ECOLOGY AND POPULATION GENETICS OF TWO LARGE MACAW SPECIES IN THE PERUVIAN AMAZON

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George Olah

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Cover image: view from a scarlet macaw nest in Candamo, Peru (2010).

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of the author's knowledge, it contains no material previously published or written by another person, except where due reference is made in the text.

George Olah

May 2016

Thesis Plan

This thesis is presented in nine chapters. References, figures and tables appear at the end of each chapter. All photographs and images are my own. Chapters 2, 3, 4, and 5 have been published in scientific peer reviewed journals, while chapters 6, 7, and 8 are presented as manuscripts intended for submission. This format necessitates some repetition of basic information between chapters and the pronoun "we" is used to represent co-authors in material intended for publication. Below I outline my specific contributions and those of my co-authors and colleagues:

Chapter 1: General introduction

I was sole author and contributor to this chapter.

Chapter 2: Ecological and socio-economic factors affecting extinction risk in parrots

I performed the literature review, conceptual development, database building from various sources, statistical analyses, and writing. Stuart Butchart and Andy Symes constructed the BirdLife database on parrots and made it available for this study. Iliana Medina Guzmán contributed to the phylogenetic control analysis and constructed Figure 3. Ross Cunningham contributed to the statistical model construction. Stuart Butchart, Donald Brightsmith, and Robert Heinsohn contributed to conceptual development of the study and provided editorial comments.

Chapter 3: Nest site selection and efficacy of artificial nests for breeding success of scarlet macaws in lowland Peru

I contributed to data collection between 2008 and 2011, constructed the database of nest preferences of scarlet macaw for 12 consecutive breeding seasons, performed the statistical analyses, and writing. Gabriela Vigo, Donald Brightsmith, and the Tambopata Macaw Project contributed to data collection. Robert Heinsohn and Donald Brightsmith contributed to conceptual development of the study and provided editorial comments.

Chapter 4: *Philornis* sp. bot fly larvae in free living scarlet macaw nestlings and a new technique for their extraction

I contributed to data collection between 2008 and 2011, performed the conceptual development, database building, statistical analyses, and writing. Gabriela Vigo, Lizzie Ortiz Cam, and the Tambopata Macaw Project contributed to data collection. Lajos Rozsa contributed to the

statistical analysis. Donald Brightsmith and Lajos Rozsa contributed to conceptual development of the study and provided editorial comments.

Chapter 5: An evaluation of primers for microsatellite markers in scarlet macaw and their performance in a Peruvian wild population

I completed the bioinformatics, developed the microsatellite markers, and conducted the microsatellite screening in the laboratory. Rod Peakall contributed to the conceptual development of the study and to the manuscript preparation. Robert Heinsohn, Jose Espinoza, and Donald Brightsmith provided editorial comments.

Chapter 6: Validation of non-invasive genetic tagging in large macaws of the Peruvian Amazon

I was responsible for sample collection and design in the field, DNA extraction, genotyping of blood and feather samples, statistical analysis, and writing. I consulted closely with Rod Peakall who developed GenAlEx for the statistical analysis. Robert Heinsohn, Donald Brightsmith, Jose Espinoza, and Rod Peakall provided editorial comments.

Chapter 7: Non-invasive genetic tagging of large macaws in the Peruvian Amazon

I performed the literature review, conceptual development, statistical analysis, and writing. Robert Heinsohn and Donald Brightsmith provided editorial comments. Rod Peakall contributed to conceptual development of the study and also provided editorial comments.

Chapter 8: Landscape genetics reveals isolation-by-elevation in scarlet macaws of the Peruvian Amazon

I performed the conceptual development, statistical analysis, and writing. Annabel L. Smith contributed to the resistance model building and provided editorial comments. Greg Asner constructed the maps that served as source for the resistance models. Donald Brightsmith provided unpublished data about the movement of satellite tagged scarlet macaws. Robert Heinsohn and Rod Peakall contributed to conceptual development of the study and provided editorial comments.

Chapter 9: Priorities for future conservation and genetic studies on parrots

I was sole author and contributor to this chapter.

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Abstract

The order *Psittaciformes* (parrots) contains 398 extant species, divided into 3 families (*Psittacidae* 374, *Cacatuidae* 21, *Strigopidae* 3 species) of which 111 (28%) are classified as threatened on the IUCN Red List. This thesis presents a wide array of interdisciplinary methods to study parrots: statistical modeling of their extinction risk, on site ecological studies of nest preferences, and population genetic techniques.

I modeled the factors associated with extinction risk in parrots, including intrinsic biological, life history and ecological attributes, external anthropogenic threats, and socio-economic variables of the countries where they occur. I found a range of significant effects on parrot conservation status including historical distribution size, forest dependency, body size, generation time, the proportion of the human population living in urban areas in the countries encompassing the parrots' home ranges, per capita GDP of the countries of occurrence, endemism to a single country, and whether the species are used as pets.

Most parrots are obligate secondary cavity nesters, and can be limited in their breeding success by the availability and quality of nest hollows. I evaluated how nesting opportunities for parrots can be increased by provision of artificial nest boxes. My results show that artificial nests can be used by conservation managers seeking to assist macaw populations where nest hollows are in short supply, and that artificial nests can contribute important data to natural history studies of species where access to natural nests is limited.

I showed that *Philornis* sp. bot fly larvae prevalence was higher in artificial nests than in natural nests. I also described a new field technique of removing *Philornis* larvae using a snakebite extractor pump.

While extended knowledge about the natural history and ecology of species is crucial for their conservation, by combining ecology and genetics we can reveal new insights not evident from either ecology or genetic studies alone. I developed species-specific microsatellite (STR) genetic markers for scarlet macaw (*Ara macao*) based on their full genome. Using these new genetic markers I validated their potential for genetic tagging by using blood samples and moulted feathers of two sympatric macaw species in the Peruvian Amazon.

I applied non-invasive genetic tagging technique to estimate population size of red-and-green macaws (*Ara chloropterus*) in the Tambopata region of southeastern Peru. These conservation genetics techniques can be implemented for other related species with higher conservation concern, while also determining population structure and measuring levels of genetic diversity.

Landscape genetics provide an extra framework to study population dynamics, revealing the landscape factors that contributed to the genetic structure. I used landscape genetic resistance models to confirm isolation by elevation due to the mountain ridges between macaw populations in Candamo (an intermountain valley) and the lowland rainforest of Tambopata. Maintaining large protected areas and giving conservation priorities for intermountain rainforest valleys are essential for conserving the current genetic diversity of scarlet macaws in Peru.

I conclude the thesis by discussing the possible future paths of parrot research including conservation genetic studies that can help conservation management planning for this highly endangered order.

Table of Contents

Chapter 1

General introduction

Pristine rainforest habitat in Tambopata, Peru (2015).

1.1 The origin of parrots

Studies based on mitochondrial DNA (mtDNA) suggest that the order *Psittaciformes* (parrots) probably originated in Gondwana about 82 million years ago (Ma) in the late Cretaceous, well before the extinction of dinosaurs and pterosaurs (Brown et al. 2008; Pacheco et al. 2011; Wright et al. 2008). Other studies based on next-generation DNA sequencing date the origin of parrots later to the Paleocene (56-66 Ma), and consider parrots as the closest living relatives of falcons and passerine birds (Prum et al. 2015; Suh et al. 2011). However, it is difficult to determine the exact link between these bird groups given the poor fossil record (Dyke and Mayr 1999; Mayr 2002). The oldest known parrot fossil, a fused jawbone is from the late Cretaceous, which seems to support the mtDNA results (Stidham 1998), while other scientists disagree with this conclusion (Dyke and Mayr 1999). The first undoubtedly parrot fossils date from about 54 Ma from the northern hemisphere (Waterhouse 2006; Waterhouse et al. 2008).

The diversification of parrots probably started about 58 Ma after the Cretaceous–Palaeogene boundary (Schweizer et al. 2011; Toft and Wright 2015). The common ancestor of *Arini* tribe (Neotropical parrots) and *Psittacini* tribe (African parrots) probably lived in Antarctica (Schweizer et al. 2011). It is hypothesized that due to the cooling climate in Antarctica they colonized Africa and South America, and then the two tribes split about 30.8 Ma in the early Oligocene (Schweizer et al. 2014) and became separated from the old Gondwanan lineages that became the Australasian parrots (Schweizer et al. 2011). *Arini* colonized the previously parrot-free Neotropics by adaptive radiation with an early concentration of size evolution, sustaining a high diversification rate due to continuously emerging new habitats (mountain orogenesis, new areas in open vegetation, wetland and river dynamics) and continuing speciation even in the Pleistocene period (Schweizer et al. 2014). The colonization of Indomalaya occurred several times from Australia about 28 Ma (Schweizer et al. 2011).

The order *Psittaciformes* contains 398 extant species today, divided into 3 families (*Psittacidae* 374, *Cacatuidae* 21, *Strigopidae* 3 species) of which 111 (28%) are classified by BirdLife International (2014) as threatened on the IUCN Red List (IUCN 2014).

1.2 Parrot extinction, ecology, and conservation issues

The worldwide decline in biodiversity requires urgent actions (Butchart et al. 2010; Pimm et al. 2014; Tittensor et al. 2014). But in order to make proper actions we first need to understand better the mechanisms behind this damage to the ecosystem, which is very complex and depends

on many biological and artificial factors and can be very diverse for different taxa (Spray and McGlothlin 2003). Although exposure to threatening processes is the ultimate cause of extinction, a species' biology determines how well it is able to resist the threats to which it is exposed.

Parrots exhibit many of the traits known to be associated with high extinction risk in present day human modified landscapes: for example, many are large-bodied and slow-breeding (e.g. macaws and cockatoos), and 70% are ecologically specialized (e.g. forest species) (Toft and Wright 2015). Such specialisations led to the possible extinction of the glaucous macaw (*Anodorhynchus glaucus*) depending on the Yatay palm, and to the Critically Endangered status of the blue-throated macaw (*Ara glaucogularis*). Several other macaw species, two Amazon parrots (*Amazona violacea, A. martiniaca*), and the Guadeloupe parakeet (*Psittacara labati*) became extinct in the Caribbean region shortly after the "discovery" of the new world (Williams and Steadman 2001). The Carolina parakeets (*Conuropsis carolinensis*) were hunted for their colourful feathers and because of the damage they inflicted on fruiting crops in the United States until their extinction. Many other parrots are island endemics and especially vulnerable to disturbance. The Mascarene Islands and the Seychelles in the Indian Ocean hosted six parrots that became extinct mainly in the 18th century (*Lophopsittacus bensoni, L. mauritianus, Mascarinus mascarin, Necropsittacus rodricanus, Psittacula exsul, P. wardi*) as a result of hunting, deforestation, and nest predation by introduced monkeys and rats (Hume and Walters 2012). The South Pacific region once had two parakeets (*Cyanoramphus ulietanus, C. zealandicus*) in the Society Islands, the Oceanic parrot (*Eclectus infectus*) in Tonga, and the Norfolk Island Kaka (*Nestor productus*), but they all became extinct due to over-hunting (Forshaw 1989; Steadman 2006). The Paradise Parrot (*Psephotellus pulcherrimus*) lived in Queensland and New South Wales, Australia, and became extinct possibly due to habitat alteration, trapping for the pet trade, and predation by introduced species (Hume and Walters 2012).

Many parrots remain prevalent in lowland tropical rainforest habitat (Forshaw 2011). Most parrots are obligate secondary cavity nesters, and can be limited in their breeding success by the availability and quality of nest hollows (Brightsmith 2005; Renton and Brightsmith 2009). Parrots often enlarge natural tree hollows and old nesting holes of other birds like woodpeckers or barbets (Forshaw 2011), but the natural formation of such nests can take decades. For this reason, the selective logging of large canopy trees can significantly deter the breeding of parrots in their natural habitat. Hence, conservation management projects using artificial nest boxes offer a feasible solution for increase breeding success (Downs 2005; Olah et al. 2014; Sanz et al. 2003; White et al. 2005; White et al. 2006).

The main anthropogenic threats to parrots today are habitat loss, degradation and fragmentation driven by unsustainable agriculture, logging and commercial and residential development, and hunting and trapping (Beissinger et al. 1992; Laurance et al. 2002; Snyder et al. 2000). Long-term scientific data on the effects of these threats are essential for appropriate management planning.

1.3 The study site: Tambopata, Peru

This study of two large macaws was conducted in the lowland rainforest of southeastern Peru, in the regions of Madre de Dios and Puno. This tropical moist forest ranges in elevation between 250–800 m and receives 3,200 mm of rain per year (Brightsmith 2004; Tosi 1960). There are two large, adjacent protected areas in this region: the Tambopata National Reserve (2,747 km^2) and the Bahuaja-Sonene National Park (10,914 km^2). Tambopata harbours some of the highest biodiversity in the entire Amazon basin.

The core area of the study was located in the forests surrounding the Tambopata Research Center (TRC: 13° 8.070' S, 69° 36.640' W). The site is surrounded by four principal forest types: *terra firme*, floodplain, palm swamp, and successional. The centre is located 880 m from a large clay lick, Collpa Colorado (Brightsmith et al. 2008; Brightsmith and Villalobos 2011; Powell et al. 2009). The Tambopata Macaw Project has been studying the breeding ecology and natural history of parrots and macaws in TRC for over 20 years.

1.4 Breeding ecology and health of scarlet macaws in TRC

In TRC a long-term research project has been monitoring natural nest hollows and two types of artificial nest (wooden and PVC) of scarlet macaws (*Ara macao macao*) for over a decade. Results of such a long study are invaluable for future conservation management planning of the same or similar species. For instance, a different subspecies of scarlet macaw (*A. macao cyanoptera*) is rapidly declining in northern Central America and knowledge about their breeding ecology is essential for their conservation (Britt et al. 2014; Schmidt 2013). Using artificial nests for management planning can be suitable in regions where the large emergent canopy trees with the best nest hollows have been removed but habitat has otherwise been maintained (Munn 1992).

It has been shown that artificial nests can also enhance scientific research and contribute important data to natural history studies of species where access to natural nests is limited

(Nycander et al. 1995; White et al. 2005). The side doors on the artificial nests provide easy access to eggs and chicks for the researchers facilitating morphological studies (Vigo et al. 2011), whereas it can be difficult to reach to the bottom of natural nests. Furthermore, artificial nests also facilitate the installation of electronic monitoring devices like microphones, censors, or cameras (Grenier and Beissinger 1999; White and Vilella 2004).

Easy access to the nests can also simplify frequent veterinary examination of the nestlings' health. For example, during nest inspections in TRC researchers found that scarlet macaw nestlings heavily infested by bot fly (*Philornis* genus) larvae showed reduced survival (Nycander et al. 1995). Bot fly larvae are obligate subcutaneous blood-feeding parasites of Neotropical birds including psittacines. *Philornis* infestations can increase bird mortality, decrease reproductive success, and affect nest site selection (Loye and Carroll 1998). They may even increase extinction risk for some avian hosts (Fessl and Tebbich 2002; Snyder et al. 1987). Motivated by this observation, we developed an easy technique to remove the parasitic larvae and to improve chick growth and fledging. The study also provided important insights into the different infestation rate between natural and artificial nests.

1.5 Individual, population, and landscape level genetic studies of macaws

While extended knowledge about the natural history, ecology, and health of species is crucial for their conservation, by combining this information with genetics we can frequently discover new insights not evident from either ecology or genetic studies alone (Peakall and Beattie 1996). For instance, the estimation of population size by traditional capture-mark-recapture (CMR) methods requires capturing and tagging individuals with rings, but captures and recaptures of the required number of individuals is far from straight forward for many species (Pollock et al. 2002; White and Burnham 1999).

Macaws in the Peruvian Amazon visit clay licks to supplement their diet with minerals and toxin-absorbing clays (Brightsmith 2004; Burger and Gochfeld 2003; Gilardi et al. 1999), and leave large numbers of naturally dropped feathers (Gebhardt et al. 2009). This environment provides an extraordinary opportunity to collect DNA samples from macaws non-invasively in order to investigate a wide range of questions about their populations via genetic markers. The first *de novo* genome assembly for the scarlet macaw (Seabury et al. 2013) provided a great advantage for developing species specific and highly variable genetic markers for the same species and for other macaws.

Genetic tagging is the unique identification of individuals by their DNA profile. Extraction methods for minute amounts of DNA enable the use of genetic tagging from non-invasive samples, like hair, scat, or feather. While microsatellite markers for genetic tagging have been extensively used for mammals, the same technique has not yet been widely adopted for birds. Here we use species specific genetic markers for macaws and apply them on non-invasively collected feathers from clay licks in order to build a genotype library of macaws in Tambopata, which leads to their individual identification. Once the individual genotypes are identified and "recaptured" in the landscape, traditional CMR statistics can be used to estimate population sizes (Coster et al. 2011; Lukacs and Burnham 2005; Petit and Valiere 2006).

Genetic studies at the population level are also important tools for understanding ongoing conservation issues (Frankham et al. 2004). Macaws have been extensively studied for decades in their natural habitat in southeastern Peru, however well designed population genetic studies have been lacking. Landscape genetics then provide a further framework to study the landscape-scale factors that have contributed to genetic structure. The scarlet macaw is a long-lived bird capable of flying large distances over lowland rainforest habitat. Most conservation genetic studies focus on species living in already disturbed patchy habitats, but our study site in Tambopata contains primary rainforest with more than 1,300,000 ha in continuous protected areas. Understanding how this natural landscape affects the genetic structure of the study population can help us further understand this complex interaction between a tropical landscape and the ecology of its large and mobile 'flagship' species.

1.6 Outline of the thesis

This thesis is divided into nine self-contained chapters, each (except 1 and 9) with a standalone introduction, methods, results, and discussion in the form suitable for publication.

Chapter 1 - General introduction

In Chapter 2 (*Ecological and socio-economic factors affecting extinction risk in parrots*) I model the factors associated with extinction risk in parrots, including intrinsic biological, life history and ecological attributes, external anthropogenic threats, and socio-economic variables of the countries where they occur.

In Chapter 3 (*Nest site selection and efficacy of artificial nests for breeding success of scarlet macaws in lowland Peru*) I evaluate how nesting opportunities for parrots can be increased by provision of artificial nest boxes.

In Chapter 4 (*Philornis sp. bot fly larvae in free living scarlet macaw nestlings and a new technique for their extraction*) I analyse twelve years of data on *Philornis* parasitism in scarlet macaws nesting in natural and artificial nests in the lowland forests of southeastern Peru.

In Chapter 5 (*An evaluation of primers for microsatellite markers in scarlet macaw and their performance in a Peruvian wild population*) I present primer designs for 41 di-nucleotide microsatellite loci identified from the full genome of the scarlet macaw.

In Chapter 6 (*Validation of non-invasive genetic tagging in large macaws of the Peruvian Amazon*) I evaluate the potential for non-invasive genetic tagging by using moulted feathers of two sympatric macaw species in the Peruvian Amazon.

In Chapter 7 (*Non-invasive genetic tagging of large macaws in the Peruvian Amazon*) I evaluate the genetic tagging technique as a method to reveal the number of different individual red-and-green macaws visiting clay licks, their sexes, relatedness, and aspects of their clay lick use in time and space.

In Chapter 8 (*Landscape genetics reveals isolation by elevation in scarlet macaws of the Peruvian Amazon*) I examine the genetic structure of scarlet macaws in the context of landscape of the Tambopata/Candamo region to see if they maintain high genetic diversity and gene flow.

In Chapter 9 (*Priorities for future conservation and genetic studies on parrots*) I discuss the possible future paths of parrot conservation including conservation genetic studies that can help conservation management planning for species in this highly endangered group of birds.

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Chapter 2

Ecological and socio-economic factors affecting extinction risk in parrots

Poaching blue-headed parrots in Chaco, Argentina (2005).

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2.1 Abstract

Parrots (*Psittaciformes*) are among the most threatened bird orders with 28% (111 of 398) of extant species classified as threatened under IUCN criteria. We confirmed that parrots have a lower Red List Index (higher aggregate extinction risk) than other comparable bird groups, and modeled the factors associated with extinction risk. Our analyses included intrinsic biological, life history and ecological attributes, external anthropogenic threats, and socio-economic variables associated with the countries where the parrot species occur, while we controlled for phylogenetic dependence among species. We found that the likelihood of parrot species being classified as threatened decreased with their historical distribution size, but increased with forest dependency, body size, generation time, and the proportion of the human population living in urban areas in the countries encompassing the parrots' home ranges. The severity of extinction risk from vulnerable to critically endangered further related to the per capita gross domestic product (GDP) of the countries of occurrence, endemism to a single country, and whether the species are used as pets. A disproportionate number of 16 extinct parrot species were endemic to islands and single countries, and were large bodied, habitat specialists. Unsustainable agriculture, hunting, trapping, and logging are the most frequent threats to parrots worldwide, with variation in importance among regions. We use multiple methods to rank countries with disproportionately high numbers of threatened parrot species. Our results promote understanding of global and regional factors associated with endangerment in this highly threatened taxonomic group, and will enhance the prioritization of conservation actions.

2.2 Introduction

The current worldwide decline in biodiversity requires urgent action (Butchart et al. 2010; Pimm et al. 2014; Tittensor et al. 2014), but determining appropriate actions is often impeded by poor understanding of the diverse causes of decline and extinction across different taxa and regions of the world (Spray and McGlothlin 2003). The factors that determine a species' extinction risk fall into two main categories: (1) intrinsic biological attributes of species, and (2) exposure to external anthropogenic threats (Fisher et al. 2003). Although exposure to threats is the ultimate cause of extinction, a species' biology often determines how sensitive it is to different threat types. Particular biological attributes can allow populations to recover rapidly from depletion and can offer a degree of resilience from external threats (Cardillo et al. 2004). Biological attributes that have been shown to decrease the risk of extinction include large geographical distribution, high population density, lower trophic level, and various life history traits including high reproductive rate, small body size, short generation length, and a low degree of habitat specialization (Owens and Bennett 2000; Purvis et al. 2000).

The order *Psittaciformes* (parrots) contains 398 extant species divided into 3 families (*Psittacidae* 374, *Cacatuidae* 21, *Strigopidae* 3 species) of which 111 (28%) are classified by BirdLife International (2014) as threatened on the IUCN Red List, i.e. in the categories of Critically Endangered, Endangered or Vulnerable (IUCN 2014). Sixty species are classified as Near Threatened and the rest (227 species) are considered as Least Concern. *Psittaciformes* is the fourth largest bird order after *Passeriformes* (5 913 spp., 10% threatened), *Caprimulgiformes* (593 spp., 9% threatened), and *Piciformes* (484 spp., 7% threatened), but it contains the second highest number of threatened bird species after *Passeriformes* (containing 611 threatened species). Data from BirdLife International underpinning assessments for the IUCN Red List show that 56% of all parrot species are in decline, 35% are stable and only nine per cent have increasing populations (IUCN 2014). These assessments relate to the observed, estimated, inferred or suspected direction of population trends, and the underlying basis and evidence for each one are specified under "trend justification" on the Data Zone factsheets (BirdLife International 2014).

Parrots are currently native to 124 countries between the latitudes N 35° and S 56°, but are mainly distributed in tropical and subtropical habitats of the southern hemisphere. They have been popular as pets throughout human history, probably due to their colourful appearance, reputedly high intelligence, and remarkable ability to mimic various sounds including the human voice (Grahl 1990). This popularity had led many species to be captured from the wild for pets. Although captive breeding has proven possible for many species (Arndt 2007; Tella and Hiraldo

2014), *Psittaciformes* remains the most common avian order reported in the wildlife trade (Bush et al. 2014).

Parrots exhibit many of the traits known to be associated with extinction: for example, many are large-bodied and slow-breeding (e.g. macaws and cockatoos), and 70% (see below) are ecologically specialized (e.g. forest species) (Forshaw 2011). The main anthropogenic threats to parrots are habitat loss, degradation and fragmentation driven by unsustainable agriculture, logging and commercial and residential development, and hunting and trapping (Beissinger et al. 1992; BirdLife International 2014; Laurance et al. 2002; Snyder et al. 2000). The socio-economic status of a country can determine the severity of anthropogenic threats affecting the species living there. For example, human population density was found to be closely related to the proportion of threatened bird species per country (Kerr and Currie 1995). Human activities such as poaching, hunting, and land clearing can be poverty driven in certain developing areas (Blaikie and Jeanrenaud 1997), but the effects of socio-economic drivers on the extinction risk of parrots have never been examined.

Although parrots have long been considered a group of especially high conservation concern (Pasquier 1980), in-depth quantitative analysis of the nature and trends in threats across the order has been lacking. Reviews to date have assessed particular threats (Beissinger and Bucher 1992; Harris et al. 2012; Laurance et al. 2009; Müller 2000; Newton 1994; Pain et al. 2006; Pires 2012), focused on individual species (Baker-Gabb 2011; Holdsworth and Starks 2006; Snyder et al. 2000; Webster et al. 2003) or assessed general trends in parrot conservation without comparative analysis (Collar 1997; Collar 2000; Collar and Juniper 1992; Martin et al. 2014). Jones et al. (2006) advocated decision tree analysis for assessing extinction risk and used the IUCN Red List data of parrots from 2000 as an example group, but did not cover extrinsic extinction threats or socio-economic traits, and nor did they control for phylogenetic dependence of data. Our aim is to use comparative analyses to enhance understanding of the extrinsic, intrinsic, global and regional factors associated with endangerment and extinction in this highly threatened taxonomic group.

2.3 Methods

We first examined trends in extinction risk during 1988-2012 for parrots (*Psittaciformes*) and other high profile ecological groups with similar numbers of species using Red List Indices (Butchart et al. 2007). These groups included waterbirds, seabirds, and raptors, each of which comprises multiple orders (see online supplementary methods). Other groups included pigeons (*Columbiformes*) and gamebirds (*Galliformes*). IUCN Red List criteria (e.g. absolute population size, range size, rates of decline, etc.) are used to assign species to Red List categories of relative extinction risk, and the Red List Indices are calculated from changes between these categories. Red List Indices for sets of species are based on the number of species in each category, and the number moving between categories owing to genuine improvement or deterioration in status, i.e. increases or decreases in population size, population trends, extent of occurrence, etc. that are of sufficient magnitude to cross the thresholds for lower or higher Red List categories (Butchart et al. 2004; Butchart et al. 2005; Butchart et al. 2007).

2.3.1 Database compilation

We then assembled a database of the biological and geographic attributes of all 398 extant parrot species using the 2014 version of BirdLife International and IUCN's database which underpins the IUCN Red List assessments for birds on the BirdLife Data Zone (BirdLife International 2014) and IUCN Red List website (IUCN 2014). Table 1 shows all explanatory variables (including sources) used in our statistical models of threat status. The online supplementary methods provide further details of the derivation of values in the database.

We used historical distribution size instead of current distribution sizes to test if species with originally small distributions are more prone to be endangered, and in order to avoid possible circularity as current extent of occurrence is a parameter used in the IUCN Red List criteria. Historical distribution is mapped using the same sources as for contemporary distribution, adding areas with historical records prior to 1980 for which the species is now judged to be extirpated owing to lack of recent records despite searches, lack of suitable habitat and informed by expert judgment (BirdLife International and NatureServe 2014). Population size and trend were not used as variables in our models for similar reasons. All the detailed underpinning data for Red List evaluation are available on the BirdLife Datazone (BirdLife International 2014). Because generation time was significantly positively correlated with body size (Table S1A), we calculated

and used the residual values from the simple linear regression of generation time versus body size and referred to this variable as 'residual generation time' following Owens and Bennett (2000).

To assess socioeconomic and demographic attributes of the countries in the parrot species' range, we used the World Economic Outlook Database (IMF 2013), the World Factbook (CIA 2013), and the database of the Food and Agriculture Organization of the United Nations (FAOSTAT 2013). We calculated the mean values of each parameter for all countries in which a species occurred (excluding vagrant records). Industrial production growth rate, unemployment rate, human population density, urban population, human population growth rate, and agriculture area were all significantly correlated with per capita gross domestic product (GDP) (Table S1B). We used residual values for these variables after regressing them against GDP.

We analysed the traits of 16 extinct species separately comparing trends with values for extant species.

2.3.2 Statistical modeling procedures

We used linear logistic regression models to identify the broad covariates of whether a parrot species is classified as threatened. 'Threatened' was defined as belonging to the IUCN threat categories 'Vulnerable', Endangered', and 'Critically Endangered', whereas 'Non-threatened' included the categories 'Least Concern' and 'Near Threatened'. We then used ordinal regression models to evaluate in more detail the covariates of the degree of threat faced by the threatened parrot species. Both logistic and ordinal regression models were initially computed separately using each of four categories of variables (Table 1), and a final universal model was computed by combining the variables found to be significant in each of the sub-models. We used GenStat 13.7 (Payne 2009) for all modeling.

To control for phylogenetic relatedness between species we downloaded 1 000 possible phylogenetic trees of *Psittaciformes* from birdtree.org. These were randomly selected to account for the branch lengths in addition to nodes separating species (Jetz et al. 2012). For each phylogeny, we ran a phylogenetic generalized least squares (PGLS) regression using the 'caper' package of R statistics (Freckleton et al. 2002). The explanatory and response variables were those from the best models per category found in the regression models. For each predictor in each model we report the modified p value accounting for phylogenetic relatedness, and we report λ (lambda transformation that improves the fit of the model to the phylogeny data) for each model. Greater values of λ indicate that the relationship between response and predictor variables is dependent on the phylogeny and trait values are more similar for closely related species. Values

of λ closer to 0 indicate that the relationship is unrelated to phylogeny. Since these analyses were repeated for 1 000 phylogenetic hypotheses, we report the mean and standard deviation of λ in each case. Additionally, we calculated in R the Blomberg´s K for threat status, which is a measure of phylogenetic signal. When values are closer to zero it indicates that the distribution of the trait is not related to phylogenetic relatedness.

We analysed the specific threats associated with high extinction risk in parrots by assessing how many parrot species are affected by each threat type globally and specifically at the regional scale in the Neotropics, Afrotropics, Indomalaya, and Australasia/Oceania. We used threat impact scores estimated from timing, scope and severity for each threat for each species, and we compared the high and medium impact threats worldwide. We also considered the most important conservation actions needed to improve the status of threatened parrots. A formal prioritization analysis has not been carried out for all species, but the most urgent actions are documented by BirdLife International and therefore useful to present a broad level analysis. We draw attention to countries with high risk of parrot conservation using two different methods. (1) We ranked countries by combining their scores for number of parrot species, number of globally threatened species, and number of endemic species. We refer to these as 'country priority' scores. (2) We calculated the residual values of the final combined linear logistic regression model accounting for all significant extrinsic and intrinsic variables. We refer to these values as 'unexplained extinction risk' hereafter.

2.4 Results

2.4.1 Red List Index for parrots and geographical differences

The Red List Index for parrots (0.825 in 2012) is lower than that for comparable groups of bird species including waterbirds (0.879), raptors (0.877), pigeons (0.868), gamebirds (0.842), and seabirds (0.828; Fig 1). However, rates of decline are broadly comparable across these different groups: 4.19% for parrots, 4.22% for seabirds, 4.30% for gamebirds, 4.39% for pigeons, and 4.49% for waterbirds and raptors (Fig 1).

A total of 16 parrot species are considered to be Extinct, 17 are Critically Endangered (one species Possibly Extinct in the Wild, and another species Possibly Extinct), 40 Endangered, 54 Vulnerable, 60 Near Threatened, and 227 are categorized as Least Concern. Species density maps

of all extant parrot species (Fig S1A) and of the threatened species (Fig S1B) are shown in the online supplementary material. The Neotropics have the highest proportion of threatened species (37% of the region's parrot species, N = 176), followed by Australasia/Oceania (22%, N = 168 spp.), Afrotropics (19%, N = 26 spp.), and Indomalaya (14%, N = 28 spp.; Fig 2). Overall, there was no significant phylogenetic signal in degree of threat (K = 0.05, *P* = 0.36), which means that closely related species are not more likely to share the same Red List category (Fig 3).

2.4.2 Linear logistic regression model

The combined linear logistic regression model showed that the probability of a parrot species being threatened decreased with larger historical distribution size (χ^2_1 = 64.89, *P* < 0.001; Fig S2A). The probability of being threatened increased significantly with: body size (χ^2_1 = 13.18, *P* < 0.001; Fig S2B), residual generation time (χ^2_1 = 16.82, *P* < 0.001; Fig S2C), extent of forest dependency (χ^2_3 = 29.47, *P* < 0.001; Fig S2D), and the percentage of the human population in the countries of occurrence living in urban areas (χ^2_1 = 28.71, *P* < 0.001; Fig S2E). There were no significant interactions among the 27 tested variables (all *P* ≥ 0.184). Table S3 lists additional variables found to be significant when analysed separately by group (A-D). All significant variables in the combined linear logistic regression model were also significant in the PGLS model controlling for phylogeny (Table S5A).

2.4.3 Ordinal regression model

The combined ordinal regression model indicated that the severity of threat among threatened parrot species increased significantly with higher per capita GDP (Table 1) of the countries of occurrence (χ^2_1 = 8.47, *P* = 0.004; Fig S3A) and when the species is endemic to a single country (χ^2_1 = 5.6, *P* = 0.018; Fig S3B). The severity of threat decreased significantly for species that are used as pets (χ^2_1 = 4.75, *P* = 0.029; Fig S3C). There were no significant interactions among the 27 tested variables (all *P* ≥ 0.140). Table S4 lists additional variables found to be significant when analysed separately by group (A-D). All significant variables in the combined ordinal regression model were also significant in the PGLS model controlling for phylogeny (Table S5B).

2.4.4 Traits of extinct species

Sixteen parrot species in 12 genera are recorded as extinct. Five of the extinct species lived in the Neotropics (mainly the Caribbean), six on islands near Africa (Mascarene Islands, Mauritius, Seychelles), and five in Australasia/Oceania (4 in Oceania and 1 in Australia). Fourteen out of 16 extinct species inhabited islands only. By comparison one quarter of all extant parrot species (96 out of 398 species) are insular suggesting an over-representation of island species amongst those now extinct ($\chi^2_1 = 28.5$, $P < 0.001$). All but one extinct species were endemic to a single country, but distribution size was highly variable (38 - 557 670 km^2) with the Carolina Parakeet (*Conuropsis carolinensis*) forming an outlier with range size of 3 167 000 km^2 (mean of 240 510 km^2 ± 198 000 SE for all 16 species). The mean of the extinct species' median latitude of occurrence was -6.96 degrees ± 5.00 SE compared with -6.6 degrees ± 0.75 SE for extant species, with no significant difference (ANOVA $F_{1,413} = 0.01$, $P = 0.936$). Extinct species were mainly large bodied with a mean length of 39 cm ± 3.5 SE (ranging between 25-70 cm) compared with a mean of 28.5 cm ± 0.7 SE (ranging between 8-100 cm) for extant parrots (ANOVA $F_{1,410} = 7.62$, $P = 0.006$). Their estimated mean generation time was 8 years ± 0.7 SE compared with 7.3 years ± 0.2 SE for extant species (ANOVA $F_{1,412} = 0.92$, $P = 0.339$). All but one of the extinct species lived in only one habitat type, mainly in forests. Out of the 16 extinct species 38% (6 species) were recorded as used as pets compared to 93% today (372/398 species). A percentage of 69% (11/16 spp.) of the extinct species were used as food compared to 23% today (90/398 spp.).

2.4.5 Threat types, conservation actions, and priority countries

Based on the threat impact scores, the greatest threat to parrots worldwide is from agriculture (impacting 35% of extant species), followed by hunting & trapping, logging, climate change & severe weather, and invasive alien species (Fig 4A). The top three threats (agriculture, hunting & trapping, logging) appear roughly similar across regions, however residential development features as a worse threat in the Neotropics and Indomalaya, and invasive alien species is a greater threat in the Afrotropics and Australasia/Oceania (Fig 4B). Whether species are threatened depends significantly on five major types of threat and their interactions: invasive alien species, agriculture, hunting & trapping, energy production & mining, and residential & commercial development (Table S6A). Higher categories of threat (i.e. Endangered, Critically Endangered) were especially associated with invasive alien species, even after controlling for phylogeny ($\chi^2_1 = 14.41$, $P < 0.001$; Fig S3D, Table S6B).

The most important conservation actions as determined by BirdLife International (2014) are presented in Fig 5. The most common actions needed in the Neotropics are site protection and management, in the Afrotropics they are legislation and ex-situ conservation, and in both Indomalaya and Australasia/Oceania they are awareness & communications, broad-scale habitat protection, and site protection (Fig 5).

Our country priority method revealed the following 10 countries with highest priority ranks: Indonesia, Brazil, Australia, Colombia, Bolivia, Ecuador, Peru, Papua New Guinea, Venezuela, and Mexico (more countries in Table 2). Examination of the unexplained extinction risk (after all significant intrinsic and extrinsic variables were accounted for in our models – see above) showed that island countries (or territories) accounted for both the lowest and highest values (Fig 6).

2.5 Discussion

Our analysis of Red List Index revealed that parrots are more threatened than other comparable taxonomic groups (Fig 1) with consistently negative trends in extinction risk over the last 25 years. The results show that conservation successes have been outweighed by the number of species being up-listed to higher categories of threat (IUCN 2014). Importantly, our analysis showed no significant effect of phylogeny on threatened status of parrots (Fig 3). Extinction risk is usually non-randomly distributed with respect to phylogeny (Fisher and Owens 2004), hence the most illuminating comparative models are those like ours that focus on relatively narrow taxonomic groups (Cardillo et al. 2008).

2.5.1 Biological attributes

We found several biological attributes associated with extinction risk in parrots. We found that species with larger historical distributions are less likely to be threatened (Fig S2A). This probably reflects the link between historical and present distribution size, and the fact that widely distributed species are often adapted to multiple habitats and less impacted by local threats (Ewers and Didham 2006; Purvis et al. 2000).

As for other taxa, large bodied parrots are more prone to extinction risk (Fig S2B) (Bennett and Owens 1997; Cardillo 2003; Cardillo and Bromham 2001; Purvis et al. 2000). Large body size correlates with many known extinction-promoting traits, for example large species tend to have

low population densities and slower life histories (Cardillo et al. 2005), and hunters are more likely to target larger species for food (Cowlishaw and Dunbar 2000). The significant effect of residual generation time also indicates that parrots with slower life history (longer generation time) are more likely to be threatened, independently of body size (Fig S2C). Bennett and Owens (1997) also found that increasing body size, and residual generation time were associated with increased extinction risk among birds when analysed at the family level. Marsden and Royle (2015) showed that larger bodied parrots are predictably uncommon in the wild.

Our study confirmed that forest dependent parrot species are more likely to be threatened (Fig S2D). Most parrots are forest dependent because of their nesting and feeding habits (Snyder et al. 2000; White et al. 2005). At least 70% of parrots are secondary tree cavity nesters (nesting in pre-existing tree cavities), hence primary forest destruction decreases the number of available nest sites and reproductive success (Newton 1994). Woodpeckers, as potentially important keystone cavity excavators in Neotropical forests (Cornelius et al. 2008), can also be threatened by anthropogenic factors that ultimately affect parrot reproduction as well. Forest destruction can also lead to the loss of key food resources, as many parrots eat mostly tree seeds and fruits and rely on large areas of suitable habitat to provide year-round sources of these temporally variable food sources (Arndt 2007; Brightsmith 2005; Forshaw 2011; Juniper and Parr 2003).

2.5.2 Anthropogenic and socio-economic factors

Our data suggest a subtle effect of the extent of economic development of the countries where the parrots live. Whereas the extent of urbanization (percentage of human populations living in urban areas) seems to relate broadly to whether a parrot species is threatened (Fig S2E), the impact of per capita GDP (i.e. developed economies) seems to have most influence by pushing parrot species into higher categories of extinction risk (Fig S3A). Urbanization can entail far-reaching transformations in itself but is also linked to broad scale environmental degradation (Cohen 2006; McKinney 2002). As populations and economies of primarily rural countries grow there is often a migration of people to urban areas, and this in turn can be followed by further economic expansion and higher per capita GDP (Chang and Brada 2006; Moomaw and Shatter 1996). Hence urbanization can occur at high levels before GDP is maximized (Brülhart and Sbergami 2009; Henderson 2003). Our results suggest that urbanization is a good variable accounting for the impact on parrots of the earlier stages of economic development, with the continuing impact of economic development best captured by GDP.

Parrots with distributions limited to one country are also more likely to belong to higher categories of endangerment (Fig S3B). Our analysis thus suggests that extinction risk of single country endemics (45% of all parrot species) is often adversely affected by either the singular history or the conservation management practices (or lack thereof) of their single country of occurrence. In the latter case, specific threats may be better controlled in some nations than others (e.g. progressive forestry laws and control of pet trade) with potential advantages to species living in more than one country (Hirakuri 2003; Pires 2012; Ribot 1999; Sunderlin et al. 2005).

Our finding that threatened species used for pets tend to belong to the lower categories of endangerment may seem counterintuitive (Fig S3C) but is supported by recent studies which show that the vast majority of species in domestic and international bird trade are non-threatened species (Pires and Clarke 2012; Pires 2012) and that utilized species in general are less threatened (Butchart 2008). This finding likely speaks much more to how increased threatened status (or decreased abundance) reduces use as pets than it does to how use for pets causes increases in threatened status. Pires and Clarke (2012) report that rare parrot species are only infrequently found in illegal parrot markets, confirming that most parrot poaching is driven by species that are easier to catch rather than by commercially based poachers searching for the rarest species.

2.5.3 Traits of extinct species

Our analysis of the traits of extinct species sheds light on some of our key results concerning extinction risk of extant species. For example, a disproportionate number of extinct parrot species (88% versus 24% for extant species) were insular, yet our models for extant species did not show that island endemism was a significant factor. This suggests that island endemism has been a strong factor in extinction risk but may have been masked in our analyses of extant species because many of the most susceptible species have already become extinct. In keeping with our models, extinct parrot species were larger on average than extant parrots, and most extinct species (94%) were single country endemics (compared to 45% for extant species).

2.5.4 Threat types, conservation actions, and priority countries

Our analysis reveals that the most important threats to parrots are agriculture, hunting and trapping, logging, climate change and severe weather, invasive alien species, and residential and commercial development (Fig 4A, Table S6A). Invasions by non-native species are often cited as

leading causes of species extinctions (Carrete and Tella 2008; Gurevitch and Padilla 2004), but our analysis suggests more complex synergistic effects in combination with other threats, including hunting and trapping, and agriculture (see interactions in Table S6A). Interestingly, 'invasive alien species' was the sole threat significantly associated with the higher categories of threat status (Fig S3D, Table S6B).

We found regional differences in the threats impacting parrots and hence the identified conservation actions required. Agriculture is a particularly important threat worldwide in terms of number of parrot species impacted (Fig 4A), and especially in the Neotropics (Fig 4B) where site protection is identified as the major conservation action required (Fig 5). In Indomalaya and Australasia/Oceania, the most significant threat to parrots is logging (Fig 4B), driving extensive deforestation in recent decades (Sodhi et al. 2010a; Sodhi et al. 2004; Sodhi et al. 2010b). Reflecting this, the most common conservation actions needed are broad-scale habitat protection, site protection and awareness raising (Fig 5). In contrast, the most common threat in the Afrotropics is hunting and trapping (primarily for the cage-bird trade and for use as pets; Fig 4B), and the most important key actions include enforcing and enhancing legislation (Fig 5).

Countries with the highest conservation priority were from all regions except the Afrotropics. Although Indonesia ranked the highest overall and Australia (ranked equal third) had the highest value amongst developed countries, 15 out of the top 20 countries were from the Neotropics (Table 2). These countries deserve high conservation interest given the high diversity and endemism of their parrot species, and the high proportion of their parrot species that are threatened. After accounting for all the variables in our model that proved to have significant effects on parrots being threatened (historical distribution size, body size, generation time, forest dependency, and percentage of urban human population in the distribution ranges), the mean residual (or unexplained) extinction risk values of each country suggested some positive and negative trends in country-level performances. Interestingly, island countries and territories have both the highest (on average more threatened species than expected; e.g. Dominican Republic, Haiti, Cook Islands) and lowest trends (less threatened species than expected; e.g. Bahamas, Comoros, Micronesia) in conservation status (Fig 6). Considering only mainland countries, the Afrotropics showed the worst trend compared to the other regions, however each region showed a wide range from low to high residual extinction risk (Fig 6). This method may prove important for identifying poorly performing countries (e.g. Dominican Republic, Haiti, Brazil, Burundi, Philippines, New Zealand, etc.) that need both extra conservation attention and further research on the nature of threatening processes.

2.5.5 Conclusion

Our study confirms that parrots are more threatened on average than comparable bird species groups, and that biological factors known to affect extinction risk in other taxa also apply to parrots. Thus parrot species with small historical distribution size, large body size, long generation time, and forest dependency are most likely to be categorized as threatened (Vulnerable, Endangered, Critically Endangered) under IUCN Red List criteria. Extinct parrot species shared most of these traits but also highlight island endemism as an important factor in the past. The extent of a country's urbanization provides a broad proxy for the major human socio-economic drivers of extinction risk in parrots. Our models also revealed that parrots are more likely to be highly threatened if their distribution falls within a single country's jurisdiction and in countries with higher per capita GDP, presumably because the higher levels of development that these factors are associated with tend to drive the major threats to parrots worldwide including agriculture, hunting and trapping, and logging.

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2.8 Figures and tables

Figure 1. Red List Indices of species survival for parrots (N = 398 non Data Deficient extant species; *Psittaciformes*), seabirds (N = 355; *Anseriformes, Podicipediformes, Phaethontiformes, Gaviiformes, Sphenisciformes, Procellariiformes, Pelecaniformes, Suliformes, Charadriiformes*), gamebirds (N = 307; *Galliformes*), pigeons (N = 350; *Columbiformes*), raptors (N = 320; *Accipitriformes, Cathartiformes, Falconiformes*), and waterbirds (N = 852; *Anseriformes, Podicipediformes, Phoenicopteriformes, Gruiformes, Gaviiformes, Ciconiiformes, Pelecaniformes, Suliformes, Charadriiformes*).

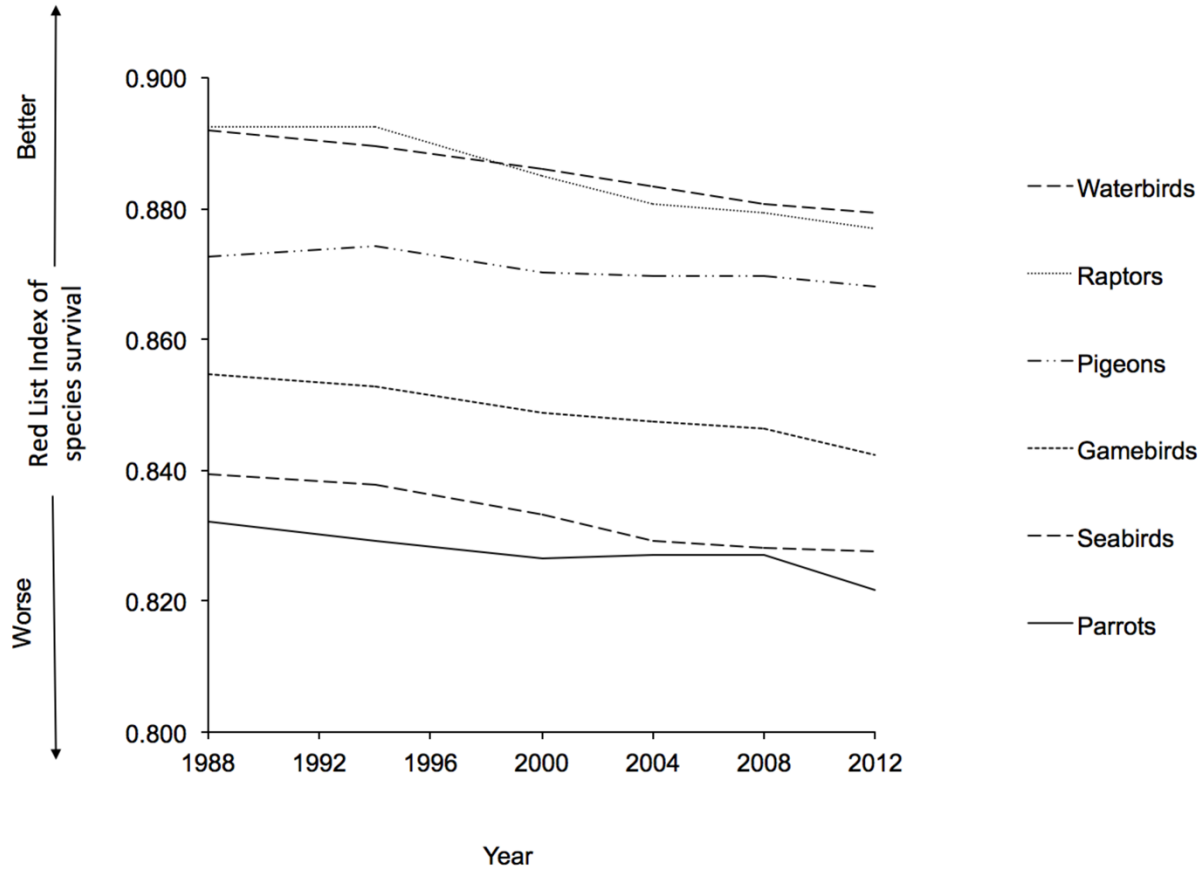


Figure 2. Proportion of parrot species in each IUCN Red List category for each region (following Croxall et al. 2012). Number of species in each category is shown.

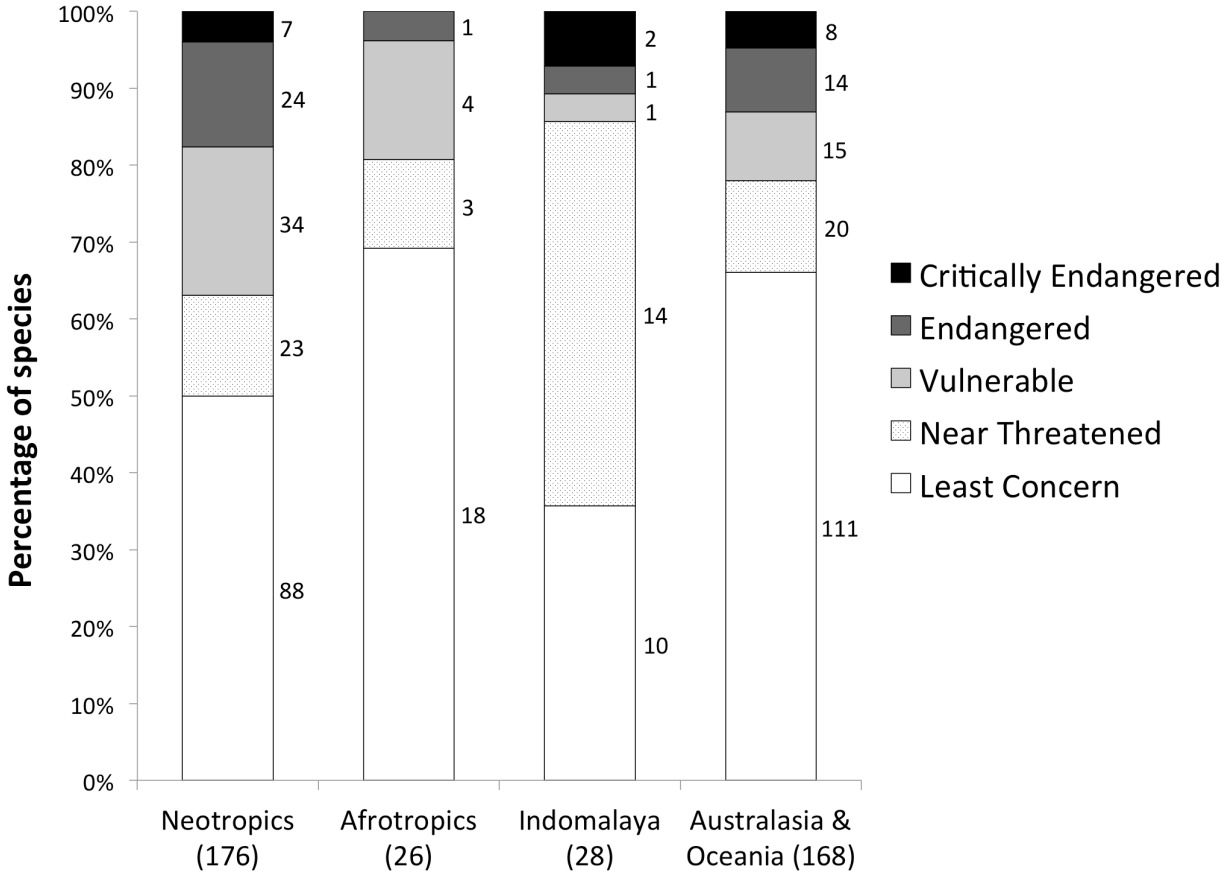


Figure 3. Phylogeny of parrots indicating IUCN Red List status of each species. Colours at the tip of the branches represent the IUCN Red List category of each species; grey shading inside the circle represent major genera and groups of related genera as labelled outside the circle. Images are taken with permission from the Handbook of Birds of the World online (Taylor 1996) and represent examples of species that are Critically Endangered. Source of phylogeny: www.birdtree.org

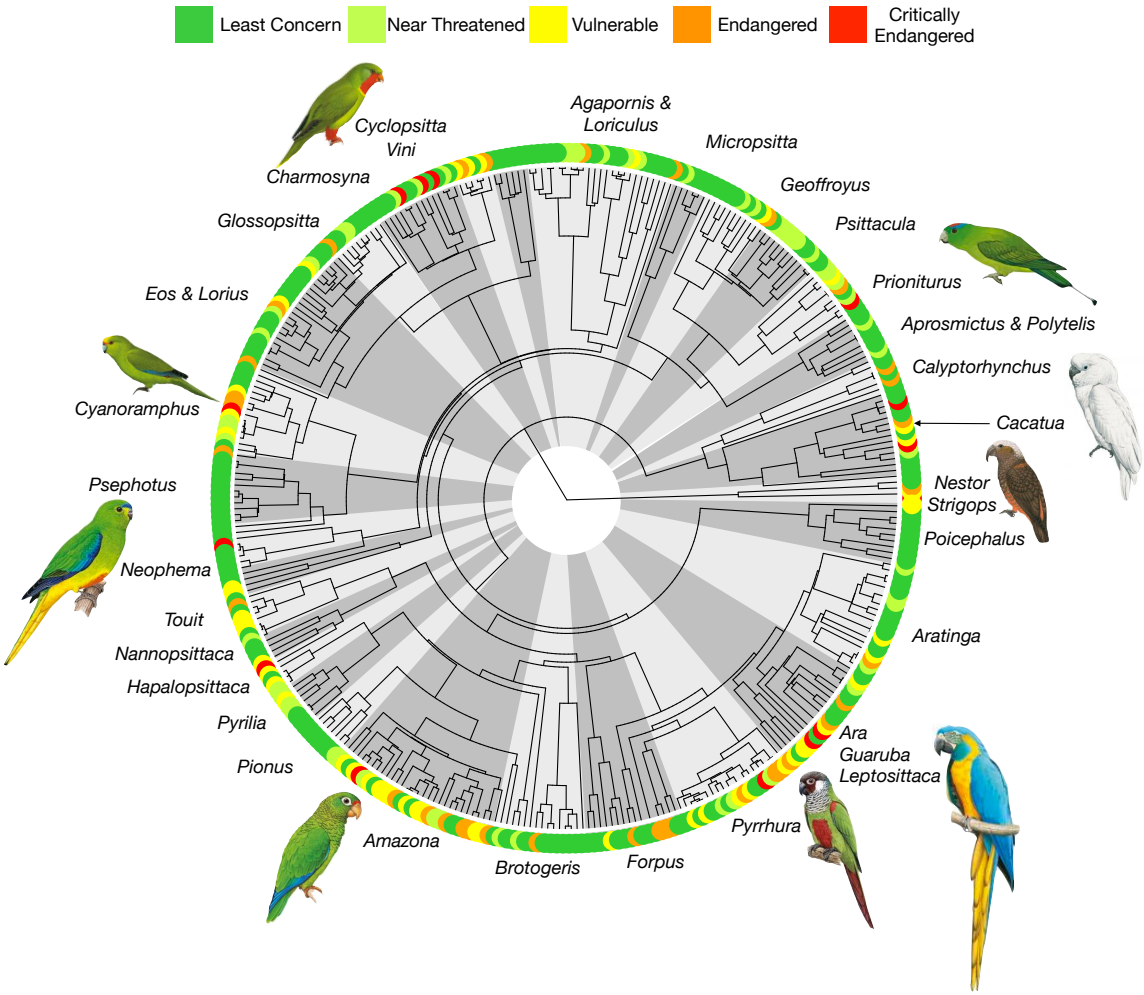


Figure 4. Threats: (A) Percentage of parrot species impacted by the 10 major threat types. Impact is calculated from scores for timing, scope and severity (see http://www.birdlife.org/datazone/info/spcthreat); (B) Percentage of species in each region impacted by each threat type. Only threats impacting >15 species were plotted.

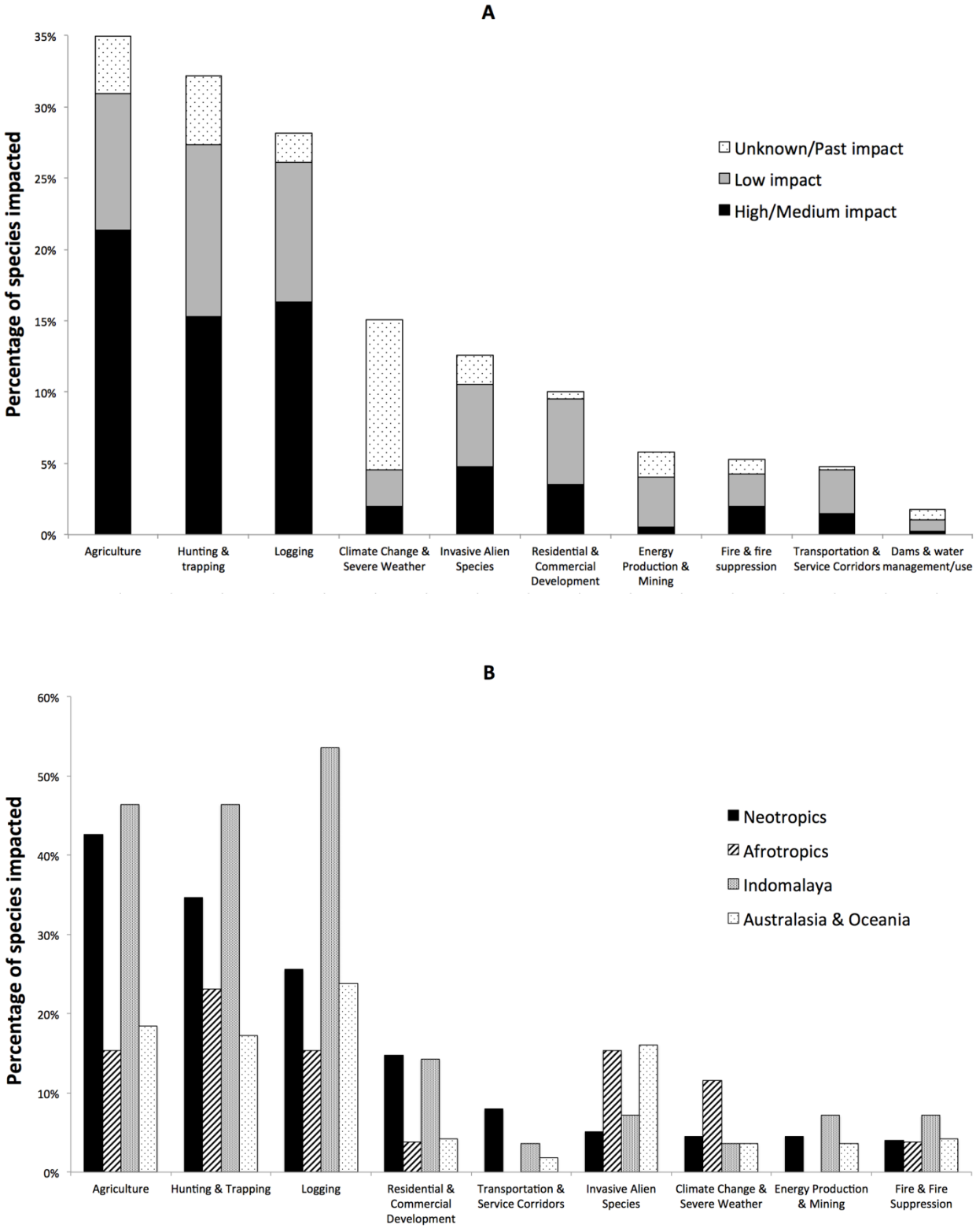


Figure 5. Most important conservation actions needed by region. The figure shows for each region the percentage of species for which each conservation action is considered required by BirdLife International (2014). Only conservation actions identified as required for >15 species across all regions were plotted. Order is given by total species showing the globally most important conservation actions first.

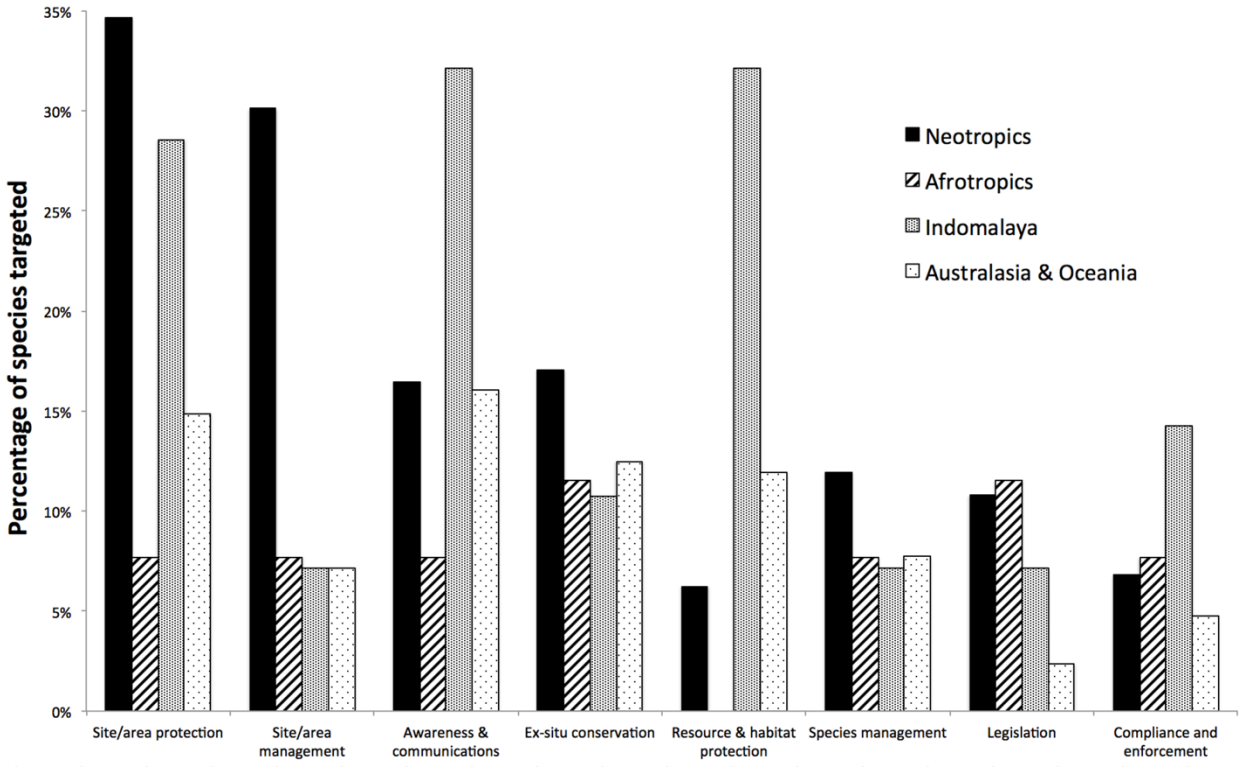


Figure 6. Unexplained (residual) extinction risk of parrots by country and region: values shown are each country's mean residuals from the combined linear logistic regression model predicting likelihood of a parrot species being threatened (VU, EN, CR). High positive values indicate high unexplained extinction risk.

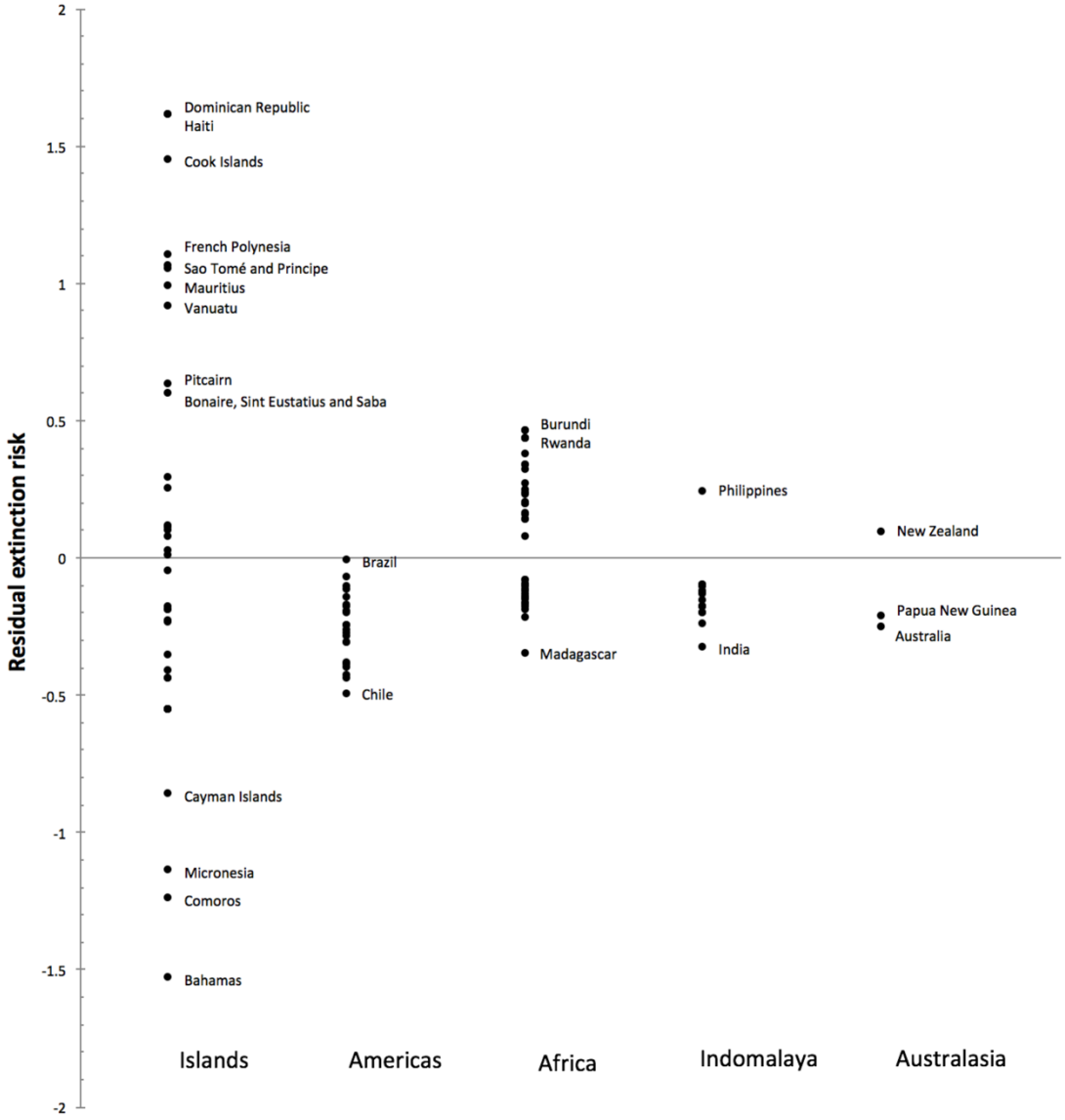


Table 1. Explanatory variables used in the statistical models. Variables marked with an asterisk were $\log_e$ transformed.

Group	Variable name	Source of data	Description	N	Median
A	Median Latitude	BirdLife International and NatureServe (2014)	Absolute value of the median latitude of the species distribution (decimal degree)	398	9.5
	Historical Distribution Size*	BirdLife International and NatureServe (2014)	Area of the historical distribution size of the species (km^2)	398	177 782
	Mean Altitude	BirdLife International (2014)	Mean elevation of the species distribution range (m)	322	322
	Region	Olson et al. (2001)	Distribution of the species: (1) Neotropic, (2) Afrotropic, (3) Indomalaya, (4) Australasia/Oceania	398	
	Island Endemism	BirdLife International (2014)	If endemic to islands smaller than 110,000 km^2 (yes/no) - Large islands were not considered as islands (New Guinea, Borneo, Madagascar, Sumatra, Celebes/Sulawesi, New Zealand, Java)	398	
B	Body Size*	Forshaw (2011)	Length of the species (cm)	398	26
	Number of Habitats	BirdLife International (2014)	Total number of habitats utilised by species (importance: major, marginal, or suitable)	398	3
	Migrant Status	BirdLife International (2014)	(0) Not a Migrant, (1) Nomadic, (2) Altitudinal Migrant , (3) Full Migrant	398	
	Main Diet	Juniper and Parr (2003)	(1) Frugivore (fruits, vegetable matters, leaves, fungi, lichens), (2) Granivore (grass seeds), (3) Nectarivore (nectar, pollen, flowers), (4) Tree seeds (hard seeds, acorns, nuts, cone seeds), (5) Specialist	398	
	Social Flocking	Forshaw (2011), Arndt (2007)	Flock size in the non-breeding season: (0) No flocks (alone or pairs), (1) Small flocks (up to 20), (2) Large flocks (more than 20)	397	
	Colony Nesting	Forshaw (2011)	yes/no	386	
	Nesting Tree Type	Forshaw (2011), Juniper and Parr (2003)	(1) hardwood trees and their branches; (2) palm trees; (3) other nest types (e.g. termite mounds, epiphytes, moss, burrows, grass, etc.)	322	
	Forest Dependency	BirdLife International (2014)	(0) Non-forest, (1) Low, (2) Medium, (3) High. Scored from published and unpublished information on the ecology of each species.	398	
	Generation Time	BirdLife International (2014)	Mean generation length in years	398	6
C	Captive Breeding	Arndt (2007)	yes/no	397	
	Used for Pets	BirdLife International (2014)	yes/no	398	
	Used for Food	BirdLife International (2014)	yes/no	398	
	Used for Accessories	BirdLife International (2014)	yes/no	398	
	Used for Sport	BirdLife International (2014)	yes/no	398	

D	Single Country Endemic	BirdLife International (2014)	If endemic to one country (yes/no)	398	
	Per capita GDP	CIA (2013), IMF (2013)	Gross domestic product based on purchasing-power-parity (PPP) per capita in USD	397	10 148
	Industrial Production Growth Rate	CIA (2013)	Mean percentage of the industrial production growth rate of countries where species is extant (%)	378	4.1
	Unemployment Rate	CIA (2013), IMF (2013)	Mean percentage of the labour force that is without jobs of countries where species is extant (%)	395	6.6
	Human Population Density	FAOSTAT (2013), CIA (2013), IMF (2013)	Mean population density of countries where species is extant (people/1000Ha)	398	315
	Urban Population	FAOSTAT (2013)	Mean percentage of population living in urban areas of countries where species is extant (%)	398	68.3
	Human Population Growth Rate	CIA (2013)	Mean population growth rate of countries where species is extant (%)	398	1.1
	Agriculture Area	FAOSTAT (2013)	Mean percentage of agricultural area of countries where species is extant (%)	398	31.3

Table 2. The highest priority countries for parrot conservation, ranked by total numbers of their (a) parrot species, (b) globally threatened species, and (c) endemic species (restricted to one country). Overall rank is derived from the sum of ranks for the three parameters.

Country	Diversity rank	Threat rank	Endemics rank	Overall Rank
Indonesia	1	2	2	1
Brazil	2	1	3	2
Australia	4	6	1	3
Colombia	3	2	6	3
Bolivia	5	4	8	4
Ecuador	8	3	9	5
Peru	6	5	9	5
Papua New Guinea	6	11	5	6
Venezuela	7	8	8	7
Mexico	13	6	8	8
Philippines	16	8	4	9
Argentina	10	9	11	10
Guyana	9	11	11	11
Panama	11	10	10	11
New Zealand	20	7	5	12
Paraguay	12	9	11	12
French Guiana	10	13	11	13
Suriname	10	13	11	13
Costa Rica	14	10	11	14
Guatemala	15	11	11	15

2.9 Supplementary methods

2.9.1 Red List Indices

We examined trends in extinction risk during 1988-2012 for parrots (*Psittaciformes*) and comparable high profile species-groups with similar numbers of species using Red List Indices. These groups included waterbirds (including species from families *Anseriformes, Podicipediformes, Phoenicopteriformes, Gruiformes, Gaviiformes, Ciconiiformes, Pelecaniformes, Suliformes, Charadriiformes*), seabirds (*Anseriformes, Podicipediformes, Phaethontiformes, Gaviiformes, Sphenisciformes, Procellariiformes, Pelecaniformes, Suliformes, Charadriiformes*), raptors (*Accipitriformes, Cathartiformes, Falconiformes*), each of which comprises multiple orders. Other groups included the largest bird orders (i.e. with more than 250 species) like pigeons (*Columbiformes*) and gamebirds (*Galliformes*), except the orders *Passeriformes*, *Caprimulgiformes*, and *Piciformes*. Cases where species were re-categorized owing to improved knowledge or revised taxonomy are excluded. We used data from the comprehensive assessments of all bird species in 1988, 1994, 2000, 2004, 2008 and 2012, updated to 2014 (Tittensor et al. 2014).

2.9.2 Database and variables

We assembled a database of the biological and geographic attributes of all 398 extant parrot species using the 2014 version of BirdLife International and IUCN's database which underpins the IUCN Red List assessments for birds on the BirdLife Data Zone (BirdLife International 2014) and IUCN Red List website (IUCN 2014). We added further data including the socio-economic and demographic attributes of the countries the parrots occur in, from various external sources (Table 1).

We used the IUCN Red List extinction risk categories of all extant species of parrots (BirdLife International 2014; IUCN 2014) as the response variables in our analyses. In tables and graphs we use the standard IUCN abbreviations for Red List categories as follows: LC = Least Concern, NT = Near Threatened, VU = Vulnerable, EN = Endangered, CR = Critically Endangered (IUCN 2014). We analysed the traits of 16 extinct species separately, and exclude hypothetical taxa that have not been confirmed as valid species (Hoyo et al. 2014). For all analyses we followed the taxonomy of BirdLife International (2014).

Because of the large number of explanatory variables, we initially divided the potential explanatory variables into four groups and performed analyses separately for each. We then combined all significant variables from each sub-analysis into a final model (see below). The groups were: (A) geographical and distributional attributes of each species; (B) biological, ecological and life history variables; (C) type of utilization by humans; and (D) socio-economic and demographic attributes of the countries where the species occur. For detailed descriptions, source of data and values of each variable see Table 1.

We used spatial analyses on the digital distribution files from BirdLife International and NatureServe (2014). ArcGIS 10.2 was used to calculate the median latitude of the distribution of each species. We calculated historical distribution size (i.e. current plus extirpated or historical ranges) from the species' shape-files. We used historical distribution size instead of current distribution sizes in order to avoid possible circularity as current extent of occurrence is a parameter used in the IUCN Red List criteria. For the same reason population size and trend were not used, to avoid circularity (IUCN 2014).

We defined whether each species was an island endemic (yes/no) depending on whether it was restricted to an island smaller than 110 000 km^2. Under this arbitrary definition, parrots of larger islands such as Borneo (743 330 km^2), Sumatra (473 481 km^2), or New Guinea (452 860 km^2) were not considered island endemics; the largest island that qualified was Cuba (109 820 km^2) (see description in Table 1). We tested the validity of this assumption by varying our definition of the island size (including larger island cutoffs) that qualified and found this made no difference to the results.

We determined the type of utilization by people (group C) from the IUCN Red List Use Classification Scheme (http://www.iucnredlist.org/technical-documents/classification-schemes) assigned into binomial variables (yes/no). Pets are defined as those species recorded as being kept in captivity, either as personal pets, or for display in zoos, collections etc.

To assess socioeconomic and demographic attributes, we used The World Economic Outlook Database (IMF 2013), The World Factbook (CIA 2013), and the database of the Food and Agriculture Organization of the United Nations (FAOSTAT 2013) as sources, and calculated the mean values of each parameter for all countries in which a species occurred (excluding vagrant records; Table 1).

Historical distribution size and body size were normalized using a $\log_e$ transformation.

2.9.3 Threats, conservation actions, and priority countries

To understand the specific threats associated with high extinction risk in parrots, we extracted data from BirdLife International (2014) who classify threats using the IUCN-CMP Unified Classification of Direct Threats (Salafsky et al. 2009, updated at http://www.iucnredlist.org/technical-documents/classification-schemes/threats-classification-scheme). We analysed threats at level 1, apart from Biological Resource Use and Natural System Modifications, which we analysed at level 2 given the fundamentally distinct processes these classes aggregate. We assessed how many parrot species are affected by each threat type globally and at the regional scale. We also considered the overall threat impact scores (which are calculated from the timing, scope and severity of each threat to each species: http://www.iucnredlist.org/documents/Dec_2012_Guidance_on_Threat_Impact_Scoring.pdf), and excluded past and unknown threats and those with no/negligible impacts.

We analysed data from BirdLife International (2014) on the most important conservation actions needed to improve the status of threatened parrots; these are coded against the IUCN-CMP Unified Classification of Actions (Salafsky et al. 2009, updated at http://www.iucnredlist.org/documents/Dec_2012_Guidance_Conservation_Actions_Needed_Classification_Scheme.pdf).

We used two methods to highlight important countries for parrot conservation. (1) We followed Croxall et al. (2012) to prioritize countries according to the sum of their ranks for the total numbers of their (a) parrot species, (b) globally threatened species, and (c) single country endemic species, and referred as 'country priority'. (2) In order to determine which countries had the highest proportion of unexplained extinction risk we calculated the mean for each country of the residuals from the combined linear logistic regression (see below) and used this to rank them in terms of the magnitude of unexplained variation remaining once all known significant causes of threatened status have been removed. We refer to this as 'unexplained extinction risk'.

2.9.4 Statistical analysis

We conducted our analyses of the likely determinants of the status of parrots at two levels, one designed to identify the broad covariates of whether a parrot species is threatened or not, and the other designed to evaluate in more detail the covariates of the degree of threat faced by parrot species. To test variables at the broader scale we assigned all species a binary response variable of 0 (Least Concern and Near Threatened) or 1 ('Threatened', i.e. Vulnerable, Endangered

or Critically Endangered), and analysed possible explanatory variables using linear logistic regression with appropriate controls for non-independence due to phylogenetic effects (see below).

To examine the possible causes of threat in further detail, we assigned numerical values corresponding to the extinction risk faced by each threatened species as follows: Vulnerable = 1, Endangered = 2, Critically Endangered = 3. We used ordinal regression models to analyse these values because of their directional numerical nature.

Linear logistic regression and ordinal regression models were initially computed using each set of variables (A-D above) separately. This was to avoid statistical issues associated with multicollinearity. The final universal model was computed by combining the variables found to be significant in each of the sub-models.

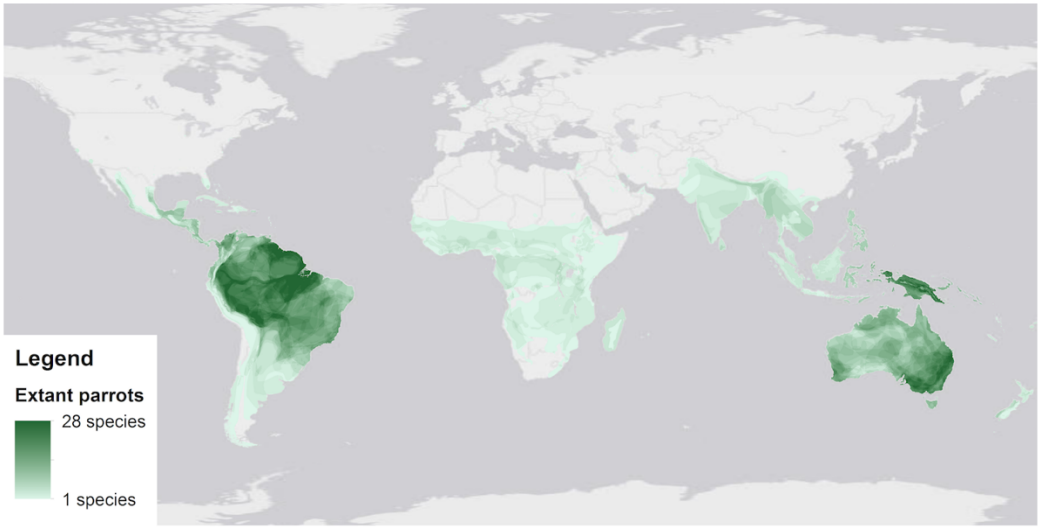
We used correlation matrices to determine whether variables within each group were correlated, and initially avoided using correlated variables in the same analysis. The following variables were excluded on this basis: mean body mass and clutch size (correlated with body size, Table S1A) and forest area of each country (correlated with area of agriculture, Table S1B). Because generation time was significantly positively correlated with body size (Table S1A), we calculated and used the residual values from the simple linear regression of generation time versus body size and referred this variable as 'residual generation time' following Owens and Bennett (2000). Similarly, we calculated residual values for industrial production growth rate, unemployment rate, human population density, urban population, human population growth rate, and agriculture area because they were significantly correlated with per capita GDP (Table S1B).

All linear logistic regressions and ordinal regression models were computed using GenStat 13.7 (Payne 2009). Akaike information criteria (AIC) and Bayesian information criteria (BIC) were used to determine the best parsimonious models containing all significant terms. Models were selected with the lowest AIC values and simultaneously having the lowest BIC values (Table S2). We also report *P*-values for each significant variable determined by its exclusion from the full models selected above. We also controlled for phylogenetic relatedness between species using phylogenetic generalized least squares (PGLS) regression.

2.10 Supplementary figures and tables

Figure S1. Global density maps of (A) all extant parrot species, and (B) threatened parrots (Vulnerable, Endangered, and Critically Endangered). Colour intensities indicate the number of parrot species.

(A)



(B)

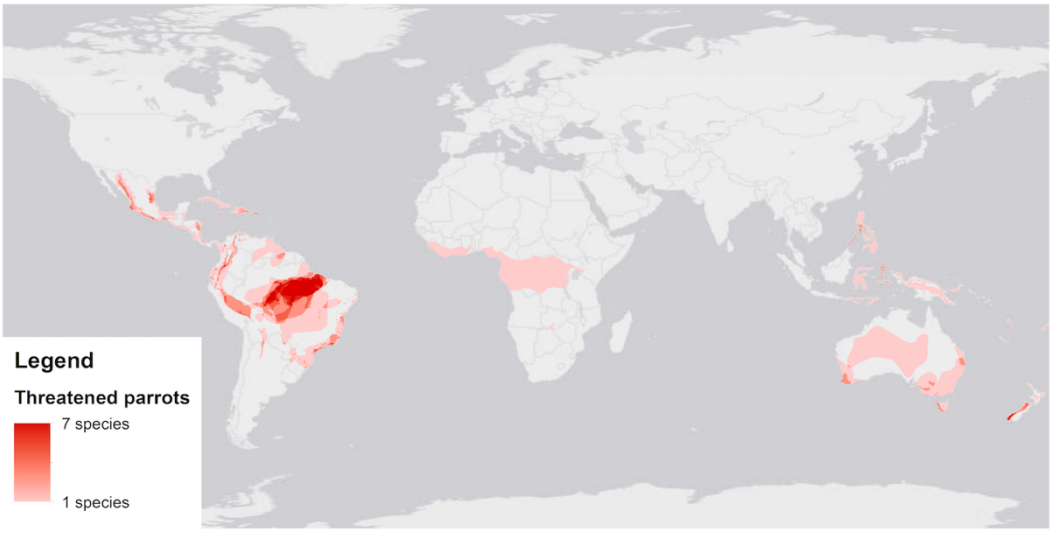


Figure S2. Predicted effect of significant variables on the probability of being threatened for parrot species according to: (A) Log_e (Historical Distribution Size, km^2), (B) Log_e (Body Size, cm), (C) Residual Generation Time (years), (D) Forest Dependency, and (E) Percentage of the human population living in urban conditions. Error bars represent standard errors.

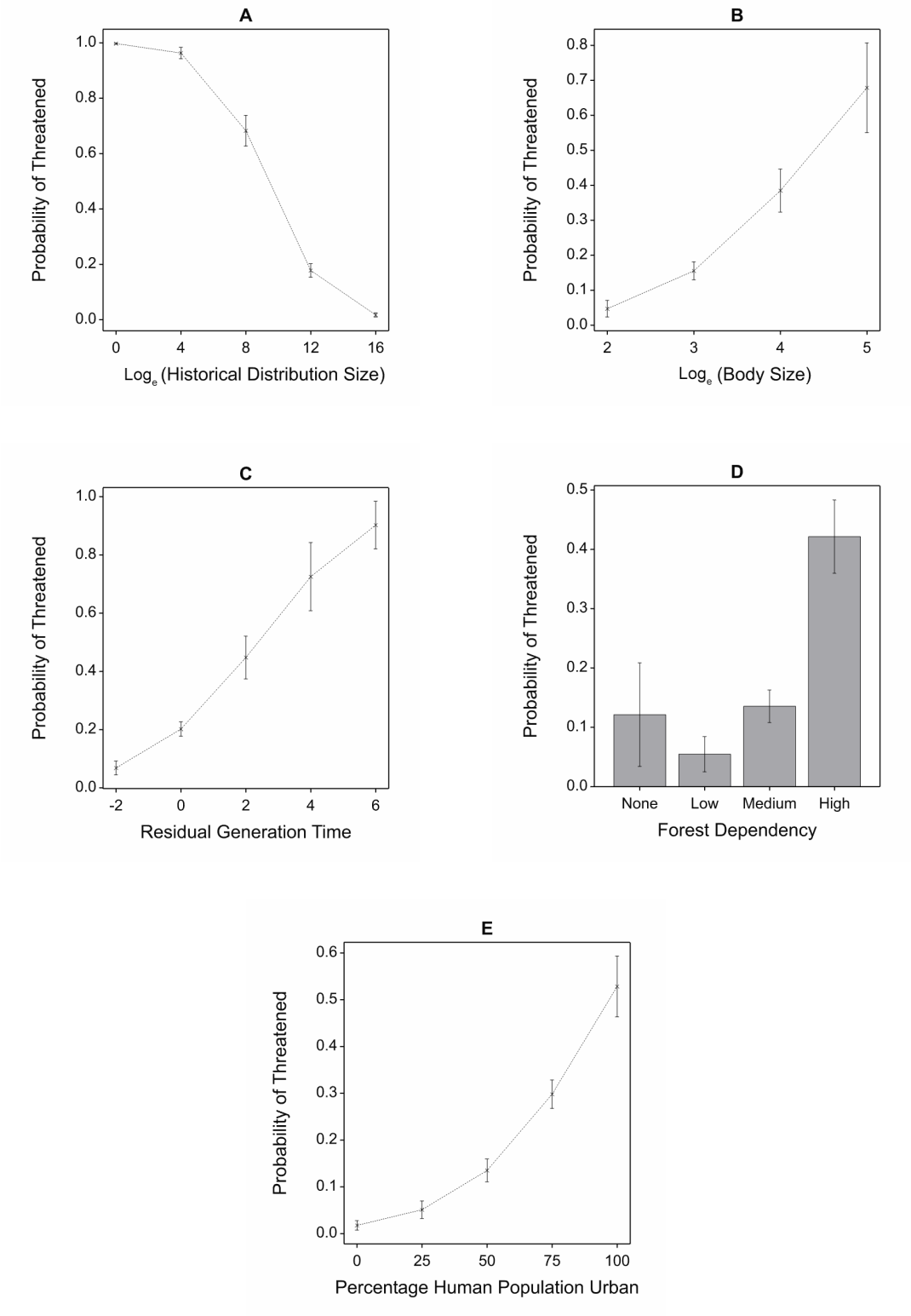


Figure S3. Proportion of threatened species in each category (VU, EN, CR) for each significant variable in ordinal regression models: (A) Per capita GDP, (B) Single Country Endemic, (C) whether Used for Pets/Display, and (D) whether threatened by Invasive Alien Species or other threats.

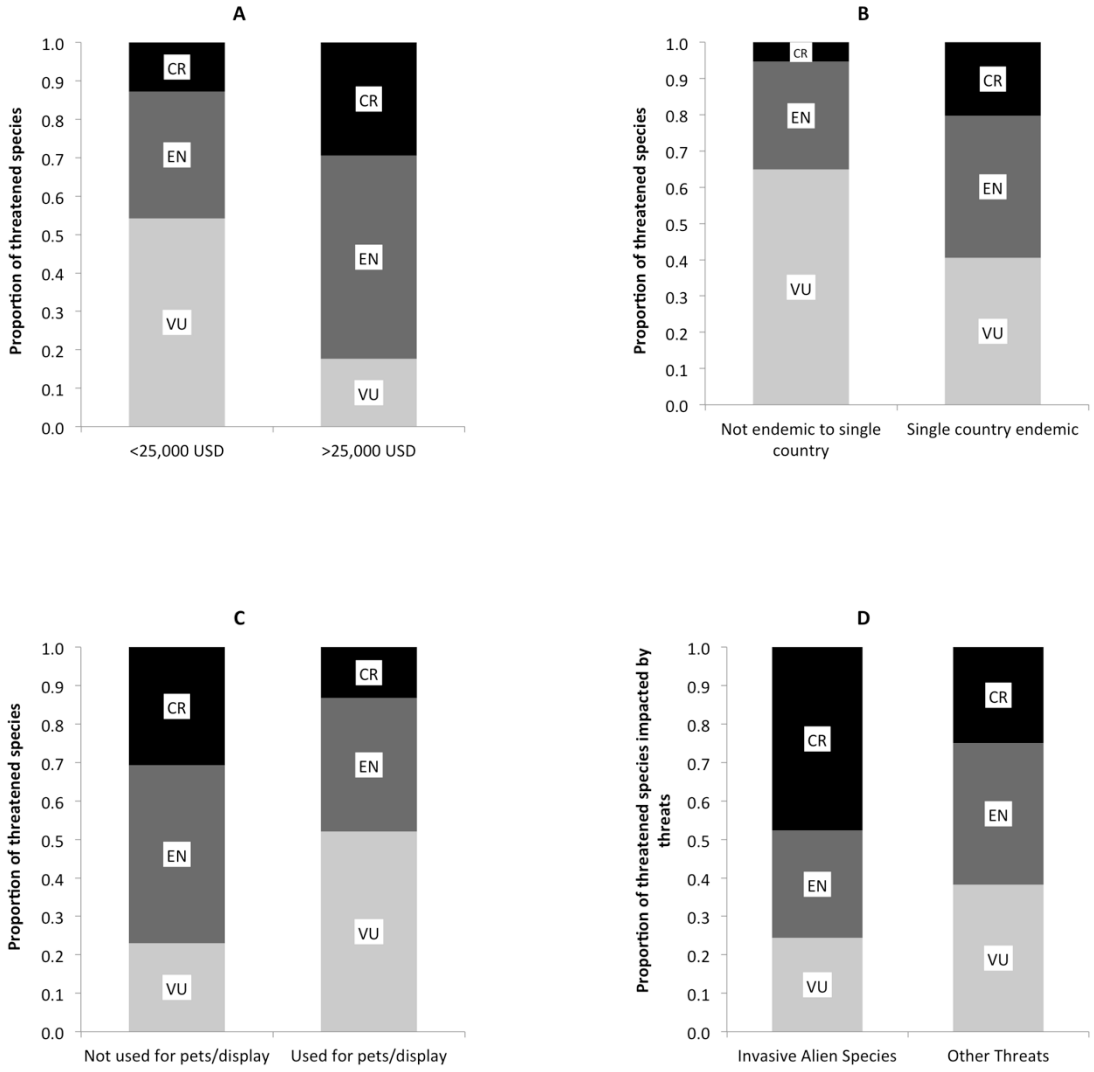


Table S1. Correlation matrices. Correlation coefficients (Pearson's) are shown below diagonal, two-sided test of correlations different from zero above diagonal.

(A) Number of species: 337

Variables	Body Size	Body Mass	Clutch Size	Generation Time
Body Size	-	<0.001	<0.001	<0.001
Body Mass	0.8447	-	<0.001	<0.001
Clutch Size	-0.2214	-0.3011	-	<0.001
Generation Time	0.6533	0.7552	-0.2535	-

(B) Number of species: 378

Variables	Per capita GDP	Industrial Production Growth Rate	Unemployment Rate	Human Population Density	Urban Population	Human Population Growth Rate	Agriculture Area	Forest Area
Per capita GDP	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Industrial Production Growth Rate	-0.5775	-	0.5399	0.1514	<0.001	<0.001	<0.001	<0.001
Unemployment Rate	-0.2218	0.0316	-	0.5357	0.0343	<0.001	<0.001	0.0019
Human Population Density	-0.373	0.0739	0.032	-	<0.001	0.0189	0.9509	0.0068
Urban Population	0.6685	-0.6265	-0.1089	-0.5035	-	<0.001	<0.001	<0.001
Human Population Growth Rate	-0.3382	0.4275	0.3233	0.1207	-0.55	-	0.7567	0.0039
Agriculture Area	0.4914	-0.5284	0.2471	-0.0032	0.4027	-0.016	-	<0.001
Forest Area	-0.5472	0.3489	-0.1589	-0.139	-0.2195	-0.1481	-0.8451	-

Table S2. AIC (Akaike information criteria) and BIC (Bayesian information criteria, also referred to as Schwarz information criterion) values for best parsimonious models containing all significant terms for each variable group (ranked by AIC values).

Model	Linear Logistic Regression		
	AIC	**BIC**	**d.f.**
Threats	249.23	273.15	6
A - Geographical and distributional attributes	281.64	304.29	6
Final combined model	292.42	324.29	8
B - Biological, ecological and life history variables	327.60	354.02	7
D - Socio-economic and demographic attributes	375.71	391.45	4
C - Type of utilization by humans	452.62	468.55	4

Model	Ordinal Regression		
	AIC	**BIC**	**d.f.**
B - Biological, ecological and life history variables	358.22	664.74	96
A - Geographical and distributional attributes	360.82	670.54	97
D - Socio-economic and demographic attributes	382.42	719.01	103
Final combined model	432.68	822.95	115
Threats	436.95	828.26	115
C - Type of utilization by humans	440.57	824.05	113

Table S3. Significant variables in the linear logistic regression models run separately for each variable group (A-D) predicting the likelihood of a parrot species being threatened (VU, EN, CR). λ values for the phylogenetic generalized least squares model (PGLS) are given in the table for each model. We give mean ± standard deviation (SD).

Group	Variable	Wald statistic	d.f.	*P* (χ^2)	Estimate ± SD (PGLS)	*P* (PGLS) ± SD	λ ± SD
A	Historical Distribution Size ($\log_e$)	71.64	1	<0.001	-0.246 ± 0.004	<0.001 ± <0.001	0.087 ± 0.083
	Median Latitude	8.67	1	0.003	0.025 ± 0.004	0.260 ± 0.080	
	Region	30.41	3	<0.001	-0.175 ± 0.027	0.032 ± 0.038	
	Median Latitude * Historical Distribution Size ($\log_e$)	5.18	1	0.023	-0.001 ± <0.001	0.581 ± 0.058	
B	Body Size ($\log_e$)	12.59	1	<0.001	0.529 ± <0.001	<0.001 ± <0.001	<0.001 ± <0.001
	Forest Dependency	50.31	3	<0.001	0.492 ± <0.001	<0.001 ± <0.001	
	Residual Generation Time	10.10	1	0.001	0.492 ± <0.001	0.006 ± <0.001	
C	Used for Pets	9.15	1	0.002	-1.162 ± 0.013	<0.001 ± <0.001	0.269 ± 0.049
	Used for Food	11.47	1	<0.001	0.353 ± 0.017	0.029 ± 0.008	
	Used for Sport	4.55	1	0.033	0.423 ± 0.019	0.242 ± 0.021	
D	Residual Human Population Density	3.90	1	0.048	0.196 ± 0.007	0.006 ± 0.001	0.324 ± 0.067
	Residual Urban Population	26.98	1	<0.001	-0.275 ± 0.013	0.006 ± 0.002	
	Single Country Endemic	26.44	1	<0.001	0.755 ± 0.011	<0.001 ± <0.001	

Table S4. Significant variables in ordinal regression models run separately for each variable group (A-D) predicting the likelihood of a species being more endangered among threatened (VU, EN, CR) parrot species. λ values for the phylogenetic generalized least squares model (PGLS) are given in the table for each model. We give mean ± standard deviation (SD).

Group	Variable	Deviance	d.f.	*P* (χ^2)	Estimate ± SD (PGLS)	*P* (PGLS) ± SD	λ ± SD
A	Region	14.42	3	0.002	0.147 ± <0.001	0.015 ± <0.001	<0.001 ± <0.001
	Island Endemic	10.45	1	0.001	-0.250 ± <0.001	0.210 ± <0.001	
	Historical Distribution Size (loge)	5.15	1	0.023	-0.016 ± <0.001	0.621 ± <0.001	
B	Main Diet	10.04	4	0.040	0.094 ± <0.001	0.078 ± <0.001	<0.001 ± <0.001
C	Used for Pets	4.55	1	0.033	-0.495 ± <0.001	0.025 ± <0.001	<0.001 ± <0.001
D	Per capita GDP	8.79	1	0.003	<0.001 ± <0.001	0.030 ± <0.001	<0.001 ± <0.001
	Single Country Endemic	5.60	1	0.018	0.404 ± <0.001	0.007 ± <0.001	

Table S5. (A) Significant variables in the combined linear logistic regression model predicting the likelihood of a parrot species being threatened (VU, EN, CR). λ value for the phylogenetic generalized least squares model (PGLS) was <0.001 ± <0.001. We give mean ± standard deviation (SD).

Variable	Wald statistic	d.f.	*P* (χ^2)	Estimate ± SD (PGLS)	*P* (PGLS) ± SD
Historical Distribution Size ($\log_e$)	64.89	1	<0.001	-0.245 ± <0.001	<0.001 ± <0.001
Body Size ($\log_e$)	13.18	1	<0.001	0.474 ± <0.001	<0.001 ± <0.001
Residual Generation Time	16.82	1	<0.001	0.200 ± <0.001	<0.001 ± <0.001
Urban Population	28.71	1	<0.001	0.014 ± <0.001	<0.001 ± <0.001
Forest Dependency	29.47	3	<0.001	0.332 ± <0.001	<0.001 ± <0.001

(B) Significant variables in combined ordinal regression model predicting the likelihood of a species being more endangered among threatened (VU, EN, CR) parrot species. λ value for the final phylogenetic generalized least squares model (PGLS) was <0.001 ± <0.001. We give mean ± standard deviation (SD).

Variable	Deviance	d.f.	*P* (χ^2)	Estimate ± SD (PGLS)	*P* (PGLS) ± SD
Per capita GDP	8.47	1	0.004	0 ± <0.001	0.024 ± <0.001
Single Country Endemic	5.60	1	0.018	0.360 ± <0.001	0.015 ± <0.001
Used for Pets	4.75	1	0.029	-0.526 ± <0.001	0.016 ± <0.001

Table S6. (A) Significant threat variables in linear logistic regression model predicting the likelihood of a parrot species being threatened (VU, EN, CR). λ value for the phylogenetic generalized least squares model (PGLS) was 0.009 ± 0.018.

Variable	Wald statistic	d.f.	*P* (χ^2)	Estimate ± SD (PGLS)	*P* (PGLS) ± SD
Invasive Alien Species	32.65	1	<0.001	1.734 ± 0.004	<0.001 ± <0.001
Agriculture	28.97	1	<0.001	1.132 ± 0.008	<0.001 ± <0.001
Hunting & Trapping	19.88	1	<0.001	1.383 ± 0.002	<0.001 ± <0.001
Residential & Commercial Development	7.13	1	0.008	0.452 ± 0.009	<0.001 ± <0.001
Energy Production & Mining	4.71	1	0.030	0.397 ± 0.010	0.009 ± 0.002
Agriculture * Hunting & Trapping	7.39	1	0.007	-0.772 ± 0.012	<0.001 ± <0.001
Hunting & Trapping * Invasive Species	6.46	1	0.011	-1.178 ± 0.002	<0.001 ± <0.001

(B) Significant threat variables in ordinal regression model predicting the likelihood of a species being more endangered among threatened (VU, EN, CR) parrot species. λ value for the final phylogenetic generalized least squares model (PGLS) was <0.001 ± <0.001.

Variable	Deviance	d.f.	*P* (χ^2)	Estimate ± SD (PGLS)	*P* (PGLS) ± SD
Invasive Alien Species	14.41	1	<0.001	0.598 ± <0.001	<0.001 ± <0.001
Agriculture	5.85	1	0.016	0.332 ± <0.001	0.063 ± <0.001

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Chapter 3

Nest site selection and efficacy of artificial nests for breeding success of scarlet macaws in lowland Peru

Artificial nest box of a breeding scarlet macaw near to the Tambopata Research Center (2008).

This chapter has been published as:

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3.1 Abstract

Psittacidae (parrots) have the most threatened species of any bird family in the world. Most parrots are obligate secondary cavity nesters, and can be limited in their breeding success by the availability and quality of nest hollows. However, nesting opportunities for parrots can be increased by provision of artificial nest boxes. The Tambopata Macaw Project has been studying the breeding ecology and natural history of the Scarlet Macaw *Ara macao macao* in the southeastern Peruvian Amazon for over 20 years by monitoring natural nest hollows and two types of artificial nest (wooden and PVC). We present data for breeding success in natural and artificial nests over 12 consecutive breeding seasons. The aims of this study were to a) determine the nesting requirements and reproductive success of breeding macaws; and b) compare the efficacy of the two types of artificial nests and natural nest cavities. Our data showed a high rate of reoccupation of successful nests in consecutive years and that nests in artificial and natural nests had very similar reproductive parameters. Our results indicate that artificial nest types can be used by conservation managers seeking to assist *A. macao* populations where nest hollows are in short supply, and that artificial nests can contribute important data to natural history studies of species where access to natural nests is limited.

3.2 Introduction

Information about nest sites and reproductive success of wild bird populations is necessary for effective conservation and management strategies (Renton, 2000). Breeding success is a crucial determinant of recruitment rates, population size and long-term population viability in many avian species (Martin & Geupel, 1993). The type of nest used by birds can determine breeding success rates and other aspects of life-history (Paredes & Zavalaga, 2001). Most parrots are obligate secondary cavity nesters (Monterrubio-Rico & Escalante-Pliego, 2006), thus their breeding success is closely related to the availability and quality of nest hollows (Collar, 1997). The number and quality of nest cavities have been shown to be limiting for various parrot species, like Eclectus Parrot *Eclectus roratus* Müller 1776 (Heinsohn et al., 2005; Heinsohn, 2008a), Palm Cockatoo *Probosciger aterrimus* Gmelin 1788 (Heinsohn et al., 2003), Swift Parrot *Lathamus discolor* White 1790 (Stojanovic et al., 2012), *Amazona* parrots (White et al., 2005), and Blue-throated Macaw *Ara glaucogularis* Dabbene 1921 (Hesse & Duffield, 2000).

Secondary cavity nesters can be assisted by the provision of artificial nest boxes (Brightsmith, 2005a). They can be placed in areas where hollow availability is low, and can be supplied in large enough numbers to facilitate statistical inference for scientific research (Major & Kendal, 1996). Their characteristics usually mimic the natural nest hollows of the target species, but artificial nests can also be more amenable to experimental manipulation as they reduce variation in nesting circumstances and improve accessibility to the nest (Villard & Pärt, 2004). They can be useful tools for supporting reintroduction and translocation of endangered bird species (White et al., 2006), and for enhancing ecotourism by increasing the numbers of nesting birds (Nycander et al., 1995). Nest boxes have made it easier to perform comparative and experimental field investigations, since they can facilitate the installation of electronic monitoring devices (Grenier & Beissinger, 1999; White & Vilella, 2004). Nevertheless, concerns have been raised about the generality and applicability of data from nest box studies (Lambrechts et al., 2012). For instance, there have been doubts about whether experimental setups of artificial nests mirror accurately enough the natural systems they attempt to model (Major & Kendal, 1996).

The family *Psittacidae* (parrots) has the highest number of threatened species of bird family (Bennett & Owens, 1997; IUCN, 2013). Approximately 30% of all the parrot species are threatened globally and 37% of the Neotropical parrots are threatened (IUCN, 2013). Despite the increase in parrot studies in the last decade (Downs, 2005; Amuno et al., 2007; Ekstrom et al., 2007; Murphy et al., 2007; Heinsohn, 2008b; Berkunsky & Reboreda, 2009; Boyes & Perrin, 2009; Theuerkauf et al., 2009; Briceño-Linares et al., 2011; Brightsmith & Villalobos, 2011; Britt, 2011; Vigo et al., 2011;

Stojanovic et al., 2012; White et al., 2012) more research is required to document basic natural history and determine the effects of processes such as deforestation, habitat fragmentation, hunting by humans for food, and trapping for the pet trade (Laurance et al., 2009).

Macaws (genera *Ara*, *Anodorhynchus, Cyanopsitta, Primolius, Orthopsittaca,* and *Diopsittaca*) are charismatic parrots that remain poorly understood in the wild (Forshaw, 1989). Currently 5 species of macaws are extinct, and of the remaining 17 species, 3 are critically endangered (CR), 4 endangered (EN), 2 vulnerable (VU), and 1 near threatened (NT). Many of these macaws are found in the Amazon Basin in South America, which also contains a highly diverse and complex globally important ecosystem. Even today, this region that includes 60% of the world's remaining tropical rainforest (Laurance et al., 2002) is little known. The large size and wide-ranging habits of macaws, together with their popularity in human society, make them suitable 'umbrella' species for conservation in the Amazon region (Roberge & Angelstam, 2004).

The Tambopata Macaw Project has been studying the breeding ecology and natural history of three large macaw species (Scarlet Macaw *Ara macao macao* Linnaeus 1758*,* Reda-and-green Macaw *Ara chloropterus* Gray 1859*,* and Blue-and-yellow Macaw *Ara ararauna* Linnaeus 1758) in the southeastern Peruvian Amazon for over 20 years (Nycander et al., 1995; Brightsmith, 2005a; Brightsmith et al., 2008a). In the Tambopata region of Peru *A. macao* is still found in abundance (Renton & Brightsmith, 2009). This natural environment provides ideal circumstances for understanding the nest preferences of this bird, which can then be applied in other locations where their populations are declining.

In this paper we examine the long-term nesting success of *A. macao macao* (hereafter *A. macao*) with emphasis on the effectiveness of providing the birds with artificial nest boxes. Our aims are to a) determine the natural nesting requirements and reproductive success of the breeding macaws; and b) examine the efficacy of two different types of artificial nest (wooden and PVC) and compare their success to natural nest cavities. We anticipate that data from both natural and artificial nest types will have important conservation applications by informing conservation managers seeking to assist macaw populations where nest hollows are in short supply.

3.3 Materials and methods

3.3.1 Study area

The study was conducted in the forests surrounding the Tambopata Research Center (TRC) in southeastern Peru (13° 8.070' S, 69° 36.640' W), in the Department of Madre de Dios, in the Tambopata National Reserve (2,747 km^2), near the border of the Bahuaja-Sonene National Park (10,914 km^2). This area, which receives an average annual rainfall of about 3,300 mm (Brightsmith, 2004), is located in tropical moist forest adjacent to subtropical wet forest (Tosi, 1960) and is surrounded by four main forest types: *terra firme*, floodplain, palm swamp, and a mixture of early and late successional forest. The research centre is located 880 m from a large clay lick, Collpa Colorado, that serves as an important source of sodium rich soil which macaws feed on year round, especially during breeding when they feed it to their chicks (Brightsmith et al., 2008b; Powell et al., 2009; Brightsmith et al., 2010; Brightsmith & Villalobos, 2011). All the studied nests were located within 3 km of TRC. The apparently high density of nests is likely due to both the provisioning of artificial nests and the close proximity of the large clay lick (Brightsmith, 2004; Brightsmith et al., 2008b). In an effort to document the breeding parameters of the normal wild population data from the nests of the hand-raised and released individuals of *A. macao* found in the study area (Nycander et al., 1995; Brightsmith et al., 2005) were excluded from the analyses presented in this paper. Data on these birds will be presented elsewhere.

3.3.2 Study species

The breeding of the *A. macao* in southeastern Peru takes place during the wet season (November-April). The species nests in hollows of emergent trees including ironwood tree *Dipteryx micrantha* Harms 1926 (*Fabaceae*), *Calycophyllum sp*. Candolle 1830 (*Rubiaceae*), *Hymenaea oblongifolia* Huber 1909 (*Fabaceae*), *Erythrina sp*. (*Fabaceae*), and barrigona palm *Iriartea deltoidea* Ruiz & Pavon 1978 (*Arecaceae*) (Brightsmith, 2005b; Renton & Brightsmith, 2009). Birds usually lay 2-4 eggs asynchronously, the incubation period is 26-28 days, and the chicks fledge around 86 ± 4 days post hatching (Forshaw, 1989; Vigo et al., 2011). Macaws like other parrots hatch asynchronously (Stoleson & Beissinger, 1997). Although 3 eggs hatch on average, in general only 1 or 2 chicks survive until fledging, mainly due to starvation of the youngest siblings (Vigo et al., 2011). We used The Clements Checklist of Birds of the World as

source of the nomenclature of avian taxa (Clements et al., 2013), the IUCN RedList database for other animal taxa (IUCN, 2013), and the International Plant Names Index for plants (IPNI, 2012).

3.3.3 Nest monitoring

We employed data collected from November 1999 - March 2011 (12 nesting seasons) to determine factors affecting the nest use and reproductive success of wild *A. macao*. The number of natural nests varied annually as new nests were found and old nests were lost due to takeover by stinging insects or through tree fall. The first artificial macaw nests were installed at the site in the early 1990's (Nycander et al., 1995). The number of artificial nests monitored each year varied as new nests were added, old nests were moved, trees containing nests fell down, and old nests were abandoned by the birds. All artificial nests were hung one per tree although distance between adjacent artificial nests varied greatly: from 31 to 425 m. They were hung at an average height of 26.8 ± 5.7 m.

Wooden nest boxes (Figure 1A) were about 1.5 m tall and made from tropical hardwoods (*Cedrela odorata, Cedrelinga catenaeformis, Calophyllum sp.*, etc.), with an approximate weight around 70 kg. PVC nests (Figure 1B) were constructed from 26 to 39 cm diameter PVC pipes lined with wire mesh and weighed 35-60 kg. Both nest types were filled to a depth of 30 cm with a mixture of sawdust, wood, and sand. A single door near to the bottom of each nest allowed investigators access to eggs and young individuals. The artificial nests were fixed to tree trunks with 2.5 cm climbing webbing or 11 mm climbing rope, and they were attached both at the top and bottom to reduce swaying and spinning.

We climbed to the nests using single-rope ascending techniques (Perry, 1978; Perry & Williams, 1981) and lowered chicks to the ground in buckets (Nycander et al., 1995). Nest visits usually took 30 to 50 minutes each from arrival until departure. For the safety of the chicks and the researchers, nests were not climbed during rain or high wind. Over the course of the 12 breeding seasons we monitored a total of 26 natural, 12 wooden and 24 PVC nests. The number of nests monitored per season was 10.1 ± 3.7 SD natural, 3.1 ± 2.2 wooden and 10.3 ± 3.3 SD PVC. Nests were monitored from October to April, and those located closer to TRC were usually monitored more intensely than those further from the centre. Nests where there was no presence of *A. macao* were climbed 1 to 2 times per week from November-January. However, where macaws were seen defending the nests, climbing frequency increased to every one to two days (on average 5 climbs before eggs were found at occupied nests). Climbing protocols changed throughout the study: from 2000-2002 we climbed every 2-3 days during incubation and from

2003-2011 once eggs were found we did not climb the nest again until the estimated hatch date to minimize the effect of human disturbance on nesting. After chicks hatched we measured them daily for the first 15 days, then two or three times per week. Climbs were increased to daily or every other day near the estimated fledging date. Overall, occupied nests were climbed an average of 28.6 (± 1.6 SE) times per season. If at least one egg was laid in a nest during the breeding season it was considered a "nesting attempt" and the nest was considered "occupied" for that season. For each nesting attempt we determined the following: 1) whether eggs were damaged, hatched, or did not hatch; and 2) whether the nest was depredated, taken over by other macaws, fell down, or successful (fledged 1 or more chicks).

3.3.4 Statistical analysis

To determine the relationship between nesting success and nest characteristics, we analysed four measures of nest use and nesting success as response variables -whether eggs were laid (yes or no), whether ≥ 1 egg hatched (y/n), whether ≥ 1 chick fledged (y/n) and number of chicks fledged. We used combinations of 43 different variables depending on the analysis. The explanatory variables examined fall into four main categories: *Nest monitoring* (10) to test if the actions of the researchers had any effect on breeding success; *Presence of adult macaws* (4) to test the influence of parental behaviour on nest success; *Nest cavity characteristics* (12) to test the importance of nest measurements on reproductive success; and *Nest site characteristics* (12) to test the effects of visibility, habitat type and distance to other nests (Table 1).

We used a statistical modeling approach with all analyses carried out in GenStat 13.2 (Payne et al., 2009). Whenever the data included repeated measures of the same nest over multiple years we assigned nest identity as a random effect, while the other factors were examined as fixed effects of interest.

We fitted generalized linear mixed models (GLMM) with a binary response (yes/no) to determine which factors influenced: a) whether eggs were laid; b) hatching success (one or more eggs hatched); and c) fledging success (one or more chicks fledged). We determined with linear mixed models (LMM) which factors affected the number of chicks fledged (continuous response variable), using all nests where eggs were laid. Due to the risk of over-parameterization, the variables of interest were tested in four separate blocks: 1) nest site characteristics; 2) nest cavity characteristics; 3) macaws present at nest; and 4) the extent of researcher monitoring activity. Once the significant variables were obtained from each block we combined these variables into a final model and reran it.

In order to determine success at the most occupied nests we performed a generalized linear model (GLM) analysis on nests that were occupied for at least 5 seasons over the study period. We used the number of years a nest was occupied as the response variable, which we compared to the the total years the same nest was observed (as binomial total of the model). We started each analysis with a full model and progressively dropped non-significant terms until the most parsimonious model containing all significant terms was obtained. Unless otherwise stated, we present data as mean ± standard error.

3.4 Results

3.4.1 Characteristics of nest trees and nest hollows

We analysed data from a total of 147 nesting attempts (n = 8 in 1999-2000, n = 9 in 2000-01, n = 15 in 2001-02, n = 12 in 2002-03, n = 10 in 2003-04, n = 11 in 2004-05, n = 14 in 2005-06, n = 12 in 2006-07, n = 14 in 2007-08, n = 15 in 2008-09, n = 8 in 2009-10, n = 19 in 2010-11) that occurred in 42 different nest sites (18 natural, 8 wooden nest boxes and 16 PVC nest boxes) over the 12 years of the study. Most of the available nests were located in floodplain forest (55%), with the remainder in upland or *terra firme* forest (28%) and successional forest (17%). The distribution of the occupied nests among habitats did not differ significantly from the distribution of available nest sites ($\chi^2_2 = 0.32$, P = 0.850, Figure 2).

Of the occupied nests, 44% successfully fledged ≥ 1 chick, 55% failed, and 1% had unknown outcome. Among the 81 nest failures, 37% were lost because the eggs were broken or disappeared before the hatch date. In 32% the eggs were cracked or failed to hatch after the anticipated hatch date. Most of these cases (18 of 26) were in PVC nests with only four in wooden nests and four in natural nests. In 15% of failed nests we found chicks which were killed by parasites, sickness, and bee or wasp attacks but without any sign of predation. For only 7.5% of failed nests (n = 6) did we suspect loss to predators. Of these 6 predation events, 4 occurred in natural nests, one in a wooden nest and one in a PVC nest. At least 6 clutches (7.5% of failed nests) failed due to fights with other pairs of macaws over nest ownership (2 in artificial and 4 in natural nest). We suspect some of the nests where eggs were cracked or did not hatch may have also failed due to fights over nest ownership. One nest (1% of failed nests) was lost because the bottom of the PVC nest box fell off. No nests were lost due to the nesting tree falling down.

3.4.2 Determinants of Reproductive Success: (1) Clutch initiation

Over the 12 seasons, 37% of the occupied nests were in natural cavities and 63% were in artificial (15% wooden and 48% PVC). Nearly half (49%) of the clutches had three eggs, 12% had one egg, 25% had two eggs, and 14% had four eggs. The mean number of eggs laid per clutch was 2.70 (± 0.08 SE) with no significant difference between natural and artificial nests (GLMM $_{\text{Nest type}}$: $\chi^2_2 = 1.66$, P = 0.441; Table 3).

A. macao laid eggs significantly more often in nests which were successful the year before (GLMM $_{\text{Outcome last year}}$: $\chi^2_7 = 21.2$, P = 0.030). In total 85% of nests successful one year were re-occupied the next year, while only 41% of nests which were not successful (no activity or failed) were occupied the subsequent year. We found no significant effect on occupancy of the distance to the nearest nest occupied by the same species (Mean $_{\text{occupied nest}}$ = 192m ± 18 SE; Mean $_{\text{unoccupied nest}}$ = 193m ± 22 SE; GLMM $_{\text{Distance to nearest nest}}$: $\chi^2_1 = 0.74$, P = 0.39). However, we found that the distance to the second nearest nest had a significant positive effect on occupancy (Mean $_{\text{occupied nest}}$ = 321m ± 22 SE, Mean $_{\text{unoccupied nest}}$ = 375m ± 29 SE; GLMM $_{\text{Distance to second nearest nest}}$: $\chi^2_1 = 4.84$, P = 0.029).

The proportion of years a hollow was occupied was dependent on the nest's inside diameter (GLM $_{\text{Diameter inside}}$: $\chi^2_1 = 8.89$, P = 0.003). The highest occupancy rate was for nests with 40-50 cm inside diameter (69% occupancy over the period each nest was monitored), while the lowest occupancy was for nests with > 50 cm diameter (26%). All other variables tested were not significant ($\chi^2_1 < 1.84$, P > 0.175; Table 1).

3.4.3 Determinants of Reproductive Success: (2) Hatching success

A. macao hatching success did not differ significantly between natural and artificial nests (GLMM $_{\text{Nest type}}$: $\chi^2_2 = 1.79$, P = 0.421; Table 3). The likelihood of hatching one or more egg increased with the number of eggs in the clutch (GLMM $_{\text{Number of eggs}}$: $\chi^2_1 = 14.63$, P < 0.001). The mean number of eggs at nests where one or more eggs hatched was 3.04 ± 0.08 SE compared with 2.21 ± 0.14 SE at nests where no eggs hatched (ANOVA $F_{1,136}$= 32.44, P<0.001). Hatching success was higher in nests with larger internal diameters (Mean $_{\text{chicks hatched}}$ = 38.5 cm ± 0.9 SE, Mean $_{\text{chicks didn't hatch}}$ = 34.4 cm ± 0.94 SE; GLMM $_{\text{Diameter inside}}$: $\chi^2_1 = 4.24$, P = 0.048). Hatching success was also higher in nests with a larger canopy minor axis (Mean $_{\text{chicks hatched}}$ = 22.50 m ± 0.58 SE, Mean $_{\text{chicks didn't hatch}}$ = 19.80 m ± 1.1 SE; GLMM $_{\text{Canopy minor axis}}$: $\chi^2_1 = 6.5$, P = 0.017). Hatching success was not related to any of the other physical characteristics of the nests or nest trees (GLMM: $\chi^2_1 < 2.63$, P > 0.150).

However, nests where eggs hatched had adults which were more likely to be present at the nest during nest inspections (Mean $_{\text{eggs hatched}}$ = 84% birds present ± 1.3 SE; Mean $_{\text{eggs did not hatch}}$ = 66% ± 2.9 SE; GLMM $_{\text{\% of birds present}}$: $\chi^2_1 = 8.07$, P = 0.005). Hatching success was not related to any of the measures of nest monitoring intensity (GLMM: $\chi^2_1 < 2.65$, P > 0.106).

3.4.4 Determinants of Reproductive Success: (3) Fledging success

For nests that successfully fledged young, the mean number of fledged chicks was 1.43 ± 0.06 SE, with no significant difference between natural and artificial nests (LMM $_{\text{Nest type}}$: $\chi^2_2 = 0.51$, P = 0.783; Table 3). The number of fledglings was significantly positively related to the number of eggs laid (LMM: $\chi^2_1 = 14.9$, P < 0.001; Figure 3). Fledging success did not vary significantly with any of the nest cavity characteristics measured (GLMM: $\chi^2_1 < 3.24$, P > 0.078).

Nests where chicks fledged had adults which were more likely to be present at the nest during nest inspections (Mean $_{\text{fledged}}$ = 85% ± 1.5 SE presence, N = 65; Mean $_{\text{no fledge}}$ = 70% ± 2.4 SE presence, N = 81; GLMM $_{\text{\% of birds present}}$: $\chi^2_1 = 10.22$, P = 0.002). The probability of fledging did not vary with the number of times nests were checked during the breeding season (GLMM: $\chi^2_1 < 1.91$, P > 0.172).

3.5 Discussion

To date most reproductive information on wild *A. macao* has been based on only a few long-term studies in the field (Nycander et al., 1995; Vaughan et al., 2003; Vaughan et al., 2009). Our long-term data provides more insights into their reproductive biology and also allows important comparisons between natural and artificial nests, and an evaluation of the latter as a potential tool for enhancing macaw conservation.

3.5.1 Nesting preferences

A. macao are secondary cavity nesters (Renton & Brightsmith, 2009) and use high and deep hollows with relatively large entrances in emergent canopy trees (*Dipteryx, Hymenaea*), isolated trees in broken canopy successional forest (*Erythrina*), and occasionally live or dead palms (*Iriartea*) (Brightsmith, 2005b). During the 12 years of this study we monitored a total of 62 nests and not all were used every year. A major determinant of nest use was whether the nest had been

successful in the previous year. A similar observation was made by Berkunsky & Reboreda (2009) for Blue-fronted Amazon (*Amazona aestiva* Linnaeus 1758) and by White et al. (2005) for Puerto Rican Amazon (*Amazona vittata* Boddaert 1783). We found that macaws preferred to use nests with larger internal diameters, but this was the only physical characteristic that we found that affected their preference.

Our analysis occurred over a fairly small area (< 9 km^2) and this may have hampered our attempts to detect significant effects of the spatial distribution of nests. In addition, the study site was quite close to the Colorado clay lick, which serves as an important sodium source for the birds at this site (Brightsmith, 2004; Brightsmith et al., 2008b; Brightsmith et al., 2010) and may have encouraged nest site clumping. However, the distance to the second nearest nest was significant with occupied nests having a closer second nearest neighbour. This variable may act as a proxy for local nest density, which may suggest that good nests are clustered in good habitat (e.g. near fruiting trees) or that the macaws prefer to breed close to each other at high density. Our anecdotal observations suggest that the latter may hold true and that there may be anti-predation benefits of nesting in close proximity. For example, neighbouring macaws were observed to respond in unison when predators (e.g. eagles, monkeys, tayras) or humans approached nests. Other parrot species are also known to nest in close proximity to each other when nest locations are available, thereby improving predator detection (Eberhard, 2002).

3.5.2 Reproductive success

A. macao, like other birds, often lay more eggs than they raise, which may act as a form of insurance in case of hatching failure or other loss during incubation (Stinson, 1979). The mean clutch size in this study was 2.70 (± 0.08 SE), similar to previous data for this species (Forshaw, 1989; Nycander et al., 1995) and similar to other parrots of similar body size. The average clutch size for *A. macao*, *A. chloropterus*, and *A. ararauna* (body mass 1015-1250g) is 2.5-2.8 (Nycander et al., 1995), whereas in large cockatoos of the genera *Calyptorhychus, Cacatua, Lophochroa,* and *Probosciger* (275-841 g) mean clutch size ranges from 1.0, e.g. *P. aterrimus* (Murphy et al., 2003), to 3.3, e.g. Pink Cockatoo *Lophochroa leadbeateri* Vigors 1831 (Rowley & Chapman, 1991).

Eggs hatched in 61% of occupied nests (Table 3), and 50% of eggs hatched successfully (hatching success). This compares to a 56% hatching success for Monk Parakeet *Myiopsitta monachus* Boddaert 1783 (Navarro et al., 1992), 64% for Red-tailed Black-Cockatoo *Calyptorhynchus banksii* Latham 1790 (Saunders, 1984), 72 % for Red-lored Amazon *Amazona autumnalis* (Enkerlin-Hoeflich, 1995), 81% for Thick-billed Parrot *Rhynchopsitta pachyrhyncha*

Swainson 1827 (Enkerlin-Hoeflich et al., 1999), and 90% for Horned Parakeet *Eunymphicus cornutus* Gmelin 1788 (Robinet & Salas, 1999). Our 61% hatching rate (% of occupied nests that produced hatchlings) is very similar to the 60% rate previously described by Nycander et al. (1995) for *A. macao* in the same study site in Peru. Nearly 70% of breeding failures occurred during incubation even though this represents only one third of the total nesting period (Vigo et al., 2011).

Hatching success was higher in nests with larger inside diameter suggesting that females do better while incubating eggs if they have more space. In support of this result, PVC nests with smaller inside diameters also had a higher rate of apparently infertile or cracked eggs. Similar results were also reported from Costa Rica, where *A. macao* preferred to nest in tubes with larger diameters (Vaughan et al., 2003).

Among the occupied nests, 44% successfully fledged at least one young (Table 3), and 49% of the hatchlings fledged (fledging success). This is an intermediate result compared to other parrots, e.g. 27% of active *E. roratus* nests were successful (Heinsohn & Legge, 2003), 22% for *P. aterrimus* (Murphy et al., 2003), 40% for *A. macao cyanoptera* Wiedenfeld 1995 in Guatemala (Boyd & McNab., 2008). Nycander et al. (1995) described a higher rate of successful fledging for *A. macao* at the same Tambopata site but with a much smaller sample size: 9 of 14 (64%) occupied nests fledged one or more young. Fledging success (percentage of hatchlings that fledged) was 91% for Burrowing Parrots *Cyanoliseus patagonus* Vieillot 1818 (Masello & Quillfeldt, 2002), 88% for Hyacinth Macaw *Anodorhynchus hyacinthinus* Latham 1790 (Guedes, 1995), 63% for *E. cornutus* (Robinet & Salas, 1999), and 50% for Pacific Parekeet *Aratinga strenua* Ridgway 1915 (Wermundsen, 1998).

Adults are usually absent more from their nest during the nestling stage because parents need to obtain more food for their rapidly growing chicks and because the chicks can thermoregulate on their own (Iñigo-Elias, 1996; Vaughan et al., 2009). Our results show that nesting success increased with the proportion of time the adults were present during nest inspections. It is unclear how this influences nesting success, but a variety of interpretations are possible. Parents that spend more time near the nest during nest inspections may be less afraid of the investigators, and by spending less time off their nests, the nest visits may not affect them. Alternatively, parents that are more efficient foragers may benefit by both bringing more food to the nest and by having more time to spend in nest attendance (Persson & Göransson, 1999; Rensel et al., 2010). This suggests that social factors, parental quality, and food availability may be important features determining reproductive success for the species. Such factors may be even more important

where predation risk from diurnal predators (like *Micrastur* sp. forest-falcons) threaten macaw chicks (R. Garcia, pers. comm.).

Our research and those of others shows that brood reduction takes place in *A. macao* as in many other parrot species (Krebs, 1999; Masello & Quillfeldt, 2002; Nycander et al., 1995; Brightsmith & Vigo unpublished data). Regardless of the number of chicks which hatch, *A. macao* pairs using natural hollows never fledged more than two chicks during our study. The fact that natural pairs did not fledge more than two young begs the question of why the birds continue to lay up to four eggs. Our results show that the average number of chicks fledged is greater with clutches of three and four eggs than with only two eggs. This presumably provides the selective pressure to maintain the average clutch size above 2.0.

The limitation of tree cavities on the reproductive rate of this and other large parrot species (Beissinger & Bucher, 1992; Newton, 1994; Nycander et al., 1995; Heinsohn et al., 2003; Legge et al., 2004; Wiley et al., 2004) can result in intense conflict over nest sites. We confirmed nest loss by nest fights in only six cases. However, we did not systematically monitor nests for conflict during this study, and recent observations suggest we may have underestimated the impact on nesting success from this source. Preliminary analysis of nest observation data suggests that increased frequency of intruding pairs of *A. macao* at nests correlates with reduced hatching success (Brightsmith & Vigo unpublished data).

Nest predation for *A. macao* in this study was relatively low with only six (4%) of the occupied nests suspected of being predated (7.5% of all failed nests). This was in spite of a diverse community of birds, reptiles, and mammals capable of taking both adults and young, and may reflect the ability of macaws to defend their nests due to their large body size (1015 g; Dunning, 2008) and strong beaks. Other smaller parrots have higher predation rates, e.g. 45% in *E. roratus* (Heinsohn & Legge, 2003) and 23% in Rose-ringed Parakeets *Psittacula krameri* Scopoli 1769 (Shwartz et al., 2009). Known nest predators of *A. macao* are Black Spider Monkey (*Ateles paniscus* Linnaeus 1758), Bolivian Squirrel Monkey (*Saimiri sciureus* Linnaeus 1758), White-throated Toucan (*Ramphastos tucanus cuvieri* Linnaeus 1758), Chestnut-eared Aracari (*Pteroglossus castanotis* Gould 1834), rodents including *Rattus* spp., and insects like cockroaches (Nycander et al., 1995). Other potential predators are Brown Capuchin Monkey (*Cebus paella* Linnaeus 1758), Tayra (*Eira barbara* Linnaeus 1758), Common Opossum (*Didelphis marsupialis* Linnaeus 1758), Crested Eagle (*Morphnus guianensis* Daudin 1800), forest-falcons (*Micrastur* spp.), and snakes including *Oxybelis fulgidus* Daudin 1803, *Leptodeira annulata* Linnaeus 1758, and *Tripanurgos compressus* Daudin 1803.

In 37% of failed nesting attempts eggs were found broken or disappeared from nests before the anticipated hatch date, and these were probably the consequences of fights for nest possession or predation. At 32% of failed nests, the eggs remained intact but failed to hatch by the hatch date. In these cases, the eggs were probably infertile, cracked during incubation, or were not incubated continuously (possibly due to nest takeover attempts in some cases). Most of these clutches occurred in PVC nests where higher internal temperatures (D.J. Brightsmith, unpublished data) and smaller internal diameters could have been important contributing factors. In 15% of failed nests we found dead chicks probably resulting from parasites, starvation, or unknown diseases. Further and more detailed investigations are needed to better evaluate the causes of nest failures.

The asynchronous nesting of *A. macao* makes it difficult to estimate the developmental stages of nestlings (Myers & Vaughan, 2004). We therefore climbed nest trees at a high frequency to determine the exact date of hatching, and to examine nestlings to determine the timing and causes of death. We did not find any significant effect of climbing rate on reproductive success at any stage of breeding. To date few studies have explicitly tested the hypothesis that researcher activities impact on reproductive success of the study species (Grier, 1969; MaCivor et al., 1990; Major, 1990), although such knowledge is clearly important especially where researchers are studying the nests of species of high conservation concern.

3.5.3 Nest boxes vs. natural cavities

There were no significant differences in success rates between artificial nest boxes and natural nest hollows at any stage of reproduction. In addition, we did not observe differences between wooden boxes and PVC tube nests. Other studies of birds have found that natural nests had higher success than artificial nests [e.g. Barrow's Goldeneyes *Bucephala islandica* Gmelin 1789 (Evans et al., 2002), Eastern Yellow Robins *Eopsaltria australis* Shaw 1790 (Zanette, 2002), or Bufflehead *Bucephala albeola* Linnaeus 1758 (Evans et al., 2002)]. Our results have important implications for conservation of macaws (Nycander et al., 1995; Vaughan et al., 2003; White et al., 2006), and follow other studies demonstrating the high value of artificial nests (Vaughan et al., 2003; Lambrechts et al., 2012; Libois et al., 2012). Our current designs with small modifications as outlined below could be useful for *A. macao* in other geographic locations where the status of the local population is of concern (e.g. in Costa Rica Vaughan et al., 2003), or with modifications for other macaw species. However, the occupancy of these artificial nests may be highly variable among different study sites even for the same species. Our project has also hung similar artificial

nests in a tourist lodge and a local community within 60 km of TRC and none of these nests were occupied over the two nesting seasons they were monitored, probably due to a lower ratio of macaws to natural nest sites (D.J. Brightsmith, unpublished data). Further studies are needed to determine the variation of acceptance of these artificial nests.

PVC nests may be preferred by researchers as they are more durable and require less maintenance than wooden nest boxes that quickly rot in humid tropical environments. In addition, the destruction of wooden nests is hastened by the incubating female macaws which chew on the inside of the box. PVC nests are not only durable for macaws but also immune to attacks by woodpeckers, termites, bees and fungi, and are relatively light, easy to make, transport, and erect (Nycander et al., 1995). Considering the preferences of *A. macao* at our site, we suggest that the diameter of artificial nests should be larger than 40 cm in future applications. However, the artificial nests in this study had smaller entrance sizes than natural nest cavities (Table 2), and this design feature should be maintained as it may help to deter predators as found in other studies (White et al., 2005; Zanette, 2002).

Artificial nests can also enhance scientific research. The low side doors on the artificial nests provide easy access to eggs and chicks for the researchers facilitating morphological studies (Vigo et al., 2011). This is in contrast to natural nests where it can be difficult to reach to the bottom and extract the chicks. Furthermore, artificial nests also facilitate the installation of electronic monitoring devices like microphones, sensors, and cameras (Grenier & Beissinger, 1999; White & Vilella, 2004).

In both a previous study (Nycander et al., 1995) and this one, many *A. macao* appeared to become accustomed to researchers climbing to check the nests. The presence of artificial nests where macaws and other species are predictable and habituated to human presence can increase the value of each bird to ecotourism (Munn, 1992; Nycander et al., 1995; Brightsmith & Bravo, 2006). So in areas where macaws are protected, the use of artificial nests and other methods to attract nesting macaws can be important for ecotourism operations (Vaughan et al., 2005).

3.5.4 Conclusion

Even with 12 years of data we were unable to isolate the attributes of *A. macao* nest trees and holes that are best for reproductive success. There are probably many factors that the birds consider each time they choose a nest cavity. Renton & Brightsmith (2009) suggest that *A. macao* might be less able to successfully compete for high-quality nests with the sympatric *A. chloropterus* leading to greater flexibility in their choice of nest sites. Our sample of known nests

is useful for describing their broad choice of nest site but clearly does not provide enough variation to isolate the factors that most affect breeding success. However, our data showed a high rate of reoccupation of successful nests in the consecutive years and anecdotal observations suggest that many of these consecutive reoccupations were by the same pairs of birds (Brightsmith, 2009; Boyd & Brightsmith, 2010). Others found that *Amazona* parrots re-used successful nests in consecutive breeding seasons (Enkerlin-Hoeflich, 1995; Berkunsky & Reboreda, 2009).

We showed that artificial and natural nests had similar reproductive parameters, suggesting that artificial nests can also contribute important data to natural history studies of species where access to natural nests is limited. This finding also supports the use of artificial nests for conservation management especially in regions where the large emergent canopy trees with the best nest hollows have been removed (e.g. due to logging) but habitat has otherwise been maintained (Munn, 1992).

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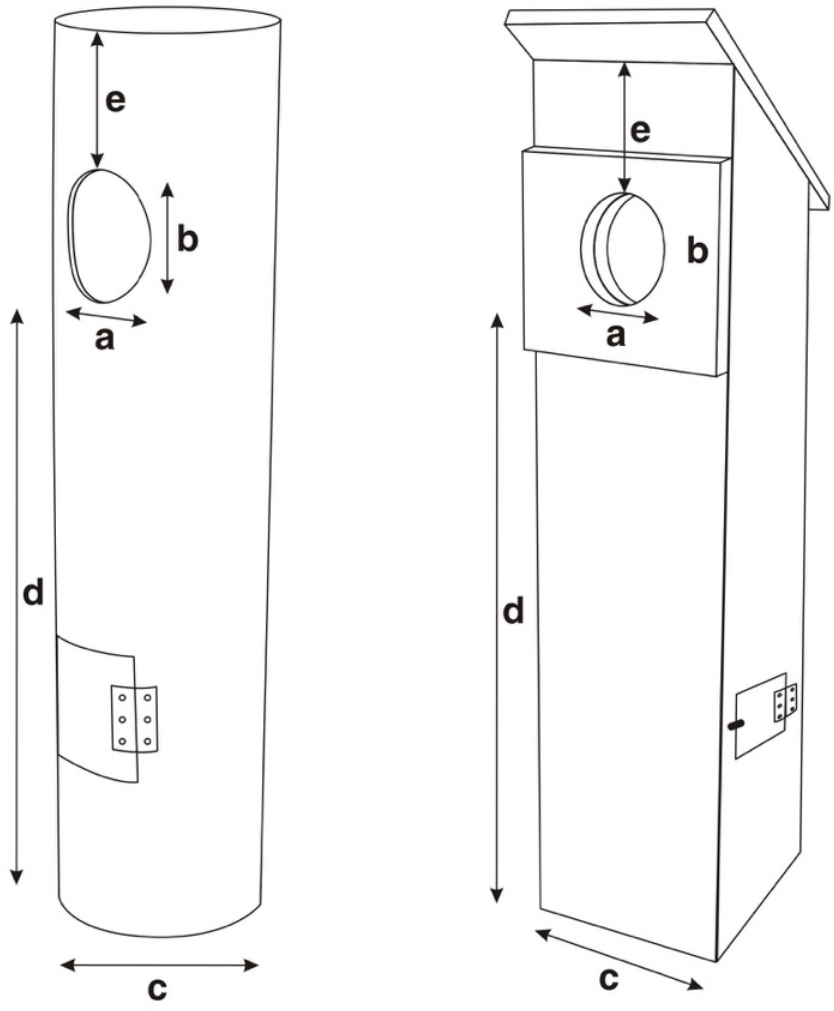
3.8 Figures and tables

Figure 1. PVC (A) and wooden (B) nest designs used in the study for *A. macao*. Main measurements (and their average values) analyzed were: a) horizontal (17.18 cm) and b) vertical diameter (18.40 cm) of the hole; c) inside diameter (36.15 cm); d) maximum depth (100.85 cm); and e) inside height (23.44 cm).



A **B**

Figure 2. Distribution of available and occupied (natural and artificial) *A. macao* nests by habitat types in the lowlands of southeastern Peru. Differences were not significant.

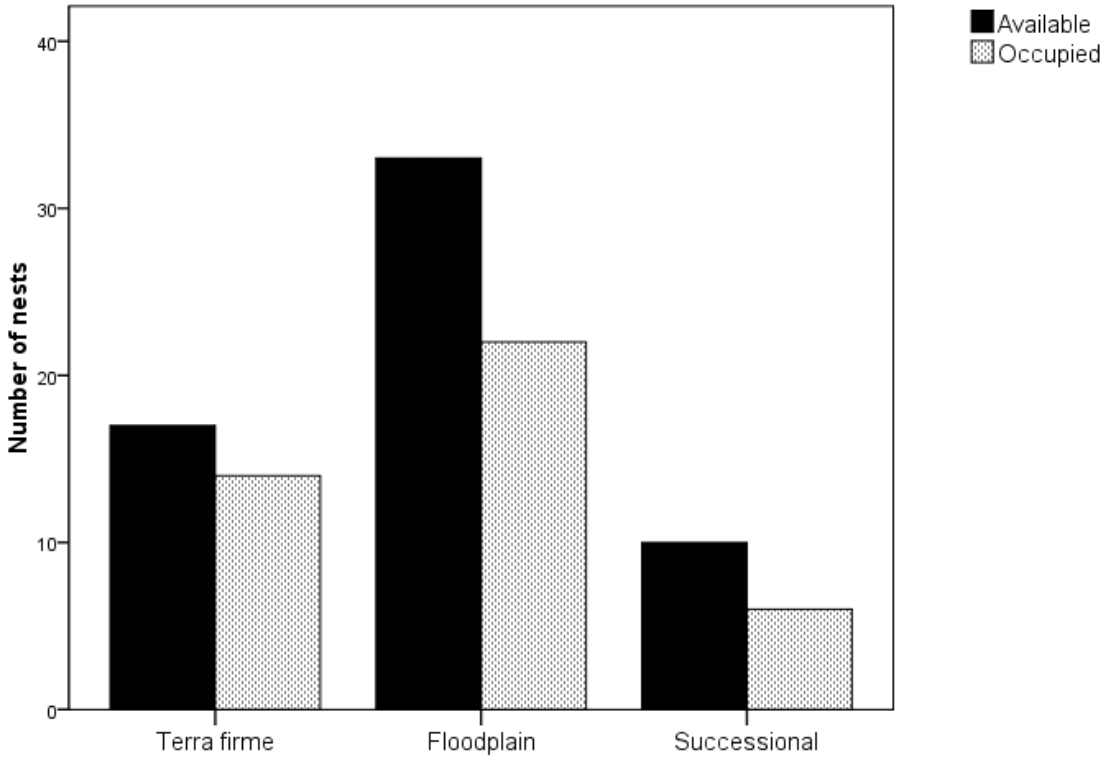


Figure 3. Predicted number (± SE) of fledged chicks versus the number of eggs at laying per *A. macao* nests in southeastern Peru. Data from 147 nesting attempts between 1999 and 2012. We used predictions of linear mixed modes (LMM) to build this graph.

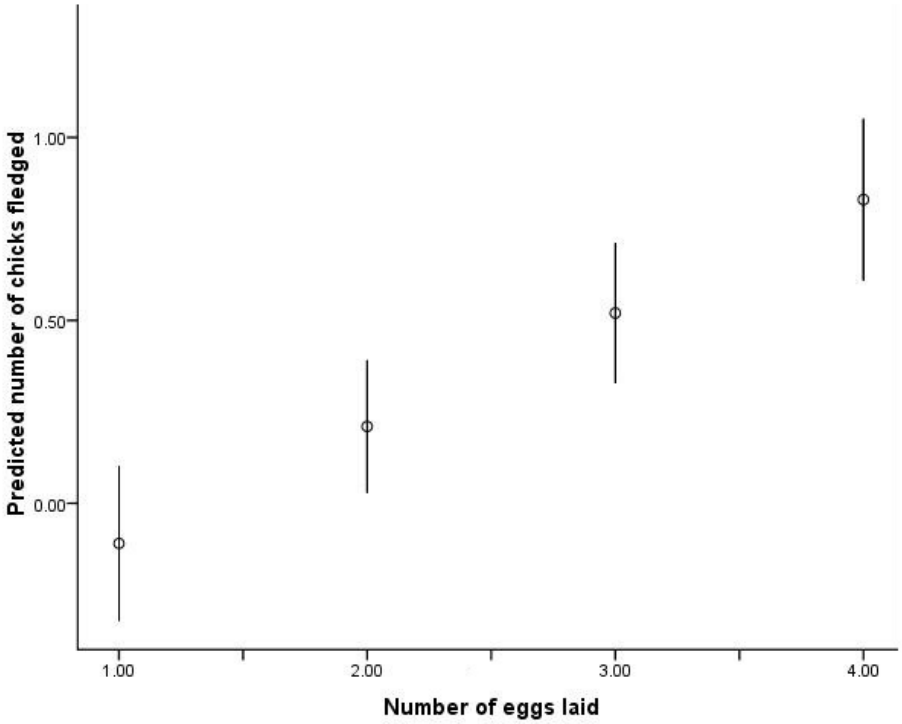


Table 1. Variables used in the regression analyses for testing the nest site selection of *A. macao* in the lowlands of southeastern Peru.

Category	Variable	Description
Breeding effort	# eggs	Number of eggs in clutch
	# hatched	Number of chicks hatched
	# fledged	Number of chicks fledged
	Outcome last year	The outcome of the nest the year before: (0) no activity or no eggs, (1) eggs hatched, (2) young fledged, (3) predation, (4) fight or takeover, (5) disappeared or cracked eggs after hatch date, (6) nest flooded, (7) nest (tree) fell, (8) unknown), (9) chick found dead in nest without any sign of predation, (10) eggs broken or disappeared before hatch date
	Occupancy rate	% of all years nest was occupied (calculated only for nests monitored ≥ 5 years)
Nest monitoring	# Climbs per season	Number of times nest was climbed in the season
	before eggs	Number of climbs before eggs were laid
	1-10 days	Number of climbs during first 10 days of incubation
	11-20 days	Number of climbs during second 10 days of incubation
	21-26 days	Number of climbs during last 6 days before expected hatching
	1st week Chick Age	Number of climbs during first 7 days after first chick hatched
	1-2 weeks Chick Age	Number of climbs from day 8-14 after first chick hatched
	2-4 weeks Chick Age	Number of climbs from day 15-28 after first chick hatched
	4-8 weeks Chick Age	Number of climbs during the 2nd four weeks after first chick hatched
	8-12 weeks Chick Age	Number of climbs during the 3rd four weeks after first chick hatched
Presence of adult macaws	% birds present	Percentage of climbs macaws were present
	Min dist	The average minimal distance between the climber and the macaws
	% birds called	Percentage of climbs macaws made an alarm call at the nest
	% birds left	Percentage of climbs macaws left the nest area

Nest cavity characteristics	Nest type	(1) artificial wooden, (2) artificial PVC, (3) natural hole in tree, (4) natural palm hollow
	Nest position	Nest faces: (1) Horizontal, (2) Vertical
	# natural in tree	Number of additional natural nests in the same tree
	# artificial in tree	Number of additional artificial nests in the same tree
	Hole vert	Vertical diameter of the hole entrance (if there is more than one hole the largest one) in cm
	Hole horiz	Horizontal diameter of the hole (if there is more than one hole the largest one) in cm
	Diameter inside	Internal diameter of cavity 30 cm from entrance in cm
	Max depth	Bottom of hole to base of the nest in cm
	Inside height	Top of hole to the roof in cm
	Hole height *	Height of hole entrance from ground in cm
	Direction hole faces	The compass bearing of the hole entrance
	Vertical angle the hole faces	Positive angle faces above horizontal (+90 straight up; -90 straight down)
Nest site characteristics	Dist to 1st	Distance (m) to the nearest nest occupied by Scarlet Macaws
	Dist to 2nd	Distance (m) to the second nearest nest occupied by Scarlet Macaw
	Macaw 1st	Distance (m) to the nearest nest occupied by either Scarlet or Red-and-green Macaw
	Macaw 2nd	Distance (m) to the second nearest nest occupied by either Scarlet or Red-and-green Macaw
	Collpa (clay lick)	Distance (m) of nest from Collpa Colorado clay lick
	Habitat	(1) *terra firme* forest, (2) floodplain forest, (3) palm swamp forest, (4) successional forest
	Tree height	Total height of the tree where the nest is situated
	Tree circumference	Circumference (at 150 cm above ground or above the buttresses) of the nest tree
	Canopy major axis	Maximum width of the canopy of the nest tree
	Lowest leaf	Height of the lowest leaf (from the ground level) of the nest tree
	Canopy minor axis	The width of the canopy measured perpendicular the maximum canopy width of the nest tree
	Tree species	Species of the nest tree

* The nest height is defined as the hole height.

Table 2. Characteristics of occupied natural and artificial *A. macao* nests in the lowlands of southeastern Peru.

Variables	Nest type	N	Minimum	Maximum	Mean (± SE)
Extra natural hollows per tree	Natural	12	0	2	1.08 ± 0.19
	Artificial	17	0	1	0.12 ± 0.08
Extra artificial nests per tree	Natural	12	0	0	0.00 ± 0.00
	Artificial	17	0	0	0.00 ± 0.00
Entrance hole vertical measurement (cm)	Natural	10	14	77	35.30 ± 6.18
	Artificial	17	14	30	18.40 ± 0.92
Entrance hole horizontal measurement (cm)	Natural	10	6	34	19.45 ± 2.60
	Artificial	17	13	37	17.18 ± 1.29
Inside diameter (cm)	Natural	9	17	66	38.56 ± 5.07
	Artificial	17	32	42	36.15 ± 0.79
Maximum depth (cm)	Natural	10	45	213	109.20 ± 20.10
	Artificial	17	14	176	100.85 ± 9.74
Inside height (cm)	Natural	10	0	155	69.60 ± 17.32
	Artificial	17	0	38	23.44 ± 2.71
Entrance hole height from the ground (m)	Natural	10	8.8	36.3	26.57 ± 2.75
	Artificial	17	19	36.1	29.34 ± 1.18
Angle the entrance hole faces	Natural	10	40	358	194.10 ± 32.09
	Artificial	17	20	340	183.18 ± 29.40
Vertical angle the entrance hole faces	Natural	11	-11	90	22.18 ± 11.50
	Artificial	17	0	0	0.00 ± 0.00
Distance to the clay lick (m)	Natural	12	450	2039	1180.50 ± 103.43
	Artificial	17	536	1927	1083.94 ± 105.40
Tree height (m)	Natural	12	28.6	58.7	44.94 ± 2.67
	Artificial	17	21.7	61.9	43.57 ± 2.75
Tree circumference (m)	Natural	12	2.8	5	3.72 ± 0.21
	Artificial	17	2	5.3	3.28 ± 0.20
Canopy major axis (m)	Natural	12	11.2	40.3	26.24 ± 2.73
	Artificial	17	15	44.9	25.28 ± 2.00
Lowest leaf height from the ground (m)	Natural	12	19.6	35.6	27.51 ± 1.56
	Artificial	17	21.2	31.2	27.13 ± 0.75
Canopy minor axis (cm)	Natural	12	2.3	32.4	18.75 ± 2.03
	Artificial	17	12	29.1	20.98 ± 1.58

Table 3. Nest occupancy and breeding success of *A. macao* in the lowlands of southeastern Peru.

	Artificial wooden nests	**Artificial PVC nests**	**Natural nests**	**Combined data for all nests**
Number of occupied nests (number of available nests) [a]	22 (37)	70 (123)	55 (NA)	147
Clutch size (N) [b]	2.73 ± 0.21 (22)	2.8 ± 0.11 (69)	2.55 ± 0.13 (47)	2.7 ± 0.08 (138)
% of occupied nests that hatch [c]	73	51	67	61
% of eggs that hatched [d]	60	42	58	50
Number of chicks which hatch per successful clutch (N) [e]	2.25 ± 0.23 (16)	2.28 ± 0.16 (36)	1.97 ± 0.12 (36)	2.15 ± 0.09 (88)
% of occupied nests that fledged [f]	50	43	44	44
% of hatchlings that fledged [g]	39	52	51	49
Number of chicks which fledged per successful nest (N) [h]	1.27 ± 0.14 (11)	1.43 ± 0.09 (30)	1.52 ± 0.12 (23)	1.44 ± 0.06 (64)

[a] Number of occupied nest years over the study period (number of total nest year where available).

[b] Average number (± SE) of eggs laid in occupied nests.

[c] Percentage of occupied nests in which hatched one or more eggs.

[d] Percentage of laid eggs that hatched (Hatching success).

[e] Average number (± SE) of hatchlings for each nest which hatched one or more eggs.

[f] Percentage of occupied nests that fledged one or more chicks.

[g] Percentage of hatchlings that fledged (Fledging success).

[h] Average number (± SE) of fledglings for each nest which fledged at least one chick.

Chapter 4

Philornis sp. bot fly larvae in free living scarlet macaw nestlings and a new technique for their extraction

Bot fly larvae removal from a scarlet macaw nestling with a venom extractor (2011).

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4.1 Abstract

Bot fly larvae (*Philornis* genus) are obligate subcutaneous blood-feeding parasites of Neotropical birds including psittacines. We analyse twelve years of data on scarlet macaw (*Ara macao*) nestlings in natural and artificial nests in the lowland forests of southeastern Peru and report prevalence and intensity of *Philornis* parasitism. Bot fly prevalence was 28.9% while mean intensity was 5.0 larvae per infected chick. Prevalence in natural nests (11%, N=90 nestlings) was lower than in wooden nest-boxes (39%, N=57) and PVC boxes (39%, N=109). We describe a new technique of removing *Philornis* larvae using a reverse syringe design snake bite extractor. We compare this new technique to two other methods for removing bots from macaw chicks and find the new method the most suitable.

4.2 Introduction

The parasitic fly genus *Philornis* (MEINERT, 1890, Diptera, Muscidae) comprises 51 species (Carvalho et al. 1993; Skidmore 1985) and has a mainly Neotropical distribution (Carvalho and Couri 2002). Their larvae are obligate subcutaneous blood-feeding parasites of nestlings of a wide range of avian hosts (Allgayer et al. 2009; Arendt 2000; Couri 1999). Larval development is rapid taking 4-6 days in furuncles with their caudal spiracles extending through the dermal openings of their avian hosts (Uhazy and Arendt 1986). *Philornis* infestations can increase bird mortality, decrease reproductive success, and affect nest site selection (Loye and Carroll 1998). They may even increase extinction risk for some avian hosts (Fessl and Tebbich 2002; Snyder et al. 1987). *Philornis* infestations have been noted repeatedly on parrot nestlings including macaws (Berkunsky et al. 2005; Nycander et al. 1995; Renton 2002).

The Tambopata Macaw Project has been studying the breeding ecology and natural history of large macaws (*Ara spp.*) in natural and artificial nests in the southern Peruvian Amazon for over 20 years (Brightsmith et al. 2008; Brightsmith 2005; Nycander et al. 1995). During nest inspections researchers found that scarlet macaw (*Ara macao*) nestlings heavily infested by bot fly larvae showed reduced survival (Nycander et al. 1995). Motivated by this observation, researchers at the site have opportunistically removed parasitic larvae to improve chick growth and fledging.

This situation gave rise to the following questions which guide the present study: (i) what are the overall rates of infestation, (ii) do different nest types affect levels of infestation, and (iii) what is the most suitable method of parasite removal in this particular host-parasite system?

4.3 Materials and methods

The study was conducted in the forests surrounding the Tambopata Research Center (TRC) in southeastern Peru (13° 8.070' S, 69° 36.640' W), in the Department of Madre de Dios, in the Tambopata National Reserve. The centre is located in tropical moist forest near the boundary with subtropical wet forest (Tosi 1960) at 350 m elevation with an average annual rainfall of 3,236 mm (Brightsmith 2004). At this site scarlet macaws nest in natural hollows (Brightsmith 2005; Renton and Brightsmith 2009) and in artificial wooden and PVC nest-boxes installed on emergent and isolated trees (Nycander et al. 1995).

We studied scarlet macaw nests in natural hollows, artificial PVC nests and wooden nest-boxes from November 2000 to March 2011 (12 breeding seasons). Nests were located within a 2.2 km radius of TRC. To determine the growth and health status of nestlings, we climbed to the nests using single-rope ascending techniques (Perry 1978; Perry and Williams 1981). We removed the chicks and lowered them to the ground in plastic buckets (Nycander et al. 1995). Once on the ground, each chick was checked visually for signs of bot flies and the number of bot flies was recorded. Chicks were also weighed and measured as part of ongoing studies (Vigo et al. 2011). On average, each of the 256 chicks involved in the study was handled 29.8 ± 1.7 SE times during the ± 86 day period of nestling development. These visits lasted about 30 – 50 minutes. The anatomic location of bot fly infestations was recorded in 89 cases.

Three different methods of killing or removing the parasitic larvae were used over the course of the study. From 2000 – 2007 all bot fly larvae were treated with Negasunt® powder. The powder was placed liberally on the swollen area caused by the larvae. Normally only a single treatment was needed as the larvae were dead and swelling reduced by the next nest inspection 1-3 days later (D.J. Brightsmith, pers. obs.). In 2007 researchers attempted to remove the dead bot fly larvae using haemostats the day after treatment with Negasunt® powder. From 2007 – 2010 bot fly larvae were removed by holding an alcohol soaked swab against the skin over the larvae for about 30 seconds to prevent the larva from breathing and forcing it to the surface. The swab was then removed and the veterinarian removed the larva with a haemostat. Sometimes after removing the larva an anti-parasite aerosol (Curabichera Spray) was applied. This technique required speed and experience and was often unsuccessful in the case of small larvae located deep in the skin.

Starting in 2010 we began to remove bot fly larvae using the Sawyer Extractor™ Pump Kit (a reverse syringe design device designed to extract snake venom). Larvae were removed by (1) cleaning the area around the bot with an alcohol soaked swab, (2) placing the head of the extractor over the larva, and (3) depressing the plunger of the extractor to start the suction. Usually within a few seconds small larvae were sucked completely out of the bird. Larger larvae only partially emerged from the wound but were easily grasped and removed with a haemostat. After bot fly removal the area was cleaned with an alcohol swab and covered with an antiseptic cream.

To quantify levels of infestations, we calculated prevalence as the percent of all chicks which had ≥ 1 larvae with 95% exact confidence intervals (CI). We also calculated the mean and median number of larvae per chick with ≥ 1 larvae (heretofore intensities). As parasites typically show an aggregated distribution across host individuals (Crofton 1971), we presented bias-corrected and

accelerated bootstrap confidence limits (CI) around the mean and median intensities. We used Fisher's exact test and Mood's median test to compare prevalences and median intensities and present 2-sided exact p-values in each case. Index of discrepancy (Poulin 1993) was used to quantify skewness of parasite distribution. For statistical analysis Quantitative Parasitology 3.0 was used (Rozsa et al. 2000).

The analyses discussed above included multiple chicks hatched and raised in the same nest. This means that our results may be influenced by pseudo replication (treating each chick as statistically independent instead of the more conservative method of treating each different nest as statistically independent). To eliminate the effects of this pseudo replication, we pooled all chicks hatched in the same nest through all years so that we created one prevalence (±SE), mean, and median intensity of its chick pool per nest. These fully independent parameters were compared across nest types using Kruskal-Wallis Tests using GenStat 13.2. Pearson chi-square test was used to compare observed and expected bot fly infestations in nests with multiple chicks.

We tested the effects of bot fly infestation on nestling growth using growth data from 45 scarlet macaw chicks studied from 2000 - 2008 as presented in Vigo et al. (2011). For each chick we determined the number of bot flies recorded during the following time periods: 0–33 days (the period of fast weight gain), 34–63 days (the period of slow weight gain) and 64 days to fledging (the period of weight loss). We used linear mixed models (LMM) of GenStat 13.2 to determine whether numbers of bot fly larvae in each of the above mentioned phases influence the (a) asymptotic size and (b) maximum growth rate and (c) age of maximum growth rate for the three biometric variables weight, wing, culmen, and tarsus.

4.4 Results

We monitored 19 natural tree cavities, 10 wooden and 19 PVC pipe boxes occupied by scarlet macaws and an average of 16.6 (±1.2 SE, range: 10-25) nesting events (laid at least 1 egg) per breeding season. We examined a total of 256 nestlings, 21.3 (± 2 SE) nestlings per breeding season (range: 10-33 chicks). In total, 372 bot flies were registered during the 12 years of the study. Bot fly larvae prevalence was 28.9% (CI: 23.4-34.9%), mean intensity was 5.03 larvae per infected chick (CI: 3.54-7.81) and median intensity was 2 (CI: 1-2) botflies per infected chick. The index of discrepancy was 0.89 indicating a rather high level of skewness, close to the theoretical maximum of 1. Larvae were most frequently located on the wings (36% of 89 reports), in open internal

cavities such as ears (10%) or nares (7%), on the feet (9%), the face (7%) or the rump (7%). Other body parts affected less frequently were the head, chin, neck, legs, and upper chest (24%). Bot fly infestations occurred from the second day to the 86th day of nestlings' age with a peak time of infestation in the first month. Bot fly infestations were not randomly distributed among chicks. In the 44 cases where there were multiple chicks in bot fly infested nests multiple chicks were infested in 50% of the cases. The probability that in nests with multiple chicks more than one chick has bot fly infestation was significantly higher than expected (chi-square=12.5, 2 d.f., P=0.002).

Larval prevalence in natural nests (11%, CI: 6-19%, N=90 nestlings monitored) was significantly lower than in wooden nest-boxes (39%, CI: 27-52%, N=57) and PVC boxes (39%, CI: 30-48%, N=109, Fisher's exact test: $p < 0.001$; Fig. 1). Mean and median parasitism intensities did not differ significantly across different nest types (Bootstrap 2-sample t-test: $p_{(\text{natural vs. wooden})}=0.219$, $p_{(\text{natural vs. PVC})}=0.431$, $p_{(\text{PVC vs. wooden})}=0.147$; Mood's median test for the 3 nest types: $p=0.125$).

When data from each nest were pooled across years, the mean of prevalence in natural nests (13%, ±5.5 SE, N=17 nests monitored) was significantly lower than in wooden nest-boxes (46%, ±8.7 SE, N=8) and PVC boxes (27%, ±6.4 SE, N=12; Kruskal-Wallis statistic = 9.5, $p<0.009$). Mean and median intensities for nestlings did not differ significantly among nest types (Kruskal-Wallis statistics < 1.9, $p>0.39$ for all three comparisons).

Over the study period we killed or removed larvae from nestlings 188 times including repeated treatments of reinfected chicks. We attempted to remove larvae using Negasunt® Powder and haemostats (N=27 cases), alcohol and haemostat (N=49) and Sawyer Extractor™ (N=112). The bot fly larvae were successfully removed from nestlings in 33% with the Negasunt® method, 80% with the alcohol and haemostat method, and 100% with the Sawyer Extractor™ method. The efficiency of the Sawyer Extractor™ method was significantly higher than the two other methods (Fisher's exact test $p<0.001$).

Asymptotic tarsus length was negatively correlated with the number of bot flies during the fast growth phase (0–33 days) (LMM $_{\text{bot flies 0-33 days}}$: $\chi^2_1 = 7.81$, $P = 0.008$). Asymptotic body mass was negatively correlated with the number of bot flies during the fast growth phase (LMM $_{\text{bot flies 0-33 days}}$: $\chi^2_1 = 6.64$, $P = 0.014$) and during the 0–63 day phase as well (LMM $_{\text{bot flies 0-63 days}}$: $\chi^2_1 = 6.59$, $P = 0.015$). Higher bot fly number also predicted lower weight of nestlings in these phases (LMM predictions; Fig. 2).

A total of 10 bot infested chicks died during the study, but only 3 were confirmed to have died due to the infestations: one died at age of 33 days due to a bot fly related ear infection, one died at 40 days old of infection after 26 larvae were detected all over its body, wings, head and nostrils, and one died at age 26 days after a single bot severed tendons in the leg and the bird was unable

to stand. In some cases, we observed the natural disappearance of *Philornis* larvae before expected emergence day. We cannot exclude the possibility that adult birds may remove some larvae from their chicks.

4.5 Discussion

Artificial nests are important tools in conservation of different parrot species. By testing different types of artificial nests compared to natural ones can result better designs for the birds. In this study we compared parasite prevalence among different nests to see whether any of the nest types results in higher bot fly infestation. Parasite prevalence was significantly lower in natural nest hollows than in either artificial wooden or PVC nests. This could be the result of the material of the nest, as usually temperature in PVC nests can raise quickly and might result in higher parasite prevalence (D.J. Brightsmith, unpublished data). However, mean and median intensity did not differ significantly among nest types. The most extreme intensities in our study (63, 40 larvae per chick) were higher than those found for other Neotropical parrot chicks: 31 larvae for a hyacinth macaw (*Anodorhynchus hyacinthinus*) nestling (Guedes 1993), >15 larvae per scarlet macaw nestling (Nycander et al. 1995), and >25 larvae in two blue-fronted amazon (*Amazona aestiva*) nestlings (Seixas and Mourao 2003). But are much lower than some reports for passerines: pearly-eyed thrashers (*Margarops fuscatus*) had a maximum of 220 larvae/nestling with an overall mean intensity of 37 (Arendt 1985).

Bot fly larvae are subcutaneous blood-feeders whose presence may facilitate secondary bacterial infections. However, we found little evidence of this as most infested chicks survived to fledging. In general, higher bot fly numbers during early development correlated with smaller overall chick size (weight and tarsus). We suspect that the bot flies are causing reduced chick size, but we cannot rule out the possibility that smaller nestlings get more bot flies for some other reason related to parental care or other unmeasured variable. Our findings that direct mortality caused by bot flies was uncommon agree with those from the literature, where the clearest direct impacts on chick health are from cases where the larvae invade sensitive locations such as sensory organs, respiratory pathways, mouth, or limbs (Arendt 2000).

Removing larvae may be helpful to the chicks, but also comes with a risk of injury, infection, impairment, and even death to the host if done incorrectly. For this reason, it is important for field personnel to use methods which maximize the benefits while minimizing risk. The Negasunt®

Powder used contains 3% Coumaphos that kills the bot fly larvae in the bird, 2% Propoxur that repels other insects from the lesion and 5% Sulfanilamide anti-bacterial. We found no gross negative effects on the chicks. However, Coumaphos is classed as highly to very highly acutely toxic to birds if consumed (Abdelsalam 1999; Abou-Donia et al. 1982; US-EPA 1996) and may be consumed by either by the parents or chicks. Therefore, we feel that using Negasunt® Powder should be avoided in wild birds.

The 'alcohol and haemostat' method reduces the risk of toxicity to the chick but it had a lower rate of success as bots that did not come to the surface of the skin were difficult to remove. The individual level of skill and veterinary training of the person using the technique also appeared to influence success. In addition, when unsuccessful, follow up attempts to remove the larvae often required incisions to remove the living or dead larvae. As a result, we do not recommend this method for extracting bot fly larvae.

By comparison, the new extractor method described here was highly efficient (100% in this study) and relatively easy for researchers of varied levels of skill and training. The age of the youngest macaw nestling we have subjected to this method was 2 days and we performed the process without complications. However, there are two concerns. When the bot is in areas where the extractor cannot get a good seal (tip of the wing, toe, etc.) suction may not be sufficient to remove the bot. In addition, the design of the extractor we used does not allow researchers to regulate the amount of suction. As a result, one must be careful when applying this method to young chicks of small-bodied species so as not to tear the skin. For this reason, researchers interested in using this technique should test it first on older individuals and monitor for bruising and skin tears before trying it on younger individuals.

Bot flies of various genera are known to infect a wide array of wild and domesticated vertebrate hosts (Angulo-Valadez et al. 2010; Cogley and Cogley 2000; Milton 1996) and this new extractor method should be effective on a wide range of taxa. If an extractor with variable suction levels was available, it would allow removal and collection of skin-dwelling arthropods from an even broader array of vertebrate hosts. Regardless, as presented, this technique should have broad application for veterinarians and scientists who wish to remove parasitic fly larvae quickly and easily without making incisions.

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4.8 Figures

Figure 1. The prevalence of *Philornis* infestations of scarlet macaw chicks in natural cavities (11%, N=90 nestlings monitored), artificial wooden nest boxes (39%, N=57), and PVC nest boxes (39%, N=109) in southeastern Peru. Vertical lines represent 95% confidence intervals around means (black dots). Columns labelled with different letters differ significantly (Fisher's exact test; $p<0.001$).

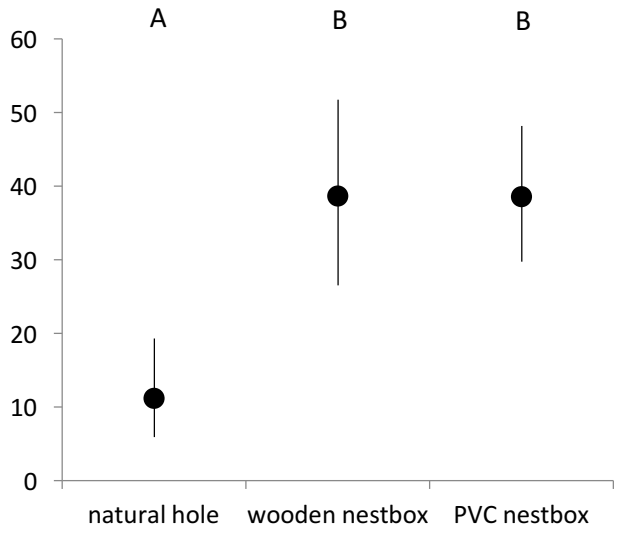
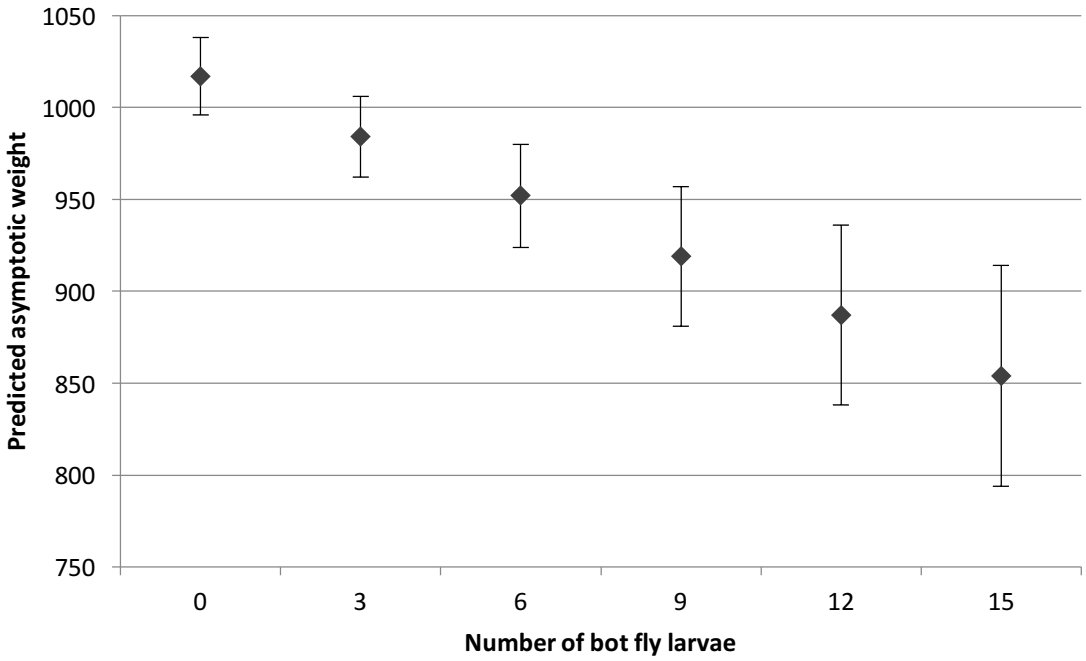


Figure 2. The predictions of linear mixed model (LMM) for the effects of bot fly number for asymptotic weight (±SE) of scarlet macaw nestlings during 0–63 days.



Chapter 5

An evaluation of primers for microsatellite markers in scarlet macaw and their performance in a Peruvian wild population

Taking samples from macaw nestlings in the Bahuaja-Sonene National Park (2010).

This chapter has been published as:

Olah G, Heinsohn RG, Espinoza JR, Brightsmith DJ and Peakall R (2015) An evaluation of primers for microsatellite markers in Scarlet Macaw (*Ara macao*) and their performance in a Peruvian wild population. *Conservation Genetics Resources* 7:157-159. doi:10.1007/s12686-014-0317-2

5.1 Abstract

Primer pairs were designed for 41 di-nucleotide microsatellite loci identified from across the full genome of the Scarlet Macaw (*Ara macao*). We present the best 30 polymorphic loci with 5–22 alleles, 3–14 effective alleles and expected heterozygosities of 0.669–0.930. These markers will facilitate population genetic and conservation genetic studies on macaws.

5.2 Primer note

Nearly 30 % of the 398 parrot species (*Psittaciformes*) are classified as threatened (critically endangered, endangered, or vulnerable) according to IUCN RedList 2014. The majority of the parrots are secondary cavity nesters hence mostly affected by habitat loss and other human disturbances. They are very popular birds in aviculture and also threatened by the illegal pet-trade.

Macaws are large and colourful Neotropical parrots that remain poorly understood in the wild. At least three species of macaws have already gone extinct in the wild with 16 species remaining (1 Critically Endangered, 3 Endangered, 3 Vulnerable, 1 Near Threatened, and 8 Least Concerned). In this study we used the Scarlet Macaw (*Ara macao*), which is considered of Least Concern, as a model for its close endangered relatives. We report here the development of 30 microsatellite markers for genetic tagging and population genetics studies. We tested the markers on a wild population from the southeastern Peruvian Amazon. Blood samples were collected from macaw nestlings and captured and released adults from natural and artificial nests (Olah et al. 2014), with the blood stored in 70% Ethanol and on FTA cards and extracted using general salting out protocol and DNAzol.

Potential target loci were identified from the first de novo genome assembly for the Scarlet Macaw (Seabury et al. 2013 - version SMACv1.1) using PHOBOS (v3.3.12) (http://www.rub.de/spezzoo/cm/cm_phobos.htm) to detect genome-wide microsatellite loci (STR). Initially, 41 autosomal di-nucleotide candidates with at least 20 repeat units were selected. We used the program iQDD (v3.1) (Meglécz et al. 2014) to design primers for these loci with PCR product lengths of 90–300 base pairs.

M13 PCR tags were attached to the forward primers (5'-3': TGT AAA ACG ACG GCC AGT) and we amplified all loci individually. PCR products were multiplexed using different fluorescent tags (Electronic Supplementary Material) and genotyped on an ABI 3130XL sequencer (Applied Biosystem) with the size standard GS500 (-250) LIZ. We used a negative and a positive control for each genotyping run. We dropped 3 loci that failed to amplify as a single locus.

To test the suitability of the remaining markers we genotyped a total of 86 samples that included 7 family groups. For population genetic analysis we analysed a subset of 40 unrelated individuals. We only included one sample per nest (excluding siblings) and if a sample was available from parents we excluded offspring of that pair. Consideration of the family groups confirmed Mendelian inheritance, but indicated null alleles at 8 loci. A MicroChecker (v2.2.3) (Van Oosterhout et al. 2004) analysis on the 40 sample set indicated departures from the Hardy–Weinberg Equilibrium (HWE) and null alleles at those same 8 loci. However, all of the other 30 loci fit expectation under HWE assumptions (Table 1). These 30 loci exhibited 5–22 alleles, 3–14 effective alleles and expected heterozygosities of 0.669–0.930 (Table 1). Checks for linkage disequilibrium in GenePop (v4.2) (http://genepop.curtin.edu.au) revealed that < 5% of all combinations showed potential departures from equilibrium.

We tested the cross species transferability of our new primers on a closely related species, the Red-and-green Macaw (*Ara chloropterus*), where all but one marker resulted to be polymorphic (Table 1). We also tested the primers on a distant relative, the Swift Parrot (*Lathamus discolor*, N=12), but only seven loci were found polymorphic (2–11 alleles, 2–6 effective alleles and expected heterozygosities of 0.278–0.840).

Our panel of 30 microsatellite markers will be highly suitable for conservation genetics studies of Scarlet Macaw, other macaws, and probably other Neotropical parrot species. Furthermore, ready expansion to 38 loci is possible by re-designing primers for the addition eight loci that exhibited null alleles.

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Table 1. Population statistics for microsatellite markers in Scarlet Macaw (*Ara macao*) and their cross transferability to Red-and-green Macaw (*Ara chloropterus*).

	Scarlet Macaw (*Ara macao*)								Red-and-green Macaw (*Ara chloropterus*)						
Locus name	N	Size range (bp)	No. alleles	No. effective alleles	H_O	H_E	P	Freq. null alleles (Oosterhout)	N	Size range (bp)	No. alleles	No. effective alleles	H_O	H_E	P
SCMA01	40	161-197	16	8.84	0.70	0.89	0.001 *	0.106 ¤	9	169-193	9	5.79	1.00	0.83	0.469
SCMA02	40	268-300	14	10.46	0.95	0.90	0.725	-0.025	9	282-292	6	4.63	1.00	0.78	0.622
SCMA04	39	251-277	14	6.84	0.82	0.85	0.079	0.024	9	267-281	6	4.38	0.78	0.77	0.688
SCMA05	40	205-235	12	7.64	0.88	0.87	0.767	-0.008	9	211-219	5	3.06	0.78	0.67	0.154
SCMA06	40	265-305	17	6.91	0.85	0.86	0.902	0.006	9	279-301	6	4.05	0.78	0.75	0.855
SCMA07	40	276-302	9	5.19	0.83	0.81	0.234	-0.010	9	298-306	5	3.77	0.56	0.73	0.652
SCMA08	40	287-307	12	8.56	0.63	0.88	0.007 *	0.148 ¤	9	287-301	6	3.77	0.89	0.73	0.578
SCMA09	40	112-136	11	5.95	0.85	0.83	0.793	-0.008	9	116-128	5	3.45	0.78	0.71	0.414
SCMA10	40	176-230	15	10.39	0.28	0.90	0.000 *	0.342 ¤	9	186-186	Monomorphic				
SCMA11	40	265-307	12	7.98	0.68	0.87	0.001 *	0.114 ¤	9	281-297	9	7.36	0.89	0.86	0.540
SCMA12	40	280-308	13	7.57	0.85	0.87	0.766	0.012	9	278-300	9	5.59	1.00	0.82	0.861
SCMA13	40	268-296	12	5.29	0.88	0.81	0.956	-0.040	9	258-286	7	2.89	0.89	0.65	1.000
SCMA14	40	222-250	11	7.73	0.93	0.87	0.441	-0.033	9	220-236	4	2.05	0.67	0.51	0.895
SCMA15	40	275-309	16	7.14	0.80	0.86	0.061	0.033	9	277-297	4	2.35	0.67	0.57	0.815
SCMA16	40	223-254	13	8.38	0.95	0.88	0.991	-0.040	9	234-246	7	5.40	0.78	0.81	0.076
SCMA17	40	261-289	8	3.54	0.75	0.72	0.624	-0.014	9	265-287	6	3.86	0.89	0.74	0.862
SCMA19	40	270-300	15	8.84	0.95	0.89	0.987	-0.036	9	276-302	9	5.59	0.78	0.82	0.588
SCMA20	40	107-143	11	7.08	0.35	0.86	0.000 *	0.291 ¤	9	129-143	6	3.52	0.44	0.72	0.037
SCMA21	40	176-200	11	4.98	0.78	0.80	0.992	0.017	9	178-196	6	4.38	0.89	0.77	0.221
SCMA22	40	114-160	18	12.36	1.00	0.92	0.842	-0.045	9	122-146	7	5.06	0.89	0.80	0.870
SCMA25	40	265-293	8	3.02	0.63	0.67	0.660	0.017	9	270-272	2	1.98	0.00	0.49	0.003 *
SCMA26	40	210-240	13	6.97	0.88	0.86	0.446	-0.008	9	224-236	7	4.76	0.89	0.79	0.711
SCMA27	40	211-245	14	10.09	0.95	0.90	0.933	-0.028	9	211-241	11	8.10	1.00	0.88	0.886
SCMA28	40	280-330	22	14.35	0.95	0.93	0.871	-0.012	9	292-318	7	3.86	0.67	0.74	0.513
SCMA29	40	238-256	8	5.04	0.73	0.80	0.246	0.047	9	241-255	7	5.06	1.00	0.80	0.784
SCMA30	40	214-246	15	7.51	0.95	0.87	0.935	-0.049	9	212-242	8	4.38	0.89	0.77	0.932
SCMA31	40	141-159	9	7.39	0.90	0.86	0.911	-0.021	9	135-165	9	5.40	0.78	0.81	0.299
SCMA32	38	181-211	12	7.72	0.84	0.87	0.381	0.017	9	175-185	4	2.79	0.89	0.64	0.282
SCMA33	40	174-206	15	8.82	0.85	0.89	0.044	0.018	9	166-200	5	2.75	0.67	0.64	0.740
SCMA34	40	159-183	12	6.99	0.88	0.86	0.489	-0.013	9	159-177	6	4.63	0.67	0.78	0.470
SCMA35	40	286-308	10	7.60	0.88	0.87	0.762	-0.005	9	274-284	2	1.91	0.56	0.48	0.613
SCMA37	40	208-220	7	4.71	0.83	0.79	0.553	-0.022	9	216-224	5	3.95	0.56	0.75	0.238
SCMA38	40	215-249	13	7.75	0.45	0.87	0.000 *	0.237 ¤	9	227-229	2	1.12	0.11	0.10	0.860
SCMA40	40	292-300	5	3.38	0.68	0.70	0.623	0.026	9	288-300	5	4.63	1.00	0.78	0.175
SCMA41	40	292-318	13	6.90	0.85	0.86	0.994	0.006	9	284-308	8	5.40	0.56	0.81	0.295
SCMA43	38	89-127	13	7.10	0.32	0.86	0.000 *	0.309 ¤	8	97-125	4	2.51	0.25	0.60	0.019
SCMA44	40	280-308	13	7.57	0.85	0.87	0.766	0.012	9	278-300	9	5.59	1.00	0.82	0.861
SCMA46	39	152-188	14	8.01	0.67	0.88	0.002 *	0.118 ¤	9	160-180	8	5.79	0.89	0.83	0.433

Number of samples (N), Observed (H_O) and Expected (H_E) Heterozygosity, probability value from Hardy–Weinberg Equilibrium test (P) calculated by GenAlEx 6.5 (Peakall and Smouse 2012); All loci were scored using Geneious R6

* significant Hardy–Weinberg disequilibrium (P<0.01)

¤ presence of a null allele calculated by MicroChecker (v2.2.3) (Van Oosterhout et al. 2004)

Microsatellite primer sequences and locus information for Scarlet Macaw (*Ara macao*)

Locus name	SMACv1.1 scaffolded contig number	Forward primer sequence 5'–3'	Reverse primer sequence 5'–3'	Repeat motif	*Fluorescent tag
SCMA01	s_1_1NewScaffoldedcontig_720	*ATGGTAGAGGGAGGCACTGA	GCATGGTATAAGGCCCATCT	$(AC)_{22}$	NED
SCMA02	s_1_1NewScaffoldedcontig_3573	*TCAACCTCCAGGTGTCTTCC	TCCTTCAGTCACCAGCTTCA	$(GT)_{21}$	NED
SCMA04	s_1_1NewScaffoldedcontig_8801	*TAAGCCCTGCTCATCAAAGG	CGACAGGAGCTGATAAGGGT	$(AC)_{20}$	VIC
SCMA05	s_1_1NewScaffoldedcontig_11956	*CAGAAAGCCAGGAGTCCAAG	TTTCTGACTTTGCTGGTTGG	$(AC)_{20}$	NED
SCMA06	s_1_1NewScaffoldedcontig_12019	*AGTCTGAGCAGGTGCAGGAT	ACAGACTCTGCACCACATGC	$(CT)_3T(TG)_{21}$	VIC
SCMA07	s_1_1NewScaffoldedcontig_14086	*CTGATGATGGTGGAAAGCCT	ATGTTCCACTGCATGTCCTG	$(TG)_{21}$	NED
SCMA08	s_1_1NewScaffoldedcontig_18034	*CTTGCCAGATGCTGACACTC	TCATGACCTTTCTGCCTTCC	$(TG)_{22}$	FAM
SCMA09	s_1_1NewScaffoldedcontig_19117	*CACTACCAGCAAGTAGCAGGC	TGAATTCTAACAAGCAGCGG	$(CA)_{20}CG(TA)_3$	VIC
SCMA10	s_1_1NewScaffoldedcontig_19611	*TCCAGGAACTGAAATACCTCAT	TGGCTTATCATTTCTTAGCCAG	$(GT)_{21}$	NED
SCMA11	s_1_1NewScaffoldedcontig_19941	*TCCTTCGTCCCTCCTTCC	AGGCAAATGACAGAACTGGG	$(AC)_{21}$	NED
SCMA12	s_1_1NewScaffoldedcontig_20383	*GGTGGAGCACATTGCTGAAA	CAAAGATGCCCACCCAAA	$(AC)_{22}$	VIC
SCMA13	s_1_1NewScaffoldedcontig_21269	*GTTGGCCACTGCTTCAGAAC	GCTGCAAGAAATTCCAGTCC	$(TG)_{21}$	VIC
SCMA14	s_1_1NewScaffoldedcontig_25432	*CGCATACTTTACACCCACCA	TTGTGACAGGGCTAGGCAG	$(AC)_{20}$	FAM
SCMA15	s_1_1NewScaffoldedcontig_26647	*GACTGGCAGTTAAGGTGGTTG	AATGACTTTCCTCTTGCTCCC	$(GT)_{20}$	NED
SCMA16	s_1_1NewScaffoldedcontig_32560	*AAAGCTTCCACACATCATGG	TTGCTTTATCCAAACATTTGTGTC	$(AC)_{20}$	NED
SCMA17	s_1_1NewScaffoldedcontig_34586	*CACAGCTGCACATTTGATCC	GCCTCATGGGTAGAACAGTTT	$(TG)_{20}$	VIC
SCMA19	s_1_1NewScaffoldedcontig_35385	*AGCGCATCTGCCTAGATGTT	TAATCCACAGCACCACCAAG	$(GT)_{24}$	VIC
SCMA20	s_1_1NewScaffoldedcontig_36691	*ATGCTTCCAAATCAGAATGC	CCAGGGACATAGTAGCTGCAC	$(AC)_{20}$	VIC
SCMA21	s_1_1NewScaffoldedcontig_41388	*TGAATTTCCGTGCCTAAAGC	TCACCCAAACAAGCAACTTTC	$(GT)_{20}$	FAM
SCMA22	s_1_1NewScaffoldedcontig_44385	*AACTGTGATGAAGTTCGTGGC	CAACGGCTACACACAGTGCT	$(TG)_{22}$	VIC
SCMA25	s_1_1NewScaffoldedcontig_58183	*AAATGCTGCCCTGAGTTCAT	TCTTATAGCTTTGTGATAGTCATTGAA	$(GT)_{20}$	FAM
SCMA26	s_1_1NewScaffoldedcontig_61104	*AGCAAAGGTAAGGAGCAGCA	GGCACCTCTATCATCTATTGCAG	$(TG)_{21}$	VIC
SCMA27	s_1_1NewScaffoldedcontig_62371	*TTCTGCAGCAGTTCCCAAA	TGGACTCTGTATTCCAGTCGC	$(CA)_{22}$	FAM
SCMA28	s_1_1NewScaffoldedcontig_65504	*GAAGGCAAAGTTCTCATGCTG	CCATTATGATCAGATTTCCGC	$(TG)_{21}$	NED
SCMA29	s_1_1NewScaffoldedcontig_66792	*GGTGGGCAGATAGCCTGAT	GTTGAATGCAAAGTGCATGG	$(TG)_{20}TA(TG)_3$	FAM
SCMA30	s_1_1NewScaffoldedcontig_68479	*TTGCCAGGTCCTTCTCTACC	ACCACCTTCTCTTGACTTGTAATTG	$(CA)_{24}$	FAM
SCMA31	s_1_1NewScaffoldedcontig_70421	*TGTGCTCCCTACAGTTCCAA	AACGCTGAACTTGGTGTGGT	$(AC)_{21}$	FAM
SCMA32	s_1_1NewScaffoldedcontig_77138	*GGCATGGCTCTTTACTTGCT	TTGCCACTGAGGCTTCTACC	$(TG)_{21}$	VIC
SCMA33	s_1_1NewScaffoldedcontig_77453	*GAGGCACTATTTCTGGCAGC	GCTAAGCAGATTTGTCTAAACATTCA	$(AC)_{21}$	VIC
SCMA34	s_1_1NewScaffoldedcontig_78919	*TTTGGCAGTAGTCGGGATTT	AACTTGGGAATACATCGCTGA	$(AC)_{22}$	VIC
SCMA35	s_1_1NewScaffoldedcontig_81206	*CTCGATCTGGACAGCACACT	GGGTTGTCTGCTGGTACTAAAG	$(GT)_{20}$	VIC
SCMA37	s_1_1NewScaffoldedcontig_91851	*TCACATGCATGAGCTGGG	CCTGTAAGGTCAGGAAGGACA	$(GT)_{20}$	VIC
SCMA38	s_1_1NewScaffoldedcontig_92645	*TCACTGAATCTCATTGCCCA	CATCCTAATCAGGCAGGGAA	$(AC)_{20}$	FAM
SCMA40	s_1_1NewScaffoldedcontig_109156	*GCCTGCACCAAATTCATACC	TTTGGTGGAAACTGGACCTA	$(AC)_{24}$	FAM
SCMA41	s_1_1NewScaffoldedcontig_115612	*AATTTGGGTAGCAATGTGGA	CAGCAGATGTGGATTCTGGTT	$(TG)_{20}$	NED
SCMA43	s_1_1NewScaffoldedcontig_127204	*GTGATCACAGAAACACGGG	GGCTGGAGAGTGCCTTACCT	$(GT)_{20}$	VIC
SCMA44	s_1_1NewScaffoldedcontig_132550	*GGTGGAGCACATTGCTGAAA	CAAAGATGCCCACCCAAA	$(AC)_{22}$	NED
SCMA46	s_1_1NewScaffoldedcontig_181852	*TGTGGCATCTCATATTGTGC	CATAAACATGCGGAGCAGC	$(AC)_{21}$	NED

Chapter 6

Validation of non-invasive genetic tagging in large macaws of the Peruvian Amazon

Searching for feathers at the Chuncho clay lick in the Tambopata National Reserve (2010).

6.1 Abstract

Genetic tagging is the unique identification of individuals by their DNA profile. This technique is well established in mammals, but it has not yet been widely adopted for birds. Extraction methods for minute amounts of DNA even enable the use of genetic tagging from non-invasive samples, like hair, scat, or feather. In this study, we evaluate the potential for non-invasive genetic tagging by using moulted feathers of two sympatric macaw species in the Peruvian Amazon. Correct species identification is critical when relying on feathers for genetic analysis, so we describe multilocus methods for species identification. We evaluate the quality of naturally shed macaw feathers in tropical environmental conditions and present new primers for molecular sexing on the damaged feather samples. We successfully validated 11 microsatellite markers for use in genetic tagging studies on large macaws and confirmed that DNA from blood and feather samples yields equivalent population genetic patterns. The techniques described here can be implemented for other birds with a higher conservation concern.

6.2 Introduction

Genetic tagging, the technique of unique identification of individuals by their DNA profile, is now well-established (Andreou et al. 2012; Palsboll 1999). Genetic tagging became feasible with the development of methods allowing access to highly variable genetic markers such as codominant microsatellites, which are still the international standard for forensic analysis despite major advances in next generation sequencing methods (Bruford and Wayne 1993; Guichoux et al. 2011; Paetkau et al. 1995; Peakall et al. 2006; Phillips et al. 2014). Genetic tagging was first used with invasively collected samples like skin biopsies of whales, fin clips of fishes, or ear tissues of small mammals (Andreou et al. 2012; Palsboll et al. 1997; Peakall et al. 2006). For non-invasive genetic tagging studies of mammals, DNA has been obtained from hair and scat samples (Arrendal et al. 2007; Coster et al. 2011; Ruibal et al. 2009; Ruibal et al. 2010; Taberlet and Luikart 1999). Genetic tagging can also provide data of value beyond individual identification. For example, it is standard practice to include a sex typing marker, since information about the sexes of individuals is needed for population demography or studies of sex-biased dispersal (Beck et al. 2008; Blackmore et al. 2011; Wright et al. 2005).

Despite its wide use in mammals, genetic tagging has not yet been widely adopted for birds (Horváth et al. 2005; Segelbacher 2002; Taberlet and Luikart 1999). Furthermore, even studies assessing the reliability of moulted feathers as a DNA source are scarce (Gebhardt et al. 2009; Segelbacher 2002), and the conclusions sometimes contradictory. Some studies have recommended avoiding the use of plucked or cut feathers due to low DNA quality of such samples (McDonald and Griffith 2011; McDonald and Griffith 2012), while others advocate their use (Katzner et al. 2012). Due to their degraded DNA content, feathers in museum samples are likely to be particularly problematic (Sefc et al. 2003). Despite the limitation of low quality and quantity of DNA, naturally moulted feathers can still provide an important source for genetic tagging when no other samples are easily available (Gebhardt and Waits 2008b; Heinsohn et al. 2007). However, damaged feather samples can still present a challenge for reliable sex typing (Gebhardt and Waits 2008b), and for genetic tagging more generally in birds.

One third of the extant parrot species are classified as threatened on the IUCN Red List (IUCN 2014; Olah et al. 2016). Capturing parrots for genetic samples often requires a large effort due to such attributes as their high mobility, preference for the forest canopy and their often remote habitats (Heinsohn et al. 2007; Masello et al. 2002; Murphy et al. 2007; Olah et al. 2015). Despite these challenges there are some population genetic studies on parrots (Brock and White 1992;

Chan et al. 2008; Heinsohn et al. 2007; Masello et al. 2011; Masello et al. 2015; Melo and O'Ryan 2007; Wenner et al. 2012; Wright and Wilkinson 2001).

Here we focus on two sympatric macaw species (scarlet macaw, *Ara macao* and red-and-green macaw, *Ara chloropterus*) from the lowland Peruvian Amazon, where they frequently visit 'clay licks' to supplement their dietary sodium by eating clay (Brightsmith and Villalobos 2011; Lee et al. 2010; Powell et al. 2009). Both species are considered as globally of Least Concern (IUCN 2014), and the availability of their shed feathers at clay licks make them a suitable test species for developing, validating, and applying genetic tagging for the first time on a large sample of wild parrots. We build on previous studies that applied genetic tagging to other species (Palsboll 1999; Peakall et al. 2006), tested non-invasive molecular sexing in parrots (Gebhardt and Waits 2008b; Presti et al. 2013), demonstrated species identification in macaws (Abe et al. 2012), and showed reliability of feather genotyping compared to blood samples (Maurer et al. 2010; Segelbacher 2002).

The goal of our study was to assess the potential for non-invasive genetic tagging with 11 microsatellite loci by using moulted feathers of macaws sampled in the wild. We (1) evaluated the DNA quality for genetic analyses of naturally shed feathers left on the ground by macaws in tropical environmental conditions; (2) described multilocus methods for species identification using DNA from feathers; (3) presented new primers for molecular sexing on damaged feather samples; (4) validated eleven microsatellite markers for use in genetic tagging studies on large macaws; and (5) confirmed that DNA from blood and feather samples yields equivalent population genetic patterns.

6.3 Methods

6.3.1 Target species and study site

The study was conducted in the lowland rainforest of Peru, in the regions of Madre de Dios and Puno, in tropical moist and subtropical wet forest from 250 m to 800 m elevation and receives 3,200 mm of rain per year (Brightsmith 2004; Tosi 1960). Our systematic collection of samples focused on two coexisting macaw species, the scarlet macaw (hereafter SCMA) and red-and-green macaw (hereafter RGMA). Both species have similar ecology (Brightsmith 2005a) and nest in emergent canopy trees during the rainy season (November-April) in Peru (Brightsmith 2005b).

A total of 1,263 naturally shed feathers were collected in each rainy season between 2009 and 2012, across 10 main clay licks spread over 1,000 km of the Piedras, Heath, Tambopata, Candamo Rivers and their tributaries (Brightsmith and Aramburú Muñoz-Najar 2004; Brightsmith and Villalobos 2011). DNA was extracted from 886 (70% of the total), and 500 (40%) of these were used in the analyses of this study after the quality screening. Although the majority (84%) of these samples were collected from clay licks, some feathers were also collected in the forest, below nesting trees, and in nest hollows. Upon collection, samples were photographed with a measuring scale and stored individually in paper envelops in airtight boxes with silica gel to avoid further degradation.

To compare population genetic results between blood and feather samples in this study, we used 33 blood samples (28 SCMA and 5 RGMA) collected from captured adults and nestlings around the Tambopata Research Center (TRC; 13° 8.070'S, 69° 36.640'W) from both species as described in Olah et al. (2015).

6.3.2 DNA extraction and genotyping

DNA extraction was performed using the Qiagen DNeasy Blood and Tissue kit (QIAGEN, California) following the manufacturer's instructions with some modifications to improve yield. These included longer incubation times, higher temperatures, and double elution on the spin columns in the last step, following Gebhardt et al. (2009). For feathers >20mm in size DNA was extracted from the blood clot from the superior umbilicus (Horváth et al. 2005). The entire shaft was used as the DNA source for small feathers after cleaning the surface with 70% ethanol.

In a pilot study of 40 moulted feather samples, consisting of DNA of varying quality, we screened 30 previously described microsatellite markers specifically designed for SCMA and also known to amplify in RGMA (Olah et al. 2015). From this pilot set of 30 loci, the 11 loci that yielded the highest amplification success across the trial DNA samples of lower quality were selected for this study. These 11 loci mainly amplified smaller fragment sizes (overall means of 122 to 284 bp). The locus SCMA 32 was found to only amplify samples of higher quality DNA. Thus amplification success at this locus was highly correlated with amplification success at the other loci. Therefore, we used this locus to pre-select samples for the full analysis.

M13 PCR tags were attached to all forward primers (Schuelke 2000) and we amplified all loci individually. PCR products of 4 loci were multiplexed in the same lane using different fluorescent tags (Table S1) and genotyped on an ABI 3130XL sequencer (Applied Biosystem) with the size standard GS500 (-250) LIZ. We used a negative control for contamination check and a positive

control to ensure consistent size scoring across all genotyping runs. Results were scored with Geneious version R6 (http://www.geneious.com, Kearse et al. 2012) and full genotypes were constructed. Most of the samples were genotyped once, with genotyping errors estimated from randomly selected samples (7-55 per locus) that yielded full genotype data for all 11 loci during the first scoring. This represents about 10% of the PCR reactions. Samples with 5 or more missing loci were excluded from the final analysis.

The following 11 microsatellite markers were used to construct the genotype data: SCMA 02, SCMA 09, SCMA 14, SCMA 22, SCMA 26, SCMA 27, SCMA 30, SCMA 31, SCMA 32, SCMA 33, and SCMA 34 (Olah et al. 2015). Given all our loci were already pre-screened for the presence of null alleles in Olah et al. (2015) from genotyping of high quality DNA from blood, we used heterozygote deficit (homozygote excess) as an indicator of DNA quality in this study. Therefore, we tested deviations from Hardy–Weinberg equilibrium in GenePop 3.4 (Raymond & Rousset 1995) by exact probability test (Markov chain parameters were set to 100 batches with 1,000 iterations per batch), and we assessed the degree of heterozygote deficit (if any). We also included blood samples that were previously genotyped during the microsatellite development for some relevant analyses (Olah et al. 2015).

6.3.3 Statistical analysis of feather quality

Feathers were provisionally assigned to the target species in the field based on their shape, size, and colour pattern. The size of each feather sample was derived from the photographs using ImageJ (http://imagej.nih.gov/ij/index.html). Each sample was also visually categorized by quality (good, medium, damaged) and whether it was covered by clay (yes/no). We also calculated the number of days between the collection and the DNA extraction dates for each feather. Finally, all samples were assigned a binary response variable of 1 (amplification of a fragment greater than 100 fluorescent units in the expected size range of SCMA 32) or 0 (failure to amplify). A linear logistic regression model was used to test the likely determinants of PCR amplification success at the SCMA 32 locus. Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC) were used to determine the best model containing all significant terms. Model was selected with the lowest AIC values and simultaneously having the lowest BIC values. Statistical models were computed using GenStat 13.7 (Payne 2009).

6.3.4 Species identification

A total of 14 parrot/parakeet (*Amazona, Pionus, Pionites, Pyrilia, Aratinga, Pyrrhura, Brotogeris, Touit, Forpus*) and 6 macaw species (*Ara, Orthopsittaca, Primolius*) are found in the study area, some of them with similar plumage patterns to our two target species, thus genetic species identification was crucial. Each feather was given a unique number and provisionally identified in the field. In the lab we used the AgGT17 locus that was expected to provide allelic differences between SCMA and RGMA (Abe et al. 2012; Gebhardt and Waits 2008a). However, these earlier studies were based on less than 30 samples. In this study, we uncovered additional species specific alleles by using a larger sample size. Therefore, to more comprehensively evaluate if the AgGT17 locus can separate our target species, we compared the identification based on this locus with assignment tests based on allele frequencies of 11 other loci (Paetkau et al. 1995; Paetkau et al. 2004).

We also applied a Bayesian approach with the program STRUCTURE version 2.3.4 to assign individual feather samples to species, based on multilocus genotype data (Pritchard et al. 2000). STRUCTURE implements the Bayesian Markov chain Monte Carlo (MCMC) method to assign individuals to *k* clusters. In order to separate clusters as species we used the no-admixture model, with independent allele frequencies among clusters. Burn-in was set to 50,000 iterations, followed by 50,000 MCMC iterations and replicated ten times for each value of *k*, from one to five. To avoid any bias in the species allocation, the AgGT17 locus was excluded from the assignment tests and STRUCTURE analysis.

6.3.5 Molecular sexing

The most widely employed method for molecular sexing of birds is based on the conserved CHD gene in the avian sex chromosomes (Ellegren 1996). In this test the primers produce one amplified fragment for males and two different size fragments for females due to retroposon insertions in the females' Z chromosome (Suh et al. 2011). In our pilot study we tested the widely used P2/P8 primers that amplify DNA fragments between 300 and 400 bp (Griffiths et al. 1998) and the 2550F/2718R primers that show much better agarose gel resolution (ranging between 400 and 1,000 bp) and higher confidence in sex determination on agarose assay over a wide range of bird taxa (Ong and Vellayan 2008). Both primer combinations showed very low amplification success on our moulted feather DNA in the pilot study, probably because of our more degraded DNA samples.

In order to achieve robust molecular sexing from degraded DNA, we therefore designed new primers for our target species that would yield results for small fragment size differences with capillary electrophoresis. Our assay targeted a 189 bp fragment of CHD-Z and a 215 bp fragment of the CHD-W yielding a difference of 26 bp (Fig S1). The primer design was based on an alignment of CHD gene sequences of SCMA from GenBank (accession numbers: KF425691, KF412778; http://www.ncbi.nlm.nih.gov/genbank). Geneious version R6 was used to obtain the alignment and optimize primer design. The sequences of the new primers (5' to 3') are:

P8_SCMA_F: TGCAAAACAGGTRTCTCT

P2_SCMA_R: GAWTAAGTAGTTCAAAGCTA

We compared the new primers for macaw samples of known sex, and on blood samples previously sexed using the 2550F/2718R primers.

6.3.6 Population genetic analyses

GenAlEx 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) was used to compute all population genetic analyses, unless otherwise stated. These calculations included allele frequencies, observed and expected heterozygosities, probability of identity (*PI*), and probability of identity for siblings (PI_{sibs}).

The *PI* value across loci provides an estimate, under the assumptions of Hardy–Weinberg equilibrium, of the average probability that two independent samples will have the same identical genotype (Waits et al. 2001). It thus provides an estimate of how many loci are needed to discriminate among individuals. The theoretical estimate of the *PI* is usually lower than the observed value, hence the calculation for PI_{sibs} was introduced in forensic science (Evett and Weir 1998), to estimate the probability when full siblings occur in the dataset that share very similar alleles. To empirically confirm how many loci were needed for recovering all genotypes, we computed the genotype recovery rates by adding increasing number of loci, in order of their effective number of alleles. Lastly, we pinpointed complete genotype matches for conspecific samples in the genetic tagging analysis. We manually checked each near match for samples that only differed at 1 to 3 loci and resolved any scoring errors.

In order to compare the genetic results between blood and feather, the genetic differentiation (F_{ST}) of feathers collected in 3 km radius around TRC was compared to previously genotyped blood samples from the same area (Olah et al. 2015) by an analysis of molecular variance (AMOVA). Allele frequencies, observed and expected heterozygosities were calculated for blood and feather samples separately. Pairwise estimate of Shannon's Mutual Information Index was also performed

(Peakall and Smouse 2012; Smouse et al. 2015), providing an alternative allele frequency based estimate of genetic differentiation.

In order to validate that samples from clay licks (N_{SCMA} = 96 feathers) give similar population genetic results to samples from nests (N_{SCMA} = 40 blood samples and 38 feathers), we also performed an AMOVA between these two types of sampling sites for SCMA.

6.4 Results

6.4.1 Feather quality, microsatellite amplification, and population statistics

The size of feathers and the number of days between collection and DNA extraction did not significantly affect the PCR amplification success (GLM $_{\text{Feather size}}$: χ^2_1 = 1.47, P = 0.225; GLM $_{\text{Days since collection}}$: χ^2_1 = 3.10, P = 0.078). However amplification success was significantly lower for poor quality feathers (GLM $_{\text{Feather quality}}$: χ^2_2 = 108.87, P < 0.001; Fig 1A) and when clay was present on feathers at collection (GLM $_{\text{Clay on feather}}$: χ^2_1 = 14.14, P < 0.001; Fig 1B).

In total 500 feather samples were genotyped across the 11 loci with only 27 samples discarded from subsequent analyses because they had 5 or more missing loci. Allele frequency and heterozygosity estimates by locus are shown in Table 1 for both target species. Across all loci the allele number (*N*a) ranged from 12 to 20 per locus for SCMA and from 9 to 18 for RGMA (Table 1). The mean expected heterozygosity (H_E) was 0.892 for SCMA and 0.772 for RGMA (Table 1). The observed heterozygosity (H_O) values across loci ranged from 0.733 to 0.908 for SCMA and from 0.553 to 0.892 for RGMA (Table 1).

The average amplification success over the 11 markers was 94% for SCMA and 95% for RGMA. The lowest overall amplification success across both species (N = 473) occurred at SCMA 31 (18.6%), SCMA 02 (14.2%), and SCMA 27 (6.3%). Scoring errors at genotype level were calculated from about 50 randomly selected samples that had no missing loci during the first scoring before the repeats. Genotyping errors occurred due to allelic dropout and false alleles at SCMA 02 (2/7 replicated samples), SCMA 27 (4/15), SCMA 14 (5/48), SCMA 32 (3/44), SCMA 30 (2/42), SCMA 26 (1/48), and AgGT17 (1/55). We found no error at SCMA 09 (0/49), SCMA 22 (0/43), SCMA 31 (0/50), SCMA 33 (0/53), SCMA 34 (0/48), and P2/P8_SCMA (0/47). Subsequently three overlapping dataset were analysed: set (1) the 6 loci with no error and low amplification failure, set (2) combination of set (1) and 3 additional loci with some error or higher amplification failure, and

set (3) combination of set (2) including two loci with both scoring error and higher amplification failure (Table 1). Using all 11 loci only eight samples showed mismatched genotypes where 8-10 loci were adequate (see below), suggesting that genotyping errors did not affect the genetic tagging analysis. Two of these samples were confirmed siblings from the same nest.

6.4.2 Species identification

The AgGT17 locus for molecular identification of the target species amplified in all but 2 samples, potentially providing a technique to separate these species. However, we uncovered additional alleles for RGMA, so we needed a more comprehensive species identification for the 500 feather samples. Based on 11 loci (excluding AgGT17), the STRUCTURE analysis (Fig S2A) and assignment tests (Fig S2B) independently allocated 18 samples into a third group probably representing a different or several different species that were not the target of this study. Most of these 18 samples also showed unusual alleles at the AgGT17 marker.

In total 142 SCMA and 313 RGMA feathers were identified and confirmed independently by the AgGT17 genetic marker, the STRUCTURE analysis (Fig S2A), and the assignment test (Fig S2B). In the field 28% of the feather samples were misidentified. Thus species ID was corrected in light of this genetic analysis.

6.4.3 Molecular sexing

The P8_SCMA_F/P2_SCMA_R primers produced two amplified fragments in females (CHD-Z and CHD-W) and one amplified fragment (CHD-Z) in males, which was easily visualized by capillary electrophoresis (Fig S1). The optimized primers yielded results matching 20 blood samples of known sex for both target species. When applied to the feather DNA samples, typing was achieved for sex in 85% of SCMA samples (66 males, 55 females, 21 unknown) and 95% of RGMA samples (183 males, 114 females, 16 unknown).

6.4.4 Probability of identity and genetic tagging

The probability of identity (among siblings) analysis was calculated using the best 6 loci (Table 1). For SCMA the five most variable loci ($PI_{sibs(5)}$ = 0.002) and for RGMA the six most variable loci ($PI_{sibs(6)}$ = 0.003) were predicted to recover all unique genotypes, given the sample sizes of this study (N_{SCMA} = 142, N_{RGMA} = 313). This prediction was supported empirically, for example we did

not recover more unique genotypes in feathers after the two most variable loci of SCMA (Fig 2A) and after the five most variable loci of RGMA (Fig 2B) when including the best six loci (similar results for 9 or 11 loci). We therefore used only these six microsatellite markers for genetic tagging.

Among the 142 feather samples of SCMA we identified five complete genotype matches (total of 137 unique genotypes). When we added 86 previously genotyped SCMA blood samples we found eight additional genotype matches between blood and feather samples (Fig S3). Out of 313 RGMA feather samples there were 23 matches (total 282 unique genotypes). As expected, across both species the most frequent type of 'recapture' was in the same location from the same sampling event (15). Further matches occurred (a) among or within nests (6), (b) between nests and clay licks (6), and (c) among or within clay licks in different time (9).

6.4.5 Reliability of non-invasive feather samples

Within the 3 km vicinity of TRC we had a comparable number of blood and feather samples from both species to test whether the invasive and non-invasive samples yielded similar population genetic results. We found similar allele frequencies, observed and expected heterozygosities between blood and feather samples of SCMA (Table 2). The genetic distance based AMOVA with the 6 most reliable loci showed no significant differentiation between the two sample types for SCMA (N = 73, FST < 0.001, P = 0.447) or RGMA (N = 23, FST < 0.001, P = 0.444), and similar results were yielded with 9 and 11 loci (Table S2). The Shannon's allele frequency based analysis also failed to detect any significant genetic differentiation (Table S2). Across all the samples we also found no significant genetic differences between samples from nests and clay licks for SCMA (AMOVA: N = 174, FST = 0.000, F'ST = 0.049, P = 0.326).

6.5 Discussion

We developed, validated, and applied a genetic tagging method for feather samples collected from wild populations of two sympatric macaw species in the southeastern Peruvian Amazon. Our results demonstrate that feathers are valuable sources of DNA for genetic tagging as tested using 11 highly variable microsatellite loci.

6.5.1 Feather sampling in a tropical environment

Feathers are a promising non-invasive source of DNA but there are some conflicting views on their utility (Katzner et al. 2012; McDonald and Griffith 2011; McDonald and Griffith 2012). In their experimental setup with feathers of domestic goose, *Anser anser domesticus*, Vili et al. (2013) showed that humidity, direct sunlight, and heat have the most degrading effect on feather DNA quality. As expected, not all feathers collected from our tropical study site yielded sufficient and high enough quality DNA for molecular sexing and genetic tagging. Gebhardt et al. (2009) found that moulted macaw feathers at clay licks provide promising DNA samples, but they also reported a high error rate in molecular sexing of samples (Gebhardt and Waits 2008b). Here we found by logistic regression analysis that damaged feathers had significantly lower amplification success over intact feathers, consistent with other studies (Gebhardt et al. 2009; Hogan et al. 2008). Despite thoroughly washing the feathers with 70% ethanol, samples with clay still had significantly lower amplification success. In addition, clay particles appear to inhibit PCR reactions as also observed by Yankson and Steck (2009). Unlike the studies of Segelbacher (2002) on a large grouse (*Tetrao urogallus*), or Gebhardt et al. (2009) on large macaws (*Ara* spp.), we found that the size of the feathers did not significantly affect the quality DNA yields.

For future genetic studies using feather samples in tropical environments we recommend (a) collecting only good quality and intact feathers, (b) collecting mainly clean feathers free of clay, and (c) considering feathers in a wide range of size, in order to maximize quality and quantity of DNA.

If feathers are stored appropriately (e.g. in dry box with desiccant), the time interval between sample collection and DNA extraction appears to be flexible, at least over a time window of 2-5 years.

6.5.2 Species and sex identifications by moulted feathers

Correct species identification remains the critical first step when relying on feathers for population genetic analysis or genetic tagging (Rudnick et al. 2007). Identification of species by the morphology and colour of feather samples can be challenging, and in our study we initially misidentified almost one third of our feather samples in the field. DNA barcoding, mainly based on mtDNA COI gene, is the standard genetic technique for species identification (Abe et al. 2012; Tavares and Baker 2008). However, in this study we were able to distinguish species using the same multilocus genotyping methods as employed for our population genetic analyses.

Although molecular sex typing of birds initially required a blood sample (Fridolfsson and Ellegren 1999; Griffiths et al. 1998) primers are now available for freshly plucked or collected feathers (Bosnjak et al. 2013; Ong and Vellayan 2008; Presti et al. 2013). Typically the molecular sexing of birds targets sex specific DNA fragments that are visualized on agarose gel electrophoresis, providing a low cost and simple laboratory assay (Miyaki et al. 1998; Ong and Vellayan 2008). For this technique the DNA rich avian blood with nucleated erythrocytes is usually used (Fridolfsson and Ellegren 1999; Griffiths et al. 1998). However, other studies have successfully applied the method to plucked feathers from captive birds (Bosnjak et al. 2013; Ong and Vellayan 2008). Gebhardt and Waits (2008b) even evaluated the performance of primer sets on moulted feathers of SCMA and reported high overall error rates and high dropout rates. Presti et al. (2013) also found that most primers did not amplify well on moulted macaw feathers and suggested the use of primers amplifying even shorter PCR fragments. With our new primers that target a shorter PCR fragment, we were able to confidently identify sexes in 84% of SCMA and 94% of RGMA moulted feather samples.

6.5.3 Genetic tagging, tracking macaws without capture

The probability of identity values calculated for siblings in our dataset of SCMA and RGMA indicated that five or six of the most variable loci were enough to recover unique genotypes for the two species respectively, given our sample sizes. We confirmed this empirically by comparing the number of unique genotypes recovered for increasing combinations of loci, including previously genotyped SCMA blood samples with many related individuals, e.g. parent/offspring and full siblings (Fig 2A).

Our study recovered a total of 36 genotype matches among samples, and according to the *PI* values and the genotype recovery rates we were confident that these were 'recaptures' of the

same individuals. Recaptures found between blood and feather samples further demonstrate the feasibility of this technique. Adult SCMAs are often observed feeding their chicks with seeds mixed with clay, and crop samples of these chicks showed high content of clay (Brightsmith et al. 2010; Cornejo et al. 2011). We suspected that adult macaws visit the nearest clay licks to their nests for sodium supplementation but no evidence has been shown to confirm this (D.J. Brightsmith, pers. comm.). In the present study we found genetic evidence that juvenile SCMAs returned to their fledging site and used the nearest clay lick (e.g. feathers of fledglings from the nests Amor & Franz were recovered at the nearest clay lick to the nests in the next year; Fig S3).

Our ability in this study to recover individual genotypes with 5-6 strategically chosen informative markers demonstrates the potential for population and individual-based genetic studies in macaws, which can help better understand their movements in subsequent analyses. We have previously observed at least four banded breeding pairs of SCMA returning to their nesting site in subsequent breeding seasons in TRC, often re-using the same nest hollows (G. Olah, pers. obs.). In this study we were able to confirm the re-use of the same nests for breeding around TRC by the genetic tagging analysis. Berkunsky and Reboreda (2009) also showed high nest fidelity of blue-fronted parrots, *Amazona aestiva*, based on observation of banded females. This behaviour of secondary cavity nesting parrots could reflect preferences for nests associated with better characteristics. SCMA has also been showed to prefer nesting in cavities (or artificial nests) with higher previous success (Olah et al. 2014).

We compared blood vs. feather samples, and samples sourced from/around nests vs. samples from clay licks, and found no genetic differentiation between these groups. These findings further show that feathers can indeed be considered as representative samples of the local population. The microsatellite markers of this study were originally designed from the full genome sequence of SCMA (Seabury et al. 2013), hence we were able to select highly variable di-nucleotide repeats for SCMA that also showed variability for the closely related RGMA (Olah et al. 2015). However, the mean numbers of alleles, effective alleles, observed and expected heterozygosity (Table 1) were lower in RGMA (cogenic species) than in the focal species (SCMA), possibly due to ascertainment bias (Ellegren et al. 1997; Peakall et al. 1998). With these possible limitations in mind, the genetic tagging technique developed for these macaws will be widely applicable to other related species of higher conservation concern. In addition, for many threatened parrots the non-invasive genetic sampling of moulted feathers may be the only available DNA source and its use can also help address the ethical concerns of catching wild individuals.

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6.8 Figures and tables

Figure 1. Predicted effect of significant variables from a linear logistic regression model on the probability of PCR amplification of SCMA32 locus: (A) Feather Quality and (B) Clay on Feather.

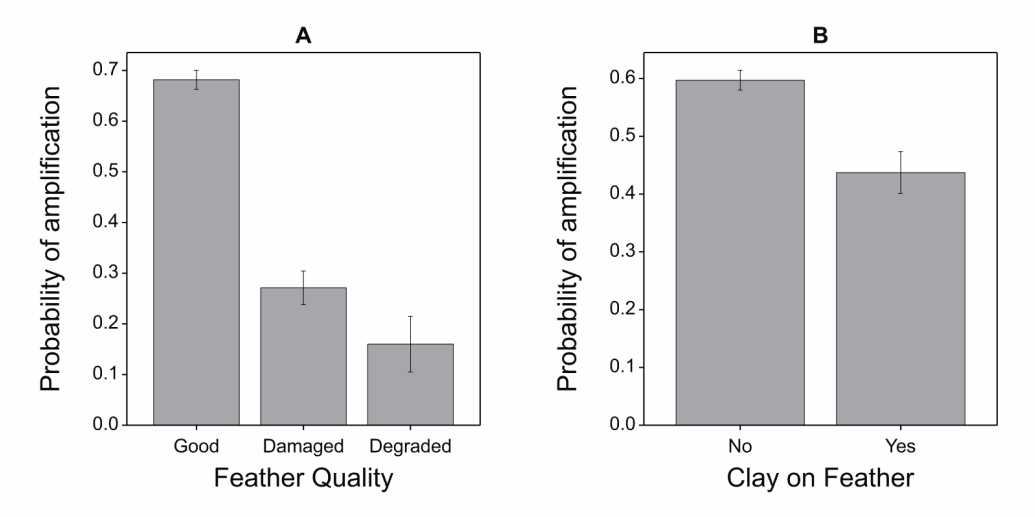

Figure 2. Recovery of unique multilocus genotypes for increasing combinations of loci for (A) scarlet macaw and (B) red-and-green macaw. The order of loci was defined by their number of effective alleles (from highest to lowest) for the two species respectively. Triangles (green line) indicate genotype recovery using only feather samples; and circles (red line) show recovery when using blood samples from related individuals (including parent/offspring and full siblings).

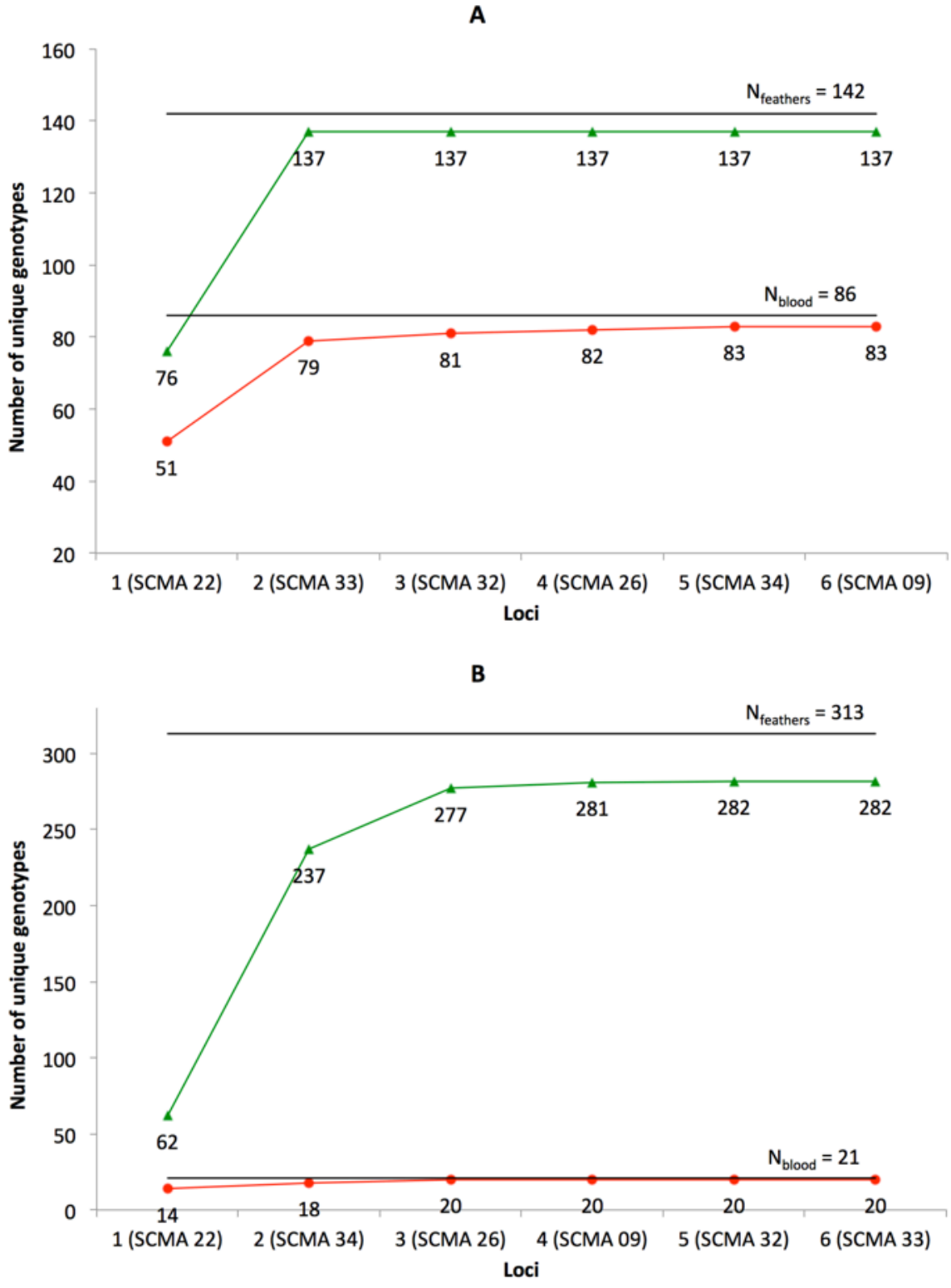

Table 1. Population statistics for microsatellite markers in non-invasive feather samples from scarlet macaw (*Ara macao*) and red-and-green macaw (*Ara chloropterus*).
Presented are species, number of locus used in the analyses, locus code, number of samples (*N*), fragment size ranges, mean fragment size (MS), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E), probability of departure from HWE (P_{HWE}), probability of heterozygote deficit (P_{HED}), and amplification failure rate (AFR).

Species	# locus	Locus	*N*	Size range (bp)	MS (bp)	N_a	N_e	H_O	H_E	*F*	P_{HWE}	P_{HED}	AFR (%)
SCMA	6 loci	SCMA 22	131	114-160	134	19	12.1	0.908	0.918	0.010	0.221	0.412	2.2
		SCMA 32	128	175-211	192	16	10.9	0.828	0.908	0.088	0.037	0.003	4.5
		SCMA 34	132	151-189	173	17	8.5	0.803	0.882	0.090	0.018	0.005	1.5
		SCMA 33	134	174-212	193	20	11.2	0.881	0.910	0.033	0.696	0.156	0.0
		SCMA 26	130	210-240	225	14	9.4	0.808	0.894	0.096	0.002	0.000	3.0
		SCMA 09	132	112-136	123	12	5.0	0.750	0.802	0.065	0.836	0.103	1.5
	9 loci	SCMA 14	131	220-252	238	14	8.8	0.733	0.886	0.173	0.000	0.000	2.2
		SCMA 30	124	206-246	229	17	9.8	0.871	0.898	0.030	0.908	0.029	7.5
		SCMA 31	108	137-169	152	16	8.7	0.861	0.885	0.027	0.235	0.197	19.4
	11 loci	SCMA 02	111	268-300	284	17	12.9	0.793	0.922	0.141	0.003	0.000	17.2
		SCMA 27	120	209-245	226	18	11.3	0.858	0.912	0.059	0.243	0.026	10.4
		AgGT17	132	102-138	119	18	6.5	0.833	0.846	0.015	0.630	0.208	1.5
		Mean			**191**	**16.4**	**9.9**	**0.827**	**0.892**	**0.069**			
RGMA	6 loci	SCMA 22	278	122-150	135	14	8.5	0.892	0.882	-0.012	0.478	0.287	1.4
		SCMA 32	279	173-199	184	11	3.1	0.631	0.677	0.069	0.005	0.001	1.1
		SCMA 34	279	157-181	169	13	5.4	0.799	0.816	0.020	0.002	0.019	1.1
		SCMA 33	280	166-190	179	10	2.4	0.575	0.586	0.019	0.571	0.269	0.7
		SCMA 26	277	222-240	231	10	5.1	0.715	0.803	0.110	0.097	0.001	1.8
		SCMA 09	280	112-132	122	11	4.0	0.739	0.751	0.015	0.889	0.382	0.7
	9 loci	SCMA 14	273	212-238	228	9	2.4	0.553	0.590	0.063	0.124	0.103	3.2
		SCMA 30	270	206-248	230	17	4.9	0.715	0.796	0.102	0.000	0.000	4.3
		SCMA 31	220	135-165	153	12	7.5	0.836	0.867	0.036	0.890	0.197	22.0
	11 loci	SCMA 02	238	260-300	283	18	5.9	0.668	0.831	0.196	0.000	0.000	15.6
		SCMA 27	267	211-245	227	17	9.0	0.760	0.889	0.145	0.000	0.000	5.3
		AgGT17	282	98-112	105	4	1.0	0.007	0.011	0.331	0.006	0.006	0.0
		Mean			**187**	**12.9**	**5.3**	**0.717**	**0.772**	**0.091**			

Table 2. Population statistics for microsatellite markers on blood and feather samples from scarlet macaw (*Ara macao*) in TRC.

Presented are number of loci used in the analysis, type of samples, number of samples (*N*), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (*F*). Numbers are mean values ±SE.

# locus	Type	*N*	N_a	N_e	H_O	H_E	*F*
6 loci	Blood	27.8 ±0.1	12.1 ±1.0	7.70 ±0.8	0.873 ±0.030	0.863 ±0.012	-0.010 ±0.025
	Feather	44.3 ±0.2	14.1 ±0.9	9.01 ±1.0	0.872 ±0.015	0.880 ±0.015	0.008 ±0.020
9 loci	Blood	27.8 ±0.1	11.8 ±0.8	7.52 ±0.5	0.892 ±0.021	0.862 ±0.007	-0.033 ±0.020
	Feather	43.1 ±1.1	13.7 ±0.7	8.88 ±0.6	0.850 ±0.023	0.881 ±0.010	0.034 ±0.027
11 loci	Blood	27.9 ±0.0	12.1 ±0.6	7.86 ±0.5	0.898 ±0.018	0.868 ±0.007	-0.034 ±0.016
	Feather	42.5 ±1.0	14.0 ±0.6	9.13 ±0.5	0.841 ±0.021	0.885 ±0.008	0.049 ±0.025

6.9 Supplementary material

Figure S1. Binding sites of primers P8_SCMA_F and P2_SCMA_R on the consensus sequence of scarlet macaw CHD Z and W genes. Geneious version 6.1 created by Biomatters. Available from http://www.geneious.com

Figure S2. Species identification tests. Red (circle) = red-and-green macaw; RGMA (N = 313), blue (square) = scarlet macaw; SCMA (N = 142), green (triangle) = unidentified bird species (N = 18).

(A) Probabilities of assignment to three genetic clusters identified by STRUCTURE.

(B) Bi-plot using pre-defined allele frequencies of the two taxa in assignment test of GenAlEx 6.5.

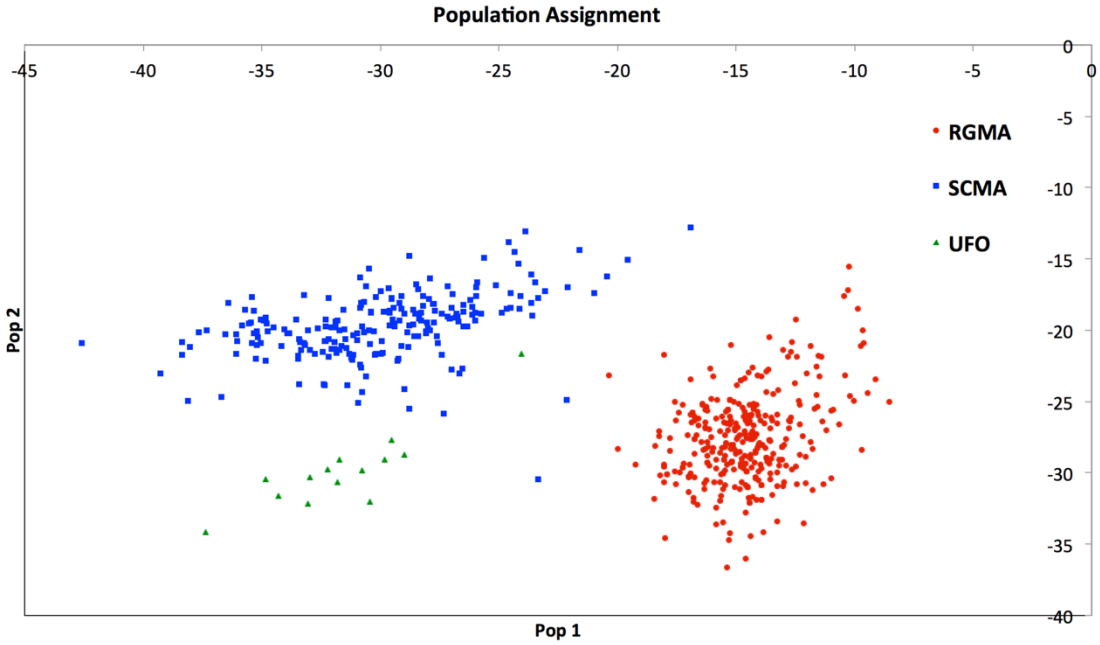

Figure S3. Complete genotype matches of scarlet macaw blood and feather samples in Tambopata, Peru.

Each horizontal line represents a unique genotype with their ID on the right.

Each marker represents a sample with the name of the location collected (black circle = blood; triangle = feather), the distance (m) between locations and the time (day) between collections on the right.

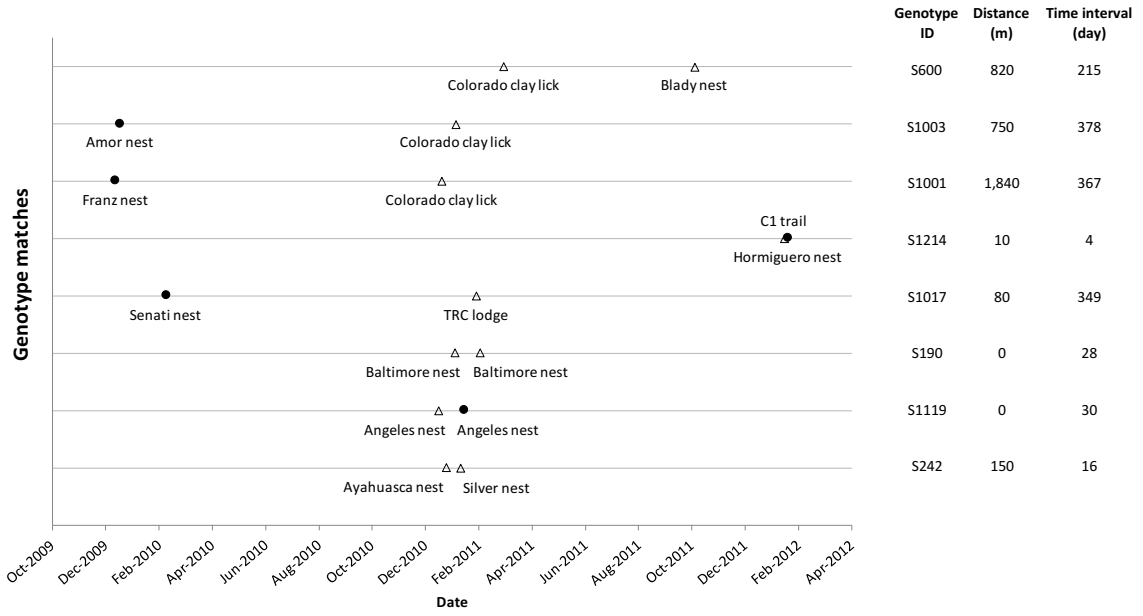

Table S1. Microsatellite primer sequences and locus information for scarlet macaw and red-and-green macaw.

Locus name	Forward primer sequence 5'–3'	Reverse primer sequence 5'–3'	Repeat motif	*Fluorescent tag
SCMA02	*TCAACCTCCAGGTGTCTTCC	TCCTTCAGTCACCAGCTTCA	$(GT)_{21}$	NED
SCMA09	*CACTACCAGCAAGTAGCAGGC	TGAATTCTAACAAGCAGCGG	$(CA)_{20}CG(TA)_3$	VIC
SCMA14	*CGCATACTTTACACCCACCA	TTGTGACAGGGCTAGGCAG	$(AC)_{20}$	FAM
SCMA22	*AACTGTGATGAAGTTCGTGGC	CAACGGCTACACACAGTGCT	$(TG)_{22}$	VIC
SCMA26	*AGCAAAGGTAAGGAGCAGCA	GGCACCTCTATCATCTATTGCAG	$(TG)_{21}$	VIC
SCMA27	*TTCTGCAGCAGTTCCCAAA	TGGACTCTGTATTCCAGTCGC	$(CA)_{22}$	FAM
SCMA30	*TTGCCAGGTCCTTCTCTACC	ACCACCTTCTCTTGACTTGTAATTG	$(CA)_{24}$	FAM
SCMA31	*TGTGCTCCCTACAGTTCCAA	AACGCTGAACTTGGTGTGGT	$(AC)_{21}$	FAM
SCMA32	*GGCATGGCTCTTTACTTGCT	TTGCCACTGAGGCTTCTACC	$(TG)_{21}$	VIC
SCMA33	*GAGGCACTATTTCTGGCAGC	GCTAAGCAGATTTGTCTAAACATTCA	$(AC)_{21}$	VIC
SCMA34	*TTTGGCAGTAGTCGGGATTT	AACTTGGGAATACATCGCTGA	$(AC)_{22}$	VIC

Table S2. Pairwise Shannon Partition Analysis and AMOVA between blood and feather samples of scarlet macaws and red-and-green macaws in TRC (999 permutations).

# loci	Species	N	Shannon		AMOVA	
			$_sH(AP)$	*P*	F_{ST}	*P*
6 loci	SCMA	73	0.095	0.564	0.000	0.447
	RGMA	23	0.079	0.799	0.000	0.444
9 loci	SCMA	73	0.093	0.663	0.000	0.481
	RGMA	23	0.065	0.847	0.000	0.455
11 loci	SCMA	73	0.051	0.709	0.000	0.475
	RGMA	23	0.069	0.919	0.000	0.440

Chapter 7

Non-invasive genetic tagging of large macaws in the Peruvian Amazon

Red-and-green macaws at the Posada Amazonas clay lick (2008).

7.1 Abstract

Genetic tagging, the unique identification of individuals by their DNA profile, has proven to be an effective method for research on several animal species. A further advantage is that non-invasive sampling can sometimes be used, eliminating the need to capture animals. Yet despite their promise, these powerful tools have rarely been applied in birds and have not yet been used for parrots. In this study we use non-invasive genetic tagging from feather samples to reveal new insights into the biology of red-and-green macaws (*Ara chloropterus*). The study was centred in the Tambopata region of the Peruvian Amazon. Here macaws frequently visit clay licks and their naturally moulted feathers provide a unique source of non-invasively sampled DNA. In a previous study we constructed 313 genotypes from individual feathers using nine microsatellite loci and found that 282 of these were unique. The remainder revealed 23 individuals which were 'recaptured' one or more times. We estimated the number of different individuals visiting clay licks within one breeding season based on the genotype matches using a capture-mark-recapture model. The population size estimates fall between 84 and 316 individuals per clay lick. Population genetic structure analysis revealed only small genetic differences among regions and clay licks, suggesting a single red-and-green macaw population. Our study confirms the utility of non-invasive genetic tagging in harsh tropical environment to obtain crucial population parameters about an abundant parrot species.

7.2 Introduction

A high proportion of the 398 extant species of parrots (28%) are classified as threatened (IUCN 2014). The major threats faced by these birds include habitat destruction and fragmentation, poaching, and invasive alien species (Olah et al. 2016; Owens and Bennett 2000; Pires 2012). Most parrots are forest dependent secondary cavity nesters, hence forest destruction decreases the availability of nesting sites and therefore reproductive success, and it can also lead to the loss of key food resources (Brightsmith 2005; Forshaw 2011). Stochastic factors in small and fragmented parrot populations can also cause extreme population decline, for example a hurricane caused significant decline of the critically endangered Puerto Rican parrot, *Amazona vittata* (Wunderle 1999). Small populations may also suffer loss of genetic diversity and inbreeding depression. These processes were identified in Spix's macaw, *Cyanopsitta spixii*, which is now extinct in the wild (Caparroz et al. 2001). The reduced viability of such small populations in the face of environmental changes could consequently drive them towards extinction (Frankham et al. 2004).

The first step towards the conservation of any species in the wild is the acquisition of information about its biology. For example, knowledge of a species' home range, dispersal pattern, and population size can be crucial for relevant management. To obtain this information by traditional methods requires individual 'tagging' of the animals for capture-mark-recapture (CMR) methods. In birds this is normally achieved by capturing and tagging individuals with leg bands, but capture/recapture of the required number of individuals is far from straight forward for many species (Pollock et al. 2002; White and Burnham 1999). There is some progress in the tracking of individual birds by satellite telemetry that is feasible in larger species (Groom et al. 2015; Limiñana et al. 2015; Webster et al. 2002), however this technique is limited by the number of birds that can be tagged affordably.

Genetic tagging, the unique identification of individuals by their DNA profile, has proven to be a highly effective method in molecular ecology, offering a powerful tool for 'tagging' that has been used for whales (Palsboll et al. 1997), fishes (Andreou et al. 2012; Sekino et al. 2005), amphibians (Ringler et al. 2015), seals (Hoffman et al. 2006), bears (Woods et al. 1999), and small mammals (Peakall et al. 2006; Ruibal et al. 2010). However, this method has not yet been widely applied in birds, where non-invasive sampling of feathers is a potential source of avian DNA (Bush et al. 2011; Horváth et al. 2005). Rudnick et al. (2005) used naturally shed feathers of eastern imperial eagle, *Aquila heliaca* to identify and monitor adult birds individually. Other studies using feathers of a large grouse species, *Tetrao urogallus* have demonstrated the value of genetic tagging for estimating local population size (Jacob et al. 2009; Mollet et al. 2015; Moran-Luis et al. 2014).

However, similar techniques have not been used to estimate population size or other demographic parameters in parrots.

Here we use non-invasive genetic tagging in combination with mark-recapture modeling to test if we can infer relevant and reliable demographic information of the red-and-green macaw, *Ara chloropterus*. In the Peruvian Amazon, where this study is based, these birds are abundant and visit clay licks to consume soils rich in sodium (Brightsmith 2004; Powell et al. 2009). However, it is not known if these clay licks act as 'magnets', in effect drawing in birds from vast areas, or whether they simply facilitate feeding aggregations of local populations. Hundreds of naturally dropped feathers are left behind at clay licks, offering excellent opportunities to obtain non-invasive DNA samples (Gebhardt et al. 2009). In turn, genetic tagging from these samples potentially allows one to obtain species-specific information on clay lick use by individuals and populations, in a species where it is not logistically feasible to capture a large number of wild birds.

In this paper we use more than 300 genetic samples collected from feathers at clay licks to (1) test for genetic differentiation and relatedness among birds using different clay licks; (2) test if we can reliably estimate the number of birds visiting clay licks; (3) compare the proportions of males and females using the clay licks over time; and (4) explore the potential of our methodology for establishing population estimates for other bird species with locally aggregated populations.

7.3 Methods

7.3.1 Target species and study sites

The red-and-green macaw (RGMA hereafter) has a large range over South America from southern Panama to southeastern Paraguay. They mainly reside in subtropical and tropical moist lowland and montane rainforest from sea level to 1,400 m (BirdLife International 2014) and nest in hollows of emergent trees (Brightsmith 2005; Renton and Brightsmith 2009).

The study was situated in the southeastern Peruvian Amazon which contains lowland rainforest habitat with an average annual rainfall of 3,200 mm (Brightsmith 2004). Our study area was distributed in both nationally protected (Tambopata National Reserve, Bahuaja-Sonene National Park) and unprotected areas (Rio Madre de Dios, Rio Las Piedras, Los Amigos Conservation Concession; Fig 1). The areas with highest human populations in the region include Puerto Maldonado and other settlements along the Inter-oceanic highway (Conover 2003; Tickell 1993).

7.3.2 Sample collection

Our study area falls within the region with the highest number of clay licks in South America (Lee et al. 2010), offering an excellent opportunity to sample moulted feathers non-invasively and repeatedly. Furthermore, many aspects of clay lick ecology have been already studied in Tambopata including the distribution of clay licks (Lee et al. 2010), parrot behaviour at clay licks (Brightsmith and Villalobos 2011; Burger and Gochfeld 2003), clay lick preference (Powell et al. 2009), soil characteristics (Brightsmith and Aramburú Muñoz-Najar 2004; Brightsmith et al. 2008), and the effect of climate on geophagy (Brightsmith 2004). However, we know very little about population sizes, sex ratio, and parrot density around clay licks.

In this study a total of 313 moulted feathers were collected from 34 sampling sites that included occasional findings in the forest, in/around nests, and six major clay licks (Fig 1) that were visited systematically (every 2-4 weeks) during each breeding (rainy) season (November-April) between 2009 and 2012. Prior to the first collection of each season we cleared the feathers of unknown age from the clay licks in order to be more confident of the dates feathers were left behind during the collection season. Upon collection, samples were stored individually in paper envelopes in airtight boxes with silica gel to avoid further degradation (Chapter 6).

7.3.3 Genotyping for genetic tagging

The genetic data for this study is drawn from our previous study where we outlined molecular methods for the non-invasive genetic tagging of large macaws in southeastern Peru (Chapter 6). Based on the full genome of scarlet macaw, *Ara macao* (Seabury et al. 2013) we previously developed 30 highly variable di-nucleotide microsatellite loci for the same species and also for red-and-green macaws (Olah et al. 2015). Nine of these markers (SCMA 09, SCMA 14, SCMA 22, SCMA 26, SCMA 30, SCMA 31, SCMA 32, SCMA 33, and SCMA 34) were selected and validated for moulted macaw feathers found at clay licks against blood samples sourced from macaw nests, and full genotypes were constructed.

The nine markers selected for this study were chosen *a priori* out of a set of 30 species specific markers in pilot studies involving Hardy-Weinberg equilibrium tests, frequencies of null alleles, tests for amplification failures and genotyping error rates (Olah et al. 2015; Chapter 6). The selected loci showed low amplification failure and very low error rates. The power of the nine loci used was also demonstrated with the estimates of probability of identity showing that only six of these loci were required to recover unique genotypes in the sampled red-and-green macaw

population ($PI_{sibs(6)}$ = 0.003), hence the probability of finding exact matches at nine loci by error is extremely low. Furthermore, all of the genotype matches were carefully evaluated locus by locus, with all near genotype matches that differed at a maximum of three loci checked manually. Finally, as a further check, we repeated the 50% of samples with full genotype matches.

Molecular sex typing of samples was also performed using the P8_SCMA_F/P2_SCMA_R primers, as part of the genotyping runs (Chapter 6).

7.3.4 Population genetic analyses

GenAlEx 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) was used to compute the population genetic analyses, unless otherwise stated. The analysis of molecular variance (AMOVA) framework was used to partition genetic variation within and among populations and regions defined *a priori*. In the analyses we define the term 'population' as groups of sampling sites within a maximum of 20 km radius from each other or from a major clay lick (Piedras, Heath, Lower Tambopata, Chuncho, Colorado, and Tavara), which also corresponds to the estimated maximum daily movement of macaws (Myers and Vaughan 2004). We define 'regions' as larger geographical units of the study site including various populations (Piedras, Heath, Tambopata, and Candamo; Fig 1).

Two different AMOVA analyses were run after exclusion of duplicates due to recapture. In the first the full data set was partitioned into four large geographical regions (Piedras, Heath, Tambopata, Candamo; Fig 1) with six populations grouped as clusters of clay licks along river systems. In the second, only samples from clay licks were used and samples were pooled across the sampling intervals (Fig 1). Moulted feathers from clay licks were partitioned into four clay licks (Heath, Chuncho, Colorado, and Tavara) and two clay lick complexes, Piedras and Lower Tambopata (Fig 1). Clay licks with sample sizes of less than 15 were excluded from this analysis. All samples from a clay lick were pooled across the sampling intervals.

The AMOVA analysis provided estimates of overall and pairwise population genetic differentiation (F_{ST}), differentiation among regions (F_{RT}) and differentiation among populations within regions (F_{SR}) following Wright (1965), Excoffier, Smouse & Quattro (1992) and Peakall, Smouse & Huff (1995), and their standardized [0,1] equivalents (Meirmans 2006; Meirmans and Hedrick 2011; Peakall and Smouse 2012). Tests for departure from the null hypothesis of no genetic differentiation were performed by random permutation (1,000 permutations). Isolation by distance across the study site was tested by using a Mantel test (Mantel 1967) with departure from the null hypothesis of no significant relationship between genetic and geographical distances

tested by random permutation (10,000 permutations) at the individual level (Smouse and Long 1992; Smouse et al. 1986).

A G-test was used to compare the proportion of males and females across clay licks (Peakall and Smouse 2012). Pairwise relatedness estimates of Lynch and Ritland (1999) *r* were calculated for each pair of individuals and mean pairwise relatedness calculated for each clay lick. Following Beck et al. (2008) we estimated the 95% confidence interval around mean pairwise *r* values of clay licks via bootstrapping. Random permutation of the data set was used to generate a distribution for the null hypothesis of no relatedness among individuals within groups and to provide a test for significance. All bootstrapping and permutation tests were performed 1,000 times. We presented the mean pairwise relatedness for the relatedness estimates and included a control group of 20 individuals from seven known family groups (nests) for control.

7.3.5 Population size estimates at clay licks

With the genetic tagging procedure we identified 23 complete genotype matches across 313 moulted feather samples in our study area, and used these genetic mark-recapture data in the subsequent analysis. Each genotype was treated as a 'capture' and when an identical genotype was found we considered it a 'recapture'. By definition, the minimum time between sampling events was 14 days. If two sampling events at the same clay lick occurred within this time frame we considered them as a single sampling event. Estimates of population sizes at clay licks (the number of individuals using the same clay lick) were derived using two approaches. As genetic recaptures occurred at various clay licks in different seasons, we used subsets of our genetic data for best fitting the assumptions of each model.

We used the program CAPWIRE (Miller et al. 2005) that allows the use of multiple detections of individuals within a single sampling session and accounts for heterogeneous feather moulting patterns among individuals. Each dataset was summarized into the total number of observations made for each individual over the sampling period (e.g. also used the total number of feathers without recaptures) and were fitted using two model types: (1) Two Innate Rates Models (TIRMs), that view populations as a mixture of two types of individuals, the seldom caught type and the often caught type. This model type is used to estimate the probability that an individual belongs to a mixture and assigns individuals to a type; and (2) the Equal Capture Probability Model (ECM), where all individuals are equally likely to be captured. A likelihood ratio test (LRT) was used to determine the most appropriate model and 95% confidence intervals about the mean were estimated with 1,000 bootstrap replicates. CAPWIRE was used to estimate the population size of

the Lower Tambopata complex and the Chuncho clay lick, where data were pooled over the whole study period.

We also used conventional closed capture–mark–recapture (CMR) models (null M0, temporal Mt, behavioural Mb, and heterogeneity Mh) in the program MARK 8.0 (White and Burnham 1999). Closed population models were selected on an a priori basis because the fundamental assumptions of demographic (births and deaths) and geographic (migration in or out) closure were reasonable for this species. We only used this model to estimate the population size at the Lower Tambopata complex, as we found the highest number of recaptures here, providing sufficient data for this analysis. Here we used data from one breeding season (December 2010 – April 2011) to avoid the violation of the assumptions of closure. Deaths are likely to be minimal during this interval in this long living species (>50 years; Brouwer et al. 2000). Migration was assumed to be negligible within the five-month interval consistent with observations that individuals are using the same areas for nesting every year (D.J. Brightsmith, pers. obs.).

7.4 Results

Our genetic data for this study are drawn from Chapter 6. Across all loci the allele number (*N*a) ranged from 9 to 17 per locus and the mean expected heterozygosity (H_E) was 0.772. The observed heterozygosity (H_O) values ranged from 0.553 to 0.892 (mean 0.717).

7.4.1 Genetic differentiation among regions and clay licks

The regional AMOVA attributed 1% of the variation among the four regions (F_{RT} = 0.008, F'_{RT} = 0.032, P = 0.001), and 0% among populations within regions (F_{SR} = 0.002, F'_{SR} = 0.009, P = 0.124). This result indicates that there were small but statistically significant genetic differences among these four regions (Table S1) but not among populations within the regions. We also detected significant genetic differentiation among the populations of macaws using each of the six major clay licks (AMOVA: N = 239, 1% among populations, F_{ST} = 0.008, F'_{ST} = 0.033, P < 0.001). The pairwise comparisons of F_{ST} values of clay licks showed that most clay licks were significantly different from each other, except the Piedras clay lick, which was not significantly different from any other clay lick (Table 1). The standardized mean F'_{ST} value (which can range from 0 to 1) was still small (0.033), although significantly different from zero (P = 0.001).

At the individual genotype level, we found low but significant isolation by distance across the whole study area (Mantel test >200 km, N = 282, r = 0.066, P = 0.036), but no significant isolation at a smaller scale within Tambopata (Mantel test <70 km, N = 186, r = 0.019, P = 0.303). It is possible that family groups may use the same clay licks. Therefore, to test for this possibility we calculated the mean pairwise relatedness from each pair of individuals within each clay lick compared to the average relatedness over all samples. The mean pairwise relatedness estimates of Lynch and Ritland (1999) did not indicate higher than expected relatedness at any clay licks, except in the control group consisting of known related individuals (Fig 2).

7.4.2 Population size estimates at clay licks

Out of a total of 313 feather samples collected from clay licks and nests we found 23 matching genotypes (Fig 3). In 17 cases with two matching feathers, in four cases with three matching feathers, and in two cases with four matching feathers, resulting in 282 unique genotypes in total. Out of the 23 samples checked for genotyping errors 21 gave a perfect match across all loci, one sample showed two and another sample showed only one allele mismatch. The choice of nine loci therefore inhibits redundancy in matching genotypes. Furthermore, it allows for matching even in those cases with some amplification failures or a mismatch of one or two alleles due to allelic dropout.

The total number of feathers collected and the number of unique genotypes identified by each sampling event at the major clay licks are shown in Table 2. Thirteen of the 23 genotype matches occurred in the same location and the same sampling event (9 males and 4 females). The other 10 genotype matches represented feathers from nine individuals (7 males and 3 females) found at different clay licks and one connection between a nest and a clay lick (Fig 3).

The temporal model (M_t) gave the most parsimonious model in MARK as determined by the Akaike information criterion (AIC). This model estimated the population size of the Lower Tambopata complex to be 84 individuals (95% CI: 47–202 birds). For the same area CAPWIRE estimated 89 individuals (95% CI: 52–122 birds). For the much larger Chuncho clay lick the CAPWIRE program provided an estimate of 316 individuals (95% CI: 221–486 birds).

7.4.3 Sex ratios of clay licks

We identified 161 males and 105 females (16 samples could not be sex typed) with unique genotypes. In total we found an overall significant bias towards males when the samples from all

clay licks were pooled (N_{males} = 161, $N_{females}$ = 105, G = 11.88, *P* = 0.001). However, we detected no significant differences in sex ratios when each clay lick was considered individually (G = 8.45, df = 10, *P* = 0.585; Table S2).

7.5 Discussion

Parrots are among the most threatened bird orders with 28% of their species classified as Vulnerable, Endangered, or Critically Endangered (IUCN 2014). Macaws are typical of parrot species facing elevated extinction risks given their high forest dependency, large body size, and long generation time (Olah et al. 2016). Reliable abundance estimates are important for successful conservation actions, however density estimates are only available for 25% of all parrot species, regardless of their conservation status (Marsden and Royle 2015). Here we discuss the efficacy of non-invasive genetic tagging for studying population structure, relatedness, estimating population size and other demographic parameters.

In this study we used 313 RGMA feather genotypes collected over three years along more than 1,000 km of riparian habitat in the Peruvian Amazon. We found 282 unique genotypes within our study region, and determined sex by genetic assay for 105 females and 161 males. Our results indicated individual genotype level isolation by distance only across large geographical scales (>200 km). AMOVA revealed low genetic differentiation (1%) among the geographical regions (on average 60 km apart). Our findings are broadly consistent with two other genetic studies also based on microsatellite markers of macaws but with much lower sample sizes. Marques (2010) reported high genetic diversity based on 64 RGMA samples from Brazil, Bolivia, and Peru, and low genetic structure with possibly high gene flow. Schmidt (2013) described 0.12% of genetic variation among core breeding sites of scarlet macaw, *Ara macao cyanoptera*, in Guatemala up to 35 km apart (*P* > 0.05).

Our results showed an overall bias towards males (161:105). As the feather samples were collected explicitly in the breeding season of the macaws, this seems to support the hypothesis that there is an overrepresentation of males at clay licks during this period as females incubate the eggs and care for hatchlings (Nycander et al. 1995). Support for this is provided by the observation that at clay licks we found twice as many genotype recaptures of males than females. Despite this overall trend, we did not find a significantly higher proportion of males when testing individual clay licks. This could also reflect that nesting females only represent a small proportion

of the population at any time, and exact incubation periods can differ even among nearby nests (D.J. Brightsmith, unpublished data).

We also found small but significant genetic differences among clay licks on a similar scale (1%) to the regional differences. This may indicate that RGMAs are not drawn completely randomly from the population when supplementing their mineral intake at clay licks. However, we did not find higher than average relatedness among birds at clay licks, rejecting the hypothesis that family groups always visit the same clay lick. This also supports the applicability of non-invasive genetic sampling at clay licks, as it gives a representation of the whole population and not just groups of relatives.

7.5.1 Unique genotypes and genetic recaptures

Given the large numbers of unique genotypes from feathers (N = 282), and despite intensive sampling effort over three years, the number of 'recaptures' was surprisingly low (N = 54 feather samples for 28 individuals). This low recapture rate may have two possible explanations. First, genotype scoring errors can lead to estimation bias. However, scoring errors would more probably produce genotype mismatches than matching genotypes at nine hyper-variable loci. Nonetheless this possible problem needs to be evaluated when assessing the accuracy of estimated individuals in the population. Scoring errors could lead to underestimation of recaptures. However, given our pre selection of the most reliable nine loci and the demonstrated low error rates on resampling (none of which affected our conclusion of a match), this risk appeared to be minimal. Any failure to detect a recapture due to genotyping error could contribute to a lower than expected capture rate, which in turn would lead to overestimation of population size via CMR techniques.

The other and more probable reason for the low recapture rate is that the RGMA populations in our study area consist of many more individuals than those we have sampled non-invasively. The average abundance of large bodied parrots (e.g. *Ara* spp.) is predicted to be <10 individuals/km^2 (Marsden and Royle 2015). Based on census data from our study region, Lee and Marsden (2012) estimated the average density of RGMA at 1.78 individuals/km^2, while Lloyd (2004) estimated their density between 1.3 and 2 individuals/km^2. The high abundance of RGMA around clay licks is further supported by some observational data. For instance, during a month of monitoring the Chuncho clay lick in December 2012 the maximum number of observed RGMA on or around the clay lick was about 50 individuals on average, and on one occasion 109 individuals were observed (D.J. Brightsmith, unpublished data).

While the low number of recaptures might not be sufficient for precise population size estimates, some interesting results were revealed from the data. The longest period between recaptures was 376 days, and the longest distance was 30 km, with a mean distance of 3.9 km. Interestingly, many of the matches occurred in the same sampling event. This result could be due to the simple process of multiple shedding of feathers at the same location. Alternatively, as macaws in the tropics seem to shed continuously over the year (D.J. Brightsmith, pers. obs.) it could indicate multiple visits to the clay lick during the sampling interval.

Our genotype data confirmed and refined previously known or suspected aspects of RGMA natural history. For example, we confirmed that the macaws re-used the same clay licks (e.g. Posada, Explorers, Chuncho, Tavara), sometimes even over long periods (e.g. 330 days at Tavara clay lick; Fig 3). RGMA often reuse the same nests over time (D.J. Brightsmith, pers. obs.), so it was assumed that they would re-use the nearby clay licks too. We repeatedly recovered feathers from the same RGMA individuals between nearby clay licks within a three km range (Posada & Explorers) and once between clay licks 30 km apart (Explores & Chuncho). The average gap between clay lick clusters is about 20 km, so the lack of longer-range recaptures suggests that the macaws at clay licks are drawn from local populations. Recaptures varied from within a month up to a year (Fig 3).

Adult scarlet macaws are often observed feeding their chicks with seeds mixed with clay, and crop samples of these chicks showed high content of clay (Brightsmith et al. 2010; Cornejo et al. 2011). Adult macaws are likely to visit the nearest clay licks to their nests for sodium supplementation but no evidence has been available to confirm this (D.J. Brightsmith, pers. obs.). Alternatively, they might visit different clay licks or clay licks with higher sodium content further away from their nesting sites. Out of the seven RGMA parental genotypes available from nests, in one case we recovered a genotype match with a feather at a clay lick nearest to that nest (Rojas nest & Colorado clay lick; Fig 3), consistent with the first hypothesis. Lee et al. (2010) argued that the distribution of parrot clay licks in South America supports the hypothesis of geophagy due to sodium deficient natural diet. Parrot geophagy was also reported in Central America (Valdéz-Peña et al. 2008), Africa (May 2001), and Papua New Guinea (Symes et al. 2006). These sites offer similar sampling opportunities to our study, suggesting that the same genetic tagging techniques could be used to study clay lick use of other species.

7.5.2 Population size estimates

Estimating population size of species in remote areas can be challenging. Capturing and later re-capturing or sighting uniquely identified macaws in a large tropical landscape is a challenging task and has not been attempted for macaws before. Techniques usually involve counting individuals or measuring density and multiplying by area. However, if individuals are not marked, all of these methods face limitations due to the high risk of under or over-counting (Lee and Marsden 2012). In our study we used individual genotypes of feathers and samples with matching genotypes to test their efficacy for CMR based population size estimates. We acknowledge that these population estimates are for the numbers of macaws visiting clay licks, which may not equate to population size estimates. Furthermore, given the very low recapture rate in our study, we only used CMR models for two clay licks with the highest number of recaptures in our dataset.

CAPWIRE was able to incorporate recapture events that occurred in the same sampling session while also modeling capture heterogeneity, but this method is considered to be less robust in situations where the maximum population size is above a thousand individuals (Miller et al. 2005). However, it has been shown by simulations that population size estimates based on single-session sampling (e.g. in CAPWIRE) are as reliable as other estimates (e.g. MARK) based on multisession sampling (Petit and Valiere 2006; Puechmaille and Petit 2007).

Both CAPWIRE and MARK estimates revealed similar population sizes for the Lower Tambopata complex (89 and 84 individuals per breeding season respectively) albeit with wide confidence intervals (47–202 and 52–122 birds respectively). At the Chuncho clay lick the population estimate was 316 individuals. Our one-month observational data from the same clay lick (see above) with the maximum number of 109 RGMA individuals observed in one event seems to support the magnitude of this estimate. However, given the large confidence intervals around these estimates more recapture data would be needed to ensure a robust estimate.

In general, our population estimate of the number of birds using a clay lick are similar to those of Munn (1992) at a clay lick in the Manu Biosphere Reserve near Tambopata. This study used close-up photographs of RGMA individuals visiting clay licks and identified them by the lines of red facial feathers, and beak shape. They estimated the total number of RGMA using that clay lick to between 241 and 282 individuals over a month interval (Munn 1992).

7.5.3 Future recommendations for non-invasive genetic tagging

The genetic CMR methods we used, provide some of the first estimates of the number of birds using clay licks over the breeding season. However, it seems that there are many more individuals in the population than those we genetically identified. With more intensive sampling focusing at only a few major clay licks it should be possible to generate better estimates with narrower confidence intervals (Petit and Valiere 2006). We need to keep in mind that the moulting intensity of wild macaws is a still unknown component of our approach. However, we assume that even if we miss recaptures because feathers are not left behind at every visitation, the average number of recaptures can still give a good estimate of the population size if the sample size of the feathers is large enough.

The amount of information gained from feather samples seems remarkable given the tropical conditions and high likelihood that DNA will degrade quickly. However, to maximize the efficacy of genetic tagging we recommend the following sampling strategy at clay licks: (a) a pilot study should be conducted to determine the most reliable locations for feathers; (b) the focus should be on only a few sampling locations with the highest number of available samples; (c) feathers should be collected on a regular basis (e.g. every week); (d) detailed location of feathers on clay licks should be recorded to be able to differentiate depositing events of feathers with matching genotypes; (e) collected feathers should be handled as recommended by Chapter 6. Furthermore, an expanded sampling effort of several clay licks will be needed in order to firm up population size estimates of the area.

Further research using satellite telemetry and genetic tagging on macaws would help calibrate these two methodologies, especially with respect to small-scale movements and restricted clay lick use indicated by the genetic tagging.

7.5.4 Conclusion

Prior to this study of RGMA we had few insights into how many different individuals use the clay licks, and whether or not populations were related. We estimated the number of different individuals visiting clay licks. We found no evidence for bias in sex ratio at clay licks, or that populations consisted of related individuals. The matching genotypes detected show that individual macaws can use multiple clay licks, and that macaw populations using various clay licks can overlap in space. However, the locations of the genotype recaptures despite sampling over a large study area were highly restricted, indicating that local movements might occur more

frequently than longer-range movements (>30 km). This corresponds to the observation about RGMA in Manu, that the birds seem to fly less than seven km after they leave the clay lick (Munn 1992).

We conclude that macaws using clay licks probably represent feeding aggregations drawn from local populations. Thus, with sufficient sampling this technique can be used for meta-population size estimates, especially for species and locations where large number of non-invasive samples are easily available (e.g. seabird colonies). Furthermore, given the small degree of genetic differentiation across the >200 km scale of our specific study, we suggest that RGMAs operate as a single population on this scale. Maintenance of large protected areas of their habitat in Peru should remain a primary conservation aim for the future.

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7.8 Figures and tables

Figure 1. Sampling locations of moulted red-and-green macaw feathers in southeastern Peru. The 34 sampling sites are grouped into six populations (Piedras, Heath, Lower Tamboapta, Chuncho, Colorado, and Tavara) and four larger geographical regions represented by the same colour/shape (Piedras: orange/square, Heath: yellow/triangle, Tambopata: green/circle, and Candamo: red/star). Balloons indicate the exact locations of clay licks the populations were named after.

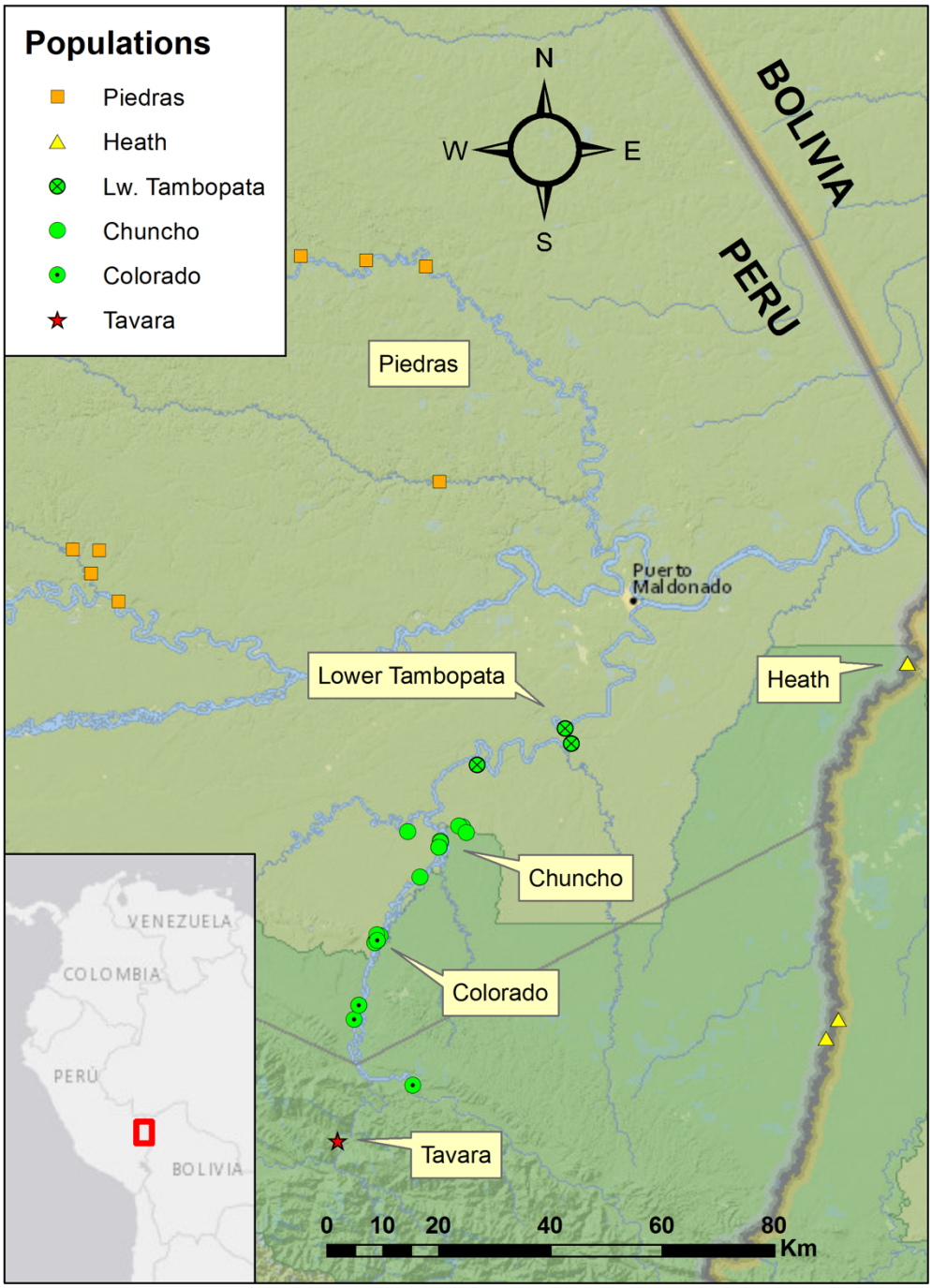

Figure 2. Pairwise relatedness results of Lynch and Ritland among clay licks of red-and-green macaw. Red lines represent permuted 95% confidence intervals – upper (U) and lower (L) – around the null hypothesis of zero relatedness and error bars represent bootstrapped confidence intervals around the mean.

* Control group includes known related individuals.

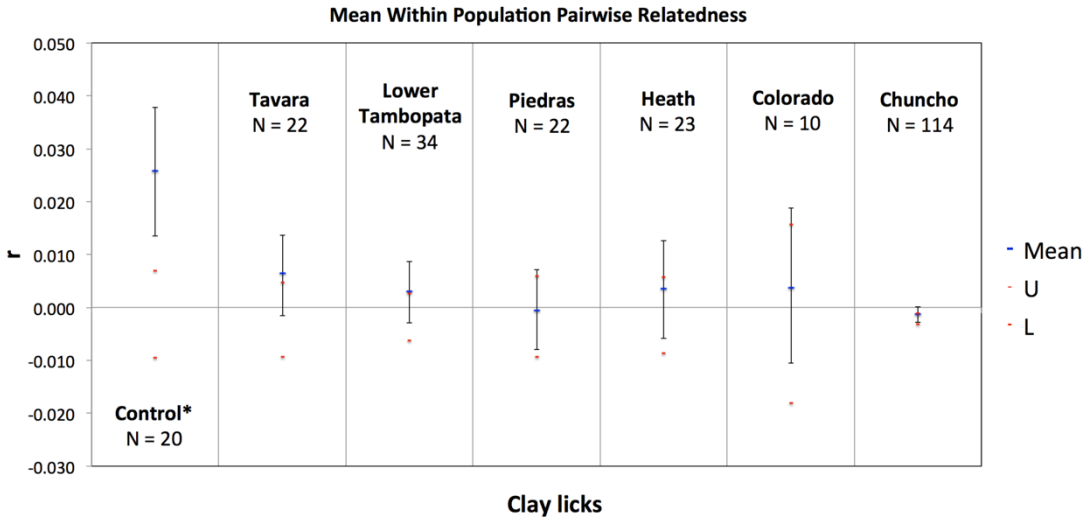

Figure 3. Complete genotype matches of red-and-green macaws showing recoveries in different sampling events, the distance (km), and the time interval (day) between recaptures in Tambopata, Peru.

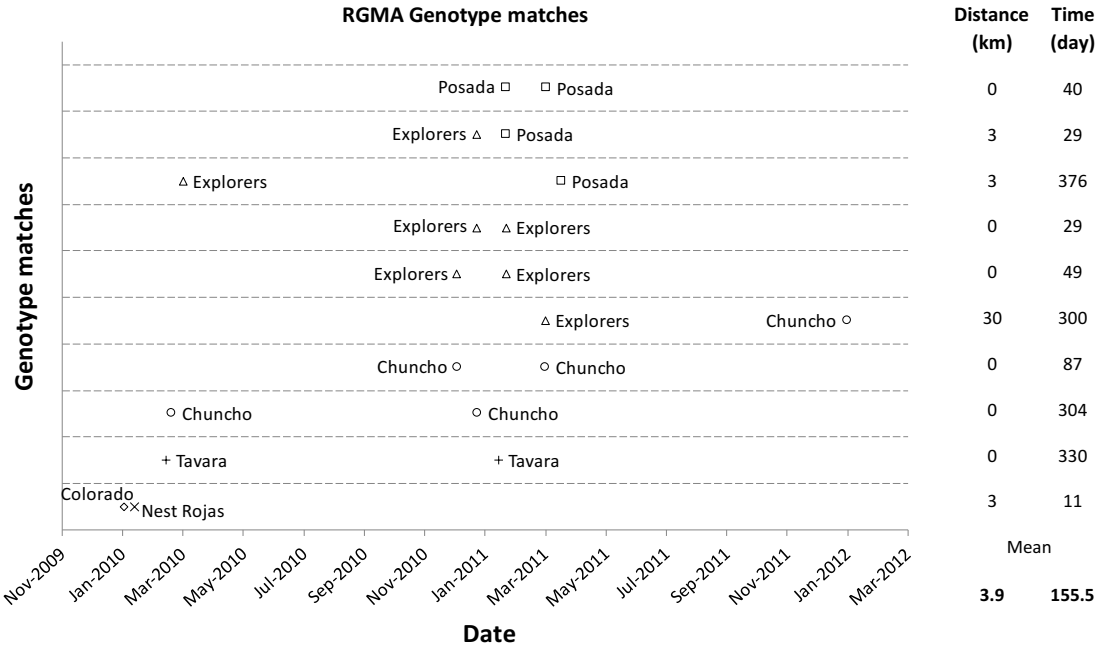

Table 1. Pairwise population genetic differentiations of red-and-green macaws between clay licks. F_{ST} values below diagonal; *P* values based on 1,000 permutations are shown above diagonal. Clay licks are in geographical order from south to north (foothills to lowland rainforest).

		P					
		Tavara	**Colorado**	**Chuncho**	**Lower Tambopata**	**Heath**	**Piedras**
		(N = 25)	(N = 15)	(N = 116)	(N = 36)	(N = 23)	(N = 24)
F_{ST}	**Tavara**	-	0.001*	0.010*	0.038*	0.008*	0.052
	Colorado	0.032	-	0.004*	0.004*	0.001*	0.093
	Chuncho	0.009	0.016	-	0.477	0.001*	0.287
	Lower Tambopata	0.008	0.021	0.000	-	0.003*	0.398
	Heath	0.015	0.041	0.013	0.016	-	0.298
	Piedras	0.009	0.010	0.001	0.000	0.002	-

Table 2. The number of red-and-green macaw feathers collected by sampling events at the major clay licks with the number of unique genotypes identified (in brackets) over the three study seasons.

If a matching genotype from a subsequent sampling event was found, it was considered as one less unique genotype only for the second event.

* indicates the sampling event where matching genotypes were found

Region	Population	Clay lick	2010			2011					2012		
			1	2	3	1	2	3	4	5	1	2	3
Tambopata	Lower Tambopata	Posada	0	0	1 (1)	2 (2)	0	6 (5)*	3 (2)*	2 (1)*	-	0	0
		Explorers	-	0	2 (2)*	4 (4)*	7 (5)*	16 (7)*	4 (4)*	0	-	0	0
		Gato	-	0	0	1 (1)	5 (3)*	-	0	-	0	-	-
	Chuncho	Chuncho	-	51 (49)*	0	1 (1)*	20 (18)*	2 (2)	39 (37)*	1 (1)	-	0	0
	Colorado	Colorado nests	1 (1)	1 (1)*	0	2 (2)	6 (4)*	0	0	0	1 (1)	5 (4)*	2 (2)
			-	4 (3)*	-	-	-	-	1 (1)	-	-	3 (3)	-
Candamo	Tavara	Tavara	-	9 (9)*	0	-	0	10 (9)*	3 (3)	0	0	4 (4)	0

7.9 Supplementary tables

Table S1. Genetic variation from four regions for nine polymorphic microsatellite loci for red-and-green macaw. Number of individuals sampled (*N*), number of alleles (*N*a), effective number of alleles (*N*e), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (*F*) are given. Populations are arranged from south to north. *Significant ($P < 0.05$) departure from HWE. Number of loci given in parentheses.

Region	*N*	*Na*	*Ne*	H_O	H_E	*F*
Piedras	44	9.00	4.64	0.729	0.750	0.027
Heath	26	8.00	4.59	0.697	0.758	0.086
Tambopata	183	10.89	4.76	0.720* (2)	0.744	0.031
Candamo	25	7.44	4.00	0.693* (1)	0.720	0.036
Mean		8.83	4.50	0.710	0.743	0.045

Table S2. Male and female red-and-green macaw ratio at clay licks based on sex typed feathers collected during the breeding season (November-April).

Region	Population	Clay lick	M	F
Piedras	Piedras	Cochacashu	6	2
		Tahuamanu	2	1
		Piedras	14	8
		Pariamanu	1	4
Heath	Heath	Inkanatura	14	7
Tambopata	Lower Tambopata	Posada	6	7
		Explorers	10	6
		Gato	3	1
	Chuncho	Chuncho	71	41
	Colorado	Colorado	12	3
Candamo	Tavara	Tavara	12	8
other (non clay lick)			10	17
		Total	**161**	**105**

Chapter 8

Landscape genetics reveals isolation-by-elevation in scarlet macaws of the Peruvian Amazon

Foothills of the Andes surrounding the Candamo valley (2010).

8.1 Abstract

Landscape genetics can assist conservation by identifying dispersal barriers or corridors that can be used for the development of conservation planning tools. Macaws have been studied for decades in their natural habitat, but we know little about their genetic structure across the landscape. We studied the landscape genetic structure of the long-lived (up to 50 years) scarlet macaw (*Ara macao*) in southeastern Peru within over 13,000 km^2 of continuous primary rainforest. Given their dispersal ability (individuals usually fly up to 15 km daily) we studied if high gene flow in scarlet macaws would eliminate signals of landscape genetic structure. We used individual- and population-level genetic analyses to examine whether natural barriers, including elevation, canopy tree height and above-ground carbon distribution influence dispersal (inferred from gene flow). Across the lowlands we found limited signals of population genetic differentiation and no sex-biased dispersal. However, a population living in Candamo, an intermountain valley of the Andes was genetically different from populations in the lowland rainforest of Tambopata. Landscape genetic resistance models showed an effect of elevation on gene flow: the mountain ridges between Candamo and Tambopata have probably restricted gene flow between these locations.

8.2 Introduction

Dispersal is essential for species persistence as it maintains genetic diversity, enables adaptation, influences spatial population dynamics, and allows individuals to locate new resources, avoid competition, and avoid inbreeding depression (Clobert et al. 2012; McDougald et al. 2012; Orsini et al. 2013; Szövényi et al. 2012). Because direct tracking to infer dispersal is notoriously difficult (Schofield et al. 2013) genetic analyses are valuable tools for understanding gene flow and dispersal (Frankham et al. 2004). In monogamous species, competition for both mates and resources is likely to affect both sexes equally, leading to predictions of equal rates of dispersal (Dobson 1982). However many monogamous birds show female-biased dispersal (Clarke et al. 1997), as predicted by the resource-competition hypothesis (Prugnolle and de Meeus 2002). Understanding the spatial genetic structures, sex-biased dispersal patterns, and the degree of isolation between populations are necessary for effective conservation planning (Keller et al. 2015). For example, habitat conservation should target areas where contemporary gene flow is restricted compared to historical gene flow due to habitat modification, to ensure that species maintain adaptive potential. Hence, recording natural gene flow in still intact natural habitats can serve as baseline data for other conservation genetic studies.

Habitat fragmentation from human activity such as habitat destruction, road construction, or urban development poses a global conservation problem by restricting species dispersal, resource availability and destroying key breeding habitats (Driscoll et al. 2013; Jaquiéry et al. 2011; Laurance et al. 2009). While many landscape features affect gene flow, structures such as roads, rivers, or mountain ridges are potentially impenetrable barriers for certain species (Storfer et al. 2006). If accompanied by a loss of genetic diversity, both human-caused or natural habitat fragmentation can reduce the adaptive potential of species (Frankham et al. 2010). On the other hand, these two separate processes can also lead to increased local adaptation and eventually speciation when habitat destruction is absent (Dias et al. 2013). Landscape genetics can elucidate interactions between landscape features and gene flow and selection (Manel and Holderegger 2013; Manel et al. 2003; Storfer et al. 2006), giving insights into dispersal barriers. Causal modeling of individual based genetic distances and multiple potential of landscape resistance provides a means to more rigorously evaluate the factors that limit gene flow (Cushman et al. 2006). Quantifying landscape effects and thresholds on gene flow is important for appropriate conservation management (Keller et al. 2015; Segelbacher et al. 2010) but these patterns are still unknown for most species.

The Amazon basin is a highly diverse and globally important ecosystem that remains poorly understood. Knowledge about genetic diversity of many Amazonian species, including the charismatic macaws (genera *Ara*, *Anodorhynchus, Cyanopsitta, Primolius, Orthopsittaca,* and *Diopsittaca*), is still lacking. Macaws have been studied extensively in their natural habitat in Tambopata, southeastern Peru for decades, providing insights about their breeding biology (Brightsmith 2005; Vigo et al. 2011), nest preferences (Olah et al. 2014), geophagy (Brightsmith 2004; Brightsmith and Aramburú Muñoz-Najar 2004), foraging ecology (Matuzak et al. 2008), parasitology (Olah et al. 2013), abundance (Lee and Marsden 2012), and reintroduction (Brightsmith et al. 2005). Comprehensive genetic studies are still lacking (Gebhardt et al. 2009) which limits our ability to identify the areas of habitat most in need of conservation. Macaws inhabit a large (more than 13,000 km^2) nationally protected lowland rainforest in Tambopata, but the rapidly growing human population along the recently built Inter-oceanic highway is a serious conservation issue in the region (Conover 2003; Tickell 1993). The still-healthy population of macaws (listed as least concern, IUCN 2014) within this area allows us to obtain baseline data for evaluation of their response to human induced habitat fragmentation. The nearby Candamo valley, a biological hotspot, is of commercial interest for oil extraction (Finer et al. 2008). Understanding the effects of natural landscape barriers to the dispersal of macaws and their present spatial genetic structure in an intact natural habitat can provide the scientific basis for local authorities to pinpoint core habitats for conservation of these birds and protect these sites from the increasing human activities in the region such as gold mining, logging, and oil extraction.

In this study we examined whether scarlet macaws maintain high gene flow in Tambopata. However, lowland rainforest (up to 900 m) is the preferred habitat of this species, so we expected that the foothills of the Andes Mountains (higher than 1,000 m) would be natural barriers to their dispersal. Since habitat suitability can also restrict gene flow (Wang et al. 2008), and scarlet macaws appear to prefer rainforest habitats with emergent trees where they can have free access to the canopy from the side (Britt et al. 2014), we also tested the effects of canopy height and carbon distribution (biomass) as proxies for habitat complexity. We used landscape resistance modeling to test the effects of these possible barriers (elevation, distance, canopy height, carbon distribution) on the genetic structure of the population. We also tested whether there was evidence for sex-biased dispersal. This is the first study to use extensive genetic data and landscape models to evaluate the population genetic structure of macaws in the Peruvian Amazon.

8.3 Methods

8.3.1 Study site and target species

This study was conducted between 2009 and 2012 in the Tambopata/Candamo region of the southeastern Peruvian Amazon, including two large protected areas: (1) the Tambopata National Reserve (2,747 km^2) with Rio Tambopata, Rio La Torre, Rio Gato, Rio Malinowsky, and the Tambopata Research Center (TRC; 13° 8.070'S, 69° 36.640'W); and (2) the Bahuaja-Sonene National Park (10,914 km^2) with Rio Heath, Rio Chuncho, Rio Tavara, and Rio Candamo. The sites are located in tropical moist forest (Tosi 1960) receiving an average annual rainfall of 3,236 mm (Brightsmith 2004). The elevation of the study site gradually increases from the Lower Tambopata (lowland rainforest with average elevation of 200 m), through Upper Tambopata (lowland rainforest closer to the foothills of the Andes Mountain, 260 m), until Candamo (intermountain valley, 350 m) surrounded by foothills of the Andes (>1,000 m) that separate this region from Tambopata (Fig 1).

The distribution of the scarlet macaw (SCMA) extends from Mexico to Bolivia (BirdLife International and NatureServe 2014). Although population sizes are decreasing in some regions, they are presently listed as Least Concern with an estimated global population size between 20,000 and 50,000 individuals (IUCN 2014). The species occupies rainforest from sea level up to 900 m (BirdLife International 2014). They are secondary cavity nesters using hollows of emergent trees (Brightsmith 2005; Renton and Brightsmith 2009) and also occupy artificial nests in our study site (Olah et al. 2014).

8.3.2 Sample collection and genetic markers

We captured adult macaws and nestlings in natural and artificial nests and sampled their DNA during the breeding season (Nov–April) each year. A small (~100 µL) blood sample was taken from the jugular vein of 22 nestlings and 18 adults and stored in 70% ethanol or on FTA paper (Whatman). We also collected 126 DNA samples non-invasively by sampling naturally shed feathers from nests, around nesting and roosting sites, and from clay licks (Fig. 1). Molecular sexing of samples was performed using the P8_SCMA_F/P2_SCMA_R primers (Chapter 6).

We used species-specific, polymorphic microsatellite markers (Olah et al. 2015) for individual identification via genetic tagging (Chapter 6). DNA was amplified at nine microsatellite loci (SCMA 09, SCMA 14, SCMA 22, SCMA 26, SCMA 30, SCMA 31, SCMA 32, SCMA 33, SCMA 34) (Olah et al.

2015). Genotyping errors were calculated from randomly selected samples that yielded full genotype data, and the markers selected in this study showed low or no error and low amplification failure (Chapter 6). Repeated genotypes were excluded from the data set. When blood samples from known family units were identified, siblings or parent/offspring samples were also excluded from the analyses (keeping samples of one parent whenever possible), so that only non-related individuals were included in the data set.

8.3.3 Population structure analysis

We used two different Markov chain Monte Carlo (MCMC) Bayesian clustering models to identify potential population genetic structure. The first model was run in STRUCTURE 2.3.4 (Pritchard et al. 2000) for which we used the admixture model, correlated allele frequencies, and no location priors (Falush et al. 2003). Burn-in was set to 50,000 iterations, followed by 50,000 MCMC iterations and replicated 10 times for each value of the number of genetic clusters (*K*) from 1 to 10. We used STRUCTURE Harvester (Earl and vonHoldt 2012) to determine *K* (Evanno et al. 2005). The second model was GENELAND 4.0.0 (Guillot et al. 2005) which includes geographical coordinates for each individual. This makes it more sensitive to weak genetic structure than STRUCTURE because spatially adjacent individuals are more likely to be in the same cluster (Guillot et al. 2012). We used the uncorrelated allele frequency model (Guillot et al. 2005) with 100,000 MCMC repetitions, and allowed *K* to vary between 1 and 10, with 5 independent runs. We set spatial uncertainty of coordinates to 15 km based on estimated daily movement.

8.3.4 Population-level genetic differentiation

We divided the study area into three *a priori* defined equal sampling sites: Lower Tambopata, Upper Tambopata, and Candamo (Fig 1). We used analysis of molecular variance (AMOVA) in GenAlEx 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) to partition genetic variation within and among these sampling sites and to estimate overall and pairwise population genetic differentiation (F_{ST}) (Excoffier et al. 1992; Peakall et al. 1995; Wright 1965). Tests for genetic differentiation were performed by random permutation (1,000 permutations).

8.3.5 Individual-level sex-biased dispersal analysis

We used analysis of spatial autocorrelation for each sex separately (Smouse and Peakall 1999) to investigate potential differences in spatial genetic structure between sexes. Pairwise genetic distance (*r*) was calculated between all pairs of individuals (Smouse and Peakall 1999) and geographic distance was calculated from UTM coordinates of each sample's location. The distance class sizes were set to match the predicted movement of macaws around the breeding sites and among clay licks (2, 15, 30, 50, and 75 km). Significance of *r* was tested by random permutation with a 95% confidence interval around *r* and also via bootstrapping (Peakall et al. 2003). We inferred significant spatial genetic structure when *r* fell outside the 95% permuted confidence interval around zero and when bootstrap estimates around *r* did not overlap zero. We also used the corrected assignment index (*AIc*) (Favre et al. 1997) to determine the expected frequency of each individual's genotype in the population from which it was sampled, corrected for population effects. Overall *AIc* values will average to zero for the population as a whole with a significant difference in the means of males and females if sex-biased dispersal occurs.

8.3.6 Individual-level landscape resistance analysis

This analysis was restricted to a subset of the data (112 samples) sourced from Upper Tambopata (lowland rainforest) and Candamo (intermountain valley) as the two closest populations isolated by the foothills of the Andes. We developed four landscape resistance models to examine factors affecting gene flow among these rainforest habitats at the individual level. The models were based on maps sourced from the Carnegie Airborne Observatory (Asner and Martin 2008; Asner et al. 2011). (1) The elevation above sea level model (Elev) investigated the effect of elevation. (2) The tree canopy height (TCH) model considered the distribution of emergent canopy trees ideal for nests. This landscape feature would probably not pose barriers to their movements but might restrict gene flow through an effect of habitat suitability. (3) The above-ground carbon distribution (ACD) represents biomass in the forest (Girardin et al. 2010) which we used as a proxy for habitat complexity. Scarlet macaws in tropical rainforest appear to prefer riverside habitats with gaps in the canopy (Britt et al. 2014), which also contain less biomass. Finally, (4) a river distance (Rio) model was calculated with a 1 km buffer zone along the river system, as the low elevations around the rivers as the preferred riverside habitat of the SCMA might act as conductance in their movements.

Because our *a priori* assumption was that the macaw population in the Candamo valley is different from the nearby lowland macaw population, we defined the extent of our study landscape with a squared map including an almost equal geographic size of Candamo (intermountain valley) and Tambopata (lowland rainforest). In this way we had an equivalent sampling frame with nearly equal geographical- and sample size from two adjacent populations. We compiled each resistance model on a separate raster grid with a 100m resolution in ARCMAP 10.2 (ESRI).

We used CIRCUITSCAPE 4.0.3 to generate values of landscape resistance between each pair of samples, taking into account all possible pathways between pairs (McRae and Beier 2007). We also generated an isolation-by-distance null model (IBD) by giving each cell a value of '1' (Cushman et al. 2006).

We used the mantel function in the 'ecodist' library (Goslee and Urban 2007) for R (R Core Team 2013) and obtained *P*-values with 5,000 permutations. For IBD models, we used simple Mantel tests and one-tailed *P*-values ($\alpha = 0.05$). For other resistance models, we used partial Mantel tests and two-tailed *P*-values to determine significant relationships between genetic distance and landscape resistance, given the spatial distance between samples (Goslee and Urban 2007). When there was a significant correlation in the first partial Mantel test, we calculated the effect of the IBD model on genetic distance while controlling for the landscape resistance model. Where the first partial Mantel test was significant and the second test was nonsignificant, we inferred that the significant effects of that landscape resistance model was genetic distance, not spatial distance (Cushman et al. 2006; Smith et al. 2014).

8.4 Results

8.4.1 Population genetic analyses

In the studied SCMA population the mean allele number (*Na*) was 13 and mean expected heterozygosity (H_E) was 0.873 (Table S1). STRUCTURE and Geneland indicated a single genetic cluster and lack of population boundaries among the overall sample (Fig S1).

Consistent with the STRUCTURE results, the AMOVA analysis revealed low levels of differentiation by attributing 0.46 % of genetic variation among the 3 populations in Tambopata/Candamo (N = 166, F_{ST} = 0.005, F'_{ST} = 0.041, P = 0.001). The Candamo population was significantly different from the other two populations (Lower Tambopata F_{ST} = 0.008, P = 0.003; Upper Tambopata F_{ST} = 0.008, P = 0.001) driving the overall significant differentiation. Upper and Lower Tambopata did not show significant genetic differentiation (F_{ST} = 0.001, P = 0.199).

We identified 74 males and 69 females (23 unknown). There was no positive fine-scale spatial genetic structure (Fig S2A) that would indicate evidence for restricted dispersal. There were no significant differences in the frequency distributions of corrected assignment indices (*AIc*) between males (mean *AIc* = -0.04 ± 0.17; N = 59) and females (mean *AIc* = 0.04 ± 0.15; N = 60; Fig S2B,C) indicating no sex-biased dispersal.

8.4.2 Landscape resistance models

There was a significant effect of elevation on genetic distance between Candamo and Upper Tambopata (r_M = 0.128, P = 0.032; Table 1). Since restriction by elevation would operate in a one-way scale, we also report one-tailed P-value (P = 0.02). Both isolation by distance (about 40 km between core regions) and elevation (max. 1,200 m) were significant explanatories of the genetic distance in a simple Mantel test (P_{IBD} = 0.021, P_{Elev} = 0.01; Table 1). However, isolation-by-distance was not significant when controlling for the elevation model (r_M = -0.104, P = 0.98; Table 1), so we inferred that the significant effects of the elevation model was genetic distance.

8.5 Discussion

Our three-year, mainly non-invasive landscape genetics study showed that SCMA in the southeastern Peruvian Amazon had low genetic structure with an absence of sex-biased dispersal. We found some evidence for isolation-by-elevation suggesting that high elevation (>1,000m) may act as natural barrier and restrict gene flow of macaws in their rainforest habitat.

8.5.1 Population structure of scarlet macaws in Tambopata

Our study site consists of two adjacent protected areas (the Tambopata National Reserve and the Bahuaja-Sonene National Park) with a total area of more than 13,000 km^2 primary rainforest. The Bayesian approaches could not detect any population structure in Tambopata, supporting our hypothesis of no genetic differentiation. Similarly, Wright et al. (2005) found no defined genetic structure among yellow-naped amazon, *Amazona auropalliata* populations over Costa Rica, despite their highly fragmented habitat. Restricted dispersal within populations results in positive local spatial genetic structure where relatedness between individuals declines with increasing geographical distance. We could not find any evidence for this in our study species, indicating no discernible restrictions in their dispersal. The low overall population genetic differentiation in these macaws probably also reflects large dispersal distances.

In Costa Rica, the two major SCMA populations show high level of genetic differentiation (F_{ST} = 0.048; P < 0.01) with only 80 km of distance (Monge et al. 2015). Similarly, Schmidt (2013) showed significant genetic differentiation between SCMA populations (20% variation) of Guatemala and Belize 170 km apart in a more fragmented landscape, however they did not find any significant differentiation among the core nesting sites in Guatemala up to 35 km apart (-0.005 < F_{ST} < 0.009). Consistent with our results, Faria et al. (2008) found no genetic differentiation between two populations of hyacinth macaw, *Anodorhynchus hyacinthinus* 100 km apart in Pantanal, Brazil, without any physical barrier, and they only found strong genetic differentiation (F_{ST} > 0.25; P < 0.05) from another population about 1,600 km away.

In the lowland rainforest of Tambopata SCMA can be found in relatively large densities of 1.47 individuals/km^2 (Lee and Marsden 2012). Given their abundance and over 13,000 km^2 of continuous and protected primary rainforest to disperse, it is therefore not surprising that strong population genetic structure was absent in our study area with a maximum Euclidean distance of 80 km. Our results indicate that large macaw species can disperse very large distances over intact habitats (Faria et al. 2008). However, fragmented habitats can restrict their gene flow resulting

genetic differentiation between populations in less than 200 km apart (Monge et al. 2015; Schmidt 2013). Our results further emphasize that maintaining large and connected protected areas is critical for the extensive gene flow of these species.

8.5.2 Absence of sex biased dispersal

Evidence for sex-biased dispersal is still unresolved in parrots, which are mainly monogamous species. Female-biased dispersal was suspected in the yellow-naped amazon (Wright et al. 2005) but male-biased dispersal has been found in blue-and-yellow macaw (Caparroz 2003). Individual-focused multilocus spatial autocorrelation analyses have proven useful for testing sex-biased dispersal and are more effective than population-level tests (Banks and Peakall 2012), like the corrected assignment index (*AIc*) that can rarely detect sex-bias if the dispersal intensities are less than 80:20 (Goudet et al. 2002). In this study we did not detect any sex-biased dispersal patterns using either individual or population level tests. Juveniles of SCMA stay with their parents in the first few months post fledging (Brightsmith DJ pers. comm.). Most probably, these juveniles of both sexes remain with or around the parents over many years, and there might be no difference between males and females in the frequency or distance of dispersal.

8.5.3 Isolation-by-elevation in macaws

Our landscape genetic model showed significant evidence for isolation by elevation between the intermountain valley of Candamo and the lowland rainforest of Tambopata separated by mountain ridges. Our results from the analysis of molecular variance also showed small but significant genetic differentiations between Candamo and the other two macaw populations outside of the valley. These results might point towards more restricted gene flow between the birds in this valley and the two lowland populations separated by high (about 1,000 m) foothills of the Andes (Fig 1).

According to a recent satellite telemetry study in our study area in Tambopata, based on 10 captured and tagged birds, SCMA have an estimated average nine-month home range of 1,730 km^2 (D.J. Brightsmith, unpublished data). These long distance movements can explain the low level of genetic differentiations found in our study system. All the captured and satellite tagged SCMAs seemed to move to the NE up to 150 km, much further than the 88 km distance from Candamo to Lower Tambopata (Fig 1). Interestingly, none of the tagged macaws were detected in

Candamo, or even flew near to the foothills that separate Candamo (D.J. Brightsmith, unpublished data).

Faria et al. (2008) showed that parrots and macaws are capable of flying long distances over large landscapes, hence their dispersal patterns are likely to reflect the selective use of habitat, the available forest for nesting, and spatial and temporal patterns of key food sources. In addition, our landscape resistance model indicates that high elevation may possibly create barriers for these species. Monge et al. (2015) argued that given the strength of the high genetic differentiation between the two large SCMA populations in Costa Rica based on seven microsatellite markers (F_{ST} = 0.048, P < 0.01), it is probably due to the montane barriers rather than recent habitat fragmentation. The central cordilleras of Costa Rica and Panama ranging in elevation from 500 to 3,800 m separate the two subspecies of SCMA (Schmidt 2013), further suggesting that mountains can act as geographic barriers for macaws.

Patterns of molecular variance are correlated with geography and climate in burrowing parrots, *Cyanoliseus patagonus* in the Andes Mountains (Masello et al. 2015). Wright et al. (2005) tested for association between genetic distance and vocal dialect in yellow-naped amazon, while controlling for geographic distance, but found that vocal dialect boundaries did not act as barriers in gene flow as they suspected (also see in Wright and Wilkinson 2001). Similar non-geography related resistance data would also be very important to test for in future analyses of gene flows in macaws as well.

8.5.4 Conclusion and implications for conservation

In our study we found no evidence of defined population genetic structure of SCMA in Tambopata or genetic differentiation among lowland rainforest sites. Apparently, gene flow in these long-lived birds is extensive in the study area. Their ability to fly over large home ranges is reflected in the absence of any dispersal patterns. When combined, the evidence points towards a single population of SCMAs in Tambopata, over a large protected area. However, our study also suggests that high elevation might act as a natural barrier to their gene flow. As the Candamo population was the most genetically isolated from the other lowland populations due to a mountain barrier, this highlights potential conservation importance. Intermountain rainforest valleys similar to Candamo can host other populations of SCMAs over their distribution range. The Candamo valley also hosts a large diversity of other species, some with much more restricted dispersal movements than large macaws. Our results suggest that maintaining connected protected areas, including intermountain rainforest valleys like Candamo, and minimizing human

disturbances like gold mining or oil extraction need to remain a conservation priority. Similar genetic studies are important to identify further areas of habitat most in need of conservation.

8.6 Acknowledgements

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8.8 Figures and tables

Figure 1. Locations of scarlet macaw (*Ara macao*) DNA samples in southeastern Peru. Balloons indicate major clay licks.

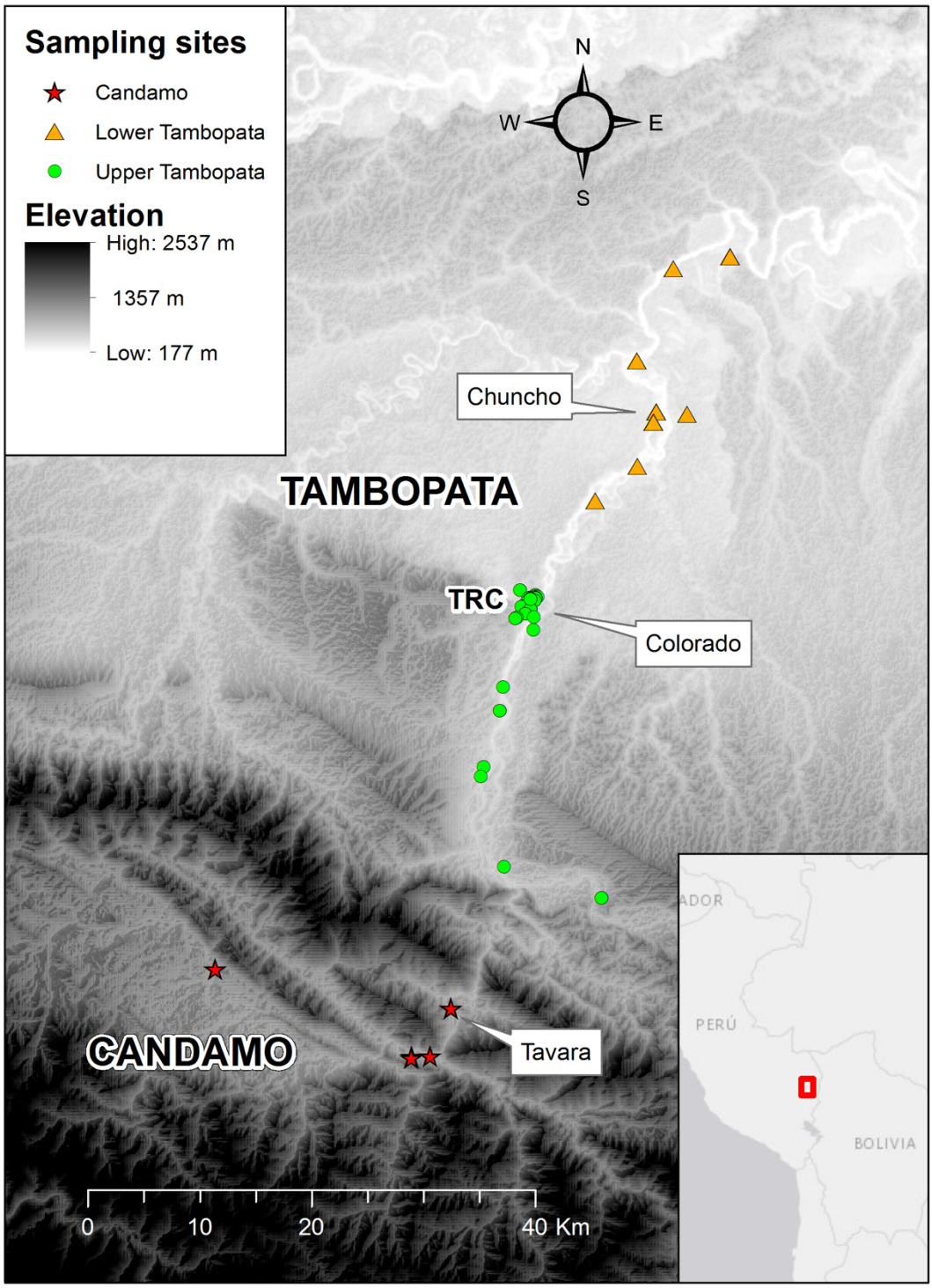


Table 1. Results from causal modeling of the effect of geographic distance (IBD) and different landscape resistances (ACD, Elev, TCH) on genetic distance in scarlet macaw (*Ara macao*). For partial mantel tests, genetic distance (y) was modeled as a function of x given z (y~x|z).

IBD = isolation by distance;

ACD = aboveground carbon distribution;

Elev = elevation above sea level;

TCH = tree canopy height;

Rio = river distance.

Test type	**Predictor**	**Mantel test**		**95% Confidence interval**	
		r_M	***P***	**Lower**	**Upper**
Simple	IBD	0.080	0.021*	0.045	0.113
Partial 1	ACD|IBD	-0.031	0.640	-0.074	0.006
Partial 1	**Elev|IBD**	0.128	0.032*	0.087	0.165
Partial 1	TCH|IBD	-0.027	0.681	-0.068	0.019
Partial 1	Rio|IBD	-0.033	0.632	-0.050	-0.007
Simple	Elev	0.110	0.010*	0.067	0.149
Partial 2	**IBD|Elev**	-0.104	0.980	-0.138	-0.066

* $P < 0.05$

8.9 Supplementary material

Table S1. (A) Average genetic variation of nine polymorphic microsatellite loci for scarlet macaw (*Ara macao*).

Locus	*N*	Size range (bp)	*Na*	*Ne*	H_O	H_E	*F*
SCMA 22	163	114-160	18	12.527	0.926	0.920	-0.007
SCMA 32	158	175-211	16	10.432	0.829	0.904	0.083
SCMA 34	164	151-189	17	7.898	0.841	0.873	0.037
SCMA 33	166	174-212	18	10.297	0.867* (1)	0.903	0.039
SCMA 26	160	210-240	14	8.994	0.831* (2)	0.889	0.065
SCMA 09	164	112-136	12	5.197	0.787	0.808	0.026
SCMA 14	163	220-252	14	8.538	0.773	0.883	0.124
SCMA 30	156	206-246	17	9.402	0.885	0.894	0.010
SCMA 31	138	137-169	15	8.264	0.877	0.879	0.002
Mean			15.7	9.061	0.846	0.884	0.042

Presented are locus code, number of samples (*N*), fragment size ranges, number of alleles (*N*a), effective number of alleles (*N*e), observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (*F*).

- Significant ($P < 0.05$) departure from HWE. The number of populations is given in parentheses.

(B) Genetic variation from three populations of scarlet macaw (*Ara macao*) for nine polymorphic microsatellite loci.

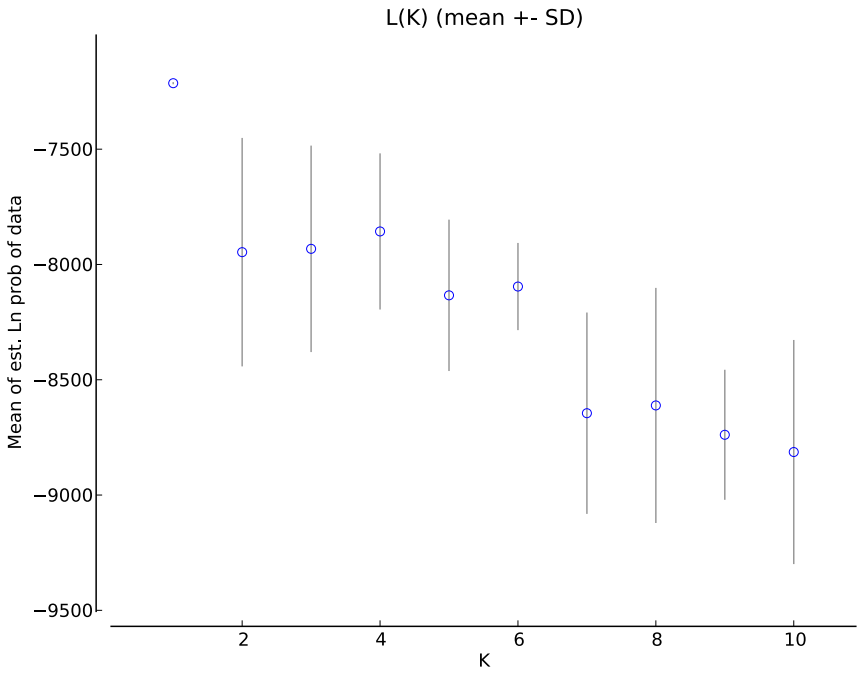
Population	***N***	***Na***	***Ne***	H_O	H_E	***F***
Lower Tambopata	54	13.78	8.69	0.833* (1)	0.875	0.048
Upper Tambopata	82	14.11	8.77	0.866* (2)	0.881	0.018
Candamo	30	11.11	7.58	0.817	0.862	0.052
Mean		13	8.35	0.838	0.873	0.040

Number of individuals sampled (*N*), number of alleles (*N*a), effective number of alleles (*N*e), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (*F*) are given. Populations are arranged from south to north.

* Significant ($P < 0.05$) departure from HWE. Number of loci given in parentheses.

Figure S1. Results from (A) STRUCTURE Harvester (Earl and vonHoldt 2012) and (B) GENELAND indicated that a single genetic cluster was most likely.

(A)



(B)

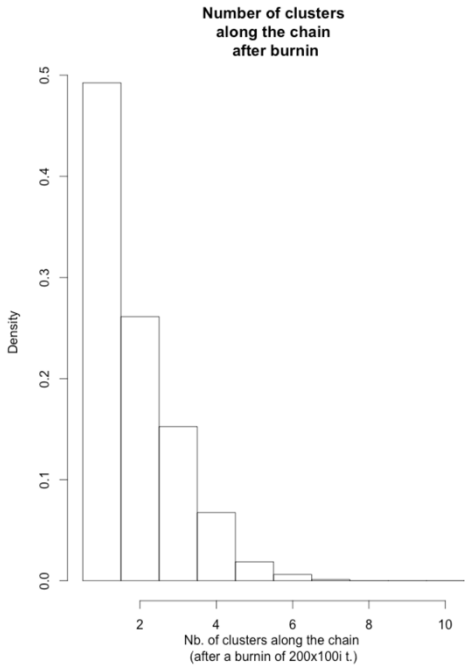
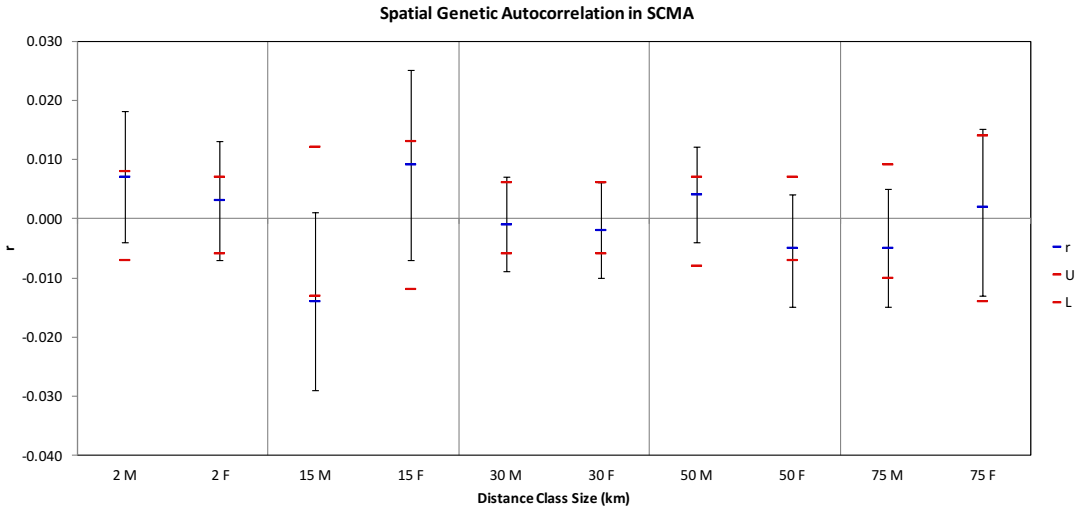
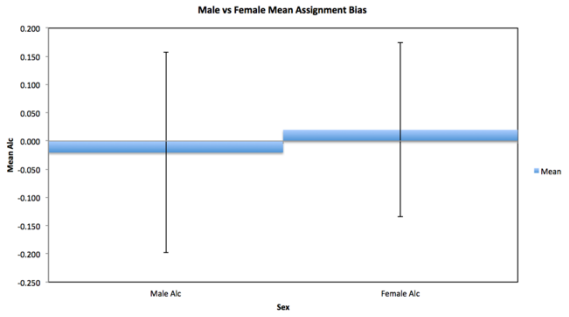


Figure S2. Sex specific analyses for scarlet macaw (*Ara macao*).

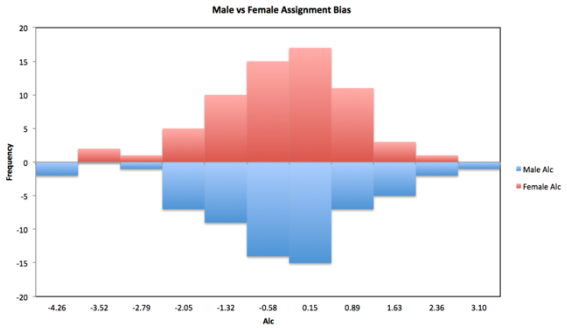
(A) Spatial Genetic Autocorrelation: graphs showing comparison of correlations for females versus males across study sites *rc* for increasing distance class sizes, 95% CI about the null hypothesis of a random distribution of scarlet macaw, 95% confidence error bars about *rc* as determined by bootstrapping.



(B) Mean Assignment Bias



(C) Assignment Indices (*AIc*) among males and females (positive values indicate individuals probably born near where they were sampled; negative values indicate individuals with a higher likelihood of being immigrants).



Chapter 9

Priorities for future conservation and genetic studies on parrots

Blue-throated macaw inspecting a possible nest hollow in Beni, Bolivia (2008).

9.1 Thesis overview

In this thesis I have employed a wide array of interdisciplinary methods to study parrots, spanning from statistical modeling and on-site ecological studies of their nest preferences, to population genetic techniques. After establishing the broad range of factors associated with extinction risk (Chapter 2) the field component of the thesis focused on two large macaw species in the Peruvian Amazon, the scarlet macaw (*Ara macao*) and the red-and-green macaw (*A. chloropterus*). While both are considered globally of 'least concern' (IUCN 2014), the various techniques developed on these widely accessible birds also provide verified tools for conservation, which I discuss below.

My investigation of the nesting ecology of macaws showed that they prefer certain nest hollows against others but in general they use artificial nests similarly to natural ones (Chapter 3). However, I found that macaw nestlings in artificial nests are more prone to parasitic bot fly larvae infestation than nestlings in natural nest hollows (Chapter 4). After developing new genetic markers for macaws (Chapter 5) and genotyping hundreds of DNA samples from wild population of the two study species (Chapters 6), I showed low genetic structure that indicates extensive gene flow in the Tambopata region. Using genetic tagging I was able to recapture matching genotypes over a large tropical study site and use this data to estimate the number of different individuals visiting clay licks, their sexes, relatedness, genetic diversity, and aspects of their clay lick use in time and space (Chapter 7). I found weak but significant genetic differentiation between the Candamo valley isolated by tropical mountain ridges (Chapter 8), and the rest of the study area, which suggests that landscape barriers may restrict free movement of these large and mobile birds.

In order to further improve our knowledge of these wild populations of macaws, satellite telemetry of individuals would be extremely useful. These data could confirm our findings concerning the recaptures using genetic tagging. Following individuals by precise GPS tags could be used for studying local fine-scale habitat use, e.g. defining the exact catchment areas around clay licks. This technique could also help to define isolated populations. For instance, capturing macaws in the Candamo valley and tracking them over a year could either confirm their restricted movement by the mountain barriers, or reveal an alternative pathway of their movement, e.g. following the river system. These and similar new techniques should be implemented through well-designed field studies on macaw populations in the Peruvian Amazon and also in other areas.

9.2 Applications to other systems

At least five key methods that were further developed or refined in this thesis should be broadly applicable to other study systems in the Amazon Basin or indeed in other tropical regions.

First, the artificial nests used in this study (Chapter 3) can easily be used for the same species elsewhere, where key nesting trees are not available anymore due to selective logging or other habitat destruction. The same nest designs can also easily be adopted for other similar size macaws or smaller size parrots with similar nesting habits. Artificial nest boxes are now used in recovery programs of the blue-throated macaws (*Ara glaucogularis*), a critically endangered species with only about 100 individuals left in the wild in Beni, Bolivia (I. Berkunsky, pers. comm.). Second, the parasitic *Philornis* fly infesting macaws in Tambopata (Chapter 4) has a mainly Neotropical distribution (Carvalho and Couri, 2002), and is a known threat to other parrots (Berkunsky et al. 2005; Nycander et al. 1995; Renton 2002). The knowledge of their biology and the novel technique we developed for their removal described in this thesis can also be used in other areas for various species. Further modifications of the artificial nests, mainly by improving thermoregulation to avoid overheating and infestation by parasites, can provide very promising tools for the conservation management of macaws and other species.

Third, I developed new species specific genetic markers for the scarlet macaw and showed similarly high variability in a closely related species, the red-and-green macaw (Chapter 5). With possible limitations, for example ascertainment bias (Ellegren et al. 1997; Peakall et al. 1998), the same markers can also be useful for other macaw species or for other Neotropical parrots. The microsatellite markers developed in this study are currently being tested in a conservation genetics study of the critically endangered blue-throated macaw (T. Wright, pers. comm.).

Fourth, I demonstrated the use of these genetic markers for individual identification via genetic tagging in macaws (Chapter 6). For many other threatened parrots the non-invasive genetic sampling of moulted feathers may be the only available DNA source. My study site contains an unusually high number of clay licks (Lee et al. 2010), which offered an excellent opportunity to sample moulted feathers non-invasively and repeatedly. Other regions might not contain similar sites where feathers can be obtained in such large quantity. However, feathers can still be found in various locations in the field, e.g. inside or below nests, below roosting sites, and fruiting trees.

Fifth, I verified the value of genotype recapture over a large landscape for gaining many important insights of the species biology, for instance the estimated number of different individuals visiting clay licks, their sexes, relatedness, genetic diversity, and aspects of their clay

lick use in time and space (Chapter 7). The genetic tagging technique developed for my study species can also be implemented for other parrots with higher conservation concern, or for other birds where access to large number of samples are easily available (e.g. see bird colonies). In the field study design, it is important to take into consideration any prior knowledge about the scale where the birds' movements might occur in order to obtain the correct information. For instance, for species with smaller predicted home range, closer sampling locations should be chosen to obtain enough genetic recaptures for population size estimates. Timing of the collection is also an important element, as parrots can show seasonal migration patterns that can affect the probability of recapture (Salinas-Melgoza and Renton 2005).

9.3 Comparison of the two study species

This study revealed new insights into the biology of two sympatric macaw species that appear to be similar in their size and ecology. I have collected several hundred samples from both species over my large study site (over 13,000 km^2 in size). I found RGMA samples in a much broader geographic area with their range extending to the river systems of Piedras, Amigos, and Heath (Chapter 7, Fig 1). In contrast, SCMA samples were more aggregated in the Tambopata region, especially around the Chuncho clay lick and the Tambopata Research Center. Thus RGMA is generally more abundant in a wide region of southeastern Peru, while SCMA populations accumulate more in some specific locations of the same region. This is further supported by the full genotype recovery data, as I found much the RGMA genotype recaptures were drawn from a wider region (up to 30 km), whereas the few SCMA recaptures occurred in a much more restricted area (up to 2 km).

The genetic analyses of both species showed relatively high genetic diversity and very low genetic differentiations among populations. Indeed, the only significant differentiation detected was between the Candamo and Tambopata populations for SCMA, probably given the mountain barrier between these two regions. The genetic results indicate that both species maintain extensive gene flow over the large, mainly protected area of southeastern Peru, with samples drawn from across this region matching Hardy-Weinberg Equilibrium expectations.

9.4 Threats to parrots and implications for their conservation

My analysis established that parrots are more threatened on average than comparable bird species groups, and a wide range of factors are associated with their endangerment (Chapter 2).

Some of these were expected, for example variables associated with life history and habitat. Extinct parrot species were larger on average than extant parrots, and most extinct species were single country endemics and/or insular species. The Neotropics have the highest proportion of threatened species, followed by Australasia and Oceania, Afrotropics, and Indomalaya.

My analysis also revealed some novel and unexpected findings concerning socio-economic factors that should be explored further. For example, the models revealed that parrots are more likely to be highly threatened if their distribution falls within a single country's jurisdiction, and in countries with higher per capita GDP. This is presumably because the higher levels of development in these countries tend to drive the major threats to parrots worldwide including logging, agriculture, hunting, and trapping. Further meta-analysis of parrots including more details about their threats, and trade in particular, should be considered for better understanding of their decline worldwide.

The most important actions to conserve parrot species are to protect their habitat from logging and illegal poaching (Carrete and Tella 2008; Pain et al. 2006; Snyder et al. 2000; Wright et al. 2001). However, habitat reforestation does not always offer an immediate remedy for secondary cavity nesters such as parrots because the tree hollows they need take many decades to form. Here, artificial nests may be crucial as outlined already.

Ecological studies have focused on the breeding biology of many parrot species, which have also contributed important information for their conservation (Brightsmith and Bravo 2006; Heinsohn 2008; Lanning and Shiflett 1983; Martuscelli 1995; Pasquier et al. 1981; Pitter and Christiansen 1995; Smith 1991; Theuerkauf et al. 2009; Tobias and Brightsmith 2007). For long-term conservation management planning, knowing the species' breeding habits and reproductive rates are not always enough. For example, for population viability analysis both deterministic factors (habitat destruction, over-exploitation, pollution, introduced alien species, etc.) and stochastic factors (demography, environmental changes, genetics, catastrophic events, etc.) need to be considered (Heinsohn et al. 2015). The importance of population genetics is now also recognized explicitly in such models (Allendorf and Ryman 2002; Reed et al. 2002).

By definition, conservation genetics is the use of genetic theory and techniques to reduce the risk of extinction in threatened species (Frankham et al. 2004). It includes the use of molecular genetic analyses to clarify the natural history of species relevant to their conservation management. Conservation genetic techniques can also highlight inbreeding depression, losses of genetic diversity, and reductions in gene flow due to human activities or barriers. It can advise the genetic management of small populations, reintroduction, and help resolve taxonomic questions (Frankham et al. 2010). Regardless of the many potential applications of genetic techniques, there

have been relatively few applications to parrot conservation (Faria et al. 2008; Leite et al. 2008; Melo and O'Ryan 2007; Raisin et al.; Taylor and Parkin; Triggs and Daugherty 1996; Triggs et al. 1989; Wenner et al. 2012).

Next-generation sequencing now routine in many fields, may help fast track the uptake of genetic analysis in parrots that seem to have been somewhat neglected, until now. For example, thousands of informative loci can be identified from a single next-generation sequencing run, and it can also provide preliminary information on genetic polymorphism if several genotypes are sequenced (Guichoux et al. 2011). This facilitates the development of new species-specific genetic markers for parrots that can be used for genetic tagging. These techniques can also be applied in wildlife forensic investigations, e.g. to uncover trading routes (Huffman and Wallace 2012). These and other novel techniques will hopefully be used more extensively among parrot researchers. A new 'Parrot Research Group' of the International Ornithologists' Union (IOU) is now well positioned to facilitate international collaboration and quick information sharing among parrot experts.

9.5 Ecotourism for conservation

A study of macaws in southeastern Peru more than 20 years ago stated that "with appropriate interpretation and marketing, almost any parrot species in the world could become a good subject for a successful ecotourism program" (Munn 1992). Motivated by this possibility, one year later a local ecotourism company (Rainforest Expeditions) was established at a nearby site in Tambopata featuring macaw research (Nycander et al. 1995). Ecotourism can be an effective way to conserve ecosystems by employing local people and funding conservation research at the same operation site (Brightsmith 2008). It can also effectively utilise volunteer tourism for conservation research projects in the area (Brightsmith et al. 2008).

My primary study site was situated in the Tambopata Research Center, operated by Rainforest Expeditions, offering me inside knowledge of how this ecotourism company helps conservation. The attitudes of people from the local communities changed dramatically, as they began to exploit new opportunities to work as field guides, research assistants, and staff at local lodges. It seems that many have concluded that "a bird in the bush is worth two in the hand" (Munn 1992), and that trees have a higher value if left standing rather than being logged and sold as wood material (K. Holle, pers. comm.). The comparison with nearby locations in the Madre de Dios region is stark, as areas outside the conservation zone have been severely affected by illegal gold miners and loggers, while Tambopata has remained relatively untouched so far under the protective dome of

intensive ecotourism. This example can serve as a model that should be implemented in many other regions with similar features.

Future conservation efforts to protect parrot species will need to employ a wide array of multidisciplinary methods such as on-site conservation management, ecological studies, reintroductions, and conservation genetics. However, success will be critically dependent on local people involved in these projects, ideally with a direct stake in the economic benefits (Barré et al. 2010; Brightsmith et al. 2008). For such involvement, local ecotourism could be a great support and also a non-academic funding source for conservation research (Brightsmith 2008).

9.6 Conclusion

This multidisciplinary study of macaws has generated many new insights into their ecology and population genetics. Artificial nest boxes designed in Tambopata proved to be successful replacements of natural nest hollows. Genetic tagging has shown some restriction of movement focused around clay licks at a scale of less than 30 km, but in general confirm an extensive gene flow across the study area. Estimates of the number of birds visiting clay licks were possible for the first time with the genetic methods developed and tested here that also have broader application to other more endangered parrot taxa. Based on rigorous comparative analyses of extinct and extant parrots, the results promote understanding of global and regional factors associated with endangerment in this highly threatened taxonomic group, which can further enhance the prioritization of conservation actions.

9.7 References

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