Coevolution between brood-parasitic honeyguides and their hosts

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Abstract

Obligate brood parasites lay their eggs in the nests of other species, foisting the costs of parental care onto the host parents. The success of the parasite and host are then at odds, with both parties evolving defences and countermeasures in an evolutionary arms race. This reciprocal influence of acting upon both species' evolution — a process known as coevolution — has forged the natural world around us. Avian brood parasites and their hosts are now model systems for studying such coevolutionary interactions between species, providing striking examples of the adaptations that arise when the life histories of two species become entangled. In this thesis I highlight the adaptations that have arisen in response to coevolutionary selection pressures in a group of understudied avian brood parasites, the honeyguides (Indicatoridae), and their hosts. This study focuses on the greater (*Indicator indicator*) and lesser (*I. minor*) honeyguides, and their respective primary hosts, the little bee-eater (*Merops pusillus)* and the black-collared barbet (*Lybius torquatus*). The interactions between honeyguides and their cavity-nesting hosts of the Old World tropics are evolutionarily ancient, contrasting with the majority of studies of avian brood parasitism which have predominantly focused on temperate brood-parasitic species targeting open cup-nesting hosts. Therefore, honeyguides and their hosts are an ideal study system in which look for novel adaptations that have not evolved in other systems. Using field observations and experimental manipulations at each stage of the parasitic life cycle — before parasitism, during egg-laying, during incubation, and during chick-rearing — I examine how honeyguides and their hosts have evolved in response to the selection pressures they exert on each other.

First, in chapter two, I consider whether the nest structure of the little bee-eater – host to the greater honeyguide – can act as a defence against brood parasitism. Experimental manipulation of the size of bee-eater nest tunnels demonstrates that bee-eaters with narrower nest tunnels are less likely to be parasitized by greater honeyguides than those bee-eaters whose nests have wider nest tunnel entrances. This study provides the first experimental evidence of a host nest functioning as a frontline defence against brood parasitism.

In chapter three, I take a comparative approach and use a phylogenetic framework to investigate, across multiple avian brood parasite species, the evolutionary drivers of rapid egg-laying. This trait is shared by most brood-parasitic birds, but not by non-parasitic birds. I find strong evidence that the egg-laying speed of avian brood parasites is ecologically and physiologically constrained, but find no evidence that variation in the costs incurred during

parasitism events have driven variation in the rapidity of egg-laying among brood-parasitic species.

In chapter four, I examine whether there are costs associated with the virulent egg puncturing behaviour of greater honeyguides, and whether honeyguides can adjust their level of virulence in accordance with these costs. I find strong support for the idea that virulence is costly to honeyguides, as bee-eater hosts are more likely to reject clutches that contained eggs punctured by honeyguides. Such punctured clutches are also more likely to be predated. Honeyguides appear to adjust how much they puncture host eggs in accordance with the severity of these costs, providing the first evidence of an avian brood parasite moderating its virulence in response to the associated costs.

In chapter five, I examine egg rejection behaviour in the black-collared barbet, a common host of the lesser honeyguide. I consider whether the (smaller) size of a parasitic egg could be used as a cue for egg rejection inside the dark environment of a cavity nest. Through observations of natural parasitism events, and experimental parasitism of host nests using different sized eggs, I demonstrate that barbets are more likely to reject a clutch of eggs when they detect a small egg within the nest. This seems to be achieved through a process of true recognition, a mechanism that involves a specific innate or learnt template of what size eggs a host should reject. Barbets do not appear to rely on discordancy – comparing all eggs within their clutch in order to reject the odd one out – in order to make rejection decisions.

Finally, in chapter six I explore whether honeyguides elicit additional provisioning from their foster parents by using vocal mimicry, and investigate why such extra food would be required. I demonstrate that both greater and lesser honeyguides mimic the sound of a brood of chicks of their respective hosts in order to receive higher levels of provisioning from their foster parents. I establish that greater and lesser honeyguides do this for contrasting reasons. Greater honeyguides require higher levels of provisioning to support their fast growth rate to a size much larger than their host siblings, whereas lesser honeyguides require more food in order to offset a sub-optimal diet provided to them by their foster parents.

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Ethical statement

This thesis involved observations and experimental manipulation of wild breeding birds. All research was conducted following the principles of the 3Rs: replacement, reduction and refinement. Options to replace animal subjects with non-animals were considered. However, the aim of this study is to understand brood parasite-host interactions in natural conditions, and to test for adaptive behaviours performed by hosts and parasites when faced with each other. For that reason, it was not possible to replace either honeyguide or host species with any other species or non-living model. All experiments used only the minimum number of animals necessary to test the hypotheses, without the loss of scientific rigour. I attempted at all times to use each nesting pair only once in any study. I performed pilot studies of each experiment, distilling each procedure and making sure no undue stress was inflicted upon study species. There was no negative effect to adults or chicks apart from the ephemeral stress of being handled. Work followed or exceeded animal care and ethical procedures in the SAFRING best practice guidelines and adhered to the University's Code of Ethics and Procedures. A brief description of the ethical considerations taken in each chapter are as follows:

In chapter two, I temporarily modified the internal dimensions of little bee-eater *Merops pusillus* nest tunnels (n= 42 nests over two years). The sizes of all modified tunnels, both small and large, fell within the natural range of bee-eater nest tunnels within my study population. Temporary modification of nest tunnel size had no observable negative impacts on nesting birds, and did not cause any birds to abandon their nest.

In chapter four, to mimic natural parasitism I experimentally punctured little bee-eater eggs, with each egg punctured seven times, at a rate of two eggs per nest (n = 34 eggs punctured, in 17 nests, over two years). Puncturing of eggs typically, but not always, leads to death of the developing embryo. Bee-eaters lay four to five eggs in a clutch. Thus, by puncturing only two eggs per clutch, I ensured that, if the bee-eater parents chose not to abandon their clutch (the response being tested in this experiment, and the natural response to honeyguide parasitism and egg puncturing), viable eggs still remained in the nest. Natural parasitism of bee-eater nests by honeyguides occurs at around 40% of nests at my field site, and host eggs are punctured in about 80% of nests that are parasitized. There is an abundant population of little bee-eaters at the field site (minimum of 80 active nests consistently located per year throughout my PhD) and high rates of natural reproductive failure occur due to nest predation, brood parasitism, and natural brood reduction due to hatching asynchrony. Therefore, puncturing experiments are expected to have had a negligible impact on the population.

In chapters four and five, I performed experiments in which I added eggs of local dove species (n= 58 emerald-spotted wood-dove *Turtur chalcospilos*, n = 11 Namaqua dove *Oena capensis*, n= 5 ring-necked dove *Streptopelia capicola)* to little bee-eater and black-collared barbet *Lybius torquatus* nests, to mimic parasitism by honeyguides. Levels of natural nest predation at my study site in Zambia are very high in these species (>90% in some cases) meaning that most eggs I removed for experiments were destined to never hatch. Such high predation rates also mean that, similar to dove species worldwide, those species whose eggs I collected all have life histories that are tuned to high levels of nest failure and rapid re-nesting. It was necessary to use real eggs in these egg rejection experiments as plastic or clay eggs are not considered to be good models of real eggs, and their use could have biased the results of my experiments.

In chapter six, I temporarily removed clutches of barbet and bee-eater chicks from their nests while conducting playback experiments. While removed from their nest, the chicks were kept in a mock nest (a straw-lined plastic bowl placed inside a cardboard box), out of sight and sound of their natal nest and in the shade, with a field assistant regularly monitoring the chicks for any signs of stress. During playback experiments, in place of the chicks that I removed, I temporarily placed a grey-headed kingfisher (*Halcyon leucocephala*) chick within the nest to act as a control. All chicks that I removed were returned to their natal nests within four hours, and all were readily accepted back by their parents. In chapter six I also temporarily hand-reared chicks of the lesser honeyguide *Indicator minor* and black-collared barbet. Chicks were kept in an incubator at 37.5°C and 70% humidity for 48 hours. After 48 hours all chicks were returned to their natal nests, where they were readily accepted back by their (foster) parents. On two occasions, lesser honeyguide chicks were returned to their natal nests before the 48-hour trial period due to signs of stress, but had fully recovered by subsequent visits.

Declaration

This thesis reports original research that I conducted under the auspices of the FitzPatrick Institute, University of Cape Town. All assistance received has been fully acknowledged. This work has not been submitted in any form for a degree at another university.

Signed: Signed by candidate Date: 28/09/2020

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CHAPTER ONE

Introduction

1.1 An introduction to brood parasites

"*It lays its eggs in the nest of smaller birds after devouring these birds' eggs….they do not sit, nor hatch, nor bring up their young, but when the young bird is born it casts out of the nest those with whom it has so far lived"*

Aristotle wrote this description of the behaviour of a cuckoo 2300 years ago (Hett 1936). Ever since, naturalists have been working to decipher the puzzling behaviour of cuckoos and other brood-parasitic birds, and to understand how they manage to trick other 'host' species into raising their young. What Aristotle may not have realised is that host parents are not helpless against brood parasites, and that an 'arms race' (Dawkins *et al.,* 1979) is occurring, with hosts establishing defences against parasitism, forcing the parasites into countering these defences through development of more elaborate and complex trickery (Davies 2000). This process, in which two or more species reciprocally influence one another's evolution, is termed coevolution (Janzen 1980). This evolutionary mechanism was apparent even at the time of Darwin, who noted in *The Origin of Species* "Thus I can understand how a flower and a bee might slowly become, either simultaneously or one after the other, modified and adapted in the most perfect manner to each other" (Darwin 1859). Coevolution is a powerful selective force and has shaped the diversity of behaviours and forms displayed throughout the natural world, from the mutualistic partnership of our gut and its bacteria (Davenport *et al.,* 2017), to the selfish exploitation of hosts by parasites (Feis *et al.,* 2016).

The tendency for novel and interesting adaptations to arise as the result of these reciprocal interactions plays no small part in the appeal of studying brood parasites. However, the ability to identify selection pressures that are often at odds with each other (host vs parasite), and directly test hypotheses derived from such observations with field experiments, means that brood parasites and their hosts are ideal systems in which to study coevolution (Rothstein 1990). Occurring in approximately 1% of all avian species, and arising independently seven times (Payne 1974), brood parasitism is also observed in both fish (Sato 1986; Kitamura *et al.,* 2012) and insects (Barbero *et al.,* 2009; Suhonen *et al.,* 2019). However, the bulk of research on the coevolutionary adaptations that arise in hosts and their brood parasites has focused on birds (Davies 2000; Thorogood *et al.,* 2019). This is because avian brood parasites are particularly suited for study, as interactions between the parasite and host are focused around a readily accessible nest, making observations and experiments logistically feasible. Furthermore, avian brood parasites generally manipulate their hosts within the sphere of visual and auditory systems (Davies 2000). This makes the study and understanding of such adaptations far more intuitive to researchers compared to, for example, the interactions that arise between parasitic and host insects, who predominantly seek to deceive each other in the chemical domain (Litman 2019).

The first step in an avian brood parasite successfully reproducing is for it to gain access to a host nest. Hosts can use nest camouflage (Saunders *et al.,* 2003) or build decoy nests (Galligan and Kleindorfer 2008) to reduce the chance a nest is detected. A neglected strategy that may be employed by avian hosts is the use of nest architecture to restrict parasite access (Freeman 1988; Rutila *et al.,* 2002). This is the focus of chapter two of this thesis, where I perform an experimental test to determine whether nest structure can act as a frontline defence against a brood parasite.

Many hosts can learn to recognize their parasite, attacking or mobbing it in order to deter it from the nest (Welbergen and Davies 2009; Gloag *et al.,* 2013). It is therefore favourable for parasites to avoid detection by the host. To accomplish this, many parasites have evolved mimetic (Feeney *et al.,* 2015) or camouflaged (Krüger 2007) plumage to reduce the efficacy of such frontline defences (Feeney *et al.,* 2012). Another strategy employed by avian brood parasites to avoid host frontline defences is to lay their eggs rapidly (Sealy *et al.,* 1995). This understudied adaptation to a parasitic lifestyle is the focus of chapter three of this thesis, where, within a comparative framework, I identify the costs and constraints that are associated with this behaviour.

The next phase of host-parasite interaction commences once the parasite has gained access to the nest and laid an egg. Host defences at this stage consist of the host detecting the parasitic egg and subsequently responding, either by rejecting the egg, the entire clutch, or simply abandoning the nest. Such egg recognition abilities are the best studied of host defences against brood parasites (Davies 2000)**.** Parasitic eggs are often recognized by cues based on egg colouration (Brooke and Davies 1988; Hanley *et al.,* 2016; Stoddard and Hauber 2017) or pattern (Lahti and Lahti 2002; López-de-Hierro and Moreno-Rueda 2010; Spottiswoode and Stevens 2010). This has in turn promoted the evolution of visual mimicry in parasitic eggs to avoid detection by the host (Brooke and Davies 1988; Stoddard and Stevens 2010). However, most of our knowledge of egg recognition abilities comes from studies that have been undertaken in hosts that construct open cup nests, where the eggs can be easily seen and inspected, and visual cues are reliable. In chapters four and five of this thesis, I explore how two different host species, that nest inside cavities, likely use non-visual cues that are more reliable inside the dark confines of an underground burrow or tree-cavity nest. Specifically, in chapter four I test whether a host can detect parasitic virulence, in the form of puncturing of host eggs, and use this as a cue for rejection. I then test whether this then imposes a cost of virulence onto the parasite itself, and how the parasite might moderate its own virulence in an attempt to avoid host detection of parasitism. In chapter five, I further explore (in a separate host-parasite system) whether a host that has larger eggs than its parasite, an unusual feature in avian brood-parasitic systems, can use this egg size difference as a cue to detect parasitism.

If a brood parasite manages to evade host defences and successfully lays its egg in a host nest, then once the parasitic chick hatches, the arms race continues. Many cuckoos throw their foster siblings out of the nest (Honza *et al.,* 2007; Rutila *et al.,* 2002), reducing competition for parental provisioning. Traditionally, it was thought that hosts had no response to such virulence, with the battle between brood parasite and host ending here (Grim 2007). However, we know now that some hosts can recognise and reject foreign chicks (Langmore *et al.,* 2003; Sato *et al.,* 2010; Noh *et al.,* 2018; Grim *et al.,* 2003), and in some cases this has prompted the evolution of parasitic visual and vocal mimicry of host chicks (Langmore *et al.,* 2008; Noh *et al.,* 2018; Langmore *et al.,* 2011). Furthermore, avian brood-parasitic offspring must obtain adequate provisioning from their host parents. This has promoted the evolution of elaborate signals in parasites that ‘tune in’ to parent-offspring communication of their hosts (Davies 2011). Many parasitic chicks abuse vocal cues to elicit sufficient provisioning in order to survive. Cowbirds outcompete foster siblings for parental care with intense begging displays (Dearborn 1998; Gloag and Kacelnik 2013), while single cuckoos call loudly and rapidly in order to mimic the vocal appearance of multiple host chicks in the nest (Kilner *et al.,* 1999). In chapter six, I explore how two parasites that eliminate their foster siblings, thereby removing any aid that host offspring might have offered in eliciting additional provisioning, are able to extort adequate provisioning from their hosts. Moreover, I ask why both these parasites require the extra provisioning that they demand from their adopted parents.

Beyond being beautiful illustrations of coevolution in action, these examples highlight that at each stage of the avian life cycle, the arms race between brood parasites and their hosts proceeds. This thesis follows a similar itinerary, questioning the adaptations that have arisen at each stage of the life cycle in a specific group of brood parasites – the honeyguides – and their hosts.

1.2 An Introduction to the honeyguides and their hosts

The Indicatoridae are a family of brood parasites of the Old World tropics consisting of 17 species, with two occurring in southern Asia, and the remainder inhabiting sub-Saharan Africa. Although plain and unremarkable in their plumage, they are both behaviourally and physiologically interesting. The honeyguides are best known (and derive their name) from the behaviour of one member of this family, the greater honeyguide *Indicator indicator*, which engages in fascinating cooperative interactions with humans (Figure 1; Spottiswoode *et al.,* 2016; Isack 1987). This cooperation between African honey-hunters and honeyguides was noted by early European explorers of southern and eastern Africa (Spaarman 1777; Dos Santos 1609), and is still exciting researchers today. Honeyguides have an unusual ability to digest wax, and in addition to hawking insects, they feed on the wax of the African honeybee *Apis mellifera* (Downs *et al.,* 2002). Honeyguides are adept at finding bees' nests (owed to a developed olfactory sense; Stager 1967; Hepburn 2009). However, the nests of honeybees are located in large trees, rock crevices, or termite mounds, which provides an obstacle for the honeyguide to obtain its prize, but which humans are good at accessing. During guiding, the honeyguide gains the humans' attention by making loud, consistent chattering calls while flying from tree to tree in the direction of the nest (Spottiswoode *et al.,* 2016; Isack 1987). The honey-hunters can communicate to the bird in their own distinctive calls, and honeyguides are able to recognize the calls of their local hunters, but do not react to other communication attempts (Isack and Reyer 1989; Wood *et al.,* 2014; Spottiswoode *et al.,* 2016). Once the nest is found, the hunter subdues the bees with fire and extracts the honey and wax (Figure 1). This mutualistic relationship greatly reduces the time required for a human hunter to find the nest (Spottiswoode *et al.,* 2016; Isack 1987), while greatly increasing the ability of a honeyguide to access the beeswax within the nest (Isack 1987).

Figure 1. The wax honeycombs of an underground beehive in Zambia. A fire was started nearby to smoke out the defensive bees, in order to extract the honey. The greater honeyguide (*I. indicator*) that guided Lazaro Hamusikili and I to the nest was rewarded with wax.

Despite, or perhaps because of these unusual and unique life history traits, the breeding biology of honeyguides has received relatively little attention. One barrier to the study of honeyguide reproduction is the difficulty of accessing the cavity nests of their hosts. These are primarily Coraciiformes (kingfishers, bee-eaters, and rollers), Bucerotiformes (hoopoes), and Piciformes (barbets and woodpeckers). However, this trait also makes them particularly interesting to study, since they are unlike most other previously researched brood parasites which generally target open cup-nesting species (Davies 2000). Specialising on cavity-nesting species could have promoted the evolution of novel adaptations, specifically tailored to these dark nests environments. Indeed, greater honeyguides fall into two distinct matrilines: those that parasitize tree-cavity nesting hosts, and those that parasitize hosts that nest in terrestrial burrows (Spottiswoode *et al.,* 2011). This has led to distinct phenotypes of honeyguide body and egg size, likely to enable access to host nests and reduce the chance the egg is detected within the nest (Spottiswoode *et al.,* 2011). Honeyguides fit the prevailing trend of tropical brood parasites, being relatively understudied in comparison to temperate brood-parasitic species

(Sorenson and Payne 2002). This is despite the fact that evolution in the tropics is thought to proceed at an accelerated rate, and so evolutionary studies in these regions have the potential to reveal particularly complex adaptations (Dobzhansky 1950; Schemske *et al.,* 2009). Moreover, interactions between honeyguides and their hosts are evolutionarily ancient. In the case of the greater honeyguide, fidelity between distinct honeyguide-host lineages has been maintained for millions of years, providing the circumstances under which unique, and previously unstudied host-specific adaptations could accumulate (Spottiswoode *et al.,* 2011). This is highlighted by the virulent behaviours that honeyguides use to monopolize the reproductive efforts of their hosts, exerting strong selection pressures for the hosts to evolve specific defences against the parasite (Spottiswoode and Colebrook-Robjent 2007; Spottiswoode and Koorevaar 2012). For all these reasons, honeyguides and their hosts are an ideal study system in which to study coevolution. This thesis focuses on the two most widespread and common honeyguide species south of the Sahara, the greater honeyguide *Indicator indicator* (Figure 2A), and lesser honeyguide *Indicator minor* (Figure 2B), and their interactions with their local primary hosts, the little bee-eater (*Merops pusillus*, Figure 2C) and black-collared barbet (*Lybius torquatus*, Figure 2D), respectively.

What little is known about greater honeyguide reproduction suggests that females are not territorial, whereas the males likely are. Males use singing trees to advertise their territories and attract females, with such trees being used for up to 20 years (Ranger 1955). After mating, the female attempts to parasitize a host nest on her own. When the greater honeyguide female attends a little bee-eater nest to lay her egg, she punctures some, or all of the host eggs with her beak or feet, typically causing death of the embryos (Spottiswoode and Colebrook-Robjent 2007). Although likely beneficial to the success of the parasites' offspring, this virulence could provide a unique and reliable cue to bee-eaters that their nest contains a honeyguide. I explore this prospect, testing whether bee-eaters can detect this virulence and then use it as a cue to reject the clutch, which would inflict a cost of virulence unto the honeyguide itself. Since bee-eaters stand to lose fitness once the honeyguide enters the nest by having its eggs punctured, it would be extremely favourable to any bee-eater that could prevent access to its nest. Little

Figure 2. The four focal species within this study. **A)** greater honeyguide *Indicator indicator* (credit: Claire Spottiswoode). **B)** lesser honeyguide *Indicator minor*. **C)** little bee-eater *Merops pusillus*. **D)** black-collared barbet *Lybius torquatus.*

bee-eaters nest in terrestrial burrows consisting of a subterranean nest chamber reached by a tunnel, and are smaller in body size than honeyguides. I therefore investigate the possibility that bee-eaters control the architecture of their nests in a way that could prevent the (larger) honeyguide from gaining access to the nest chamber. If the honeyguide is successful in accessing and parasitizing a host nest, it lays a single white egg that is larger than a bee-eaters, which is then incubated by the host along with its own clutch. The honeyguide then hatches with a specialized bill-hook which it then uses to kill its foster siblings and thus monopolize the parental care within the nest (Figure 3; Friedmann 1955; Ranger 1955; Spottiswoode and Koorevaar 2012). The parasitic chick is much larger, and now is faced with the challenge of eliciting provisioning from its foster parents without the aid of nest mates (Davies *et al.,* 1998). The parasitic chick is likely required to manipulate the parents in some manner to obtain enough resources for growth, and within the dark confines of the subterranean nest this is likely to be done in the auditory channel. I therefore examine whether the greater honeyguide uses vocal mimicry of bee-eater chick begging calls in order to evoke bee-eaters to feeding more regularly, thus fuelling the growth of the large parasitic chick. Once the honeyguide is ready to fledge, it is believed to flee the nest under the cover of darkness, and has only rarely been seen being fed by hosts once it has left the nest (Bryan and Jamie 2019).

Figure 3. The bill-hook of a newly hatched lesser honeyguide chick (*I. minor*).

Even less is known about the breeding biology of the lesser honeyguide *I. minor*. As in *I. indicator*, males attract the attention of females by singing at a favourite tree (Friedmann 1955). After mating, the female attempts to parasitize the host nest by herself. The main host at my study site is the black-collared barbet, a primary cavity nester that builds nests in the dead branches of trees. Barbets are extremely aggressive towards lesser honeyguides that are near their nest, viciously chasing and mobbing the parasite. If caught by the defenders, the honeyguide can even be killed (Cambray and Cambray 2014; Moyer 1980). The honeyguide darts around and into the nest with amazing speed, a trait shared by many other brood parasites (Sealy *et al.,* 1995). I investigate what has driven the evolution of this rapid egg-laying in brood parasites, an idea promoted by observations of lesser honeyguide manoeuvres during egg-laying at host nests. If the honeyguide accesses the nest successfully, it deposits a single, off-white egg that is smaller in size to a barbet egg. This size difference between honeyguide and barbet egg could provide an important cue for barbets when deciding whether or not they are parasitized. Within the dark confines of their tree-cavity nest, barbets are unlikely to be able to visually inspect their eggs in a way that provides reliable information. I take advantage of this to test whether barbets can make rejection decisions based on the small size of an egg, a cue that could be detected via touch alone. If the egg is accepted and hatches, the honeyguide chick then uses its bill-hook to eliminate any barbet chicks within the nest, as is expected from any of the Indicatoridae. Thus, the lesser honeyguide also must elicit provisioning from its foster parents on its own. Contrasting with the greater honeyguide, the lesser honeyguide is smaller than its host. However, the chick must still procure enough resources to grow, and again I consider vocal manipulation as the mechanism that honeyguides use to elicit adequate provisioning. Interestingly for a brood parasite, the lesser honeyguide targets a host that utilizes a very different diet than its own; barbets are primarily frugivores, a food source not likely to be appropriate for the developing honeyguide chick (del Hoyo *et al.,* 2019). I therefore examine whether lesser honeyguides require extra food in order to offset an ill-suited diet provided to them by their hosts.

1.3 Objective of this thesis

The objective of this thesis is to illuminate the suite of adaptations that have arisen in both honeyguides and their hosts as a result of coevolution. Using data on patterns of natural parasite-host interactions observed over four breeding seasons, and coupled with experimental manipulations to investigate specific hypotheses, this thesis progresses as a logical story. I start by considering the interactions between host and brood parasite that precede the actual parasitism event, and end with those that occur after parasitism has taken place and a parasitic chick is being reared by its unwitting foster parents. I focus on two tropical brood parasites and their hosts, that to date have received little research into their breeding biology.

1.4 Aims of this thesis

I address this objective in five data chapters, with the following aims and research questions:

Chapter 2 Nest architecture as a frontline defence against brood parasitism by honeyguides

Aim: to establish whether host nest structure can be used as a frontline defence against brood parasitism.

- Are little bee-eaters with narrower or shorter nest tunnels less likely to be parasitized by greater honeyguides?
- What causes variation in the size of a little bee-eaters nest tunnel?

Chapter 3 The evolution of rapid egg-laying by avian brood parasites

Aim: to investigate the evolutionary drivers of rapid egg-laying in brood parasites.

- What are the benefits of laying eggs rapidly in host nests?
- Are there constraints that impede rapid egg-laying?

Chapter 4 Violence is not (always) the answer: moderation of virulent egg-puncturing by brood-parasitic honeyguides

Aim: to assess whether there are costs associated with parasitic virulence, and to examine whether parasitic virulence can be moderated in response to any such costs.

- Do little bee-eaters reject clutches that contain eggs puncture by greater honeyguides more frequently?
- Are clutches punctured by greater honeyguides predated more frequently?
- Do greater honeyguides moderate their puncturing effort in response to the costs associated with rejection and predation?

Chapter 5 Black-collared barbets *Lybius torquatus* think little of lesser honeyguide *Indicator minor* eggs: true recognition of small parasitic eggs by a cavity-nesting host

Aim: to investigate whether the size of a parasitic egg can be used as a cue for egg rejection in a cavity-nesting host.

- Are eggs that are small more likely to be rejected by black-collared barbets than those that are large?
- How does the black-collared barbet detect this small egg?

Chapter 6 Divergent pressures lead to convergent adaptations: lesser and greater honeyguide (*Indicator* spp.) nestlings both mimic the begging calls of an entire brood of host chicks, but for different reasons

Aim: to establish whether honeyguides elicit provisioning from their foster parents using vocal mimicry, and to determine why such extra food would be required.

- Do single honeyguides get more provisioning than a single host chick, but similar to a brood of host chicks?
- Is this achieved through vocal manipulation, as would be expected in a cavity-nesting host?
- Do greater honeyguides grow at faster rates and to greater mass than host chicks do (and therefore require more food)?
- Do lesser honeyguides require extra provisioning to offset a sub-optimal diet provided to them by their hosts?

1.5 Introduction to the study site

This study was conducted in the Choma District of Zambia's Southern Province (Figure 4; 16°46'S, 26°54'E) from 2015 to 2018, predominantly on two private farms, Semahwa and Musumanene farms. The field site is a ~280 ha mosaic of mixed woodlands (predominantly broad-leafed miombo, dominated by *Julbernardia* and *Brachystegia* spp.), grassland, and agricultural land (primarily tobacco and maize). This site is located on the Choma plateau, with an elevation of ~1300 m above sea level.

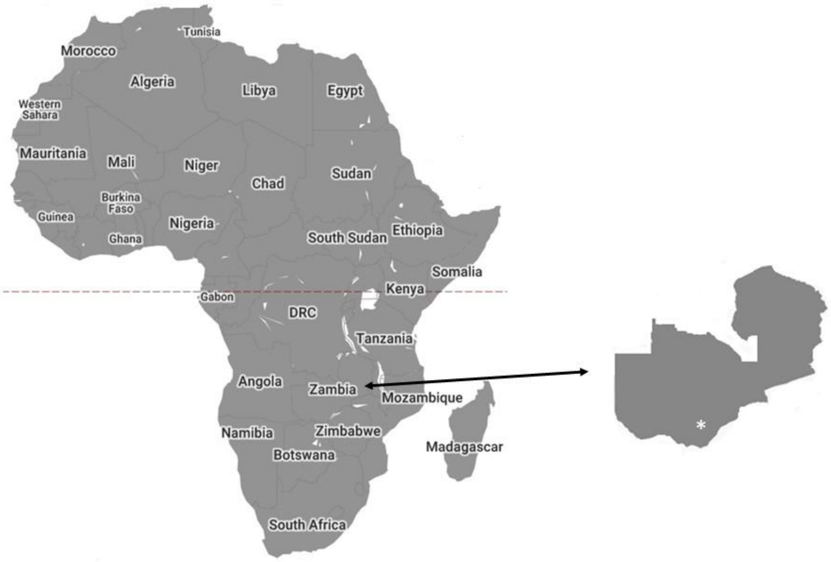


Figure 4. A map of Africa, with a red dashed line indicating the equator. The arrow indicates Zambia. The * indicates the location of the field site near Choma, in Zambia's Southern Province.

The greater and lesser honeyguides are the only two species of *Indicator* to occur at the site (Leonard 2005), although two other members of the Indicatoridae (brown-backed honeybird *Prodotiscus regulus* and green-backed honeybird *P. zambesiae*) also occur. The site is an ideal location in which to study interactions between brood parasites and their hosts. In addition to the four Indicatoridae found at the site, there are nine representatives of the Cuculidae cuckoos, and six *Vidua* finches. The breeding season for honeyguides and their hosts runs primarily from September to November, and is characterised by hot, dry conditions (Figure 5A). Outside of these times the field site becomes cooler and wetter, and is temporarily flooded, with much more vegetation present during the wet season of January to April (Figure 5B).

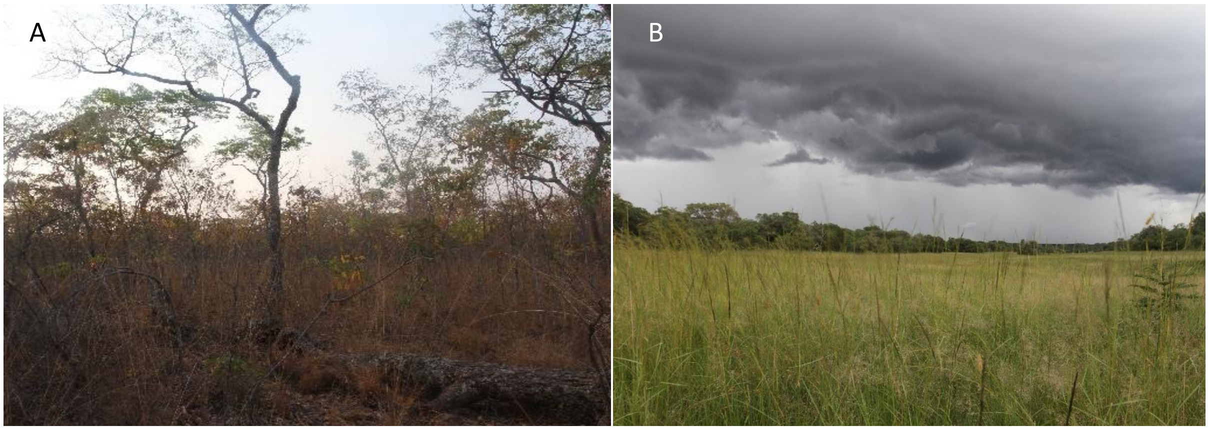


Figure 5. A) Typical miombo woodland during the hot dry season of August to December, when honeyguides and their hosts breed. **B)** A dambo (temporarily flooded grassland) during the wet season of January to April.

Members of the FitzPatrick Institute of African Ornithology at the University of Cape Town and the Department of Zoology at the University of Cambridge have conducted research at this field site since 2006. The research conducted here is owed in no small part to the late Major John Colebrook-Robjent, whose fascination with brood parasites established the groundwork for this and all other studies conducted here. His knowledge was passed to members of the local community, who now aid in finding nests and performing experiments, and without whom this research would not be possible (Figure 6).

Figure 6. The fieldwork conducted for this thesis would not have been possible without the aid of the local community in Zambia. **A)** Some of the nest finders at Semahwa Farm **B)** Lazaro Hamusikili enjoying some honeycomb **C)** Lazaro Hamusikili accessing a black-collared barbet nest **D)** Collins Moya re-building a bee-eater nest after excavation.

CHAPTER TWO

Nest tunnel entrance size as a frontline defence against brood parasitism

2.1 Abstract

Avian brood parasites are detrimental to the reproductive success of their hosts as soon as they gain access to the nest. Therefore, there is strong selection pressure for hosts to prevent parasite access in the first place. One way that hosts could do this is to construct their nests in a manner that either prevents access to, or escapes the notice of, prospecting brood parasites. However, the possible role of host nest architecture as a frontline defence against brood parasitism is not clear. Here, I tested whether the structure of little bee-eater *Merops pusillus* nests can act as a defence against a virulent brood parasite, the greater honeyguide *Indicator indicator*. Specifically, I asked whether the dimensions of the entrance tunnel to the bee-eater's subterranean nest could act as a barrier to greater honeyguides trying to access the nest chamber. I found that bee-eaters that had narrow tunnels as entrances to their nests were less likely to be parasitized by honeyguides than those with wider tunnel entrances. I was able to replicate the results of this observation through experimental manipulation of bee-eater nests. Together, these results imply that the nest tunnel entrance of little bee-eaters can act as a frontline defence against parasitism by greater honeyguides. Finally, I found evidence of corresponding selection on honeyguides, as small honeyguide eggs were found in nests with narrow tunnels, and large honeyguide eggs in nests with wide tunnels. This result implies that the size of bee-eater nest entrance tunnels is selecting for smaller-bodied honeyguide females, since it suggests that honeyguides with larger eggs were unable to gain access to nests with smaller entrance tunnels. This study provides the first experimental evidence that host nest structure can act as a defence against a brood parasite.

2.2 Introduction

Avian brood parasites exert strong selective pressures on their hosts. Day-old common cuckoos *Cuculus canorus* throw their foster siblings out of the nest (Rutila *et al.,* 2002; Honza *et al.,* 2007), cowbirds outcompete foster siblings for parental care with intense begging displays (Dearborn 1998; Gloag and Kacelnik 2013) and honeyguide chicks kill host offspring with a specialized bill hook (Spottiswoode and Koorevaar 2012). Such virulent behaviours lead to strong selection on hosts to develop defences against being parasitized. Perhaps the best studied of these defences are those that attempt to salvage the hosts' own breeding attempt once parasitism has been detected, such as rejecting foreign eggs (Brooke and Davies 1988; Langmore *et al.,* 2005) or chicks (Langmore *et al.,* 2003; Sato *et al.,* 2010). However, such defences are costly if recognition errors occur, such that a host subsequently ejects its own offspring (Stokke *et al.,* 2002; Davies *et al.,* 1996). In addition, many brood parasites reduce host fitness as soon as they have gained access to the nest and before the host has had the opportunity to detect that parasitism has even taken place. For example, many cuckoo species eat or remove a host egg while laying their own (Chance 1922; Brooker and Brooker 1989), and cowbirds puncture host eggs during laying, to prevent hatching (Massoni and Reboreda 1999; Gloag *et al.,* 2013). Brood-parasitic species also often have hard-shelled eggs, that may damage host eggs during laying (Soler *et al.,* 1997; López *et al.,* 2018). These virulent adaptations of brood parasites are all costly to hosts and cannot be prevented by host defences that only occur after parasitism has taken place. Selection is therefore predicted to favour host defences that prevent parasitism in the first place.

Such 'frontline' defences (Welbergen and Davies 2009) are less well studied than subsequent defences, but have the potential to be both the most effective, and least costly, to the host (Feeney *et al.,* 2012). Examples of frontline defences include increased nest surveillance and mobbing of brood-parasitic individuals that approach the nest (Røskaft *et al.,* 2002; Welbergen and Davies 2009). Hosts may also try to reduce the chance that a parasite finds their nest in the first place, by nesting further from vantage sites that a brood parasite might use (Øien *et al.,* 1996), by concealing their nest (Moskát and Honza 2000), or by nesting as part of a larger colony (Brown and Lawes 2007). An additional and under-studied form of frontline defence to deter or prevent brood parasitism is nest architecture. For example, many African weaverbirds (Ploceidae) build nests with a narrow entrance tube, which may provide defence against parasitic diederik cuckoos *Chalcites caprius*. Weaverbird species that are cuckoo hosts are more likely to build nests with entrance tubes than those that are not currently parasitized (Freeman 1988), and diederik cuckoos can even become trapped within the tube during attempted parasitism (Davies 2000). Similarly, a host-race of the common cuckoo suffers heavy costs of poorly placed eggs during attempts to parasitize redstarts *Phoenicurus phoenicurus* breeding in nest boxes, suggesting that nest-box design can prove to be a happy accident for anti-parasitic defence (Rutila *et al.,* 2002; Thomson *et al.,* 2016). Both these host-parasite systems suggest that appropriate nest design can reduce parasitic success. However, since no direct experimental test has been undertaken of whether nest architecture can act as a frontline defence against brood parasitism, it remains uncertain whether such defensive features of nest architecture might have arisen in another context, such as deterrence of predators.

In this study, I carried out such an experimental test using the brood-parasitic greater honeyguide *Indicator indicator* (hereafter “honeyguide”) and its host, the little bee-eater *Merops pusillus* (hereafter ‘bee-eater’). Three aspects of this system make it ideal to ask whether nest architecture can be used as a frontline defence against an avian brood parasite. First, the costs of parasitism in this system are very high. Honeyguides are highly virulent brood parasites, and during parasitism female honeyguides puncture host eggs in order to prevent hatching (Spottiswoode and Colebrook-Robjent 2007). Eighty-five per cent of parasitized bee-eater nests in my study population suffer this fate (chapter three). The newly hatched honeyguide then kills any host chicks that do hatch (Spottiswoode and Koorevaar 2012), ensuring that a little bee-eater that fails to recognise it has been parasitized will suffer total reproductive loss during that breeding attempt. Bee-eaters mob honeyguides (Tong *et al.,* 2015), but given the extreme fitness costs associated with parasitism in this system, any additional frontline defences that can eliminate the likelihood of parasitism occurring in the first place should be strongly favoured by selection. Second, the design of the bee-eater nest suggests that it could function in defence against parasitism by the honeyguide. Bee-eaters nest in subterranean chambers that are reached via long, narrow, entrance tunnels (Figure 1). They excavate their nests in ustic, sandy soils that are loose enough to dig into, yet cohesive enough to prevent tunnel collapse or unwanted erosion (Heneberg 2013). Such properties likely allow for fine control over the size of the nest tunnel and ensure that its dimensions remain relatively constant after construction. Third, little bee-eaters are much smaller than the parasitic honeyguide (13–19 g and 40–57 g respectively; del Hoyo *et al.,* 2019). Importantly, this means that bee-eaters could excavate nest tunnels that are sufficiently wide to allow their own passage yet narrow enough to exclude the larger honeyguide. The length of the nest tunnel could also act as a defence against honeyguide parasitism. Longer tunnels could increase the time required for a honeyguide to parasitize the nest by increasing the distance the parasite is required to traverse to access the nest chamber, thus making the honeyguide more susceptible to detection by hosts. Additionally, a longer nest tunnel could make the nest chamber less apparent or difficult to access. Thus, the potential exists for nest architecture to act as a frontline defence in this system.

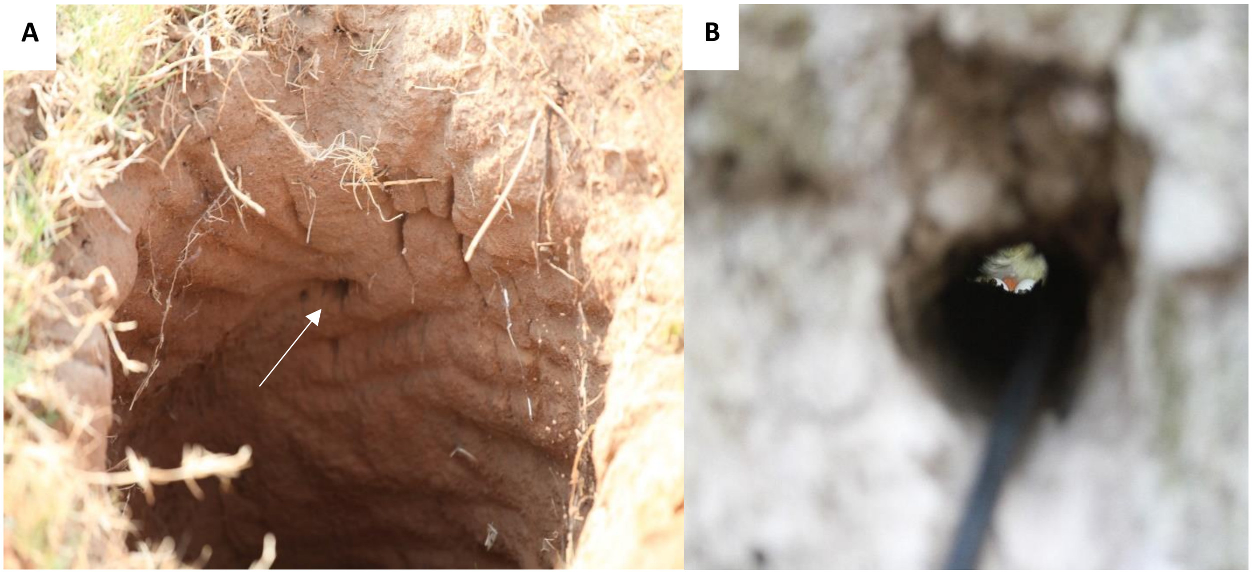


Figure 1. Bee-eaters build their nests in subterrenean burrows. **A)** shows the view of the entrance to an aardvark burrow, with a bee-eater nest tunnel in the roof (arrow indicates the entrance of the bee-eater tunnel). **B**) shows the view of a bee-eater tunnel straight on, where a light is being shone down the length of the nest tunnel into the nest chamber. The breast feathers of a bee-eater can be seen, sitting on two eggs and a chick.

In this study I asked whether bee-eaters could reduce the risk of their nests being parasitized by constructing nests with entrance tunnels that are too narrow, or too long for a honeyguide to pass through, thus restricting honeyguide access to their nest**.** These hypotheses predict that, on average, bee-eaters with narrow nest tunnel entrances, and/or long nest tunnels will be parasitized less frequently than those with wide nest tunnel entrances and/or short nest tunnels. First, I collected data on natural parasitism of bee-eater nests by honeyguides, to establish whether there was a relationship between nest tunnel entrance size and/or nest tunnel length, and parasitism risk. Second, to distinguish the mechanism underlying any such relationship with tunnel entrance size, I experimentally manipulated the diameter of the nest tunnel of bee-eater nests by inserting either a narrow or wide pipe at the mid-point of the nest tunnel. I then tested whether nests that had the nest tunnel either kept naturally narrow, or experimentally altered to be narrow, were less likely to be parasitized than those with wide nest tunnels. If the architecture of bee-eater nests does act as a frontline defence against honeyguide parasitism, then bee-eaters with naturally narrow entrance tunnels should already benefit from reduced parasitism risk. In that case, experimental addition of a narrow pipe should have little or no effect on the parasitism rate of these nests. It should have a much greater effect on those bee-eater nests with naturally wide tunnel entrances, since these will now contain a narrow pinch point that should restrict honeyguide access to the nest chamber. Therefore, the addition of narrow pipes to bee-eater nest tunnels directly tests the hypothesis that nest tunnel size (width and/or height) restricts honeyguide access. By contrast, the addition of a wide pipe to a nest tunnel should not alter honeyguide access to the nest chamber, and acts solely as a control to confirm that honeyguides do not avoid nests with experimental pipes. This is because nests with naturally wide nest tunnels should still be accessible to honeyguides after a wide pipe has been added, while naturally narrow tunnels will still have narrow entrances that could restrict honeyguide access, even if the mid-point of the tunnel has been experimentally altered to be wide. Accordingly, the narrowest point in the nest tunnel after an experimental pipe has been added (either the natural tunnel entrance or the experimental pipe) is predicted to be negatively correlated with the incidence of honeyguide parasitism. Since narrower nest tunnel entrances and longer nest tunnels could also reduce access to the nest chamber by potential predators (Wesołowski 2017), I also recorded nest survival until hatching of all nests within the study and asked whether nest tunnel entrance size or tunnel length predicted rates of predation. If nest architecture does reduce predator access, then bee-eaters with narrower nest tunnel entrances and/or longer nest tunnels are predicted to be predated at lower rates than those with wider tunnel entrances and/or shorter tunnels.

In addressing the hypothesis that bee-eater tunnels can be used as a defence against parasitism or predation, I also tested for two possible constraints on nest design. First, the structure of avian nests can be influenced not only by the behavioural decisions of the builder, but by also the materials available to them (Muth and Healy 2014; Bailey *et al.,* 2014). Soil sediment properties are important for burrow-nesting birds (Smalley *et al.,* 2013), as the soil must be soft enough to allow the nest-builder to dig a tunnel or nest chamber, yet must remain stable once excavated (Heneberg 2013). Nesting substrate for bee-eaters is a mix of sand, silt, and clay, and nest sites excavated in soil containing varying proportions of these materials could yield different structural properties for the nest tunnel. Sandier soils are more likely to deteriorate and crumble (Mitchell and Soga 2005), which could result in loss of fine control of nest construction by bee-eaters. I therefore asked whether the soil type micro-habitat of bee-eater nests influenced the size of their nest tunnel, predicting that wider nest tunnel entrances will be found in nests built in sandier soils, compared with nests built in soils of higher clay and silt proportions. Second, the structure of avian nests may be influenced by individual variation in body size of the builders. Bee-eaters require access to their own nest, therefore providing a physical limitation on the size of the entrance tunnel for each pair. I therefore asked whether the body size of the nesting pair predicted the size of the tunnel entrance they built, such that smaller bee-eaters, or those that laid smaller eggs (using egg size as a proxy for adult body size; Christians 2002), built narrower nest entrance tunnels.

If bee-eater nest entrance size acts as a defence against parasitism by reducing honeyguide access, then there should be selection on honeyguides to evolve smaller body size. Using egg size as a proxy for body size (Christians 2002), I tested whether smaller honeyguides were better able to access bee-eater nests with smaller nest entrances than were larger-bodied honeyguides. If so, this would imply selection for smaller body size in honeyguides.

2.3 Materials and methods

2.3.1 Field site and study system

Fieldwork was conducted on Musumanene and Semahwa farms in the Choma district of southern Zambia (16°46’S, 26°54’E), in a patchwork of miombo woodlands, dambos (seasonally flooded grassland) and agricultural land. The bee-eater and honeyguide breeding season is characterised by dry, hot conditions, from September to November, with a peak of active nests in October. Bee-eater nests are built into sand banks or, more commonly at my study site, in the entrance to tunnels and excavations dug by aardvarks *Orycteropus afer* or bushpigs *Potamochoerus larvatus*.

2.3.2 Measuring natural bee-eater nests sizes

To document whether rates of honeyguide parasitism are predicted by host nest tunnel entrance size, I monitored naturally nesting bee-eaters during the 2017 and 2018 breeding seasons (n = 63 nests in 2017, n = 33 nests in 2018). Upon discovery of an active bee-eater nest, the width and height of the nest tunnel entrance within the first 5 cm of the tunnel was measured to the nearest 0.01 mm with a pair of modified callipers. These consisted of standard digital callipers where the lower jaws had been extended by attaching two rigid, 5 cm-long nails. The extended jaws were then inserted into the tunnel entrance, allowing both the width and height of the first 5 cm of the tunnel to be measured. Nest tunnel entrances were not perfectly round, and I had no *a priori* assumption as to whether the width, height, or cross-sectional area of the tunnel would be more important for determining whether a honeyguide could enter or not. Therefore, I separately tested each of these measurements in my statistical models. I calculated the elliptical area ($ab\pi$) using measurements of both width (a) and height (b). Models based on all three measures (width, height, elliptical area) produced the same patterns (see supplementary material, Table 1) but the model using elliptical area ($ab\pi$) produced the lowest AIC values, and results from this model are therefore presented in the main text. Since I modelled tunnel cross-sectional area as an ellipse, when reporting tunnel sizes in the results section, I list both the cross-sectional area of the tunnel, and the smaller of either the height or the width (since this is what may constrain honeyguide access). For conciseness, I refer to this second measurement as ‘diameter’. The length of the nest tunnel was measured using a tape measure to the nearest cm (n = 62 of 96 nests). To examine the contents of the nest, I used the same approach as Spottiswoode and Koorevaar (2012) and excavated a vertical access shaft directly in front of the nest chamber at the end of the nest tunnel. This access shaft was rebuilt at each visit using a slab of compressed earth, thereby preventing the shaft from collapsing in on the nest tunnel (Figure 2). All nest tunnel measurements were taken before I excavated the nest for the first time.

To confirm that my measurements of the nest tunnel taken at the external entrance were an accurate and reliable estimate of the tunnel dimensions throughout its length, I took an additional set of measurements of the tunnel diameter at the internal end of the nest tunnel, where it joined the nest chamber (Figure 2; n = 13 nests). These measurements were taken after excavation of the vertical access shaft, and I used the same procedure as at the external tunnel entrance to measure the minimum tunnel height and width. I refer to this tunnel size measurement as the nest chamber entrance size. Nest tunnel entrance size and nest chamber were significantly positively correlated ($R^2 = 0.669$, $\beta = 0.393 \pm 0.078$, $t_{11} = 5.023$, $p < 0.001$), showing that the diameter of the nest tunnels were consistent throughout their length, and

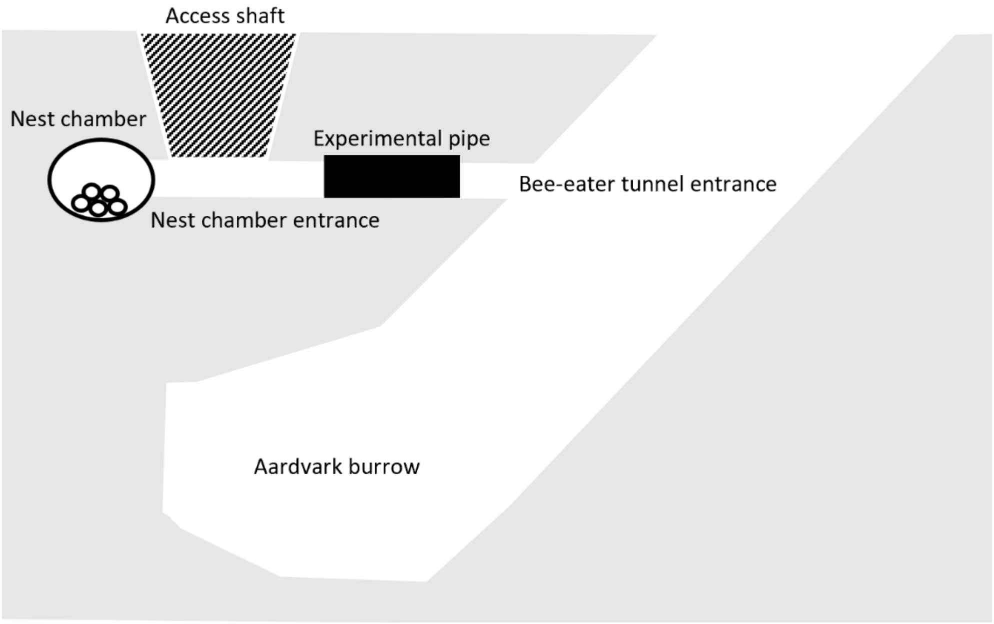


Figure 2. A cross-sectional view of an aardvark burrow containing a bee-eater nest. The soil in the hatched area is removed and replaced upon each visit, allowing access to the nest chamber (black oval) and its contents. Grey areas indicate the soil, whereas white areas indicate the area of the burrows. The black rectangle indicates the placement of the experimental pipe within the bee-eater nest tunnel.

validating my use of nest tunnel entrance size to represent the entire internal dimensions of the nest tunnel.

As honeyguides can parasitize bee-eater nests at any point during bee-eater incubation (Spottiswoode and Colebrook-Robjent 2007), I monitored nests every 2–3 days throughout the incubation period. I considered nests to have been visited by a honeyguide (hereafter, ‘parasitized’) if they contained a greater honeyguide egg (recognised by its larger size and shinier shell surface than bee-eater eggs) and/or contained punctured host eggs (Spottiswoode and Colebrook-Robjent 2007). This is justified for this question since the presence of punctured eggs indicates that a honeyguide was able to reach the nest chamber. To avoid pseudoreplication, the nest of a breeding pair was measured only once (pairs identified through GPS location, with nests in 2016 within approximately 1 km of nests in used in 2015 not included in the final data set). I recorded all instances of nest predation that occurred until hatching. As bee-eaters often abandon parasitized clutches, I defined predated nests as those that were found empty, with no sign of honeyguide parasitism and/or host egg rejection (eggs or eggshells within nest or below the nest tunnel entrance). Some nests that I recorded as predated may have been first abandoned, and only subsequently predated. However, as I monitored all nests at similar intervals throughout incubation, any such error should be equal across groups.

2.3.3 Measurement of soil characteristics

I sampled the composition of the soil in which bee-eater nests were located using simple sedimentation measurements. I sampled a subset of little bee-eater nests (n = 18 nests in 2017, n = 25 nests in 2018), selected from different areas of the study site. Soil samples were collected from nests concurrently with tunnel measurements and stored in plastic zip-lock bags. I then conducted a ‘jar test’ (Sitton and Story 2016) as follows: I desiccated each soil sample for 12 hours at 140°C in individual metal dishes, then weighed the sample to the nearest 0.1g to obtain a dry weight. Ten grams of dry soil was then added to a 50 ml centrifuge tube that was filled with water to a final volume of 50 ml. Samples were left for 48 hours in an upright position to allow sedimentation to occur. Sediment layers were then measured to the nearest mm using a ruler, as sand, silt or clay. I then used a soil sample triangle (Figure 3) to categorize the soil type of my samples based on their composition, and ranked each of the categories in order of their proportions of sand (Cosby *et al.,* 1984).

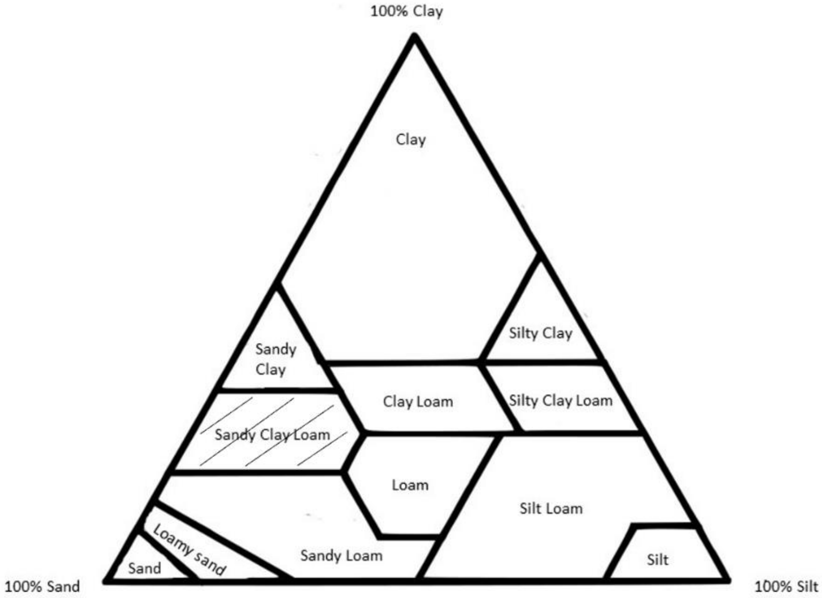


Figure 3. Soil sample triangle used to classify soil substrates of bee-eater nests within the study (n = 43 nests). The majority of bee-eater nests within the study were located in soils of sandy clay loam (hatched region), which contains a high percentage of sand, but relatively little clay or silt. Adapted from USDA soil classifications (Cosby *et al.,* 1984).

2.3.4 Measurement of bee-eater body size

I captured nesting bee-eaters during the 2017 and 2018 field seasons using mist nets (n = 31 birds at 27 nests). Adults were only captured towards the end of chick rearing, to minimize disturbance. Mass and tarsus length were measured to the nearest 0.1 g and 0.01 mm respectively. In most cases (n = 27 nests), only one bird was caught, and since the sexes are alike, I do not know whether this was the larger or smaller of the pair. Therefore, at nests where multiple birds were caught (n = 4 nests), for analysis I randomly selected measurements for the smaller of the two birds at two nests, and the larger of the two at the other two nests.

2.3.5 Measurement of host and brood parasite egg size

I measured the width and length of bee-eater and honeyguide eggs to the nearest 0.01 mm using digital callipers, and calculated egg volume as described in Hoyt (1979).

2.3.6 Experimental manipulation of nest tunnel size

During the 2017 and 2018 breeding seasons, I experimentally manipulated a subset of active bee-eater nests to alter the internal dimensions of the nest tunnel (Figure 2). Original tunnel sizes were measured before excavation (as above). Then, approximately halfway along the length of the nest tunnel, I excavated an additional vertical access shaft, using the same methods as previously described. I then inserted into the nest tunnel a pipe of one of two sizes: ‘narrow’ (2290 mm^2 cross-sectional area, 27 mm diameter, n = 20 nests), or 'wide’ (4300 mm^2 cross-sectional area, 37 mm diameter, n = 22 nests). These values represent the 25th and 75th quartiles of nest tunnel entrance sizes based on measurements from 16 nests measured in 2016. Nests were randomly assigned to one of the two experimental treatments. The pipes were made of cardboard, with the inside surfaces roughened with sandpaper to reduce the smoothness of the material, and thus more closely resemble the texture of the natural tunnel floor. Pipes were 10 cm long, comprising on average 16% of the natural tunnel length (range 10–26%). To securely insert a pipe into the natural nest tunnel, I backfilled the space around the pipe with soil, as necessary, thus ensuring that access to the nest chamber was only possible by passing through the pipe. The vertical access shaft was also refilled with soil, and the area returned to normal. From the outside, it should not have been possible for a honeyguide or potential nest predator to detect that the internal dimensions of the nest tunnel now differed from those at the immediate entrance to the nest tunnel. Both narrow and wide tunnel sizes were within the natural range of bee-eater nest tunnels measured in 2016 (cross-sectional area: range = 768–6831 mm^2, median = 2665 mm^2; diameter: range = 15–48 mm, median = 27 mm), and natural parasitism has occurred in nests with tunnel entrances of these sizes (Figure 4A). Nests were then monitored for parasitism and predation until hatching, at which point the cardboard pipes were removed. Nests that were predated (n = 4) were not included in further analyses on parasitism rates.

2.3.7 Statistical analyses

I conducted all analyses in R v. 3.1.3 (R Core Team 2019), using the packages nlme4 (Bates *et al.,* 2014) and MuMIn (Bartoń 2019) to generate generalised linear models (GLM). Visualisations of the results were generated with ggplot2 (Wickham 2019b). Model selection was performed using the Akaike information criterion (AIC (Wagenmakers and Farrell 2004), with models of >2 AIC units lower than a less complex model considered the most parsimonious

(Burnham *et al.,* 2011). All models were found to meet the assumptions of normality and homogeneity of variance, as evaluated from residual plots of fitted models. Model goodness of fit was evaluated with a Hosmer-Lemeshow test (Hosmer and Lemeshow 2000) and McFadden pseudo R^2 (McFadden 1974) in the case of GLMs.

To test whether natural bee-eater nest tunnel entrance size predicted the probability of the nest being parasitized by a honeyguide, I constructed generalised linear models, using data on unmanipulated nests only. Nest fate was treated as a binary response (parasitized or unparasitized). This was fitted to the model with a logit link to account for the binomial error distribution, with nest tunnel entrance width, height, or elliptical area as continuous predictors in separate models, to avoid collinearity. I included the year, and month within the year as ordinal predictors in this logistic regression, to confirm that neither bee-eater tunnel size nor honeyguide parasitism rates changed throughout the season. For the experimental dataset, I ran separate models to test the effect of the following fixed effects on the probability of a nest being parasitized: the original nest tunnel entrance size, the experimental pipe size, tunnel length, the measurement of the narrowest point after experimental pipe addition (either the size of the experimental pipe or the size of the natural tunnel entrance), and the year of the experiment (2017 or 2018). I then compared AIC values to determine the best model(s). To evaluate whether nest tunnel entrance size predicted predation rates, separate models were generated with the same predictors but with predation instead treated as the binary response variable (predated or not predated). To evaluate whether addition of narrow experimental pipes had a greater effect on parasitism or predation rates of bee-eater nests that had wide natural tunnels to begin with (i.e. those nests made narrower by addition of a narrow pipe), compared to nests that already had naturally narrow nest tunnels, all models were then rerun on two subsets of data: (i) only bee-eater nests with naturally narrow (below 2290 mm^2 cross-sectional area; 27 mm diameter) nest entrance tunnels, and (ii) only bee-eaters with naturally wide (above 2290 mm^2 cross-sectional area; 27 mm diameter) nest entrance tunnels.

To test whether nest site soil type explained any variance in bee-eater nest tunnel entrance size or length, I ran a linear model with nest entrance tunnel size or length as a continuous response, and with soil type as an ordered categorical variable (ranked by sand content). To test whether the size of the nest tunnel entrance was determined by the size of the bee-eater that excavated it, I used a linear model with nest tunnel entrance size included as a continuous response variable, and tarsus length, mass, and average host egg volume within the clutch included as continuous predictor variables. To test whether smaller honeyguides parasitized bee-eater nests with smaller nest tunnels, I used a linear model with the size of the tunnel entrance of the bee-eater nest as a continuous response variable, and the volume of the honeyguide egg found within that nest as a continuous predictor.

2.4 Results

2.4.1 Does bee-eater nest tunnel entrance size act as a defence against parasitism or predation?

2.4.1.1 Unmanipulated nests

The mean diameter of bee-eater tunnels was 27.40 ± 0.90 mm (range = 14.19–77.25 mm), and the mean length 63 ± 2.95 cm (range = 39–99 cm). The mean elliptical area of bee-eater tunnels was 2567 ± 164 mm^2 (range = 900–7586 mm^2, Figure 4A), and this did not differ between the two study years ($\beta = -90.050 \pm 347.100$, $t_{61} = 0.26$, $p = 0.80$), or between months within the years ($\beta = 0.421 \pm 0.542$, $t_{94} = 0.242$, $p = 0.73$). There was no relationship between tunnel entrance size and tunnel length ($\beta = -0.002 \pm 0.003$, $t_{61} = -0.537$, $p = 0.60$). Of 96 nests monitored in 2017 and 2018, 47 (49%) were parasitized, with 30 and 17 clutches parasitized in 2017 and 2018 respectively. Across both years, 20 nests (21%) were predated, ten of which (50%) were parasitized by a honeyguide beforehand. There was no difference in parasitism ($\beta = 0.393 \pm 0.760$, $Z_{95} = 0.517$, $p = 0.61$) or predation ($\beta = -0.693 \pm 1.05$, $Z_{95} = -0.656$, $p = 0.51$) rates between 2017 and 2018. There was a positive relationship between bee-eater nest tunnel entrance size and the incidence of honeyguide parasitism ($\beta = 0.001 \pm 0.001$, $Z_{95} = 3.828$, $p < 0.001$, Figure 4B). A 15% increase in tunnel size (equivalent to increasing the cross-sectional area by 385 mm^2, or the diameter by 4.11 mm, for a nest of mean size), increased the odds of parasitism by 1.6, or the probability by 62%. There was no relationship between nest tunnel entrance size and predation rate ($\beta = -0.002 \pm 0.001$ $Z_{95} = -0.937$, $p = 0.35$).

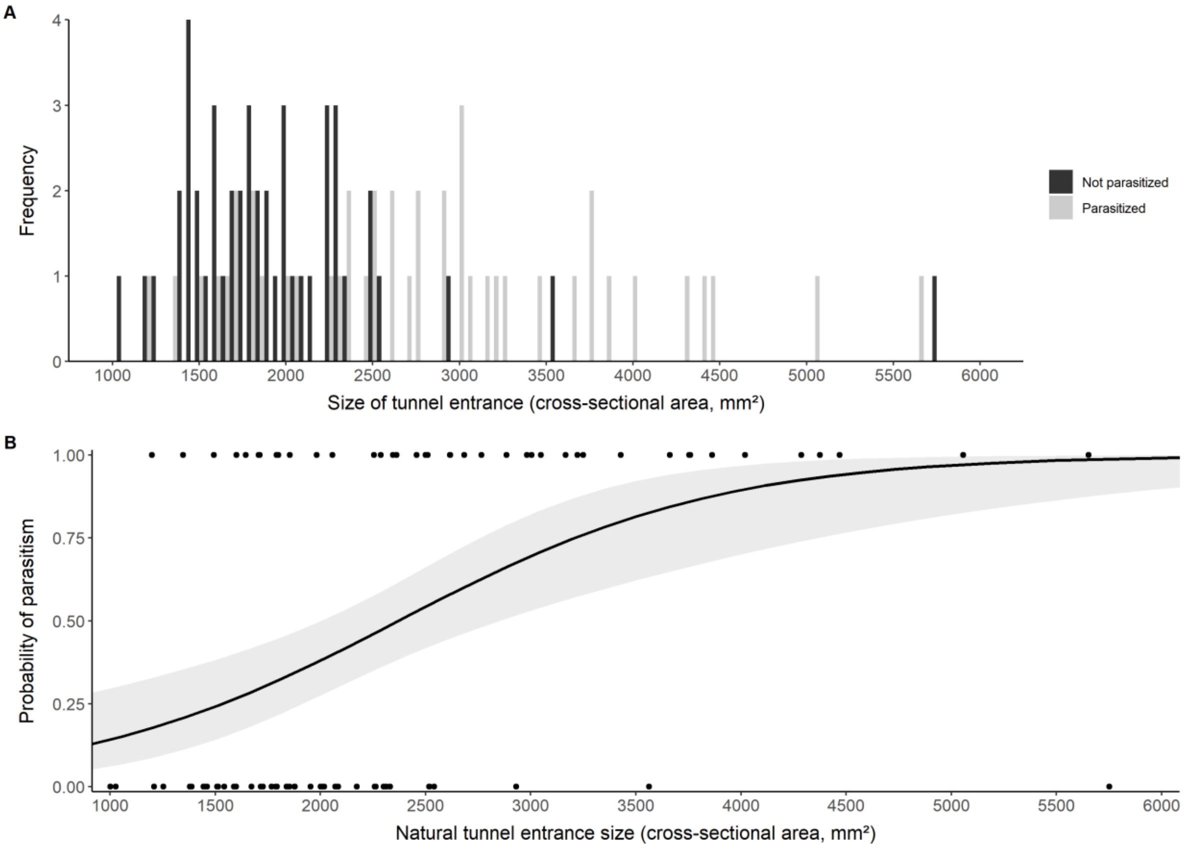


Figure 4. A) The frequency distribution of bee-eater nest tunnel sizes (cross-sectional area) within the study. Black bars indicate unparasitized nests and grey bars indicate parasitized nests. **B)** The probability of parasitism of a bee-eater nest as predicted by the cross-sectional area of its nest tunnel entrance (mm^2). Black circles indicate raw data (n = 96 nests). The black line indicates the estimated logistic regression between parasitism probability and bee-eater nest entrance tunnel size. The grey bands indicate 95% CI.

2.4.1.2 Experimental nests

For those nests with wide experimental pipes added, nine of 22 nests were parasitized, with eight of these parasitism events occurring in nests where the tunnel diameter remained wide after experimental addition of a pipe. By contrast, only one nest out of 20 with a narrow experimental pipe added suffered parasitism (Fischer’s exact test, $p < 0.001$). Similarly, only one of 22 bee-eater nests that had naturally narrow tunnel entrances (and therefore where the nest tunnel was always narrow, regardless of addition of the experimental pipe) was parasitized.

Thus, bee-eater nests with wide experimental pipes were more likely to be parasitized than those with narrow pipes (β = 2.545 ± 1.200, Z_{38} = 2.274, p = 0.023), equivalent to 12.6 times greater odds, or a 92% greater likelihood of being parasitized compared to a nest with an experimentally narrowed nest tunnel. Furthermore, the strongest negative correlation found was between the narrowest point in the nest after addition of the experimental pipe and the probability of honeyguide parasitism (β = -0.002 ± 0.001, Z_{38} = -2.568, p < 0.01; Figure 5), regardless of whether the narrowest point was natural or experimental.

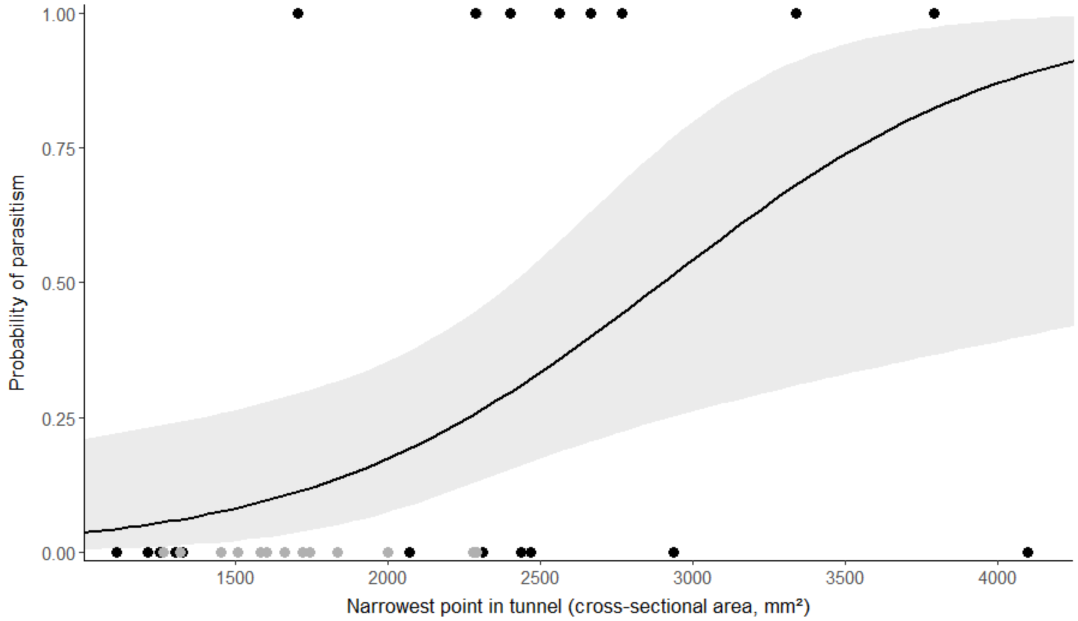


Figure 5. The probability of honeyguide parasitism predicted from the narrowest point in the tunnel after addition of an experimental pipe (regardless of whether this was the natural tunnel entrance, or the experimental pipe). Black circles indicate nests with wide experimental pipes added to the nest tunnel (n = 21). Grey circles indicate nests with narrow experimental pipes added to the nest tunnel (n = 18). The black line indicates the logistic relationship between parasitism rate and the narrowest point in the tunnel.

As expected, the pattern of small experimental pipes being parasitized at lower rates was more pronounced, although not statistically significantly so, in bee-eater nests that had naturally wide tunnels before experimental manipulation (β = 2.262 ± 1.221, Z19 = 1.857, p = 0.061; Figure 6). For bee-eater nests that had a narrow natural entrance tunnel (before manipulation), there was no effect of experimental pipe size (narrow or wide) on probability of parasitism (β = 18.487 ± 4917.520, Z_{21} = 0.004, p = 0.99). As was the case for unmanipulated nests, tunnel length was not associated with parasitism rate in experimental nests (β = 0.026 ± 0.760, Z_{23} = 0.694, p = 0.50). Rates of parasitism did not differ between experimental nests in 2017 and 2018 (β = 0.393 ± 0.76, Z_{38} = 0.517, p = 0.61).

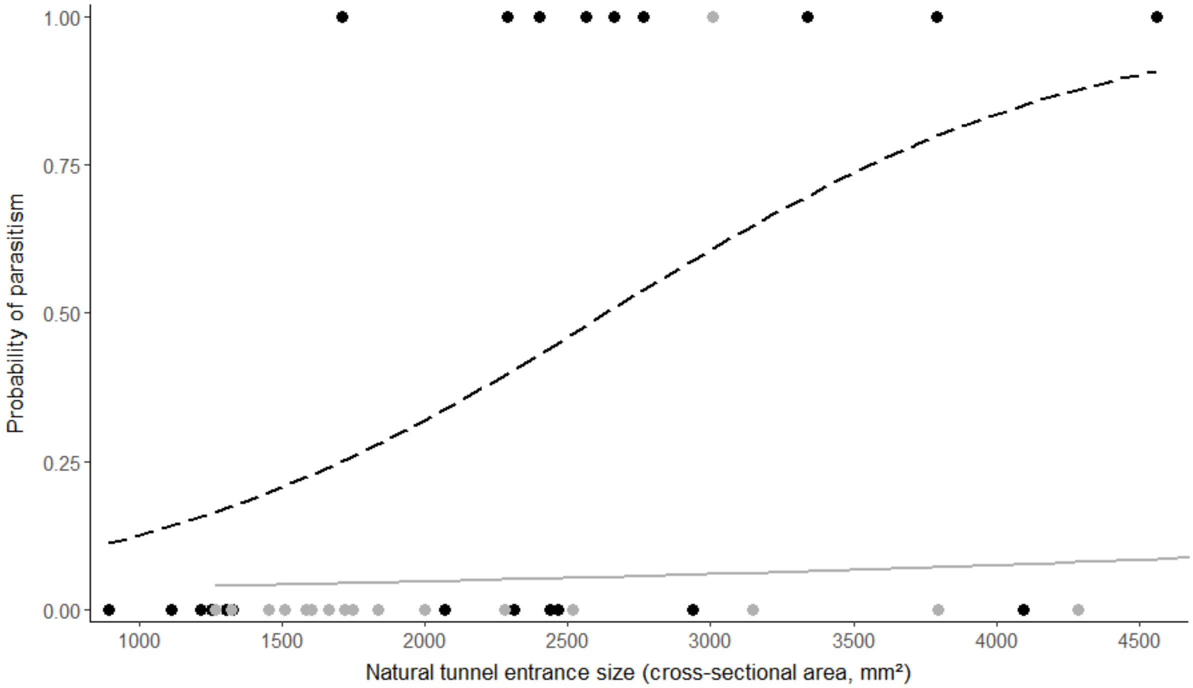


Figure 6. The probability of honeyguide parasitism predicted from both natural bee-eater tunnel size, and experimental pipe size. Black circles indicate nests with wide experimental pipes added to the nest tunnel (n = 21). Grey circles indicate nests with narrow experimental pipes added to the nest tunnel (n = 18). The dashed black line indicates the relationship between probability of parasitism and natural nest entrance size for nests with wide experimental pipes. The solid grey line indicates this same relationship for nests with narrow experimental pipes. Nests with narrow experimental pipes added were significantly less likely to be parasitized than those with wide pipes.

Four of 43 experimental nests (9.3%) were predated during the incubation period, two of which had large experimental pipes added, and two with small experimental pipes added. There was no relationship between experimental nest tunnel size and predation rate (β = 0.154 ± 1.05, Z_{42} = 0.155, p = 0.89).

2.4.2 Is bee-eater tunnel size correlated with soil type or builder body size?

Of 41 bee-eater nests sampled for soil composition, 34 (83%) were located in sites classified as sandy clay loam, four in soils classed as clay (10%), and three (7%) in soils categorized as clay loam. I found no evidence that bee-eater nest tunnel entrance size ($\beta = 1.792 \pm 41.12$, $t_{40} = -0.446$, $p = 0.97$) or tunnel length ($\beta = 3.778 \pm 3.300$, $t_{22} = 1.155$, $p = 0.27$) were associated with the composition of the soil in which the nest was excavated. I also found no evidence that bee-eater body size determined the size of the entrance to the nest: nest entrance size was correlated with neither the tarsus length ($\beta = -33.44 \pm 149.130$, $t_{25} = -0.434$, $p = 0.65$) nor the mass ($\beta = 8.996 \pm 46.630$, $t_{25} = -0.173$, $p = 0.873$) of the bee-eater that excavated it. There was also no relationship between bee-eater nest tunnel entrance size and the size of the bee-eater eggs within the nest ($\beta = 0.005 \pm 0.007$, $t_{67} = 0.739$, $p = 0.46$).

2.4.3 Is bee-eater tunnel size correlated with honeyguide egg size?

Nests with small entrance tunnels were still sometimes parasitized by greater honeyguide eggs, but only honeyguide eggs in the lowest 25th percentile by size were found in nests with tunnel entrances of elliptical area less than 1800 mm^2 or diameter less than 24 mm (34% of bee-eater nests fell below this threshold). There was a positive relationship between bee-eater nest tunnel entrance size and the size of the honeyguide egg found within the nest ($\beta = 1.565 \pm 0.592$, $t_{25} = 2.646$, $p = 0.01$; Figure 7). Small honeyguide eggs were found in nests with narrow nest tunnel entrances, while larger parasitic eggs were found only in nests that had wide nest tunnel entrances.

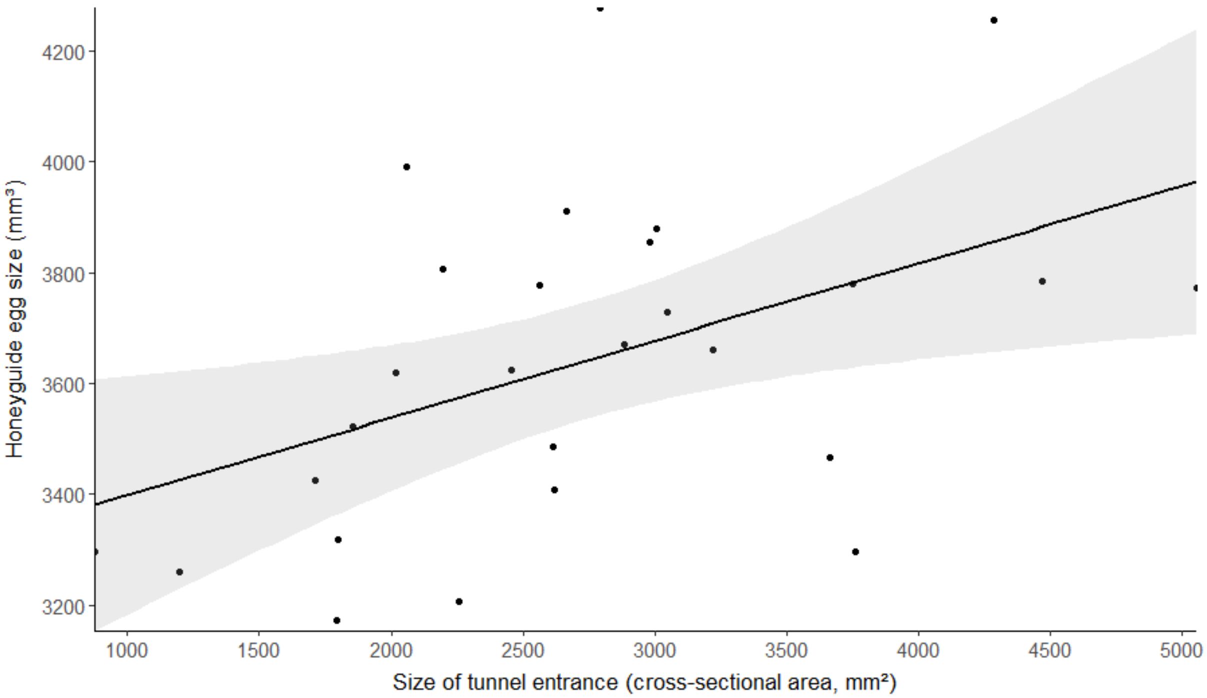


Figure 7. The relationship between the size of a bee-eater nest tunnel entrance (cross-sectional area) and the size (volume) of the honeyguide egg found within that nest (n = 27). Smaller honeyguide eggs were found in nests that had narrow nest tunnel entrances, whereas larger honeyguide eggs were found only in nests with wide nest tunnel entrances. The black line indicates the estimated regression between the size of the honeyguide egg and the nest entrance tunnel size of the bee-eater nest that the honeyguide egg was found within. The grey bands indicate 95% CI.

2.5 Discussion

2.5.1 Bee-eater tunnel size as a defence against brood-parasitism

My results suggest that, in the little bee-eater, nest tunnel entrance size can act as a frontline defence against brood parasitism by the greater honeyguide. Studying a population of little bee-eaters, I found that nests with narrower tunnel entrances were parasitized by greater honeyguides less often than those with wider nest tunnel entrances. Experimental manipulation of nest tunnel size showed that bee-eater nests where a narrow pipe was added to the nest tunnel were parasitized less often than those fitted with a wide pipe. However, this effect was stronger in bee-eater nests that naturally had a wide nest entrance tunnel, suggesting that those nests with a naturally narrow nest tunnel entrance were already sufficiently protected from parasitism, and the addition of a narrow pipe did nothing to improve upon this. Providing further support for this hypothesis, the narrowest point in the nest after the addition of the pipe, regardless of whether this was the entrance of the nest tunnel or the experimental pipe, was the best predictor of the likelihood of honeyguide parasitism. Nonetheless, bee-eaters with narrow tunnels were not completely impervious to honeyguide parasitism, as I occasionally found nests with very narrow tunnels that were still successfully parasitized (the narrowest nest parasitized had a tunnel entrance size of 1347 mm^2 cross-sectional area, or 21 mm diameter). However, unlike in the diederik cuckoo-weaver system (Davies 2000), a greater honeyguide 'stuck' in a nest entrance tunnel has never been encountered in this study population, which has been monitored annually since 2008. Conversely, of those nests in the top 10% for widest nest tunnel entrance (those with nest tunnel entrances above 3600 mm^2 cross-sectional area, or 34 mm diameter), nine out of ten were parasitized, suggesting that the dimensions of the little bee-eater nest tunnel do provide defence against parasitism by greater honeyguides.

In other host-brood parasite systems, there is evidence that parasitism may be reduced by host nest camouflage, such as the amount of foliage around the nest (Saunders *et al.,* 2003), or by deception through use of 'decoy' nests (Galligan and Kleindorfer 2008). However, my experiment rules out these explanations in the bee-eater honeyguide system, since only the inside of the nest tunnel was manipulated, and not the outward appearance of the nest tunnel entrance. It is therefore unlikely that the observed reduction in parasitism in bee-eater nests with narrow experimental pipes was because these nests were less conspicuous to honeyguides, or because honeyguides avoided nests with this experimental manipulation. Instead, I interpret my findings as supporting the hypothesis that the architecture of the little bee-eater nest – specifically the cross-dimensional area of the nest tunnel – can act as a physical barrier to honeyguide parasitism and is a form of frontline defence.

2.5.2 Bee-eater nest tunnel size as a defence against predators

Brood parasitism is not the only selection pressure that might shape nest architecture, and predators can also shape the nest structures of their prey (Wesołowski 2017). However, I found no evidence of this in little bee-eaters, since narrower nest tunnels were not associated with lower rates of predation. The most common predators of bee-eater eggs and chicks at my field site include snakes (most likely Mozambique spitting cobras *Naja mossambica* and boomslangs *Dispholidus typus*), ants, and humans. These are all predators against which a narrow nest tunnel entrance would provide little defence. These considerations and my results suggest that little bee-eaters have likely evolved narrow nest entrance tunnels specifically in response to selection pressures from greater honeyguide parasitism. Conversely, it is also possible that wider nests are safer for adult bee-eaters, increasing the chance they can escape themselves when a predator enters the nest. This would lead to evolutionary trade-offs between the safety of the breeder and the safety of the brood.

2.5.3 Ecological correlates of bee-eater tunnel size

Since I found evidence that bee-eaters that excavated narrower nest tunnels were parasitized at a lower rate, I next asked why all bee-eaters do not build narrow tunnels, by assessing whether certain ecological factors might limit tunnel design. First, I tested for a possible constraint of soil type, but found no relationship between either nest tunnel entrance size or tunnel length and the soil substrate in which the nest was built. The majority of nests (80%) were built in sandy clay loam substrates, with the remaining nests built into substrates that differed only slightly in composition. Whether such consistency of nesting substrate in bee-eaters is a product of nesting-pair site choice, or because of habitat availability, is unknown. However, other species of bee-eater have been noted to have specific soil-type requirements to excavate their nests (Smalley *et al.,* 2013), particularly favouring areas of loess, a form of wind-blown silt (Donahue 1965). Therefore, finding virtually no difference in soil composition among bee-eater nests is perhaps unsurprising. The distribution of loess soils is suggested to drive the breeding distribution of burrow-nesting birds more generally (Smalley *et al.,* 2013) but see (Heneberg 2013). Loess soils are absent at my field site, and in Zambia as a whole (Li *et al.,* 2019). However, while the soil substrates observed at my field site were of similar clay compositions to those observed in loess soils (20% clay content), they comprised much less silt and more sand. To identify whether bee-eaters were selective in their nesting locations at my study site, further sampling of soil in areas where no bee-eater nests are found (but where they might nonetheless be expected) must be undertaken. If the soil composition in these unoccupied sites is different from that of locations where bee-eaters do nest, this would provide correlative evidence that soil composition is an important factor in determining where these birds choose to nest (Smalley *et al.,* 2013).

A second possible constraint on nest tunnel size is body mass of the nest-builder. White (2005) described a positive relationship between body mass and burrow cross-sectional area across multiple taxa, including birds. However, although there is surely a physical limitation to how narrow a bee-eater can make its tunnel, I found no relationship between the size of the nest tunnel entrance and the size of the bee-eater that excavated it. This was the case regardless of whether I used body mass or other structural measures of adult body size, or the size of bee-eater eggs within the nest as a proxy for adult body size. This observation could arise if neither egg size nor adult body size are indicative of the cross-sectional area of a bee-eater. An alternative explanation for the maintenance of variation in bee-eater tunnel size could be the degree of experience of the nesting pair. In other avian systems, more experienced individuals have been found to be more efficient nest builders (Bailey *et al.,* 2014; Walsh *et al.,* 2010). Unfortunately, I have no information on age or experience of individual birds in my study population. However, Tong *et al.* (2015) found that, in the same study population, over half of bee-eater pairs did not react to a model honeyguide. This suggests they did not recognise the brood-parasitic threat, which is a skill thought to improve with experience (Hauber *et al.,* 2006). Although speculative, these observations suggest that there are naïve hosts present within my study population. Further studies are required to test this hypothesis, and until then it remains unclear as to why all little bee-eater nests do not build narrow nest tunnels.

2.5.4 Evolution of greater honeyguide size in response to bee-eater nest tunnel size

Brood parasites can evolve counterstrategies to overcome frontline defences. For example, many brood parasites lay their eggs rapidly and secretively (chapter three; Chance 1922) or have evolved cryptic plumage (Krüger 2007) to avoid the costs associated with being detected and mobbed. As bee-eaters that dig narrower tunnels are less likely to be parasitized, this could exert selection on honeyguides to evolve smaller body size, to be able to gain access to those bee-eater nests with narrower nest tunnels. If so, then we should expect to see evidence of selection for small body size in honeyguides. Partially consistent with this expectation, I found small honeyguide eggs in bee-eater nests with narrow nest tunnel entrances and large honeyguide eggs in nests with wide nest tunnel entrances. Assuming that the size of an egg is indicative of the size of the female that laid it (although note that female body size explains only a small proportion of egg size variance; (Christians 2002; Deeming 2007)), this observation implies that smaller honeyguides can fit through narrower nest entrance tunnels, while larger honeyguides cannot. We might expect, however, that while honeyguides laying larger eggs would only be able to access nests with wide tunnels, small-bodied honeyguides (and by proxy, those that lay small eggs) should be able to access bee-eater nests with both narrow and wide tunnels. This was not the case, and so this result is therefore somewhat surprising.

Nonetheless, the variance in honeyguide egg size was equal across the range of values for bee-eater nest tunnel size (as assessed by a heteroscedasticity test of my linear model), confirming the validity of this result. This pattern is also consistent with a previous finding: greater honeyguides that parasitize larger, tree-cavity-nesting hosts (such as green woodhoopoes *Phoeniculus purpureus*) are larger in body size, and lay larger eggs, than the ancient and genetically-distinct matriline of greater honeyguides that parasitize smaller-bodied hosts breeding in terrestrial burrows, such as the bee-eaters studied here (Spottiswoode *et al.,* 2011). Whether body size in honeyguides is the result of phenotypic plasticity, with larger hosts providing more resources and thus fledging larger parasitic chicks, or is genetically controlled, remains unknown. Cross-fostering experiments moving honeyguide chicks from nests of the smaller terrestrial hosts (such as the little bee-eater) to those of the larger tree-nesting hosts (such as the green woodhoopoe) could discriminate between these two hypotheses. Alternatively, if, as previously hypothesized, bee-eater nest tunnel size is correlated with the builders' experience, then honeyguides with larger eggs (who themselves could be older, more experienced breeders; Hipfner *et al.,* 1997; John-Jaja *et al.,* 2016) could instead be targeting naïve bee-eaters, whereas smaller honeyguides (who could be younger, less experienced breeders) would be forced to target more experienced bee-eater pairs. In this scenario, honeyguides could identify such naïve and experienced hosts by their behaviour at the nest, with hosts that recognize and actively defend the nest being more likely to be experienced breeders, and therefore likely to have a narrow nest tunnel.

2.5.5 Conclusion

This study provides the first experimental evidence of a novel frontline defence against brood parasites. This is likely not a unique strategy employed only by bee-eaters. Other avian hosts of brood parasites, such as the weavers (Freeman 1988) and the common redstart (Rutila *et al.,* 2002) use nests that also likely function as a defence against parasitism. Furthermore, nests, and brood parasitism, are not exclusive to birds. Many insects build nests inside structures as diverse as shells, plant stems, and tree cavities (Litman 2019) and can become hosts to parasites such as larger-bodied, brood-parasitic Hymenoptera (Cervo 2006). Thus, nest structures across species likely have the potential to act as a frontline defence against brood parasitism. I suggest that nest structures could be particularly useful as defence against brood parasites in hosts that (i) are smaller than their parasite, (ii) build enclosed nests, and (iii) have fine control over the structure of their nest, an ability which is likely to improve through phenotypic plasticity. If these prerequisites are met, then the magnitude of the effect found here (a 15% reduction in nest tunnel entrance size leading to a 62% reduction in parasitism) suggests that nest structure can be an extremely valuable defence that is effective in reducing the fitness costs to the host associated with parasitism of their brood.

2.6 Supplementary material

2.6.1 Models using different calculations for tunnel size

Nest tunnel entrances were not perfectly circular, and I had no *a priori* assumption as to whether the width or height of the tunnel would be more important for determining whether a honeyguide could enter the nest or not. Therefore, I ran separate models examining the effect of tunnel width, height, and elliptical area on the probability of parasitism, using a GLM with a logit function. To calculate the elliptical area, I used measurements of both width and height (width x height x π). All models predicted the same patterns (supplementary table 1), but the model using the area calculated as an ellipse had the lowest AIC value. I also assessed whether tunnel entrance height and width were correlated using a linear model. Bee-eater tunnels with larger tunnel widths also had larger tunnel heights ($R^2 = 0.669$, $\beta = 0.862 \pm 0.232$, $t_{95} = 8.84$, $p < 0.001$).

Supplementary table 1. Model outputs of the three measurements used for bee-eater tunnel size calculations (n = 96 nests). All three measurements were positively correlated with the probability of parasitism by a greater honeyguide. The best model (selected via AIC) involved the area of the tunnel as calculated as an eclipse (πab), and was therefore used for all analysis presented in the main text.

Tunnel calculation	Estimate	Z value	p value	AIC
Elliptical area (πab)	0.001	3.829	< 0.001	109.6
Height	0.176	3.917	< 0.001	112.8
Width	0.177	3.584	< 0.001	116.8

CHAPTER THREE

The evolution of rapid egg-laying by avian brood parasites

3.1 Abstract

Among avian brood parasites, the process of parasitizing a host nest, including laying a parasitic egg, can take place remarkably quickly, often within a few seconds. This rapid egg-laying is thought to be an adaptation to reducing the costs, such as mobbing, that a parasite may suffer when visiting a host nest. However, the precise selection pressures that have given rise to this phenomenon remain poorly understood. I tested multiple hypotheses for rapid egg-laying by examining variation in laying speed across 20 species of brood parasite within a phylogenetic framework. I found little evidence that the rapid egg-laying speeds of brood parasites have evolved in response to the costs of host defences, but instead found evidence that they have been primarily shaped by ecological and physiological constraints. I tested five main hypotheses and found the strongest support for those hypotheses proposing that the speed at which a brood parasite lays its egg is dependent on (i) the size of the parasitic egg, as larger eggs were laid more quickly, and (ii) the ease of access to host nests, since species parasitizing nests within cavities laid more slowly. These results provide insight into an under-explored aspect of the coevolutionary arms-race between parasites and their hosts. I also document for the first time the laying behaviour of the lesser honeyguide *Indicator minor*, which accomplishes egg-laying in less than one second, making this to my knowledge the fastest-laying bird species ever recorded.

3.2 Introduction

In the summer of 1920, a cuckoo revealed her egg-laying secret to Edgar Chance when he observed her to deposit an egg within a meadow pipit nest in just a few seconds (Chance 1922). This ability to rapidly parasitize host nests is a trait shared among many avian brood parasites (Sealy *et al.,* 1995) which in general appear to lay their eggs much more rapidly than non-parasitic birds that typically take minutes to hours to perform this task (Sealy *et al.,* 1995). This is despite the fact that egg-laying visits by brood parasites also requires them to access the host nest, avoid host defences, and perform any associated laying behaviours in addition to actually laying the egg. However, even among brood parasites there is much variation in egg-laying visit speeds between species (Sealy *et al.,* 1995). We still know very little about what selective pressures drive this behaviour. Why do brood parasites lay their eggs so quickly compared to non-parasitic species, and what drives variation in egg-laying speed among parasitic species, and even among different host-races of a single parasitic species (Chance 1922; Moksnes *et al.,* 2000)? Since all birds do not lay their eggs rapidly, it seems likely that there are costs associated with laying eggs fast. For example, the risk of damaging an egg might be higher when it is laid rapidly, which may also explain why brood parasites generally have thicker egg shells than non-parasites (Brooker and Brooker 1991; López *et al.,* 2018). Moreover, it may be costly to evolve the correct physiology to allow eggs to be laid rapidly. In the case of brood parasites, it would appear that the benefits of rapid egg-laying can outweigh such costs, with parasites expected to optimise the trade-offs between these costs and benefits. Here, I examined host and parasite life history traits within a comparative framework in order to test the potential drivers of rapid egg-laying by avian brood parasites. As the traits that best explain the variation in egg-laying visit speeds among brood parasites are the most likely evolutionary drivers for this phenomenon, examining the potential importance of these traits will help to shed light on why avian brood parasites lay their eggs faster than non-parasitic species.

First, I considered a series of hypotheses for the adaptive benefits of rapid egg-laying speed. By parasitizing nests rapidly, parasites may minimise inadvertently advertising the location of the host nest, or their presence at it. Such unwanted attention could alert predators (Wyllie 1981) and conspecific competitors (Gloag *et al.,* 2013), or give hosts the opportunity to block a laying attempt either through mobbing or by obstructing the nest (Hobson and Sealy 1989; Welbergen and Davies 2009). Moreover, some hosts are more likely to reject a foreign egg if they see a parasite beside the nest, bringing subsequent costs to a parasite that is detected while laying (Davies *et al.,* 1996). Therefore, parasites that target hosts which refine their egg rejection based on whether or not they see a parasite at their nest would be predicted to have faster egg-laying speeds than those that do not. By laying rapidly, the parasite could thus reduce the chance of detection and increase the chance that their egg is accepted. Rapid parasitism could also reduce the chance that a host injures (Brooke and Davies 1988; Welbergen and Davies 2009) or even kills (Molnár 1944; Moyer 1980; Gloag *et al.,* 2013) the parasite. If this is the case, then parasites of relatively larger hosts (which could inflict more damage) might be expected to spend less time in the act of parasitizing a host nest compared to brood parasites of relatively smaller hosts. Parasites attempting to lay in the nests of hosts that nest cooperatively (Feeney *et al.,* 2013) or colonially (Brown and Lawes 2007) likely also face increased costs of frontline defences (Welbergen and Davies 2011). The additional defenders at cooperatively and colonially breeding host nests increase the risk of the parasite being mobbed or detected. Parasites targeting such hosts would therefore be expected to lay eggs more quickly than those that target pair-breeding hosts. Finally, many brood parasites carry out virulent behaviours when attending host nests, such as puncturing host and parasitic eggs (chapter four; Spottiswoode and Colebrook-Robjent 2007; Gloag *et al.,* 2013) or removing host eggs (Brooke and Davies 1988). While these behaviours provide fitness benefits to the parasite, they may increase the time that such virulent species need to spend at the nest compared to parasites that are more benign during egg-laying. Therefore, brood parasites that remove or puncture host eggs during parasitism events are expected to have slower egg-laying visit speeds.

Second, I considered whether variation in laying visit speed might be limited by ecological and physiological constraints. As enclosed nests such as terrestrial burrows or cavity nests are more difficult for brood parasites to access (chapter two; Davies 2000; Rutila *et al.,* 2002), brood parasites that target hosts using such nests would be predicted to have slower laying visit speeds. The size of the egg being laid (Krüger and Davies 2004), or the physiology of the reproductive tract (Birkhead *et al.,* 2011) could also affect a brood parasite’s ability to parasitize a nest quickly. Since large eggs could require more time to pass through the reproductive tract or cloaca (Joy and Divya 2014), brood parasites that lay large eggs may be expected to lay their eggs more slowly than those that lay small eggs. However, this is likely to be affected by the size of the egg relative to the size of the brood parasite laying it. If, as we might speculate, an egg is less likely to become stuck or slowed in the cloaca when it is small relative to the size of the bird laying it, then brood parasites that lay small eggs for their body size should lay eggs faster than those that lay large eggs for their body size.

This study takes a comparative approach to test these hypotheses by examining egg-laying visit speeds in 20 brood-parasitic bird species, drawn from six of the seven independent lineages of avian brood parasites.

3.3 Methods

3.3.1 Collection of data and definition of the variables

I obtained data on ‘laying visit speed’ (time, in seconds) for 20 species of brood parasite. For five of these species, data were available for multiple host species, resulting in 11 data points contributed by *Clamator jacobinus, Cuculus canorus, Chalcites lucidus, Molothrus bonariensis* and *Molothrus ater*. All data were included in the analysis (details of phylogenetic methods for multiple host-races below). I defined laying visit speed as the total time the brood parasite spent at the nest, from start to finish, including entering the nest, the act of egg-laying itself, and all associated behaviours (inspecting host eggs, puncturing, egg removal) if these were carried out. Although facultative parasites of conspecifics also lay more rapidly than non-parasites (Sealy *et al.,* 1995), I included only obligate interspecific brood parasites in my dataset as they are likely under different selection pressures to facultative, conspecific brood parasites. Laying visit speeds were sourced from the literature, my own data, and personal communication with field workers (Table 1). If more than a single observation of a parasite-host pair existed, the mean value was taken. I also obtained information on egg rejection rates for a subset of hosts that have had their rejection behaviour observed after experimental presentations of a model parasite at the nest (n = 20 of 31 parasite-host combinations). I used this information to categorize these hosts as either those that either increase their egg rejection rate upon sight of a parasite at the nest, or those that do not. Life history and ecological data were gathered from del Hoyo *et al.* (2019). Female body mass (g) was used for brood parasite mass, since only female brood parasites lay eggs, while an average of male and female mass was taken as host mass, (since both sexes typically participate in nest defence; Gill and Sealy 1996; Honza *et al.,* 2010). I then calculated the absolute size difference between the parasite and the host, and the relative size difference by calculating the ratio of parasite to host mass after log-transforming both variables. Hosts were assigned as either group breeders (colonial or cooperative, since both imply shared nest defence by more than two individuals) or pair breeders. To test whether brood parasites with virulent laying behaviours require more time at the nest, brood parasites were assigned as those that punctured host eggs during laying, and those that did not, and brood parasites that removed a host egg(s) during laying and those that did not. To assess whether there are ecological constraints on brood-parasitic egg-laying visit speeds, host nest types were classified as either ‘open’ (cups) or ‘enclosed’ (cavity or dome). Egg size (mm^3) was calculated from egg dimensions using the formula in Hoyt (1979). To assess whether relative egg size (to the brood parasite laying it) is a better predictor of laying visit speed than the absolute size of the parasitic egg, I also calculated the ratio of brood parasite mass to egg volume after log-transforming both variables (Huxley 1927).

3.3.2 Phylogenetic analysis

As species are not statistically independent, I modelled laying visit speeds while considering the phylogenetic histories of the species in my analyses (Freckleton *et al.,* 2002). Phylogenetic generalized least-squares (PGLS) regression models were created using the packages caper (Orme *et al.,* 2019) and ape (Paradis *et al.,* 2004), which incorporate the phylogenetic relatedness of species into the model's error term. The phylogenetic signal within the data was estimated as Pagel’s λ, calculated with the phytools package (Revell 2012; Revell 2010). A majority-rule consensus tree (Holder *et al.,* 2008) was generated (Figure 1) from a sample of 1000 phylogenies downloaded from birdtree.org (Jetz *et al.,* 2012) which uses a backbone phylogeny from Ericson *et al.,* (2006). To incorporate multiple laying visit speeds from a single species, the majority-rule consensus tree was then modified in the program Mesquite (Madison and Madison 2018) and visualized using Figtree (Rambaut 2010), as follows. I treated species with records of laying visit speeds in multiple hosts as separate taxa, subtended by branch lengths estimated by dividing the original branch length for the parent species by the number of tips present in the clade (Grafen 1989; see supplementary material 1 for this extended phylogeny). The black-headed duck *Heteronetta atricapilla*, which is distantly related to all other parasitic lineages, was included as an outgroup to the rest of the phylogeny. I then added each predictor variable into a univariate PGLS model, with egg-laying visit speed as the predictor. This was done because missing data meant that not all predictors could be simultaneously included in the same model. All significant (or marginally insignificant) variables were then added into increasingly complex models, which were then compared using the Akaike information criterion (AIC; Wagenmakers and Farrell 2004), with models of lower AIC (>2 AIC units lower than a less complex model) considered the most parsimonious (Burnham *et al.,* 2011). All statistical analyses were performed in R (R Core Team 2019). Model normality and homogeneity of error was evaluated from residual plots of fitted models, and models were visualized using ggplot2 (Wickham 2019b).

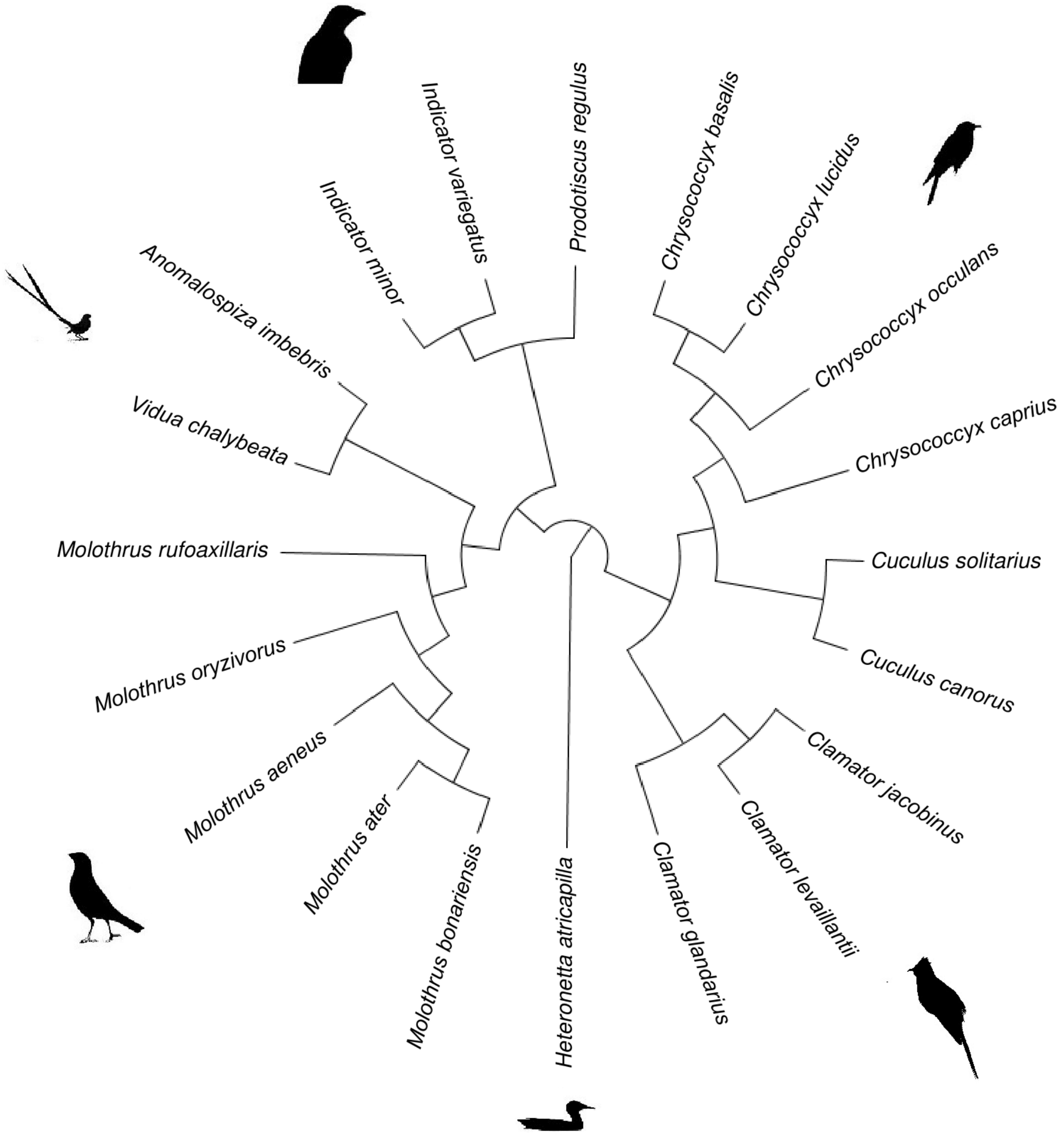


Figure 1. A phylogeny of all 20 species of brood parasite in my dataset. The brood-parasitic black-headed duck *Heteronetta atricapilla* was set as the root of the tree. Silhouettes indicate the six different independent parasitic lineages these represent.

3.4 Results

3.4.1 Variation in egg-laying visit speeds among brood parasites

The laying visit speeds of all the brood parasites in the dataset were rapid relative to published records of non-parasites (which are measured in minutes rather than seconds; Sealy *et al.,* 1995), ranging from 0.8 seconds in the lesser honeyguide *Indicator minor* to 180 seconds in the village indigobird *Vidua chalybeata*. The mean (32.7 seconds) and median (18 seconds) laying visit speeds suggested that most are very fast. Lesser honeyguides were the fastest egg-layers of any species in this study — and to my knowledge the most rapid of any bird recorded — since they were able to lay an egg in less than one second (Figure 2; supplementary videos 1 and 2). There was a strong phylogenetic signal in egg-laying visit speed (λ = 0.82), indicating trait evolution according to Brownian motion (Pagel 1999).

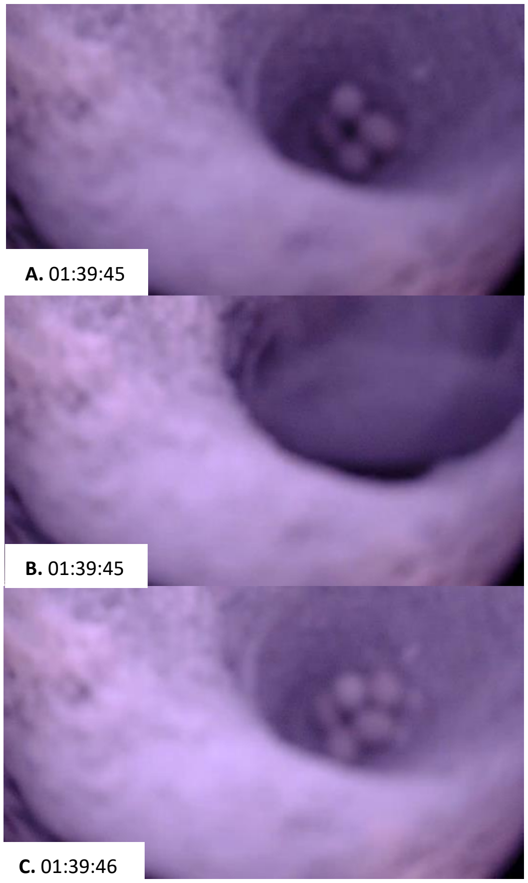


Figure 2. Lesser honeyguides *Indicator minor* are to my knowledge the fastest egg-layers of any bird in the world. This female deposited her egg within the cavity nest of a black-collared barbet *Lybius torquatus* in under a second (0.8 s). In panel **A**, four barbet eggs can be seen in the nest with a time-stamp of 01:39:45. Panel **B** shares the same time-stamp (that is, was within a second of the first) and shows the female lesser honeyguide inside the nest, obscuring the eggs. Panel **C** with a time-stamp of 01:39:46, shows the same four host eggs plus an additional parasitic egg at top right (see supplementary videos 1 and 2).

3.4.2 Are rapid egg-laying visits associated with the costs of host defence?

I found little evidence to support hypotheses proposing that laying visit speed in brood parasites is shaped by the costs of being detected at the nest. Parasites of hosts that are known to increase their rates of rejection upon seeing a parasite at their nest did not have faster egg-laying visit speeds than parasites targeting hosts that have been demonstrated not to increase rejection upon seeing the brood parasite at their nest ($\beta = -7.76 \pm 19.20$, $t_{18} = -0.40$, $p = 0.69$). Similarly, I found no evidence of a relationship between parasitic laying visit speed and the absolute body size of either the host ($\beta = -0.05 \pm 0.15$, $t_{29} = -0.34$, $p = 0.74$) or the parasite ($\beta = -0.22 \pm 0.18$, $t_{29} = -1.26$, $p = 0.22$). I found no evidence that parasites that are relatively ($\beta = -6.06 \pm 20.64$ $t_{29} = -0.29$, $p = 0.71$) or absolutely larger ($\beta = -0.26 \pm 0.23$, $t_{29} = -1.16$, $p = 0.25$) than their hosts have faster egg-laying visit speeds. Brood parasites of group-breeding (cooperative or colonial) hosts did not have faster egg-laying visit speeds than those of pair-breeding hosts ($\beta = -14.20 \pm 15.67$, $t_{29} = -0.91$, $p = 0.37$). Parasites that carry out virulent behaviours during egg-laying did not have slower egg-laying visit speeds than more benign parasites: parasites that puncture host eggs ($\beta = -73.72 \pm 15.54$, $t_{16} = -1.26$, $p = 0.23$) or remove one or more host eggs ($\beta = -6.33 \pm 13.42$, $t_{15} = -0.47$, $p = 0.64$) during parasitism did not have slower egg-laying visit speeds than parasites that do not.

3.4.2 Are rapid egg-laying visits associated with ecological constraints?

I found stronger evidence that ecological and physiological constraints act upon egg-laying visit speed. Species parasitizing hosts with enclosed nests had faster egg-laying visit speeds than those parasitizing hosts with open nests ($\beta = 48.10 \pm 15.09$, $t_{27} = 3.19$, $p = < 0.01$, Figure 3A). Moreover, there was a significant negative relationship between laying visit speed and the absolute size of the parasitic egg: parasites with larger eggs had faster laying visit speeds than those with smaller eggs ($\beta = -0.01 \pm 0.01$, $t_{27} = -2.38$, $p = 0.02$; Figure 3B), contrary to expectation. While there was a non-significant trend for parasites that laid relatively smaller eggs for their body size to have faster egg-laying visit speeds ($\beta = - 243.00 \pm 150.13$, $t_{29} = 1.62$, $p = 0.12$), this was not retained by AIC-based model selection.

The final model that best predicted laying visit speeds of brood parasites contained the absolute size of the parasitic egg and host nest type; taken together, these explained 26.6% of the variance in brood-parasitic laying visit speed (Table 2).

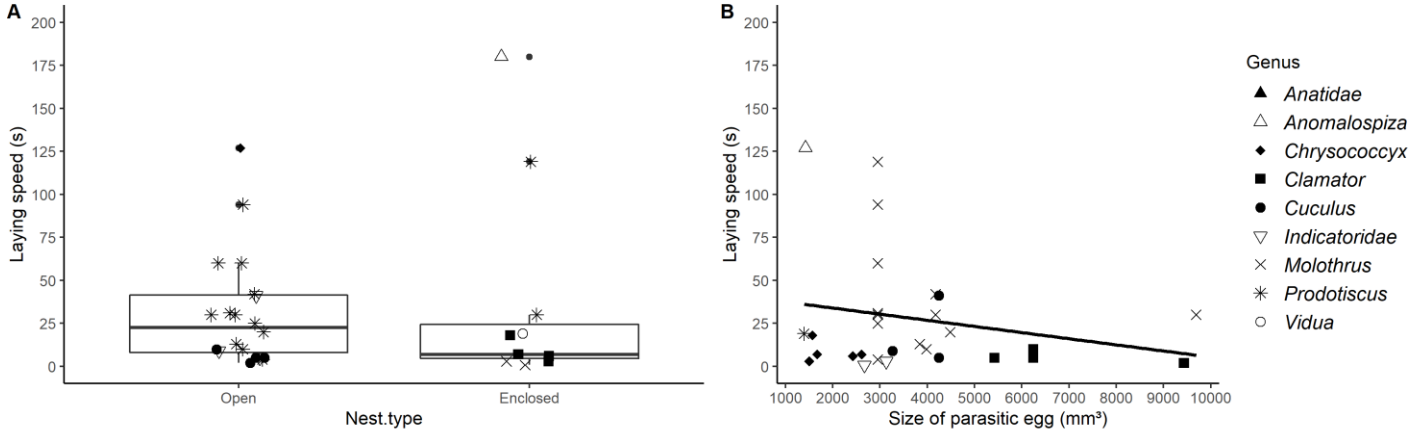


Figure 3. A) Parasites of enclosed nesting hosts had faster egg-laying visit speeds than open-nesting hosts. **B)** Parasites that laid larger eggs had faster egg-laying visit speeds. The black lines indicate the regression slopes. The points indicate the raw data (n = 31), with shapes illustrating the different genera of brood parasites within the study

Table 2. The most parsimonious model for predicting the laying visit speed of the 20 species of brood parasite compared within this study included (i) the volume of the parasitic egg (mm^3), and (ii) the type of nest the host constructs (open or closed). The table shows the intercept, t and p values, and partial R^2 of each variable when included in the full model.

Model variable	Intercept	t value	p value	R^2
Volume of parasite egg	-0.001	-2.382	0.0243	0.06
Nest type	48.104	15.087	< 0.001	0.13

3.5 Discussion

My results support the hypothesis that the rapid egg-laying visit speed of brood parasites is primarily constrained by ecological and physiological limitations. I found that the time taken to parasitize a host nest was best predicted by two main host and parasite ecological and physiological traits: (i) the type of nest built by the host, and (ii) the absolute size of the parasitic egg. These findings provide little support for the hypothesis that variation in the speed of egg-laying visits among brood-parasitic birds has been driven by variation in the potential costs to the parasite incurred during laying, but strong support for the hypothesis that rapid egg-laying visits are under ecological and physiological limitations. I now discuss each of these in turn.

I found the strongest support for the idea that rapid parasitic egg-laying visits are shaped by ecological constraints. Parasites laying eggs within the nests of cavity-nesting hosts had faster egg-laying visit speeds than those targeting open cup-nesting hosts. This at odds with the idea that the structure of a host's nest can act as a defence against parasites, reducing parasite ease of access (see chapter two of this thesis, on the greater honeyguide *Indicator indicator* and its commonest host the little bee-eater *Merops pusillus*). However, my data suggest that parasites targeting enclosed nesting hosts have faster egg-laying visit times. Perhaps this is because many parasites do not need to enter the nest to lay their egg, instead 'squirting' the egg into the nest by positioning their cloaca at the entrance. This behaviour could arise for two, non-mutually exclusive reasons i) the nest entrance is too small for the parasite to enter (see chapter two of this thesis), or ii) the cost of being caught inside the nest during laying by the host is too great.

Further to the possible constraints imposed by host nest structure, I found that parasites with larger eggs had quicker egg-laying visit speeds than parasites with smaller eggs, suggesting that the absolute size of the parasitic egg can constrain its speed of laying. This trend is opposite to my expectation that larger eggs would require more time to pass through the reproductive tract, and therefore require more time to be laid. An alternative interpretation is suggested by the positive relationship between egg size and eggshell thickness across birds (Spaw and Rohwer 1987; Juang *et al.,* 2017). Brood-parasitic eggs in general tend to have thicker shells than the eggs of similarly-sized non-parasitic species (Brooker and Brooker 1991), which has been suggested to result from selection to resist damage during rapid egg-laying (Lack 1968; Rothstein 1990; López *et al.,* 2018). Since larger parasitic eggs tend to have thicker eggshells than smaller ones (Spaw and Rohwer 1987), the pattern I found could be explained if smaller eggs are more susceptible to damage during laying, perhaps limiting the speed at which small eggs can be laid. This hypothesis predicts that only larger-egged brood parasites would be able to safely accomplish very rapid laying visits, as observed: for example, the *Clamator* cuckoos in this study were some of the fastest egg-layers, and all lay large (thick-shelled) eggs (Brooker and Brooker 1991).

I found less evidence in support of the hypothesis that variation in rapidity of egg-laying visits in brood parasites has been shaped by the costs associated with parasitizing a host's nest. Parasites that target hosts of larger mass than themselves (either absolute or relative size difference) did not have faster egg-laying visit speeds than those parasites that target hosts of smaller mass than themselves. However, the size difference between parasites and their hosts only takes into account costs associated with mobbing, whereas hosts have multiple lines of defence in which rapid egg-laying visits could be useful in avoiding, so this index is likely an over-simplification of the potential costs incurred during laying visits. I also found no evidence to support the hypothesis that egg-laying visit speed is influenced by the risk of the host rejecting either the entire clutch or the parasitic egg, should they detect a brood parasite at or near their nest. A possible explanation for this null result is that parasites generally attempt to lay at times of day when the host is least likely to be active at the nest (Davies and Brooke 1988; Gloag *et al.,* 2013), or have evolved additional behaviours that may discourage nest attendance or parental attentiveness around the time the nest has been parasitized, such as Batesian mimicry (York and Davies 2017). Such strategies may be more advantageous (and less risky due to potential damage incurred by host mobbing) than attempting to lay quickly when the host is nearby. Moreover, I also found no evidence to support the hypothesis that brood parasites utilizing cooperative and colonial breeding hosts have evolved faster egg-laying visit speeds due to selection from increased nest attentiveness, and increased opportunity for nest defence by hosts. A link between cooperative breeding by hosts and brood parasitism has been well-established, as brood-parasitic hosts are more likely to be cooperative breeders than expected by chance (Feeney *et al.,* 2013). However, I did not find that parasites targeting group-breeding hosts laid their eggs more rapidly than those targeting hosts that breed solely as pairs, suggesting that parasites have not responded to selection from group defence of nests by evolving faster laying. I also found no support for the idea that parasitic species that employ virulent laying behaviours such as egg puncturing or removal of host eggs need to spend more time at host nests when they lay their own egg. The absence of such a pattern might instead suggest either that these virulent behaviours are themselves extremely fast (and thus do not increase the total time the parasite requires at the host nest), or that these virulent parasites are particularly specialised at being rapid in the specific act of laying, to compensate for the extra time needed for adaptive virulence at host nests. In support of the first suggestion, observations show that removal of a host egg can be very quick in cuckoos (Moksnes *et al.,* 2000; Šulc *et al.,* 2016). Finally, I found little support for the hypothesis that the speed with which an egg can be laid is constrained by the reproductive physiology of the parasite, since there was only a weak and non-significant trend for parasites that lay eggs that are relatively larger for their body size have slower egg-laying visit times than those parasites with small eggs relative to their body size. Therefore, it appears that larger eggs are not more difficult to pass rapidly through the reproductive tract, an effect that was predicted to be especially pronounced in parasites that lay eggs large relative to their body size. Many parasites lay eggs that are smaller than expected for their body size (Payne 1974; Krüger and Davies 2004), which has been attributed to hosts recognising and rejecting eggs that differ in size from their own (Krüger and Davies 2004). Therefore, perhaps even the largest brood-parasitic eggs in this study are not large enough to constrain rapid egg-laying. In the absence of evidence that egg-laying speed is related to the relative size of the parasitic egg, my findings suggest that host recognition remains the best hypothesis to explain why parasites tend to lay small eggs.

The slowest egg-laying speed in my dataset was that of the village indigobird *V. chalybeata*, which spent on average three minutes at its red-billed firefinch *Lagonosticta senegala* hosts' nest. This species lays a small egg which exemplifies the overall pattern observed across species in my study, but parasitized a enclosed-nesting host, which is contrary to the overall trend. Interestingly, the species with the fastest act of parasitism in the study, the lesser honeyguide *I. minor*, is a clear exception to both main patterns observed. This species lays a small egg and parasitizes a cavity-nesting host. Remarkably, lesser honeyguides are capable a laying visit in less than one second (Table 1; supplementary videos 1 and 2), the fastest in my dataset and indeed across all birds, being more than twice as fast as the next fastest layer, the great spotted cuckoo *Clamator glandarius*. Notably, black-collared barbets *Lybius torquatus*, the most common host of lesser honeyguides, have highly aggressive front-line defences, ferociously mobbing any parasites detected at the nest and at times even killing female honeyguides (Moyer 1980; Cambray and Cambray 2014). The extreme costs of being detected at a host nest in this system may have helped drive the evolution of the exceptional egg-laying visit speed of lesser honeyguides.

My comparative analysis suggests that the extent to which rapid laying visits are achievable by brood parasites has been primarily shaped by ecological constraints, providing little evidence suggesting that costs incurred during laying in a host nest have shaped variation in rapid egg-laying visits among brood parasites. However, it remains possible that the overall faster egg-laying visit speeds of brood parasites versus non-parasitic birds has been shaped by such costs. In this study I found strongest support for the importance of ecological constraints, since parasites targeting enclosed nests appeared to require less time for laying visits, and since larger eggs were laid more rapidly, which I propose may be permitted by their stronger shells. I suggest that these likely drivers of natural selection shaping variation in laying visit speed among parasites help to explain why brood parasites have evolved this behaviour, and why it is not observed in non-parasites. Non-parasites are not constrained by access to their own nest, but the costs of potential damage while laying an egg quickly — which could be especially prevalent in the thinner-shelled eggs of non-parasites (Rothstein 1990) — could promote the opposite trend, forcing non-parasitic birds to lay their eggs slowly to avoid damage to their own eggs. Although I found little evidence that any single cost incurred during laying visits in a host nest has promoted rapid egg-laying, taken together they might have influenced the evolution of rapid egg-laying visits from a slow non-parasitic laying ancestral state, with ecological constraints preventing all parasites from evolving rapid laying speeds.

Table 1. The 20 species of brood parasite considered for analysis in this study, plus 11 additional records from species duplicates in different hosts, with the life history traits of both parasites and hosts.

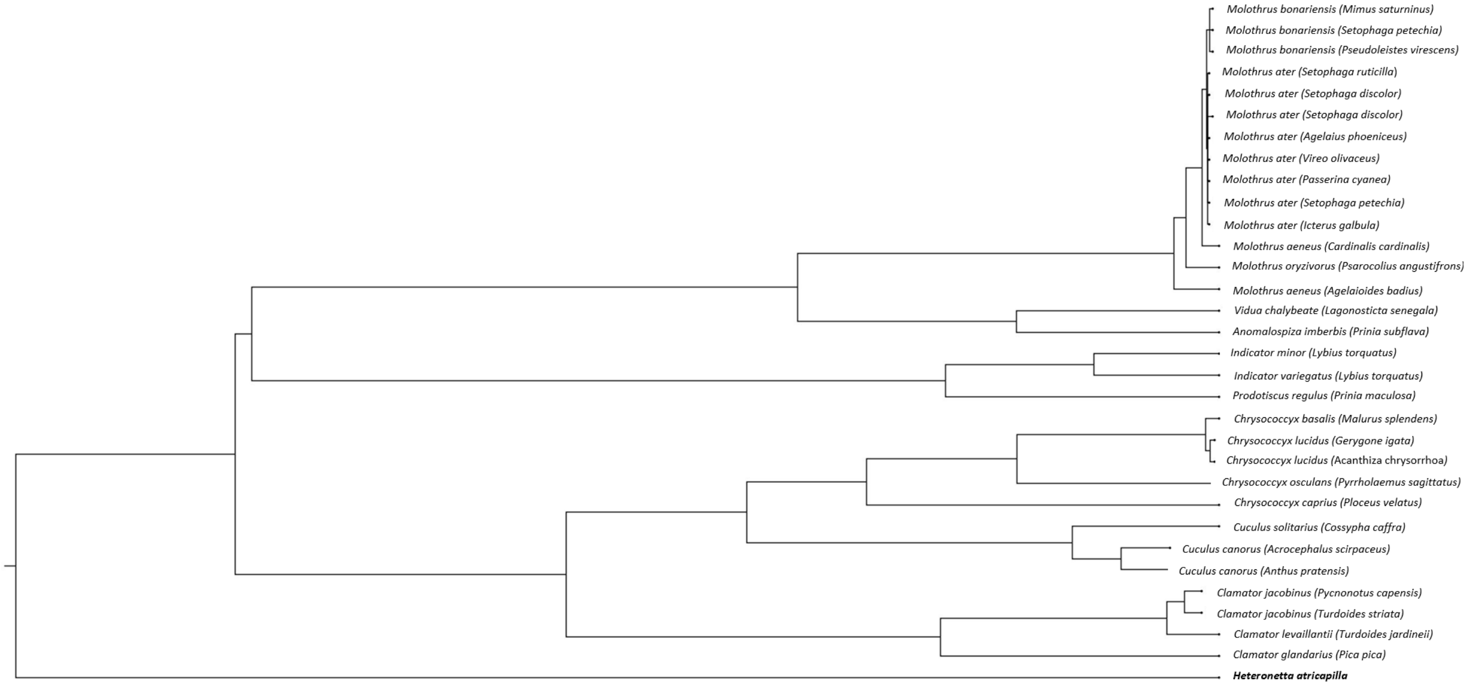
Brood parasite	Host	Laying visit speed (s)	n	Size of egg (mm^3)	Removes egg	Puncture	Size of parasite (g)	Size of host (g)	Size difference (g)	Host rejection	Group breeding host	Nest type	Laying speed source	Method
Indicator minor	*Lybius torquatus*	0.8	2	2669.9	No	No	28.0	55.0	-27.0	NA	Yes	Enclosed	Personal observation	Video at nest, direct observation
Indicator variegatus	*Lybius torquatus*	3.0	1	3133.0	No	No	47.8	55.0	-7.2	NA	Yes	Enclosed	Newby-Varty (1946)	Direct observation
Clamator levaillantii	*Turdoides jardineii*	5.0	1	5421.6	No	No	150.0	68.0	82.0	NA	Yes	Open	Steyn (1973)	Direct observation
Clamator jacobinus	*Pycnonotus capensis*	10.0	1	6247.2	Yes	No	67.0	39.0	28.0	NA	No	Open	Liversidge (1971)	Direct observation
Clamator jacobinus	*Turdoides striata*	5.0	1	6247.2	Yes	No	67.0	34.0	33.0	NA	Yes	Open	Gaston (1976)	Direct observation
Clamator glandarius	*Pica pica*	2.75 [1–41.5]	21	9433.1	Yes	No	170.0	180.0	-10.0	Yes	No	Open	Soler *et al.* (2014)	Video at nest
Cuculus solitarius	*Cossypha caffra*	5.0	1	4242.7	Yes	No	75.0	28.0	47.0	NA	No	Open	Rowan (1969)	Direct observation
Cuculus canorus	*Acrocephalus scirpaceus*	41.2	14 [7–158]	4242.7	Yes	No	75.0	12.0	63.0	Yes	No	Open	Mosknes *et al.* (2000)	Video at nest
Cuculus canorus	*Anthus pratensis*	9.0	1	3266.9	Yes	No	120.0	15.0	105.0	Yes	No	Open	Chance (1922)	Video at nest, direct observation
Chalcites lucidus	*Gerygone igata*	7.0		1670.4	Yes	No	24.8	6.5	18.3	No	No	Enclosed	Brooker *et al.* (1988)	Video at nest
Chalcites lucidus	*Setophaga coronata*	18.0	1	1568.3	Yes	No	22.9	9.0	13.9	No	No	Enclosed	Brooker *et al.* (1988)	Video at nest
Chalcites basalis	*Malurus splendens*	3.0	1	1503.5	Yes	No	22.9	9.0	13.9	No	Yes	Enclosed	Brooker *et al. (1988)*	Video at nest
Chalcites osculans	*Pyrrholaemus sagittatus*	7.0	1	2610.7	Yes	No	30.6	13.0	17.6	NA	Yes	Enclosed	Chisholm (1973)	Direct observation
Chalcites caprius	*Ploceus velatus*	6.0	1	2425.3	Yes	No	35.0	23.8	11.2	Yes	Yes	Enclosed	Macdonald (1980)	Direct observation
Vidua chalybeata	*Lagonosticta senegala*	180.0	1	723.3	No	No	13.2	8.0	5.2	No	No	Enclosed	Morel (1959)	Direct observation
Molothrus rufoaxillaris	*Agelaioides badius*	13.0	1	3837.8	No	Yes	50.2	46.6	3.6	Yes	No	Open	Fraga (1983)	Direct observation
Molothrus bonariensis	*Mimus saturninus*	19.7	259	4485.6	No	Yes	47.0	70.0	-23.0	No	No	Open	Gloag *et al.* (2013)	Video at nest
Molothrus bonariensis	*Setophaga petechia*	30.0	1	4172.7	No	Yes	47.0	11.0	36.0	Yes	No	Open	Wiley and Wiley (1980)	Direct observation

Table 1. continued.

Brood parasite	Host	Laying visit speed (s)	n	Size of egg (mm^3)	Removes egg	Puncture	Size of parasite (g)	Size of host (g)	Size difference (g)	Host rejection	Group breeding host	Nest type	Laying speed source	Method
Molothrus bonariensis	*Pseudoleistes virescens*	42.0 [0.5–2.0]	13	4172.7	No	Yes	47.0	72.9	-25.9	Yes	No	Open	Lopez *et al.* (2018)	Video at nest
Molothrus ater	*Piranga olivacea*	4.0	1	2949.2	No	Yes	38.8	25.0	13.8	NA	No	Open	Prescot (1965)	Direct observation
Molothrus ater	*Setophaga discolor*	31.0 [22–40]	3	2949.2	No	Yes	38.8	8.0	30.8	Yes	No	Open	Nolan (1978)	Direct observation
Molothrus ater	*Agelaius phoeniceus*	94.0	1	2949.2	No	Yes	38.8	85.0	-46.2	No	No	Open	Sealy (1995)	Direct observation
Molothrus ater	*Vireo olivaceus*	60.0	1	2949.2	No	Yes	38.8	18.0	20.8	Yes	No	Open	Grave (1949)	Direct observation
Molothrus ater	*Passerina cyanea*	30.0	1	2949.2	No	Yes	38.8	16.0	22.8	Yes	No	Open	Morgan (1976)	Direct observation
Molothrus ater	*Setophaga petechia*	25.0	1	2949.2	No	Yes	38.8	11.0	27.8	Yes	No	Open	Sealy *et al.* (1995)	Direct observation
Molothrus ater	*Setophaga ruticilla*	60.0	1	2949.2	No	Yes	38.8	7.0	31.8	NA	No	Open	Sealy *et al.* (1995)	Direct observation
Molothrus ater	*Icterus galbula*	119.0	1	2949.2	No	Yes	38.8	42.0	-3.2	Yes	No	Enclosed	Sealy *et al.* (1995)	Direct observation
Molothrus oryzivorus	*Psarocolius angustifrons*	30.0	1	9690.6	No	Yes	162.0	172.0	-10.0	NA	Yes	Enclosed	Robinson (1988)	Direct observation
Anomalospiza imberbis	*Prinia subflava*	127.0	1	1423.2	Yes	No	19.6	9.1	10.5	Yes	No	Open	Personal communication W.E Feeney & C. N. Spottiswoode	Video at nest
Prodotiscus regulus	*Prinia maculosa*	19.0	1	1392.6	Yes	No	14.0	10.0	4.0	NA	No	Enclosed	Personal communication J. Walton	Video at nest

3.6 Supplementary material

3.6.1 Extended phylogeny used in comparative analysis



Supplementary figure 1. An extended phylogeny of brood-parasitic species included in the study, including multiple records of the same parasitic species parasitizing different host species (n = 31). Host species are given in brackets beside each brood parasite. Briefly, brood-parasitic species with records of laying visit speed in different hosts were duplicated and added to the topology of the tree. Branch lengths in these split topologies were calculated by dividing the original branch length for the single species by the number of tips present in the clade (Grafen 1989). The black-headed duck *Heteronetta atricapilla* (in bold) was used as a phylogenetic outgroup.

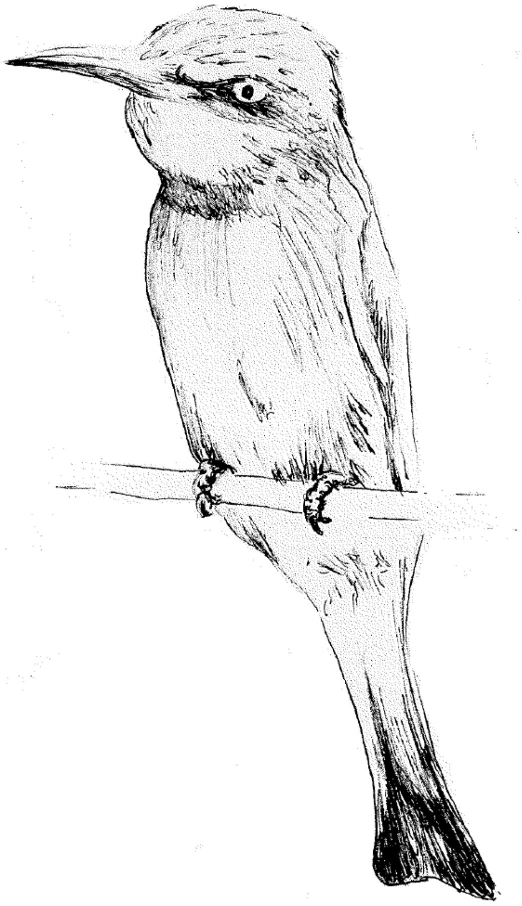
3.6.2 Videos of the fastest egg-layer in the world

Supplementary videos 1 and 2.

Lesser honeyguides *Indicator minor* are (to my knowledge) the fastest documented egg-layers of any bird in the world. These videos show this rapid egg-laying ability. **Video 1** shows a lesser honeyguide female deposit her egg within a black-collared barbet *Lybius torquatus* nest in less than one second, recorded using an infrared camera (modified BrightTea 808#16 keychain camera, BrightTea, China) placed inside the cavity nest of the host. **Video 2** shows another parasitism event at a different barbet nest, viewed from outside the cavity and recorded using a Canon 7d DSLR (Canon, Huntington, USA). The female enters the nest, lays her egg (confirmed through visually inspecting the eggs after this event), and exits the nest in just over a second.

CHAPTER FOUR

Violence is not (always) the answer: the evolution of virulent puncturing in brood-parasitic honeyguides



4.1 Abstract

In pathogens, it is well established that virulence has both costs and benefits to the parasite, but these trade-offs are little studied in the context of brood parasitism. Greater honeyguides *Indicator indicator* are virulent avian brood parasites that reduce host hatching rate by puncturing host eggs when they lay their own. I studied the potential costs to honeyguides of puncturing the eggs of its host, the little-bee eater *Merops pusillus*, and the evolution of honeyguide behaviour in response to these costs. My primary finding is that puncturing of host eggs by honeyguides is costly to the parasite, in two ways. First, by monitoring host populations over an eight-year period, I show that parasitized bee-eaters in southern Zambia reject punctured clutches at a higher rate than unpunctured clutches. By simulating honeyguide parasitism at bee-eater nests, I replicated this pattern experimentally. Clutches that were experimentally punctured early on in incubation were rejected at higher rates than those that were punctured closer to hatching, suggesting that bee-eaters incorporated information about the reproductive value of their clutch when making rejection decisions. Second, I found that nests containing punctured eggs suffered elevated rates of predation, providing a further cost to honeyguides of their own virulence. Finally, I show evidence consistent with the hypothesis that honeyguides strategically adjust their puncturing behaviour in relation to host clutch size and incubation stage, suggesting that honeyguides temper their virulence in accordance with these trade-offs. These findings are consistent with a coevolutionary scenario in which hosts defend themselves against elevated parasitic virulence, which in turn promotes the evolution of less virulent parasites.

4.2 Introduction

Obligate parasites are dependent on their hosts for fitness and have evolved myriad mechanisms to improve their own reproductive success, often to the detriment of their hosts. This reduction in host fitness caused by parasitism is termed virulence (Read 1994). While virulence brings benefits to parasites, these trade off evolutionarily against its costs. Studying the trade-offs involved in maintaining parasitic virulence is key for understanding the coevolution of host defences and parasitic counter-adaptations, and has been particularly well applied in the contexts of disease-causing pathogens (Griffin *et al.,* 2004; de Roode *et al.,* 2005; de Roode *et al.,* 2008; Kamada *et al.,* 2012). By contrast, in brood parasites, which exploit the parental care of their hosts, the trade-offs that may shape the evolution of brood-parasitic virulence remain mostly unstudied (Kilner 2005; Grim 2006) despite the fact they have long been known as model systems in which to study coevolution (Rothstein 1990).

Avian brood parasites lay their eggs in the nests of other birds and have evolved a wide array of virulent behaviours. For example, day-old common cuckoos *Cuculus canorus* throw their foster siblings out of the nest (Jenner 1788; Honza *et al.,* 2007; Rutila *et al.,* 2002), cuckoo finches *Anomalospiza imberbis* replace host eggs and typically outcompete any remaining host young (Vernon 1964), and brown-headed cowbirds *Molothrus ater* revisit host nests for post-laying visits to puncture eggs (Massoni and Reboreda 1999). These behaviours can likely be explained by the benefits that they provide to brood parasites in reducing competition for incubation (Peer and Bollinger 1997) or chick provisioning (Mock and Parker 1998; Carter 1986). However, like pathogens (Le Clec'h *et al.,* 2012), brood parasites are variable in their virulence (Kilner 2005), suggesting that these behaviours also have associated costs to the parasite. For example, superb fairy-wrens *Malurus cyaneus* often stop provisioning at nests that contain only a single chick, conferring a high cost on a parasitic Horsfield's bronze-cuckoo *Chalcites basalis* chick that has ejected all other chicks from the nest (Langmore *et al.,* 2003). Kilner (2003) found that chicks of the brood-parasitic brown-headed cowbird *Molothrus ater* that are raised alone in the nest receive less provisioning than those raised alongside host siblings, demonstrating that host-killing behaviours can be costly if they reduce a parasitic chick's ability to elicit provisioning from foster parents. However, we lack quantitative evidence that brood parasites adaptively adjust their virulence in accordance with the trade-offs involved. In many cases, virulence may be inflexible as parasitic offspring are trapped in a winner-takes-all game with their hosts, which precludes any down-regulation of virulence. For example, in the case of cuckoo species that eject host eggs and chicks, there is little scope for a cuckoo chick to temper its virulence, as it cannot eject half a foster sibling, and likely suffers reduced fitness when forced to compete with its nest mates (Hauber and Moskát 2008; Grim *et al.,* 2009). Consequently, the potential risk of the host parent abandoning the single cuckoo chick is less likely to act as an evolutionary constraint on the cuckoo chick's virulence, since the costs of not ejecting outweigh the costs of ejecting. Forms of virulence with the potential to be facultatively adjusted, such as the egg puncturing performed by some brood-parasitic birds including certain species of cowbird (Gloag *et al.,* 2013; Massoni and Reboreda 1999; Peer 2006) and honeyguide (Spottiswoode and Colebrook-Robjent 2007), may provide a better model system for understanding the trade-offs shaping observed levels of virulence.

The greater honeyguide *Indicator indicator* (hereafter 'honeyguide'), is a brood parasite most familiar for its mutualistic relationship with human honey-hunters (Spottiswoode *et al.,* 2016). During laying, female honeyguides puncture some or all of the host eggs present in the nest they are parasitizing (Figure 1; Friedmann 1955), typically leading to the death of the host embryo (Spottiswoode and Colebrook-Robjent 2007). Unlike in other brood-parasitic systems (Gloag *et al.,* 2013), honeyguides do not appear to visit host nests specifically to puncture outside of laying events, but egg puncturing during laying still has several possible advantages: it can (i) induce a host to re-nest such that it synchronises with parasitic egg-laying (Arcese *et al.,* 1996), or (ii) provide the parasite with information on the state of incubation of a host's clutch (Massoni and Reboreda 1999). Additionally, reduction in the number of viable host eggs could (iii) reduce competition for incubation (Lerkelund *et al.,* 1993), (iv) reduce the number of nestlings the parasitic chick has to kill in order to monopolise host parental care (Spottiswoode and Colebrook-Robjent 2007), or (v) allow parasites more freedom in the timing of laying by preventing host chicks from hatching first (Spottiswoode and Colebrook-Robjent 2007). However, there is large variation in degree of honeyguide puncturing between parasitism events (Spottiswoode and Colebrook-Robjent 2007), suggesting that there are costs to honeyguide virulence.

In this study I investigated the potential costs to honeyguides of their own virulence. Successful parasitism by honeyguides always leads to complete reproductive loss for the host (Spottiswoode and Koorevaar 2012). Therefore, there is strong evolutionary pressure for hosts to develop defences against honeyguide parasitism. At my field site in southern Zambia, the main host of the greater honeyguide is the little bee-eater (*Merops pusillus*; hereafter 'bee-eater'). Bee-eaters that detect that they have been parasitized respond by either abandoning the nest (leaving the eggs intact; Spottiswoode 2013), or by ejecting the entire clutch from the nest (supplementary material 1; Spottiswoode 2013). However, the cues that bee-eaters use to prompt these defences remain unclear. Despite recognising and mobbing honeyguide models near their nests, bee-eaters did not use this as a cue to reject eggs, even when in combination with the presence of a foreign egg in their nest (Tong *et al.,* 2015). Most bee-eater clutches that are parasitized by a honeyguide at my study site suffer puncturing of their eggs (Spottiswoode and Colebrook-Robjent 2007; Spottiswoode 2013; Spottiswoode and Koorevaar 2012). Therefore, in this study I asked whether bee-eaters are more likely to reject clutches that contain punctured eggs, thus imposing a cost on parasitic virulence. As heavily punctured eggs are less likely to survive to hatching (Spottiswoode and Colebrook-Robjent 2007), selection should favour bee-eaters that can identify punctured eggs within their nest and then reject (eject or desert) the entire clutch, since in cases where all host eggs have been punctured, rejecting only the foreign egg would not avert reproductive failure. A greater number of punctures may be easier for a bee-eater to detect and so provide a more reliable cue of the presence of a honeyguide egg. Accordingly, the chance of a clutch being rejected should be greater when parasitism is accompanied by higher levels of puncturing of host eggs (Spottiswoode & Colebrook-Robjent 2007).

Figure 1. A bee-eater egg that has suffered honeyguide puncturing. Each dent and mark on this egg which, due to the punctures, now contains a dead embryo, was made by a female greater honeyguide as she laid her own egg in the host nest.

However, life-history considerations may further modulate rejection decisions. The reproductive value to the host of its eggs increases as the clutch develops (Albrecht and Klvana 2004; Osiejuk and Kuczyński 2007), as if they do survive honeyguide puncturing, more developed bee-eater eggs have a better chance of hatching before the parasitic chick does (Spottiswoode and Colebrook-Robjent 2007). This makes potential rejection errors (where a host incorrectly identifies its own clutch as containing a parasitic egg and subsequently reject it; Davies *et al.,* 1996; Lyon 2003) more costly as the development of the clutch advances, which would be expected to make hosts more risk-averse in making rejection decisions. I therefore asked whether bee-eaters modulated their rejection behaviour based on the reproductive value of their clutch. If more advanced clutches have greater reproductive value, then the probability that bee-eaters will reject a punctured clutch should decrease as incubation stage of the clutch increases. Alternatively, since the cost of honeyguide parasitism is so high (Spottiswoode and Colebrook-Robjent 2007; Spottiswoode and Koorevaar 2012), the optimal decision for a bee-eater once it has detected honeyguide parasitism could still be to reject the clutch, regardless of incubation stage.

In addition to the potential cost of eliciting host rejection, puncturing may also increase predation rates, given that damaged eggs in avian nests can attract predators (Kemal and Rothstein 1988). This hypothesis predicts that bee-eater nests containing clutches which have received a greater amount of egg puncturing by a honeyguide are more likely to be predated than those that have received lower levels of puncturing, and that parasitized clutches should be more likely to be predated than unparasitized clutches.

Finally, honeyguides may modulate their egg-puncturing behaviour in accordance with the fitness trade-offs involved. Some host clutches are completely destroyed by honeyguide puncturing, while some are not punctured at all (Spottiswoode and Colebrook-Robjent 2007). Such variation in the intensity of parasitic virulence suggests that, in some cases, the costs of puncturing host eggs, relative to any benefits gained, are sufficient to reduce it to zero. Honeyguides may be able to facultatively reduce this first line of virulence, since they have a second line of virulence: in addition to egg-puncturing, honeyguide chicks kill all host young upon hatching, using their specially adapted bill hook (Spottiswoode and Koorevaar 2012). This virulent behaviour at the chick stage may give scope for flexibility at the egg stage, allowing honeyguides to reduce levels of puncturing in response to a trade-off with its costs. In addition to simply puncturing less, honeyguides could puncture strategically to reduce the chances of the puncturing being detected. For example, reducing the total amount of puncturing within the clutch, and only puncturing each egg a few times, may make the virulence less obvious. If so, then honeyguides should puncture each egg within a clutch, ideally below a threshold level of bee-eater detection, such that the variance in puncturing within the clutch will not increase as host clutch size increases. By contrast, if honeyguides puncture indiscriminately within a clutch, then as clutch size increases, some eggs should receive substantially more punctures than others. Strategic spreading of puncturing effort would imply that honeyguides trade off an increased likelihood of avoiding detection of their parasitic behaviour against a potentially reduced likelihood of being successful in halting host embryonic development. Lastly, if bee-eaters are more likely to reject clutches that have been punctured early on in incubation (when these clutches have lower reproductive value), then selection should favour honeyguides that reduce virulence when parasitizing freshly laid clutches. This predicts that host clutches parasitized earlier in host incubation should be accompanied by lower levels of puncturing than are clutches parasitized later on in incubation.

I tested these hypotheses by monitoring natural honeyguide parasitism of bee-eaters, and through experimental manipulation of nests. I show that there are costs to honeyguide egg-puncturing, and that these costs of virulence have likely shaped the egg puncturing behaviour of honeyguides.

4.3 Materials and methods

4.3.1 Field site and study system

I conducted fieldwork on greater honeyguides and their little bee-eater hosts predominantly on two adjacent farms, Musumanene and Semahwa, near Choma, southern Zambia (16°46’S, 26°54’E). The dominant vegetation at my study site consists of miombo woodlands, dambos (temporarily flooded grassland) and agricultural land. Bee-eaters nest in terrestrial burrows built into sand banks or, most commonly at my study site, aardvark *Orycteropus afer* or bushpig *Potamochoerus larvatus* excavations. The breeding season is dry and hot, and both host and parasite breed from September to November.

4.3.2 Monitoring of nests to document interactions between bee-eaters and honeyguides

Parasitism and egg puncturing by honeyguides have been monitored over eight breeding seasons by other observers (2008–2011) and myself (2015–2018; n = 161 parasitized clutches across both study periods). Following Spottiswoode and Koorevaar (2012), I gained access to bee-eater nests by excavating a vertical access shaft above the nest chamber at my first visit, which was then temporarily filled in and allowed for easy access to the nest chamber at each subsequent visit. As honeyguides can parasitize host nests at any point during host laying and incubation (Spottiswoode and Colebrook-Robjent 2007), nests were monitored until hatching. Parasitism events were defined as the discovery of a honeyguide egg within or outside of the nest, identified by its larger size, more elongated shape, and shinier shell surface compared to bee-eater eggs. Upon detection of a parasitic egg, I inspected all host and parasite eggs for punctures. Puncture holes in each host egg within the clutch were individually marked with a pencil and counted. If a host egg was destroyed as a result of puncturing (n = 32 of 161 parasitized clutches), making counting punctures impractical, the clutch was removed from the dataset. Punctures in parasitic eggs are the result of multiple parasitism by different females (Spottiswoode 2013). Multiple parasitism (two or more honeyguide eggs laid by different females) occurred in 13.6% of nests in this dataset (n = 22 of 161 parasitized nests with puncturing data). Multiply-parasitized clutches were removed from the dataset as I was unable to determine how much each honeyguide contributed to puncturing of host eggs. After removing clutches with completely broken host eggs or multiple parasitism, there were 108 parasitism events with honeyguide puncturing recorded. Parasitic eggs were considered to be rejected if the host abandoned or ejected the clutch from the nest after 5 days of completing its clutch any eggs were ejected from the nest or the clutch was abandoned within this time. Each puncture mark (most often a pin-prick, sometimes a crack or dent) was scored as one puncture, regardless of size. The size of punctures may be a feature that bee-eaters use to identify punctured eggs, as larger punctures presumably inflict more damage to the egg and are easier to detect. However, I have no data with which to quantify the damage caused by different sized punctures. I scored individual nests for puncturing only once, as there is no indication that honeyguides visit host nests for pre- or post-laying puncturing visits, as established in other brood-parasitic systems (Gloag *et al.,* 2013). Eggs within a clutch were scored on the first visit when a honeyguide egg was found in or outside the nest. As such, the exact timing of honeyguide laying could not be determined, and since honeyguides parasitize host nests throughout the laying and incubation period, some host eggs that had no punctures may have been laid after the honeyguide had laid its egg. This is not a problem in my observational test of my hypothesis of host rejection behaviour, because it is unlikely that a bee-eater would decide to reject, but first lay a fresh egg before doing so; therefore, any unpunctured eggs found within a rejected clutch would have been used as a cue by the bee-eater parent making the rejection decision. Similarly, it is likely that the actual rate of host rejection is higher than estimated by my observational dataset, because active nests were easier to find. Bee-eater nests that were found with punctured clutches, but no honeyguide eggs, were not included in the dataset because I could not determine with certainty whether the nest had been parasitized in case punctured eggs could arise from causes other than honeyguides. Nests checked during incubation and found to be empty with no sign of host or parasitic eggs were considered predated; I did not record predation rates after the chicks hatched. In 2017 and 2018 I additionally monitored unparasitized nests (n = 72) to examine whether there was a difference in predation rates between parasitized nests that were accepted by the host, and unparasitized nests. I considered any honeyguide eggs that were still present in an active nest five days after host clutch completion to have been accepted by the host, following Spottiswoode (2013). To determine whether the reproductive value of the clutch influences host rejection behaviours, I estimated the state of incubation of all eggs by 'candling' (Lokemoen and Koford 1996) at the time that a parasitic egg was first discovered. This was done by shining a bright torch through the white egg. All eggs were then ranked on a scale from 0 to 5 based on the development of the embryo, following Spottiswoode and Colebrook-Robjent (2007). Eggs ranked as 0 had no visible embryonic development and those ranked 5 were close to hatching. I used the mean candling score for all host eggs in the nest at the time of parasitism as an index of the reproductive value of the host clutch.

4.3.3 Experimental test of the effect of egg puncturing on bee-eater rejection

I performed an experiment during 2015–16 to test whether bee-eaters detect and reject clutches that contain punctured eggs. To do so, I manipulated bee-eater clutches by adding a single foreign egg simulating a honeyguide egg, concurrently with one of two treatments: (i) puncturing of two eggs seven times each with a clean 22-gauge needle (n = 17 nests), (ii) no puncturing (n = 23 nests), for a total of 40 manipulated nests. Treatment (i) replicates the average number of punctures per egg in natural parasitism events (Spottiswoode and Colebrook-Robjent 2007) and therefore simulated a natural honeyguide laying event as closely as possible. Host eggs were candled to determine their incubation stage at the time of experimental parasitism. All experimental puncturing was performed upon the first visit to the nest, to avoid any confounding effect of previous disturbance. I experimentally punctured eggs in nests at various states of development, as would occur naturally, since honeyguides parasitize bee-eater nests throughout laying and incubation. Half of experimentally punctured clutches were stage 0 (undeveloped embryo), 30% were stages 1 or 2 (partially incubated), and 20% were stage 3 or later (mid-late stage development). Following previous studies, I used emerald-spotted wood-dove *Turtur chalcospilos* eggs to simulate honeyguide parasitism, because they closely approximate greater honeyguide eggs in appearance and dimensions (Spottiswoode 2013; Tong *et al.,* 2015). I monitored nests for five days for rejection of the foreign egg. As for naturally parasitized nests, rejection was considered to have occurred if any eggs were ejected from the nest or the clutch was abandoned within this time. I recorded each bee-eater pair once, using GPS location to avoid pseudoreplication within and between years, with nests in 2016 within approximately 1 km of nests in used in 2015 avoided for the puncturing experiment.

4.3.4 Statistical Analyses

In the observational dataset, I first tested whether puncturing was costly to honeyguides. I used a generalized linear mixed model (GLMM) with logit link function to test whether honeyguide puncturing rate predicted the rejection behaviour of bee-eaters. Models were generated with the rejection decision of a bee-eater pair as a binary response (yes or no), and the average number of punctures per egg, the total number of punctures in the clutch, the proportion of the clutch punctured at the time of discovery, and the incubation stage of the clutch as fixed effects. The year of study was included as a random effect. To test whether egg puncturing was associated with predation rates, I re-ran each model with predation fate (yes or no) during the incubation period (until hatching) included as a binary response. As the average and total number of punctures within a clutch are collinear, models containing both measures were excluded from model selection. Using a separate observational dataset of both unparasitized and parasitized nests, I used a generalized linear model with logistic regression to test the effect of parasitism on predation rate. Predation outcome (yes or no) was modelled as a binary outcome, and parasitized or not as a binary predictor.

In the experimental dataset, I modelled rejection decision (yes or no) as a binary response variable in a logistic GLMM framework. The experimental puncturing treatment (yes or no), the proportion of the clutch punctured, and incubation stage were treated as fixed effects. Models with each combination of predictor variables were explored. I naturally generated variation in the proportion of the clutch that I experimentally punctured by always puncturing two eggs. However, clutches punctured at the beginning of incubation were more likely to have a higher proportion of the clutch punctured, as not all eggs had necessarily been laid (e.g. if only two eggs were present, I punctured 100% of the clutch). Clutches punctured once the full number of eggs had been laid were more likely to have a lower proportion of the clutch punctured, as most bee-eaters lay four to five eggs within a clutch (e.g. if a clutch of four eggs was laid, I punctured 50% of the clutch). Therefore, the proportion of the clutch punctured is confounded with incubation stage, and since we cannot distinguish between the effects of these two variables, models containing both measures were excluded from model selection. The year of experiment, and nest ID were included as random effects in a mixed model, but were subsequently dropped as they did not explain any more variance in the model; therefore the final model used was a generalized linear model (GLM).

Finally, I used the observational dataset of natural parasitism events to test whether honeyguides modulate their virulent behaviour in accordance with the costs of puncturing. To investigate whether honeyguides punctured clutches as inconspicuously as possible to reduce detection, I used a linear model to test for a relationship between the variance in puncturing among eggs in a clutch (calculated as the standard deviation of puncturing within the clutch) and the number of eggs in the clutch. To test whether honeyguides punctured host eggs more heavily in clutches with more advanced embryonic development, I modelled incubation stage as an ordinal response variable, and the average number of punctures within the clutch, the total number of punctures in the clutch, and the proportion of the clutch punctured as predictor variables.

I conducted all analyses in R v. 3.1.3 (R Core Team 2019). Models were checked for assumptions of normality and heteroscedasticity with use of diagnostic plots and the lmtest package (Zeileis and Hothorn 2002). Mixed effects models were implemented using the lme4 package (Bates *et al.,* 2014). Goodness of fit and quality of model fit for GLMs were assessed using DescTools (Signorell *et al.,* 2020) and ResourceSelection (Lele *et al.,* 2019). Ggplot2 (Wickham 2019b) and cowplot (Wilke 2019) were used to produce graphs. Model selection was performed using the Akaike information criterion (AIC; Wagenmakers and Farrell 2004), with models of lower AIC considered the most parsimonious when they were >2 AIC units lower than a less complex model (Burnham *et al.,* 2011).

4.4 Results

4.4.1 Observations of honeyguide puncturing in bee-eater clutches

Greater honeyguides punctured at least one host egg in 87 of 108 (80%) parasitism events, with host eggs receiving a mean ± SE of 6.13 ± 0.65 punctures per egg (range = 0–31). The total number of punctures per clutch was, on average, 18.45 ± 2.04 (range 0–85, n = 309 host eggs in 96 clutches with complete data on egg puncturing). Most nests were parasitized at the start of bee-eater incubation, with 68% of parasitism events occurring in nests containing eggs at incubation stage 0 (73 of 108 clutches). Only 16% (17 of 108 clutches) of parasitism events were recorded in host nests that contained eggs in the later stages of development (incubation stages 3–5). On average, 67% of the eggs within the clutch were punctured. In 50% (n = 54 of 108 clutches) of parasitism events, honeyguides punctured every egg within the clutch; in 19% (21 of 108 clutches), no puncturing was detected.

4.4.2 Costs of puncturing to honeyguides

4.4.2.1 Are naturally punctured clutches more likely to be rejected?

Bee-eaters rejected clutches containing a honeyguide egg in 48 of 108 (44%) parasitism events with complete data on egg puncturing. Bee-eaters that were parasitized by a honeyguide but that did not suffer any puncturing rejected their clutch on only 15% (n = 3 of 20 nests) of occasions. Conversely, heavily punctured clutches (when each egg within the clutch was punctured 10+ times) were rejected on 65% of occasions (n = 15 of 23 nests). Clutches containing more than 20 punctures per egg, or more than 60 punctures in total, were always rejected by bee-eaters (Figures 2A and 2B). There was a positive relationship between the incidence of rejection and both the average number of punctures ($\beta = 0.221 \pm 0.592$, $Z_{99} = 3.726$, $p < 0.001$; Figure 2A) and the total number of punctures ($\beta = 0.029 \pm 0.011$, $Z_{107} = 2.622$ $p < 0.01$; Figure 2B) in the clutch, and with the proportion of the clutch that was punctured: clutches that contained a greater proportion of punctured eggs were more likely to be rejected ($\beta = 0.027 \pm 0.006$, $Z_{107} = 2.921$, $p < 0.001$; Figure 2C). There was a negative relationship between the incubation stage of the host clutch and the probability of rejection ($\beta = -0.946 \pm 0.249$, $Z_{99} = -3.919$, $p < 0.001$; Figure 2D). The most parsimonious model contained the average number of punctures per egg within the clutch, and the incubation stage of the clutch, with this model predicting 27% of the variation in bee-eater rejection response.

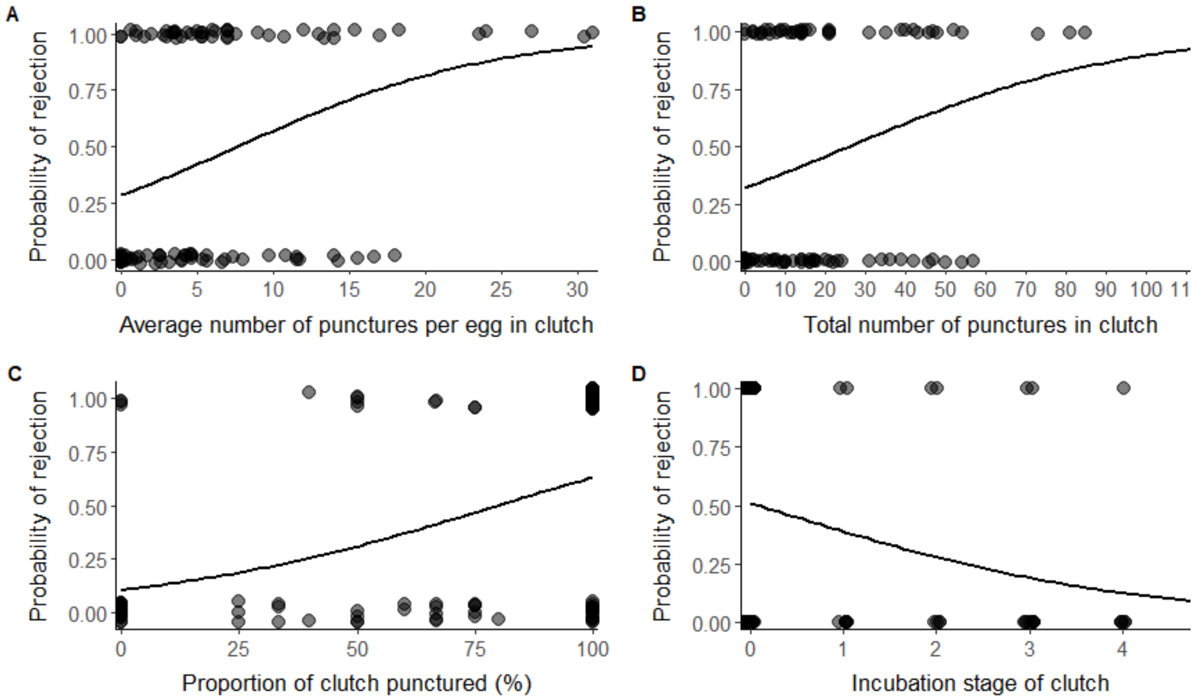


Figure 2. Observational data on bee-eater rejection behaviour in relation to honeyguide puncturing of their clutch at n = 161 nests. **A)** The probability that a bee-eater rejected its clutch was positively correlated with the average number of punctures per egg within that clutch. **B)** The probability that a bee-eater rejected its clutch was positively correlated with the total number of punctures in that clutch. **C)** The probability that a bee-eater rejected its clutch was positively correlated with the proportion of the eggs in the clutch that was punctured. **D)** The probability that a bee-eater rejected its clutch was negatively correlated with the incubation stage of the clutch at the time of parasitism. The proportion of the clutch punctured and incubation stage are likely confounded, as host nests parasitized at the beginning of incubation may not have yet completed their full clutch. Black lines indicate the model fits of logistic regression. Points indicate the raw data (darker circles indicate overlapping data points) and have been jittered.

4.4.2 Are naturally punctured clutches more likely to be predated?

Of 21 honeyguide eggs accepted after parasitism and with known hatching fate, 14 failed to hatch. Of these 14, 13 were predated during incubation, and one was in a nest that was parasitized a second time (and subsequently punctured again), which prompted the hosts to abandon the clutch. Nests containing clutches that had a higher proportion of punctured eggs were significantly more likely to be predated ($\beta = 0.019 \pm 0.009$, $Z_{39} = 2.194$, $p = 0.028$). There was no observed increase in predation rate with an increase in the total number of punctures ($\beta = 0.028 \pm 0.022$, $Z_{39} = 1.286$, $p = 0.20$), and a marginally non-significant trend for predation rate to increase with a higher average number of punctures ($\beta = 0.144 \pm 0.076$, $Z_{39} = 1.905$, $p = 0.057$). There was a strong positive association between the stage of host egg incubation when the nest was parasitized and the likelihood of predation ($\beta = 0.990 \pm 0.297$, $Z_{39} = 3.329$, $p < 0.001$), such that nests that were parasitized early on in incubation were more likely to be predated than those that were parasitized late in incubation.

Finally, parasitized nests were significantly more likely to be predated than non-parasitized nests ($\beta = 1.980 \pm 0.795$, $Z_{71} = 2.491$, $p = 0.013$): of 72 nests with complete records, I found that 29 of 50 (58%) unparasitized nests suffered predation, while 20 of 22 (91%) parasitized nests were predated.

4.4.3 Are experimentally punctured clutches more likely to be rejected?

Bee-eaters that received experimental puncturing were significantly more likely to reject their clutch than those whose eggs were not experimentally punctured ($\beta = 3.188 \pm 0.952$, $Z_{38} = 3.349$, $p < 0.001$, Figure 3A). There was a positive relationship between the proportion of eggs within the clutch that were punctured and the incidence of bee-eater rejection ($\beta = 0.05 \pm 0.016$, $Z_{39} = 3.330$, $p = 0.025$, Figure 3B), which was mirrored by a non-significant trend for more incubated clutches to be rejected less often ($\beta = -0.530 \pm 0.351$, $Z_{37} = -1.510$, $p = 0.131$); note that these are confounded because since I always punctured two eggs from each clutch, clutches punctured at the start of incubation (before the clutch was completed) had a larger proportion of their clutch punctured than those nests where the clutch was already complete. Two models were selected as equally good (within 2 AIC units of each other) at explaining the variation in bee-eater rejection in experimentally punctured clutches: (i) whether or not the clutch was punctured, and (ii) whether or not the clutch was punctured and the incubation stage of the host clutch at time of puncturing (Table 1). The less complex model (i) was therefore selected as the best model.

4.4.4 Do honeyguides modulate their puncturing behaviour according to its expected costs?

The degree of honeyguide puncturing of bee-eater clutches was highly variable throughout the study, with some honeyguides puncturing as much as 85 times across a clutch, and some not puncturing at all. However, variance in puncturing across eggs in a clutch did not differ with host clutch size ($\beta = 19.67 \pm 106.87$, $t_{57} = 0.184$, $p = 0.86$). There was a positive relationship between incubation stage and the average number of punctures within the clutch ($\beta = 1.403 \pm 0.439$, $t_{103} = 3.196$, $p < 0.001$), replicating previous findings (Spottiswoode and Colebrook-Robjent 2007), as well as the total number of punctures within the clutch ($\beta = 5.581 \pm 1.340$, $t_{103} = 4.163$, $p < 0.01$). There was a non-significant trend for the proportion of the clutch that was punctured upon discovery to increase with the incubation stage of the clutch ($\beta = 5.118 \pm 2.771$, $t_{103} = 1.847$, $p = 0.068$).

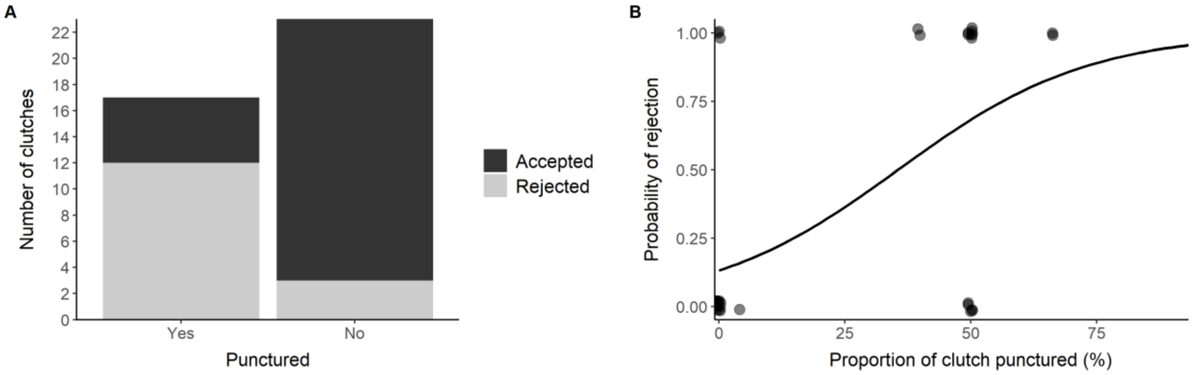


Figure 3. Experimental manipulations of egg puncturing at n = 40 bee-eater nests. **A)** The results of experimental puncturing showing the difference in rejection rate between experimentally punctured and unpunctured clutches. Experimentally punctured clutches were significantly more likely to be rejected than experimentally unpunctured clutches. **B)** The probability that a bee-eater rejected its clutch was positively related to the proportion of the eggs within the clutch that were experimentally punctured. However, this is likely confounded with incubation stage, as smaller clutches within the dataset were generally at earlier stages of incubation, since most bee-eaters lay four to five eggs in a clutch and begin incubation before the clutch is complete. Black lines indicate the model fits of logistic regression. Points indicate the raw data (darker circles indicate overlapping data points) and have been jittered.

Table 1. Top models for analysis of bee-eater rejection after experimental puncturing. Model support table (AIC) for the top ranked GLM models (>2 AIC units lower than next best models). Model 1 was selected as the best model, since it is less complex.

Model #	R^2	AIC	Model variable	Intercept	Z value	p value
1	0.29	42.409	Punctured (yes/no)	2.773	3.396	< 0.001
2	0.33	41.790	Punctured (yes/no)	3.188	3.349	< 0.001
			Incubation stage of clutch	-0.530	-1.510	0.131

4.5 Discussion

Parasites vary greatly in virulence between and among species (Kilner 2005), suggesting that virulence must be involved in evolutionary trade-offs with other traits associated with parasitic behaviour. Here I identified two different costs to virulence in the brood-parasitic greater honeyguide, one mediated by hosts and the other by predators, and explored how these may have consequently shaped the evolution of honeyguide puncturing behaviour.

4.5.1 Costs of puncturing to honeyguides: rejection by bee-eaters

I found evidence for the costs of parasitic virulence to honeyguides. Many hosts of brood-parasitic birds have evolved egg rejection abilities (Langmore *et al.,* 2005), and this is the most extensively researched area of parasite-host interactions (Grim 2007). These abilities often rely on recognition of differences between host and parasitic eggs in terms of size (chapter five; Mason and Rothstein 1986; Marchetti 2000; Meshcheryagina *et al.,* 2018), or colouration and pattern (Brooke and Davies 1988; Spottiswoode and Stevens 2010) as a means by which hosts can detect parasitic eggs, and so as a cue that their nest has been parasitized. I provide evidence that little bee-eaters use the presence of punctured eggs as a cue of honeyguide parasitism. In natural observations at host nests, bee-eaters that had been parasitized were more likely to reject their clutch if it contained punctured eggs. The presence of a honeyguide egg alone, in the absence of punctured eggs, was generally insufficient to trigger clutch rejection, as has previously been shown experimentally (Tong *et al.,* 2015; Spottiswoode 2013). Bee-eaters rejected clutches more often when their eggs were more heavily punctured. Additionally, bee-eaters rejected clutches more often when a greater proportion of the eggs within the clutch (at the time of discovery) was punctured. However, this is likely confounded with incubation stage, since clutches parasitized at the beginning of host incubation tended to contain fewer eggs, therefore leading to a higher proportion of the clutch being punctured. Despite these clear patterns, in some cases bee-eaters did accept heavily punctured clutches, mirroring the findings of Spottiswoode and Colebrook-Robjent (2007), using a different dataset from the same study area. For example, a bee-eater nesting in 2017 suffered a total of 23 punctures to its clutch of three eggs at the start of incubation (incubation stage 0) yet still accepted the parasitic egg.

A potential alternative explanation for these patterns in the observational dataset of natural puncturing behaviour by honeyguides is that bee-eaters responded to a different cue correlated with extent of puncturing. For example, if more puncturing requires more time at the nest, it may be associated with a higher chance of a honeyguide being detected while laying. While this remains a possibility, my experimental manipulations simulating honeyguide egg-puncturing behaviour nonetheless confirm that bee-eaters do specifically use punctured eggs as a cue for rejection. Experimentally parasitized and punctured clutches were much more likely to be rejected than those that only received a foreign egg but no experimental puncturing.

Yet, despite most bee-eaters rejecting punctured clutches, some did not. Why some bee-eaters do not respond to punctured eggs as a cue of parasitism is intriguing. It is possible that more experienced individuals are better at recognising punctured eggs, and subsequently rejecting the clutch. Unfortunately, I have no data on the breeding experience of individual bee-eater pairs at my study site, or on whether particular birds have been parasitized previously. Nonetheless, a previous study suggests that many birds at my site may be naïve to parasitism by greater honeyguides, despite the high parasitism rates that are reported here and in previous studies at this site (Spottiswoode 2013; Spottiswoode and Colebrook-Robjent 2007; Spottiswoode and Koorevaar 2012). In 2015, when presented with a stuffed greater honeyguide at their nest, around 50% of pairs that were tested showed no mobbing response, suggesting that they failed to recognize the model as a potential threat (Tong *et al.,* 2015).

Although bee-eaters can use egg puncturing as a cue to reject the clutch, and thus as a defence against honeyguide parasitism, my experiment does not identify whether bee-eater egg rejection behaviour has evolved specifically as a response to honeyguide virulence. Detection of broken eggs can generate a similar response in a nest sanitation context (Kemal and Rothstein 1988; Guigueno and Sealy 2012b). However, my observations of bee-eaters throughout the incubation and nestling phases suggest that little bee-eaters do not respond to other cues of unsanitary nests. Rotting eggs and the bodies of dead chicks are often left untouched in the nest chamber (Spottiswoode and Koorevaar 2012). Chicks sit on a bed of insect remains and faeces, which often become infected with fly larvae that feed upon the detritus, a situation similar to that reported in European bee-eaters *Merops apiaster* (Krištofík *et al.,* 2016). Future studies could investigate how species that are closely related to little bee-eaters, yet naïve to honeyguide parasitism and egg puncturing (such as several other bee-eater species in this region; Spottiswoode and Colebrook-Robjent 2007) might react to punctured eggs within their nest. If these species were to show little or no reaction to punctured eggs, then the rejection behaviours of little bee-eaters in response to egg puncturing could be considered a defence that has evolved specifically in response to honeyguide virulence.

Given that ejection or abandonment of the clutch is very costly if the host is mistaken and the nest is actually unparasitised, I asked whether bee-eater rejection decisions were mediated by the reproductive value of the clutch. Less developed clutches are of lower reproductive value to the host (Cooper and Voss 2013), and therefore bee-eaters should be more willing to reject parasitized clutches early on in incubation since the cost of losing the entire clutch is reduced. I found that in natural situations, bee-eaters were more likely to reject parasitized clutches containing punctured eggs if they were at the start of incubation. Although I found a similar trend during experimental puncturing of bee-eater eggs, this relationship was not statistically significant and so was dropped from the final model in favour of a less complex model. The natural results could suggest that bee-eaters optimize their rejection strategy by making decisions based on the perceived reproductive value of their clutch. By using information on the incubation stage of their clutch, bee-eaters could optimize the trade-offs involved in egg rejection in three ways. First, later-developed clutches have a higher chance of reaching hatching, since compared to a little-incubated clutch, relatively less time remains until the eggs should hatch, thereby reducing the likelihood that the clutch will be predated or parasitized before hatching. Second, more developed eggs may require more puncturing to cause embryo failure (Spottiswoode and Colebrook-Robjent 2007), and therefore the chance that a bee-eater might gain some offspring from a damaged clutch is higher in developed clutches than in those that are less developed. Finally, clutches that are parasitized but that contain well-developed eggs will have a higher chance of the host young hatching out before the parasitic chick, which would in turn reduce the chance that the honeyguide chick is able to kill its host siblings and thus monopolize parental care. As bee-eaters were only more likely to reject at the start of incubation compared to late incubation in the natural dataset, but not in the experimental dataset, conclusive evidence that bee-eaters adjust their rejection decisions based on the reproductive value of their clutch is still lacking.

4.5.2 Costs of puncturing to honeyguides: increased predation risk

Two lines of evidence suggest that parasitism affected the predation rate of bee-eater clutches. First, bee-eater nests that suffered parasitism, and where the clutch was not rejected by the hosts, were significantly less likely to fledge any chicks as a result of predation compared to unparasitized nests. Second, successfully parasitized bee-eater clutches that contained punctured eggs were more likely to suffer predation than successfully parasitized nests where no host eggs were punctured. Although I found no relationship between the total quantity of punctures in a clutch and its chance of predation, clutches that contained a higher proportion of punctured eggs were more likely to be predated. Predatory snakes and ants (the most common predators of bee-eaters eggs and chicks in my study population, in addition to humans) have highly developed olfactory systems, which are used in foraging (Shivik and Clark 1997; Threlfall *et al.,* 2013; Huber and Knaden 2018). An increase in the number of punctures on a single egg may not increase the olfactory cue for predation, whereas a small number of punctures to multiple eggs in the clutch could do so. In support of this idea, there was a non-significant trend for clutches that contained a higher average rate of puncturing per egg to suffer higher predation rates.

An increased risk of predation associated with honeyguide parasitism and with puncturing of host eggs could also help to explain why bee-eaters do not reject individual honeyguide eggs, but instead abandon or eject the whole clutch from the nest (even though they appear to be capable of picking up individual eggs within the nest; supplementary material 2). Such behaviour is costly since it involves complete loss of the clutch, loss of time and energy in re-laying the eggs, and often the added time of excavating a new nest. However, clutches containing punctured eggs are more likely to be predated even if the parasitic egg could be removed alone, promoting the evolution of rejection or abandonment of entire clutches. Additionally, nests that were parasitized late in host incubation and where the parasitic egg was accepted were more likely to be predated than nests parasitized at the beginning of incubation. This could arise for two reasons. First, if the parasitic egg is laid at the end of host incubation, and if puncturing prevents any host chicks from hatching, then the total incubation time for that clutch is increased by a larger amount than if the parasitic egg is laid at the start of host incubation. This is because, like many other species, the incubating bee-eater will remain sitting on her eggs well past their expected hatching date, and will only stop incubating after a prolonged period of time, or when the hatching of the parasitic egg prompts her to stop incubating and start chick rearing. This prolonged incubation could increase the chance the nest is detected and consequently predated. Second, fresher, less incubated eggs may provide a less obvious olfactory cue to predators than more developed decomposing embryos. The increased chance of nest predation at nests where the clutch is parasitized in late incubation could therefore be a strong selection pressure on honeyguides to lay their eggs at the start of host incubation.

4.5.3 Adaptive puncturing by honeyguides

Increased predation risk and an increased risk of rejection impose costs on parasite virulence, and should therefore select for a response in honeyguide behaviour. First, since bee-eaters reject conspicuously punctured clutches, honeyguides could avoid detection by puncturing host eggs in the least conspicuous manner possible. I found some support for this. The within-clutch variance in puncturing intensity did not differ between small and large clutches. If honeyguide puncturing were indiscriminate, I would expect the variance of puncturing within a clutch to increase as clutch size increased, due to random chance. Instead honeyguides appeared to distribute their puncturing effort evenly across the clutch, which should minimise cues of parasitism to a host parent.

This adaptive puncturing could bring two potential benefits. By avoiding excessive puncturing, honeyguides can likely reduce the chance that the clutch (and their egg) will be rejected. However, by puncturing each egg within the nest, honeyguides also potentially damage all potential foster siblings, reducing both the initial level of competition for host care that their offspring will experience, and the total number of host nestlings that the honeyguide chick will ultimately need to kill in order to monopolise host care (Spottiswoode and Koorevaar 2012). These data therefore supports the idea that honeyguides modulate virulence, but it is not clear which of these two pressures (increased chance of host rejection, or competition with foster nest mates) has driven these patterns. Notably, nests containing a higher proportion of punctured eggs were more likely to be predated. There could therefore be a trade-off between the combined benefits of inconspicuous puncturing and reduction of potential competitors, and the cost of increased likelihood of predation. As honeyguides still choose to puncture each egg within the clutch, it appears that the net benefit of puncturing more eggs within the clutch outweighs the cost of potentially increasing the risk of predation.

In the observational dataset, bee-eaters were less willing to reject clutches containing punctured eggs if their eggs were closer to hatching. As expected, honeyguides responded to this, and appeared to adjust their puncturing intensity appropriately in order to maximize the benefits that they can gain from puncturing host eggs. Honeyguides increased the total number of punctures per clutch, the average number of punctures per egg, and the proportion of the clutch that was punctured, as host incubation stage increased. This replicates results found in Spottiswoode and Colebrook-Robjent (2007) with a different dataset from the same study area. Three non-mutually exclusive mechanisms could contribute to this pattern: (i) the benefits to the honeyguide of increased puncturing are higher for more incubated clutches, because more developed host eggs pose a greater future cost to the parasitic chick once hatched; (ii) the costs of puncturing are lower, because bee-eaters are less likely to reject more developed clutches; and (iii) more developed host embryos may require a greater number of punctures to be successfully killed. Swan *et al.* (2015) found a similar relationship between incubation stage and puncturing rates in the brood-parasitic brown-headed cowbird. Although increased rates of cowbird egg puncturing were associated with hosts re-nesting, and not with eliminating potential chick competition, hatching before host chicks is key for parasitic success in both systems.

Adaptive puncturing in response to host incubation stage requires honeyguides to have a mechanism to assess the development of host eggs. How a honeyguide perceives host egg incubation stage remains unknown. Visual cues are unlikely in the dark cavity nest of a bee-eater, but possible indirect cues might include monitoring of host behaviour at the nest (Banks and Martin 2001), host clutch size (White *et al.,* 2007), or information gained in the act of puncturing itself (Massoni and Reboreda 1999). Indirect cues seem less likely to be informative in a nest cavity as opposed to an open nest with its visible contents, and therefore it seems that the most likely scenario is that honeyguides gain information on host incubation stage while parasitizing the nest, presumably by egg puncturing. Such modulation of puncturing is possible because greater honeyguides have a backup method of virulence, where the newly hatched, and still blind and naked, honeyguide nestling uses its sharp bill hook to kill all competing host offspring within the nest (Spottiswoode and Stevens 2012). Thus, if greater honeyguide females temper their egg-puncturing virulence, their offspring can still salvage the breeding attempt by killing the remaining host young.

4.5.4 Benefits of puncturing to honeyguides

This study provides evidence that parasitic virulence can be costly to the parasite as well as to the host, and that this can promote adaptive variation in virulence. To gain a more complete picture of the evolution of honeyguide virulence, the benefits of this behaviour must also be more closely studied. Because of high rates of rejection in clutches containing punctured eggs, and those that are accepted being more likely to be predated, the benefits of egg-puncturing probably do not arise at the egg stage, but instead likely enhance the success of the parasitic chick. Future experiments are required to investigate this possibility, which could arise via several non-mutually exclusive mechanisms. First, chick-killing is presumably an energetically costly activity, as has been suggested in cuckoos (Anderson *et al.,* 2009). Minimising the energetic costs for the honeyguide offspring right after hatching may bring strong benefits, as this early post-hatch time could be important in determining the chick's future survivability and growth potential (Christensen 2009; Maiorka *et al.,* 2006). This 'ghost of killings past' could affect the fitness of honeyguide chicks even after they become sole occupants of host nests (Rutila *et al.,* 2002; Kleven *et al.,* 1999), although in the common cuckoo, the cost of ejecting hosts is transient and soon recovered (Anderson *et al.,* 2009). A test of this hypothesis would involve experimental manipulations of the number of host young in the nest when the honeyguide chick hatches, predicting that honeyguide chicks that are not required to kill host chicks should grow faster and be more likely to fledge than honeyguides that were forced to kill multiple host chicks. Second, although honeyguide chicks can kill host nest mates, they cannot evict them due to the cavity nest and extended tunnel entrance of bee-eater nests. Nests with dead host chicks could suffer higher predation than those nests without dead chicks. If punctured eggs are less likely to attract predators to the nest than are dead chicks, then this could be another benefit of egg puncturing. Finally, observations suggest that honeyguides that fail to eliminate host offspring within the clutch (either through egg puncturing or chick-killing) soon disappear from the nest (personal observations). This suggests that honeyguide chicks suffer large costs when forced to compete with host young for resources, which is consistent with patterns observed in other chick-killing brood parasites (Petrescu and Beres 1997; Geltsch *et al.,* 2012). Such intense competition, even if only for the initial few days after hatching (before the honeyguide chick kills its foster siblings), would be costly to parasitic growth. Taken together, this suggests that multiple potential benefits may counterbalance the costs of virulence documented here.

4.5.5 Conclusion

Brood parasite-host interactions are often referred to as textbook examples of coevolution (Rothstein 1990). Understanding the trade-offs that shape the extent of parasitic virulence is key to predicting the outcomes of such coevolutionary arms races, since virulence is a powerful selective pressure on both parties (Kilner 2005). Here, I provide evidence that avian brood-parasitic virulence, like pathogenic virulence, has associated costs, and that these costs promote variation in the extent of virulent behaviour. Although egg puncturing is present in other avian brood-parasitic systems (Massoni and Reboreda 1999; Gloag *et al.,* 2013), this is the first study to show that virulent puncturing can be costly to the parasite (by increasing the chance of rejection or predation), as well as to the host. Brood-parasitic virulence has been demonstrated to be costly to the parasite in at least one other system (Kilner *et al.,* 2004), but this study provides the first evidence of adaptive modulation of virulence by parasites in response to these costs. In pathogens, costs of virulence arise primarily because excessively exploitative pathogens risk killing their host, and therefore themselves (Day *et al.,* 2007). Although the nature of these costs differs between pathogens and the brood-parasitic greater honeyguide, in both cases virulence carries a cost.

4.6 Supplementary material

4.6.1 Video of bee-eater rejecting eggs from nest chamber

This video, taken on 10/10/2017 at a little bee-eater *Merops pusillus* nest, shows a bee-eater parent inspecting its clutch, detecting that it has been parasitized, and subsequently rejecting each egg from the clutch by grasping/pushing the egg along the tunnel and out of the nest entrance.

CHAPTER FIVE

Black-collared barbets *Lybius torquatus* think little of lesser honeyguide *Indicator minor* eggs: true recognition of small parasitic eggs by a cavity nesting host

5.1 Abstract

Avian brood parasites escape the costs of parental care by forcing other species to raise their young. Hosts suffer high costs as a result of parasitism and have therefore evolved defences to prevent or deter parasite success. The most common of these defences is egg rejection, whereby hosts detect a parasitic egg and subsequently reject it. However, there are few studies that have examined egg rejection abilities in cavity-nesting hosts, where low light levels mean cues such as the size of the egg are likely to be more effective than cues of egg pattern or colour. By adding experimental eggs to host nests, I tested whether the black-collared barbet *Lybius torquatus*, a cavity-nesting host of the brood-parasitic lesser honeyguide *Indicator minor*, can use the size of a foreign egg as a cue for making rejection decisions. Parasitic eggs were rejected if they were small, regardless of the size of other eggs within the clutch, and confirming observations of rejection decisions made at naturally parasitized nests. This suggests that rejection decisions in barbets are based on true recognition and not discordancy, with parasitized individuals using a template of egg size (reject eggs that are small) to recognize when they have been parasitized, rather than simply rejecting the smallest egg in their clutch. These results demonstrate that a brood-parasitic host can evolve egg rejection abilities in the likely absence of visual cues inside a cavity nest, using parasitic egg size as a cue for rejection. This study highlights that true recognition of foreign eggs can evolve when the parasitic egg is smaller than the host egg, as there is no potential for the host to possess a template that could cause it to erroneously reject its largest, most valuable eggs.

5.2 Introduction

Avian brood parasites lay their eggs in other birds' nests, leaving the care of their offspring to the recipient host (Davies 2000). Brood parasites impose heavy costs on their hosts (Øien *et al.,* 1998; Hauber and Ramsey 2003), which promote the evolution of defences against parasitism. Some hosts defend themselves with frontline defences, attacking or mobbing a parasite to deter it from the nest (Welbergen and Davies 2009; Gloag *et al.,* 2013), or using nest architecture to restrict access to the nest by the brood parasite (chapter two; Rutila *et al.,* 2002). Other hosts can recognise and reject foreign chicks (Langmore *et al.,* 2003; Sato *et al.,* 2010; Noh *et al.,* 2018). However, the best-studied host defences involve the rejection of parasitic eggs (Rothstein 1975; Davies and Brooke 1989; Langmore *et al.,* 2005). Recognition of parasitic eggs commonly uses cues based on egg colouration (Brooke and Davies 1988; Hanley *et al.,* 2016; Stoddard and Hauber 2017) or pattern (Lahti and Lahti 2002; Spottiswoode and Stevens 2010; López-de-Hierro and Moreno-Rueda 2010). However, in some cases, the size of the egg can be a stronger cue for rejection than differences in colouration or maculation, with evidence suggesting that hosts can discriminate against eggs both larger and smaller than their own (Mason and Rothstein 1986; Marchetti 2000; Guigueno *et al.,* 2014) . This is particularly so in situations where visual cues are less reliable, such as in the nests of hosts that breed in cavities, as size can be detected through touch when incubating or by probing the eggs (Guigueno and Sealy 2012a; Guigueno *et al.,* 2014).

I studied egg rejection decisions in the black-collared barbet *Lybius torquatus* (hereafter 'barbet'), specifically focusing on whether parents can detect and reject eggs based on their size. The barbet is a common host of an Afrotropical brood parasite, the lesser honeyguide *Indicator minor*. Like other species of honeyguide , lesser honeyguides (hereafter 'honeyguide') are extremely virulent brood parasites (Spottiswoode and Koorevaar 2012). The newly-hatched parasitic chick uses a hook on the end of its bill to kill all other chicks in the nest (supplementary material, video 1), and thus monopolizes the parental care of its hosts. Selection should therefore favour barbets that evolve specific defences against parasitism by honeyguides. Correspondingly, barbets aggressively chase and mob honeyguides detected near the nest (Moyer 1980; Cambray and Cambray 2014). Clutches containing parasitized eggs are also often ejected from the nest, or completely abandoned, suggesting that barbets can detect and respond to parasitic eggs, but are unable to selectively reject individual eggs within a clutch. Black-collared barbets and lesser honeyguides are an ideal study system in which to study egg size as a cue for rejection, for two reasons: first, both barbets and honeyguides lay white, immaculate eggs inside a dark tree cavity nest, where it is unlikely that visual cues are informative, but tactile cues could be

(Guigueno and Sealy 2012a; Guigueno *et al.,* 2014). Second, honeyguide eggs are much smaller than barbet eggs (Table 1). This means that egg size could provide a reliable cue of parasitism for barbets, but also contrasts with many of the more commonly studied host-brood parasite systems in which the parasite typically lays a larger egg than its host (Krüger and Davies 2004). In such systems where the parasite is larger, it is less likely that hosts will evolve rejection mechanisms in which egg size is used, since any erroneous decision to reject large host eggs is especially reproductively expensive as the host is ejecting its most valuable egg (Nisbet 1978; Christians 2002; Davies 2000). By contrast, a barbet that mistakenly rejects its own, smallest egg in place of a small honeyguide egg, is likely rejecting a runt egg anyway, and so the costs of making such a mistake are correspondingly much lower.

There are two mechanisms by which host species could assess and recognise a smaller egg by size alone. The first mechanism is known as 'true recognition' (Hauber and Sherman 2001), and requires host species to have an internal template that defines what one of their own eggs should look or feel like. Any eggs that deviate from this internal template could then be rejected. This template could be innate, learnt, or a combination of both. By following a rule that honeyguide eggs are smaller than the host's own eggs, barbets could defend against parasitism by rejecting any egg that is outside of the size range of their specific template. This mechanism of egg recognition has the benefit of not requiring comparison to other eggs within the clutch (Hauber and Moskát 2007), which is likely difficult within a cavity nest with low light conditions (Guigueno *et al.,* 2014). The second mechanism by which host species might assess and recognise smaller eggs is known as 'discordancy'. This involves making rejection decisions based on assessment of variation in egg phenotypes within the clutch (Sealy and Underwood 2012). In this case, a host that suspects it has been parasitized would compare each egg in its nest to all other eggs present and use the information gained from examining the entire clutch to distinguish any aberrant egg(s). This discordancy rule is an intuitive method by which hosts could identify parasitic eggs and does not rely on a specific template (Rothstein 1974b). An advantage of using discordancy is that it allows a female to use information provided by her own individual egg phenotype (Marchetti 2000). For example, if a honeyguide lays a particularly large egg in the nest, then a template-based rule for recognition that relies on parasitic eggs being 'small' might not result in recognition of the honeyguide egg as parasitic. A discordancy-based rule for recognition of foreign eggs would not be constrained in this way, and could simply make use of the fact that the parasitic egg, even though bigger than normal, was still smaller than the host's own eggs. True recognition appears to be the most common form of egg recognition among hosts of brood parasites, although both mechanisms may also be used together

(Lotem *et al.,* 1995; Lyon 2007; Stevens *et al.,* 2013), but so far no host has been reported to use template-based recognition using the size of an egg.

I used a combination of observations of natural parasitism, and experimental additions of foreign eggs to simulate honeyguide parasitism at barbet nests, to establish the mechanisms by which barbets identify and respond to parasitized clutches. If barbets use a template-based, true recognition mechanism to make rejection decisions, then this predicts that they will be more likely to abandon or eject clutches containing foreign eggs that are small, regardless of how such eggs compare in size to other eggs within the nest. Alternatively, if barbets instead use discordancy to decide that their clutch has been parasitized, then the relative size difference between the parasitic egg and other eggs within the clutch should best predict whether or not they abandon or eject their clutch.

Abandonment of the entire clutch is costly to barbets, forcing them to search for a new nest site, excavate a cavity, and produce a new clutch. Even in cases where barbets eject the clutch from the nest rather than simply abandoning it in-situ, thus allowing them to reuse their nest cavity (as sometimes occurs, personal observations), they must still re-lay and incubate their new clutch. An additional cost of egg rejection occurs if a host incorrectly rejects its own egg (Davies *et al.,* 1996), or in the case of barbets, the entire clutch. Since the likelihood that the clutch survives till hatching increases as incubation progresses, more developed clutches have a higher reproductive value (Albrecht and Klvana 2004; Osiejuk and Kuczyński 2007). The costs associated with a barbet parent making a rejection error are therefore higher for developed clutches compared to fresh clutches. Given that making an error during rejection of honeyguide eggs is so costly to barbets, I asked whether barbet rejection behaviour was influenced by the reproductive value of the clutch. This would predict that barbets show higher levels of rejection at the beginning of incubation than they do in the later stages of incubation.

5.3 Methods

5.3.1 Study area, study species

This study was conducted on the Semahwa and Musumanene farms in the Choma district of Zambia’s Southern Province (16°46’S, 26°54’E). The field site consists of a diverse habitat of miombo woodlands, dambos (temporally flooded grassland) and agricultural land. The barbet-honeyguide breeding season is characterised by dry, hot conditions, spanning September to December. Black-collared barbets are primary cavity nesters, excavating nests in the trunks or dead branches of (amongst others) muwombo *Brachystegia boehmii*, wild fig *Ficcus burkei*, masuku *Uapaca kirkiana* and mbula *Parinari curatellifolia* trees. They lay clutches of 3–5 white eggs. Lesser honeyguide females enter the nest cavity at any time after egg laying has commenced, and while the hosts are absent lay a single, white egg that is 75–84% of the volume of the host's eggs (Table 1). Unlike greater honeyguides (chapter four; Spottiswoode and Colebrook-Robjent 2007), lesser honeyguides do not routinely puncture host eggs when they lay their own, although they occasionally do so (personal observations: Luke McClean, Nicholas Horrocks, Claire Spottiswoode).

Table 1. Summary of egg sizes within this study, both naturally laid eggs and those used in the experiment, showing mean egg volume, length and width (± SE).

Egg type (species)	**Volume (mm^3)**	**Length (mm)**	**Width (mm)**	***n***
Naturally laid eggs				
Black-collared barbet *Lybius torquatus*	3583 ± 469	23.70 ± 1.34	17.20 ± 0.58	353
Lesser honeyguide *Indicator minor*	2843 ± 210	20.85 ± 1.20	16.34 ± 0.50	47
Experimental eggs				
Little bee-eater *Merops pusillus*	2283 ± 184	18.14 ± 0.80	15.79 ± 0.40	16
Namaqua dove *Oena capensis*	2405 ± 355	18.72 ± 2.37	15.38 ± 0.40	11
Emerald-spotted wood-dove *Turtur chalcospilos*	3450 ± 401	23.22 ± 1.68	15.84 ± 4.40	16
Ring-necked dove *Streptopelia capicola*	6250 ± 656	27.12 ± 0.66	21.12 ± 0.91	5

5.3.2 Field observations of barbet rejection behaviour

I monitored 163 barbet nests during 2015–2019. To inspect the contents of barbet nests, I cut a small hole into the side of the nest cavity, just above where the eggs were housed, creating a 'hatch' that could be removed and replaced at each visit (Figure 1). I followed each nest until hatching, and monitored parasitism throughout host incubation. In addition to their smaller size, honeyguide eggs were identified by their yellowish yolk (visible through the white shell) compared to the pinkish-orange yolk of barbets (Friedmann 1955), and their shinier shell surface. Each egg in the nest was marked with pencil and its length and width were measured (± 0.01 mm) with digital callipers. The volume of each egg was then calculated following Hoyt (1979). Any host or parasitic eggs discovered during subsequent visits were measured in the same manner. If a honeyguide egg remained in an active host nest for five days it was scored as accepted. Parasitic eggs were scored as rejected if the clutch was found cold in the nest after parasitism (abandonment), or if the clutch was found below the nest outside of the cavity (whole clutch rejected), or if the individual parasitic egg was rejected. Nests found completely empty, with no sign of eggs below the nest, were scored as predated. It is possible that some among these had been abandoned and then predated.

Figure 1. To enable the contents of the nest to be accessed on multiple visits with minimal disturbance to the hosts, a small hatch is cut into the side of this tree cavity nest belonging to a black-collared barbet. Here, the hatch has been removed to allow a black-collared barbet chick to be removed for growth measurements.

5.3.3 Experimental parasitism of barbet nests

To corroborate any patterns derived from natural observations, in 2015 and 2016, experimental manipulations were performed at 57 barbet nests (n = 26 nests in 2015, n = 31 nests in 2016). After measuring all host eggs, the clutch was returned to the nest with the addition of a single experimental egg of another species, thereby mimicking laying behaviour by lesser honeyguides, which do not remove an egg when they lay their own. The species of experimental egg was randomly assigned before the nest was checked, and came from one of four species: little bee-eater *Merops pusillus*, Namaqua dove *Oena capensis*, emerald-spotted wood-dove *Turtur chalcospilos* (Figure 2), or ring-necked dove *Streptopelia capicola*.

Eggs from these species were chosen because, superficially at least, they all resemble barbet eggs: they are of a similar shape, are immaculate, and are shades of off-white in colour. However, they vary in size, such that they are respectively much smaller than, slightly smaller than, similarly sized to, and larger than barbet eggs (Table 1). All experimental nests received one additional egg, mimicking a parasitism event by a single honeyguide (multiple parasitism by multiple females does also occur in this system). Naturally parasitized nests, or nests that became parasitized during the experiment, were excluded from the final experimental dataset. As there were no instances where experimental eggs were rejected or abandoned more than five days after being added, I considered any experimental egg that was still present in an active nest after five days to have been accepted by the hosts (as for the observational dataset).

To determine whether the reproductive value of the clutch predicted host rejection behaviour, upon first discovery of a parasitic egg, or addition of an experimental egg, all eggs were ‘candled’ by placing a bright torch next to the egg surface and shining it through the egg (Lokemoen and Koford 1996). Eggs were then ranked on a scale from 0 to 5 based on the development of the embryo, following Spottiswoode and Colebrook-Robjent (2007). Eggs ranked 0 contained no visible embryonic development and those ranked 5 were close to hatching. Once the incubation stage of parasite and host eggs was established at the time of discovery, I was then able to determine the incubation stage of the host eggs at the time when the parasitic egg was laid. I assumed that incubation stages progressed linearly, such that subtracting the incubation stage of the parasitic egg from the mean incubation stage of the host clutch yielded an estimate of the incubation stage of the host clutch at the time of parasitism.

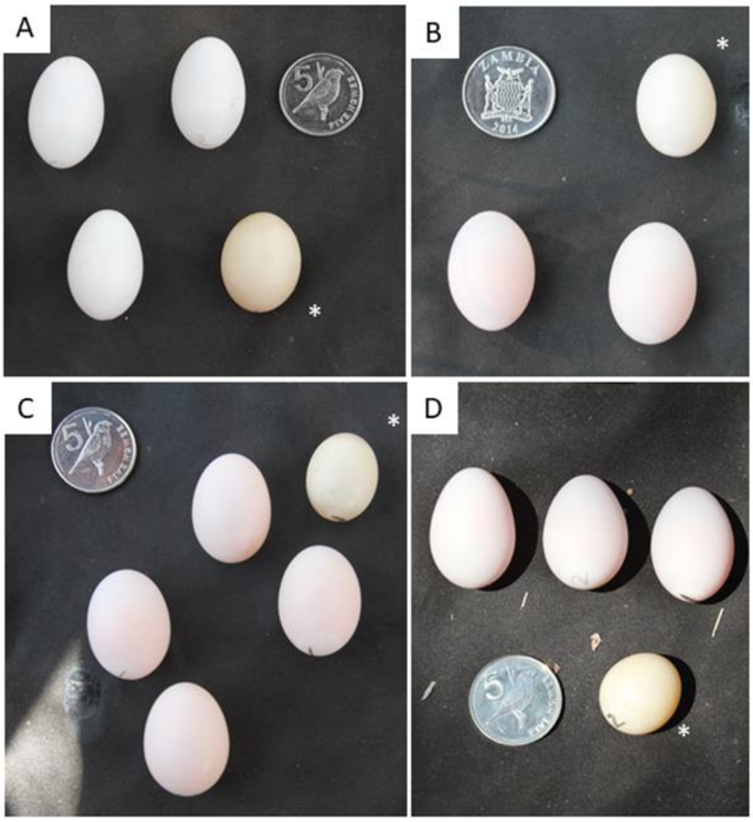


Figure 2. Examples of experimentally and naturally parasitized black-collared barbet clutches. Experimental parasitic eggs are marked in each photo with *. A Zambian five Ngwee coin is included for scale (19mm diameter). **A)** Emerald-spotted wood-dove **B)** Namaqua dove **C)** Little bee-eater **D)** Lesser honeyguide.

5.3.4 Statistical Analyses

I created models to test my hypotheses, separately assessing (i) the observational dataset of natural parasitism, and (ii) the experimental dataset. I excluded all experimental nests from the natural observation dataset. To test the hypothesis that barbets use a template when deciding to reject eggs, which predicts that the absolute size of the parasitic egg is the best predictor of barbet egg rejection, I built generalized linear models (GLM) with the rejection decision of the barbet treated as a binary outcome. I then fitted separate models with the volume, the length, or the width of the honeyguide/experimental egg as continuous predictors. I also tested whether barbets used discordancy between eggs as a cue for rejection. This hypothesis predicts that the difference between parasitic and host egg size is a better predictor of barbet rejection decisions than the absolute size of the egg. To test this, I again used logistic regression, using separate models (to avoid collinearity among predictors) containing barbet rejection decision as a binary outcome, and the absolute difference (in volume, length, or width) between the parasitic (honeyguide or experimental) egg and the host eggs (average value within the clutch) as continuous predictors. The incubation stage of the clutch when parasitized was included as an ordinal predictor in all models. All models were initially run as generalized linear mixed models (nlme4 package; Bates *et al.,* 2015), with the year that the data was collected included as a random effect. However, this was later omitted after comparisons with a Likelihood Ratio test (Luke 2016), and so all final models were run as GLMs, as described.

I conducted all analyses in R v. 3.1.3 (R Core Team 2019). Model selection was performed using the Akaike information criterion (AIC; Wagenmakers and Farrell 2004), with models of lower AIC (>2 AIC difference) considered the most parsimonious (Burnham *et al.,* 2011). Model normality and homogeneity of error was evaluated from residual plots of fitted models. Over-dispersion was assessed using residual deviance and degrees of freedom (Noriszura and Jemain 2007). I then evaluated the model goodness of fit with Nagelkerke pseudo r^2 (Nagelkerke 1991), which was calculated with use of the piecewiseSEM package (Lefcheck 2016), using methods described in Nakagawa and Schielzeth (2013) and Johnson (2014).

5.4 Results

5.4.1 Naturally parasitized nests

Of 106 unmanipulated nests, 32 (30%) were naturally parasitized by lesser honeyguides, and six of these (19% of parasitized nests) were rejected by barbets. Of these six rejections, four were by ejection of the entire clutch, and two by abandonment of the nest. The absolute size of a lesser honeyguide egg predicted the likelihood of the parasitized clutch being abandoned or ejected: smaller parasitic eggs were more likely to be associated with clutch rejection than larger parasitic eggs (Figure 3). This negative relationship between size and rejection probability was apparent when modelling egg size as either the volume, length, or width of the honeyguide egg, but was only statistically significant in the case of egg volume (volume: $\beta = -0.001 \pm 0.005$, $Z_{31} = -2.169$, $p = 0.03$, Figure 3A; length: $\beta = -0.739 \pm 0.499$, $Z_{31} = -1.481$, $p = 0.138$, Figure 3B; width: $\beta = -2.790 \pm 1.457$, $Z_{31} = -1.915$, $p = 0.055$, Figure 3C). However, the size difference between the host eggs and the parasitic honeyguide egg did not predict the probability of clutch abandonment or ejection. This was the case whether size difference between the parasitic egg and the average of the host eggs was modelled using egg volume ($\beta = 0.007 \pm 0.066$, $Z_{31} = 0.102$, $p = 0.92$), egg length ($\beta = -0.232 \pm 0.241$, $Z_{31} = -0.963$, $p = 0.34$), or egg width ($\beta = -0.508 \pm 0.512$, $Z_{31} = -0.991$, $p = 0.32$).

All natural occurrences of honeyguide parasitism took place at the beginning of host incubation, when the mean within-clutch incubation stage of the host eggs was either incubation stage 0 (n = 24), 1 (n = 6), or 2 (n = 2). There was no relationship between the incubation stage of the barbet clutch when it was parasitized, and the likelihood of the clutch being rejected ($\beta = -0.669 \pm 1.012$, $Z_{31} = 0.661$, $p = 0.50$). Thus, when considering only naturally parasitized nests, I found that the best model for predicting whether a parasitized clutch was abandoned or rejected included only the volume of the honeyguide egg.

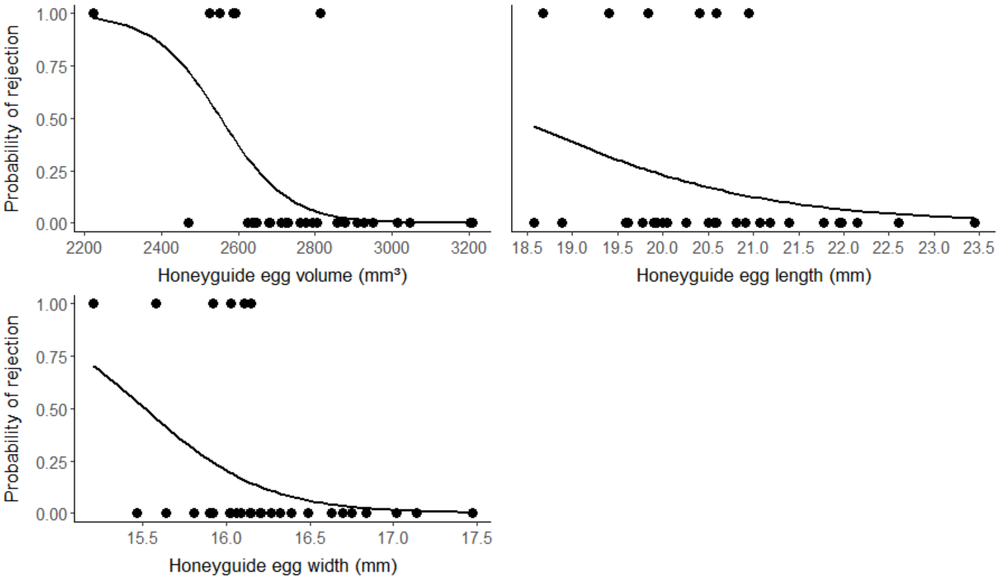


Figure 3. Naturally parasitized black-collared barbet clutches containing small lesser honeyguide eggs were more likely to be rejected than those containing large honeyguide eggs. This relationship was consistently found for **A)** volume; **B)** length; and **C)** width of the parasitic egg. The solid black line indicates the relationship between the egg size and the probability of rejection based on logistic regression models. Black points indicate the raw data points. Only the relationship between egg volume and probability of rejection was statistically significant at the 0.05 level.

5.4.2 Experimentally parasitized nests

Barbets rejected clutches containing experimental eggs on 31 of 57 occasions (54%). Fifteen of 19 (79%) experimental bee-eater eggs (the smallest experimental eggs) were rejected, six of 11 (55%) Namaqua dove eggs (the experimental eggs that were most similar in size to the eggs of lesser honeyguides) were rejected, 8 of 21 (38%) experimental emerald-spotted wood-dove eggs (the experimental eggs that were most similar in size to barbet eggs) were rejected, whereas 2 of five (40%) of the largest ring-necked dove eggs were rejected. There was a significant negative relationship between the volume of the experimental egg added and the probability of rejection (Figure 4A; β = -0.001 ± 0.001, Z_{56} = -2.011, p = 0.044), such that smaller experimental eggs were more likely to prompt clutches to be rejected than were larger experimental eggs. A similar negative relationship between rejection rate and egg size was also evident if using the length or width of the experimental egg, rather than the volume. This relationship was significant for egg length (Figure 4B; β = -0.250 ± 0.99, Z_{56} = -2.635, p < 0.01), but non-significant for egg width (Figure 4C; β = -0.311 ± 0.183, Z_{56} = -1.733, p = 0.083).

Neither the difference in volume, nor in width, between host and parasitic eggs were significant predictors of clutch abandonment or ejection by hosts (volume: β = -0.043 ± 0.025, Z_{56} = -1.757, p = 0.079; width: β = 0.155 ± 0.121, Z_{56} = -1.279, p = 0.20). However, the difference in length between host and experimental eggs was a good predictor of egg rejection: experimental eggs that were much smaller in length than the hosts eggs had an increased chance of causing hosts to reject their clutch (β = -0.243 ± 0.100, Z_{56} = -2.609, p < 0.01). Experimental eggs added at the beginning of host incubation were not more likely to be rejected than those added at the end of host incubation (figure 4D; β = -0.244 ± 0.182, Z_{56} = -1.343, p = 0.180).

Two models were identified (Table 2; both models >2 AIC units lower than all other models, but within <2 AIC units of each other) that best predicted barbet rejection behaviour in the experimental dataset: (i) the length of the experimental egg that was added (shorter eggs were more likely to cause clutch rejection), and (ii) the difference in length between experimental and host eggs (experimental eggs that differed most in length to host eggs were more likely to cause clutch rejection).

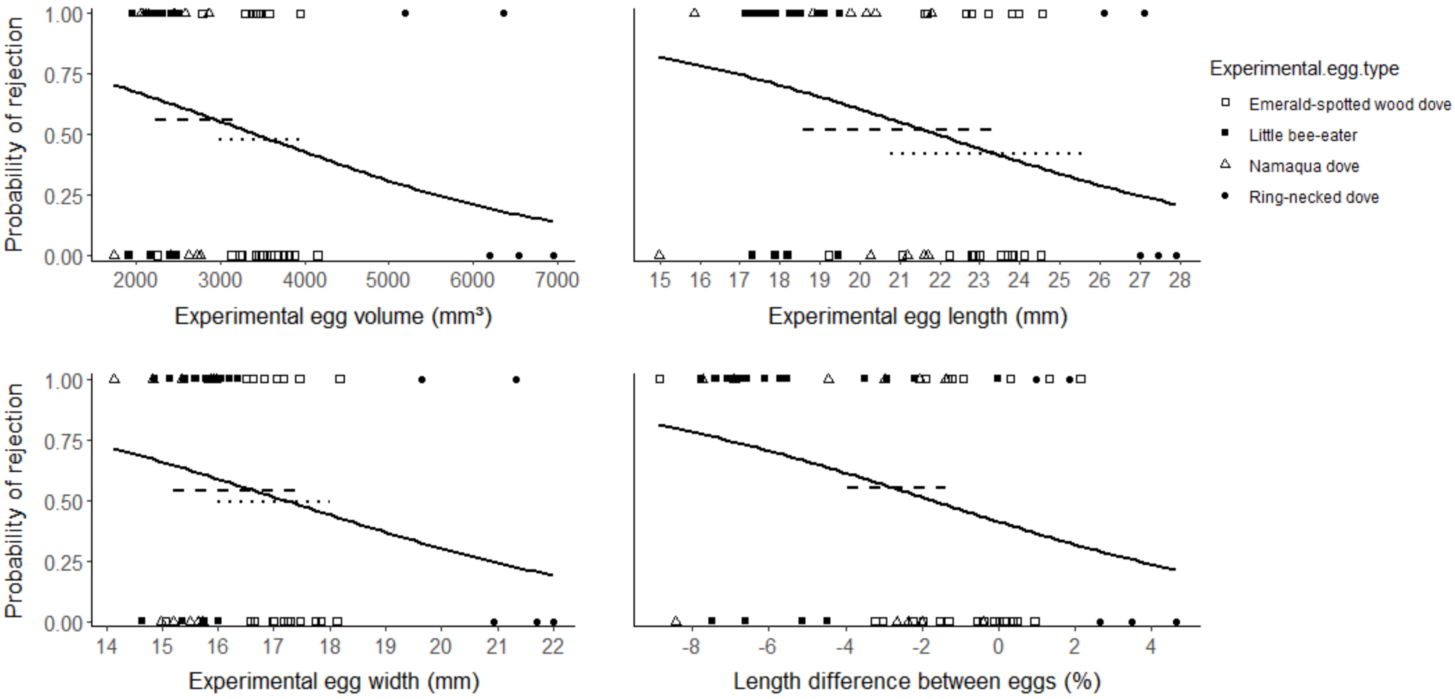


Figure 4. Black-collared barbet clutches with small experimental eggs added were more likely to be rejected than those that had large experimental eggs added to them. This relationship was consistently found for parasitic egg **A)** volume, **B)** length, and **C)** width. Experimental eggs that were most different in length from host eggs were the most likely to be rejected **D)**. The solid black line in each figure indicates the relationship between egg size and the probability of rejection based on logistic regression models. The dashed black line in each figure indicates the size range of lesser honeyguide eggs, whereas the dotted black line in figures **A)**-**C)** indicates the size range of black-collared barbet eggs. Data points represent the results of experimental additions of eggs, and are coded by symbol, according to the species of egg that was added.

Table 2. Top models for analysis of barbet rejection after experimental addition of eggs. Model support table (AIC) for the top ranked GLM models (>2 AIC units lower than next best models).

Model #	R²	AIC	Model variable	Intercept	t value	p value
1	0.13	75.80	Experimental egg length	-0.24	-2.48	< 0.01
2	0.10	74.93	Difference in length between experimental and host eggs	-0.24	-2.61	< 0.01

5.5 Discussion

5.5.1 Recognition and rejection of small eggs by barbets

Most studies of egg rejection abilities in hosts of brood parasites have focused on host species that build open nests (Davies and Brooke 1989; Langmore *et al.,* 2005), where visual cues can be used for egg recognition, and where the parasitic egg is larger than the host's eggs. This study uses a contrasting system, in a cavity-nesting host, where visual cues are unlikely to be reliable when making egg rejection decisions, and where the parasitic egg is smaller than those laid by the host species. My observational and experimental results demonstrate that black-collared barbets can recognize and reject clutches containing lesser honeyguide eggs based on the small size of the parasitic egg. Eggs similar in size to or larger than their own were mostly accepted. The results suggest that either the volume of the parasitic egg (according to the observational data) or the length of the parasitic egg (according to the experimental data) are the best predictors of barbet rejection decisions. I found no evidence that rejection decisions made by black-collared barbets are mediated by the reproductive value of the clutch.

Black-collared barbets were more likely to reject clutches containing a small honeyguide or experimental egg, regardless of the size of the other eggs within the nest. In both natural and experimental datasets, the absolute size of the egg was a better predictor of barbet rejection decisions than the difference in size between host and parasitic eggs. Thus, barbets appear to use true recognition (that is, relying on an internal template of egg size to recognise parasitic eggs) to identify that their clutch has been parasitized, rather than a discordancy-based rule (that is, directly comparing the parasitic egg to their own eggs). The chances of rejection were nearly threefold greater for eggs of comparable size in experimentally parasitized nests compared to those naturally parasitized. I interpret this as being a consequence of the fact that barbets abandon or eject their clutches soon after they recognise that they have been parasitized. Thus, many naturally parasitized nests were likely abandoned or ejected before I was able to observe them, leading to a bias in my observational dataset.

Although true recognition appears to be the most common form of egg recognition among hosts of brood parasites (Lotem *et al.,* 1995; Lyon 2007; Stevens *et al.,* 2013), true recognition of parasitic eggs by hosts based on size, rather than on visual cues such as colour or maculation, has yet to be reported. In other brood-parasitic systems, where the brood parasite is typically larger than its host, such as occurs with hosts of the common cuckoo *Cuculus canorus*, hosts are less strongly selected to reject the larger parasitic egg, as any recognition error would mean that instead of rejecting the virulent parasitic egg, the host would throw out their largest egg (or clutch containing their largest eggs). Given that larger eggs result in larger chicks, which are likely to have the highest reproductive value, such eggs would also be the most valuable within a clutch (Christians 2002; Krüger and Davies 2004). In such systems the potential cost of an error should therefore act as a constraint on the evolution of a recognition template based on the size of the parasitic egg. This selection pressure against rejection of large eggs is exemplified by a host of the closest relative to the lesser honeyguide, the greater honeyguide *I. indicator*. The presence of a greater honeyguide egg alone is not enough to stimulate rejection by its little bee-eater *Merops pusillus* host, despite the egg being noticeably larger than the host's own eggs, likely because of the cost of making a rejection error (Spottiswoode 2013; Tong *et al.,* 2015). In the case of barbets and lesser honeyguide eggs, any erroneous rejection of clutches containing smaller eggs would be less costly to barbets, as the loss of runt eggs would have a smaller impact on host fitness. Thus, for barbets, an inherited template that resulted in a rule of 'reject any egg that is small' or 'reject any egg that is not large', might be sufficient to avoid maladaptive decisions.

One potential drawback of a template-based approach to egg recognition is that naïve individuals that are parasitized during the first breeding attempt may learn to recognize parasitic eggs as their own (Victoria 1972; Rothstein 1974a). This incorrect template could then lead to acceptance of parasitic eggs, or maladaptive rejection of their own eggs in future breeding attempts. Hosts using true recognition could escape the costs of this mis-imprinting by inheriting this template, rather than learning it on the first breeding attempt. How such a recognition template could be inherited requires future study. However, an innate template could still be refined through experience (Lotem *et al.,* 1995; Moskát and Hauber 2007), which in barbets might occur if parasitized individuals examine their clutch outside of the nest, when rejecting it, although this seems unlikely since many eggs break when dropped outside of the nest. Refining the innate template, however achieved, would allow the host to accommodate for the variability in egg size between clutches of different years (Hauber and Sherman 2001), thus improving their template for subsequent breeding attempts (Moskát *et al.,* 2014).

If barbets were able to compare between eggs and then pick the odd egg out, we might also expect the evolution of specific egg rejection – that is, rejection of only the odd egg out. That barbets do not do so lends further support to the idea that discordancy-based recognition is not used to make rejection decisions in this host. Despite being less common than true recognition, there is evidence for discordancy-based egg recognition in other host-brood parasite systems, particularly in hosts that use the size of the egg as a cue for rejection. In North America, Hume's leaf warbler *Phylloscopus humei* reject eggs depending on the degree to which a given egg is larger than other eggs in the clutch (Marchetti 2000), and rufous horneros *Furnarius rufus* reject cowbird eggs that are narrower than their own (Mason and Rothstein 1986). One proposed pitfall of the discordancy hypothesis is that if a host population suffers high rates of multiple parasitism, then rejecting through discordancy can be maladaptive, as rejecting the 'odd egg out' could mean rejecting your own egg (Rothstein 1974b). Although multiple parasitism of barbet nests by honeyguides does sometimes occur, it only occurred in 3% of all nests in this study, perhaps due to the aggressive frontline defences of barbets, which at their most extreme can lead to honeyguides being killed (Cambray and Cambray 2014; Moyer 1980). However, rates of multiple parasitism rates at this site have been much higher in the past (unpublished data: Claire Spottiswoode and John Colebrook-Robjent) and could therefore have reduced the selection pressure for barbets to evolve a discordancy-based mode of rejection.

5.5.2 Method of rejection by barbets

Barbets are indiscriminate rejecters, discarding their entire clutch upon recognition of parasitism (supplementary video 2), despite this 'all or nothing' approach leading to loss of the entire breeding attempt. That barbets do not reject single eggs could suggest that they are unable to differentiate between eggs within the confines of a dark cavity nest. Despite this, such an approach is presumably still less costly than raising a parasitic honeyguide chick, since this consumes the entire breeding effort of the barbets for that season. Barbets that detect parasitism and subsequently reject their entire clutch still stand to gain fitness if they are then able to successful lay and raise another brood. It is uncertain why barbets sometimes ejected the clutch from the nest, but at other times deserted the clutch without ejecting it from the nest cavity. Of 31 rejection events after experimental addition of foreign eggs, in 20 cases barbets rejected by clutch abandonment, in 10 cases they threw all the eggs from the nest, and in one case they removed only the experimental egg from the nest. It has been proposed that desertion can be adaptive for a host when it is unable to reliably recognize the parasitic egg within the clutch, but nonetheless knows that it has been parasitized (Avilés *et al.,* 2005). While an intuitive hypothesis, experimental evidence is currently inconclusive (Šulc *et al.,* 2019), and thus how this might apply to making decisions on whether to eject the entire clutch or simply to abandon it is unclear.

One plausible benefit of ejecting eggs from the nest is that barbets would be able to re-use their nest cavity, and thus save the time and effort required to excavate a new one. Barbets re-excavate new nests each year, usually in the same branch immediately above their old nest, but do appear to re-nest in the same nest cavity within a season (personal observations: Luke McClean, Nicholas Horrocks and Claire Spottiswoode). Therefore, the availability of potential re-nesting sites could affect host rejection behaviour (Svennungsen and Holen 2010). For example, if there is low availability of suitable nest sites, or not enough time to re-excavate a new cavity, then barbets might be prompted to eject the entire clutch from their nest to enable reuse of the cavity. A second possible benefit is that ejection of the clutch outside of the nest could perhaps allow barbets to visually inspect the eggs, and thus allow learning for future breeding attempts (Moskát *et al.,* 2014), although this would be difficult as most of the eggs ejected in this manner break when dropped outside the nest. Finally, these rejected eggs (shells) could also possibly act as a signal to the honeyguide that their deception has been discovered. Honeyguides frequently visit barbet nests, including those that they have already parasitized (personal observations; Friedmann 1955). Signalling that a parasitism attempt has been discovered could allow barbets to indicate to prospecting honeyguides that they are not naïve hosts, and therefore not worth parasitizing. A test of this could be to place experimental clutches of eggs below the nests of barbets and monitor the rate of honeyguide parasitism. If nests with experimental clutches below the nest were less likely to be parasitized, this would provide evidence that this acts as a signal to honeyguides.

5.5.3 Detection of small parasitic eggs by barbets

Although barbets rejected smaller eggs, how they detected these eggs as being smaller remains unclear. As visual cues are likely difficult to assess in the darkness of the cavity nest, tactile cues seem most likely, as has been suggested in other host-brood parasite systems. For example, yellow warblers *Setophaga petechia* that probed cowbird eggs after being parasitized by brown-headed cowbirds were more likely to reject the parasitic egg than those that did not (Guigueno and Sealy 2012a). There is also evidence of birds using tactile stimuli in contexts such as foraging (Cunningham *et al.,* 2007) or incubation (Baerends *et al.,* 1970). Therefore, it is perhaps unsurprising that, under the extreme selection pressures exerted by honeyguide parasitism, barbets might evolve the use of such cues in making egg rejection decisions.

5.5.4 Mediation of rejection decisions based on incubation stage of the clutch by barbets

Contrary to my initial hypothesis, I found no evidence that barbets mediated their rejection decisions based on the reproductive value of the clutch. As the likelihood that a clutch will survive until hatching increases as incubation progresses, fresh clutches are of lower reproductive value. I hypothesised that this trade-off could be used by barbets to mediate their rejection responses. Tuning rejection behaviour in this way would have the advantage of avoiding the risk of costly rejection errors – incorrectly deciding that the clutch has been parasitized and subsequently rejecting it late in incubation (Davies *et al.,* 1996) – when eggs are of higher value (Albrecht and Klvana 2004; Osiejuk and Kuczyński 2007). Additionally, any parasitic egg that is laid too late into the incubation period of the host clutch is unlikely to hatch out before its foster-siblings and so will be unable to outcompete them. In this case the best decision of the host should be to accept the parasitic egg instead of abandoning/ejecting the clutch. I suggest that barbets may not mediate their rejection decisions based on the reproductive value of the clutch as honeyguides primarily only parasitize fresh-laid clutches, which would provide only a weak selection pressure for barbets to mediate their decisions based on incubation stage, since hosts rarely encounter variation in the reproductive value of a parasitized clutch.

5.5.5 Conclusions

My results demonstrate that the black-collared barbet, a cavity-nesting host of a virulent brood parasite, is able to recognize parasitism events and reject parasitic eggs based on their size, but does not adjust its egg rejection decisions based on the reproductive value of the clutch. Barbets appear to have an innate or learned template of what constitutes a parasitic egg that is based on egg size (either egg volume or egg length). These results add to the growing body of evidence suggesting that hosts recognise parasitic eggs using template-based recognition, rather than by a discordancy-based detection process. I report for the first time evidence of a rejection template based on parasitic egg size, present in a system in which it would be expected – where the parasitic egg is smaller than those of the host.

5.6 Supplementary Material

Video S1. Lesser honeyguide chick-killing in action. Within the host cavity nest a lesser honeyguide chick (closest to the bottom of the screen) attacks a black-collared barbet chick. The honeyguide chick is four days old, while the three host barbet chicks are two days old. The host chick seen top right has already succumbed to the attacks of the parasite.

Video S2. Black-collared barbets are capable of ejecting eggs, even those of sizes larger than lesser honeyguide eggs. Here, a barbet is observed to reject its own eggs after a parasitic chick has hatched, presumably assuming that the remaining eggs in the nest are infertile.

CHAPTER SIX

Divergent pressures lead to convergent adaptations: lesser and greater honeyguide (*Indicator* spp.) nestlings both mimic the begging calls of an entire brood of host chicks, but for different reasons

6.1 Abstract

Chicks of the parasitic honeyguides (Indicatoridae) are virulent brood parasites that kill their foster siblings and are then raised alone by the host parents. The chick then faces the challenge of obtaining enough food from its hosts to ensure its survival. Many brood parasites mimic the begging displays of their host siblings or call rapidly and loudly to induce provisioning. Whether honeyguides use similar mechanisms to elicit adequate provisioning is unknown, and this study undertakes to answer this question in the lesser *Indicator minor* and greater *I. indicator* honeyguides. First, I show that in both parasitic systems, a single honeyguide elicits similar rates of provisioning from host parents as do a brood of their own young, which in turn are fed at higher rates than is a single host chick. Second, using playbacks of host and parasite vocal displays, I show that the begging calls of the parasitic chicks are integral to their ability to elicit these greater-than-expected levels of provisioning. The results suggest that honeyguide begging calls mimic the sound of a whole brood of host chicks, and thus stimulate the host parent to provision at a high rate. Finally, hand-rearing experiments of *I. minor* chicks, which are smaller than their hosts, suggested that they are ill-adapted to the diet of their hosts, requiring extra provisioning to offset inadequate digestion of fruit material. By contrast, *I. indicator* chicks required higher rates of provisioning to fuel their rapid growth and large size relative to their hosts. This study provides further evidence that vocal begging displays are a focal point in the coevolutionary arms race between parasites and their hosts at the chick stage. These results add to the growing evidence that vocal mimicry of hosts is a common phenomenon in avian brood-parasitic lifestyles, but suggests for the first time that selection on brood parasitic chicks to evolve vocal mimicry can be driven by reasons distinct from simply requiring greater provisioning for growth.

6.2 Introduction

Avian brood-parasitic offspring face the challenge of obtaining enough food from foster parents, who have evolved parental behaviours by interacting with their own young, rather than those of the brood parasite. In the case of virulent brood parasites that kill or remove foster siblings (Brooke and Davies 1988; Peer *et al.,* 2013; Spottiswoode and Koorevaar 2012), the challenge of being sufficiently fed is especially acute, since a parasitic chick must elicit provisioning without the aid of host offspring. Vocal begging is the primary means by which chicks obtain food from parents (Budden and Wright 2001; Madden *et al.,* 2005), and in order to receive adequate care, brood parasites have evolved elaborate signals that 'tune in' to the parent-offspring communication of their hosts (Davies 2011). In a classic study by Davies *et al.* (1998), single common cuckoo *Cuculus canorus* chicks were found to be able to tap into this parent-offspring communication, calling more rapidly and loudly than host chicks, and thereby mimicking the combined vocal stimuli of multiple host chicks in the nest. This mimicry of a harmless model (aggressive mimicry) in turn induced the host parents to deliver more food to the parasitic chick than, as a single chick in the nest, it would otherwise have been expected to receive. Begging displays that manipulate host parental provisioning behaviour have since been documented in many other avian brood parasites (reviewed in Jamie and Kilner 2017). Some parasitic species use nonspecific acoustic features, calling more loudly or more rapidly to induce host parents to deliver more food than they would to their own young (Dearborn 1998; Lichtenstein and Sealy 1998; Gloag and Kacelnik 2013), while other parasitic nestlings exploit host-specific vocal signals, mimicking the acoustic structure of host chick begging calls (Davies *et al.,* 1998; Langmore *et al.,* 2008). Here, I studied the begging behaviours of two brood-parasitic species from the Indicatoridae family, the lesser and greater honeyguides *Indicator minor* and *I. indicator*.

The honeyguides are a group of woodland birds inhabiting Asia and sub-Saharan Africa. Honeyguides inflict costs upon their hosts (members of the orders Piciformes, Bucerotiformes and Coraciiformes) through egg puncturing (this thesis, chapter 3; Spottiswoode and Colebrook-Robjent 2007) and chick killing (Spottiswoode and Koorevaar 2012). Thus, honeyguide chicks always become the sole occupier of the nest. While this virulent behaviour enables the parasitic chick to monopolise host care, it may also come at a cost, since hosts should reduce their provisioning rates to match the requirements of a single chick (Grundel and Dahlsten 1991). However, both lesser and greater honeyguides receive food at a higher rate than that of a single host chick (this chapter; personal observations: Luke McClean, Jeroen Koorevaar). At my study site, the commonest hosts of these species are the cavity-nesting black-collared barbet *Lybius torquatus* and little bee-eater *Merops* *pusillus*, respectively. Given the dark interior of a cavity nest, it is unlikely that honeyguides exploit visual cues to elicit extra food from their foster parents. Here I test the hypothesis that, instead, honeyguides mimic the begging calls produced by chicks of their respective hosts in order to elicit additional food. Honeyguide begging calls have previously been subjectively suggested to mimic a host brood (Jubb 1966; Fry 1974), but this is the first experimental test of this hypothesis. Using both observations at nests and experimental manipulations, I assess whether both species have evolved vocal mimicry to elicit sufficient provisioning. I then ask why each species requires that level of provisioning.

First, I quantified provisioning rates at both barbet and bee-eater nests to confirm that single honeyguides are provisioned at a higher rate than a single host chick in the nest, and at a similar rate to an entire brood of host chicks. I then performed experiments using playback of the begging calls of parasitic chicks or different numbers of host chicks to evaluate host responses to chick begging displays, when only a single chick occupied the nest. If honeyguide chicks mimic the call of a host brood in order to elicit more food than they would otherwise be expected to receive from their hosts, then hosts exposed to the begging calls of either a honeyguide chick, or of a brood of host chicks, should provision at a higher rate compared to when exposed to the begging calls of a single host chick. An alternative hypothesis is that, rather than using mimicry of a host brood, parasitic chicks elicit additional provisioning through non-specific vocal cues such as call amplitude or frequency (Lichtenstein and Dearborn 2004; Gloag and Kacelnik 2013), resembling a brood of begging chicks because they produce a vocal signal of higher amplitude and/or frequency than the calls of a single chick (Kilner *et al.,* 1999; Wright and Leonard 2002). In this case, honeyguides could be eliciting extra food from their hosts simply because they produce a begging call that sounds like multiple chicks, rather than specifically sounding like multiple host chicks. To test this, for each host species I included an additional playback treatment, consisting of the begging calls of a brood of an unrelated cavity-nesting species. Thus, while similar in amplitude and frequency to a brood of host chicks, the begging calls in this treatment did not mimic those of a host brood. If honeyguides elicit additional provisioning through mimicry, then hosts exposed to the begging calls of a brood of chicks of an unrelated species (that do not sound similar to a host brood) should not be stimulated to provision at a higher rate. However, if hosts are stimulated to provide elevated provisioning simply by hearing a begging display of high amplitude or frequency (as would be produced by a brood of chicks, regardless of species identity), then playback calls of a brood of chicks from an unrelated species should stimulate similar rates of provisioning to the begging calls of a brood of host chicks, or of a single honeyguide chick.

High levels of begging are energetically expensive (Chappell and Bachman 2002) and can increase the likelihood of predation (Dearborn 1999; Haskell 2002; Husby 2019). Therefore, I expected honeyguide begging displays to provide an honest signal of how much food the chick requires (Kilner and Johnstone 1997). When parasitic chicks are larger and take longer to reach fledging than host chicks, requiring more food to fulfil energetic needs, the need for extra provisioning is clear (Davies *et al.,* 1998). However, this explanation is likely not valid for lesser honeyguides (21–39 g), which are smaller in body size than their barbet hosts (45–80 g; del Hoyo *et al.,* 2019). Why then would lesser honeyguides require extra provisioning? Suitable food is crucial for proper nestling development (Ricklefs 1968), and parasites are fundamentally constrained by the life history of their hosts (Grim *et al.,* 2011). Host diet, in particular, has often been proposed to be an important constraint on host selection by brood parasites (Davies and Brooke 1989; Grim 2007; Grim *et al.,* 2017). For example, cuckoos and cowbirds frequently parasitize insectivorous hosts, but do not target species that raise their young on fruit and seeds, presumably because their diet makes them unsuitable as hosts (Moksnes and Røskaft 1995). Honeyguides primarily feed on wax as adults (Downs *et al.,* 2002; Friedmann 1955), and although they also eat insects, they are not known to consume fruits or seed (del Hoyo *et al.,* 2019; Friedmann 1955; Isack 1987). Barbets, by contrast, are primarily fruit eaters (del Hoyo *et al.,* 2019). Therefore, even if barbets feed a mixture of fruit and insects to their chicks, rather than just fruit alone, a lesser honeyguide chick in a barbet nest faces the potential problem of receiving a sub-optimal diet and so might not grow properly. One solution to this problem is for lesser honeyguide chicks to stimulate extra provisioning from their foster parents, receiving more food overall to offset the sub-optimal diet they are provided. To test the hypothesis that lesser honeyguides require extra feeding in order to compensate for an inadequate diet, I initially assessed the type of food (insect or fruit) provided by barbets to honeyguide chicks, and their own. I then used hand-rearing experiments to assess whether honeyguides can assimilate a fruit diet. If barbet chicks can utilise a fruit diet but honeyguide chicks cannot, then barbet chicks should gain weight when fed a diet of either fruit or insects, while lesser honeyguide chicks should only gain weight when fed insects, and not when fed fruit. By contrast, bee-eaters – hosts of the greater honeyguide – are purely insectivorous (Douthwaite and Fry 1982), and so greater honeyguide chicks are unlikely to have a problem with the diet they are fed by their hosts. Greater honeyguides are, however, at least double the weight of a bee-eater (40–57 g vs 13–19 g; del Hoyo *et al.,* 2019). Therefore, a greater honeyguide chick likely requires extra provisioning to sustain its growth in order to fledge, similar to a common cuckoo chick that also exploits the care of hosts much smaller than itself (Davies *et al.,* 1998). If greater honeyguides elicit extra provisioning from their foster parents to sustain higher growth than their host's chicks, then greater honeyguide chicks would be predicted to grow at faster rates and fledge at larger mass than bee-eater chicks in unparasitized nests. However, if lesser honeyguides elicit extra provisioning from their foster parents to offset a sub-optimal diet, then lesser honeyguide chicks should not grow at faster rates than black-collared barbets, and nor should they necessarily fledge at larger mass.

6.3 Methods

6.3.1 Study site and species

This study was conducted primarily on the Semahwa and Musumanene farms in the Choma district of Zambia's Southern Province (16°46'S, 26°54'E) from 2015 to 2018. The host and honeyguide breeding season lasts from September to November and is dry and hot. Black-collared barbets, the hosts of lesser honeyguides, are primary cavity nesters, excavating nests in the dead branches of (amongst others at my study site) muwombo *Brachystegia boehmii*, musuku *Uapaca kirkiana*, wild fig *Ficus burkei*, and mubula plum *Parinari curatellifolia* trees. Little bee-eaters, the commonest host of greater honeyguides at this site, nest in terrestrial burrows built into sand banks or, more commonly here, in the entrance to tunnels and excavations dug by aardvarks *Orycteropus afer* or bushpigs *Potamochoerus larvatus*.

6.3.2 Video recording of host nests and assessment of provisioning rates

To examine the contents of bee-eater nests, I excavated a vertical access shaft above the nest chamber, which was rebuilt at each visit, following Spottiswoode & Koorevaar (2012; and chapters two and four of this thesis). To inspect the contents of barbet nests, I cut a small hole into the side of the cavity, just above the nest chamber, creating a 'hatch' which could be removed and replaced at each visit, and secured in place with wire (as in chapter five). I was then able to monitor nests on a regular basis with minimal disturbance to hosts. Once the chicks had hatched, I assessed parental provisioning rate by using infrared nest cameras (modified BrightTea 808#16 keychain camera, BrightTea, China) fixed to the interior of the cavity nest (Figure 1).

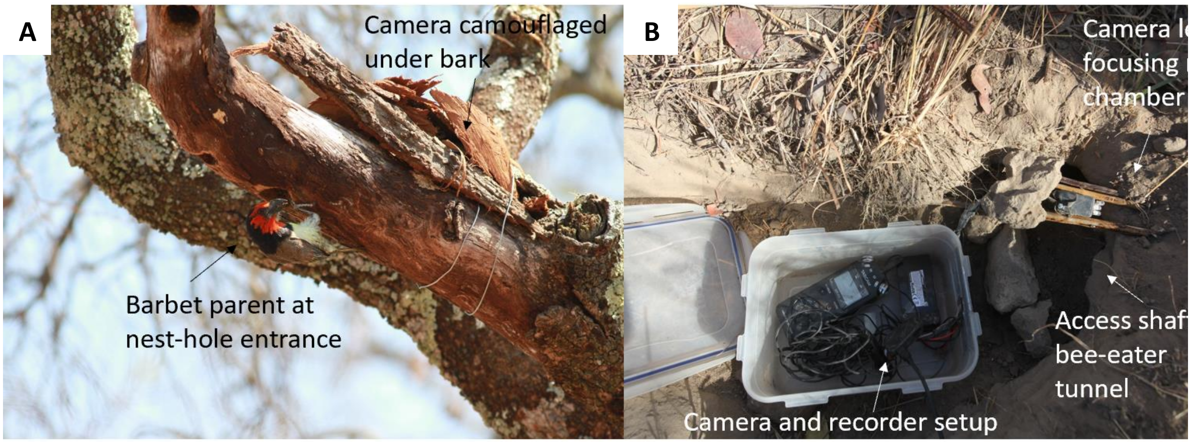


Figure 1. Camera setups to record provisioning rates at nests of **A)** black-collared barbets; **B)** little bee-eaters. Cameras were camouflaged at nests to minimize disturbance and left to record provisioning rates to host and parasitic chicks.

I recorded the provisioning rates of barbets at nests naturally containing either a single honeyguide chick (n = 10), or a brood of three barbets chicks (n = 30) for 6.0 ± 1.8 hours (mean ± SE, range = 1.45—9.7, n = 304 hours total) per nest; and at bee-eater nests naturally containing a single honeyguide chick (n = 6), or a brood of four bee-eater nestlings (n = 9) for 4.0 ± 1.2 hours (mean ± SE, range = 1.63—5.7, n = 90 hours total) per nest. For video recordings of single barbet chicks (n = 13), and single bee-eater chicks (n = 7), I was required to temporarily remove all but one chick from the nest for the duration of the video recording (and then returned), as both species typically raise more than one chick. Using the videos, I counted each time a parent visited the nest with food. When it was visible, I also scored the fullness of the beak and recorded the time taken to feed, in seconds. Beak fullness was scored on an ordinal scale from 1–5, with 1 being barely full, and 5 a full beak. I calculated provisioning rates as the average number of times the chick(s) were fed, per hour.

6.3.3 Recording and processing of begging calls

I recorded the begging calls of natural broods of 7–14 day-old chicks (n = 15 brood of three barbets, n = 16 single lesser honeyguides; n = 13 brood of four bee-eaters, n = 13 single greater honeyguides) inside nests at the point when the parents visited the nest to feed them. For begging call recordings of single barbets (n = 16) and single bee-eaters (n = 13), I was again required to remove all but one chick from the nest for the duration of the recording. I additionally recorded the begging calls of natural broods of three miombo blue-eared starling chicks *Lamprotornis elisabeth* (n = 7) and four grey-headed kingfisher *Halcyon leucocephala* chicks (n = 10) at the ages of 10–14 days to be used as alternative controls in experimental playbacks at barbet and bee-eater nests respectively. These control species were chosen because they are common cavity-nesting species that share the honeyguide breeding season. The begging calls were recorded in 44.1kHz, 16-bit WAV format with a Tascam DR-05 (TEAC Corporation, Los Angeles, USA) recorder and an Audio-Technica ATR3350 (Audio-Technica, Shinjuku, Japan) tie-clip microphone.

6.3.4 Playback experiments

To test whether honeyguide begging calls were sufficient to change the provisioning behaviour of host parents, I conducted a playback experiment at active, unparasitized barbet (n = 16) and bee-eater (n = 13) nests. To avoid parents trying to brood their chicks during playbacks, I targeted nests containing chicks that were old enough not to require brooding. These were 7–10 day-old chicks in bee-eaters, and 10–15 day-old chicks in barbets. For playback trials at barbet nests, I removed all but one host chick from the nest. Lesser honeyguide chicks are of similar size to barbet chicks (Fig. 4A), so using a barbet chick as a model meant that host provisioning rate was unlikely to be influenced by differences in visual stimuli caused by one species being larger than the other. Similarly, a barbet chick was unlikely to become satiated during playback trials. By contrast, since little bee-eater chicks are much smaller than greater honeyguide nestlings from the moment of hatching (Fig 4B), to prevent satiation of a single bee-eater nestling due to over-feeding, I removed all host chicks and temporarily placed a foster chick (a grey-headed kingfisher) inside the bee-eater nest during playback trials. The kingfisher (35–61 g) is an ideal model for this purpose as it is similar in size to a greater honeyguide (40–57 g), is insectivorous, and is a terrestrial burrow nester, and so kingfisher chicks were therefore not perturbed by being placed in bee-eater nests. At both barbet and bee-eater nests, the same model chick was used for consecutive playback trials, but no chick was used at more than one nest. All natal host chicks that were removed from the nest during playbacks were kept out of sight in a model nest (a small cup lined with cotton and grass, housed within a dark, aerated box) and checked regularly for signs of distress (but not fed, and no chicks were observed to be stressed) throughout the playback trial. For each playback trial I placed a speaker next to host nests, connected to an iPhone (Apple, Cupertino, USA). At barbet nests these were a JBL Clip Bluetooth speaker (JBL Corporation, Los Angeles, USA) and at bee-eater nests a wired Gigaware 40-218 speaker (RadioShack Corporation, Forth Worth, USA). Playbacks were created from begging calls of chicks recorded previously. I used the software Audacity 2.1.2 (Audacity Team 2019) to extract two 10-second episodes of chick begging from each recording. I then applied a low-cut noise filter at 1000 Hz with a 6.0 dB corner frequency to the chosen sections to reduce low frequency noise, normalised the peak amplitude to 4.0 dB, and converted the recordings to stereo by duplicating the mono track. Finally, the first and last second of each recording was faded in/out to reduce the abruptness of recording initiation.

Before each playback trial, I standardized all playbacks to a maximum amplitude (at 10 cm distance) of 50 dB over the playback, by placing the speaker 10 cm away from a Maplin N05cc sound-level meter (Maplin Electronics, Rotherham, UK; measurements validated against an iTest sound level meter, Studio Six Digital, Santa Cruz, USA: repeatability = 0.995 p = < 0.01, n =10) and adjusting the volume on the device. The repeatably (Nakagawa and Schielzeth 2010) between the iTest and Maplin sound level meter measurements was calculated using the rptR package (Stoffel *et al.,* 2017). An amplitude of 50 dB was chosen based on sound level measurements (at 10 cm) of chicks at barbet nests and is approximately similar to that produced by a single lesser honeyguide chick while begging (mean ± SD = 53 ± 3 dB, n = 5). This is higher than that of the begging call of a single host chick (mean ± SD = 38 ± 5 dB, n = 4), and lower than the amplitude of begging calls from a complete brood of barbet chicks begging naturally (mean ± SD = 59 ± 8.5 dB, n = 5). Due to the subterranean nature of bee-eater nests, I was unable to obtain sound level measurements of natural chick begging in this species. Therefore, all recordings for playback trials at bee-eater nests were set to the same 50 dB amplitude as used at barbet nests. For each host species, once the speaker was in place at the nest, I retreated to a hide placed in line of sight within 10 m of the nest. Each time a host parent entered the nest, the 10 s playback exemplar was played once. Since I was unable to view inside the nest, I assumed that any visit to the nest by an adult bird constituted a feeding event (which appears to be true, based on video recordings of natural feeding events). Hosts were allowed to visit the nest three times, with 10 s of recorded begging calls played at each visit, to allow both hosts and chicks to adjust to the new situation and to provide sufficient opportunity for both parents to hear the playback stimulus. Once a parent left the nest after the third visit, a one-hour trial period began. During this time, the playback was initiated every time a parent entered the nest, and each visit that a parent made to the nest was scored as one provisioning event. At the end of the hour the trial was ended, the speaker was removed, and all chicks were returned to their natal nests. I returned to the same nest for three subsequent days until all four playback treatments (begging calls of single honeyguide chick, single host chick, host brood, or unrelated brood of chicks) had been completed, with the order of playback treatments randomly assigned among nests. In almost all cases I was able to use playback recordings from unique nests. In two cases a starling brood playback was reused, and in three a kingfisher brood playback was reused.

6.3.5 Measurement of growth rate and fledging weight

To test whether parasitic honeyguide chicks were able to assimilate the food provided to them by their foster parents, and utilise it for growth, throughout 2015–2018 I monitored host nests. At every visit weighed both host and parasitic chicks using a Pesola MS500 electronic balance (Pesola Corporation, Schindellegi, Switzerland), accurate to 0.1 g. I then calculated the mass change between repeated measures of the same chicks over the time period recorded (full description of calculations in statistical analysis below; n = 24 lesser honeyguide chicks, n = 254 barbet chicks in 36 broods, n = 9 greater honeyguide chicks, n = 114 bee-eater chicks in 19 broods). Based on the differing fledging ages of host and parasite species (observed in this study), I considered fledging weight to be the mass of a chick at day 30 for barbets (n = 20), day 14 for bee-eaters (n = 39), and day 35 for both lesser (n = 5) and greater (n = 9) honeyguides.

6.3.6 Diet analysis and hand-rearing of lesser honeyguides and barbets

I collected faecal samples from barbet (n = 56 in 27 broods) and lesser honeyguide (n = 12) chicks during the 2016 field season. Chicks were not forced to defecate, and faecal samples were collected only when the opportunity arose. When produced, samples were collected immediately into an Eppendorf tube, and saturated with salt. Samples were then stored at room temperature until analysis, approximately three months later. For analysis, each sample was washed in water, and a dissection microscope was used to identify and separate the contents into fruit and insect material. Not all material was identifiable as either fruit or insect, and this was similarly separated. After sorting, items were dried and weighed to the nearest 0.01 g. Although faecal samples may overestimate the proportion of arthropods in the diet of frugivorous/insectivorous birds (Nazaro and Blendinger 2017), I was not able to accurately assess prey items provided to chicks through videos as prey items were not clearly visible (Grim *et al.,* 2017) and chick-collars were impractical (Grim and Honza 2001). However, as the procedure for diet analysis for both honeyguides and barbets was the same, I expect any noise that might arise due to my sampling protocol to be similar in both species.

In 2018 I temporarily hand-reared lesser honeyguide and barbet chicks for two days (n = 9 for each species, each from a different brood) and on each day fed them exclusively either a fruit-only, or an insect-only, diet. I removed a single lesser honeyguide or barbet chick from their natal nests before sunrise, and immediately weighed them to the nearest 0.01 g. In the case of lesser honeyguides, to ensure that the parents did not abandon the otherwise empty nest, I temporarily replaced the removed parasitic chick with a barbet chick of a similar age and size from a nearby, unparasitized nest. Chicks were housed for two days in a Brinsea Octagon egg incubator (Brinsea Products Inc., Titusville, USA) maintained at 37.5°C and 70% humidity. Over two days, between sunrise and sunset (approximately 06:00–18:30) I fed each chick every 15 minutes until satiation, recording to the nearest 0.01 g the quantity of food consumed by the chick. Chicks were stimulated to beg by tapping the end of the beak with either tweezers or a syringe, but were not forced to eat. The following morning, after allowing all food to be assimilated and passed, at approximately 06:00, and before starting the next diet trial, I weighed each chick (± 0.01 g). I then recommenced feeding, following the same 15-minute regime, but this time with the alternative diet. Thus, if on day one a chick was fed a fruit diet, on the other day they received an insect diet (and vice versa). The order of the diets was randomised for each chick. The fruit diet consisted of 100 g of mashed ripe bananas mixed with 82 ml of water (adapted from Denslow *et al.,* 1987), while the insect diet was composed of dried mealworms, but not mixed with any water. Table 1 shows the standard values of protein, fat and carbohydrate content of each diet, as well as their calorific content. On the third morning, after having been weighed (± 0.01 g) for a third time, chicks were returned to their natal nests. All chicks were readily accepted back by their (foster) parents.

Table 1. Nutrient composition comparisons of fruit and insect diets used in hand-rearing experiments. Nutritional contents were sourced from standard producer values.

Diet	Protein (%)	Fat (%)	Carbohydrate (%)	Calories (/g)
Insect	46	33	21	5.65
Fruit	5	1	94	3.21

6.3.7 Statistical analysis

To test whether host parents differed in their provisioning rate to nests containing different chicks (host versus parasitic), I used linear mixed models with planned comparisons (Ruxton and Beauchamp 2008) between groups: (i) one host chick compared to both one honeyguide chick and a brood of host chicks, (ii) one honeyguide chick compared to a brood of host chicks. If honeyguides elicit extra food, there should be a significant difference in provisioning rates between the compared groups in (i), but not in (ii). I used separate models for lesser and greater honeyguides. I included nest identity as a random effect in each model as I recorded provisioning rates at most nests more than once. As larger chicks are likely to be fed more food (Krebs 2001) I included the mass of the chick (or mean mass of chicks in the case of complete broods) as a continuous variable within the model, but this was dropped during AIC-based model selection. To confirm that only the rate and not the quality or quantity of feeding changed with brood composition, I included the beak fullness score and average time spent at the nest per feeding event as continuous predictors in each model. I modelled the results of playback experiments in a similar manner, using planned comparisons between three groups: (i) one host and a brood of control chicks (starlings or kingfishers) compared to one honeyguide chick and a brood of host chicks, (ii) one host chick compared to a brood of control chicks, and (iii) one honeyguide chick compared to a brood of host chicks. If honeyguides elicit extra provisioning by using vocal mimicry of host chicks (and not by simply making a lot of noise), I expect there to be a significant difference between the comparisons in contrast (i), no difference between the comparisons in (ii), and no difference between the comparisons in contrast (iii). I included the same independent variables as were used to model natural provisioning rates, with the final model again retaining nest identity as a random effect, and chick mass in the case of bee-eaters only.

To compare growth rates of parasites and hosts (lesser honeyguide and barbet, or greater honeyguide and bee-eater), I created a linear mixed model with the rate of mass gain as the response variable, with the chick type (parasite or host) as a binary predictor. To account for multiple measures of chicks within a single nest, I included chick ID nested within nest identity as random effects. As the gain of mass in chicks is a sigmoidal curve, I included only chicks at ages within the linear phase of growth (lesser honeyguide, black-collared barbet, and greater honeyguide 5–18 days, little bee-eater 5–14 days). To test for differences in fledging weights between parasites and hosts, I created an additional linear mixed model with the same predictors, but with the fledging weight of chicks as the response.

I used generalized linear mixed models (GLMM) with logit links when assessing the differences in the proportions of insect, fruit and unidentified material present in lesser honeyguide and barbet faeces. As I collected repeat samples at some nests (n = 25 of 66 nests), I included nest identity as a random effect, but this was later dropped from subsequent models based on a likelihood ratio test (Luke 2016). I next used linear regression to test whether there was a difference in mass gained by barbets or honeyguides in feeding trials when provided (i) a fruit diet, or (ii) an insect diet. I included the change in mass over one day as a continuous predictor, with chick type (honeyguide or barbet), and diet (insect or fruit) as categorical predictors, and the interaction between chick type and diet. If host and parasite differ in their ability to assimilate the two diet types, then this interaction term is expected to be significant. Additionally, to confirm the directionality of results from this interaction term, I split the data into two subsets: (i) insect diet, and (ii) fruit diet, and then compared the mass change between the two species of chicks. The model initially included the quantity and calorific content of food fed to chicks, but both of these terms were subsequently dropped from the final model, as neither explained any additional variation.

All analyses were conducted in R v. 3.1.3 (R Core Team 2019), using the packages lme4 (Bates *et al.,* 2014), lmerTest (Kuznetsova *et al.,* 2017) and nlme (Pinheiro *et al.,* 2013). I used ggplot2 (Wickham 2019b), cowplot (Wilke 2019), and forcats (Wickham 2019a) to generate visualisations of the data. I selected models using the Akaike Information Criterion (AIC; Wagenmakers and Farrell 2004), with models of >2 units lower AIC than a less complex model considered the most parsimonious (Burnham *et al.,* 2011). I assessed the homogeneity of variance and normality of each model using residual plots of fitted models, with all models meeting assumptions.

6.4 Results

6.4.1 A parasite that is smaller than its host: lesser honeyguides

6.4.1.1 Chick provisioning in natural broods

Lesser honeyguide chicks were fed at approximately double the rate at which a single barbet chick was fed, and at a similar rate as a full brood of host chicks (single barbet chick = 3.0 ± 0.3 feeds hr^{-1}, range = 1.6–4.1 feeds hr^{-1}; single lesser honeyguide chick = 7 ± 0.9 feeds hr^{-1}, range = 3.18–11.32; brood of barbet chicks = 8 ± 0.43 feeds hr^{-1}, range = 3.8–12.9; Figure 2A). Thus, both a single lesser honeyguide chick and a brood of barbet chicks were provisioned at a higher rate than were single barbet chicks ($\beta = 4.551 \pm 0.701$, $t_{49} = 6.657$, $p < 0.01$). The provisioning rate of single lesser honeyguide chicks did not differ significantly from that of a brood of barbets ($\beta = -0.381 \pm 0.395$, $t_{49} = -1.245$, $p = 0.22$). There was no difference in the duration of provisioning visits to single honeyguide chicks in comparison to single barbet chicks ($\beta = -0.1022 \pm 6.677$, $t_{33} = -0.015$, $p = 0.99$) or barbet broods ($\beta = -2.756 \pm 3.282$, $t_{33} = 0.109$, $p = 0.91$), and nor were there differences in the size of prey barbets provided to single honeyguide chicks when compared to single barbet chicks ($\beta = 0.905, \pm 0.639$, $t_{27} = 1.416$, $p = 0.170$) or barbet broods ($\beta = 0.003 \pm 0.250$ $t_{27} = 0.011$, $p = 0.99$).

6.4.1.2 Playback experiments

Playbacks of chick begging calls explained 42% of variation in barbet provisioning rates (Figure 3A). Playbacks of the begging calls of either a lesser honeyguide chick or a brood of host chicks stimulated barbets to provision the nest at a rate of 7.6 ± 3.17 (range = 2–12) and 7.5 ± 2.92 (range = 4–15) feeds hr^{-1} respectively. Playbacks of the begging calls of a single barbet chick stimulated barbets to provision the nest at only 2.3 ± 0.86 (range = 1– 4) feeds hr^{-1}, while the playbacks of a brood of starlings provisioned the nest at 3.6 ± 2.52 (range = 1–12) feeds hr^{-1}. There was no difference in provisioning rate to playback of a single honeyguide or a brood of barbets ($\beta = -0.184 \pm 0.357$, $t_{52} = -0.514$, $p = 0.61$), but these were provisioned at significantly higher rates than playbacks of a single barbet or a brood of starling chicks ($\beta = -4.961 \pm 0.560$, $t_{52} = -8.863$, $p < 0.001$). There was no difference in provisioning rate between the latter two treatments ($\beta = 0.837 \pm 0.428$, $t_{52} = 1.963$, $p = 0.060$).

6.4.1.3 Host vs parasite growth

Lesser honeyguide and barbet chicks gained mass at a similar rate (Figure 4A; $\beta = 0.003 \pm 0.179$, $t_{170} = 0.196$, $p = 0.844$; mean mass gain for barbets = 1.74 ± 1.67 g day^{-1}; lesser honeyguides = 1.81 ± 1.46 g day^{-1}). However, barbets fledged at higher masses than lesser honeyguides did ($\beta = -0.349 \pm 0.153$, $t_{24} = -2.905$, $p < 0.01$).

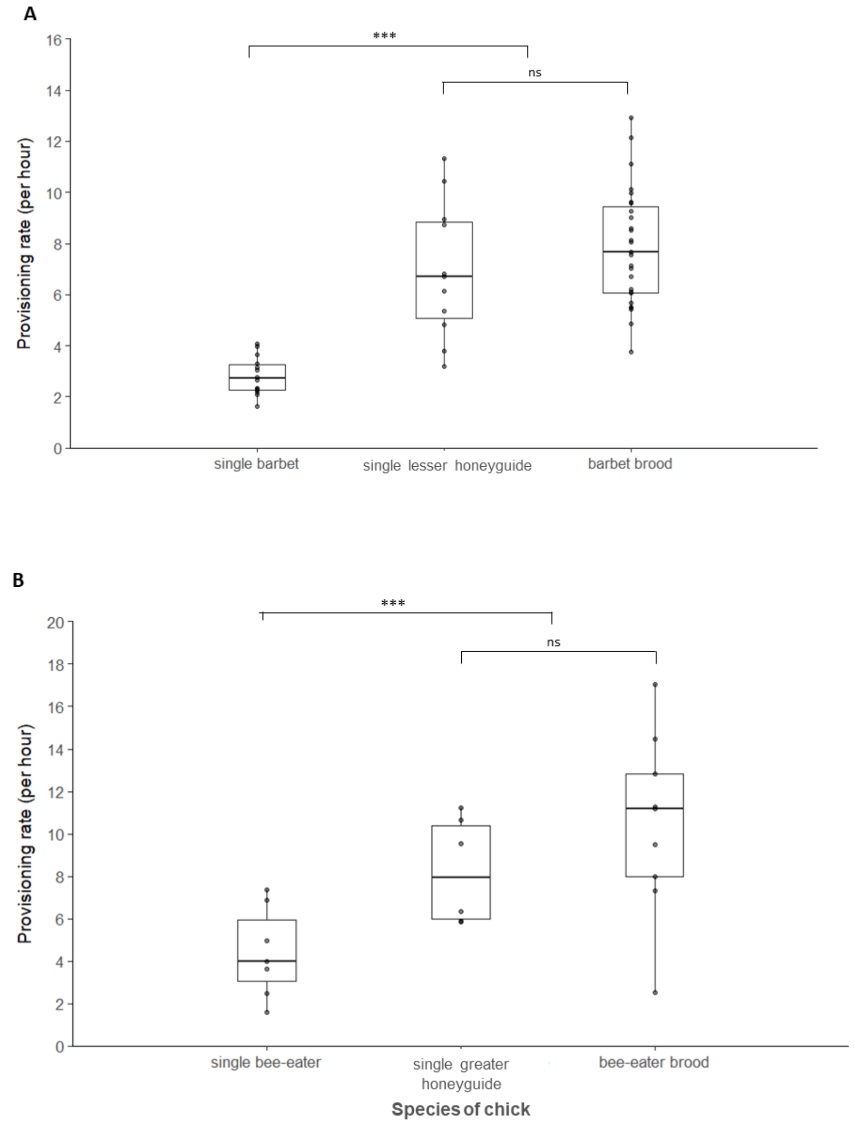


Figure 2. I recorded the begging calls of natural broods of 7–14 day-old chicks (n = 15 brood of three barbets, n = 16 single lesser honeyguides; n = 13 brood of four bee-eaters, n = 13 single greater honeyguides) inside nests at the point when the parents visited the nest to feed them.Single honeyguides of both species were fed at similar rates to whole broods of host chicks, but at significantly higher rates than that of single host chicks. A) Provisioning rates (feeds hr^{-1}) to barbet nests containing a single lesser honeyguide, a single barbet, or a brood of barbet. B) Providing rates (feeds hr^{-1}) to bee-eater nests naturally containing a single greater honeyguide, a single bee-eater, or a brood of bee-eater chicks. Bars indicate the significance between the groups in the planned comparisons (*** for < 0.001, ns for non-significance). Black dots indicate raw data from camera observations of provisioning rates, where nests are independent data points. The black line within the box indicates the average feeding rate, with the whiskers extending from the box indicating the 25th (lower) and 75th (upper) quartiles of provisioning rates.

6.4.1.4 Host vs parasite diet

Barbets fed both insects and fruit to chicks. Insects were predominantly darkling beetles (Tenebrionidae) and fruit chafer beetles (Scarabaeidae), but also soldier flies (Stratiomyidae) and wasps (Formicidae). Fruit consisted of mainly figs *Ficus burkei* (occasionally in honeyguide faces), but larger items including seeds from musuku *Uapaca kirkiana*, mbula plum *Paranari curatellifolia*, and lubangeni *Senna singueana* trees that were commonly found in barbet chick faeces were never found in honeyguide faeces.

Lesser honeyguides had significantly higher proportions of insects in their faeces compared to barbets ($\beta = 0.062 \pm 0.020$, $Z_{65} = 2.477$, $p < 0.01$; Table 2), and significantly less fruit (Table 2; $\beta = -0.065 \pm 0.21$, $Z_{65} = -2.569$, $p < 0.01$). There was no difference in the quantity of unidentified material in the faeces between lesser honeyguides and barbets ($\beta = -0.001 \pm 0.03$, $Z_{65} = 0.684$, $p = 0.49$).

Table 2. I collected faecal samples from black-collared barbet (n = 56) and lesser honeyguide (n = 12) chicks. The table shows the proportional content of insect and fruit material found in chick faeces of each species. The average percentage of each material is given (± standard deviation), with the percentage range below. Honeyguide faeces contained significantly less fruit material than barbet faeces.

	Food type	
	Insect	**Fruit**
Barbet	58.6% (± 27.4)	42.5% (± 27.9)
	0–100%	0–100%
Honeyguide	87.6% (± 26)	12.4% (± 26)
	23–100%	0–*77%*

6.4.1.5 Host vs parasite feeding experiments

During hand-rearing, lesser honeyguides and barbets ate comparable quantities of insects (Table 3; $\beta = 0.506 \pm 1.712$, $Z_{35} = 0.296$, $p = 0.77$), fruit (Table 3; $\beta = -0.002 \pm 0.022$, $Z_{35} = -$

0.109, $p = 0.913$) and calories (Table 3; $\beta = 0.008 \pm 0.014$, $Z_{35} = - 0.109$, $p = 0.91$), with both species consuming a larger volume of food during fruit trials than during insect trials (honeyguides: $\beta = -0.415 \pm 0.211$, $Z_{17} = -2.043$, $p = 0.041$, barbets: $\beta = -22.624 \pm 0.341$, $Z_{17} = -2.028$, $p = 0.043$). This is likely because the fruit diet contained far fewer calories, and more water. Honeyguides gained 1.99 g ± 0.96 per 24-hour trial when fed insects, but lost 2.02 g ± 1.61 per 24-hour trial when fed fruit (Figure 5). Barbets gained 1.58 g ± 1.34 when fed insects, and 1.55 g ± 1.20 when fed fruit (Figure 5). In my model containing an interaction between chick type and diet type as an interaction, honeyguides gained significantly less weight than barbets; this effect was much greater when fed fruit rather than insects ($\beta = 3.49 \pm 0.999$, $Z_{32} = 3.432$, $p < 0.01$). In the split dataset honeyguides gained significantly more mass when fed insects than when fed fruit ($\beta = 1.329 \pm 0.590$, $Z_{17} = 2.250$, $p = 0.024$), but in barbets there was no detectible difference in mass gain between the two diet types ($\beta = 0.030 \pm 0.400$, $Z^{1}_{7} = 0.074$, $p = 0.94$). Honeyguides gained significantly less mass when fed fruit compared to barbets ($\beta = 1.084 \pm 0.518$, $Z_{16} = -2.094$, $p = 0.036$), but gained a similar amount of mass when fed insects ($\beta = -0.673 \pm 0.419$, $Z_{17} = -1.606$, $p = 0.108$).

Table 3. The mean, and (range) of quantity and calorie content of food consumed by black-collared barbet and lesser honeyguide chicks ($n = 9$ for each species) during hand-rearing experiments. Barbet and lesser honeyguide chicks ate comparable quantities of insects, fruit, and calories during trials. Both barbet and lesser honeyguide chicks ate a larger quantity of food during fruit trials than during insect trials (to offset the lower calorie-density of the diluted fruit diet compared to the insect diet).

		Quantity of	
	Insect (g)	**Fruit (g)**	**Calories**
Black-collared barbet	9.12 (4.66–20.46)	31.75 (22.50–54.00)	76.73 (26.26–173.74)
Lesser honeyguide	8.38 (4.32–21.39)	28.59 (17.50–54.00)	69.59 (24.40–163.33)

6.4.2 A parasite that is larger than its host: greater honeyguides

6.4.2.1 Chick provisioning in natural broods

Single greater honeyguide chicks were also fed at approximately double the rate of single bee-eater chicks, and at a similar rate to bee-eater broods (Figure 2B; single honeyguide chick 8.2 ± 1 feeds hr^{-1} (range = 5.9–11.2); single bee-eater chick 4.4 ± 0.8 feeds hr^{-1} (range = 1.6–7.4); brood of bee-eater chicks 9.3 ± 1.4 feeds hr^{-1} (range = 2.5–17.1)). A single greater honeyguide chick, or a bee-eater brood of four chicks, were fed at significantly higher rates than a single bee-eater chick ($\beta = 1.647 \pm 0.501$, $t_{19} = 3.261$, $p < 0.01$). There was no difference in provisioning rate between single honeyguide chicks and bee-eater broods ($\beta = -1.105 \pm 0.866$, $t_{17} = -1.276$, $p = 0.22$). Bee-eaters spent similar quantities of time provisioning nests containing a greater honeyguide as they did to a single bee-eater ($\beta = -14.030 \pm 13.310$, $t_{14} = -1.055$, $p = 0.309$), or a brood of bee-eaters ($\beta = 13.500 \pm 24.310$, $t_{14} = 0.555$, $p = 0.59$). Bee-eaters provided greater honeyguides with similar sizes of prey as they did to single bee-eaters ($\beta = 9.989 \pm 34.722$, $t_{15} = 0.288$, $p = 0.78$) and bee-eater broods ($\beta = -60.863 \pm 60.705$, $t_{15} = 1.003$, $p = 0.33$).

6.4.2.2 Playback experiments

The playback treatment type played to bee-eaters at the nest explained 64% of the variation in the rate that they provided food to the nest (Figure 3B). Bee-eaters hearing playbacks of begging calls of a brood of bee-eater chicks provided 9.4 ± 0.7 (range = 6–15) feeds hr^{-1}. Those hearing a single bee-eater chick begging provisioned at 4.8 ± 0.5 (range = 2–7) feeds hr^{-1}, and those hearing the begging calls of a brood of kingfishers at 3.2 ± 0.4 (range = 1–5) feeds hr^{-1}. Bee-eater parents that heard the begging calls of a whole brood of bee-eaters, or of a single greater honeyguide chick, provisioned at significantly higher rates than those that heard the begging calls of either a single bee-eater chick or a brood of kingfisher chicks ($\beta = -2.635 \pm 0.295$, $t_{48} = 8.927$, $p < 0.001$). Hosts provisioned at similar rates when hearing the playbacks of a single honeyguide chick or a brood of bee-eaters ($\beta = 0.077 \pm 0.417$, $t_{48} = 0.184$, $p = 0.85$). There was no significant difference in provisioning rate between bee-eater parents that were exposed to begging calls of a single bee-eater chick or a brood of kingfisher chicks ($\beta = -0.808 \pm 0.417$, $t_{48} = -1.84$, $p = 0.059$), despite a trend for lower provisioning rate in response to playback of a kingfisher brood. Bee-eaters provisioned foster chicks of large mass at higher rates than those of small mass ($\beta = 0.149 \pm 0.056$, $t_{47} = 2.488$, $p = 0.016$).

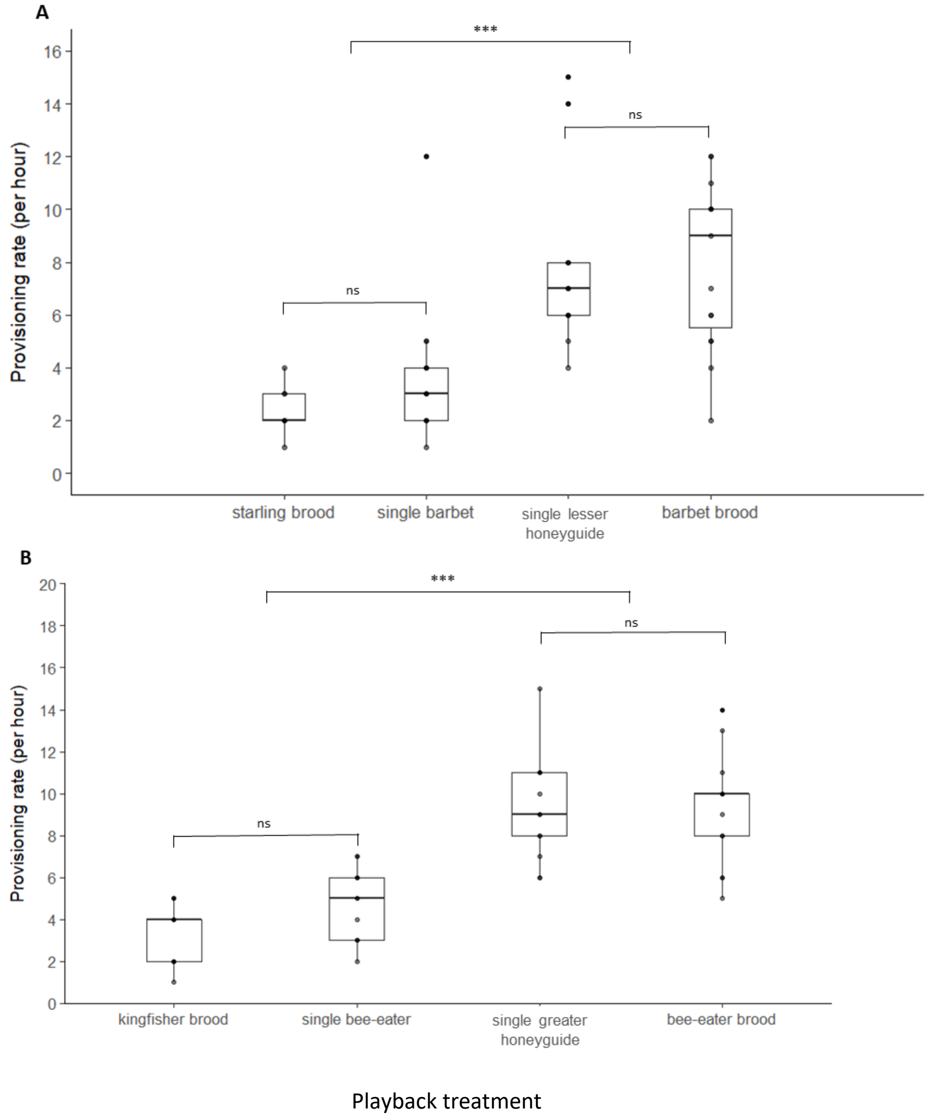


Figure 3. I conducted a playback experiment at active, unparasitized barbet (n = 16) and bee-eater (n = 13) nests. Provisioning rates observed during experimental playbacks. In both host-parasite systems, a chick presented alongside the begging call of a single honeyguide was fed at similar rates to those accompanied by the begging call of a whole brood of host chicks, but at significantly higher rates than those accompanied by the begging call of a single host chick. **A)** Provisioning rates (feeds hr^{-1}) to barbet nests containing a single barbet chick during experimental playbacks of a single lesser honeyguide, a single barbet, a brood of barbets, or a brood of starling chicks. **B)** Provisioning rates (feeds hr^{-1}) to bee-eater nests containing a single kingfisher chick during experimental playbacks of a single greater honeyguide, a single little-bee eater, a brood of little bee-eaters, or a brood of grey-headed kingfisher chicks. Bars indicate the significance between the treatments (*** for < 0.001, ns for non-significance). Black dots indicate raw data points from playback experiments. The black line within the box indicates the average feeding rate, with the whiskers extending from the box indicating the 25th (lower) and 75th (upper) quartiles of provisioning rates.

6.4.4.3 Host vs parasite growth

Greater honeyguides gained weight at a faster rate than did bee-eater chicks (Figure 4B: β = 0.592 ± 0.270, t_{24} = 2.905, $p < 0.001$; mean weight gain rate bee-eater = 0.82 ± 0.96 g day^{-1}; mean weight gain rate greater honeyguide = 2.0 ± 1.4 g day^{-1}), and fledged at significantly higher masses (β = 29.223 ± 1.097, t_{42} = 29.121, $p < 0.001$; bee-eater = 14.1 ± 2.0 g; greater honeyguide = 43.4 ± 2.0 g).

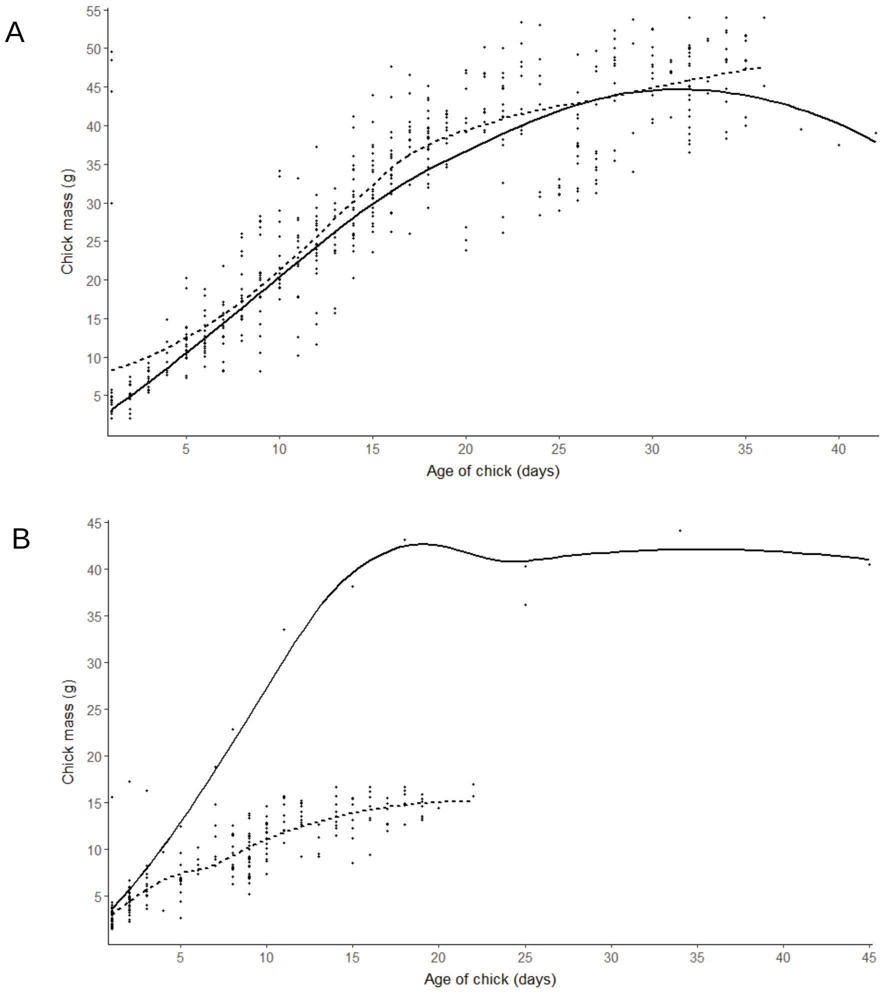


Figure 4. Raw data plot of the mass of host and honeyguide chicks in unmanipulated nests. **A)** The mass of black-collared barbets ((n = 23 chicks, dotted black line) and lesser honeyguides (n = 7 chicks, solid black line) as a chick ages. Lesser honeyguides and barbets gain mass at similar rates, but barbets fledge at greater mass than lesser honeyguides. **B)** The mass of little bee-eaters (n= 20 chicks, dotted black line) and greater honeyguides (n = 4 chicks, solid black line) as the chicks age. Greater honeyguides gained mass at faster rates and fledged at significantly larger masses than little-bee-eaters. Curves were fitted using loess, with chick ID nested within nest ID as random effects, to account for repeated measurements of nests/chicks.

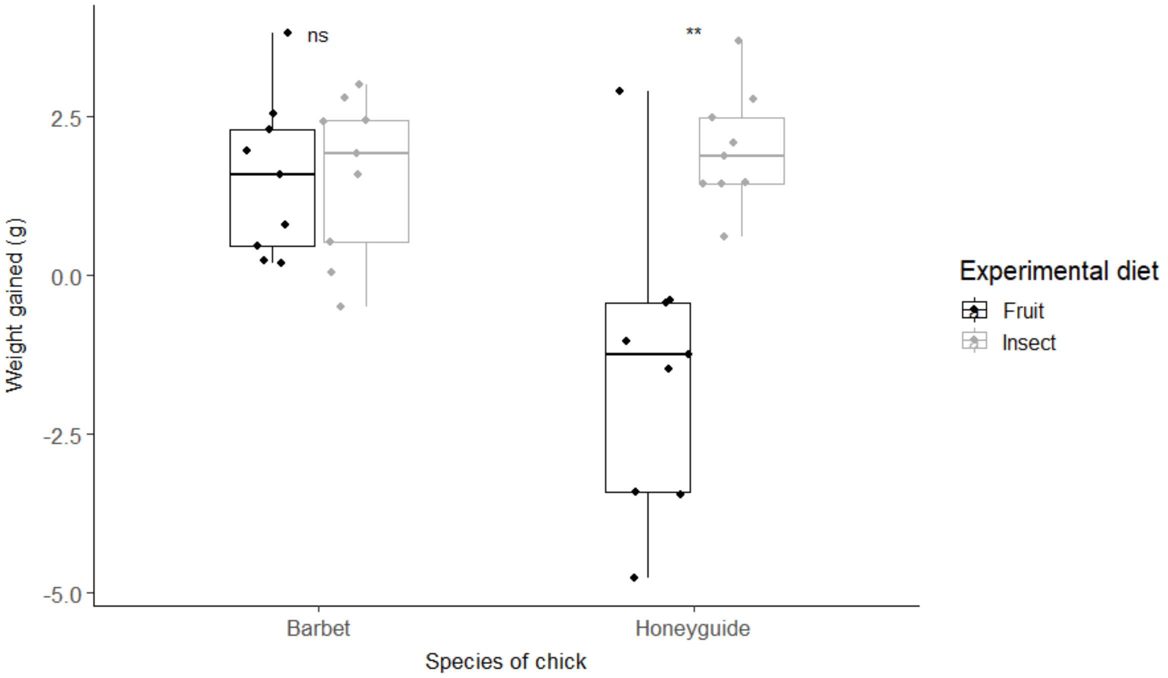


Figure 5. Barbets had similar mass gains when experimentally fed diets consisting entirely of either insects or fruits (n = 9 chicks). By contrast, honeyguides lost mass when experimentally provisioned with the fruit diet than when fed the insect diet (n = 9 chicks). Additionally, honeyguides and barbets gained mass at similar rates when provisioned with the insect diet, but barbets gained significantly more mass than honeyguides did on the fruit diet. Black points and boxes indicate mass changes during fruit trials, and grey points and boxes indicate mass changes during insect trials. The black line within the box indicates the median mass gain, with the whiskers extending from the box indicating the 25th (lower) and 75th (upper) quartiles. The significance level of differences between the experimental diets is shown above the bars (** for < 0.01, ns for non-significance).

6.5 Discussion

6.5.1 Honeyguides elicit increased provisioning from their hosts

In this study, I first showed that single, brood-parasitic lesser and greater honeyguide chicks are provisioned at higher rates than are single host chicks in the nest. The mechanism underlying these observational findings was confirmed with experimental playback of chick begging calls, which showed that the hosts of both lesser and greater honeyguides provisioned at higher rates upon hearing the begging calls of a single honeyguide chick than when they heard the playback of a single host chick. There was no difference in provisioning rate of either host species in response to experimental playback of begging calls of a single parasitic honeyguide or an entire host brood. These results are consistent with the hypothesis that honeyguide begging calls mimic the sound of a brood of begging host chicks. By contrast, the provisioning rate of host species in response to the begging calls of entire broods of closely-related cavity nesting species (starling or kingfisher for barbets and bee-eaters respectively) was similar to that of a single host chick. This suggests that common call structure cues used by chicks to signal parents, such as call amplitude, length, or regularity (Leonard and Horn 2001) were not responsible for the increased provisioning observed in parasitic or host brood playbacks; in other words, honeyguides are specifically mimicking several host chicks, rather than simply sounding like several chicks (see supplementary material 1).

These findings match predictions of the hypothesis that honeyguide begging calls have evolved to tune into parent-offspring communication systems in order to manipulate host parents to supply them with enough food (Davies *et al.,* 1998; Kilner *et al.,* 1999). Honeyguides kill all chicks in the nest, and therefore do not need appropriate begging displays to compete with host offspring, as in other systems (Hauber and Moskát 2007; Pagnucco *et al.,* 2008), but instead require them to elicit adequate levels of provisioning. The begging call similarity between host and parasitic chicks could arise if some hosts use the vocal begging displays of chicks for identification, rejecting chicks that do not make appropriate begging calls and thus selecting for parasitic mimicry (Langmore *et al.,* 2008). However, as neither barbets nor bee-eaters were observed ever to reject parasitic chicks, this selection pressure is unlikely to have driven the evolution of the observed similarities in begging calls. However, in both hosts, experimental playback of the begging calls of control chicks (starlings and kingfishers) elicited provisioning at low rates, despite the high regularity of the calls. This result suggests that honeyguide mimicry of their specific host species is indeed important in order to elicit sufficient provisioning.

The similarity in begging calls between honeyguide nestlings and their hosts could be a product of their phylogenetic history (Rivers *et al.,* 2014; Grim 2005), if common features of avian begging calls are shared by honeyguides and their hosts. However, grey-headed kingfishers are closer phylogenetic relatives to little bee-eaters than are honeyguides, and begging calls of a brood of kingfisher chicks did not stimulate host provisioning to the same levels as did the calls of either a honeyguide chick or a brood of host chicks. This would suggest that it is rather selection for mimicry as an adaptation to brood parasitism in honeyguides that has promoted the evolution of matching chick begging calls.

What mechanism allows honeyguides to mimic the chick begging calls of the appropriate host? As honeyguide nestlings kill host chicks within days of their hatching, there is little or no potential for social learning (Redondo and Reyna 1988; Madden and Davies 2006). However, vocal behaviour is much more plastic than are visual signals (Jamie and Kilner 2017; Langmore *et al.,* 2008), and similarities in begging calls can arise very quickly without the need for generations of evolution. Honeyguides could learn by 'testing out' various begging calls, and then tuning in to which vocal displays elicit the desired parental behaviour (Langmore *et al.,* 2008). Under this scenario, the begging calls of honeyguides could potentially be innate, learnt, or a combination of both (Rojas Ripari *et al.,* 2019). Cross-fostering of honeyguide (or other parasitic) chicks between hosts could provide insight into the mode of parasite begging call development. Indeed, although my study supports the hypothesis that honeyguides mimic host broods in order to elicit sufficient provisioning, the key part(s) of the call structure that host parents respond to remains unknown. Some hosts are sensitive to features of begging displays that their own young do not produce (Gloag and Kacelnik 2013), or to only a small portion of the call structure (Madden and Davies 2006).

I cannot be sure that the model chick within the nest begged consistently throughout all playback treatments. Previous studies have shown that in brood-parasitic species where the parasitic chick does not kill or remove its foster siblings, the parasitic chick can influence the begging intensity of its host nest mates, which in turn may influence provisioning rates (Pagnucco *et al.,* 2008). However, in the experimental playback trials at bee-eater nests, where a kingfisher chick was used as a model, it is unlikely that an increase in intensity of begging by the chick would cause the differences I observed, as the begging calls of a whole kingfisher brood only prompted low provisioning rates. Interestingly, I observed similar rates of provisioning in response to playback trials as I did under natural, unmanipulated conditions. This suggests that the playbacks accurately simulated the brood composition they were designed to represent, and that host parents did not integrate both the begging displays of the model chick and the playback calls to determine how much food to bring to the nest. Rather, hosts seem to have relied primarily or solely upon the information provided in the playback begging calls, and ignored any signal provided by the model chick. This could have arisen because of the high amplitude at which experimental playbacks were given (50 dB). This amplitude was chosen to match the amplitude at which lesser honeyguide chicks beg under natural conditions but may have 'drowned out' the vocal display of the single chick left in the nest.

6.5.2 Lesser and greater honeyguides require this provisioning for different reasons

Despite being provisioned at twice the rate of a single barbet chick, lesser honeyguides did not grow faster, or grow to larger masses than barbet chicks did, which is expected since they are smaller in adult body size than barbets are. Faecal sampling showed that barbets provisioned chicks with both insects and fruit material throughout chick development. I found significantly more fruit and seed material in barbet chick faeces than in honeyguide chick faeces. In fact, I found no large seeds (e.g. musuku *U. kirkiana, Lubangeni S. Singueana*, mbula *P. curtellifolia*) within the faeces of honeyguides, which were commonplace in barbets, with all fruit material identified in honeyguide faeces being small fig seeds. This could have arisen because barbets fed honeyguide chicks less fruit than they fed barbet chicks, and/or because honeyguide chicks are more selective in the food items that they accepted from their host parents, and/or because honeyguide chicks are more selective in the food items they digested. There was no difference in the size of prey items, or the time spent feeding (per provisioning event) that hosts provided to their own chicks compared to honeyguide chicks. This suggests that hosts brought similar food quantity and type to the nest regardless of the call they heard, only changing the rate at which they provisioned. Therefore, it seems more likely that honeyguides are selective in the food that they eat.

In support of the hypothesis that honeyguides are selective, honeyguides appear to regurgitate seeds much more frequently than barbets did (supplementary video 1), which may indicate an attempt to free up the digestive tract of such material to make room for more, ideally insectivorous, food. The rate at which birds can consume fruit may be limited by the rate at which their guts can process the fruit, and this can cause digestive bottlenecks when large amounts of indigestible seeds or fruit are consumed (Levey and Grajal 1991). Although the fruit diet provided during hand-rearing contained significantly fewer calories per gram than did the insect diet, both barbets and honeyguides ate significantly more fruit than insects during hand rearing. The effect of this was to counter-balance any differences in calorific content, such that overall both species received a similar quantity of calories from each diet. Fruits generally represent an inferior diet for any bird (Castro *et al.,* 1989). Despite this, barbets gained mass when fed fruit or insects, offsetting the diluted, lower calorie content of the fruit diet by eating larger volume. By contrast, honeyguides gained significantly more mass on days they were fed the insect-based diet compared to when they were fed fruit, despite eating larger quantities of fruit. On two occasions, trials of feeding fruit to honeyguides were halted (not included in analyses) and the chicks returned to their natal nests, because the chick appeared to be under stress. These results highlight that while barbets can digest and assimilate fruit, honeyguides appear unable to do so. I believe that the best explanation for this pattern, when combined with the increased provisioning rates that lesser honeyguides elicit from their barbet hosts, is that lesser honeyguide chicks require additional provisioning to offset an inadequate diet for growth.

The reasons why a fruit diet seems to be sub-optimal for lesser honeyguides currently remain unknown, but could arise because their digestive physiology is poorly adapted to a fruit diet (Kozlovic *et al.,* 1996), or because of potentially poisonous substances contained in fruits (Rothstein 1976). Of the two, the former seems most likely. Despite the digestive organs of birds being remarkably dynamic (Karasov 1996; Levey and Karasov 1989), honeyguides seem unable to undergo the required changes in digestive structure and function to assimilate a frugivorous diet efficiently. Adult honeyguides subsist predominantly on a diet of wax. The digestive tract of honeyguides is extremely long (Downs *et al.,* 2002), presumably to help with absorption of lipids, which are assimilated more slowly than carbohydrates (Karasov and Hume 1997). Although I have no physiological data on barbets, frugivorous birds generally have a short digestive tract (Levey and MartÍnez del Rio 2001). This allows large volumes of food to be passed through the gut rapidly (Worthington 1989). In addition to the variety of biochemical and physiological adaptations required for digestion, bacterial assemblages play an important role in the digestive process (Hooper *et al.,* 1998). Gut microbiota can differ between brood parasite and host species (Ruiz-Rodríguez *et al.,* 2009) and this could affect the ability of a brood parasite to digest the diet provided to it by its host. Therefore, the potential physiological differences between honeyguides and their hosts likely explain the inability of honeyguides to adequately digest fruit material. The diet provided to parasitic nestlings is central to host selection and has been proposed to be a major driver for the absence of parasitism of granivorous passerines by cuckoos (Rothstein 1976; Moksnes and Røskaft 1995). Parasitic chicks that end up in nests of hosts that provide unsuitable diets almost always starve in the nest or die shortly after fledging (Yang *et al.,* 2013; Grim *et al.,* 2017). To my knowledge, the lesser honeyguide is the only brood parasite to consistently and successfully parasitize a host that provides, at least in part, an unsuitable diet. We might speculate that this is made possible by a combination of its relatively small body size and its ability to elicit high levels of provisioning through vocal mimicry.

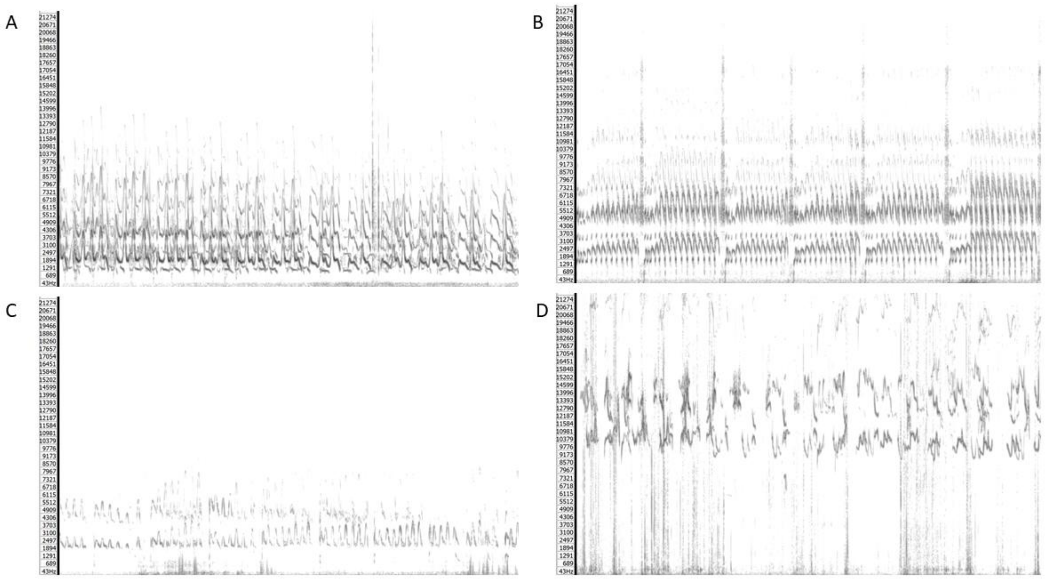
In contrast to lesser honeyguides, greater honeyguides appeared to be able to utilize the extra provisioning that they elicited from bee-eaters, since they grew faster and to larger fledging masses than bee-eater chicks. Greater honeyguides therefore appear to elicit extra provisioning in order to acquire adequate nutrition for their faster and sustained growth to a considerably larger size than bee-eaters. The ability of a single greater honeyguide chick to elicit high rates of provisioning may have been a key adaptation in allowing the parasite to utilize hosts that are smaller than themselves, increasing the range of potential hosts available to them, and analogous to the situation with common cuckoos and their hosts (Kilner *et al.,* 1999). We might reason that the greater honeyguide could not parasitize a host with an ill-suited diet with the same success as the lesser honeyguide achieves, as its large body size could require unfeasibly large quantities of unsuitable food.

6.5.3 Conclusion

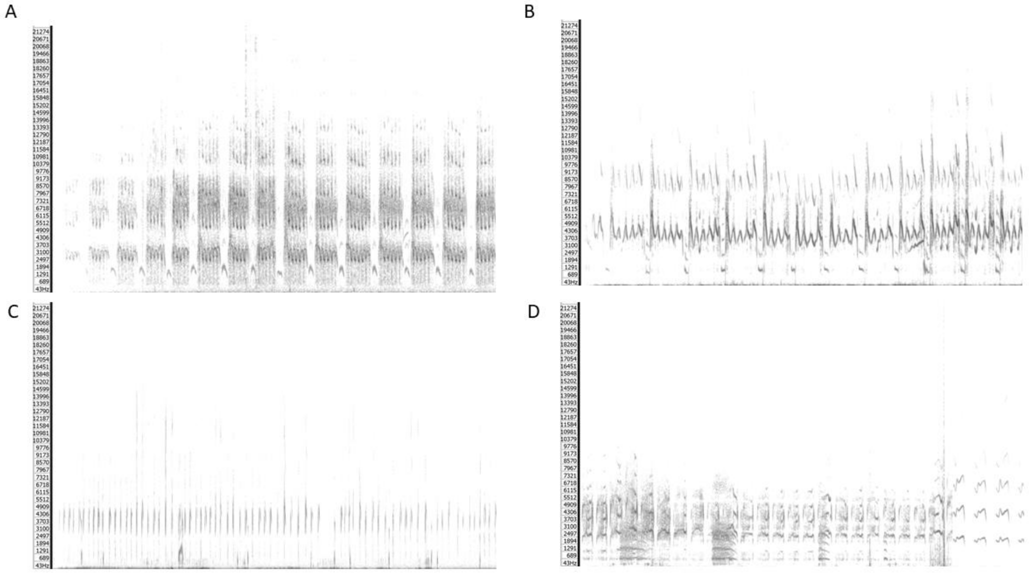
Begging call similarity has been reported in six of seven avian brood parasite transitions (Jamie and Kilner 2017), with the exception of the precocial black-headed duck *Heteronetta atricipilla* which does not need to solicit food from its foster parents (Lyon and Eadie 2013). This study adds to the body of evidence showing that mimicry of host chick begging calls is a convergent strategy employed by nest-mate-evicting parasites. Both honeyguide species in this study share vocal mimicry of their hosts in their arsenal of parasitic adaptions, but this adaptation appears to have evolved due to different selection pressures. The use of vocal mimicry to elicit additional provisioning in greater honeyguides mirrors earlier studies of other brood parasites (Kilner *et al.,* 1999) and reflects the larger size of the brood parasite compared to their host. Lesser honeyguides, which are smaller than their hosts, appear to have evolved to elicit extra provisioning to exploit a host that provides a sub-optimal diet. Further to this study, knowledge of host responses to the begging calls will inform our understanding of the evolution of parental care to brood parasites, and the outcomes of coevolutionary interactions between parasites and hosts at the nestling stage. The next stage of research in honeyguides might therefore look at which structural features of the chick begging call are used by hosts in determining provisioning rate.

6.6 Supplementary material

6.6.1 Spectrograms of honeyguide and host begging calls



S1. Spectrograms of the begging displays over five seconds of **A)** single lesser honeyguide chick, **B)** brood of barbet chicks, **C)** single barbet chick, **D)** brood of starling chicks. The Y axis indicates the frequency (Hz). The honeyguide chick display appears to most closely resemble a brood of barbet chicks, and is most dissimilar to that of a brood of starling chicks.



S2. Spectrograms of the begging displays over five seconds of **A)** single greater honeyguide chick, **B)** brood of bee-eater chicks, **C)** single bee-eater chick, **D)** brood of kingfisher chicks. The Y axis indicates the frequency (Hz). The honeyguide chick display appears to most closely resemble a brood of barbet chicks, and is most dissimilar to that of a brood of starling chicks.

6.6.2 Video of lesser honeyguide chick regurgitating fruit material

Supplementary video 2. Lesser honeyguide chicks were often found to regurgitate large seeds after being provisioned by their barbet foster parents, as can be seen in this footage. While a barbet chick was observed to regurgitate seeds once, this behaviour was much more common in lesser honeyguides. This lends support to the idea that lesser honeyguides are selective in the food they accept from barbets, and likely explains why barbet faeces often contained large seeds, whereas I never found such large seeds in the faecal samples of honeyguides.

CHAPTER SEVEN

Discussion and Synthesis

7.1 Overview

It has been known since Darwin that co-evolutionary interactions such as mutualism, competition, predation and parasitism shape the diversity of behaviours and forms found in nature (1859). Coevolutionary pressures are especially likely to lead to reciprocal adaptations when the actions of both parties in the interaction are antagonistic. Avian brood parasites and their hosts fit these credentials: if parasites do not effectively parasitize their host they do not pass on their genes, whereas if hosts do not defend themselves from parasitism, they stand to lose all progeny. Therefore, there are strong selective pressures on both the parasite and the host, making avian brood parasitism an ideal system to test hypotheses concerning coevolution (Rothstein 1990). These antagonistic interactions promote both the exploiters and their victims to evolve greater phenotypic diversity and behaviour (Davies 2000). Adaptations that arise due to such interactions can have profound implications for the physiology (Krüger and Davies 2004; Garamszegi and Avilés 2005; Birkhead *et al.,* 2011), behaviours (Welbergen and Davies 2009; Feeney *et al.,* 2013), and life histories (Brown and Lawes 2007; Canestrari *et al.,* 2009) of brood parasites and their hosts. This thesis supports this central message, displaying how interactions between honeyguides and their hosts have led to a suite of interaction-specific adaptations. Specifically, this thesis establishes, as demonstrated in the different chapters, that these adaptations and counter-adaptations have occurred at all stages of the breeding process: host nests that prevent honeyguide access, rapid laying of parasitic eggs by honeyguides to avoid detection, recognition and rejection of parasitic eggs by hosts, and manipulation of host feeding behaviours by parasitic chicks to increase growth. In addition to describing novel defenses and countermeasures, I identified general evolutionary patterns that could apply outside the context of avian brood parasitism. I first summarize my findings of each experiment performed in this thesis. I will then address the broader significance of these results. Finally, I will speculate on future research questions that could be pursued.

7.2 Review of key findings

Following on from my introduction, I began in chapter two by establishing if host nest structure can be used as a frontline defence against brood parasitism. I found that bee-eaters that had narrow entrances to their nest tunnels were less likely to be parasitized by honeyguides than those with wide tunnel entrances. I was able to replicate the results of this observation through experimental manipulation of bee-eater nests, implying that the nest tunnel entrance of little bee-eaters can act as a frontline defence against parasitism by greater honeyguides. I next asked why all bee-eaters do not build narrow tunnels, since it provides such an obvious benefit against parasitism. However, I was unable to explain the variation in bee-eater tunnel size, as it was neither related to the size of the bee-eater that excavated the tunnel, nor the properties of the soil in which the nest was built. An untested hypothesis for why there is such variation in bee-eater tunnel entrance size is that tunnel size could be related to the experience of the builder. Evidence of this would further strengthen the idea that more experienced hosts are better at defending themselves from brood parasites (Hauber *et al.,* 2006; Moskát *et al.,* 2014). I found evidence consistent with the idea that bee-eater nest architecture is selecting for smaller-bodied greater honeyguides, as small honeyguide eggs were found in nests with narrow tunnels, and large honeyguide eggs only in nests with wide tunnels. This study provides the first experimental evidence that host nest structure can act as a defence against a brood parasite, contributing a novel frontline defence in avian brood parasitism.

In chapter three, I examined the variation in egg-laying speeds of brood parasites. My aim was to investigate the evolutionary drivers of rapid egg-laying in brood parasites by asking what the trade-offs involved with laying eggs rapidly in host nests are, and if there are constraints that impede rapid egg-laying. I highlighted two major constraints on rapid egg-laying in parasites: (i) the host nest type and (ii) the size of the parasitic egg being laid. The first constraint suggests that hosts that nest in enclosed, dome/cavity nests are more difficult to access, requiring more time to be successfully parasitized. The second finding suggests that larger eggs can be laid more rapidly, perhaps because of the reduced risk of damage during laying that a small (thin shelled) egg might suffer if rapidly laid. Despite testing multiple hypotheses concerning whether rapid egg-laying is related to the potential costs incurred during parasitism events, such as mobbing, or differences in body size between parasite and host, I found none were strongly supported. I found little evidence that any single cost incurred during laying in a host nest has promoted rapid egg-laying. Nonetheless, taken together these costs might have influenced the evolution of rapid egg-laying from a slow non-parasitic laying ancestral state, with ecological constraints preventing all parasites from having very fast egg-laying speeds.

In chapter four, I assessed whether parasitic virulence can be moderated in response to the associated costs. I established that bee-eaters reject punctured clutches at a higher rate than unpunctured clutches. I found that in natural (but not experimental) settings, bee-eaters were more likely to reject parasitized clutches containing punctured eggs if they were at the start of incubation. This could suggest that bee-eaters optimize their rejection strategy by making decisions based on the perceived reproductive value of their clutch. I also found that punctured nests were more likely to be predated. These findings show that virulent puncturing has costs to the honeyguide, but this study is the first evidence of moderation of virulence by brood parasites in response to costs. Honeyguides adjust their puncturing behaviour in relation to host clutch size and incubation stage, supporting the hypothesis that honeyguides moderate their virulence in accordance with trade-offs.

In chapter five, I investigated whether the size of a parasitic egg can be used as a cue for a host to recognize parasitism. Barbets appear to use true recognition (that is, relying on an internal template of egg size to recognise parasitic eggs) to identify that their clutch has been parasitized, rather than a discordancy-based rule (that is, directly comparing the parasitic egg to their own eggs). Black-collared barbets were more likely to reject small honeyguide and experimental eggs, regardless of the size of the other eggs within the nest. This suggests that the difference between a parasitic egg and their own eggs was not used to detect parasitism, but rather the absolute size of the egg was. These results add to the growing body of evidence that suggests that template-based, rather than discordancy-based recognition, is used by hosts to recognise parasitic eggs (Lotem *et al.,* 1995; Lyon 2007; Stevens *et al.,* 2013). However, true recognition using the size of an egg has, to my knowledge, not yet been identified in any other brood parasitic host — but was found in this study in a system where it would be expected to — when the parasitic egg is smaller than those of the host. This highlights the importance of studying interactions between species with a range of different life-histories (Thorogood *et al.,* 2019)

In chapter six, my objective was to establish whether honeyguides elicit provisioning from their foster parents using vocal mimicry, and to determine why such extra food would be required. Using playbacks of host and parasite vocal displays, my experiments show that the begging calls of the parasitic chicks are integral in their ability to elicit provisioning from host parents. The results suggest that honeyguide begging calls mimic the sound of a whole brood of their host chicks, and thus stimulate the host parent to provision at a high rate. Hand-rearing experiments of *I. minor* chicks suggested that they are ill-adapted to the diet of their hosts, requiring extra provisioning to offset inadequate digestion of fruit material. By contrast, *I. indicator* chicks required higher rates of provisioning to fuel their rapid growth and large size relative to their hosts, comparable to other brood parasitic systems (Davies *et al.,* 1998). Despite these divergent selection pressures, both species of honeyguide converged upon the same adaptation of chick mimicry, a common phenomenon in avian brood-parasitic lifestyles (Jamie and Kilner 2017).

7.3 Methodological considerations

The research conducted in this thesis builds upon the legacy of Major John Colebrook-Robjent. The field site that he established has furthered our knowledge of coevolution between parasites and their hosts (Jamie 2017; Spottiswoode 2013; Spottiswoode and Colebrook-Robjent 2007; Spottiswoode and Koorevaar 2012; Spottiswoode and Stevens 2010; Spottiswoode *et al.,* 2011; Stevens *et al.,* 2013) , in addition to broader avian topics such as camouflage (Wilson-Aggarwal *et al.,* 2016; Stevens *et al.,* 2017; Troscianko *et al.,* 2017) and migration (Sorensen *et al.,* 2016b; Sorensen *et al.,* 2016a). The skills he instilled are still present in the local community today, and without this expertise, the experiments conducted in this study would not be possible. Thanks to the experience of my Zambian friends and colleagues, I was able to repeatedly and safely access nest cavities in order to conduct experiments. Furthermore, many of the hypotheses tested here originated from discussions on the observations of people living on the site. I further expanded on their methods, using new technologies to advance our understanding of the brood-parasitic systems that live here. In order to monitor behaviour and provisioning rates at otherwise unobservable cavity nests, I designed and built small, unobtrusive infrared cameras that could be useful in other systems where breeding is hard to observe. In addition, I used Bluetooth speakers to remotely perform playback experiments, reducing the overall impact to the birds I studied, promoting natural behaviours in an experimental setting. I was also able to successfully manipulate the structure of an avian nest in the wild, a feat that to my knowledge has not yet been achieved and could be useful in tests of a wider range of hypotheses. Long term studies have been invaluable in providing insight into animal social systems (Caro 1996), behaviours (Alberts 2019), and ecology (Lindenmayer *et al.,* 2012), and the development of this site promises similar outcomes in the studies of brood parasites.

7.4 Significance of this thesis

7.4.1 Coevolution in the tropics

The relatively warm, aseasonal climates typical of tropical have been present throughout most of Earth's history, maintaining the relationships between the species that live there (Brown 2014). The resulting selection pressures have had notable effects on avian behaviour and ecology: tropical birds have smaller clutches (Cardillo 2002), slower nestling growth-rates (Ricklefs 1968), longer periods of dependence on their parents (Russell *et al.,* 2004), and live longer (Wiersma *et al.,* 2007) than birds living in more temperate climates. This has important implications for the evolution of host defence and parasitic countermeasures. Research on tropical brood parasites has lagged behind the classical brood parasitic systems of the more temperate regions, but a recent burst of studies is rapidly reducing this discrepancy. For example, Gloag *et al.* (2014) presented a novel mechanism by which a brood parasite can reduce detection of its eggs in a host nest, by laying a pigment-coated cryptic egg. Langmore *et al.* (2003) discovered that superb fairy wrens *Malurus cyaneus* can recognize and reject Horsfield's bronze cuckoo *Chalcites basilis* chicks from their nests, while De Mársico et al. (2012) present evidence that arms races can proceed beyond the nest. Each of these studies highlight novel defences that not only broaden our understanding of the interactions between parasites and hosts, but generate further questions regarding the routes that coevolution may proceed along. In this thesis I provide further evidence for the value of studies of tropical brood parasites, identifying three adaptations undescribed in previously studied systems: (i) host nest architecture as a defence against brood parasites, (ii) use of parasitic virulence as a cue for egg-rejection, (iii) evolution of an egg-recognition template based on the size of the parasitic egg. Evolution in the tropics is thought to proceed at an accelerated rate and therefore studies of interactions among tropical species have the potential to reveal especially complex adaptations (Dobzhansky 1950; Schemske *et al.,* 2009). In support of this hypothesis, I found that the life of honeyguides and their hosts are completely entwined, illustrating adaptations before parasitism (rapid egg laying and nest architecture), at the egg-stage (egg rejection by hosts), and in the nestling stage (vocal mimicry of host chick begging calls).

7.4.2 Importance of life-histories in coevolutionary interactions

Detailed understanding of both host and parasite life-history traits is required in order to interpret and predict how the arms races between species proceeds. This also applies in reverse: selection pressures can promote shifts in the behaviour, physiology and ecology of hosts and parasites. Thus, a grasp of the both the interactions between species and the lifestyles of the species within this interaction can help explain the natural world.

In chapter two, I found that nest structure can act as an antiparasitic defence in a cavity-nesting host. Not only does this finding highlight the utility of studying systems with distinct breeding strategies, it also suggests that brood parasitism could act as a selection pressure on the structure of host nests. Nest structure is under strong selection from the pressures of predators (Mainwaring *et al.,* 2014; Collias 1997), temperature (Windsor *et al.,* 2013; Heenan 2013) and radiation (Englert Duursma *et al.,* 2018). Although there is evidence that hosts may assess parasitism risk and adjust where they build their nest (Øien *et al.,* 1996; Moskát and Honza 2000), so far the effect of brood parasitism on the structure of nests has been untested. Building parasite-proof nests has the potential to be a highly effective strategy for hosts, and could reduce the costs of parasitism to zero (Feeney *et al.,* 2012). It is also a strategy that need not apply only to birds. For example, although nest structure could not act as a host defence in the cuckoo-catfish that parasitizes mouth-brooding cichlids (Blažek *et al.,* 2018), it could be used in nest-building insects who are hosts to brood parasitic Hymenoptera (Litman 2019). I suggest that defensive nest structures are most likely to evolve in hosts that build enclosed nests, are smaller than their parasite and can control the structure of their nest. If these requirements are fulfilled, then nest structure could act as defence mechanism against brood parasites in both avian and insect systems. The weaverbirds (Ploceidae) of Africa fulfil these prerequisites, and observations and experimental manipulations at weaverbird nests would likely provide further evidence of defence via nest-structure (Freeman 1988; Davies 2000). Although secondary cavity nesting hosts such as the prothonotary warbler *Prototaria citrea,* and the common redstart *Phoenicurus phoenicurus* do not often build their own nests, they could take advantage of nest structure as a frontline defence by preferentially utilizing nest-boxes or cavity nests with smaller nest entrances that reduce access of the relatively larger birds that parasitize them.

Studies of host egg rejection have noted that some hosts choose to eject only the parasitic egg while others choose to abandon the entire clutch (Šulc *et al.,* 2019). The reasons for this variance remains puzzling (Servedio and Hauber 2006), with suggestions that predation risk (Gloag *et al.,* 2013; Arcese *et al.,* 1996), or confidence in the identification of parasitism (Brooke and Davies 1988) promote one strategy over the other. Both barbets and bee-eaters in this study rejected honeyguide parasitism by abandonment of their nest, or by fully ejecting the whole clutch (chapters four and five). This is costly to the host, forcing them to search for a new site, excavate the nest, and produce a new clutch. However, host renewal of breeding attempts once parasitized is more likely to be favoured in the tropics, as breeding periods are longer, so hosts that desert still have enough time to re-nest. Such a strategy is less likely to evolve in temperate hosts where breeding seasons are short (Baker 1939; Morais *et al.,* 2019).

7.4.3 Constraints on brood parasite host choice

Brood parasitism is absent in many potential hosts (Grim *et al.,* 2011). Host quality is invoked as crucial for parasite success and a main constraint on host choice (Stokke *et al.,* 2018; Davies and Brooke 1989; Grim *et al.,* 2017). The two most common explanations for why brood-parasites do not utilize certain hosts invoke (i) an unsuitable diet provided by the host (Grim *et al.,* 2017), and (ii) the presence of inaccessible nests (Davies 2000; Rutila *et* *al.,* 2002; Thomson *et al.,* 2016). In this study, I highlighted mechanisms in which brood parasites could evolve to circumnavigate these ecological restrictions.

The results of chapter six show that lesser honeyguides are able to exploit a seemingly ill-suited host, extorting greater provisioning from black-collared barbet parents in order to offset the partially frugivorous diet they are provided with. This is in contrast to other well-studied systems such as brown headed cowbirds *Molothrus ater* (Middleton 1977) and common cuckoos *Cuculus canorus* (Glue and Morgan 1972), where provisioning of predominantly plant material (an ill-suited diet for these species) leads to the death of the parasitic chick. The host diet likely is restrictive in these species, and not likely to be overcome, as cowbirds and common cuckoos are larger than their hosts and therefore would require excessive levels of provisioning that could not be feasibly supplied by hosts. Great-spotted cuckoos *Clamator glandarius* are fed different prey items by their magpie *Pica pica* host, perhaps acting as a form of nestling discrimination (Soler 2008). Despite being smaller than host chicks, great-spotted cuckoos are able to elicit the lions share of provisioning from their magpie hosts, diverting food away from foster siblings (Soler *et al.,* 1995b), perhaps highlighting an evolutionary response to the lower-quality food items provided to them by their foster parents.

Another constraint to parasite host choice commonly cited is the type of nest that the host constructs. The common cuckoo exploits only one cavity nesting host (Rutila *et al.,* 2002; Thomson *et al.,* 2016), where it is prevented from accessing the nest directly and suffers major costs to its fitness when attempting to drop the egg into the nest cup. Cuckoos form distinct host races or gentes (Gibbs *et al.,* 2000), and if the cuckoo parasitizing this cavity nesting host maintained host fidelity over a prolonged evolutionary time period, we might expect the cuckoo to evolve to be physically smaller, as appears to have occurred in the greater honeyguide. Honeyguides have been coevolving with their hosts for many magnitudes longer than cuckoos have with their hosts. A consequence is that in honeyguides, the matriline for terrestrial cavity nesters is distinct from the race that nests in larger arboreal cavities, with associated differences in body size (Spottiswoode *et al.,* 2011). Furthermore, the honeyguides likely evolved from a cavity-nesting ancestor, promoting the evolution of adaptations in utilizing cavity nesting hosts. Interestingly, the *Chalcites* cuckoos of Africa and Australia also parasitize enclosed nesting hosts, which could have led to their relatively smaller body size in order to grant access to their hosts nests (Krüger and Davies 2004).

7.5 Future experiments

Coevolution between species can vary across geographical space (Thompson 2005). Parasitism rates (Soler *et al.,* 2011), host use (Campobello and Sealy 2009) and occurrence of mobbing or egg-rejection can differ between populations of the same host species (Welbergen and Davies 2009; Lindholm and Thomas 2000). The genetic and phenotypic variation among populations of brood-parasites and their hosts can encourage the evolution of specific gentes (Spottiswoode *et al.,* 2011; Starling *et al.,* 2006), with parasite interactions occurring in geographic isolation promoting different evolutionary trajectories. Greater honeyguides that parasitize larger, tree-cavity-nesting hosts lay larger eggs and are larger in body size compared to smaller races of honeyguides which target burrow-nesting bee-eaters (Spottiswoode *et al.,* 2011). Whether this difference in body size between honeyguide lineages is the result of phenotypic plasticity, with larger hosts providing more resources, and therefore fledging larger parasitic chicks, or is genetically controlled, remains unknown. Cross-fostering experiments moving honeyguide chicks from nests of the smaller terrestrial hosts (such as the little bee-eater) to those of the larger tree-nesting hosts (such as the green woodhoopoe) could discern between these two hypotheses.

In recent years, reliable phylogenetic methods and robust phylogenies have enabled the use of comparative analyses to investigate research questions at the macroevolutionary level (Thorogood *et al.,* 2019). In the context of brood parasitism, comparative analyses have proven fruitful by identifying general patterns in the evolution of egg-rejection (Medina and Langmore 2015) and parasitic egg physiology (McClelland *et al.,* 2019). Chapter three in this thesis further confirms that comparative approaches can be useful in identifying broader patterns of brood parasite evolution. Future studies could take advantage of these methods to reveal patterns in the physiology (Garland *et al.,* 2005), speciation (Kesäniemi *et al.,* 2012) and breeding systems (Weller and Sakai 2003) of brood parasites.

Many brood parasites are virulent, inflicting costs to host fecundity (Spottiswoode and Colebrook-Robjent 2007; Spottiswoode and Koorevaar 2012; Honza *et al.,* 2007; Massoni and Reboreda 1999). In chapter four, I showed that brood-parasitic virulence can have costs to the parasite, and this can subsequently generate variation in virulence. Although virulence has been demonstrated to be costly to the parasite in other systems (Kilner *et al.,* 2004), further examples of such moderation of virulence have yet to be identified. Variation of virulence in response to trade-offs is well reported in disease causing pathogens (Griffin *et al.,* 2004; de Roode *et al.,* 2005; de Roode *et al.,* 2008), and adopting the frameworks already established in these areas of research to the studies of brood parasites promises to provide exciting results. For example, many species of cowbirds *Molothrus sp.* also puncture host eggs during parasitism (Gloag *et al.,* 2013; Massoni and Reboreda 1999), and here too an evolutionarily stable state in which the trade-offs of puncturing to both host and parasite would be expected to be maintained in an equilibrium (Smith 1982).

The results of playback experiments in chapter six lend further support to the idea that vocal mimicry is a common adaptation in brood parasites to exploit their hosts at the chick stage (Jamie and Kilner 2017). However, as of yet no study has identified what the defensive countermeasure in hosts might be. When parasites become good mimics of egg characteristics, evolution can favour reduced intra-clutch variation of egg phenotype, while increasing the inter-clutch variation becomes higher, thus making it more difficult for a parasite to match a wide range of host phenotypes within a given population (Øien *et al.,* 1995; Soler and Pape Møller 1996). I propose that this could also apply in the context of nestling begging calls. A reduction in intra-brood, but increase in inter-brood variation of host chick begging displays could aid nestling discrimination by hosts. The superb fairy wren, who ejects cuckoo chicks who sound different from their own chicks (Langmore *et al.,* 2012), would make an excellent system in which to test this prediction. Another potential evolutionary response to parasitic mimicry involves a change in the breeding strategy of the host. Evolving a reduced brood size could reduce the effectiveness of brood mimicry by brood parasites, as the innate response to increase provisioning to the sound of multiple chicks within the nest would be curtailed. Brood parasitism can lead to increased or decreased clutch size (Soler *et al.,* 1995a; Brooker and Brooker 1996), in other contexts, as those hosts with smaller clutches lose less of their offspring when parasitized compared to those host with large clutches (Hauber 2003), and thus illustrates this potential response.

7.7 Final statement

My observations of natural history blended with experiments revealed novel adaptations not only in honeyguides and their hosts, but for all brood parasites. The information within this thesis underlines the importance of evolutionary biology studies of lesser-known systems. I hope that the concepts presented in this thesis stimulate original ideas and encourage further research into the interactions between brood parasites and their hosts.

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