS4 (Adobe Systems complex, San José, CA, US). For each section of the olfactory bulb all mitral cells were counted using the Adobe Photoshop CS4 counting tool. The total number of mitral cells resulted from the additive value of mitral cells per section volume. Counts of the mitral cells of eight different parrot species were obtained: for kakapo, sulphur-crested cockatoo, galah, three specimens of Eastern rosella, crimson rosella, Australian king parrot, rainbow lorikeet and red-rumped parrot. Three separate counts were conducted in random order of species. Because most of the material was received as high-resolution photo-micrographs, I decided to conduct the mitral cell count based on the photos. In order to validate the counts though, independent cell counts (three times) using the corresponding histological slides were conducted in kakapo, sulphur-crested cockatoo and the three specimens

of Eastern rosella. These resulted in comparable numbers compared to counts using photo-micrographs with only minimal differences. The results were listed in Table 4.6, which includes examples from the literature (birds and mammals). Additionally, the number of mitral cells was compared to the mitral cell layer volume in all parrot species used here. Therefore, the results obtained above were averaged and $\log_{10}$ transformed prior to all analyses and compared with the standardized mitral cell layer volume (that is the volume of the mitral cell layer was compared with the olfactory bulb volume minus the volume of the mitral cell layer volume) of the parrots used here.

4.3.13 Average mitral cell length

Mitral cells for kakapo, sulphur-crested cockatoo, galah, three specimens of Eastern rosella, crimson rosella, Australian king parrot, rainbow lorikeet and red-rumped parrot were identified by their large amoeboid to triangular shape. Another criterion for identifying them was the presence of a nucleolus. High resolution photo-micrographs of the olfactory bulb of eight different parrot species, obtained as indicated in section 4.3.1, were loaded into Adobe Photoshop CS4 (Adobe Systems complex, San José, CA, US). Three slides in the central part of the olfactory bulb were chosen for the measurements. The mean length of mitral cells was evaluated by measuring 30 mitral cells with visible nucleolus; that is, 10 mitral cells on each of the three slides were chosen per species. Measurements of the amoeboid to triangular shaped cells were made by measuring the longest diameter, regardless of the shape of the cell using ImageJ (National Institutes of Health, US, http://rsb.info.nih.gov/ij/) (Figure 4.10). Additionally, the length of mitral cells was compared to the mitral cell layer volume in all parrot species used here. Therefore, the results were obtained above were averaged and $\log_{10}$ transformed prior to all analyses and compared with the standardised mitral cell layer volume (as in section 4.3.8) (Figure 4.11b).

4.3.14 Principal component analysis (PCA).

Principal component analysis (PCA) was used to correlate the number and length of mitral cells with the volume of the mitral cell layer. The data were reduced to principal components (PC) that represented correlations of peak areas between the number and length of mitral cells and the volume of the mitral cell layer in all eight parrot species.

4.3.15 *Measurement of the olfactory bulb using Bang and Cobb's (1968) method.*

Ratios of the olfactory bulb versus the cerebral hemisphere were calculated three separate times in the kakapo and the sulphur-crested cockatoo, as described in Bang and Cobb (1968), but using photo-micrographs (as in Figure 2.1). The ratio of the olfactory bulb and the cerebral hemisphere was measured as indicated in Figure 3.5 using ImageJ (National Institutes of Health, US, http://rsb.info.nih.gov/ij/). The results were compared with those obtained from Hagelin (2004) (Table 4.2).

4.4 Results

4.4.1 Olfactory bulb size

The overall size of the olfactory bulb in kakapo and other parrots was evaluated. In order to account for allometric effects of the varying size of brains, the relative relationship between the olfactory bulb against: (1) brainstem volume, (2) telencephalon volume and (3) total brain volume was analysed. The data were regressed and a 95% confidence interval was fitted using SigmaPlot12 (Systat Sofware Inc. San José, CA, US). Regardless of the scale the olfactory bulbs were contrasted with, the kakapo's olfactory bulb was significantly larger than the bulbs of all other parrots and exceeded the 95% confidence interval (Figure 4.3). Although the olfactory bulb of a kakapo comprises only about 0.32% of the whole brain, it appeared as a strong outlier (Figure 4.3).

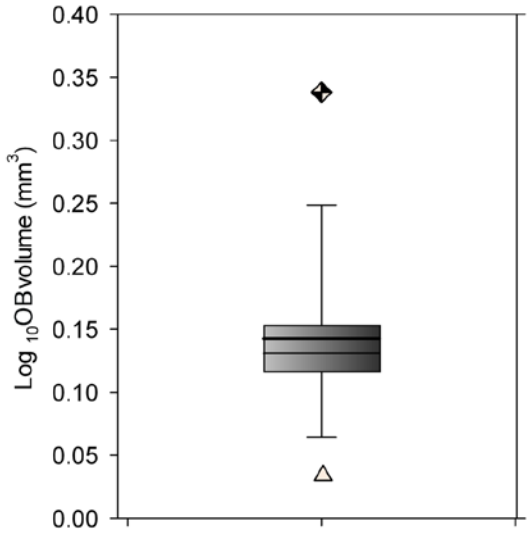


Figure 4.3 Box plot of the olfactory bulb volume in kakapo and nine Australasian parrots (log_{10} and corrected for brain size and in mm^3). The box plot shows the smallest observation (sample minimum), lower quartile (Q1), median (Q2), upper quartile (Q3), and largest observation (sample maximum). Outliers are kakapo (at the top) and cockatiel (at the bottom).

Apart from kakapo, the only other parrot with a relatively larger olfactory bulb than its brain size would suggest was the Australian king parrot, although this was by no means as pronounced as in kakapo (Figure 4.4). Only in one case, the cockatiel does the olfactory bulb appear relatively smaller

than expected (Figure 4.3 and 4.4). Close to the upper boundary and the 95% CI were the rainbow lorikeet and the crimson rosella, whereas the galah and the Eastern rosella were at the lower limit and therefore had slightly smaller olfactory bulbs than would be expected. The ground parrot, the sulphur-crested cockatoo and the red-rumped parrot all had olfactory bulbs whose sizes were in the expected range (Figure 4.4). Table 4.3 lists all volumetric measurements of the brain and of the olfactory bulb.

Finally, percentages of the olfactory bulb within the cerebral hemisphere was calculated as described in Bang and Cobb (1968) and Figure 4.5 and using photo-micrographs (as in Figure 3.1). While Hagelin (2004) reported a percentage of 30.2% in kakapo, I calculated a percentage of 21.1% for kakapo and 2.1% for sulphur-crested cockatoo (Table 4.4).

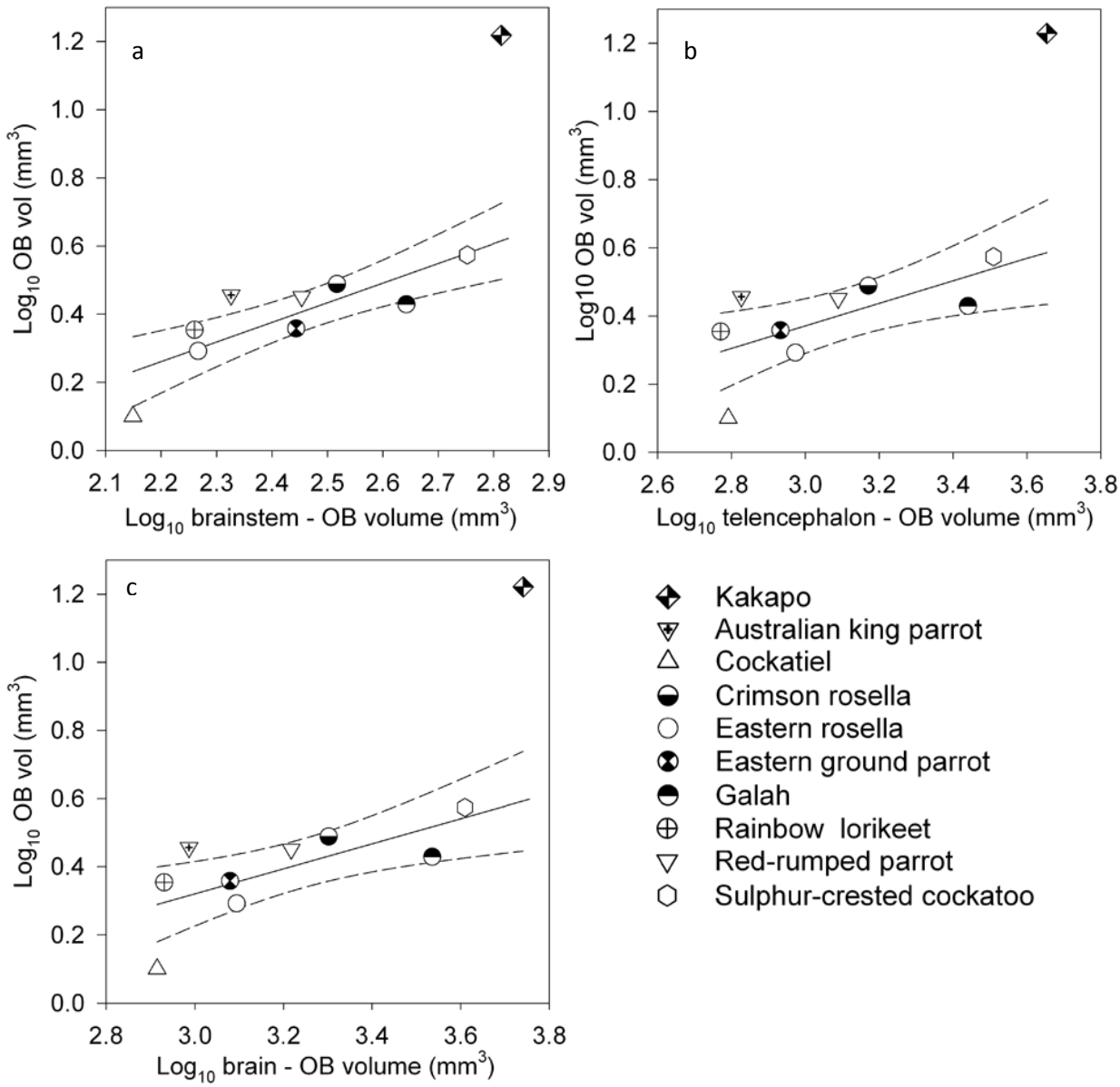
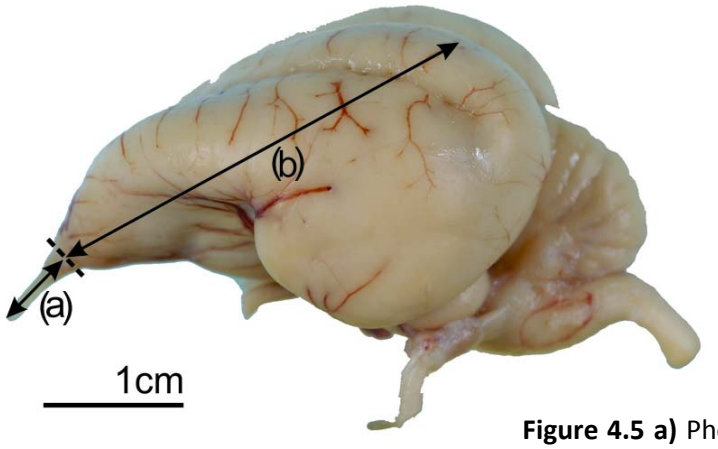


Figure 4.4 Scatter-plots of the olfactory bulb measured against a) brainstem volume b) telencephalon and c) total brain volume. The solid lines indicate the least-squares linear regression lines and dotted lines indicate the 95% confidence interval. The diamond: ✥ represents the kakapo.

Table 4.3 Volumetric measurements of the brain, olfactory bulb, olfactory bulb layers and visual centres (see Chapter Five) in mm^3. Brain volumetric measurements pertain to the whole brain. The brainstem included the hindbrain, midbrain and thalamus. Abbreviations used: T: telencephalon; Ce: cerebellum; E: entopallium; nRt: nucleus rotundus; TeO: optic tectum; Ha: hyperpallium apicale / Wulst; OB: olfactory bulb; ONL: olfactory fila; Gl: glomerular layer; EPL: external plexiform layer; MCL: mitral cell layer; IPL: internal plexiform layer; GCL: granule cell layer; PL: periventricular layer; E: ependym; V: ventricle. Mcells counted: number of mitral cells counted on all mitral cell layers: Mitral cells / mm^3: number of mitral cells calculated based on the mitral cell count and the mitral cell layer volume. Numbers in brackets symbolise different individuals measured and their averaged values (av).

	Brain measurements																			
	Brain volumetric measurements				**Vision measurements**				**Olfactory bulb measurements**										**Mitral cell counts**	
Species	**Brain**	**Brainstem**	**T**	**Ce**	**E**	**nRt**	**TeO**	**Ha**	**OB**	**ONL**	**GL**	**EPL**	**MCL**	**IPL**	**GCL**	**PL**	**EP**	**V**	**Mcells counted**	**Mitral cells/mm^3**
Kakapo	**5517.80**	**669.38**	**4546.58**	**303.05**	**4.68**	**0.98**	**75.75**	**487.34**	**17.39**	**2.20**	**1.95**	**1.95**	**0.53**	**0.42**	**3.66**	**5.07**	**0.91**	**0.70**	**5576**	**253904.00**
Kea	5002.20		4546.58		8.92	2.06	197.47	418.08												
Sulphur-crested cockatoo	4071.05	567.66	3237.02	265.81	7.39	4.52	93.97	265.72	1.25	0.30	0.21	0.11	0.02	0.11	0.34	0.16			57.00	71594.27
Galah	3435.97	439.90	2760.45	235.59	5.92	3.92	82.38	203.60	1.69	0.13	0.41	0.18	0.05	0.19	0.26	0.46			630.00	135524.58
Crimson rosella	2006.64	330.20	1483.20	193.23	8.57	3.18	79.71	114.36	2.08	0.25	0.58	0.24	0.08	0.28	0.21	0.43			231.00	28262.28
Rainbow Lorokeet	1650.58	285.08	1229.67	135.73	3.47	0.84	50.31	108.38	1.83	0.43	0.32	0.23	0.03	0.11	0.28	0.40			43.00	18413.00
Eastern rosella (2)	1393.80	222.00	1021.73	150.08	0.66	0.90	51.21	73.19	0.70	0.05	0.12	0.15	0.02	0.11	0.22	0.02			50.00	24277.81
Eastern rosella (1)	1283.08	174.85	1007.41	100.82	2.96	1.29	48.85	71.21	1.21	0.17	0.23	0.24	0.02	0.14	0.38	0.13			117.00	24943.37
Eastern rosella (3)	1053.64	157.44	791.88	94.93	3.77	1.02	38.79	60.76	0.96	0.09	0.21	0.19	0.07	0.08	0.25	0.07			59.00	23689.54
Eastern rosella (av)	1243.51	184.76	940.34	115.28	2.46	1.07	46.29	68.39	0.96	0.10	0.19	0.19	0.04	0.11	0.28	0.07			75.33	24303.58
Eastern ground parrot	1200.83	278.14	857.48	65.21																
Australian king parrot	971.08	212.77	672.32	85.95	4.84	1.73	43.99	22.39	1.86	0.44	0.37	0.25	0.14	0.14	0.30	0.21	0.01	0.01	416.00	59441.90
Red-rumped parrot	852.92	182.35	590.72	79.87	1.37	1.87	46.04	45.09	1.26	0.13	0.31	0.30	0.08	0.10	0.25	0.11			91.00	18316.39
Cockatiel (2)	1169.52	191.37	889.45	88.68	2.10	1.75	68.20	68.20												
Cockatiel (1)	474.13	88.90	346.06	37.51	1.56	0.85	15.65	13.72												
Cockatiel (av)	821.82	140.13	617.75	63.10	1.83	1.30	41.92	40.96												



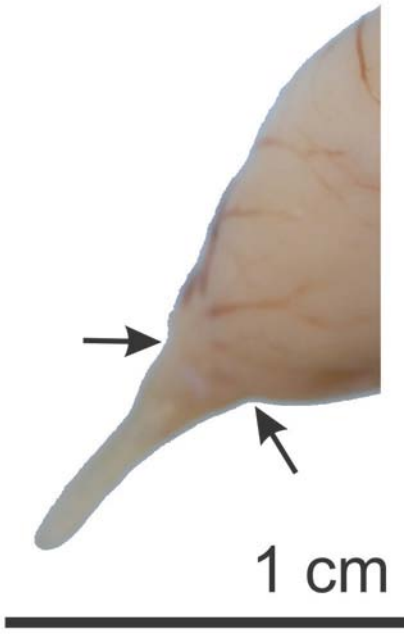


Figure 4.5 a) Photo of the kakapo brain and the ratio between (a): largest diameter of the olfactory bulb versus (b): largest diameter of the corresponding cerebral hemisphere as indicated in Bang and Cobb (1968), encompassing the area from the olfactory bulb to the hippocampus.

b) Magnified view of the olfactory bulb of a kakapo. Arrows indicate the typical groove, which indicates the proximal limit of the bulb according to Bang and Cobb (1968). Photos by Nick Duggan (Manager, AMRF Medical Sciences Learning Centre – *Whakaaro Pai,* Department of Anatomy with Radiology, The University of Auckland, Auckland, New Zealand)

Table 4.4 Olfactory bulb ratios in the kakapo and the sulphur-crested cockatoo

Olfactory bulb diameter (mm±sd), brain hemisphere diameter (mm±sd) and olfactory bulb ratio (%) of a male kakapo and a male sulphur-crested cockatoo. Kakapo** measurements are from Hagelin (2004). The ratio of the olfactory bulb versus the cerebral hemisphere was measured as indicated in Bang and Cobb (1968) and Figure 4.5.

Common name	Species	Bulb dia. (mm)	Hemisphere dia. (mm)	OB ratio (%)
Kakapo	*Strigops habroptilus*	7.25±0.04	34.35±0.16	21.1
Kakapo**	*Strigops habroptilus*	10.2±0.2	33.8±0.7	30.2
Sulphur-crested cockatoo	*Cacatua galerita*	0.76±0.03	34.88±0.16	2.1

4.4.2 Configuration and composition of the olfactory bulb layers

Only the birds with significantly larger olfactory bulbs (the kakapo and the Australian king parrot; Figure 4.4) showed evidence of the ventricle and the ependym (Figures 4.7a&b). Birds with smaller brains and a smaller olfactory bulb lacked those two layers (Figs.4.7 c-h). Therefore the majority of the parrots studied (nine out of 11) had olfactory bulbs with only seven layers composing the main olfactory bulb, in contrast to the kakapo and the Australian king parrot, which had nine layers and therefore distinguished themselves by having not only the main olfactory bulb, but also an associated olfactory bulb.

The olfactory filaments present themselves in all species as clearly visible, white, streamlined bundles of cells at the tip of the olfactory bulb. The glomerular layer was clearly identifiable in all parrots and had a dense tangle of axon terminals and adjacent glomerular cell bodies. The adjacent, thin external plexiform layer was in some species difficult to distinguish from the mitral cell layer and sometimes the two layers were merged (as in Figure 4.7d: the red-rumped parakeet). In other birds, the mitral cell layer stood out as a distinctive layer of closely packed, amoeboid to triangular shaped cells (in kakapo, Australian king parrot, rainbow lorikeet, crimson rosella and galah: Figure 4.7a-c, f&g). Sometimes, it formed groups of less densely packed cells of typical, amoeboid to triangular shape (as in red-rumped parrot, eastern rosella and sulphur-crested cockatoo: Figure 4.7d, e&h). As with the external plexiform layer, the thin internal plexiform layer was also in some cases merged with the mitral cell layer (as in Figure 4.7e: Eastern rosella), whereas in other cases it was clearly defined as an independent layer (such as in Figure 4.7a-c, f&g: kakapo, Australian king parrot, rainbow lorikeet, crimson rosella and galah). The granules in the granule cell layer were easily discerned in all specimens due to their large cell bodies. The ependym is lighter coloured than the granule cell layer and resembled a sponge with small cell bodies encasing the ventricle.

The line diagrams also show the grooves where Bang and Cobb (1968) would have measured the ‘longest diameter’ of the olfactory bulb (Figure 4.6). However, they are not the actual margin, where the bulb physiologically ends. In kakapo for example the granule cell layer and the plexiform layer extend considerably over this fictional border and are in contact with the nidopallium (Figure 4.6), and almost also with the mesopallium (Figure 4.7a) of the brain.

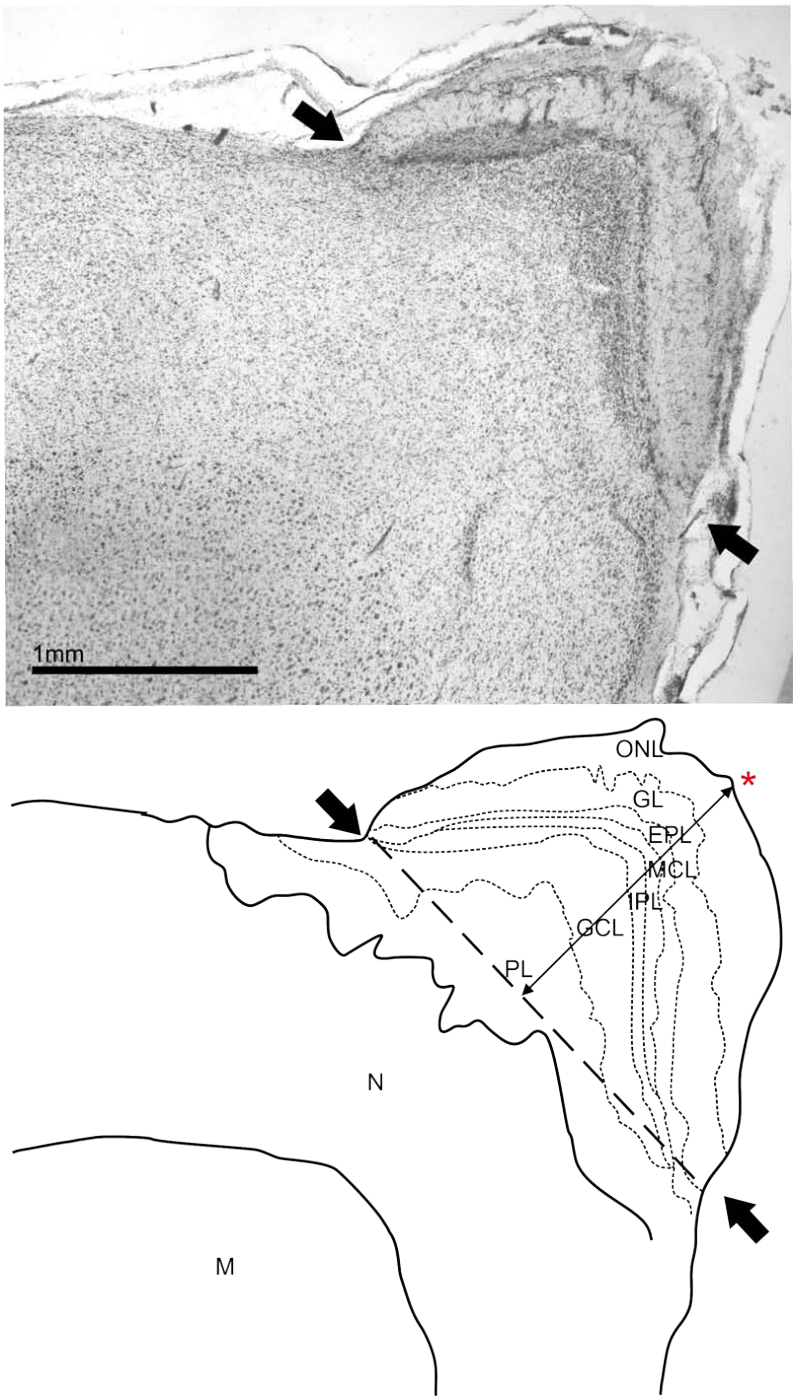
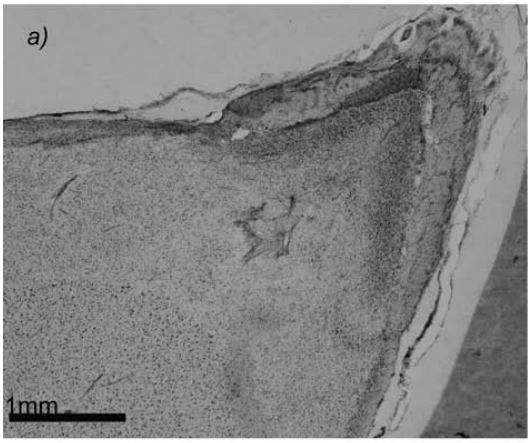
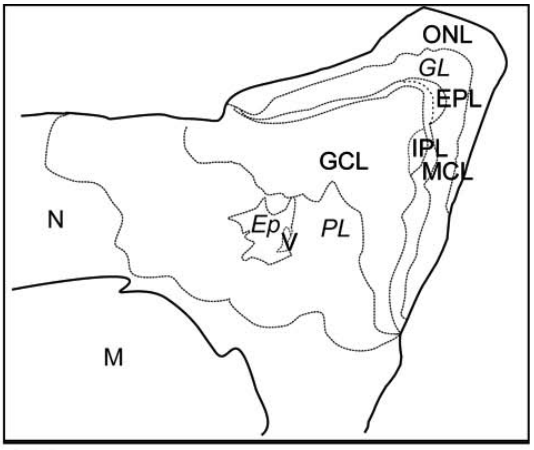
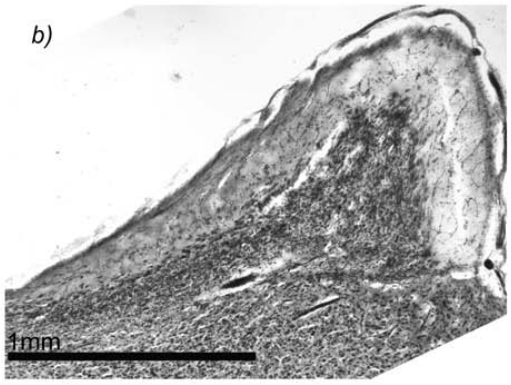


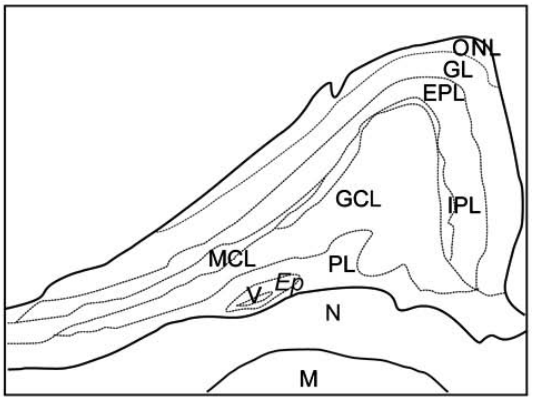
Figure 4.6 Photo-micrograph of the olfactory bulb of the kakapo and line diagram indicating the different layers. Abbreviations used: ONL: olfactory fila; GL: glomerular layer; EPL: external plexiform layer; IPL: inner plexiform layer; MCL: mitral cell layer; GCL: granule cell layer; PL: periventricular layer; Ep: ependym; V: ventricle; N: nidopallium and M: mesopallium. Scale bar: 1mm. Arrows and star indicate the location from where Bang & Cobb (1968) would have measured the 'longest diameter' of the olfactory bulb.



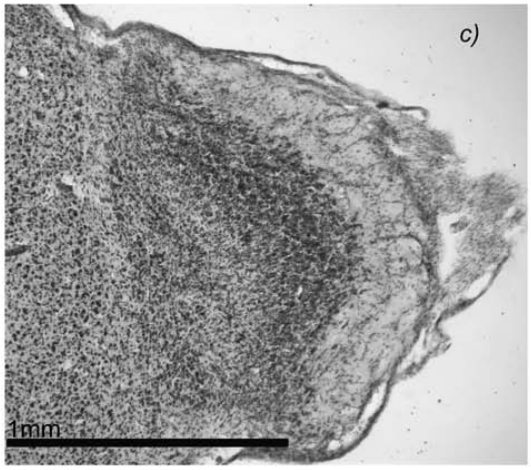


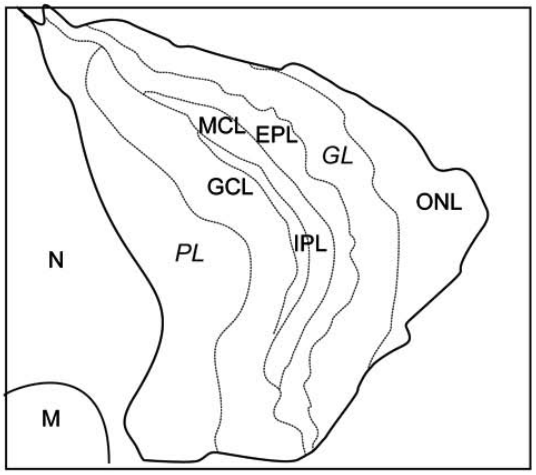
Kakapo



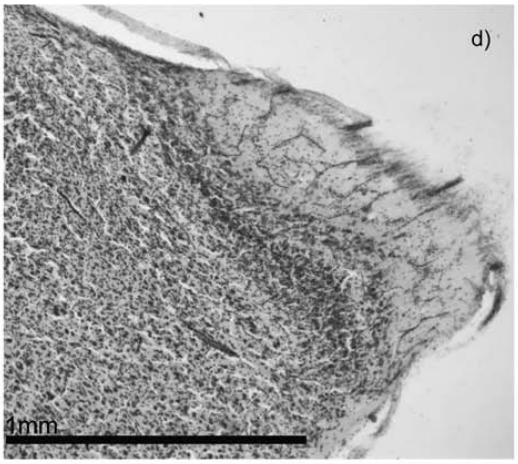


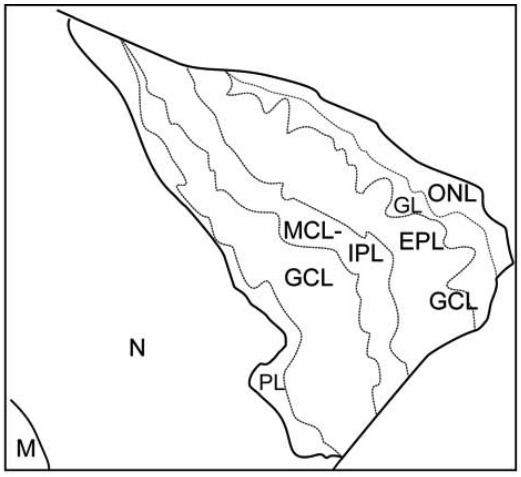
Australian king parrot



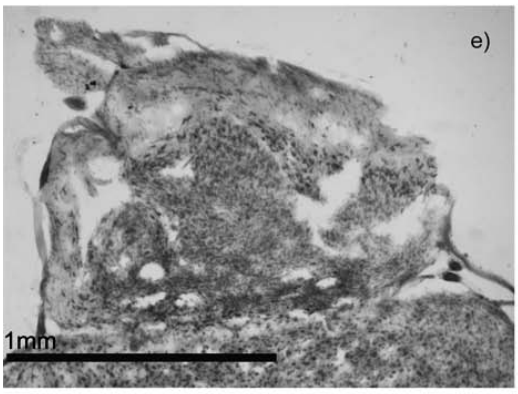


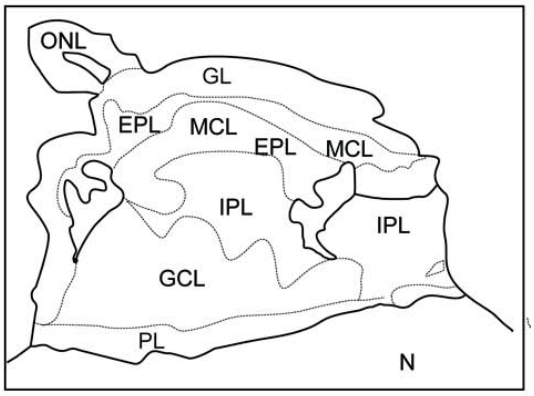
Rainbow lorikeet



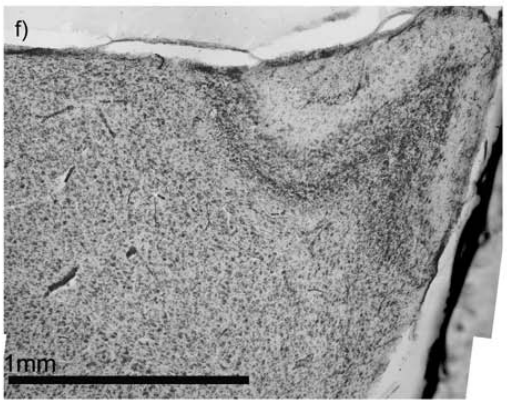


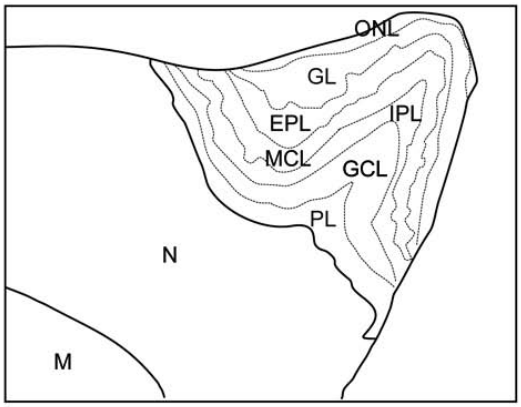
Red-rumped parrot





Eastern Rosella





Crimson Rosella

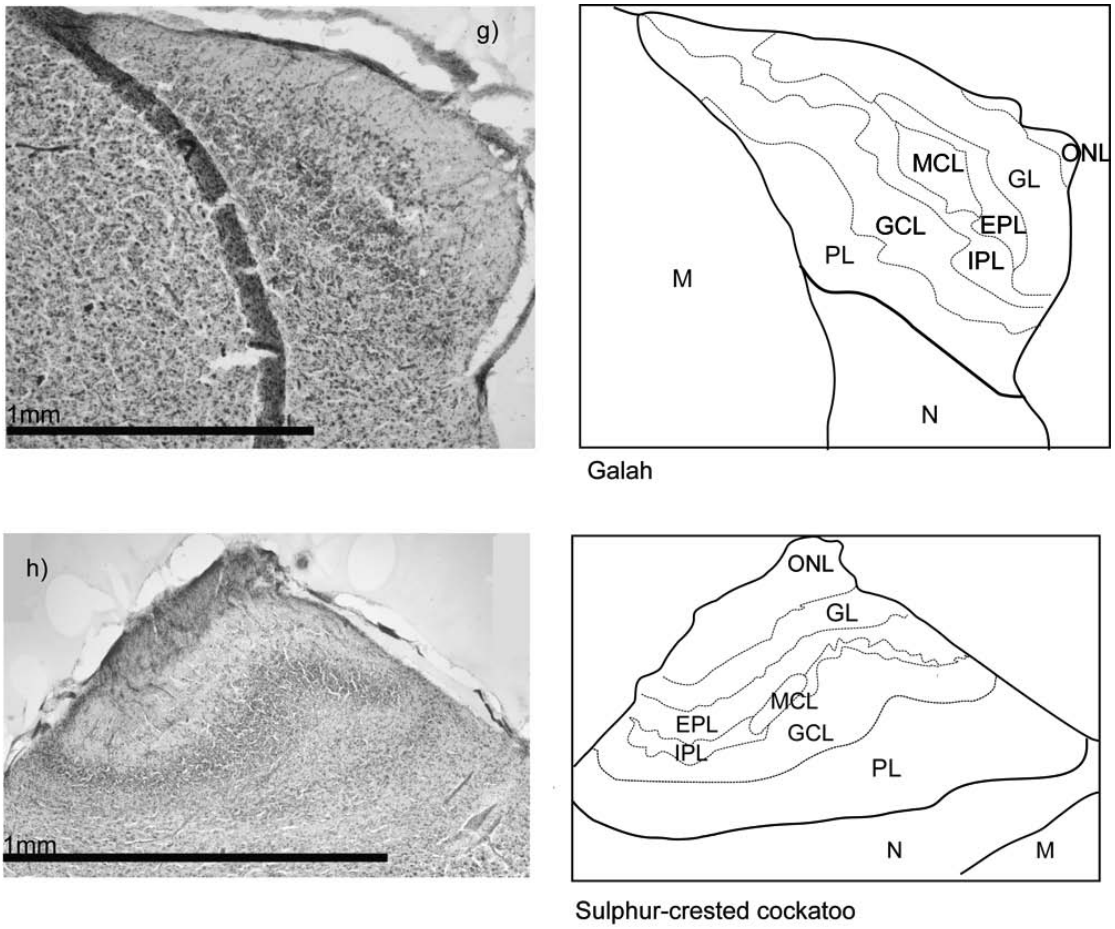


Figure 4.7 Photo-micrographs of the olfactory bulb and line diagrams of the layers identified in the olfactory bulb of a) kakapo; b) Australian king parrot; c) rainbow lorikeet; d) red-rumped parrot; e) Eastern rosella; f) crimson rosella; g) galah and h) sulphur-crested cockatoo. Figures ordered according to bulb size with the largest first. Abbreviations used: ONL: olfactory fila; GL: glomerular layer; EPL: external plexiform layer; IPL: inner plexiform layer; MCL: mitral cell layer; GCL: granule cell layer; PL: periventricular layer; Ep: ependym; V: ventricle; N: nidopallium and M: mesopallium.

4.4.3 Volumetric assessment of the olfactory bulb layers

The volumes of the olfactory bulb layers varied greatly among the eight parrot species (Figure 4.8 and Table 4.5). The periventricular layer had the largest variation among species, followed by the glomerular layer and the olfactory fila. The mitral cell layer had the smallest range of all layers and the lowest variability. Both, the periventricular layer and the granule cell layer, had the highest variability among all layers examined. External and internal plexiform layer were between those extremes (Figure 4.8). Kakapo was notable in having a significantly larger mitral cell and periventricular layer compared to all other parrots.

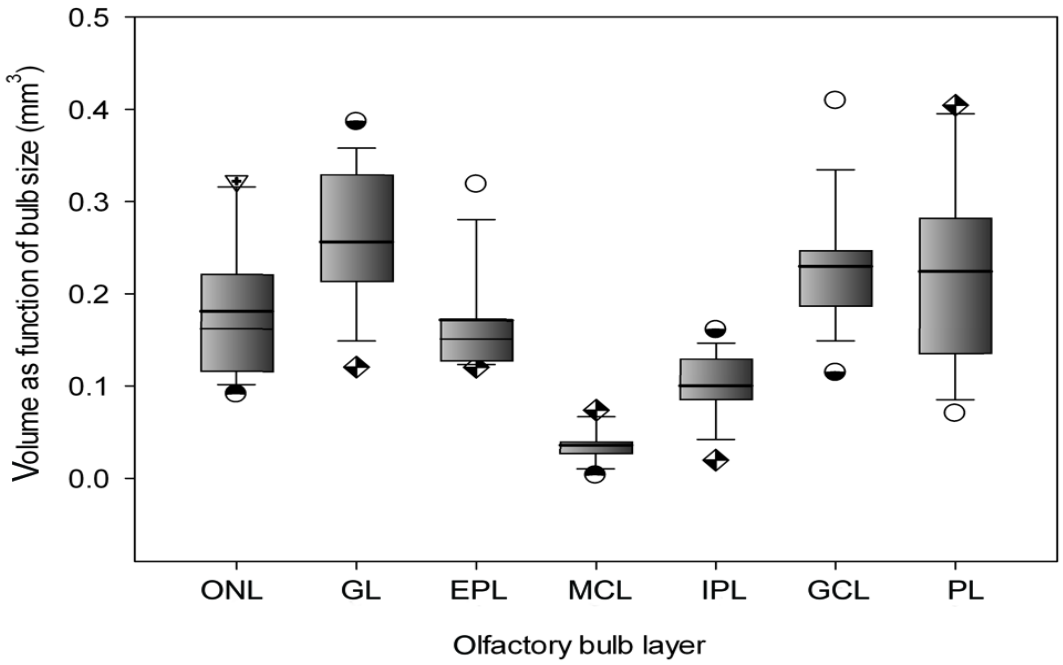


Figure 4.8 Box plot of the volumetric differences in seven layers of the olfactory bulb as a function of the olfactory bulb volume (in mm^3) across eight species of parrots. The box plot shows the smallest observation (sample minimum), lower quartile (Q1), median (Q2), upper quartile (Q3), and largest observation (sample maximum). Outliers: Australian king parrot: ⛛, galah: ◓, crimson rosella: ◒, Eastern rosella: ○, kakapo: ✥. Abbreviations used: OL: olfactory fila; GL: glomerular layer; EPL: external plexiform layer; MCL: mitral cell layer; IPL: inner plexiform layer; GCL: granule cell layer; PL: periventricular layer.

In fact, half of the olfactory bulb in kakapo is composed of just the periventricular layer (29%) and the granule cell layer (21%) (Table 4.5). In spite of having an overall significantly larger olfactory bulb compared to the other parrots (Figure 4.3 and 4.4), internal, external and glomerular layers were all significantly smaller in kakapo compared to the other parrots (Figure 4.8).

Table 4.5 Volume of the olfactory bulb layers (as a function of bulb size) in kakapo and other Australasian parrots. The size is shown as the volume of the layer divided by the volume of the bulb. 95% confidence intervals (CI) were fitted (mean ± lower and upper limit). Values that are above the 90% percentile were indicated with >, and values below the expected range with <.)

	Kakapo	Australian king parrot	Sulphur-crested cocka-too	Galah	Crimson rosella	Eastern rosella	Red rumped parrot	Rainbow lori-keet
Olfactory fila	**0.139**	0.321	0.221	0.091	0.142	0.111	0.115	0.309
CI: (0.18 ± 0.05)		>		<				>
Glomerular layer	**0.120**	0.260	0.177	0.329	0.386	0.236	0.329	0.214
CI: (0.26 ± 0.05)					>		>	
External plexiform layer	**0.120**	0.162	0.127	0.126	0.134	0.242	0.318	0.140
CI: (0.17 ± 0.04)						>	>	
Mitral cell layer	**0.074**	0.017	0.037	0.003	0.027	0.060	0.039	0.030
CI: (0.04 ± 0.01)	**>**	<		<				
Internal plexiform layer	**0.020**	0.087	0.132	0.129	0.161	0.123	0.085	0.065
CI: (0.10 ± 0.03)	**<**				>			<
Glomerular layer	**0.260**	0.198	0.241	0.187	0.115	0.409	0.246	0.183
CI: (0.23 ± 0.051)					<	>		
Periventricular layer	**0.404**	0.135	0.152	0.386	0.265	0.071	0.100	0.282
CI: (0.22 ± 0.08)	**>**			>		<		
Ependym	**0.050**	0.012	-	-	-	-	-	-
CI: (0.007 ± 0.01)	**>**	**>**						
Ventricle	**0.037**	0.012	-	-	-	-	-	-
CI: (0.006 ± 0.01)	**>**	**>**						

4.4.4 Mitral cell count

Kakapo had the highest number of mitral cells per bulb followed by the galah. The sulphur-crested cockatoo had fewer mitral cells per bulb than the northern fulmar, but more than the rock dove and king parrot (Wenzel & Meisami 1987). Rats, mice and rabbits (Smith 1928; Schönheit 1971; Meisami & Safari 1981; Royet *et al.* 1998) have between ca 40,000 and 60,000 mitral cells per bulb. All other parrots, the two rosella, the red-rumped parrot and the rainbow parrot, had a lower number of mitral cells per bulb than the mammals reported (Table 3.4).

Table 4.6 Number of mitral cells per bulb, showing the species and the source.

Species	**Number of mitral cells per bulb**	**Source**
kakapo	253,904	this thesis
galah	135,525	this thesis
northern fulmar	120,000	Wenzel & Meisami (1987)
sulphur-crested cockatoo	71,594	this thesis
rock dove	60,000	Wenzel & Meisami (1987)
common rabbit	59,600	Royet *et al*. (1998)
Australian king parrot	59,442	this thesis
norway rat (wild)	56,200	Meisami & Safari (1981)
norway rat (dom.)	55,000	Smith (1928)
house mouse	38,000	Schönheit (1971)
norway rat	37,000	Smith (1928)
crimson rosella	28,262	this thesis
eastern rosella	24,304	this thesis
rainbow lorikeet	18,414	this thesis
red-rumped parrot	18,316	this thesis

4.4.5 Mitral cell length

The average length of the mitral cells is shown in Figure 4.9. Kakapo had the longest mitral cells, followed by the northern fulmar (Wenzel & Meisami 1987), the Eastern rosella, the galah, the red-rumped parrot and the Australian kings parrot, whose mitral cells all had approximately the same length. The rainbow lorikeet and the crimson rosella had slightly smaller mitral cells, followed by the rock dove (Wenzel & Meisami 1987). The sulphur-crested cockatoo had the comparatively smallest mitral cells.

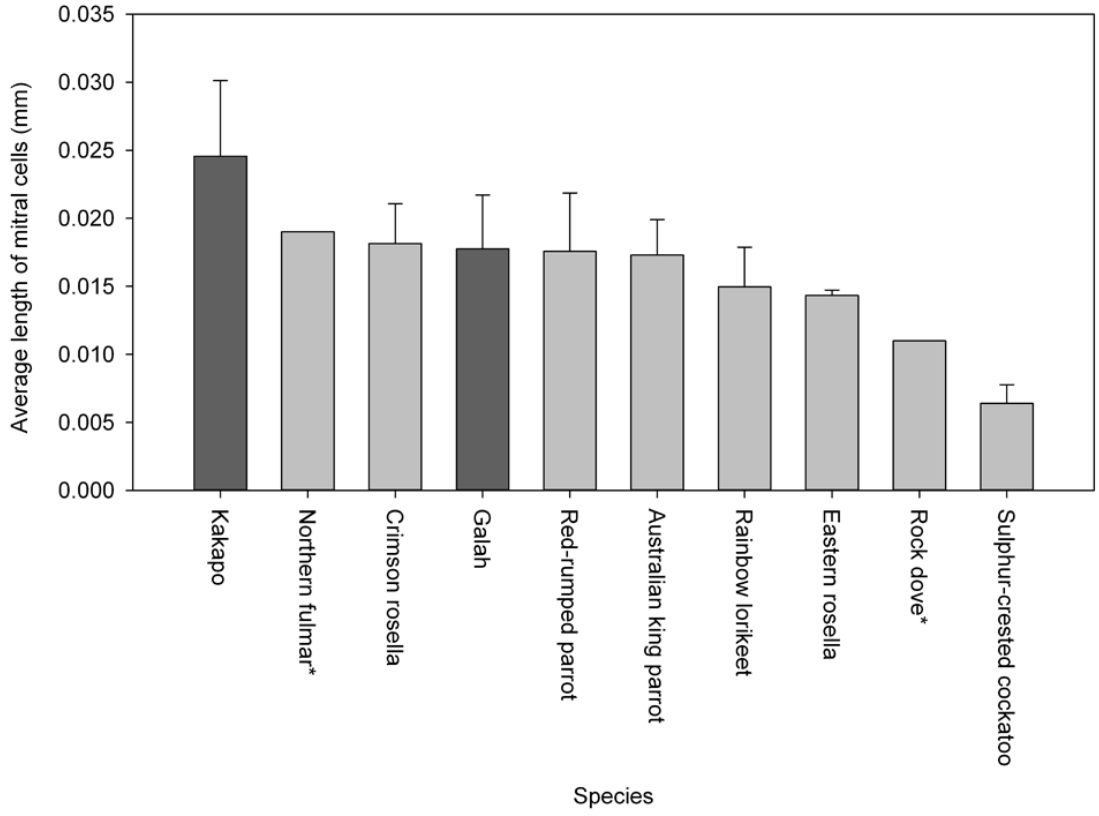


Figure 4.9 Bar-diagram of the average size and standard error of mitral cells in eight parrot species (this thesis) and two species* (*Fulmar glacialis* and *Columba livia*) taken from Wenzel and Meisami (1987).

Additionally, the number and the length of the mitral cells, was compared to the mitral cell layer volume. As indicated above, kakapo had the highest number of mitral cells and the longest mitral cells of the parrots with which it was compared (Figure 4.10a&b), even when controlling for mitral cell layer volume.

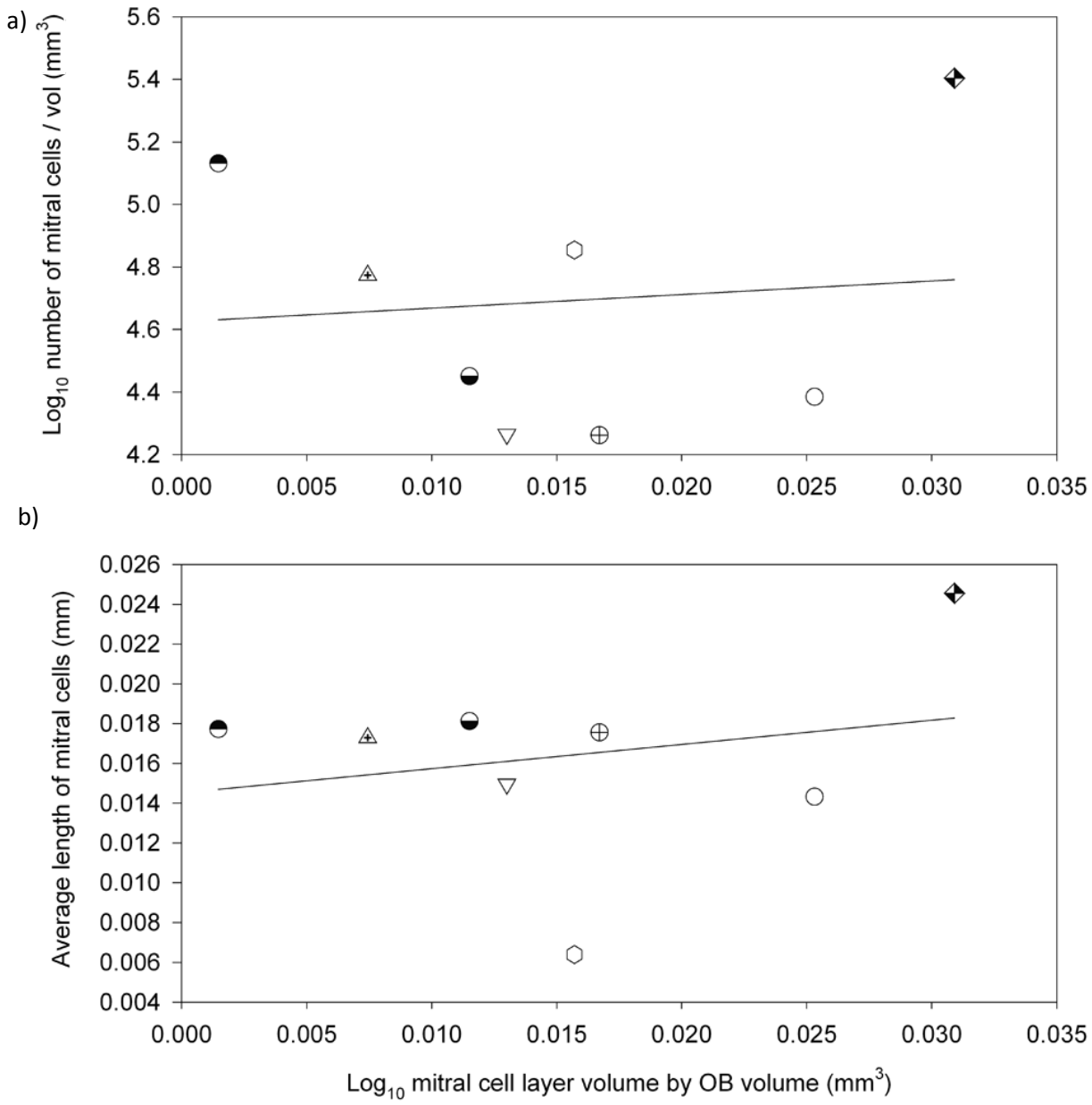


Figure 4.10 a) Scatter plot of the number of mitral cells per mm^3 in the olfactory bulb measured against the mitral cell layer. The diamond: represents the kakapo.

b) Scatter plot of the averaged length of mitral cells in mm measured against the mitral cell layer. The diamond represents the kakapo.

(Symbols used: Australian king parrot: ; sulphur-crested cockatoo: ; galah: ; crimson rosella: ; Eastern rosella: ; red-rumped parrot: ; rainbow lorikeet:)

4.4.6 Principal component analysis (PCA)

A principal component analysis (based on normalised data and Euclidean distances) was performed on the average length of mitral cells, the number of mitral cells and the volume of the mitral cell layer in the eight species of parrots. Multidimensional scaling of the data showed a separation between: (a) kakapo and galah; (b) from the sulphur-crested cockatoo and the Australian king parrot; as well as from (c) the remaining parrots. This pattern was largely driven by the number of mitral cells, which is explained by the Eigenvector Figure 4.11 that reached a value of 1. PC 1 represented 98.9% of variation among the eight species, and PC 2 represented a variation of 1.1%, whereby smaller mitral cell layer volumes had negative values and larger ones positive values.

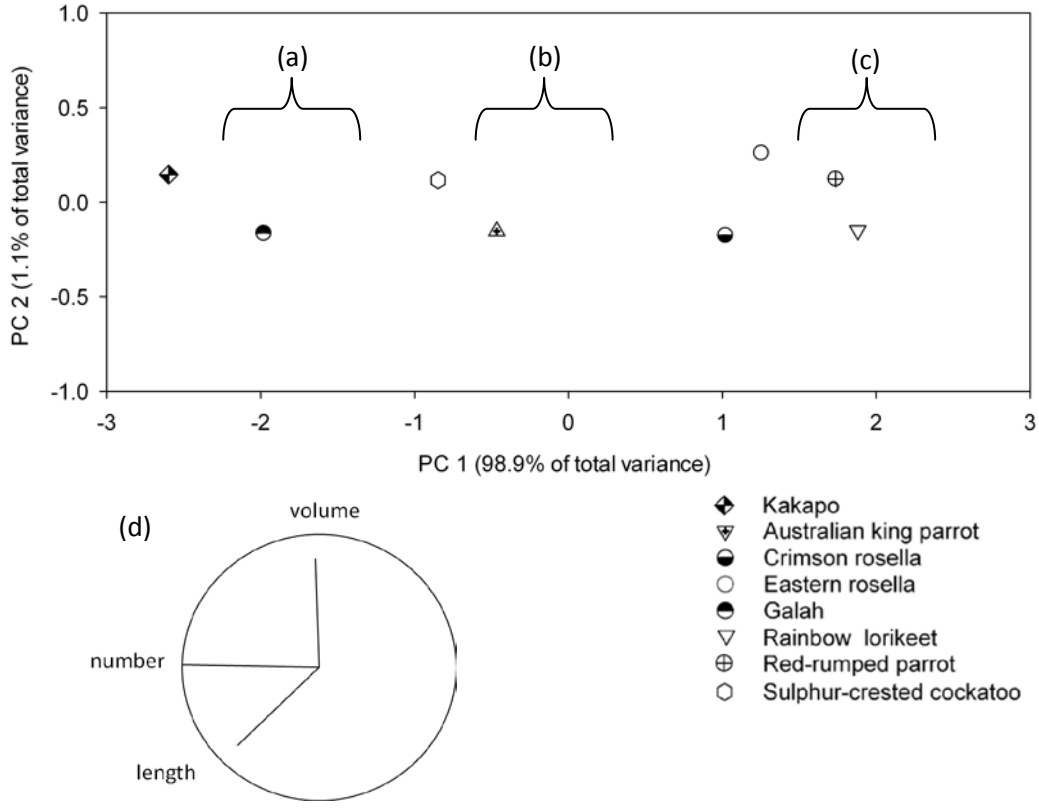


Figure 4.11 Principal component analysis (PCA) of the number of mitral cells and their averaged length. PC 1 accounted for much of the divergence of the kakapo and the galah (a) from the sulphur-crested cockatoo and the Australian king parrot (b), as well as from the remaining parrots (c). The plot is based on normalised variables and Euclidean distances. The Eigenvectors (d) show the variables: volume, number and length of mitral cells, whereby the number of mitral cells is the most important and determinant variable with a value of 1.

4.5 Discussion

4.5.1 Olfactory bulb size

In spite of numerous descriptions of the kakapo's strong body scent, little is known about its sensory abilities. The unique opportunity to conduct a comparative study of the anatomy of the brain, and in particular, of the olfactory bulb and its layers in a male kakapo led to new insights. The kakapo had a significantly larger olfactory bulb than any parrot it was compared to (Figures 4.3 and 4.4). This result was achieved regardless of the scale (telencephalon, brainstem or the whole brain) used to make the comparison. The only other bird in this study that had a significantly larger olfactory bulb than expected was the Australian king parrot. There are no accounts of the Australian king parrot using olfaction, and it does not emit a smell. The cockatiel had a significantly smaller olfactory bulb than would have been expected (Figure 4.4), which is not surprising for a bird living on grains and in an arid habitat. Living in areas where water is a limiting factor and any loss of water is likely to be reduced, olfaction might not be the first sensory choice for cockatiels, because a fairly moist lining of the epithelium is necessary for odours to be picked up (Kratskin 1995). The olfactory bulb in the remaining parrots all had values as expected for their size. This included the galah, the Eastern rosella and the ground parrot, which also have been reported to emit strong smells (McFarland 1991b; McFarland 1991a; Juniper & Parr 1998). Although it could be assumed that parrots with significantly larger olfactory bulbs have a better sense of smell compared to parrots with smaller bulbs, the ability to process olfactory cues will ultimately depend on the expression and qualities of the layers of the olfactory bulb and in particular the number and the density of mitral cells in the mitral cell layer.

4.5.2 Descriptive assessment of the olfactory bulb

The mitral cells had the most distinctive shape within the olfactory bulb. As described by Wenzel (1987), they were of triangular to amoeboid shape and stained densely with cresyl violet. The number of mitral cells in my specimens varied greatly (Table 4.4). In some species the mitral cell layer appeared only rudimentary, in other species it presented as a thick layer of dense clusters of mitral cells (Figures 4.7a-h). A well established mitral cell layer with bands of neurons one to two layers thick was found in kakapo, the Australian king parrot, the crimson rosella and the galah. Other species, such as the rainbow lorikeet, the red-rumped parrot, the Eastern rosella and the sulphur-crested cockatoo, had less well defined mitral cell layers, that were partly merged with adjacent layers, as has been described for sparrows and small parakeets (Huber & Crosby 1929). Hence, it is apparent that in parrots with a smaller bulb size, the

mitral cell layer is less distinct and in part merged with the external or internal plexiform layer, which fits well with observations made by Crosby and Schnitzlein (1982) and Wenzel (1987). Instead of forming a clear border, the mitral cells in these parrots were spread over the layer, although still clearly distinct by their shape. An exception was the galah (*Cacatua roseicapilla*). Although it has a comparatively small olfactory bulb and the smallest mitral cell layer among all parrots examined here, its mitral cell layer showed a well defined band of mitral neurons closely strung together. A less structured mitral cell layer might suggest a less sensitive olfactory sense. Merging layers between the mitral cell layer and the internal, as well the external, plexiform layer can also occur in mammals (Kratskin 1995), but it is not known as to whether their performance is altered as a result. The thin internal plexiform layer underlies the mitral cell layer. It has a low density of cell bodies and is composed of axons and axon collaterals of mitral cells followed by the more pronounced granule cell layer. In all the parrots studied here, the cell bodies of the densely populated granule cell layer gave this layer a dim appearance and contrasted strongly with the lighter coloured periventricular layer. Only in larger brained birds such as the kakapo and the Australian king parrot, did the plexiform layer embed both an ependym and a ventricle. Even though there was considerable variability in terms of the layer size among the parrots investigated, the kakapo stood out by having a significantly larger mitral cell and periventricular layer (Figure 4.8). In fact, the periventricular layer made up a quarter of the kakapo's olfactory bulb, and the granule cell and the periventricular layer together accounted for half of the olfactory bulb (Figure 4.9). The periventricular layer extends the olfactory bulb in kakapo deep into the nidopallium (Figure 3.6) and nearly touches the mesopallium in the centre of the bulb (Figure 3.7a). Therefore the physiological end of the olfactory bulb lies much further caudal than the location of the external grooves indicated by Bang and Cobb (1968) (Figures 4.5b, 4.6 and 4.7a). Hence, the olfactory bulb is much larger than suggested by Bang and Cobb (1968).

The reason for the large periventricular layer and granule cell layer remains speculative and might have to do with the maintenance of the kakapo's large olfactory bulb. Any qualitative assessment of the functionality of the layers would have to come from neurophysiologically studies, which are not feasible in such a highly endangered species. Alternatively, cell counts can reveal more about the importance and functionality of the layers, as the mitral cell counts prove. Mitral cell counts showed an unexpected result in that the kakapo has by far the highest number of mitral cells per bulb followed by the galah. Both had a higher number of mitral cells than any bird or mammal reported to date (Table 3.4).

Generally, it is assumed that the relative size of an organ is positively correlated with its functional importance (Mace *et al*. 1980; Healy & Rowe 2007). However, as Wenzel and Meisami (1987) noted, the situation might be more complicated. They found that the olfactory bulb of the northern fulmar is 20 times larger than that of the rock pigeon, but it exceeded the pigeon in terms of mitral cells only by a factor of six. Quantity does not ultimately determine the quality with which an organ responds. Even a bird with a comparatively small olfactory bulb and mitral cell layer volume and/or a comparatively small number of mitral cells could still have an excellent sense of smell. Since mitral cells form the main link between the olfactory bulb and higher brain regions, they can essentially be counted as the main port for the neural dissemination of olfactory information. It then can be assumed that the number of mitral cells reflects the degree to which information of the olfactory bulb is transmitted to higher brain centres, such as the hippocampus and the amygdala in mammals or the nucleus taenia in birds. The more mitral cells that are present, the more information can be transferred simultaneously (Mackay-Sim & Royet 2006). Lin (2005) fractioned the compounds present in the urine of mice using gas chromatography, and she was able to show that out of the hundreds of compounds present in mice urine mitral cells react solely to specific compounds. A study in aging humans found that olfactory sensitivity as well as the number of mitral cells in the mitral layer decrease with age (Bhatnagar *et al*. 1987). An equivalent situation could apply to the size of the mitral cells in birds, and birds with comparatively fewer mitral cells might only have limited olfactory abilities.

Comparatively larger sized mitral cells presumably process more information compared to smaller sized neurons. On the other hand, it is known that smaller neurons are less costly than larger ones, as they can be maintained with fewer resources. At the same time, they need a higher electric voltage to be discharged compared to larger neurons (Henneman 1957). Although it is not known whether smaller mitral cells transmit less information than larger mitral cells, there is some indication that the size of mitral cells does change according to the diversity of odours an individual experiences. An exposure to monotonous olfactory cues early in life results in a degeneration of mitral cells. At the same time the acuity of mitral cells diminishes and their ability to perceive and detect unfamiliar odours decreases (Laing & Panhuber 1978; Meisami & Noushinfar 1986; Panhuber & Laing 1987). From these results it can be assumed that olfaction is unlikely to be important in species with a comparatively small number of mitral cells and / or comparatively small mitral cells.

Kakapo exceeded all birds examined in not only having the largest mitral cell volume compared to all other birds, but by having the longest mitral cells and the highest density of mitral cells compared to any species studied to date. The northern fulmar has a well documented and pronounced olfactory bulb (Matochik *et al*. 1991), but only half as many mitral cells as the kakapo (Wenzel & Meisami 1987). Judging by these results and the numerous accounts of seabirds in general using olfactory cues in their daily life, it can be assumed that kakapo also uses olfactory signals. Northern fulmar emit a strong smell, mainly due to their rancid smelling stomach oil, which they discharge in response to potential mammalian predators (Rosenheim & Webster 1927; Armstrong 1951). They also have been observed to find their main food source by flying upwind towards it (Hutchison & Wenzel 1980); however, Nevitt (1999) did not find them more attracted to krill-scented than unscented slicks. Pigeons, in contrast, have approximately four times fewer mitral cells than the kakapo, yet they have been shown to rely on olfaction for homing (Benvenuti *et al*. 1973; Papi 1991; Waldvogel & Phillips 1991). Therefore, birds with lower numbers of mitral cells compared to kakapo seem to be able to sense olfactory cues. Based on this evidence, there is strong anatomical support that kakapo is able to process olfactory information.

Surprisingly, the galah was found to have a relatively small olfactory bulb, a comparatively small mitral cell layer and yet a very large number of mitral cells (Figures 4.10a&b). The relationship between the volume of the mitral cell layer and the size as well as the number of the mitral cells in the species studied was illustrated with a principal component analysis (PCA) (Figure 4.11). In this analysis, the variables are ordered alongside multiple scales (multidimensional scaling), and the distance between the objects reflects their dissimilarity (Faith *et al.* 1987; Clarke 1993; Clarke & Ainsworth 1993; Clarke *et al.* 2006). The PCA revealed that the number of mitral cells, which accounted for 98.9% of the variance between the eight species in PC1, clearly separated the kakapo and galah from the remaining parrots. Both of these parrots have reportedly used olfaction. Since this result was achieved based on only two birds, it would be interesting to see whether the relationship between birds that use smell and those that do not could be established more strongly with a larger sample that use olfaction in their daily life.

4.5.3 Making use of Museum specimens

Calculations of the olfactory bulb / cerebral hemisphere size in kakapo by J. Hagelin and in the present study resulted in ratios of 30.2% and 21.1%, respectively (Table 4.2). Hagelin conducted her measurements on a specimen stored in the Museum of New Zealand TePapa Tongarewa, Wellington, NZ (Hagelin 2004). My measurements, in contrast, were based on a photograph. Three-dimensional objects naturally

appear distorted when placed on a two-dimensional photograph, making exact measurements almost impossible. Additionally, Hagelin's kakapo was a nine year old male, while my bird was approximately 90 years of age. The hippocampus and therefore the cerebral hemisphere of more experienced birds could vary in size compared to less experienced birds (Abbott *et al*. 1999). Kakapo are lek breeders and during the breeding season, adult males defend display arenas or bowls that are interconnected with tacks (Chapter One and Merton *et al*. 1984). A mature kakapo for example may have a larger hippocampus as the demands for spatial memory in a bird with an established track-and-bowl system will be larger. A younger and not yet established bird is likely to have a less strongly developed spatial memory, since young male kakapo do not have allocated booming sites and are still searching for the perfect spot. Adult kakapo are faithful to their bowling systems and will return regularly to the same site, which they also defend against intruders or younger males (pers. comm. R. Moorhouse and Merton *et al*. 1984). Hence, it could be that Hagelin's (2004) young kakapo had a smaller hippocampus, explaining the slightly smaller cerebral hemisphere (Table 43.2). In contrast, Hagelin (2004) had a much larger measurement for the olfactory bulb compared to my result. Individual variation does occur and so do hemispheric differences (Riddle & Purves 1995), but they are unlikely to be as high as in this case (Table 4.2). Methodological difficulties may need to be accounted for this difference. Bang and Cobb (1968) already pointed out the difficulties posed by the need to accurately measure non-spherical objects. For example, results will vary with the choice of the hemisphere, as there are slight size differences between the right and the left hemisphere (Kolb *et al.* 1982). Results will also depend on the exact angle from which brain measurements are taken.

I recommend that comparative studies aiming to compare the olfactory bulb ratio with measurements reported in Bang and Cobb (1968) need to be conducted on completely dissected brains only. They also should consider including control measurements for specimens used in Bang and Cobb (1968) in order to ensure that the best possible methodology is followed. All too often valuable specimens are preserved and available in Museum collections but they have not been preserved appropriately to allow for a histological examination of the brain. While they would still be suitable for general olfactory bulb ratio measurements as suggested by Bang and Cobb (1968), it would be useful to compare measurements taken from Museums specimens with histological measurements of the olfactory bulb where possible. This way an 'error' could be calculated by which olfactory bulb volumes evaluated with Bang and Cobb's method could be adjusted. This would not only allow us to get a better idea about olfactory bulb sizes in a wider range of birds, but also help to make better use of specimens stored in museums.

4.5.4 Low sample sizes and their value?

"*No two brains are the same. Their shape. Their size. The way they are organized*" (quote by: John Mazziotta, Frances Stark Chair of Neurology and head of the Department of Neurology at UCLA's David Geffen School of Medicine). There seems to be no limit to the plasticity and variation between brains, not only between species but also within the same species. Genetic and environmental factors influence the development of the brain. Song learning centres in songbirds, for example, are larger in males that perform more songs, which is associated with genetic and epigenetic factors (Brenowitz *et al.* 1995). The exposure to scent early in life contributes and determines the extent to which olfaction is used (Meisami & Noushinfar 1986). The brain is known for its high degree of plasticity, and the ability to accommodate to changes required by environmental demands has been shown in several studies across species. The posterior hippocampus in taxi drivers for example is significantly larger compared to control groups, reflecting the higher navigational demands (Maguire *et al.* 2000). Likewise, the hippocampal volume in nocturnal Leach's storm petrels is related to the nesting habitat and is significantly larger in birds that stay in the darker and navigationally more demanding woods than in birds that nest in open meadows (Abbott *et al*. 1999). There also exist differences in performance between the two brain hemispheres. Pigeons whose right nostril was blocked during homing experiments performed worse than controls or pigeons whose left nostril had been blocked (Gagliardo *et al*. 2011).

Apart from the functional differences, there also exist volumetric differences between the two hemispheres. For example, it has been shown that the right olfactory bulb is significantly larger than the left one in albino rats (Heine & Galaburda 1986). The right hemisphere of rats, mice, rabbits and cats were all larger and heavier than the left one, even though there were no significant differences between the hemispheres in cross sectional sections of the hippocampi (Kolb *et al*. 1982). Finally, there are individual differences between brains of the same species, as well as age and sex related differences (Raz *et al.* 2005). A study on 12 bird species discovered that the brains of males were significantly larger when compared to brains of their female counterparts (Sahin *et al.* 2001). However, as the volumetric and cell density differences in the mitral cell layers in my parrots showed, often only the evaluation of the number of neurons in these brain compartments will provide a better idea about the extent of interhemispherical differences and associated functional capacities. Environmental, genetic, epigenetic and sexual differences are only a few examples of factors that help shape the brain. Even though my results are based on one old, male kakapo only, there are strong trends visible. Judging by the size of its olfactory bulb and the sheer number of mitral cells in its mitral cell layer, it can be concluded that the kakapo

seems to have an excellent sense of smell and is likely to use olfaction in a much wider fashion than assumed so far.

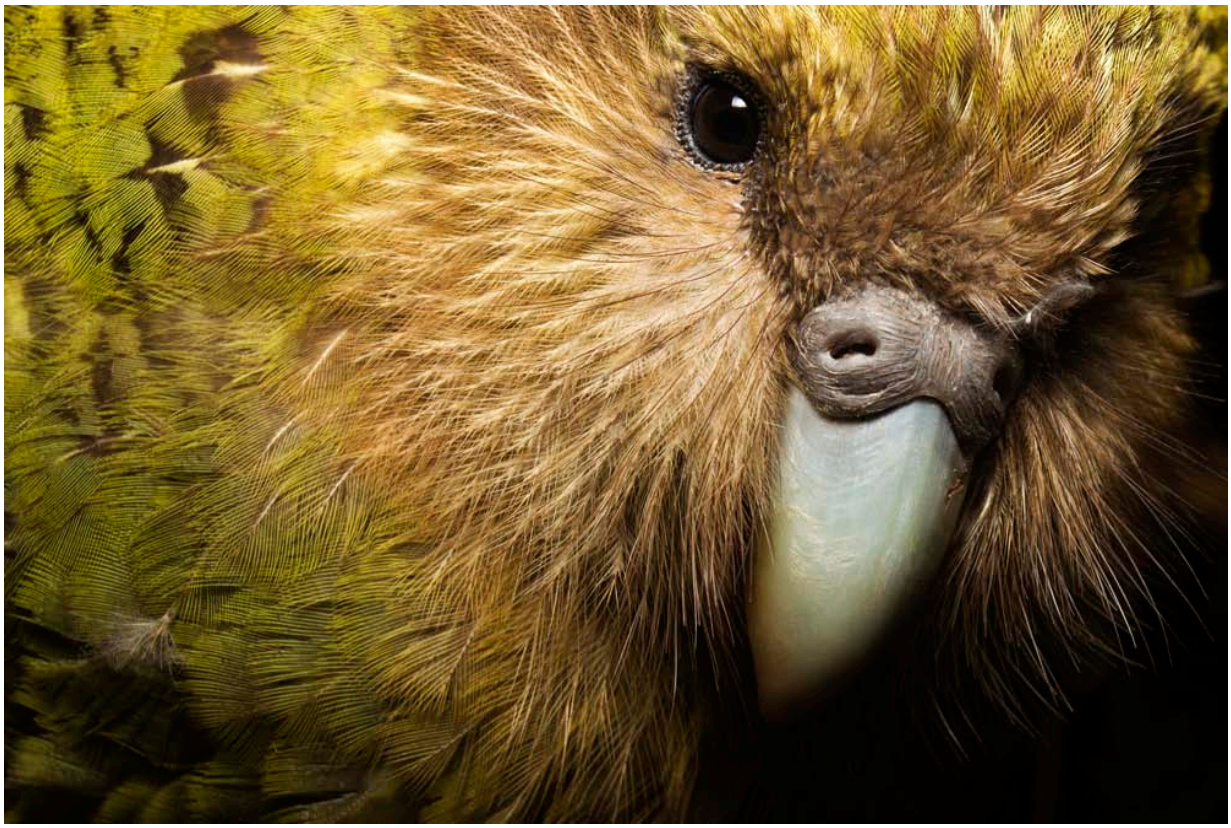
These results should encourage the establishment of behavioural experiments to address in what ways kakapo use olfactory cues and might prove helpful in the endeavour to save this critically endangered species. In addition, as these results also exemplify, anatomical studies comprise a useful tool to better understand behavioural associations.

CHAPTER FIVE

ANATOMY AND HISTOLOGY OF THE VISUAL SYSTEM IN THE KAKAPO (*STRIGOPS HABROPTILUS*) IN COMPARISON TO OTHER BIRDS

Chapter reference:

Corfield J., Gsell A.C., Brunton D.H., Monica A., Heesy C.P., Hall M.I., Iwaniuk A.N. 2011 Anatomical specialization of the visual system in the endangered and nocturnal Kakapo (*Strigops habroptilus*). *PLoS One* **6**(8), e22945.



5.1 Abstract

The endangered kakapo (*Strigops habroptilus*) is a parrot endemic to New Zealand that possesses several unique traits compared to related species, such as a folivorous diet, flightlessness and nocturnality. Its nocturnal lifestyle, combined with its owl-like facial ruff, earned this species the moniker 'owl parrot', but nothing is known about its visual system. This study provides a detailed morphological analysis of the brain and the retina of the kakapo and comparisons with other parrots and birds. The tectofugal vision pathway, specifically the optic tectum, nucleus rotundus and entopallium, were significantly reduced in size relative to the rest of the brain. There was no apparent reduction to the thalamofugal visual pathway, namely the Wulst. Finally, the retina morphology shows features for both functioning in a nocturnal and diurnal environment, suggesting a retina that is specialised for a crepuscular niche. Overall, this suggests that the kakapo has enhanced light sensitivity but also has relatively poor visual acuity. Based on these results, it seems that kakapo represents a species that possesses a visual system similar to that of both nocturnal and diurnal birds and therefore does not adhere to the traditional view of the evolution of nocturnality in birds.

5.2 Introduction

Living in a scotopic, or low light environment poses significant challenges for the visual system. In contrast to photopic, or well-illuminated environments where the chances of a photon hitting the retina are extremely high, in scotopic environments where light levels are typically about a million times lower (Land & Nilsson 2001) the visual apparatus relies on specialised tissues to attain vision. As a result, the visual systems of animals that live in scotopic environments have evolved in one of two ways. Firstly, they can evolve mechanisms to increase the sensitivity of the eye to light. Examples of this include increasing the size of the eye, cornea and/or density of photoreceptors in the retina (Walls 1943; Ritland 1982; Martin *et al.* 2004; Hall & Ross 2007). Alternatively, animals can decrease their emphasis on the visual system and enhance the sensitivity of other sensory systems to provide equivalent information about their environment (Martin 2007). Kiwi (*Apteryx* spp.), moles (Talpidae) and naked mole-rats (*Heterocephalus glaber*) are all prime examples of this second strategy. These species have relatively small eyes and visual brain regions, but greatly enlarged somatosensory systems and tactile specialisations in their extremities (Ritland 1982; Catania 2000; Crish *et al.* 2006; Martin 2007; Nemec *et al.* 2008; Corfield 2009). Thus, shifting from a diurnal to a nocturnal lifestyle can either be associated with the enlarge-

ment of the visual system to enhance light sensitivity or the reduction of the visual system combined with the enlargement of other sensory systems. Although in fishes and mammals there are numerous examples of both strategies, the extent to which individual species evolve one strategy or the other in birds is not well understood. Nocturnality has evolved multiple times in otherwise diurnal avian lineages (Ericson *et al.* 2006; Braun & Huddleston 2009) and, in the case of owls, diurnality has evolved several times within an otherwise nocturnal lineage (König & Weick 2008). One of the most profound shifts in activity pattern from diurnality to nocturnality has occurred in the critically endangered New Zealand parrot, the kakapo, a parrot unlike any other in many aspects (Powlesland *et al*. 2006; Ballance 2010). It is the largest and heaviest parrot worldwide; it is nocturnal and flightless and an obligate herbivore with a strong body-odour (Butler 1989; Hagelin 2004; Eason *et al*. 2006). Its nocturnal lifestyle, combined with its owl-like facial ruff, earned this species the moniker: 'owl parrot' (Turbott 1967; Juniper & Parr 1998). In order to successfully conserve this enigmatic species, there is a strong need to understand the sensory abilities of this species and their unique nocturnal lifestyle.

Pettigrew (1978) commented that the kakapo could have visual specialisations similar to that of owls based on its nocturnal activity pattern and the presence of a facial ruff. Hall *et al*. (2009) compared the orbits among 177 avian families and across 27 different orders of diurnal and nocturnal birds and suggested that the optic foramen size fell well within the range of nocturnal birds. However, the analyses of the eyes or brain of the kakapo have not been carried out. Here I provide a detailed comparative examination of the size and shape of the brain as well as the eyes of this enigmatic species. I compare my data with closely related parrots such as the kea (*Nestor notabilis*), a sister taxa of kakapo (Wright *et al*. 2008), as well as more distantly related parrots.

Because very little is known about retinal morphology in parrots, the retinal morphology of the kakapo is compared with that of two diurnal parrot species, the sulphur-crested cockatoo (*Cacatua galerita*) and the Eastern rosella (*Platycercus eximius*) as well as the nocturnal morepork (*Ninox novaeseelandiae*). Additionally, results of Corfield *et al*. (2011) on the barn owl (*Tyto alba*) and the diurnal chicken (*Gallus domesticus*) will make my comparison stronger. Much is known about the visual systems of both barn owls and chickens (Meyer & May 1973; Hart 2001; Harmening *et al.* 2007) and they exhibit a retinal morphology that is typical of nocturnal and diurnal birds, respectively. By comparing retinal morphology across these species, I will be able to determine whether the kakapo has a retina typical of parrots and diurnal birds or one that is more similar to that of nocturnal birds, like morepork and barn owl.

5.2.1 Retina

The retina consists of two types of light sensitive photoreceptors: rods and cones. Rods function mainly in dim light situations and create a black and white picture of poor visual acuity, while cones support daytime vision of high visual acuity and the perception of colour (Wässle & Boycott 1991; Tovee 1996; LaCour & Ehinger 2006; Schwartz 2010). The retina of nocturnal animals is generally much thicker than the retina of diurnal birds. Their outer nuclear layer tends to have multiple rows, while their inner nuclear and ganglion cell layers are thinner compared to diurnal species. The retina of diurnal animals has a much lower proportion of rods (~20%). Cones though are typically much thicker than rods and a multilayered and thicker outer nuclear layer is created in order to place their nuclei against the external limiting membrane. Additionally, diurnal species need an increased number of horizontal, amacrine and ganglion cells in order to create the high resolving power needed for colour vision in bright light conditions. Therefore their inner nuclear and ganglion cell layers are thicker compared to nocturnal species (Walls 1943).

5.2.2 Two pathways for retinal information

Retinal information is conveyed over two major pathways: the thalamofugal pathway and the tectofugal pathway. In birds the thalamofugal pathway ends in the telencephalon and information is fired towards a structure in the dorsal nidopallium called the visual Wulst, a visible protuberance at the dorsal hemisphere of the brain comparable to the primary visual cortex of mammals (Karten *et al*. 1973). This structure is rather pronounced in nocturnal species such as owls and kiwi (Iwaniuk & Hurd 2005; Iwaniuk & Wylie 2006; Iwaniuk *et al*. 2008) and plays a key role in stereoscopic vision in these taxa (Pettigrew 1978; Pettigrew 1986; Wagner & Frost 1993; Nieder & Wagner 2000; Nieder & Wagner 2001), although its role in modulating stereopsis in other taxa has been debated (Iwaniuk *et al*. 2008; Martin 2009).

The tectofugal pathway in contrast leads information firstly to the highly laminated tectum opticum (TeO), the primary efferent target of retinal ganglion cells in the avian brain (Mpodozis *et al*. 1996) and sends a large projection to the thalamic target, the Nucleus Rotundus (nRt). The optic tectum in turn, projects information to the entopallium (E) of the telencephalon (T) and together these three brain regions comprise the tectofugal pathway (Benowitz & Karten 1976; Husband & Shimizu 2001) (Figure 5.1).

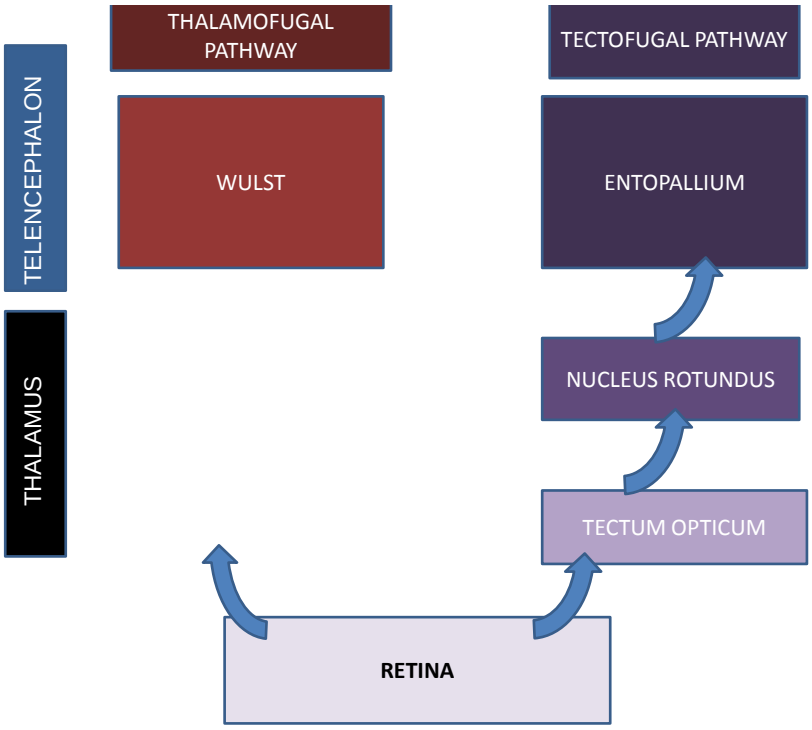


Figure 5.1 Schematic diagram of the two pathways through which visual information is conveyed. Adapted from (Shimizu & Karten 1993).

5.2.3 Outcomes

In principle there are two outcomes that can be expected. If the kakapo has enhanced light sensitivity in a comparable fashion to nocturnal owls, then from an anatomical perspective it should have relatively large eyes, a larger cornea relative to the axial length of the eye, more rods in the retina and more convergent (i.e., similar facing) orbits (Bowmaker & Martin 1978; Martin 1986; Brooke *et al.* 1999; Garamszegi *et al.* 2002; Thomas *et al.* 2002; Martin 2007; Hall & Ross 2007; Burton 2008; Iwaniuk *et al*. 2008). Similarly, if the kakapo has stereoscopic abilities comparable to that of nocturnal owls, the brain of the kakapo should have: 1) an enlarged Wulst (Iwaniuk & Wylie 2006); 2) a markedly reduced optic tectum (Iwaniuk & Hurd 2005; Martin 2007; Iwaniuk *et al*. 2010) and 3) correspondingly smaller forebrain targets of the optic tectum (Iwaniuk *et al*. 2010). Alternatively, if the kakapo has diminished its reliance on vision, it should have relatively small eyes, few rods in its retina, little change in corneal diameter and optic tectum size and a relatively small Wulst. My data suggests that the kakapo has undergone profound changes to the morphology of its visual system. Some features of the kakapo visual system are typical of nocturnal birds, the brain morphology is vastly different from that of other parrots and other aspects of the visual system are intermediate with respect to diurnal and nocturnal birds.

5.3 Methods

5.3.1 Specimens and modelling of brain regions

Brains of specimens (see 4.3.1 and 4.3.2; Table 4.1) were prepared and stained for histology and sectioned (as in 4.3.3 and Table 4.2).

5.3.2 Volumetric measurements of visual centres:

I measured four regions, all of which are involved in visual processing: optic tectum (TeO), nucleus rotundus (nRt), entopallium (E) and the Wulst. The nRt is readily defined by the presence of large, intensely Nissle stained cells of low density relative to adjacent structures and the borders of the entopallium were defined by the description of Nissl stained tissue outlined in Krützfeldt & Wild (2004) and Krützfeldt & Wild (2005). Identification (see 3.3.4), labelling, modelling and analysis are as outlined in sections: 4.3.7, 4.3.8 and 4.3.9. Details of the brain region volumes are provided in Table 4.3.

5.3.3 Retina

The anatomical structure of the kakapo retina was compared with that of other parrots and birds, all of which were processed in similar conditions. For this purpose I used material from kakapo, sulphur-crested cockatoo, Eastern rosella, pigeon and morepork. Permits for the parrots used are as outlined in sections 3.3.1 and 4.3.2. Pigeon eyes were received from Nils Krützfeldt at the Department of Anatomy with Radiology, School of Medical Sciences, Auckland University, Auckland, New Zealand. The morepork was a specimen that had died from a road accident and was received from Sylvia Durrant (SPCA Bird-Wing, Auckland, New Zealand).

The eyes were dissected and the anterior part and lens was removed prior to placing the tissue in 4% paraformaldehyde (PFA) for 30 minutes. The posterior cup was then washed and stored in 0.1M phosphate buffer (PB) for one week. The eyes were cryoprotected in a series of 10% and 20% sucrose in PB solutions for ten minutes each and then left overnight in a 30% sucrose PB solution. The posterior eye cup was embedded in TissueTek medium, frozen and cut perpendicular to the equator on a LEICA cryostat (Germany) at a thickness of 16-20 µm. Sections were mounted onto glycerine coated slides, stained with cresyl violet, dehydrated and cover slipped with DePeX (Serva GmbH) from xylene. Images of central and peripheral areas of retina from kakapo, sulphur-crested cockatoo, Eastern rosella, rock pigeon as well as morepork were obtained using a LEICA DC 500 camera. A 40x objective and 10x ocular

was used to render a final magnification of 400x to measure total retinal thickness and thickness of the outer and inner retina. Central retina was defined as a 2mm linear area around the optic nerve. Peripheral retina was 5mm or more away from the optic nerve. Quantification consisted on measuring total retinal thickness, inner nuclear layer, inner plexiform layer and photoreceptors (Table 5.1).

5.4 Results

5.4.1 Brain morphology

The adult kakapo brain has a length of 5.2 cm and a width of 3.5 cm. Both, the olfactory bulb and the Wulst are prominent, however the optic lobe is extremely small and partially obscured by the lateral part of the cerebral hemisphere (Figure 5.2). In contrast, the kea brain is relatively wider than the kakapo with a length of 4.2 cm and a width of 3.8 cm. The olfactory bulbs were missing in the kea, one of the sister taxa to the kakapo (Wright *et al*. 2008), and the brainstem was slightly damaged. However, the optic lobes were prominent, as they are in other parrots, such as the sulphur-crested cockatoo.

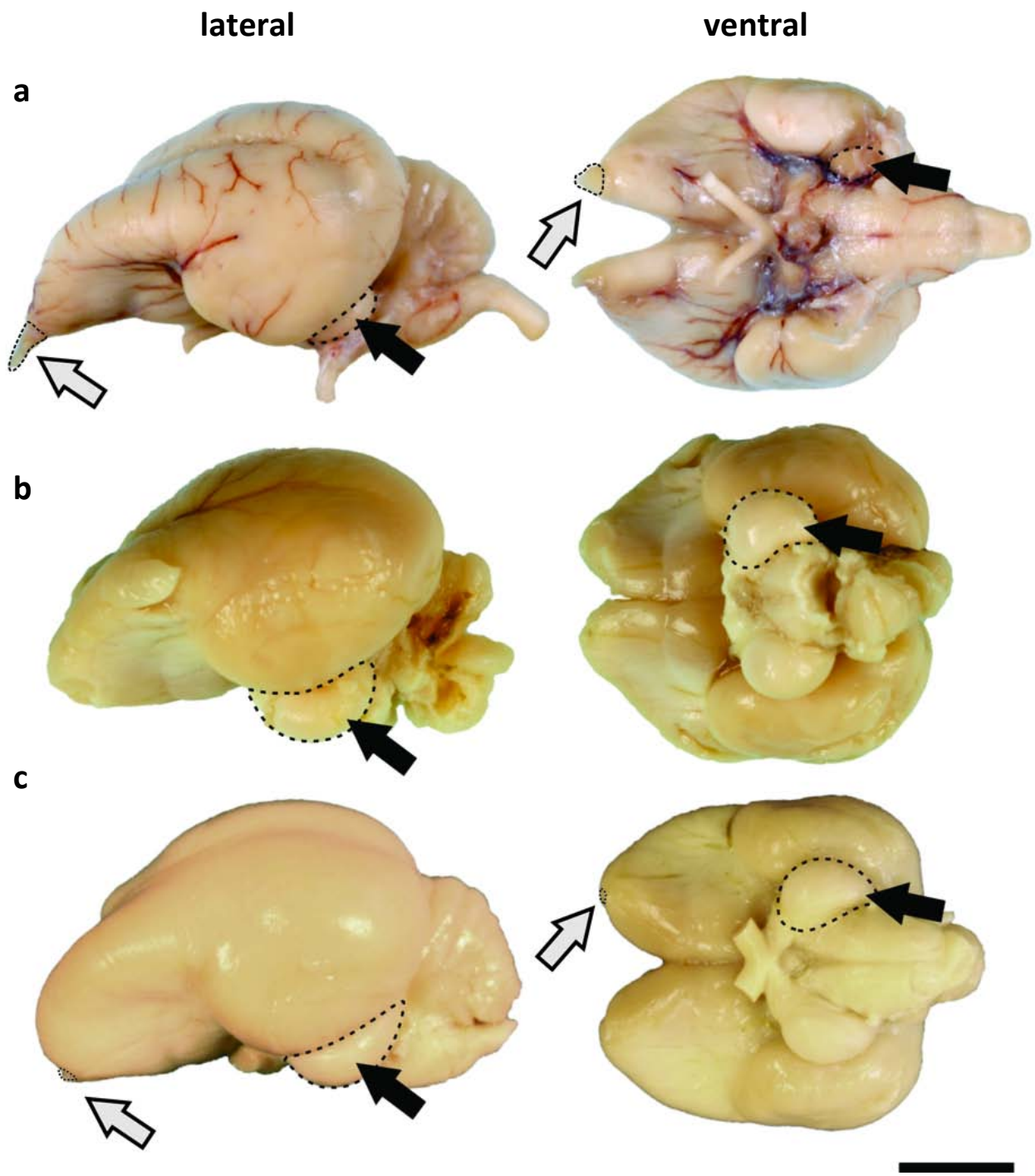


Figure 5.2 Photo of the kakapo (a), kea (b) and sulphur-crested cockatoo (c) brain. The dotted lines indicated by the black arrow outline the left optic lobe. The dotted lines indicated by the open arrow outline the left olfactory bulb Scale bar = 10 mm. Photo by Nick Duggan (Manager, AMRF Medical Sciences Learning Centre – *Whakaaro Pai,* Department of Anatomy with Radiology) using a Nikon D2Xs and a 105mm f/2.8D AF Micro-Nikkor lens.

5.4.2 Brain volumetric measurements

As suggested by the external appearance of the kakapo brain, the TeO is significantly reduced in size relative to the total size of the brain (Figure 3.1a). The same is also true of the other two tectofugal regions, nRt and E; both of them are significantly smaller in the kakapo compared to other parrots (Figure 5.3b&c). This overall reduction in the tectofugal pathway does not, however, extend to the Wulst (Figure 5.3d), which is similar in relative size across all parrots examined. See also data in Table 4.2.

5.4.3 Retina

The kakapo had a markedly different retinal morphology to that of the diurnal Eastern rosella and chicken and also the nocturnal barn owl, but was similar to that of the cockatoo (Table 5.1). In the kakapo, the overall width and proportion of the retina that the photoreceptor layer occupies is larger compared to the other species examined in this study, but is most similar to the barn owl (Figure 5.4, Table 5.1). The outer nuclear layer (ONL), formed by the rod and cone photoreceptor nuclei, has the highest relative thickness in the barn owl and is relatively thin in the chicken and Eastern rosella (Figure 5.4c-e and Table 5.1). The relative thicknesses of the ONL in the kakapo and cockatoo are similar and have an intermediate thickness (Figure 5.4a&b and Table 5.1). Also, the relative thickness of the inner nuclear layer (INL), which contains the cell bodies of the amacrine and bipolar cells, was much thinner in the kakapo compared to the chicken and Eastern rosella and similar to that of the barn owl. Finally, the staining of the ganglion cell layer of the kakapo revealed relative few cells compared to the other species examined.

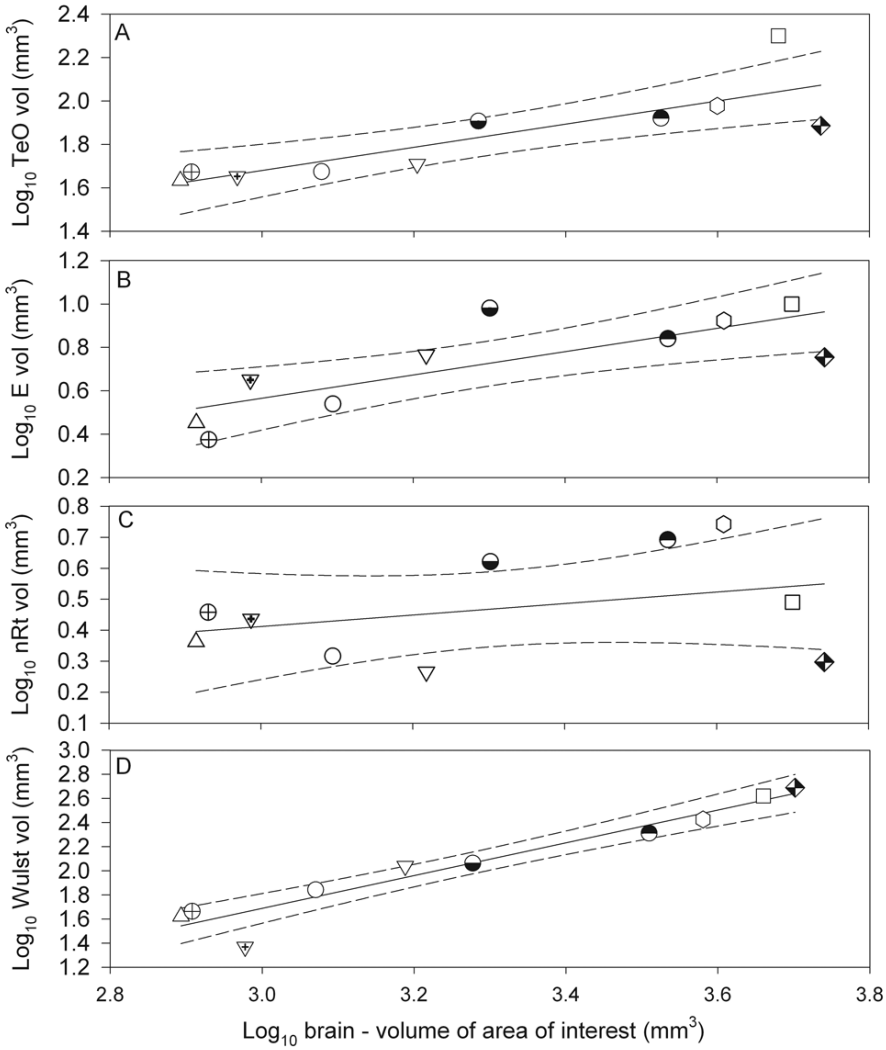


Figure 5.3 Scatter plots of each of the four visual brain regions measured against total brain volume. A: optic tectum (TeO); B: Nucleus Rotundus (nRt); C: Entopallium (E); and D, Wulst. The solid lines indicate the least-squares linear regression lines and dotted lines indicate the 95% confidence intervals.

Table 5.1 Average thickness for central and peripheral retina and retinal layers in diurnal and nocturnal species. Values are expressed in micrometers. Highlighted values indicate best similarities with the kakapo retina. The barn owl and the morepork are representative of a nocturnal species and the chicken, rock pigeon, cockatoo and Eastern rosella are diurnal species (Juniper & Parr 1998). Abbreviations: total: total thickness of the retina (microns), OS/IS: outer segment/inner segment, ONL: outer nuclear layer, INL: Inner nuclear layer, GCL: Ganglion cell layer. GCL cells/mm indicates the number of cells per mm in the GCL. All species with an asterix (*) are borrowed from my co-authored paper: (Corfield *et al*. 2011).

	total	**OS/IS**	**ONL**	**INL**	**GCL**	**OSIS/tot**	**ONL/tot**	**INL/tot**	**GCL/tot**	**GCL**
Kakapo central*	214.34	99.59	22.01	34.41	10.03	0.46	0.10	0.16	0.05	89
Kakapo peripheral*	211.215	101.25	22.14	34.1	11.86	0.48	0.10	0.16	0.06	87
Morepork central	**195.33**	60.66	13.00	**33.66**	15.00	0.3	0.07	**0.17**	0.08	**86**
Morepork peripheral	115.67	24	7.33	14.67	16.67	0.21	0.06	0.13	0.14	**70**
Barn owl central*	178.42	**73.97**	28.81	29.97	6.89	**0.41**	0.16	0.17	**0.04**	82
Barn owl peripheral*	170.71	70.12	29.15	31.81	6.03	0.41	0.17	0.19	0.04	96
Cockatoo central*	158.43	54.80	**19.55**	36.84	**14.59**	0.35	**0.12**	0.23	0.09	124
Cockatoo peripheral*	137.03	46.40	16.41	31.50	**13.46**	0.34	**0.12**	0.23	0.10	98
Rosella central*	232.89	28.88	17.30	55.94	15.31	0.12	0.07	0.24	0.07	285
Rosella peripheral*	**225.21**	42.69	**21.30**	62.72	15.11	0.19	0.09	0.28	0.07	333
Pigeon central	192.00	17.67	16.00	43.67	38.67	0.09	0.08	0.23	0.30	429
Pigeon peripheral	161.67	15.00	16.67	**37.67**	18.3	0.09	0.10	0.23	0.11	98
Chick central*	235.60	25.52	13.04	101.19	19.59	0.11	0.06	0.43	0.08	354
Chick peripheral*	234.53	27.97	12.15	107.39	15.39	0.12	0.05	0.46	**0.07**	348

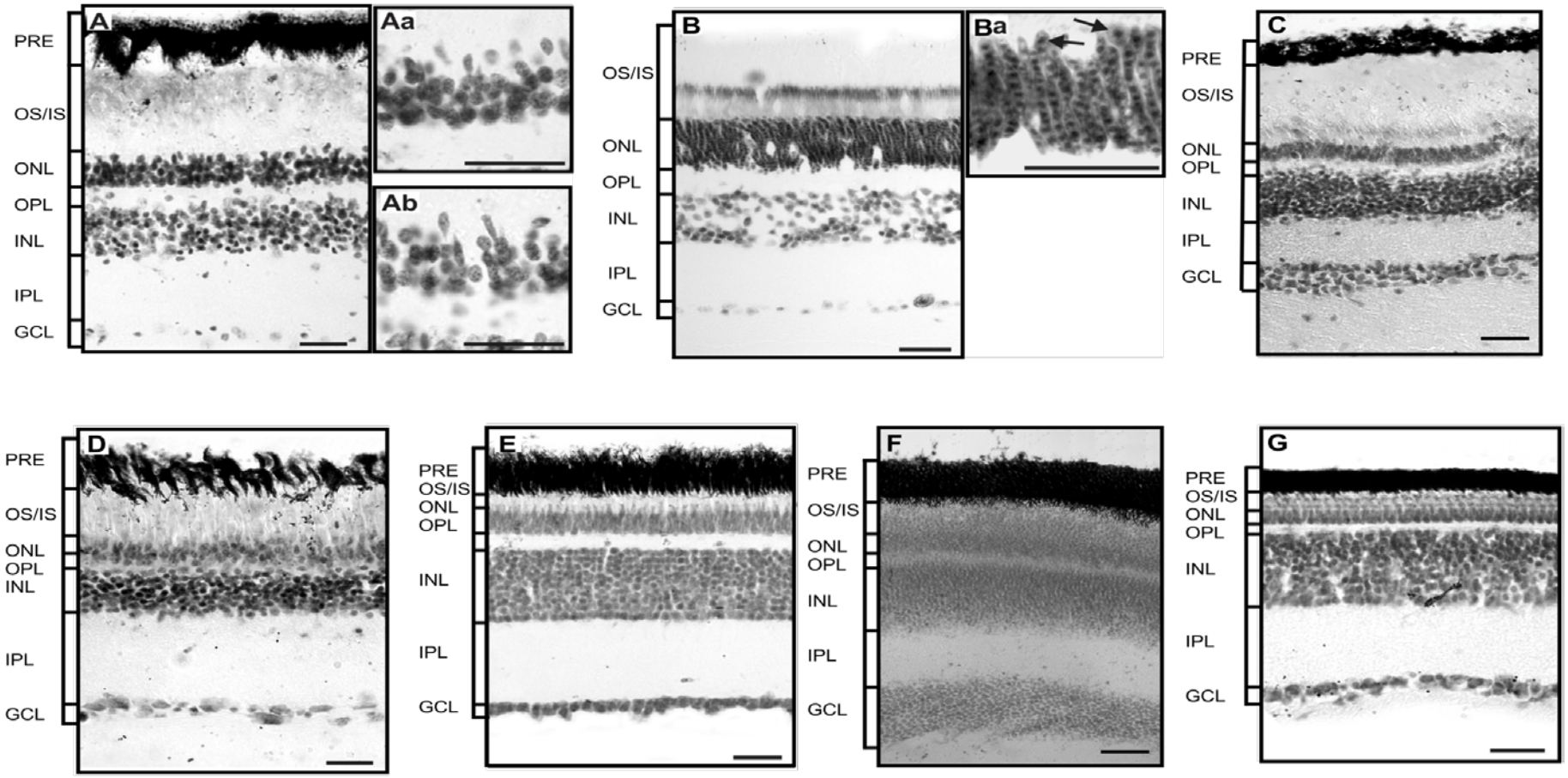

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Figure 5.4 Photomicrographs of transverse sections through the retina of seven species of birds. A, kakapo; B, barn owl*; C, morepork; D, sulphur-crested cockatoo; E, Eastern rosella; F, rock pigeon and G, chicken*. Aa and Ab are photomicrographs of peripheral and central photoreceptors respectively in the kakapo retina at 100 times magnification. Ba, is a higher magnification image of the photoreceptors in the Barn Owl. The arrows indicate cone photoreceptor cells. Retinal tissue was stained with cresyl violet. Abbreviations are as follows: RPE, retinal pigmented epithelium; OS/IS, outer segment/inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 25 mm. All species with an asterix are borrowed from my co-authored paper: (Corfield *et al*. 2011).

5.5 Discussion

The kakapo is likely to have become nocturnal after the split from his sister taxa, kea and kaka (Wright *et al*. 2008), which are both partially but not obligate nocturnal parrots (pers comm. R. Moorhouse and D. Brunton). This shift has driven the evolution of mechanisms to either enhance light sensitivity or decrease the reliance on vision in the kakapo (Walls 1943). Instead of either of these extremes, the kakapo has a unique combination of traits including an advanced reduction in the overall size of the tectofugal pathway, whereby the morphological appearance of the retina very much still shows features of a diurnal bird. This interesting combination of traits speaks for the kakapo's unusual phylogenetic position as the single species to evolve nocturnality in an otherwise entirely diurnal group, necessitating comparisons both within parrots and with unrelated nocturnal birds.

Adding to the features discussed are the reduction in the relative size of the optic nerve and convergently oriented orbits (Corfield *et al*. 2011) as they are common for nocturnal vertebrates. This trait indicates that the kakapo likely has a larger binocular visual field, which could confer enhanced light capture by increasing the quantum catch probability within the expanded region of overlap (e.g. Warrant 2004; Warrant 2008). Additionally, the kakapo has an eye size and shape that is within the range of the diurnal parrots but is also within the range of other nocturnal birds (Hall *et al.* 2009; Corfield *et al*. 2011), including the nightjars, nighthawks, and owls (Hall & Ross 2007). The observed paucity of retinal ganglion cells compared to other parrots could be indicative of relatively poor visual acuity (Heesy 2004; Iwaniuk *et al*. 2008; Hall *et al*. 2009; Heesy & Hall 2010). Orbit orientation is correlated with the number of binocular overlap in the visual field of both birds and mammals (Heesy 2004; Iwaniuk *et al*. 2008). The significantly greater amount of orbital convergence in the kakapo could therefore be taken as an indication of a wider binocular visual field, as predicted by

Pettigrew (1978). The orientation of the orbits is not, however, solely responsible for the width of the binocular field. Indeed, eye movements make a significant contribution to the shape of the visual fields of many birds (Martin 2007). Thus, the extent to which degree of binocularity can be inferred during various activities in the kakapo is limited. Similarly, it is difficult to comment on the suggestion that the kakapo has stereoscopic abilities (i.e., depth perception) similar to that of owls (Pettigrew 1978; Pettigrew 1986). Unlike owls, the kakapo does not have an enlarged Wulst, and Wulst hypertrophy is not necessarily a robust predictor of either the binocular visual field or stereopsis (Iwaniuk *et al*. 2008; Martin 2009). Determining these features of the kakapo visual system will depend on behavioural testing as neurophysiological studies are unlikely to be feasible in such a highly endangered species.

The sensitivity and acuity of the kakapo's visual system can be inferred from data obtained in Corfield *et al*. (2011). In most tetrapods, eye size and shape varies according to activity pattern. In general, nocturnal species tend to have broader corneas, relative to the axial length of the eye, than either crepuscular or diurnal species (Hall & Ross 2007). The purpose of these changes in eye size and shape is to increase the sensitivity of the eye. Corneal diameter is associated with the light gathering ability of the eye. The axial length of the eye is associated with visual acuity; the longer the axial length, the larger the projected image on the retina becomes (Walls 1942; Hughes 1977; Martin 1993). An eye shape with a large corneal diameter relative to the axial length of the eye is typical of nocturnal birds, including owls, nightjars and nighthawks and the oilbird (*Steatornis caripensis*) (Hall & Ross 2007). Although the size and shape of the kakapo eye is within the range of other parrots, the kakapo is also within an area of overlap with many nocturnal birds (Hall & Ross 2007). Therefore, it can be suggested that the eye shape of the kakapo is consistent with the typical nocturnal eye shape of birds. Based on eye morphology alone, I would therefore predict that the kakapo has enhanced visual sensitivity with concomitantly poor visual acuity.

Both, the enhanced sensitivity and poor visual acuity of the kakapo, relative to other parrots, are reinforced by the structure of the retina. The kakapo retina is characterised by a broader photoreceptor layer and an increased length of the outer and inner segment (Table 5.1). The outer segment of photoreceptors is the area where the photo pigment is located and in the inner segment the metabolic and biosynthesis of molecules for the outer segment occur (Young 1976). Thus, increased outer and inner segment length may suggest increased retinal sensitivity. The histological analysis does not reveal specialised photoreceptors cells. However, the moderately thick outer nuclear layer in the kakapo and the presence of round nuclei located in the most outer part of the ONL, suggestive

of cones, indicates that rods and cones are well represented in the retina as in other nocturnal birds (Rojas *et al.* 2004). A narrower inner nuclear layer and fewer ganglion cells likely reflect a strategy for increasing retinal sensitivity, although with very poor resolution power (Dowling 2012). A relatively small number of retinal ganglion cells is also supported by the small size of the kakapo's optic foramen (Corfield *et al*. 2011). The optic nerve, which passes through the optic foramen, is largely comprised of retinal ganglion cell axons (Corfield *et al*. 2011). A smaller optic foramen, therefore, reflects few retinal ganglion cells and is typical of nocturnal species (Hall *et al*. 2009). More photoreceptors per retinal ganglion cell, referred to as increased retinal summation, would then provide enhanced light sensitivity, but poor visual acuity, similar to other nocturnal birds (Rojas *et al*. 2004; Corfield 2009). Based on measurements of the optic nerve (Corfield *et al*. 2011) and examination of retinal sections, it would appear that the kakapo has the requisite morphology of a bird with enhanced low light (mesopic) vision.

The morphology of the kakapo's brain also provides insight into its visual abilities. Parrots possess relatively small visual regions (Iwaniuk & Hurd 2005; Iwaniuk *et al*. 2010), although the kakapo has taken this reduction in the tectofugal pathway to an extreme. In fact, apart from kiwi (Martin 2007; Corfield 2009), the kakapo appears to have the smallest tectofugal brain regions of any bird examined to date. This reduction in the visual system in the kakapo is not, however, universal. The Wulst, the telencephalic target of the thalamofugal pathway, is similar in size to that of other parrots and not enlarged as it is in owls or some caprimulgiformes (Iwaniuk & Hurd 2005; Iwaniuk & Wylie 2006). The huge reduction in size of the tectofugal pathway combined with no change in Wulst volume strongly suggests a decreased reliance on vision in the kakapo in a similar fashion to what has occurred in kiwi (Martin 2007; Corfield 2009). The evolution of flightlessness, folivory and nocturnality on a largely predator-free island may have reduced the kakapo's reliance on vision in favour of enhancing other sensory modalities (Chapter Three and Hagelin 2004).

Overall, I conclude that the kakapo has a unique visual system unlike that of other parrots or any other bird examined to date. The kakapo is a highly unusual animal that evolved nocturnality in the context of its phylogenetic background as a parrot, and as such it almost certainly had a diurnal ancestor. Therefore, in order to interpret the suite of nocturnal characteristics exhibited by the kakapo, it must be compared to both parrots / diurnal birds and nocturnal birds. Indeed, kakapo seems to possess traits consistent with nocturnal birds, including owls (retina, eye size and shape and orbit orientation), caprimulgiformes (eye size and shape), and kiwi (brain morphology), and also diurnal birds (eye size and shape). Based on this suite of traits, the kakapo likely has somewhat reduced its

overall reliance on vision. However, its visual abilities are characterised by the larger binocular visual field, enhanced low light sensitivity and poor visual acuity usual for nocturnal birds. In doing so, the kakapo breaks the dichotomy typical of the evolution of nocturnality in birds and mammals and illustrates that the visual system can evolve in a mosaic rather than a strictly concerted fashion by exhibiting individual nocturnal traits found in a variety of other unrelated nocturnal birds.

CHAPTER SIX

OLFACTORY SENSITIVITY IN KEA AND KAKA

Chapter reference:

Gsell A.C., Hagelin J., Brunton D. 2012 Olfactory sensitivity in Kea and Kaka *Emu*.

Kea exploring a scent placed on a wooden stack. Photo by A.C.Gsell

6.1. Abstract

The olfaction capabilities of birds have been vastly underestimated. I investigated the sense of smell of a captive group of two New Zealand endemic parrots and found strong support for the hypothesis that both species can detect odour. The aim was to assess whether kea (*Nestor notabilis*) and kaka (*N. meridionalis*) display varying behavioural responses to different types and concentrations of scent in comparison to controls. Video monitoring was used to measure parrot visits to scent stations compared to controls and to assess any tendency to explore novel odours. Although the sample sizes were small and individual responses varied, both species showed an ability to distinguish between scents and controls and to detect novel scents. It is likely that both kea and kaka have functional olfactory abilities, and I hypothesise that scent plays a significant role in their ecology.

6.2. Introduction

The use of scent and olfaction in birds has gained considerable attention in the last two decades and avian scent use is associated with behaviour such as orientation, homing, sexual advertisement, partner and individual recognition and foraging (Nevitt *et al.* 1995; Bonadonna & Bretagnolle 2002; Bonadonna & Nevitt 2004; Hagelin 2007; Hagelin & Jones 2007; Balthazart & Taziaux 2009; Bonadonna 2009). Olfactory preference in parrots was first established by Roper (2003) who found that yellow-backed chattering lories (*Lorius garrulus flavopalliatus*) can distinguish between different fruit odours. Hagelin (2004) later reported that a flightless, nocturnal New Zealand endemic parrot, the kakapo (*Strigops habroptilus*), has an olfactory ability which it uses to find food.

Here, for the first time the chemosensory ability of kea and kaka, diurnal members of the distinct parrot family (Nestoridae) that includes the kakapo are examined. Scent responses have never been specifically tested in kea or kaka although chemosensory ability has been suggested. Studies exploring the viability of bird repellents as a wildlife management strategy (Spurr 1979) found that cinnamon scent added to food baits appear to deter both species (Spurr 1979; Udy & Pracy 1981; Orr-Walker *et al.* 2012). However, older baits reportedly did not have the same effect. Additionally, both parrot species have a repertoire of active olfactory genes, albeit not as extensive as in the kakapo (Steiger *et al*. 2009b).

Although kea and kaka are closely related to kakapo they contrast strongly from kakapo and each other in their behaviour, diet and ecology. Unlike the nocturnal kakapo both species are diurnal although kaka can be active on moonlit nights (pers. comm. R. Moorhouse,D. Brunton). Kea are restricted to the South Island of New Zealand where they inhabit podocarp forest on the West Coast of

the South Island and alpine beech forests and mountains throughout the Southern Alps. Kaka are found in low numbers throughout New Zealand but prefer lower altitude continuous native forest tracts and forested offshore islands (Worthy & Holdaway 2002). Kaka are highly neophobic (Wilson *et al.* 1991) and predominantly forage on nectar and honeydew (Beggs & Wilson 1988), which they collect with their specially modified tongue (Kirk *et al*. 1993). However, they will also consume nuts, seedlings, fruits and insects. Kea are truly omnivorous and have been documented as consuming over 100 different food types including insects, fruit, carrion, and plants (Temple 1996; Diamond & Bond 1999). Kea demonstrate extreme inquisitive behaviour (Huber *et al.* 2001; Schloegl *et al.* 2009) and their behavioural flexibility and opportunistic foraging is thought to have evolved in response to the scarcity of food in the kea's mountain habitat (Diamond & Bond 1999).

The aim of this study was to experimentally assess whether kea and kaka can detect or distinguish between different scents. I also measured their tendency to explore novel odours and how birds behaved towards different concentrations of the same odour.

6.3. Methods

Scent experiments were conducted using kea and kaka held in non-display aviaries in the Native Fauna section of Auckland Zoo, New Zealand. Captive kea and kaka show little or no sign of stress related to human presence (Pullar 1996, Collen and Pullar 2010). Cages (6 x 3 x 2.5 m) ran parallel to each other, and all had a concrete floor, a section of overhead shelter, plants, perches and food and water bowls. I worked with three kaka and one kea. A pair of kaka (male and female 1) were housed together and a male kea and female kaka (female 2) were each housed separately. The paired kaka could be individually distinguished by plumage and colouration. The daily routine for all cages was cleaning with sprayed water (circa 09:00 h) followed by replacement of food bowls with fresh fruit and a selection of grains. Twice a day (circa 11:00 h and 14:00 h) the birds received a behavioural enrichment which remained in the cage until the following day's cleanup.

6.3.1 *Scent experiment set-up*

The scent apparatus consisted of a 1 x 1 m plywood base with upright plastic pipes (55 cm high and 5 cm diameter) at each corner. Two pipes contained scent and two were controls so that birds approaching the platform from any direction had a dyadic choice of a scent or control pipe. Each plastic pipe penetrated the base and supported the platform 10 cm above ground. Pipes were sealed at the top but had two 7 cm long slot openings at 28 and 40 cm above the platform. A narrower tube within each pipe was used to hold vials (small plastic screw top containers) at a height of 27 cm, just

below the lower slot opening and out of reach and sight of the birds (Table 6.1a). Control vials were empty and the lids were not perforated, whereas scent vials had a 6 mm perforation in the screw top lid and contained either 15 ml of liquid scent essence in 0.25 g of 100% cotton wool or 1 g of feathers.

Three different scent trials were conducted: 1) fruit (orange and strawberry), 2) mint (weak and strong concentrations), and feather (heterospecific and conspecific). All trials followed the same sequence: day 1: scent (orange, weak mint, or heterospecific feathers), day 2: control, day 3: scent (strawberry, strong mint, or conspecific feathers), then a 1 month period before the next trial. For feather scent, kea and kaka males were presented with their own feathers and the female kaka (female1 & female2) were presented with feathers from the kaka male (housed with female1). Heterospecific feathers were obtained from Australasian Shoveler (*Anas rhynchotis*) housed at the Zoo. The control day between scent presentations within a trial allowed us to test for residual effects (or habituation). At the end of each day all pipes were washed to remove residual scent using a cleaning detergent (Trigene©). A video-camera was placed 2.5 m above the platform outside the cage. Each 'day' of the trial consisted of the 24 hours between when the cages were cleaned. A total of 16 hours of daytime video-recordings per 24 hours (ca 9:00 - 21:00 and 6:00 - 9:00 h DST) were used for analysis.

6.3.2 Measuring parrot responses

The following variables were recorded for each pipe: 1) total frequency of visits made by a bird, 2) total time spent (min) by each bird at a pipe, and 3) number of times a bird touched a pipe with its beak or climbed the pipe. Birds occasionally crawled underneath the platforms and in these cases entry and exit positions were recorded and the time halved to create two events when entry and exit were in different quarters. Future experiments should use closed sides or lower platforms.

Two types of controls were used: trial controls (during scent presentation) and control days between scent presentations. These sets of controls were compared using two-tailed Friedman chi-squared tests (Q) with individual birds as subjects (Agresti 2002). Overall trial controls were not significantly different to control days in the number of visits (Q = 8.716, df = 6, P = 0.190), the total time spent at pipes (Q = 7.178, df = 6, P = 0.305) or the frequency of touches (Q = 8.898, df = 6, P = 0.179). Therefore, it could be concluded that residual scents were not detectable to the birds. Due to the small number of birds used in the experiments, responses to control and scent pipes during each trial were compared using one-tailed Friedman chi-squared tests (Q) with individual birds as subjects (Agresti 2002). A stronger response to scent treatments compared to controls was assumed (Roper

2003, Hagelin 2004, 2007). One-tailed tests were used to compare responses between parrot and duck feathers, predicting stronger responses to conspecfic feathers. 2-tailed Friedman chi-squared tests were used to compare between different scents. All statistical analyses were performed using SAS System for Windows, version 9.1 (SAS Institute, Cary, NC, USA) and a significance level of 5% was used for all tests.

6.4. Results

All birds visited scented pipes significantly more often than control pipes: (mint-scent: Q = 11.1, df = 3, P= 0.011; feather-scent Q = 9.90, df = 3, P = 0.010; fruit-scent Q =7.920, df = 3, P = 0.024; Figures 6.1a-c). During the three scent trials, all birds spent significantly more time at scent-pipes versus control pipes: (mint-scent Q = 12.00, df = 3, P = 0.004; feather-scent Q = 10.8, df = 3, P = 0.007; fruit-scent Q = 9.60, df = 3, P = 0.011; Figs.. 6.2a-c). Finally, scent pipes were touched significantly more often than control pipes for mint-scent (Q = 7.97, df = 3, P = 0.024) and feather-scent pipes (Q = 7.34, df = 3, P = 0.031), but not for fruit scent (Q = 5.31, df = 3, P = 0.076; Figs.. 6.3a-c).

When all scent trials were compared there were no significant differences in time spent at pipes (Q = 0.08, df = 2, P = 0.960), visits to pipes (Q = 0.29, df = 2, P = 0.866) or number of touches (Q = 2.25, df = 2, P = 0.325). (Figures 6.1-3). When responses of birds to the two mint concentrations were compared, significantly more visits were made to low concentration pipes (Q = 4.00, df = 1, P = 0.04; Figure 6.1a), but no differences were found between the two concentrations for time spent (Q = 3.00, df = 1, P = 0.083; Figure 6.2a) or number of touches (Q = 1.00, df = 1, P = 0.317; Figure 6.3a).

No significant differences were found, when comparing responses to duck versus conspecfic feathers (time spent, Q = 0.00, df = 1, P = 1.00; visits, Q = 1.00, df = 1, P = 0.158; number of touches, Q = 1.00, df = 1, P = 0.158; Figures 6.1b-3b). However, individual differences were pronounced in the feather-scent trials and suggest that individual scent preferences are important. Both female kaka reacted strongly towards both types of feather-scents, whereas both males showed little interest in either type of feather-scent. Kaka female2 demonstrated extreme interest when presented with feather-scents; she climbed and stood on top a scent pipe that contained weak mint-scent. The male kea was generally less interested in scent than the kaka; an observation confirmed by the Zookeepers (Pers. comm. M. Whybrow).

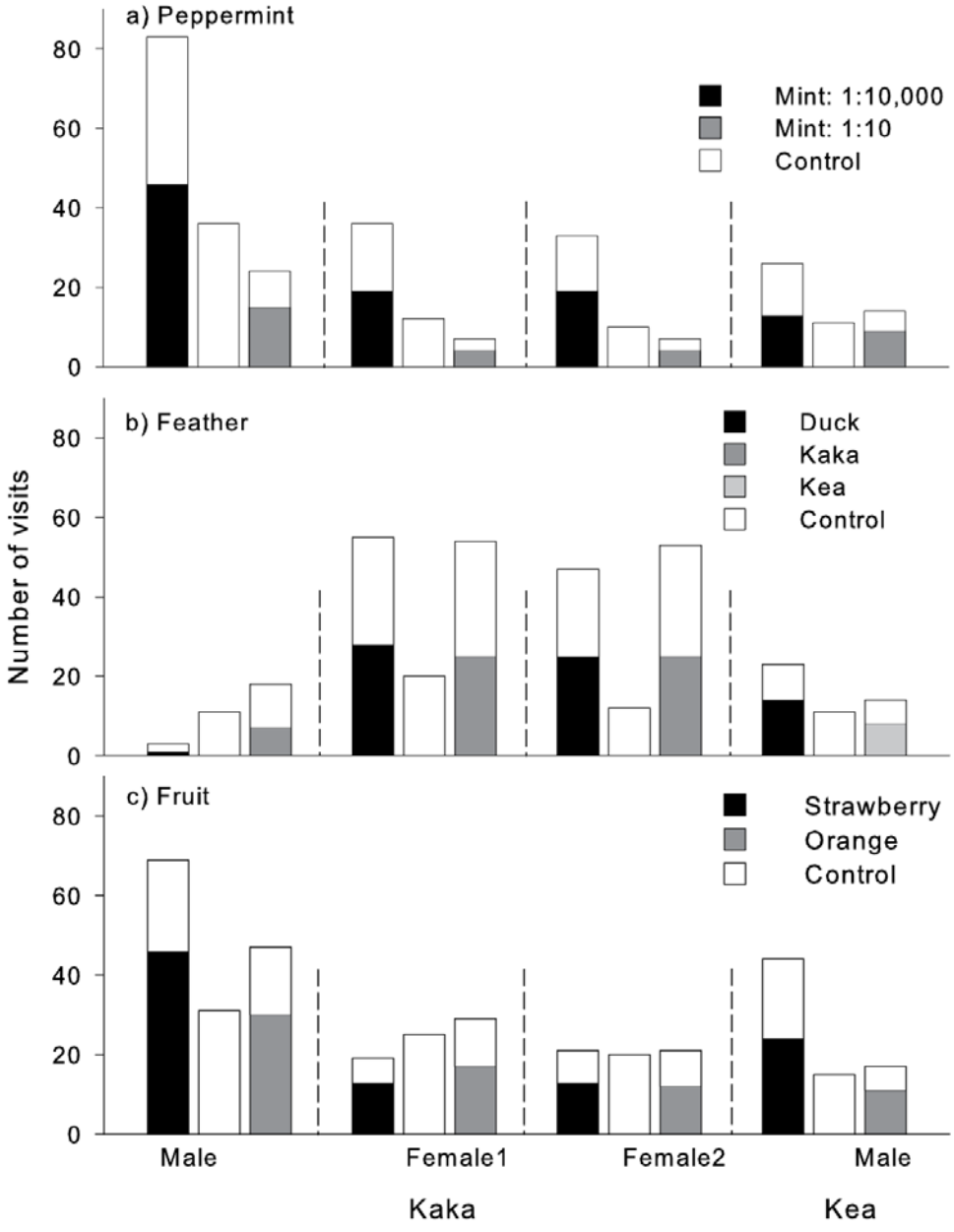

Figure 6.1 The frequency of visits to scent pipes per day (16hrs) for each of three days (scent, control, scent) of the three different scent experiments: mint (diluted with water at 1:10,000 or 1:10), feathers (duck and conspecifics parrot), and fruit (strawberry and orange). The white bars represent controls during scent presentation (above the trial bars) and control days between scent presentations (between trial bars). Note that feathers from the male kaka and kea were used hence both males were presented with their own feathers. For details of the temporal sequence of trials and the dates see Table 6.1.

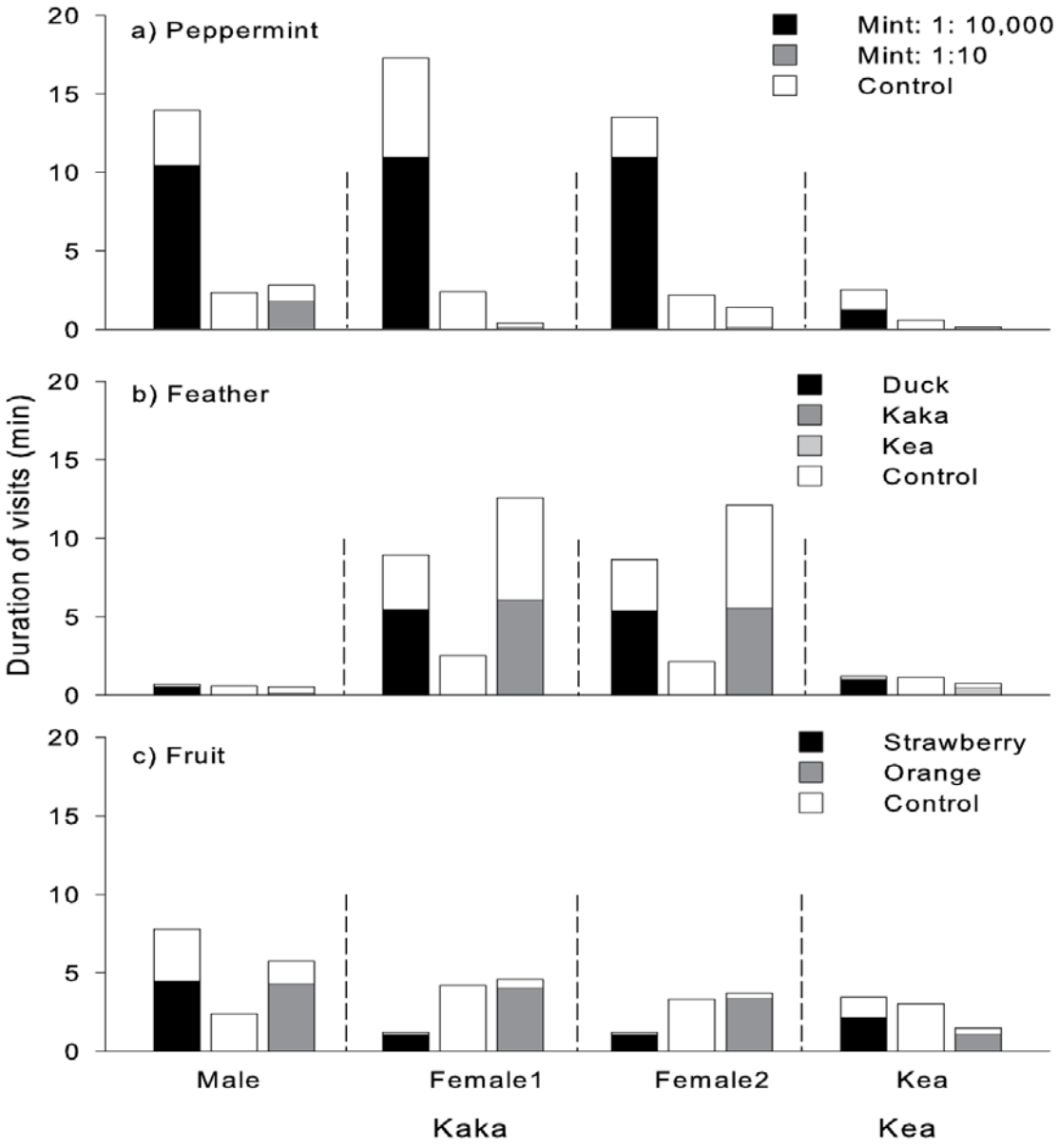

Figure 6.2 The time spent (min) by each bird at scent pipes per day (16hrs) for each of three days (scent, control, scent) of the three different scent experiments: mint (diluted with water at 1:10,000 or 1:10), feathers (duck and conspecifics parrot), and fruit (strawberry and orange). The white bars represent controls during scent presentation (above the trial bars) and control days between scent presentations (between trial bars). Note that feathers from the male kaka and kea were used hence both males were presented with their own feathers. For details of the temporal sequence of trials and the dates see Table 6.1.

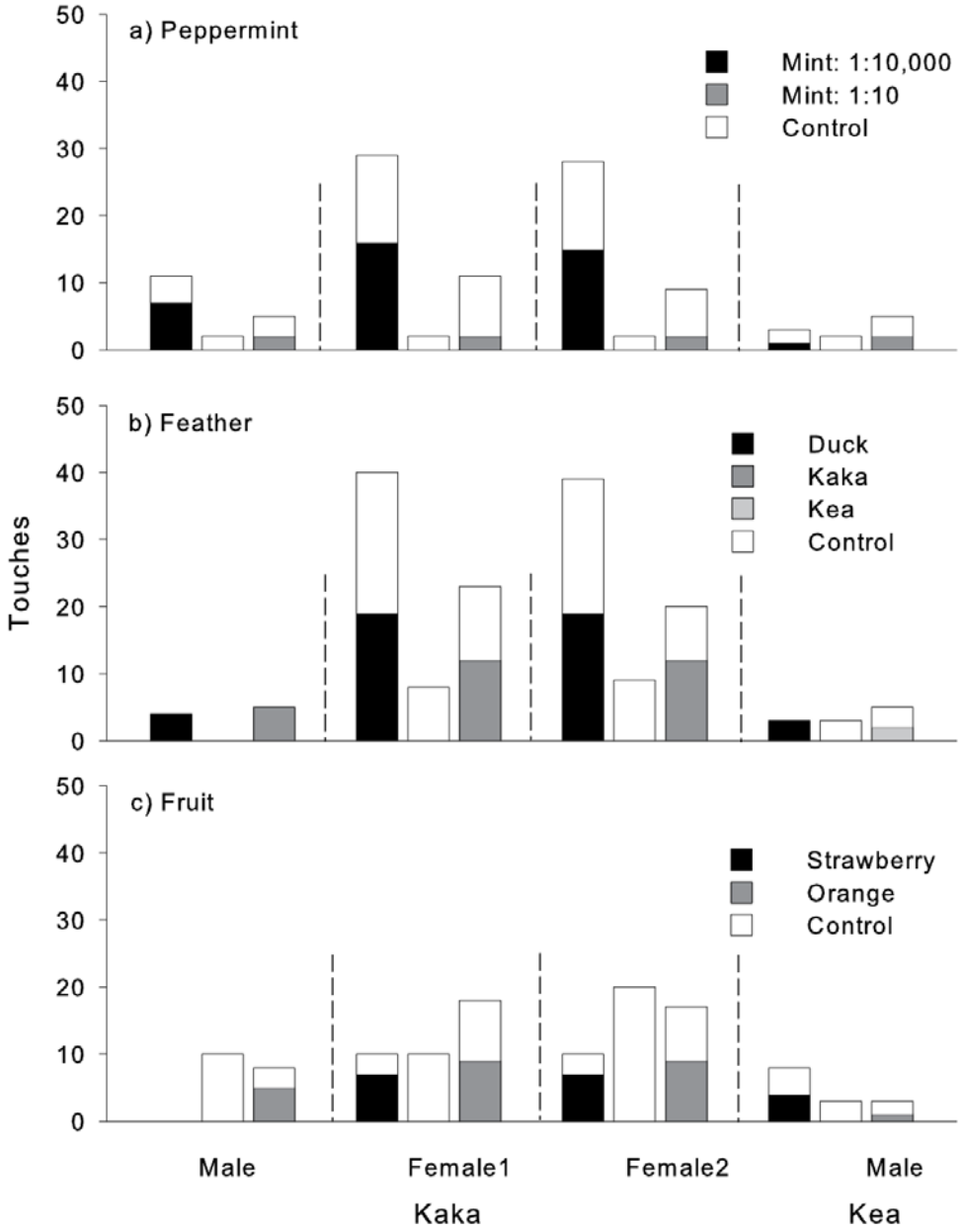

Figure 6.3 The frequency of touches of scent pipes per day (16hrs) for each of three days (scent, control, scent) of the three different scent experiments: mint (diluted with water at 1:10,000 or 1:10), feathers (duck and conspecifics parrot), and fruit (strawberry and orange). The white bars represent controls during scent presentation (above the trial bars) and control days between scent presentations (between trial bars). Note that feathers from the male kaka and kea were used hence both males were presented with their own feathers. For details of the temporal sequence of trials and the dates see Table 6.1.

Table 6.1 Summary of scent trial schedules and the scents used. Photo a) shows the experimental apparatus. Photo b) shows a kaka investigating a scent pipe.

Trial	Day 1	Day 2	Day 3	Dates
Fruit scent	Strawberry[1]	Control	Orange[1]	7/12-9/12, 2010; Recording time: 9:00h - 21:00 h & 5:00h – 21:00 h. Total 16hrs
Mint scent	Mint[1] (diluted 1:10,000)[2]	Control	Mint[1] (diluted 1:10) [2]	3/1–5/1, 2011; Recording time: 9:00h - 21:00 h & 5:00h – 21:00 h. Total 16hrs
Feather scent	Duck feathers Australasian shoveler (*Anas rhynchotis*)	Control	Conspecific feathers: male kea (*Nestor notabilis*) or male kaka (*N.meridionalis*)	4/2-6/2, 2011; 8:00h - 20:00 h & 6:00h – 20:00 h. Total 16hrs

a) Video view **b) kaka smelling pipe**

[1] Hansells© Natural Food Essence

[2] diluted using tap water

6.5. Discussion

The results presented support the conclusion that kea and kaka have functional olfactory abilities. When kea and kaka are given a choice of scent or controls they spend more time at scent pipes and visit and touch them more frequently. Little interest was exhibited in the platforms during the control days between trials (Figures 6.1-3). Despite the study being limited to four parrots, the results are conclusive and the parrots show strong individual preferences to scents.

All three kaka reacted strongly towards low concentrations of mint, but appear to avoid high concentrations (Figures 6.1-3a). Mint is a strong flavour and works not only though the olfactory nerve, but also triggers the trigeminal nerve and can be perceived as pain when at high concentrations (Behrendt *et al.* 2004; Müller-Schwarze 2006). Hence, a mint dilution of 1:10 may have generated an aversive stimulus (Mason & Silver 1983). Both male kea and kaka were presented with their own feather samples (Figures 6.1-3b). Their "avoidance" response to self-odour could be explained by habituation to their scent or from kin-recognition and inbreeding avoidance (Boyse *et al.* 1991; Mardon & Bonadonna 2009; Zhang & Zhang 2011). Self-odour avoidance has been found in petrels (Mardon & Bonadonna 2009) and my results suggest it also occurs in parrots, a completely different taxonomic group. Both female kaka in contrast reacted strongly towards all feather-scents (Figures 6.1-3b). The singly house female (kaka female2) exhibited an extreme response, but the precise trigger is unknown. Kaka female2 will shortly join a breeding programme and is sexually mature. The scent experiments were conducted during the parrot breeding season and both females may have increased olfactory sensitivity comparable to the seasonal olfactory acuity found in female starlings (Clark & Clark 1990). Although all birds showed generally more interest in fruit-scented pipes compared to control pipes, their reactions were less intense to fruit-scent compared to the other scents (Figures 6.1, 2a-c). These parrots were fed daily with fresh fruit and may be more familiar with fruit-scents than novel scents such as mint. However, the chemistry of the fruit-scent essences used is unlikely to be identical to natural fruit and reduced responses to artificial fruit-scents may be based on the lower volatility of artificial scents (Figures 6.1-3c).

Overall, kea and kaka distinguish between types of scent, behave differently towards different concentrations of the same odour and show a tendency to explore novel odours. However, these two species may be sensitive to scent for different ecological reasons. Kea are primarily diurnal and inhabit alpine regions. The lower temperature and humidity found at high altitudes may result in the release of fewer volatile components, which could mean that the kea's sense of smell is directed to a different spectrum compared to kaka. In contrast, kaka are sometimes nocturnal and use the forest

canopy. Activity in such low light situations makes olfaction a good signal and it has been shown that birds in these scenarios have enlarged olfactory bulbs and are likely to rely on scent (Healy & Guilford 1990; Hagelin 2004). The research presented provides a promising foundation for future work on olfaction in kea and kaka including its role in social behaviour. Furthermore, it may be useful to explore olfactory deterrence as a means of decreasing losses of kaka and kea in poison pest control operations. The control of pest-species is imperative in trying to reduce the decline of many New Zealand native bird species including kea and kaka (Moorhouse *et al.* 2003). Until new and cost-effective ways of controlling invasive pests and detecting reinvasions (Gsell *et al.* 2010) using targeted baits and or monitoring systems (Booth *et al.* 2001) have been found, poison pellets need at least to be made undesirable and deterrent for non-target species without losing their attraction (Devine & Cook 1998) to target species. Therefore, the strong smell of mint could work as a deterrent flavour. Finally, this research has shown that kea and kaka can help trial behavioural experiments for kakapo, a critically endangered New Zealand endemic parrot, with a large olfactory bulb and a distinct and differentiated plumage-odour (Chapter Two and Four and Hagelin 2004).

CHAPTER SEVEN

CONCLUSION AND OUTLOOK

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7.1 Introduction

The main motivation for this thesis was to contribute to the growing field of avian olfaction by examining the role of the kakapo's strong scent. The study of avian olfaction has come far in the last twenty years and showed that birds employ olfaction in similar ways to mammals. Birds use scent for individual and partner recognition, territorial marking, sexual attraction and mate choice (Ralls 1971; Johnson 1973; Roper 1999; Hagelin & Jones 2007). However, studies of avian olfaction thus far are restricted to a small number of species, primarily seabirds, known for their large olfactory bulb and their strong, obnoxious smell (Bonadonna & Nevitt 2004; Nevitt 2008). There is a need to study more species in order to better understand whether scent is a more universal signal and when and why birds use or produce olfactory cues. Additionally, studies to date of avian olfaction have generally been confined to one season, and the field lacks studies that observe individual changes in the scent patterns over multiple seasons (pers. comm. J. Hagelin).

Kakapo show unique combinations of behavioural patterns and life history traits that make them an interesting model to study olfaction. Kakapo plumage emits a strong, sweet smell that lasts for days following contact with a bird (pers. comm. J. Rickett). The scent also persists for years on sampled feathers, even when samples are held at room temperature (pers. comm. J. Hagelin). Additionally, kakapo is nocturnal; a scenario where olfaction is a good media for signalling (Healy & Guilford 1990). Males set up a network of tracks and bowls during the breeding season, which they use for their lek breeding displays. These displays consist of flamboyant dances and rocking movements that are combined with flapping and persistent long ranging low frequency calls (Merton *et al*. 1984).

In this thesis, I have explored the kakapo's ability to sense smell by examining the anatomy of the brain and, in particular, the olfactory bulb of the kakapo. Additionally, I analysed the quality of information kakapo emits with its feather scent by studying the overall chemical composition of the scent of feather samples from individuals of different sex, age and at different seasons. Moreover, the ability of kea and kaka to ascertain scent and different concentrations of scent was examined. This concluding chapter recalls and summarises my findings and synthesises how my findings contribute to the science of avian olfaction and the knowledge of the olfactory and visual abilities of a critically endangered species, the kakapo. Finally, I critique this research and suggest ideas for future work.

7.2 Main findings

7.2.1 The brain of the kakapo as a window to its sensory abilities

Surprisingly, the examination of the anatomy of the brain showed that the kakapo's visual abilities are limited in that kakapo has enhanced light sensitivity but a relatively poor visual acuity. The tectofugal vision pathway, specifically the optic tectum, nucleus rotundus and entopallium, are all significantly reduced in size relative to the rest of the brain. These findings differ from Pettigrew's (1978) observation, who commented that the kakapo could have visual specialisations similar to that of owls based on its nocturnal activity pattern and the presence of a facial ruff, which gained the parrot the moniker 'owl parrot'. There is however, no apparent reduction to the thalamofugal visual pathway, namely the Wulst. While the function of the Wulst is still not entirely clear (pers. comm. M. Wild), it may have to do with the ability to see in a binocular format. Finally, the retina morphology shows features for both functioning in a nocturnal and diurnal environment, suggesting a retina that is specialised for a crepuscular niche and therefore does not follow the traditional view of the evolution of nocturnality in birds. A reduced visual acuity can only be compensated with the improved acuity by another sense, olfaction being one of them. My research found that kakapo has a significantly larger olfactory bulb compared to other Australasian parrots. In particular, the number of mitral cells in the mitral cell layer was higher than in any other avian or mammalian species examined to date. The findings suggest limited visual acuity, but an acute sense of smell.

7.2.2 The quality of the chemical composition of kakapo scent

In the light of an acute sense of smell, the chemical composition of the feather scent from 67 birds of different sex, age and during different seasons was analysed using gas chromatography-mass spectrometry. Feather samples of booming males had a significantly greater diversity and individual variation of chemical components compared to feather samples of non-booming males. Feather samples collected from non-booming males during the breeding season also had a significantly greater individual variation of chemical components compared to feather samples of males collected during the non-breeding season. Females, in contrast, maintained high levels of individual variation of chemical compounds in their body odour year round. There was also a strong indication from my data that plumage odour from juvenile kakapo have a different chemical profile compared to plumage odour of adult birds, which could signify that olfactory cues might play a role in individual and chick recognition. Greater variation of chemical components present in booming males and the seasonal pattern to male odour is consistent with the use of scent as a sexual signal. The chemically diverse, year-round odour of females is harder to explain, but may relate to competition for high qual-

ity territories during the non-breeding season and the competition for males during the breeding season.

7.2.3 The use of scent in the kakapo's closest relatives, the kea and the kaka

Behavioural and experimental analyses can only be conducted in limited ways in a critically endangered species, like the kakapo. Therefore, I experimentally tested the ability of the kakapo's closest relatives, the kea and the kaka, to discriminate between different scents and concentration of scents. The two New Zealand endemic parrots offered a suitable alternative to kakapo, and the opportunity to study olfaction in two other bird species. Even though samples sizes were low and limited the efficacy of the experiment, it nonetheless showed that both parrots, kea and kaka, do have a strong sense of smell (Gsell *et al*. 2012). Not only were they able to distinguish between different scents, but they also showed strong behavioural differences when confronted with different concentrations of the same smell. A number of studies have shown behavioural and physiological similarities between closely related species, which, to some degree, justifies using kea and kaka as a model for kakapo (Shubin *et al.* 2009). Hagelin (2004) found that kakapo can find food based on scent and my scent experiments with kea and kaka allow me to conclude that these two species can serve in future as surrogate test models for kakapo, especially when trialling future experiments for kakapo.

7.3 Future Research

My research into the feather scent currently lacks the specific identification of the chemical components that characterise the scent. At the moment, the data presented are based on the retention times of the chemical components (the characteristic time a particular chemical constitute takes to pass through the system (from the column inlet to the detector) under set conditions (Sparkman *et al*. 2011)). Once their identification is resolved, I will be able to comment on what chemicals the sex and season based differences in the feather scent relate to. This will open new options, as it will then be possible to relate specific chemicals to food intake for example, especially as the diet of the individual birds is carefully recorded by the Kakapo Recovery Team, Department of Conservation, New Zealand. Additionally, it will be possible to compare and relate the chemicals found on the body odour of kakapo with those found on other birds and reported in the last two decades (Campagna *et al.* 2012).

Sample sizes are always an issue, when working with critically endangered species. Nonetheless, access was obtained to feather samples from half of the population that makes up this species, even though the study would have profited by having more samples from breeding females and juvenile

birds. However, future work is planned that will help to gain more feather samples from birds currently not included in my dataset, and this will help to get a more powerful analysis in regard to the breeding versus non breeding females as well as samples from juveniles versus adult birds.
Furthermore, this study showed that much information can be gained by examining the brain anatomy. However, my research was limited by having only one sample, the brain of an old, male kakapo. It would be interesting to look at the brain anatomy of birds from different ages and sexes when samples become available.

Future research should especially address what role scent plays in kakapo's social interactions and where the scent originates. I suggest three approaches:

7.3.1 *Can bird odour signals indicate a male's quality?* [1] *Are these signals shaped by sexual selection in the same way as ornaments such as colourful plumage or complex song*?

Sexual selection theory involves intra-sexual competition for access to mates and mate choice (Andersson 1994; Andersson & Simmons 2006). The significance of mate choice has been disputed (Trivers 1972; Andersson & Simmons 2006). However, in lek mating systems the role of female mate choice is explicit: the only resources females receive from the males they select are gametes (Ryder *et al.* 2010). Kakapo males exhibit an 'exploded' lek system with widely distributed displaying males (Merton *et al*. 1984; Payne 1984). Some males are 'attractive' and have extraordinary reproductive success; other 'unattractive' males fail to breed at all. Despite the kakapo's endangered status it is an ideal model for understanding olfaction and mate choice because the vocal behaviour, genetics, reproductive success and movement of all individuals is monitored very closely by New Zealand's Department of Conservation. Most significantly, my research has found sexual and individual variation in the feather scent of kakapo and the histological examination of a kakapo brain revealed a comparatively large olfactory bulb, indicating an acute sense of smell (Chapter Two and Four). Additionally, kakapo have limited vision and their retina shows elements of both diurnal and nocturnal birds, which means that visual signals of quality can be ruled out (Chapter Five and Corfield *et al*. 2011).

[1] This research is currently in discussion in collaboration with Assoc. Prof. Dianne Brunton (Massey University, Auckland, New Zealand), Prof. Dave Greenwood (The School of Biological Sciences, Auckland University, Auckland, New Zealand), Prof. Tom Goodwin (Hendrix College, Conway, Arkansas, USA) and Assoc. Prof. Dr. Julie Hagelin (Institute of Arctic Biology, University of Alaska, Fairbanks, Alaska, USA).

7.3.2 Do kakapo display MHC-dependent mate choice based on individual-specific scent? [2]

The major histocompatibility complex (MHC) is a highly polymorphic cluster of genes that plays an important part in the immune response of individuals to pathogens (Edwards & Hedrick 1998; Milinski 2006; Chaix *et al.* 2008). MHC genes are also linked to reproductive and social behaviours, such as mate choice and kin selection (Penn & Potts 1998; Jacob *et al.* 2002; Beauchamp & Yamazaki 2003; Boehm & Zufall 2006). Future research could examine whether kakapo displays MHC-dependent mate choice based on individual-specific scent.

Preliminary research of the kakapo MHC has successfully sequenced the variable peptide binding region (PBR) of the exon 2 of the class II *B* MHC gene (Robertson 2006 and Robertson unpubl. data). All individual kakapo possess a maximum of two alleles indicating that there is only a single class II *B* locus in kakapo and no pseudogenes, as found in other parrots and non-passerine birds (Hughes *et al.* 2008). With my data on individual scent profiles presented in this thesis as well as additionally won samples from the remaining birds (as in Chapter Two) missing in my dataset, and the MHC genotypes for all kakapo, the research team will be in a position to examine whether mate choice in kakapo is MHC-dependent and/or based on individual scent. Research by the Kakapo Recovery Team has reliably documented all kakapo matings since 1996 and the Dr. B. Robertson has resolved the paternity of the successful matings (Robertson 2006; Robertson *et al.* 2009). Based on these data, it is possible to use two metrics to investigate whether mated pairs were less similar at the MHC Class II *B* exon 2 than expected under random mating (disassortative mating : Juola & Dearborn 2012): an allele-sharing index and an index that captures the magnitude of differences between alleles.

2 This research is planned in collaboration with Prof. B. Roberston (Otago University, Otago, New Zealand), Assoc. Prof. Dianne Brunton (Massey University, Auckland, New Zealand) and Prof. Dave Greenwood (The School of Biological Sciences, Auckland University, Auckland, New Zealand). Some funding has been secured and work will start in August 2012.

7.3.3 Chemical composition and origin of scent responsible for sexually attractive body scent in kakapo?[3]

Olfaction is an important signal for mate-choice decisions in mammalian species and has recently been recognised as a significant aspect of communication for several bird species (Chapter One). Although the pathways by which olfactory cues are produced are still obscure, it is known that microbes alter olfactory communication in animals by changing chemical signals either by degrading or adding volatile metabolites (Bradbury & Verencamp 1998). Feather-scent in kakapo revealed significant individual variation, especially between booming and therefore sexually active males and non-booming males (Chapter Two). If olfaction is derived from or altered by distinctive microbes associated with individuals, then reproductively active birds should have a different microbe community compared to reproductively inactive birds. Microbial communities may provide insights that could perhaps explain observed differences in the scent profiles.

3 This research is in discussion in collaboration with Dr. Peter Deines and Assoc. Prof. Dianne Brunton (both at Massey University, Auckland, New Zealand) as well as Dr. Mike Taylor (The School of Biological Sciences, Auckland University, Auckland, New Zealand).

7.4 Implications

It has generally been assumed that the sense of smell in birds has decreased during the evolution of birds (Turner 1892; Edinger 1951; Wenzel 1971a; Pearson 1972, Zelenitsky *et al.* 2009), presumably because birds have good eye sight, produce loud and complex songs and display a large repertoire of stereotypical social behaviours, making the role of olfaction appear to be minor in comparison. A recent study by D. Zelenitsky, however, showed that the importance of olfaction increased during the early evolution of birds (Zelenitsky *et al.* 2011). By having an enhanced olfactory acuity and improved navigation and foraging skills, for example, olfaction might have helped modern birds to survive the late Cretaceous mass extinction, which is marked as a time of great diversification for birds (Cooper & Penny 1997). Kakapo belong to a very distinct and ancient lineage of parrots (Nestoridae) (Wright *et al*. 2008), and stand out with a markedly large olfactory bulb compared to other parrots (Chapter Four and Zelenitsky *et al*., 2011). The advanced cognitive abilities and especially the use of tools (Lefebvre *et al*. 2002; Lefebvre *et al*. 2004) in more modern, phylogenetically more diverged Psittacciformes (Emery 2006), might have lead in these orders to a divergence from olfactory to more visual capacities for example, which fits with the findings in this thesis (Chapter Four and Five), in that kakapo had the largest olfactory bulb and the highest number of mitral cells, whereas the other Australasian parrots in my data set stood out with a rather well developed visual system.

Selective pressures for increased (or at least not decreasing) olfactory acuity in kakapo, might have come along with the bird becoming nocturnal (Healy & Guilford 1990; Healy 1996), as the efficiency of visual perception is reduced in low light conditions. This theory is supported by the kakapo's unusual visual system that is characterised by traits consistent with nocturnal birds, including owls (retina, eye size and shape and orbit orientation), caprimulgiformes (eye size and shape), kiwi (brain morphology), and also diurnal birds (eye size and shape). Based on this suite of traits, kakapo likely have somewhat reduced their overall reliance on vision. However, their visual abilities are characterised by a large binocular visual field, enhanced low light sensitivity and poor visual acuity compared to other nocturnal birds. In doing so, the kakapo breaks the dichotomy typical of the evolution of nocturnality in birds and illustrates that the visual system can evolve in a mosaic rather than a strictly concerted fashion by exhibiting individual nocturnal traits found in a variety of other unrelated nocturnal birds. The importance of olfaction in kakapo is not only marked by its large olfactory bulb, but also by an increased number of olfactory receptor genes and the proportion of intact olfactory receptor genes in their genome (Steiger *et al*. 2009a). Additionally, kakapo has the largest number of mitral cells counted in the mitral cell layer of the olfactory bulb in any bird or mammal counted to date (Chapter Four). The number of mitral cells is positively correlated with olfactory

acuity (Mackay-Sim & Royet 2006) and therefore indicates a fully functional and highly developed olfactory system in kakapo. The mitral cell layer serves as port between incoming and outgoing olfactory signals in the olfactory bulb and ultimately is responsible for all reactive processes to olfactory cues (Nieuwenhuys 1966). In rabbits, spatially separated mitral cells have been found to decode specific molecules, which could mean that the number of mitral cells also represents the ability of an animal to identify different molecules (Mori *et al.* 1992; Nagayama *et al.* 2004). Mitral cell numbers generally diminish with age (Hinds & McNelly 1977; Hinds & McNelly 1979; Bhatnagar *et al.* 1987). Considering that the kakapo individual studied here was approximately 100 years old, it would be interesting to know the mitral cell numbers for younger birds.

Yet, it is not clear why kakapo possess such a highly developed olfactory sense. Hagelin (2004) was able to demonstrate that kakapo sense food using olfactory cues. Apart from foraging, olfaction might, however, play a role in behaviours of social relevance. In particular, as this study found significant differences in the individual variation of feather samples from booming versus non-booming males as well as between non-booming males during the breeding season versus males during the non-breeding season. Even though I was unable to discern the identity of the specific chemicals responsible for these differences, it is possible that the variation of the chemical composition observed acts as some mode of chemical information or signal that is of fundamental role for these birds.

As with all signals, there is a trade-off between the incurring costs and the benefits gained by having a specific signal (Andersson 1994; Maynard Smith & Harper 2003). Costs and benefits of having a smell are two-fold. Firstly, costs and benefits can incur by producing a scent (production cost). Secondly, they can incur by emitting a scent, if that scent reveals the sender's location and makes it more vulnerable to predators. For example, scent marking mice not only advertise their sexual status by scent marking, but simultaneously reveal their location to potential predators (Roberts *et al.* 2001). Red knots (*Calidris canutus*), for instance, undergo a seasonal change from more volatile monoesters of lower molecular weight, to the less volatile diesters of higher molecular weight (Piersma *et al*. 1999) when migrating from their wintering grounds to their breeding grounds in the high Arctic. Olfactory-searching dogs had great difficulty in finding mixtures of diesters compared to mixtures of monoesters, suggesting that the diesters have a cryptic role and therefore reduce the risk of predation to the nest (Reneerkens *et al*. 2005). In an experiment with captive birds, Reneerkens *et al*. (2007) showed that birds with limited food supply would not undergo an olfactory change, indicating that the production of diesters is costly. Likewise, sick birds do not show a change in their chemical profile (pers. comm. J. Reneerkens). However, the bird's cost of producing the less volatile

diesters might be offset by the benefits of having a lowered risk of losing their clutch. New Zealand originally did not have mammalian predators (Wilson 2004), and therefore birds like kakapo and kiwi possibly were able to use olfaction as a tool of communication, without having the danger of being predated (Clavero *et al*. 2009). Today, the strong smell make these birds an easy target for invasive mammalian species.

Kakapo are lek breeders and females would, as in all lek breeding animals, assess specific signals expressed by the male that can indicate genetic qualities of a male and help them choosing a mate. Peahens (*Pavo cristatus*) for example prefer males with larger and more elaborated trains and will lay more eggs for them (Petrie *et al*. 1991; Petrie & Williams 1993). Trains are hypothesised to have evolved due to female sexual selection, but also to act as an 'honest signal' indicating the male's genetic and health constitution to the female. It is currently not known, what kind of signal females assess in kakapo males. During the breeding season males excavate shallow bowls, which are interconnected with tracks (Merton *et al*. 1984). Bowls are preferably excavated against overhanging banks, rock faces or trees, as these create a sound wall to reflect their low frequency booming calls (Merton *et al*. 1984). Males call throughout the night, up to 8000 times, to attract females from afar as calls are heard up to five kilometres. To date no correlation has been found between booming rates and mate-choice (pers. comm. G. Harper). When females approach, they are welcomed with flamboyant dances (Merton *et al*. 1984). However, due to their limited visual acuity the birds would not see much, and I suggest that kakapo males use olfaction as a tool of sexual advertisement at close range. While flapping their wings they are able to broadcast their signal, while approaching females can assess the smell.

For a scent to be a reliable sign of quality its production would have to bear a cost, as mentioned above. The costs incurring by an olfactory signal are generally very difficult to assess, as many factors are at play (pers. comm. J. Reneerkens). However, it could be that healthier and qualitatively better males have a larger home range with more suitable and high quality food available compared to males of less quality, which both may translate into the chemical composition of their body scent. Even though it is known that food intake can alter the quality of the body smell (Kodricbrown 1989; Ferkin 1999), there are only few studies about the impact of the diet on body odour and its attractiveness. For example, the attractiveness of the body odour of males living on meat in humans (Havlicek & Lenochova 2006). However, it would be reasonably easy to conduct controlled dietary experiments, using captive kea and kaka as Chapter Six has shown, to determine the effect of a controlled diet on plumage scent. Scent can also change according to the birds' hormonal status. Female

mallards emit a specific smell indicating their readiness to males (Balthazart & Schoffeniels 1979). Estradiol treated female mallards, in the non-mating season, start producing fatty acids in their uropygial gland, identical to those which they produce during their mating season (Bohnet *et al.* 1991), indicating that their smell is hormonally induced. Male kakapo do exhibit an annual cycle of gonadal growth and regression, which has been shown to be linked with annual faecal testosterone/estradiol levels In kakapo (Cockrem 1995). It may well be that the hormonal changes in kakapo are translated into changes of the plumage scent in kakapo.

Based on my results, it is therefore tempting to speculate that male kakapo have multiple indicators of 'quality' to attract females: booming calls to draw females from long distances and olfactory signals at close range. The male's individual variation in the body scent may serve the females as a platform to choose from, and based on the male's chemical profiles, females may ascertain a male's genetic quality. The scent may perhaps even inform females about the male's abilities to compete against other males, or their health condition.

Der Duft

Wer bist du, Unbegreiflicher: du Geist,
wie weißt du mich von wo und wann zu finden,
der du das Innere (wie ein Erblinden)
so innig machst, daß es sich schließt und kreist.

Der Liebende, der eine an sich reißt,
hat sie nicht nah; nur du allein bist Nähe.
Wen hast du nicht durchtränkt als ob du jähe
die Farben seiner Augen seist.

Ach, wer Musik in einem Spiegel sähe,
der sähe dich und wüßte, wie du heißt.

Rainer Maria Rilke, 1875-1926

Sep 2004

Re-
becca Wu

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APPENDIX A: STATEMENT OF CONTRIBUTION 1

Figure A1 Statement of contribution for a publication partly reffered to in Chapter Three: Corfield, J., **Gsell, A. C.,** Brunton, D. H., Monica, A., Heesy, C. P., Hall, M. I. & Iwaniuk, A. N. 2011 Anatomical specialization of the visual system in the endangered and nocturnal Kakapo (*Strigops habroptilus*). *PLoS ONE* **6**, e22945.

DRC 16

MASSEY UNIVERSITY
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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anna Gsell

Name/Title of Principal Supervisor: A/P Dianne Brunton

Name of Published Research Output and full reference:

Corfield, J., Gsell, A. C., Brunton, D. H., Monica, A., Heesy, C. P., Hall, M. I. & Iwaniuk, A. N. 2011 Anatomical specialization of the visual system in the endangered and nocturnal Kakapo (Strigops habroptilus). PLoS ONE 6, e22945.

In which Chapter is the Published Work: Chapter Three

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate 65%
  and / or
- Describe the contribution that the candidate has made to the Published Work:

Anna Gsell — Digitally signed by Anna Gsell DN: cn=Anna Gsell, o=INS, ou=Ecology and Conservation Group, email=a.c.gsell@massey.ac.nz, c=NZ Date: 2012.05.18 16:32:12 +12'00'

Candidate's Signature — 18.05.2012 — Date

Dianne Brunton — Digitally signed by Dianne Brunton DN: cn=Dianne Brunton, o=Massey University, ou=INS, email=d.h.brunton@massey.ac.nz, c=NZ Date: 2012.05.21 11:16:30 +12'00'

Principal Supervisor's signature — 21 May 2012 — Date

GRS Version 3– 16 September 2011

APPENDIX B: STATEMENT OF CONTRIBUTION 2

Figure B.1 Statement of contribution for a publication partly referred to in Chapter Six: **Gsell, A. C.**, Hagelin, J. & Brunton, D. 2012 Olfactory sensitivity in Kea and Kaka. *Emu* **112**, 60-66.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anna Gsell

Name/Title of Principal Supervisor: A/P Dianne Brunton

Name of Published Research Output and full reference:

Gsell, A. C., Hagelin, J. & Brunton, D. 2012 Olfactory sensitivity in Kea and Kaka. Emu 112, 60-66.

In which Chapter is the Published Work: Chapter 7

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate 80%
  and / or
- Describe the contribution that the candidate has made to the Published Work:

Anna Gsell — Digitally signed by Anna Gsell DN: cn=Anna Gsell, o=INS, ou=Ecology and Conservation Group, email=a.c.gsell@massey.ac.nz, c=NZ Date: 2012.04.20 17:09:44 +12'00'
Candidate's Signature

20 April 2012
Date

Dianne Brunton — Digitally signed by Dianne Brunton DN: cn=Dianne Brunton, o=Massey University, ou=INS, email=d.h.brunton@massey.ac.nz, c=NZ Date: 2012.05.18 15:27:57 +12'00'
Principal Supervisor's signature

18 May 2012
Date

GRS Version 3– 16 September 2011

APPENDIX C: DETERMINATION OF PEAKS

Figures C1-3 Assessment of peaks in the chromatograms (see 2.4.1 and APPENDIX E). An example is provided and the different steps are explained in Figures 1-4.

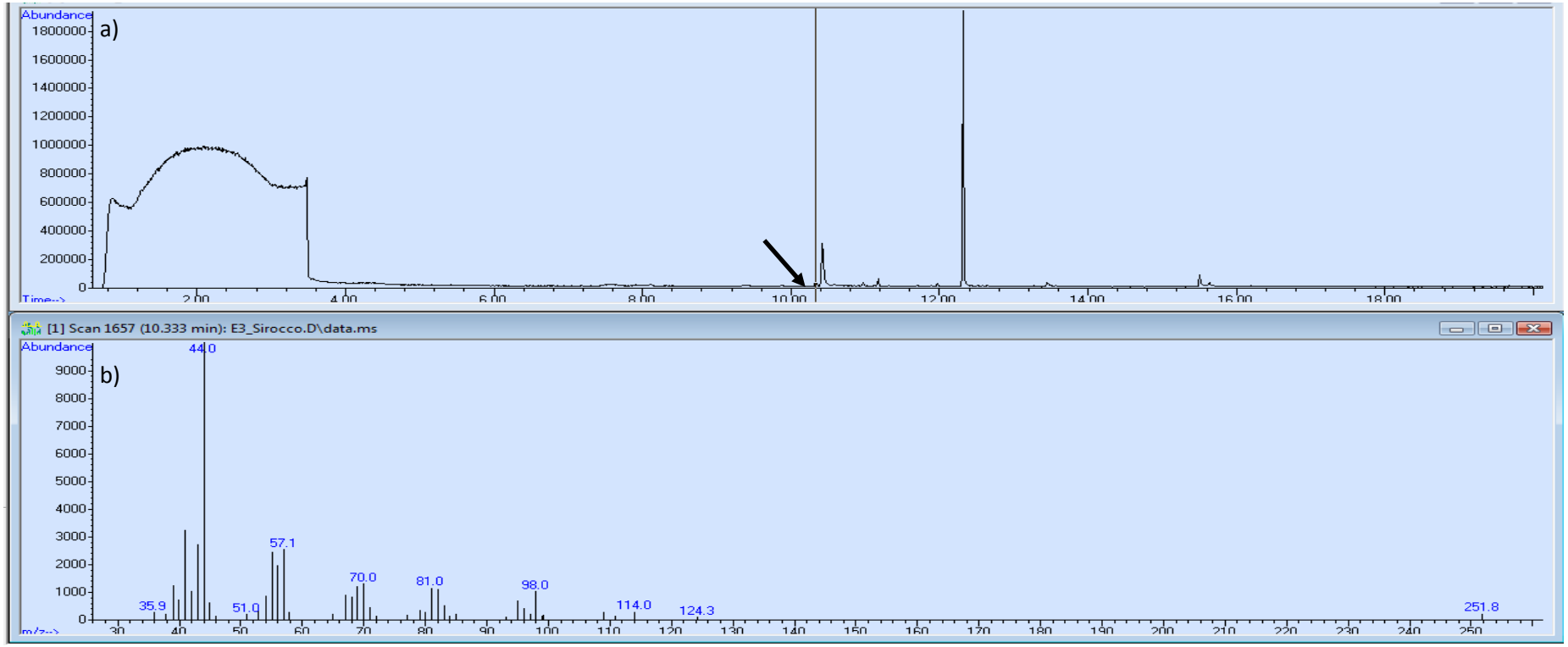


Figure C1a Chromatogram with a peak at 10.3 minutes, indicated by the cursor/arrow. Time (min), on the x-axis and Abundance of the peak (nanoamps) on the y-axis. **Figure C1b** Fragmentation series of compounds found at the area of interest with the mass of the molecules indicated as numbers.

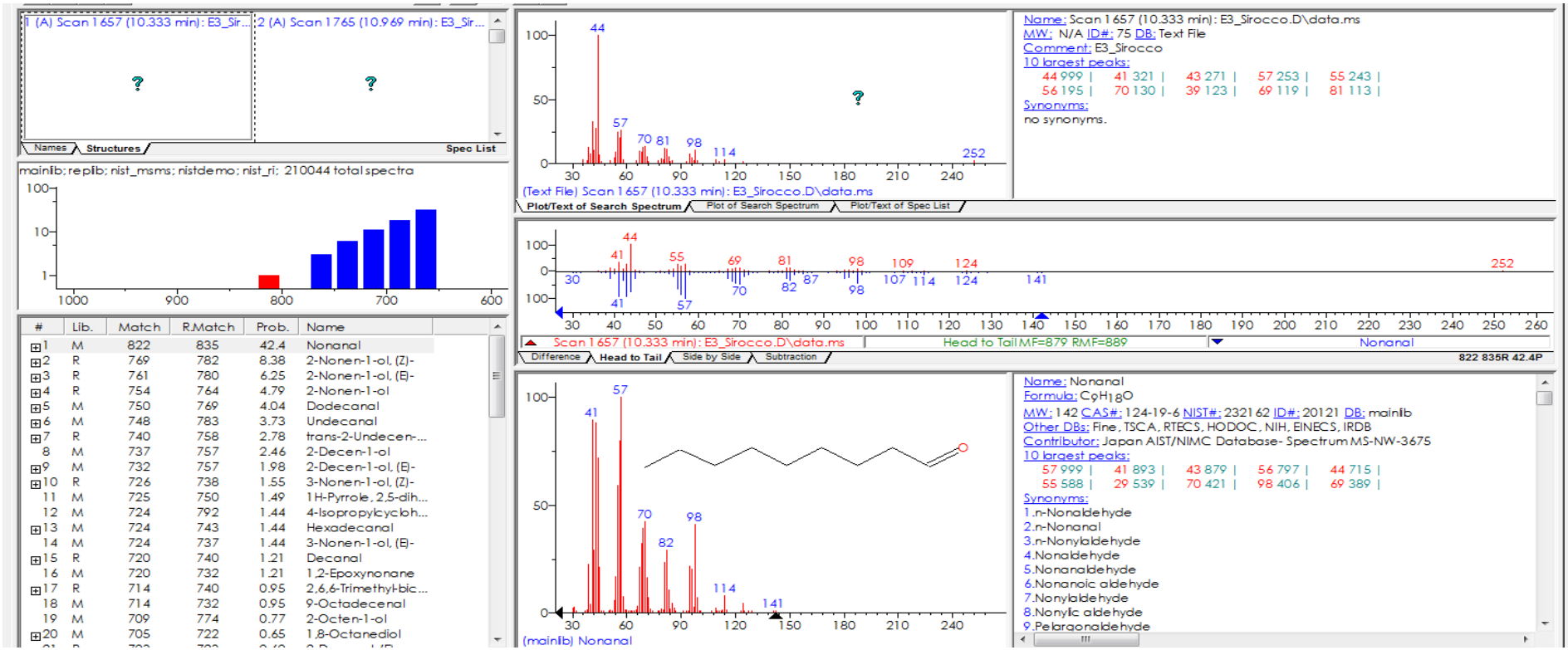


Figure C2 An Agilent 6890 Gas Chromatography was used for the analysis (see 2.3.3). The computer intern program contains the Automated Mass Spectral Deconvolution and Identification System (AMDIS). AMIDS is designed to extract spectra for individual components in the data file and identifies target compounds by matching these spectra against a reference library, the National Institute of Standards and Technology (NIST) (Watson & Sparkman 2007; NIST 2008). The AMIDS search page shows that, in this particular case (at 10.3min), the highest Match (822/1000) is achieved with Nonanal. However, matches above 700 are usually of interest, and the expertise of a chemist is needed to exactly identify the relevant chemical components.

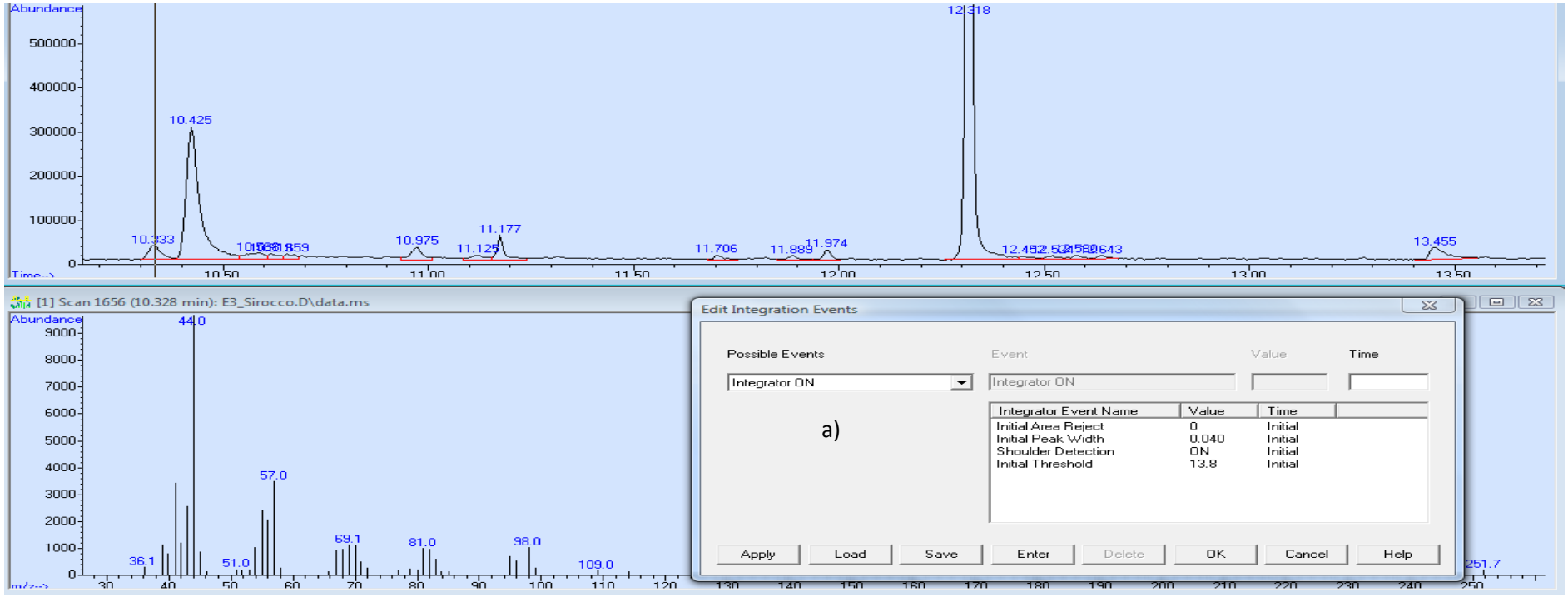


Figure C3 The sensitivity by which peaks are recognised by the system also depends on the voluntary choice of the threshold, which is the height of the Abundance. The choice of the threshold must be equivalent for all samples run on a specific day. In this example, the threshold (a) is 13.8 (nanoamps).

Table C1 Examples of an output data file, received from the Agilent 6890 Gas Chromatograph and AMIDS. The area + RT times (in bold) and peak heights were copied into an Excel file and alignments were made (i.e. manually). Only peaks (peak height) and retention times (R.T. min), that had a peak height above 700 nanoAmps (in bold) were included into the analysis.

Area Percent Report

Data Path : C:\msdchem\1\DATA\GCMS\20110308Analysis\
Data File : 03.D
Acq On : 8 Mar 2011 18:35
Operator : Zum
Sample : 03
Misc :
ALS Vial : 8 Sample Multiplier: 1

Integration Parameters: autoint1.e
Integrator: ChemStation

Method : C:\msdchem\1\METHODS\default.m
Title :

Signal : TIC: 03.D\data.ms

peak #	**R.T. min**	first scan	max scan	last scan	PK TY	**peak height**	corr. area	corr. % max.	% of total
1	**5.159**	83	97	104	BV 8	688	10699	0.32%	0.191%
2	**5.890**	200	222	230	PV 8	**909**	59083	1.75%	1.057%
3	**5.951**	230	232	244	VV 6	**932**	32251	0.96%	0.577%
4	**6.038**	244	247	250	VV 4	**1011**	17526	0.52%	0.313%
5	**6.514**	322	328	340	PV 5	437	12032	0.36%	0.215%
6	**6.831**	369	382	387	VV 5	**782**	31449	0.93%	0.562%
7	**6.882**	387	390	403	VV 6	667	20949	0.62%	0.375%
8	**7.062**	414	421	433	PV 3	413	13710	0.41%	0.245%
9	**7.175**	433	440	443	VV 4	454	6828	0.20%	0.122%
10	**7.449**	456	487	491	PV 7	**2607**	169695	5.03%	3.035%
11	**7.509**	491	497	509	VV 8	**2836**	164165	4.87%	2.936%
12	**7.609**	509	514	518	VV 7	**2605**	79294	2.35%	1.418%
13	**7.646**	518	520	567	VV 8	**2602**	248205	7.36%	4.439%
14	**8.359**	634	641	651	VV 3	521	14386	0.43%	0.257%
15	**8.613**	677	684	700	VV 7	**940**	40183	1.19%	0.719%
16	**8.794**	700	715	728	VV 7	698	53072	1.57%	0.949%
17	**8.891**	728	732	735	VV 4	**825**	13678	0.41%	0.245%
18	**8.941**	735	740	756	VV 3	**785**	24732	0.73%	0.442%
19	**9.536**	823	841	845	PV 5	329	8117	0.24%	0.145%
20	**11.553**	1173	1184	1187	PV 5	**1674**	30355	0.90%	0.543%
21	**11.596**	1187	1192	1203	VV 4	**2060**	64437	1.91%	1.152%
22	**12.665**	1368	1373	1376	PV 4	595	7369	0.22%	0.132%
23	**12.693**	1376	1378	1397	VV 6	549	34082	1.01%	0.609%
24	**13.004**	1397	1431	1436	VV 8	633	68461	2.03%	1.224%
25	**13.070**	1436	1442	1458	VV 6	534	20101	0.60%	0.359%

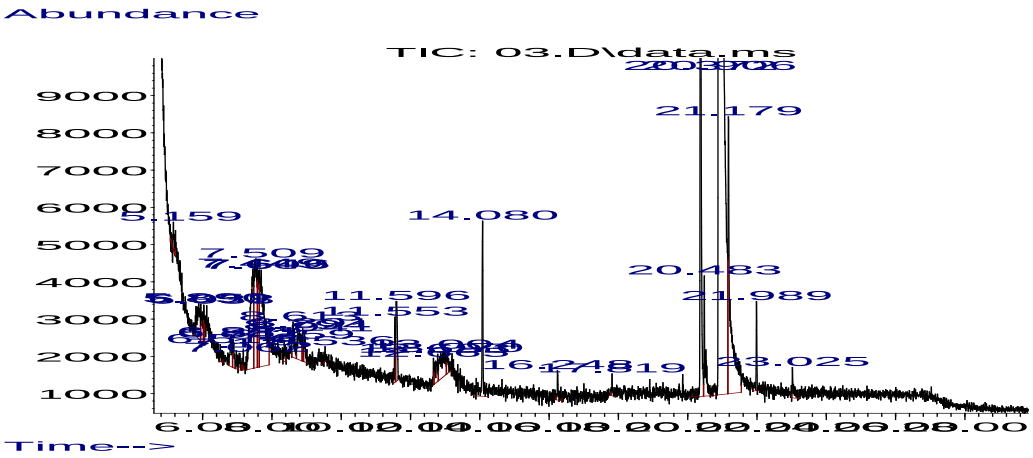
List continued

peak R.T. first max last PK peak corr. corr. % of

#	min	scan	scan	scan	TY	height	area	% max.	total
26	**14.080**	1570	1614	1642	PV 5	**4489**	109221	3.24%	1.953%
27	**16.248**	1974	1983	2004	VV 6	725	20979	0.62%	0.375%
28	**17.819**	2242	2250	2266	BV 4	536	10806	0.32%	0.193%
29	**20.372**	2643	2684	2698	PV	**13729**	348089	10.32%	6.225%
30	**20.483**	2698	2702	2732	VV 7	2980	72146	2.14%	1.290%
31	**20.906**	**2732**	**2774**	**2818**	**PV**	**58536**	**3373794**	**100.00%**	**60.334%**
32	**21.179**	2818	2821	2883	VV 7	**745**4	353049	10.46%	6.314%
33	**21.989**	2941	2959	2969	VV 3	**2280**	34546	1.02%	0.618%
34	**23.025**	3131	3135	3167	PB 4	**777**	24376	0.72%	0.436%

Sum of corrected areas: 5591866

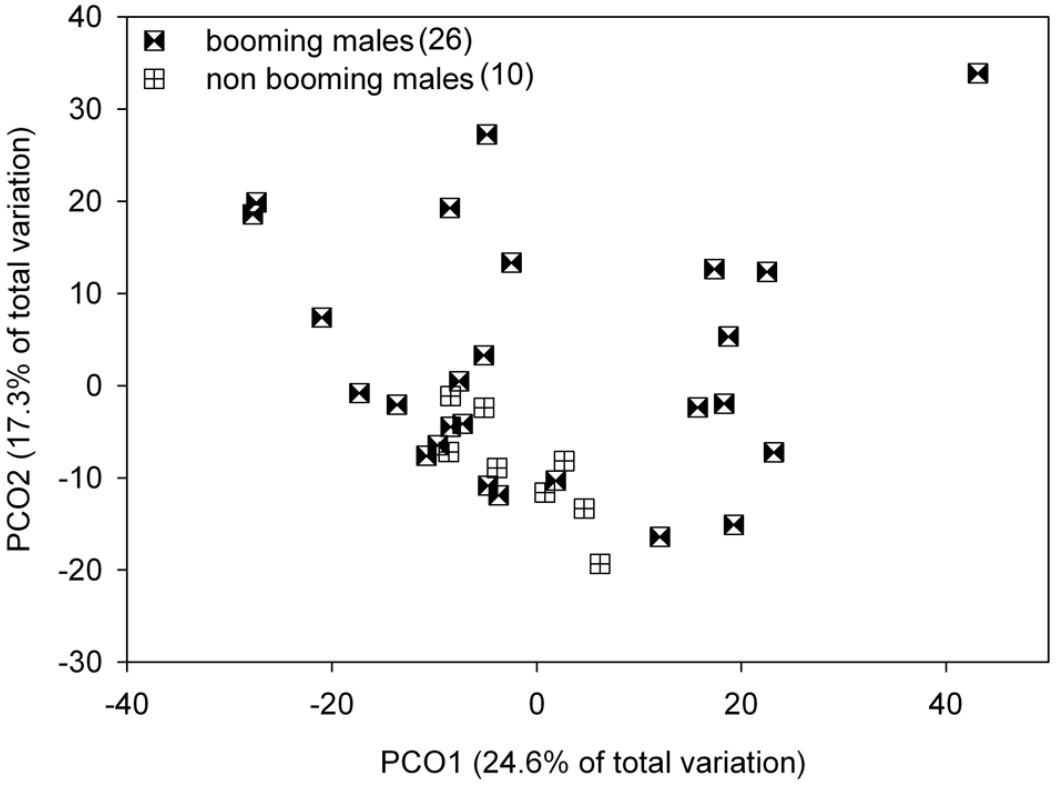
default.m Thu May 05 15:49:28 2011



APPENDIX D

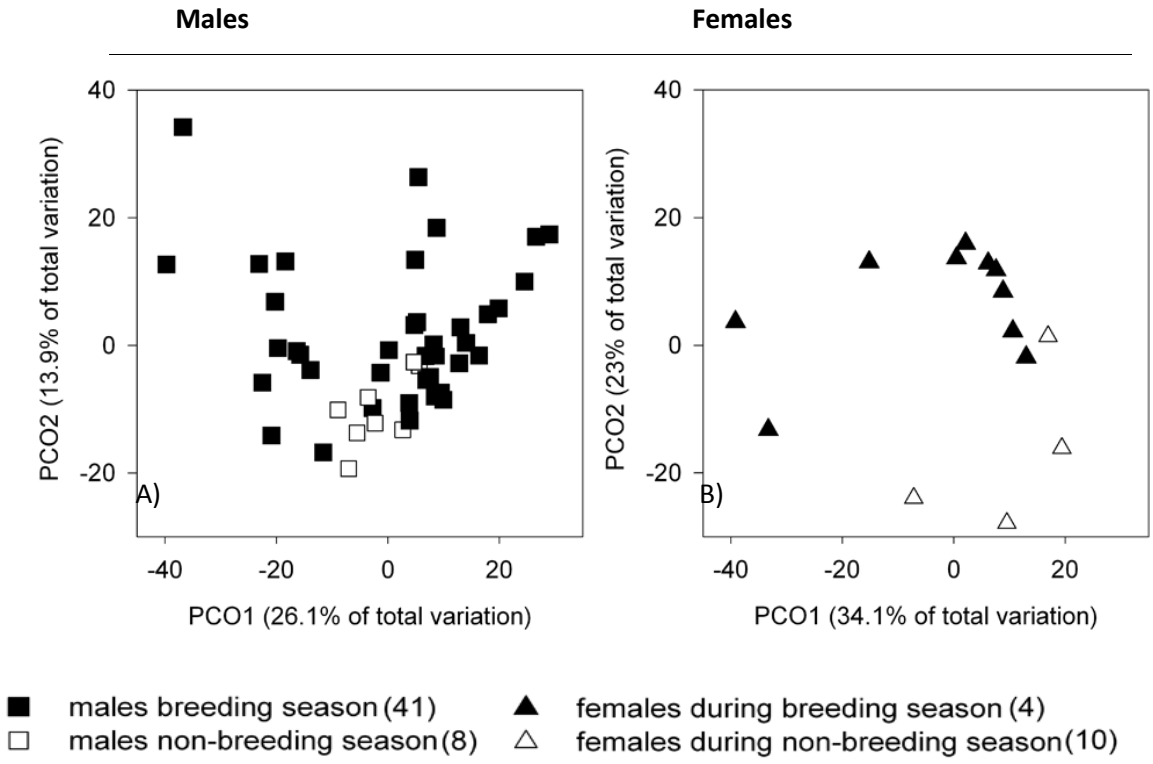
BOOMING VERSUS NON-BOOMING MALES

Figure D1 Principal coordinate analysis (PCO) of booming versus non-booming males. Feather samples were collected during the breeding season (November to March). The individual variation in the feather scent of booming birds is significantly higher (see Table 2.2) compared to non-booming birds. Sample size in brackets.



APPENDIX E WITHIN SEX COMPARISIONS

Figure E1 Principal coordinate analysis (PCO) of **A)** Males during the breeding and non-breeding. **B)** Females during the breeding and non-breeding.



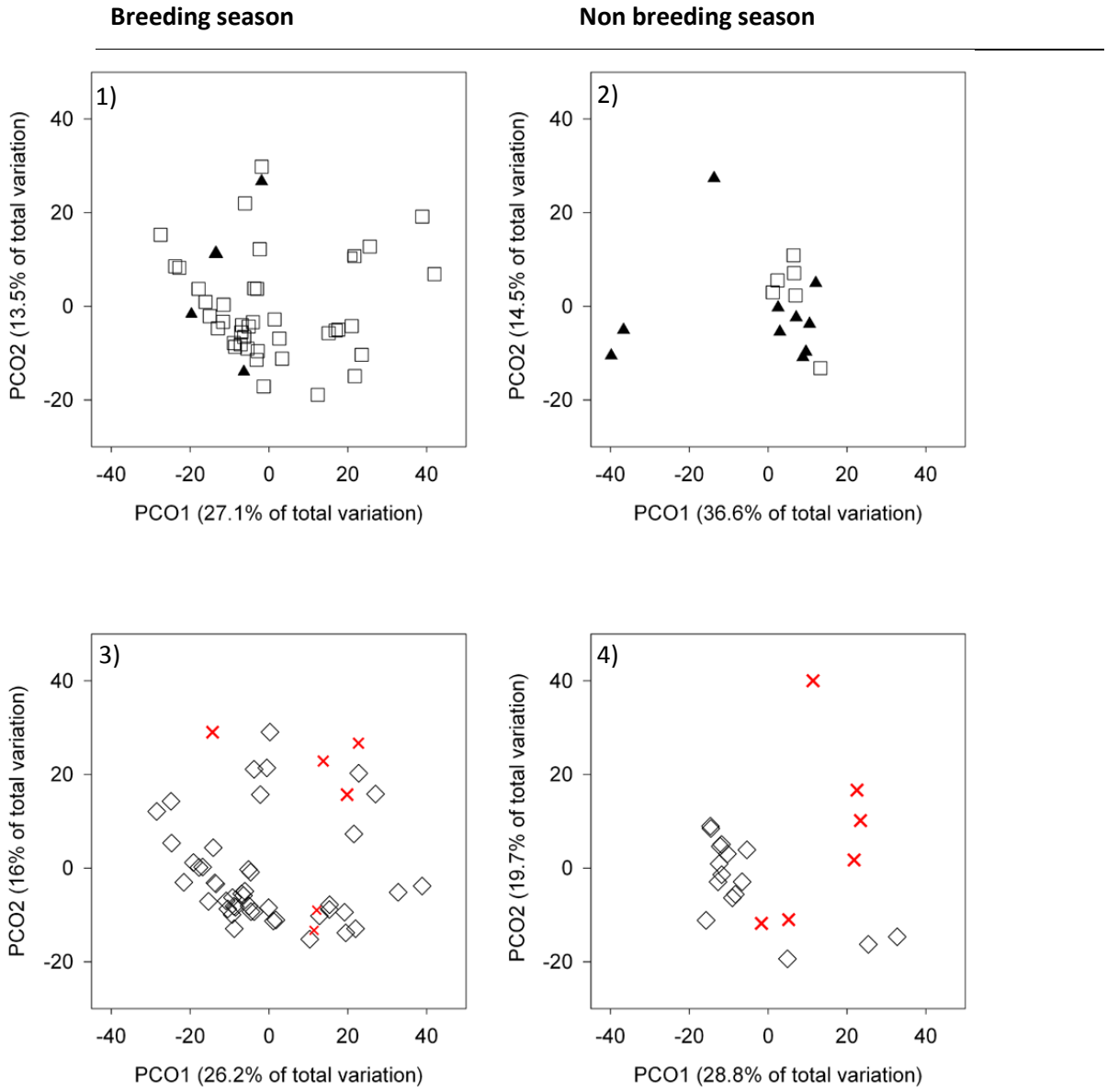
APPENDIX F GROUP COMPARISONS

Figure F 1&2 Sexual differences during the breeding- and non-breeding season

Principal coordinates analysis of feather samples from adult males (41: □) versus adult females (4: ▲) (Figure F 1&2)

Figure F 3&4 Age related differences within and outside the breeding season

Principal coordinates analysis of feather samples from adult birds (45: ◇) versus juvenile birds (6: ×) (Figure F 3&4).



APPENDIX G LIST OF FEATHER SAMPLES

Table G1 Birds sampled for the feather scent analysis (main set). Indicated are the name of the bird, its sex, in which year it was born, whether it was an adult or a juvenile bird (<6 years of age) at the time of sampling, the date the feather-sample had been collected, the weight of the feather sample, whether the sample had been collected during the breeding season (yes) or not (no), and as whether the birds were booming at the time of sampling (yes) or not (no) or not applicable (-).

Nr	Name	Sex	Year born	Adult/ Juvenile	Date sampled	Feather weight (g)	Breeding season	Booming males only)
1	Barnard	m	unkn	adult	04.02.2009	0.0158	yes	yes
2	Basil	m	unkn	adult	16.02.2006	0.0351	yes	no
3	Basil	m	unkn	adult	23.01.2008	0.0516	yes	yes
4	Basil	m	unkn	adult	28.01.2009	0.0303	yes	yes
5	Ben	m	unkn	adult	12.02.2006	0.0354	yes	no
6	Bill	m	unkn	adult	11.02.2006	0.0356	yes	yes
7	Bill	m	unkn	adult	24.01.2008	0.0274	yes	yes
8	Bill	m	unkn	adult	26.03.2008	0.0901	yes	yes
9	Blades	m	unkn	adult	23.06.2006	0.0252	no	no
10	Blades	m	unkn	adult	10.02.2006	0.0752	yes	unkn
11	Boss	m	unkn	adult	03.02.2009	0.0473	yes	yes
12	Felix	m	unkn	adult	09.11.2008	0.1082	yes	yes
13	Gulliver	m	1998	adult	16.02.2006	0.0332	yes	no
14	Gumboots	m	unkn	adult	29.07.2006	0.0165	no	no
15	Jimmy	m	unkn	adult	13.02.2006	0.0498	yes	no
16	Jimmy	m	unkn	adult	04.03.2008	0.0132	yes	yes
17	Lionel	m	unkn	adult	28.01.2009	0.0147	yes	yes
18	Merty	m	unkn	adult	23.06.2006	0.0207	no	no
19	Merty	m	unkn	adult	06.03.2008	0.0156	yes	yes
20	Merty	m	unkn	adult	31.01.2009	0.0338	yes	yes
21	Merv	m	unkn	adult	15.02.2006	0.0366	yes	unkn
22	Merv	m	unkn	adult	25.01.2008	0.0221	yes	yes
23	Merv	m	unkn	adult	31.01.2009	0.0189	yes	yes
24	Nog	m	unkn	adult	12.02.2006	0.0524	yes	unkn
25	Nog	m	unkn	adult	23.01.2008	0.0462	yes	yes
26	Nog	m	unkn	adult	04.03.2009	0.0108	yes	yes
27	Ox	m	unkn	adult	11.02.2006	0.0367	yes	Unkn
28	Ox	m	unkn	adult	23.06.2006	0.0108	no	no

Nr	Name	Sex	Year born	Adult/ Juvenile	Date sampled	Feather weight (g)	Breeding season	Booming males only)
29	Ox	m	unkn	adult	10.02.2007	0.0472	yes	yes
30	Ox	m	unkn	adult	24.01.2008	0.0613	yes	yes
31	Piripi	m	unkn	adult	13.02.2006	0.06	yes	unkn
32	Piripi	m	unkn	adult	03.02.2009	0.021	yes	yes
33	Ralph	m	unkn	adult	28.01.2009	0.0149	yes	yes
34	Richard Henry	m	unkn	adult	02.08.2006	0.0282	no	unkn
35	Sass	m	unkn	adult	10.02.2007	0.013	yes	unkn
36	Sass	m	unkn	adult	02.01.2009	0.0255	yes	yes
37	Sinbad	m	1998	adult	16.02.2006	0.0681	yes	unkn
38	Sinbad	m	1998	adult	04.02.2009	0.0452	yes	yes
39	Sirocco	m	1997	adult	14.01.2007	0.0388	yes	unkn
40	Sirocco	m	1997	adult	06.03.2008	0.0503	yes	yes
41	Smoko	m	unkn	adult	13.02.2006	0.0617	yes	unkn
42	Smoko	m	unkn	adult	10.02.2007	0.0175	yes	unkn
43	Smoko	m	unkn	adult	04.03.2008	0.0239	yes	yes
44	Stumpy	m	1991	adult	04.03.2008	0.0193	yes	yes
45	Waynebo	m	unkn	adult	19.06.2006	0.0152	no	unkn
46	Whiskas	m	unkn	adult	11.02.2006	0.0426	yes	unkn
47	Whiskas	m	unkn	adult	24.01.2008	0.0212	yes	yes
48	Ellie	f	1999	adult	17.06.2006	0.0217	no	-
49	Flossie	f	unkn	adult	03.04.2008	0.0497	yes	-
50	Flossie	f	unkn	adult	19.06.2008	0.0152	no	-
51	Hoki	f	1992	adult	17.06.2006	0.0377	no	-
52	Jane	f	unkn	adult	13.02.2006	0.0579	yes	-
53	Jane	f	unkn	adult	10.02.2007	0.2043	yes	-
54	Jasmine	f	unkn	adult	20.06.2006	0.2033	no	-
55	Jean	f	unkn	adult	21.10.2006	0.0313	no	-
56	Lisa	f	unkn	adult	31.08.2006	0.0151	no	-
57	Nora	f	unkn	adult	27.03.2008	0.0522	yes	-
58	Kuia	f	1998	adult	26.06.2006	0.0285	no	-
59	Sandra	f	unkn	adult	16.06.2006	0.0294	no	-
60	Solstice	f	unkn	adult	19.06.2006	0.0227	no	-
61	Zephyr	f	unkn	adult	19.06.2006	0.0227	no	-
62	Jovi=Jem	f	2008	juvenile	19.06.2008	0.0295	no	-
63	Totiti	f	2008	juvenile	19.06.2008	0.0334	no	-

Nr	Name	Sex	Year born	Adult/ Juvenile	Date sampled	Feather weight (g)	Breeding season	Booming males only)
64	Weheruatanga-o-te-po	f	2008	juvenile	19.06.2008	0.0119	no	-
65	Elwin	m	2008	juvenile	19.06.2008	0.0152	no	unkn
66	Jester	m	2008	juvenile	19.06.2008	0.0371	no	unkn
67	Roostar	m	2008	juvenile	19.06.2008	0.0153	no	-

APPENDIX H

ACCOMPANYING CD OF KAKAPO CHROMATOGRAMS

The CD accompanying this thesis contains all chromatograms of the 67 birds that were used in Chapter Two.