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„Investigating the behavior and larval development of the invasive parasitic fly, *Philornis downsi*, in different host nests in the Galapagos Islands“

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**Dedicated to my loving parents,
Gene (1928-2011) &
Glenda Pike**

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It is not the most intellectual of the species that survives; it is not the strongest that survives; but the species that survives is the one that is able best to adapt and adjust to the changing environment in which it finds itself.

~Charles Darwin

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General Introduction

Introduced species & parasitism

Introduced and invasive species have become a topic of global concern as they may affect their new environment in various ways (Elton, 1958; Baur and Schmidlin, 2007; Nentwig, 2007). Establishment and spread of introduced species, including parasites, may be influenced by numerous factors such as habitat and climatic suitability, availability of resources, interactions with native species, and suitable host species (Williamson and Fitter, 1996; Sakai et al., 2001). For many introduced parasite – host systems, there is limited information on which factors influenced successful introduction and invasion, how the parasite is adapting in the novel environment and its impacts on native species. How an introduced parasite interacts with native or endemic species can also influence its invasion success (Kondoh, 2006). Therefore, its invasion success may be highly dependent/influenced by the co-evolutionary processes occurring between the parasite and its novel hosts (Feis et al., 2016). Thus, it is important to understand the biological processes that aid in successful invasion and how native species are adapting. Furthermore, more detailed knowledge of the general biology and behavior of an invasive parasite and its current and prospective hosts in its new environment are vital for developing successful management and control measures.

Behavior of a newly introduced species can greatly influence the ability of it to establish and become invasive (Weis, 2010). Some introduced parasites, including parasitic insects, may arrive with ‘preadaptations’, such as higher behavioral flexibility that aids in invading novel environments and adapting to multiple host species (Sol, 2007; Khokhlova et al., 2022). However, a parasite may also adapt its behavior to parasitize a novel host and/or utilize the host’s resources (Harkonen and Kaitala, 2015). To parasitize multiple novel host species and respond to hosts’ behavioral adaptations, introduced parasites may develop generalized adaptations, such as changes in behavior for foraging and avoidance of predation, (Härkönen & Kaitala, 2015), demonstrating behavioral plasticity or flexibility (Sol, 2007; Weis, 2010).

Hosts may also fall under higher selection pressure as invasiveness of an introduced parasite increases. When this occurs, hosts may respond to this parasitism with counterstrategies, including behavioral and defensive adaptations such as grooming, nest sanitation, food compensation, resistance (via an immune response), and tolerance (Mason, 1944; Wilson and Cotter, 2013). As the parasite changes and adapts to a host, the host must also adapt. As a driving force of selection, those that cannot or do not adapt appropriately

may have reduced fitness or may not survive themselves. Thus, in order to determine the threat level a new parasite imposes on its new hosts, it is imperative to determine whether hosts can adapt to the new parasite and how.

Study system

Focal species

The Avian Vampire Fly, *Philornis downsi*, is a Dipteran fly with a parasitic larval stage that is native to regions in South America and the Caribbean (as reviewed in Pike, 2015), where it parasitizes over 20 avian species (McNew & Clayton, 2018). In the Galapagos Islands, this fly has been present in the islands since at least 1960's (Causton et al., 2006), where it is now considered invasive. It is currently present on at least 13 islands in the archipelago (Causton et al., 2013), in both humid and arid habitats. Many avian species in the Galapagos are afflicted by this parasitism, including 20+ landbird hosts (Fessler et al., 2018; Anchundia and Fessler, 2020; Coloma et al., 2020). Many of these hosts are novel for the Avian Vampire Fly, as they are endemic to the Galapagos Islands. Since the first report of this parasite in birds' nests in 1997 (Fessler et al., 2001), extensive studies have been conducted on various aspects of its life history and control (Causton et al., 2013; Fessler et al., 2018; McNew & Clayton, 2018; Common et al., 2020).

Life cycle

Adult avian vampire flies are non-parasitic, with their diet generally consisting of fruits and decaying matter (Couri, 1999). Its larvae are obligatory parasites, requiring avian hosts as food sources (Couri, 1985; Fessler et al., 2001). Female flies seek out active birds' nests to lay their eggs. Once the flies' eggs in the nests hatch, the first instar larvae mainly live in the nasal cavities and ear canals of their hosts (nestlings). The second and third instar larval stages stay in the nest base during the day and exhibit nocturnal feeding behavior, with larvae feeding off blood and tissue from their hosts (Fessler et al., 2006b). Larvae develop a cocoon and pupate in the nest base material for ~10 days until they emerge as adult flies (Fessler et al., 2006b; Lahutte et al., 2016). The parasitic behavior of the larvae causes body wounds, contusions, deformities and reduced haemoglobin levels, among other effects on its hosts (Dudaniec et al., 2006; Fessler et al., 2006a, 2006b; Knutie et al., 2016). Moreover, Avian Vampire Fly parasitism may also take a toll on future reproductive success of parents (McNew et al., 2020) and there may also be long-lasting effects from larval parasitism for nestlings that do survive, such as beak deformations that can affect foraging behavior and mating signals (Fessler et al., 2006b; Kleindorfer et al., 2019, 2022).

Adult behavior

Female flies seek out and detect active birds' nests, where the flies lay their fertilized eggs, generally in the nest material. There is limited information about how the Avian Vampire Fly locates its hosts' nests; however, detection may be via auditory, visual, and/or olfactory cues, among others. Adult avian vampire flies have been reported to be attracted to yeast volatiles, however, this attraction may be related to food source seeking behavior, and not host nest seeking behavior for oviposition (Cha et al., 2016). Additionally, a prior study investigating nest odors and scent of whole flies as attractants yielded no evidence of adult fly attraction (Doherty, 2012). Reports of Avian Vampire Fly parasitism in nests built in artificial nest structures suggests female flies do not require visual cues of nests for locating them (Bulgarella et al., 2015; Lincango et al., 2015). However, flies may still rely on visual bird behavioral cues for locating nests. Female flies may also use other cues to find host nests for egg laying, but further research into how they detect these resources is needed.

A shift in oviposition behavior of the Avian Vampire Fly in the Galapagos has been recently documented. Until 2012, Avian Vampire Fly larvae were rarely detected in abandoned nests with eggs, with most parasites found in nests with nestlings (Common et al. 2020). Over the past decade, however, female flies have been ovipositing earlier in the bird breeding cycle, with reports of a higher prevalence of parasitized nests in the early nestling phase and incubation phase (Kleindorfer et al., 2014; reviewed in Common et al., 2020; Cimadom and Tebbich, 2021). This also suggests the Avian Vampire Fly is shifting its developmental life history to include brooding females as hosts. This change in female oviposition behavior may be driven by resource competition, given host nests are a limited resource. Female avian vampire flies need to ensure their larvae will have sufficient hosts available as a food source to ensure proper development before nestlings fledge (Kleindorfer et al., 2014). Thus, this timing may influence competition between females and drive earlier oviposition (Kleindorfer et al., 2014).

Host choice/ quality

Varying parasitism levels and earlier oviposition in host nests may indicate host preferences by the Avian Vampire Fly. Additionally, host quality may be influenced by the vulnerability of hosts to Avian Vampire Fly parasitism, which varies between host species in the Galapagos Islands. In mainland Ecuador, a native range of the Avian Vampire Fly, one host species had much higher parasitism levels than other host species studied, suggesting a host preference by egg-depositing females (Ramirez et al., 2022). In its invasive range, Avian

Vampire Fly parasitism intensity per nest ranges from zero to 182 parasites per nest and mean intensity varies between host species (Fessler and Tebbich, 2002; Cimadam et al., 2014; Fessler et al., 2018; McNew and Clayton, 2018; Common et al., 2020). Furthermore, a range of nestling mortality levels influenced by Avian Vampire Fly parasitism has been reported in prior studies, with up to 100% brood loss (reviewed in Fessler et al., 2018; Cimadam et al., 2019; Mosquera et al., 2022). The size of the host may play a role in vulnerability to parasitism, as high fledging success (60-80%) for parasitized Galapagos Mockingbird (*Mimus parvulus*) and Vegetarian Finch (*Platyspiza crassirostris*) nests suggest these larger-bodied species can tolerate Avian Vampire Fly parasitism (Knutie et al., 2016; Heimpel et al., 2017). However, most smaller-bodied Darwin finch species and flycatchers in Galapagos suffer from lower breeding success attributed to Avian Vampire Fly parasitism (reviewed in McNew and Clayton, 2018; Cimadam et al., 2019; Mosquera et al., 2022). The critically endangered Mangrove Finch, for example, is vulnerable to extinction, in part from the effects of Avian Vampire Fly parasitism (Fessler et al., 2010). Additionally, recent prior studies on sympatric Darwin finches have found significantly lower breeding success for parasitized Small Tree Finch compared to the Green Warbler Finch and suggest other environmental factors and host adaptations may play a role in host vulnerability to Avian Vampire Fly parasitism (Cimadam et al., 2014, 2019; Cimadam and Tebbich, 2021). The quality of a host species may also influence larval developmental times, as suggested by Cimadam and Tebbich (2021). The low breeding success for some endemic species in the Galapagos is concerning and conservation initiatives may focus on species of concern first. Thus, it is important to determine both the adaptive capacity of these species and how they are currently reacting to Avian Vampire Fly parasitism.

Host adaptations

Furthermore, hosts' adaptations, thus far, are not well understood for the Avian Vampire Fly and many of its novel hosts in Galapagos. From the host perspective, video recordings at host nests provided insights into host behavior to avoid parasitism, recording nestlings with larval parasitism preening and repositioning and females preening parasitized nestlings (O'Connor et al., 2010). Host tolerance and resistance studies in Galapagos have found limited evidence of host adaptation to Avian Vampire Fly parasitism via parental compensation (O'Connor et al., 2014; Knutie et al., 2016; McNew et al., 2019; Heyer et al., 2021). Furthermore, Medium Ground Finch females produced antibodies against the larvae (Huber et al., 2010; Koop et al., 2013), indicating adult females can mount an immune

response to Avian Vampire Fly parasitism. However, no immune response to Avian Vampire Fly parasitism was detected in nestlings of the Galapagos Mockingbird nor Medium Ground Finch (Knutie et al., 2016; Koop et al., 2013), which suggests nestlings may not be resistant. However, these studies are species-specific and further studies are necessary to determine how other host species are responding to this parasitism.

Knowledge gaps

Despite extensive studies over the last two decades, there are still substantial gaps in the understanding of the basic ecology of the fly, such as how it locates host nests, behavior of flies at host nests, mating behaviors and the location of mating. Key information on the factors that influence host preference, host quality, and larval development in hosts' nests is still missing. Additionally, while there have been a few studies on host response to this parasitism in Galapagos, information is still lacking for specific species. Further studies on fly behavior at host nests and its interactions with hosts can build on studies from O'Connor et al. (2010) and Lincango et al. (2015), which reported the first insights into Avian Vampire Fly and host behavior at host nests. This knowledge, along with a better insight into Avian Vampire Fly mating behavior, is pertinent to planning further management efforts and planning control initiatives (Causton et al., 2013). Information on fly activity at host nests may inform fly trapping methods, while a better understanding of mating conditions and host quality may aid in improving laboratory conditions for achieving mass rearing for long-term control initiatives. Furthermore, new insights into host adaptations would provide essential information that can be utilized in strategic conservation efforts for endemic hosts, including the critically endangered Mangrove Finch (Causton et al., 2013).

Aims of this PhD thesis

In my PhD thesis, I aim to close knowledge gaps in basic ecology of the Avian Vampire Fly, host quality, and host response in the Galapagos Islands, its non-native range. More specifically, my thesis focuses on elucidating fly behaviors and interactions between the fly and host at host nests and determining whether flies use host nests as a mating resource. I also aimed to provide a better understanding on host response to Avian Vampire Fly parasitism and examine host characteristics that may influence the quality of the host for larval development.

Therefore, the main questions I aimed to answer included:

I. What are the temporal patterns of the Avian Vampire Fly infestation at nests of a cavity-nesting host species?

II. Do potential factors influencing larval growth of the Avian Vampire Fly differ between host species in field conditions?

III. Does the Green Warbler Finch behaviorally adapt to higher Avian Vampire Fly larval parasitism via parental compensation?

To answer the questions above, I conducted projects in varying settings, including filming, observations, and nest manipulation in the field and stable isotope and amino acid analyses.

In **Chapter 1**, I aimed to determine the temporal patterns of Avian Vampire Fly infestation at nests of a cavity-nesting host species. I monitored nests of the Galapagos flycatcher for Avian Vampire Fly visitation and behavior in the Galapagos Islands. The Galapagos Flycatcher was chosen as an initial focal species for methodological reasons. Nests were filmed with both external and internal cameras, recording over 1,400 hours of video. I aimed to determine the timing of infestation throughout the day and test for differences in visitation rates throughout the breeding cycle (in incubation and feeding phases). Additionally, I hoped to identify whether bird visitation rates affect fly visitation rates. I predicted that there would be a higher rate of fly visitation in the feeding phase than incubation and I predicted that lower bird visitation rates would be correlated with higher rates of fly visitation. Furthermore, I was interested to determine whether mating took place (after emergence) at host nests. Using these nest videos to record fly and bird activity, I gained insightful information into behavior of the Avian Vampire Fly and one of its host species. I determined peak hours of fly visitation to nests, correlation of flies entering the nest and bird absence and recorded evidence of flies mating outside the nest cavity. I discuss the results in terms of host seeking behavioural adaptations, predator avoidance, and implications of our findings for future rearing and control.

In **Chapter 2**, I examined factors that may influence larval growth and their variations between hosts. More specifically, I investigated whether there were variations in nest climate or nutritional quality of host blood between two host species, the Small Tree Finch and Green Warbler Finch. I also wanted to know whether host differences in nutritional quality were reflected in Avian Vampire Fly larvae from each host's nests. My results suggest nestling

blood quality is similar between the Small Tree Finch and Green Warbler Finch, but Small Tree Finch nests may be better at buffering against external climatic influences. I discuss these findings in relation to host specificity and quality for generalist parasites.

In **Chapter 3**, I aimed to determine whether a host can respond to Avian Vampire Fly parasitism via behavioral adaptations. Specifically, I investigated if the Green Warbler Finch, *Certhidia olivacea*, as a host can parentally compensate for Avian Vampire Fly parasitism, as measured via higher food provisioning to parasitized nestlings. I discuss from an evolutionary perspective how this avian host may exhibit specific adaptations to nest parasitism; however, parental compensation may not be one of these adaptations.

Overall, this research provides a unique, up-close perspective of behavioral adaptations of an invasive species and its novel hosts in its non-native range and factors influencing host quality. Furthermore, this study offers invaluable information that may be utilized for breeding the Avian Vampire Fly and development or improvement of control methods to minimize the effects of this parasitism on its hosts in the Galapagos Islands.

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List of Publications

CHAPTER 1

Published

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CHAPTER 2

To be submitted

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Author Contribution -- Idea formulation: ST; Methodology: ST, HR, and RHN; Data collection: **CLP** and BK; Laboratory analyses: **CLP** and RHN; Statistical analyses: **CLP** and BK; Writing—original draft preparation: **CLP**; Writing - review and editing: **CLP**, BK, RHN, HR, and ST.

CHAPTER 3

Published

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CHAPTER 1

Behavior of the Avian Parasite *Philornis downsi* (Diptera: Muscidae) in and Near Host Nests in the Galapagos Islands

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Photos credit: Courtney Pike

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Abstract The Avian Vampire Fly, *Philornis downsi*, has invaded the Galapagos Islands, where it causes high mortality of endemic and native landbird species, including most species of Darwin's finches. Control methods are under development, but key information is missing about the reproductive biology of *P. downsi* and the behavior of flies in and near nests of their hosts. We used external and internal nest cameras to record the behavior of *P. downsi* adults within and outside nests of the Galapagos Flycatcher, *Myiarchus magnirostris*, throughout all stages of the nesting cycle. These recordings showed that *P. downsi* visited flycatcher nests throughout the day with higher fly activity during the nestling phase during vespertine hours. The observations also revealed that multiple *P. downsi* individuals can visit nests concurrently, and that there are some interactions among these flies

within the nest. Fly visitation to nests occurred significantly more often while parent birds were away from the nest than in the nest, and this timing appears to be a strategy to avoid predation by parent birds. We report fly mating behavior outside the nest but not in the nest cavity. We discuss the relevance of these findings for the adaptive forces shaping *P. downsi* life history strategies as well as rearing and control measures.

Keywords Insect behavior · Ectoparasite · Invasive species · Nest visitation · Life history · Host-parasite interactions

Introduction

Island ecosystems are increasingly challenged by invasive species that have been introduced by humans (Simberloff 2010; Bellard et al. 2017; Spatz et al. 2017; Lenzner et al. 2020). These invasive species threaten the health and survival of often naïve endemic species through interactions such as predation, competition, parasitism, or as vectors of parasites and pathogens (Causton et al. 2006; Reaser et al. 2007; Blackburn and Ewen 2016; Russel et al. 2017). While there is extensive research on the impacts attributed to alien plants and vertebrates (Medina et al. 2011; Pyšek et al. 2012; Spatz et al. 2017), less is known about introduced parasites and how they interact with biodiversity in these novel environments

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and what makes them successful invaders (Poulin 2017).

At least 499 species of insects have been introduced to and established in the Galapagos Islands, including 35 species that are parasitic on animals or plants, and additional surveys will likely reveal more (Toral-Granda et al. 2017). One of the most devastating introduced insects is the Avian Vampire Fly, *Philornis downsi*, a muscid whose larvae are ectoparasites of nestling birds (Fessl and Tebbich 2002). While the first report of *P. downsi* in bird nests occurred in 1997 (Fessl et al. 2001), records in museums date back to the 1960 s (Causton et al. 2006; Fessl et al. 2018). This fly is native to mainland South America and Trinidad (Bulgarella et al. 2015; Fessl et al. 2018; Koop et al. 2020), but it now also has a widespread distribution and broad host range in Galapagos, infesting nests of at least 21 small land-bird species (Fessl, et al. 2018; Anchundia and Fessl 2020; Coloma et al. 2020). The introduction of this parasite has been highly detrimental to the reproductive success of small Galapagos landbirds, including various species of Darwin's finches (Kleindorfer and Dudaniec 2016; Koop et al. 2016; Fessl et al. 2018; McNew and Clayton 2018). Indeed, *P. downsi* is now regarded as a leading causal factor in the observed population declines of passerine species in Galapagos, and, as such, it is a priority for conservation organizations to develop tools for its management (Cunninghame et al. 2012; Causton et al. 2013). Control options that are being evaluated include insecticidal treatment of nests and trapping as stop-gap methods and biological control and the Sterile Insect Technique as long-term options (Knutie et al. 2014; Heimpel 2017; Bulgarella et al. 2017; Fessl et al. 2018; Boulton et al. 2019; Bulgarella et al. 2020; Ramirez et al. Accepted).

The behaviors exhibited by *P. downsi* in and around nests of its hosts have implications for the development of control strategies for this fly and prior studies have provided information on nest-associated behaviors and interactions. Video recordings from within the nests of three species of Darwin's finches from 2008 revealed that adult *P. downsi* visit nests during the incubation and nestling phases of bird development (both during the day and night), and may typically occur when adult birds are absent (O'Connor et al. 2010). These observations also revealed that adult *P. downsi* tend to oviposit on nest

material near nestlings and that larval feeding is primarily nocturnal. Other observations of *P. downsi* behavior outside of an active nest of the Galapagos Flycatcher, *Myiarchus magnirostris*, showed that adult flies landed outside the nest and walked into it once a parent flycatcher had exited the nest (Lincango et al. 2015). Some use of defense strategies by parents or nestlings against *P. downsi* has been documented in Darwin's finches, including preening of *P. downsi* larvae by nestlings or female parents, and antibody production against *P. downsi* by female parents (Huber et al. 2010). However, these appear to do little to protect nestlings from larval feeding by this parasite (O'Connor et al. 2010; Koop et al. 2013).

Despite these advances, there are still considerable gaps in our knowledge about how and when *P. downsi* adults locate and interact with their bird hosts. Furthermore, nothing is known about the courtship and mating behavior of *P. downsi*. In this study, we observed active nests of the Galapagos Flycatcher in artificial structures as a means of obtaining additional information on the behavior of *P. downsi* and its host inside and outside host nests. We also provide the first data on the interactions between *P. downsi* and its host over the entire bird developmental cycle – from incubation to fledging. Our main aims were to determine (i) if *P. downsi* preferentially visits host nests at certain times of the day or night and during certain stages of the reproductive cycle of birds, (ii) if multiple adult *P. downsi* can be found in host nests at the same time and if there are any interactions among them, (iii) which factors influence nest visitation by *P. downsi*, (iv) if Galapagos Flycatcher hosts exhibit defense strategies against *P. downsi* parasitism, and (v) if host nests are used by *P. downsi* as mating rendezvous sites. Additionally, we aimed to identify any natural enemies of *P. downsi* that might be present within host nests. Addressing these questions will aid researchers in developing techniques for controlling this fly, including techniques that could disrupt host or mate finding. This information will also aid efforts underway to develop a laboratory rearing system for *P. downsi*, which is needed to evaluate control methods (Lahuate et al. 2016; Sage et al. 2018), in particular how to stimulate flies to mate and lay eggs.

Methods

Study Site and Host Species

This study was conducted in the arid zone of the southern part of Santa Cruz Island, Galapagos, Ecuador. The field site, El Barranco (0° 44' 14.0" S; 90° 18' 4.1" W, ~8 m (m) above sea level), is located in the Galapagos National Park, adjacent to the Charles Darwin Research Station (CDRS). This site is a deciduous dry forest, consisting of *Opuntia* and *Jasminocereus* cacti, palo santo (*Bursera graveolens*) and *Acacia* spp. trees, and a variety of herbaceous shrubs (Hamann 2011).

The Galapagos Flycatcher is widespread throughout Galapagos (Harris 1973) and is susceptible to *P. downsi* parasitism (Fessler and Tebbich 2002; Lincango et al. 2015). The incubation and nestling phases of the Galapagos Flycatcher are generally between 14 and 17 days each (C. Pike, unpublished data). This species is the only Galapagos landbird that readily nests in artificial structures (Lanyon 1978; Ervin 1994), including bamboo towers with cavities that have been constructed to study the biology of this bird and that were used in this study. The bamboo nest towers (3 m tall x 20–35 cm wide), each contained 3 to 11 cavities with entrances that measured approximately 10.5 cm high x 7.5 cm wide and nest boxes made of wood (25 cm high x 16 cm x 16 cm) with one entrance hole (3.75 cm radius).

Video Recording

We filmed interactions between the Galapagos Flycatcher and *P. downsi* in three active nests built in three of the artificial bamboo cavities (two in 2015 and one in 2016). Filming of the first nest began on 23 March 2015. The three nestlings in the nest were estimated to be four days old (see below). The nest was filmed for 12 days - one day after all nestlings had fledged (Table 1). Filming of the second nest began on 3 June 2015. This nest contained three nestlings that were estimated to be seven days old. This nest was filmed for six days; the nestlings were found dead after the sixth day (Table 1). The third nest was filmed from 8 January to 20 February 2016. This nest contained four eggs and was estimated to be at day three of the incubation phase at the beginning

of filming. Filming continued from this day until 17 days after fledglings had left the nest, a total of 47 days (Table 1). The three nests were filmed externally, focusing on the entrance of the nest and surrounding area, using a GoPro Hero 3+ camera (GoPro Inc., San Mateo, CA, USA). Filming was generally continuous between 6:00 and 18:30 with batteries and SD cards replaced once daily at noon (see Supplementary Table 1). The cameras were secured to a nearby *Opuntia* tree or to the bamboo tower with a flexible clamp mount with the camera aimed at the opening of the nest (Fig. 1). The cameras were 30–50 cm away from the entrance, allowing free entrance and exit of the flycatcher parents and additionally enabling the filming of *P. downsi* flight activity near the nest.

The nest studied in 2016 was also filmed from inside the nest cavity using an infrared nest box spy camera (3.5 mm lens, Bunker Hill Security), which ran for 24 h/day. The camera was positioned on the ceiling inside of the bamboo cavity at 27 cm from the base of the nest and approximately 25–26 cm from the nest material, to include most of the nest material in camera view. The nest camera was connected to a DVR Receiver (Spy Camera Cctv.com Model W720, Camarillo, CA, USA). Twelve-Volt sealed lead batteries and solar panels were used to provide energy continually. Internal recordings were made based upon a motion detection setting controlled by the DVR in which clips including approximately five seconds of filming before the initiation of movement were incorporated into a saved clip that continued until the motion stopped. Pilot tests showed that the video camera was sensitive enough to be triggered by a fly walking into view, a gecko entering the nest, and nestlings moving within the nest. The internal nest camera recorded activity from 9 January to 23 February, 2016. This included 17 days post-fledging to record the behavior of flies emerging from pupae (Table 1, Supplementary Table 2).

Video Analysis

The external camera video recordings collected in 2015 were viewed and analyzed using Windows Media Player by one observer. The external and internal nest camera video recordings from 2016 were analyzed using the BORIS behavioral software program (Friard and Gamba 2016, v. 2.81) by multiple

Fig. 1 External video camera set-up for filming a nest in a bamboo tower with an extended-life battery, protective casing and flexible clamp mount facing the entrance of an active Galapagos Flycatcher nest. Arrow indicates active nest cavity. Photo: I. Ramirez



observers. This program was used to standardize observations and minimize observer bias from multiple observers. Flies observed within the nests were characterized as either (i) extending their ovipositors, (ii) engaging in other oviposition-like behavior, or (iii) doing neither of these things.

Confirmation of Fly Identification

The video images were of insufficient quality to identify flies definitively as *P. downsi*. For this reason, we conducted supplementary studies during 2017 – 2019 to identify flies entering Galapagos Flycatcher nests. We captured flies visiting four Galapagos Flycatcher nests in three bamboo towers and one nest box for species identification during 14–18 April 2017 (nest 1), 21–30 April 2017 (nest 2), 13–23 March 2018 (nest

3), and 18–20 March 2019 (nest 4), from 16:30 to 18:10. Nests 1 and 2 were in the nestling stage, while nest 3 was in the incubation phase. Nest 4 was also in the incubation phase on the first day of fly trapping, but with newborn nestlings the following two trapping days. The four nests were monitored for 85–90 min per day for 26 days in total (11 days during the incubation period and 15 days during the nestling phase). We trapped the flies after they entered the nest cavity (when birds were not present), by placing a custom-made flytrap or a large Ziploc bag (26.8 × 27.3 cm) over the nest entrance. The custom flytrap consisted of a modified insect cage (30 × 30 cm) with a 10 cm diameter hole in one side, which was surrounded with part of a bicycle inner tube to ensure that the cage entrance fit securely against the entrance in the bamboo. Following trapping, each fly was identified to

Table 1 Information on Galapagos Flycatcher nests filmed in 2015 (using external cameras) and in 2016 (using external and internal cameras)

Nest*	2015 March	2015 June	2016 Jan-Feb**
Video recordings			
Hours of recording (EX)	169 (N)	67 (N)	78 (I)/ 165 (N)/ 142 (PF)
Hours of recording (IN)	-	-	233 (I)/ 318 (N)/ 329 (PF)
Number of days filmed (EX)	12 (N)	6 (N)	12 (I)/18 (N)/14 (PF)
(IN)			11 (I)/18 (N)/17 (PF)
Bird information			
Number of eggs	n/a	n/a	4
Number of nestlings	3	3	4
Number of dead nestlings	0	3	0
Number of fledglings	3	0	4
Fly information			
Number of visits by flies to nest (EX)	44	107	9 (I)/ 161 (N)/ 6 (PF)
Mean fly visits per day filmed	3.70 ± SE 1.84	17.80 ± 2.86	0.75 ± 0.37 (I)/ 9.00 ± 2.53(N)/ 0.36 ± 0.23 (PF)
Highest number flies in nest at one time	1	5	11
Total <i>P. downsi</i> found in nest:	87	135	114
1st instar larvae	0	4	0
2nd instar larvae	6	13	0
3rd instar larvae ^a	2	38	0
Unemerged puparia ^b	77	77	0
Empty puparia	2	3	114

See Supplementary Tables 1 and 2 for a summary of the amount of time spent filming. * (EX) = external nest camera, (IN) = internal nest camera. ** (I) = Incubation phase, (N) = Nestling phase, (PF) = Post-fledging phase

^a Larvae pupated and emerged in laboratory setting

^b For the June 2016 nest, 18 puparia were parasitized with emergence hole sizes common to *Nasonia* (Hymenoptera: Pteromalidae) or *Exoristobia* (Hymenoptera: Encyrtidae) parasitoids

species following Couri (1999). In total, one individual was caught during the incubation phase and eight individuals during the nestling phase. All flies were identified as female *P. downsi*.

Nest Check and Nest Collection

Nests of the Galapagos Flycatcher were observed every morning for activity of parent birds. If a nest did not exhibit activity for two hours, it was checked using an endoscopic fiber-optic camera with a wireless monitor (dnt Findoo, shaft 17 mm diameter, fiber-optic cable length 91 cm) mounted on a pole to determine the status of the nest. Final nest status was categorized as “fledged,” when the nest no longer contained nestlings and fledglings were seen outside the nest or “with dead nestlings,” when only dead

nestlings were found in the nest. In 2015, once each nest was determined to be inactive, it was collected and inspected at the CDRS *P. downsi* laboratory the same day of collection to quantify *P. downsi* larvae and puparia. The larvae were categorized as first, second or third instars based upon the size and spiracular slit morphology (Fessl et al. 2006b). We categorized pupal casings (puparia) as either emerged, unemerged, or parasitized by Hymenoptera. In 2016, filming continued for 17 days post-fledging after which the nest was inspected in the CDRS laboratory.

Statistical Analyses

We used generalized linear mixed models (GLMMs) using *lme4* in RStudio (R Core Team 2013) with Poisson error structure to test whether the number

of *P. downsi* nest visits per hour (the dependent variable) during the nestling phase was significantly affected by (i) the time of day, (ii) the amount of time adult birds spent outside the nest per hour, and (iii) the number of times the adult birds visited the nest per hour, as fixed variables. Nest identity was coded as a random effect and the number of observations was added as a second random effect as well to account for overdispersion of the data. Additionally, we tested for correlations between the continuous GLMM predictors using linear regressions.

We used a two-tailed log-likelihood goodness-of-fit (G) test with William's correction factor (Sokal and Rohlf 1981) to determine whether the presence of parent flycatchers within the nest affected the propensity of *P. downsi* flies to enter the nest cavity. This test was done separately for nests in the incubation and nestling phases and observed proportions were compared to expected, or null, proportions. Under the null hypothesis, *P. downsi* visits are assumed equally likely when adult birds are present within the nest or not. We calculated the expected number of *P. downsi* visits under each condition based on the proportion of time adult birds spent in or out of the nest (27 % and 73 % of time, respectively, pooled for the nestling phase from all three nests, and 40 % and 60 %, respectively, for the incubation phase of the nest filmed in 2016). We also used a Kruskal-Wallis test to test whether the time a fly spent in the nest differed significantly between phases (incubation, nestling, and post-fledging phases). We used XLSTAT in Microsoft Excel (2016) to conduct these analyses. Lastly, we used a Chi-square contingency table test in RStudio (R Core Team 2013) to determine whether instances of ovipositor extension or apparent oviposition

behavior were more likely to be observed during the incubation or nestling phases.

Results

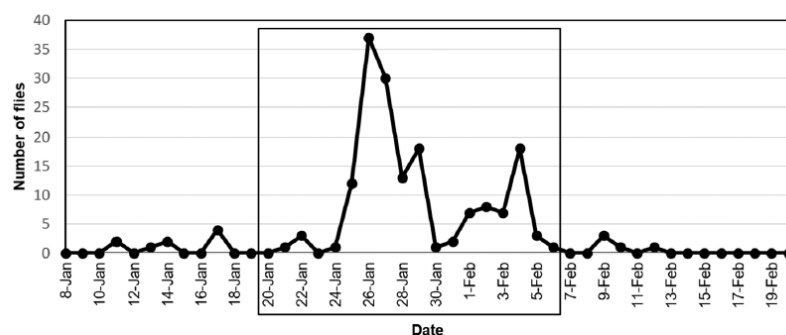
Galapagos Flycatcher Fledging Success and Parasite Load

Each filmed nest contained three or four nestlings. While all nestlings fledged successfully from the March 2015 and the 2016 nest, fledging success was zero in the June 2015 nest (Table 1). The three nestlings in this nest died during the last day of filming, when they were estimated between 11 and 13 days old. The number of *P. downsi* recovered from each of the three nests ranged from 87 to 135 (Table 1). Both larval and pupal stages of *P. downsi* were found in the nests from 2015, but only empty puparia were found in the 2016 nest, which was collected 17 days post-fledging (Table 1). We did not find any larvae or pupae of insect species other than *P. downsi* in the nests.

Fly Visitation of Nests

Fly visits were recorded during all three nesting phases of the 2016 nest, with 0.12 visits/ hour filmed during incubation, 0.98 visits/ hour filmed (91 % of total visits) during the nestling phase, and 0.04 visits/ hour filmed in the post fledging phase (see Supplementary Table 1 for total hours of filming). For this nest, peak visitation occurred when the nestlings were six days old (2.94 fly visits/ hour filmed, 37 visits, Fig. 2). For the nests filmed in 2015 during the nestling phase only, peak visitation occurred when

Fig. 2 Fly visits per day over the reproductive cycle of a Galapagos Flycatcher nest filmed with an external camera in 2016. The box indicates dates when nestlings were present in the nest with dates to the left of the box indicating the incubating phase and areas to the right of the box indicating the post-fledging phase



the nestlings were nine days old (0.82 fly visits/ hour filmed, 10 visits) and eight days old (2.40 fly visits/ hour filmed, 29 visits), in March and June respectively. Additionally, no flies were seen entering the June nest on the last day of filming, during which the nestlings died. Both the time of day and the presence of parent birds had effects on *P. downsi* nest visitation behaviors. Most visits during the nestling phase occurred in the afternoon, with peak visitation by flies between 17:00 and 18:00 h. Trends for visitation times during the incubation or post-fledging phases were not discernable because of the low number of fly visits (both phases) combined with fewer hours of video recording (incubation phase; see Supplemental Table 1) (Fig. 3). No nocturnal visits were recorded by the internal nest camera.

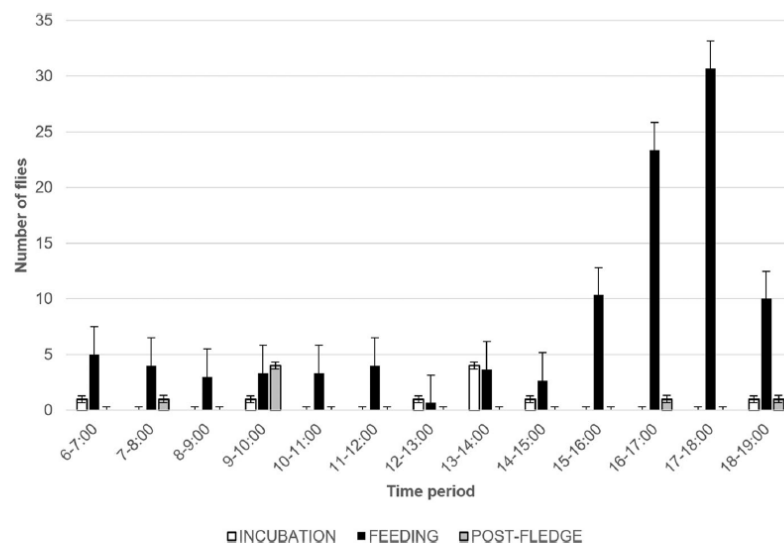
The GLMM on fly visitation (visits/hour) during the nestling phase showed a highly significant effect of time of day and marginally significant effects for both the number of adult bird visits per hour and the time they spent outside the nest per hour (Table 2). In addition, the Goodness-of-Fit test showed that the fly visitation rate (visits/hour) was significantly higher when parent birds were absent from the nest during the nestling phase ($G = 122.86$, d.f. = 1, $p < 0.0001$, only nine of 312 flies entered while the parent bird was inside the nest cavity; Fig. 4). There was no significant correlation between time of day and the number of adult bird visits ($F_{1,209} = 0.64$, $P = 0.425$) or

the time that birds spent outside of the nest ($F_{1,209} = 1.28$, $P = 0.259$), which suggests that different bird behavior over the course of the day did not drive the results of bird behavior affecting fly visitation rates.

The timing of fly entrance into nests once flies had landed adjacent to nests was associated with the behavior of parent birds as well. During the incubation phase, flies landed near the nest on average 1.76 ± 0.42 min. after the parent bird had left ($n = 8$), and flies entered the nest cavity on average 0.22 ± 0.05 min. after arrival ($n = 9$). These values were 1.45 ± 0.11 min. ($n = 318$) and 0.17 ± 0.01 min. ($n = 307$) for the nestling phase. Flies normally walked into the nest cavity (95% of all fly entrances ($n = 327$)), but exited the nest cavity flying (94% of all exits ($n = 318$)). Flies that landed but did not enter the nest ($n = 68$ observations) flew away less than five seconds after landing. The average time spent in the nest cavity did not significantly differ between nest phases ($H = 2.771$, d.f. = 2, $p = 0.25$; incubation phase 1.78 ± 0.54 min. ($n = 9$); nestling phase 1.38 ± 0.12 min. ($n = 271$); post fledgling stage 1.14 ± 0.65 min. ($n = 6$)). Lastly, four of the six visits to the nest during the post-fledging phase took place on the same day that fly emergence from puparia occurred (on days three and day six post-fledging).

While the majority of observations using the external camera involved a single *P. downsi* adult visiting a nest at a time, there were some instances

Fig. 3 Fly visitation to Galapagos Flycatcher nests recorded at hourly intervals using an external camera. Values shown indicate the number of flies that entered nest cavities per hour filmed $\pm$ Standard Error. For the nestling phase, the number of flies entering are hourly averages from the three nests combined



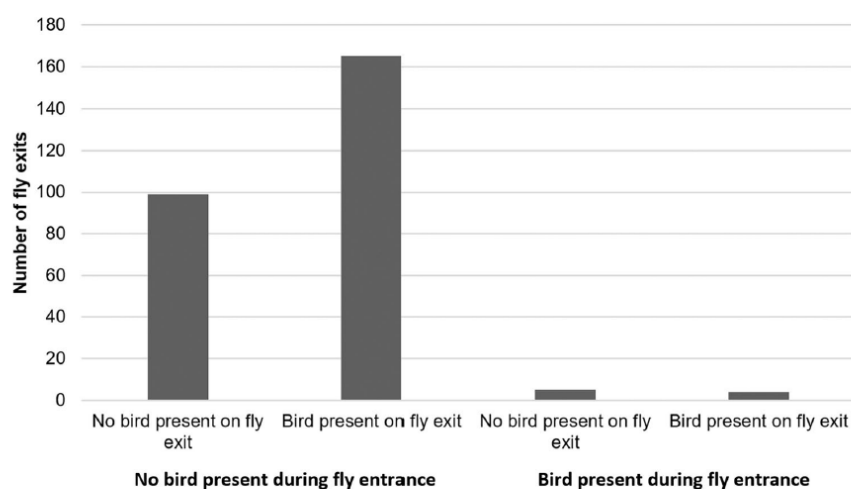


Fig. 4 Number of fly entrances and exits when compared to bird presence and absence at the nests. Data from the three nests combined (two filmed in 2015 during the nestling phase and one filmed in 2016 during the incubating and nestling phases combined)

Table 2 Results of a generalized linear mixed model assessing the effects of time of day, the amount of time parent birds spent away from the nest per hour, and the number of bird vis-

its to the nest per hour on the rate of *P. downsi* visitation (also per hour) during the nestling phase, with nest identity and the number of observations per nest as random effects

	Estimate	Std. Error	Z	P
(Intercept)	-3.64255	0.70271	-5.184	2.18e-07 ***
Time of day	0.20631	0.02812	7.336	2.21e-13 ***
Time bird away from nest	-0.05739	0.03070	-1.869	0.0616
Number of bird visits to nest	0.06334	0.03296	1.921	0.0547

The analysis included data from all three nests

of multiple adult flies being present in a nest at the same time. Four events in which between two and five flies were registered in a nest cavity simultaneously were recorded during the nestling phase from the June 2015 nest. In the 2016 nest, multiple flies were also recorded in the nest cavity at the same time, with a maximum of 11 flies recorded on day seven of the nestling phase (Table 1).

Fly Activity Within the Nest

Adult flies were observed walking on flycatcher eggs and nestlings as well as on various substrates within the nesting cavity. During the incubation phase, adult *P. downsi* spent up to 73.5% of the time observed on eggs. Flies that walked on host eggs spent a mean of

15.4 ± 2.08 s. ($n = 27$, max 33 s.) on eggs per observation. Flies were only seen walking across or standing on eggs; no instances of flies noticeably palpating them with their mouthparts were observed. During the nestling phase, adult flies were observed walking on nestlings of ages ranging from less than one day to fifteen days old (two days before fledging). The flies also walked on the nest base underneath the nestlings' bodies while the nestlings were sitting and standing. No visible reaction by the nestlings to the flies was observed.

We did not observe flies ovipositing on eggs or nestlings, but noted behaviors that suggested that flies were laying eggs in the nesting material; such as ovipositor extension or, "oviposition-like behavior," in which the abdomen was pointed down towards

the nest material and a bobbing movement anteriorly - posteriorly was noted. Ovipositor extension or oviposition-like behavior was observed in 76% of the observations of *P. downsi* in the incubation phase ($n = 25$) and 71% of observations during the nestling phase ($n = 248$). No significant differences were found between the incubation and nestling phases for the proportion of times *P. downsi* adults exhibited this behavior ($\chi^2 = 1.19$, d.f. = 2, $p = 0.5505$).

We recorded 17 instances of multiple flies present in the nest cavity using the internal nest camera, all from nests with 4–9 day old nestlings and in six of these cases, interactions between flies were observed. Three of these interactions were particularly notable. In one of these interactions, one fly encountered a second fly that appeared to be in the process of ovipositing; the second fly responded to the first fly by pushing it with its legs. In another interaction, one fly appeared to oviposit, then the second fly walked toward it, and the first fly walked forward ~ 2 mm. The first fly again appeared to initiate oviposition, but the second fly approached it again, pushing it with its legs. The second fly then appeared to oviposit in the location of the first fly's apparent oviposition attempt. In a third interaction, one fly walked into a second fly and the second fly walked forward. Then, both appeared to oviposit within 1–2 cm of each other.

Larval Activity in the Nest

Philornis downsi larvae (first, second and third-instars) were first seen via the internal nest camera when the nestlings were five days old, two days after the parent stopped staying in the nest overnight. From then on, adult flycatcher visits to the nest overlapped with larval activity only from 17:40 until dark (~18:40) and in the mornings from sunrise (~05:20) until 07:00. On day six of the nestling phase, early instar larvae were seen protruding from the nasal cavities and ear canals of two nestlings and the first large second or third instars were seen in the nest base, near the nestlings. By day seven, many larvae were seen on the surface and the sides of the nest at night. Throughout the nestling phase, larvae were observed on multiple body parts, including within the nares (35.0%) or ear canals (2.5%), or externally on the wing (22.5%), head and neck (17.5%) or back, abdomen or chest (22.5%) of the nestlings. The longest time a larva was seen

attached to a nestling was 101 min. on the back of the head of a nine-day old nestling. This appeared to be a third instar larva. When the larva detached, the nestling had a visible lesion. Mature larvae were observed with part of their bodies emerging from the nest material during nighttime hours for six days after the nestlings fledged.

Fly Predation and Removal

Both external and internal cameras recorded instances of *P. downsi* predation and removal. External cameras revealed adult Galapagos Flycatchers preying on flies outside or near the nest entrance four times during the nestling stage (once in 2015 and three times in 2016). On all occasions, the bird was arriving or standing on the outside of the nest cavity and successfully caught and either ate the fly or fed it to a nestling. In one of these events, the fly was standing on the outside of the bamboo, while in all other instances the fly was exiting the cavity and was in flight when it was caught. The internal nest camera recorded multiple occasions in which adult birds were observed pecking at the nest material and eating unidentified objects, but we could only once confirm that a *P. downsi* larva was taken in this way. Additionally, in one instance, an adult Galapagos Flycatcher was observed pecking at the nostril of a nestling, but the adult did not appear to remove anything. The removal of fly larvae by nestlings themselves via flapping and preening was observed during the hours 1:00–4:00 and 18:00–21:30 when nestlings were 9, 10, and 14 days old but it was not clear that these preening behaviors were acts of predation. Additionally, nine to 13 day old nestlings used their feet to 'scratch' their heads during the night (20:00–01:00) and morning (~7:45) hours but this behavior did not necessarily remove larvae.

In 2016, we observed geckos inside the nest cavity during incubation (five times), nestling development (13 times), and after fledging (24 times). These were all identified as the introduced Mourning Gecko, *Lepidodactylus lugubris* and may have represented repeat visits by the same individuals or multiple individuals. Gecko visits occurred only between 18:39 and 05:35. Over this period, six successful predation events on *P. downsi* larvae were observed, all of which took place after nestlings had fledged.

Fly Emergence from Nest Material and Mating Behavior

Adult *P. downsi* emerged from their puparia within the nest material over a period of 11 days, starting one day after the nestlings fledged. An average of 6.09 ± 1.65 flies emerged per day with a maximum of 20 flies emerging on day 11 post-fledging (Fig. 5a); all recorded fly emergences took place between 03:00 and 09:00 (Fig. 5b). Although we found 114 open puparia in the nest material, we only recorded 67 instances of fly emergence, potentially due to the internal camera malfunctioning 18% of the time during the post-fledging phase (see Supplementary Table 2). Thirty flies were registered exiting the nest cavity by the external camera during this time period. The remaining flies may have exited either during periods of the day when the camera failed (see Supplementary Table 1) or during nighttime hours when the camera was not on. Flies exited the nest cavity between 06:48 and 13:47 with the highest number of 19 flies exiting the nest cavity between 07:00 and 09:00. By comparing video recording from the internal and external nest cameras, we found that some flies stayed in the nest cavity after emergence, in one case for up to 11 h.

Behaviors that appeared to be mating by presumed *P. downsi* flies were observed outside the nest cavity on four occasions during the post-fledging phase in the 2016 nest. The first of these mating events occurred on the day that the last fly emergence was recorded, one day after the highest number of flies

(20) was recorded emerging from the nest. The remaining three mating events took place two days after this. No flies were filmed exiting the nests on these days. The first mating event occurred 12 days post-fledging, at 17:35, and began when a fly landed just below the nest cavity. This was followed by a second fly landing nearby, at which point the first fly (the “resident” fly) walked toward the arriving fly. The two flies then walked in circles following each other before the resident fly mounted the arriving fly for ~ 42 s. After this, the flies backed away from each other, and the arriving fly flew away. The second event took place 14 days post-fledging, at 17:59 when a fly landed a few inches below another fly that was positioned just beneath the entrance to the nest cavity. Wing fanning by both individuals was observed, and the fly that had been present earlier (the “resident” fly) moved toward the arriving fly and mounted it for six sec. This was followed by the flies chasing each other for 35 s., after which the fly that had been mounted flew away. The third event occurred at the same time as the second event. A fly landed above the nest cavity and was approached by another fly already present on the bamboo. This resident fly appeared to mount the arriving fly for seven sec., after which the arriving fly flew away. The fourth instance also occurred 14 days post-fledging at 18:17. One fly was standing ~16 cm below the nest entrance, when a second fly landed approximately 2 cm away from the resident fly. The resident fly fanned its wings and walked to and mounted the arriving fly for 34 s. After this, the arriving fly flew away, while the resident fly

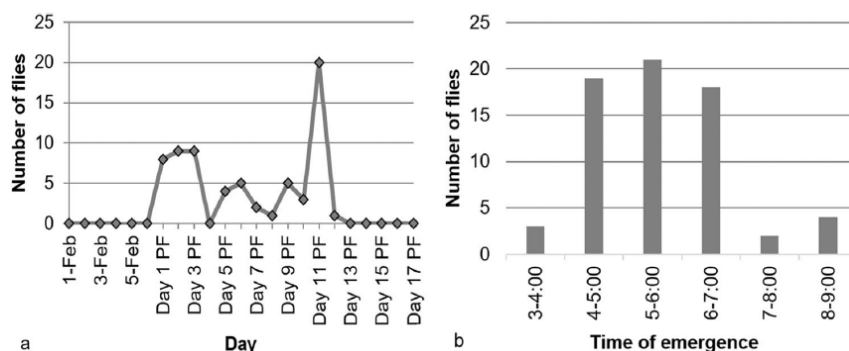


Fig. 5 Number of *P. downsi* adult flies emerging from material of a Galapagos Flycatcher nest filmed with an internal nest camera in 2016 (a) per day and (b) by time period. PF indi-

cates post-fledging phase. *P. downsi* was not seen emerging outside of the times displayed

stayed on the bamboo. A number of consistencies can be discerned from these four instances of attempted or actual mating. First, they all occurred around dusk. Second, mating appears to have been initiated by the resident rather than the arriving fly. Third, mounting was short, never exceeding one minute.

Discussion

Video recordings taken outside and within nests of the Galapagos Flycatcher that were heavily infested with *P. downsi* have provided new insights into the behavior of this invasive parasitic fly, though results should be interpreted with some caution given the low sample size (three nests) and the limited data collection period for two nests. By tracking three nests during the nestling phase and monitoring fly activity from the onset of the incubation phase to post-fledging in one of these, we determined that *P. downsi* visitation of nests appears to be highly dependent on the time of the day and is influenced by the presence of adult birds; when birds are in the nest, flies rest outside the nest until the birds leave. This suggests an adaptation of *P. downsi* to nesting behaviors of its hosts. The highest visitation rates of *P. downsi* to bird nests were during the nestling phase during late afternoon/dusk. Nests were visited by high numbers of flies (up to 37 visits occurred during daylight hours in a single day) with multiple individuals (up to 11) in the nest cavity at the same time. Larvae were observed feeding on all parts of the nestlings, however, active late-instar larvae were also observed six days after the nestlings had fledged, suggesting that larvae can survive for long periods without a live host before pupating. While no mating was observed inside the nest cavity, observations suggest that *P. downsi* rendezvous to mate outside the nest cavity. Lastly, low level parasitism of puparia by parasitoid Hymenoptera and predation of *P. downsi* larvae by introduced geckos were recorded, which, in addition to fly captures by adult Galapagos Flycatchers provides some mortality of *P. downsi*, but likely not enough to reduce numbers substantially.

Although we could not categorically confirm that all flies observed in our recordings were *P. downsi*, the evidence collected in this and prior studies strongly supports the hypothesis that they were indeed *P. downsi* for the following reasons: (1) We did not find

larvae or pupae of other Diptera in the nests filmed in our study. This suggests that flies of other species were not ovipositing in the nest material; (2) Muscid larvae and pupae have not been reported in the more than 1500 + nests that have been inspected in the last eight years by scientists (P. Lahuatte, pers. comm) or in the literature - a prior report of an unidentified muscid in nests (Fessler and Tebbich 2002) was later confirmed by B.F. to be *P. downsi*; (3) The only other flies that have been found in nests other than *P. downsi*, are sarcophagids (Fessler et al. 2006a). These species are typically associated with nests containing dead nestlings - two of our nests (March 2015 and 2016 nests) did not contain dead nestlings; the third nest did not contain dead nestlings during recorded fly visitation, as no flies visited the last day of filming when the nestlings died; (4) Trapping of flies entering bird nests in 2017–2019 led to captures of only *P. downsi* and these flies exhibited similar behaviors to those filmed in 2015–2016.

Most observed fly visits took place during the nestling phase in the last hour of daylight, with the highest number of fly visits occurring in the middle of the nestling phase, when the nestlings were six to nine days old. This suggests that fly visitation may be influenced by the age of nestlings rather than other factors, such as weather. Young (1993) found the highest number of *P. carinatus* larvae on nestlings between six to nine days old, similar to our findings, although he suggested that *P. carinatus* and other bird-parasitic flies do not time egg laying to nestling age. The vespertine activity of *P. downsi* (i.e. activity during late afternoon/dusk, but not at dawn), on the other hand, could be explained by several adaptive hypotheses, including seeking optimal abiotic conditions, tracking resource availability and/or gaining safety from diurnal predators, such as parent birds. *Philornis downsi* may be active during dusk to avoid higher temperatures, as found in other insects (May 1979), or their evening activity may be correlated with lower light intensity, as shown in other species (Rieger et al. 2007; De et al. 2013). Alternatively, *P. downsi* may visit nests more often at dusk due to a genetic and/or hormonal-based internal rhythm related to egg laying (as reviewed in Howlader and Sharma 2006). The absence of activity at nests during dawn, which is also characterized by cooler temperatures, could reflect a prioritization for seeking food resources such as nectar, pollen or plant exudates

(see Fessl et al. 2018) during morning hours before they have dried up or been consumed by competitors. Lastly, while nighttime visits to nests would appear to be advantageous from the standpoints of both temperature and safety from parent birds (in our study adults stopped brooding at night when nestlings were three days old), we did not record *P. downsi* visiting nests at night as found by O'Connor et al. (2010).

Philornis downsi adults were more likely to enter nest cavities when adult birds were absent. This suggests that *P. downsi* can discriminate nests with and without adult birds and tend to avoid the former, thus reducing the risk of predation. In addition, fly visitation during the nestling phase was negatively correlated with long absences of adult birds from the nests and positively correlated with the number of bird visits to the nest. Both of these results suggests that the presence of adult bird activity in or near nests attracts *P. downsi* to the vicinity of nests. We therefore propose that *P. downsi* adults use cues (noise, odor, movement or infrared radiation) associated with the presence of adult birds to locate active nests while avoiding predation. This includes detecting a nest and waiting outside of it until a parent bird exits for the fly itself to enter, which was a common behavior of the flies recorded in our study. The few predation events that we observed occurred when a fly entered a nest while an adult bird was present or when a fly exited a nest while a bird had just landed at the nest entrance. The rarity of these events is consistent with predation avoidance by *P. downsi*.

Evidence of female fly visitation was recorded by the internal nest camera and allowed for descriptions of oviposition behavior by *P. downsi*. Observations of extended ovipositors and apparent oviposition behavior during the incubation and nestling phases suggest that at least 70% of the flies recorded in the nest camera were female; however, it must be noted that some of the recorded events may have been individual flies re-entering the camera viewing area. Additionally, up to three confirmed female flies (with ovipositors extended) were seen in a nest at one time, which is consistent with previous population genetic studies indicating that multiple *P. downsi* females oviposit per nest (Dudaniec et al. 2010), and provides new evidence that oviposition events by several flies can take place simultaneously. Evidence of female flies in the nest cavity during the incubation phase supports prior reports of *P. downsi* oviposition during the

incubation phase in nests of Darwin's finches (Cimadom et al. 2016; Common et al. 2019; Cimadom and Tebbich 2020). On the other hand, observations of fly visitation and oviposition-like behavior during the late nestling phase concur with findings of first instar *P. downsi* larvae in host nests with nestlings close to fledging age (C. Pike, unpublished data; Kleindorfer et al. 2014). Oviposition at this state of bird development is somewhat paradoxical since fly eggs would appear to be deposited with insufficient time for completing larval development. However, these observations contribute to gathering evidence of such late oviposition by *P. downsi* females over the last decade, which may be a response to high population densities of flies and/or low availability of hosts (Kleindorfer et al. 2014; Causton et al. 2019; Cimadom and Tebbich 2020). As expected, there was no evidence for oviposition by visiting flies during the post-fledging phase and fly visitation was considerably lower in general. Given this, it is possible that the fly visits observed during the post-fledging phase could be either males or females seeking mates. Lastly, given that more visitation events were recorded than the total number of *P. downsi* parasites found in the nest, it may be that visiting females do not lay eggs during every nest visit. Alternatively, more *P. downsi* larvae may have been present, but did not survive to pupation due to larval competition, and/or predation by birds or geckos.

We also documented times of activity and feeding sites for *P. downsi* larvae inside the nest cavity. Larval feeding took place from dusk to dawn; however, since this study we have also seen *P. downsi* larvae feeding on Galapagos Flycatcher nestlings during the day (personal obs., C.L.P.). We report larval feeding on most body parts of the nestlings, including on the head, neck, back, abdomen, and chest, congruent with prior reports of contusions on the abdomen and back of nestlings (e.g. Fessl et al. 2006b; Koop et al. 2011). Larval feeding mainly during the nighttime hours may be an adaptive strategy (Gold and Dahlsten 1989), as nestling activity is lower and Galapagos Flycatcher parents do not stay overnight after nestlings are approximately three days old.

By filming nests after nestlings departed, we were able to observe the timing of *P. downsi* emerging from pupae in the nest material. Multiple peaks of emergence may indicate multiple egg cohorts laid on different days, by one or multiple female flies, as

reported in *P. carinatus* in Costa Rica (Young 1993) and consistent with reports of Dudaniec et al. (2010) for *P. downsi*. Additionally, our finding that adult flies emerged within host nests only during early morning hours suggests there may be an eclosion rhythm based on factors such as temperature or the circadian clock (Pittendrigh 1954), potentially providing an adaptive benefit, such as meeting potential mates, avoiding predation, and/or avoidance of desiccation (Cloudsley-Thompson 1960). Peak hours of adult eclosion from puparia have been previously reported in other avian nest parasites, including *Protocalliphora*, which took place during daylight hours, from 09:00–15:00 (Gold and Dalhsten 1989).

Insects commonly use hosts as rendezvous sites for mating (“resource-based mating,” Wilkinson and Johns 2005). We recorded four events of flies similar in size to *P. downsi* mating outside the nest cavity during the post-fledging phase around the time that flies were emerging from pupae. These events took place one to three days after the day that the highest numbers of flies were observed emerging from the nest material, but only the first event coincided with a known emergence event. Studies suggest that male *P. downsi* use pheromones to attract females (Collignon 2011; Mieles 2018). Given the ephemeral nature of host nests, typically available during the hot, wet season (January to April/May, Grant 1986), it may be that male *P. downsi* use nests to locate and attract females for mating. Since active nests may pose risks of predation by adult birds, nests in the post-fledging period out of which females are emerging may be a favored site for male pheromone release. Male courtship in flies that use resource-based mating strategies is generally minimal and can involve a repetitive action like wing waving, fanning (to disseminate pheromones) or wing vibrations (Burk 1981; Wilkinson and Johns 2005), such as was seen near the Flycatcher nest.

In our study, *P. downsi* density was high in all three nests and mortality imposed by natural enemies appeared to be minimal. There are currently seven parasitoid species (Hymenoptera) reported to sporadically use *P. downsi* as a host in the Galapagos Islands (Fessl et al. 2018). We found a small number of pupae that appeared to be parasitized by a member of the parasitoid families Pteromalidae or Encyrtidae, but we did not observe any parasitoids visiting the nest. Geckos were recorded preying upon *P. downsi*

larvae, and were observed in all of the nest phases. Additionally, there was evidence of a low number of predation events by adult flycatchers. Furthermore, *P. downsi* individuals from the Galapagos Islands have been found to host endoparasites with unknown effects; however, further studies are required to determine whether they affect fly populations (Pike et al. 2020).

Implications for Management

This study provides new information for researchers and conservationists attempting to control *P. downsi* in the Galapagos Islands and could also help to inform the management of other *Philornis* species that are affecting endangered bird species (Bulgarella et al. 2019). While this study focuses on only three nests, each nest provided a substantial amount of information and, importantly, showed consistent fly behaviors across years and nests. This suggests that similar behaviors occur at other nests of the same species. Additionally, while our study focused on the activity of *P. downsi* in and around Galapagos Flycatcher nests, it is probable that similar behaviors occur in association with nests of other Galapagos landbirds, including Darwin’s finches, in whose nests fly activity has been previously recorded (O’Connor et al. 2010; Koop et al. 2013). The observations on the timing of the nest visits during the reproductive cycle of the birds and the time of day visited have helped guide additional experimental studies on life history and control of *P. downsi*; for example, the timing of insecticide treatment in nests (Causton et al. 2019). This study also provides clues about the mating and courtship behaviors of *P. downsi*, which are critical for establishing captive colonies and developing methods such as sterile male releases (Lahuatte et al. 2016; Fessl et al. 2018). Using data from this study, mating and egg laying experiments may be timed more effectively, especially taking into consideration that the late afternoon is the time of day that *P. downsi* is most active at host nests.

Lastly, although interactions of *P. downsi* with the Galapagos Flycatcher could differ from interactions with other Galapagos birds that do not engage in predation while flying, the inferences regarding cue use for host finding might aid in directing future studies into how female *P. downsi* find their hosts. This, in turn, may lend to the development of management

tools such as auditory, visual or olfactory decoys that could manipulate the fly's behavior thus reducing parasitism pressure on at-risk bird populations (Sutherland 1998).

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Authors' Contributions Conceptualization: George E. Heimpel, Charlotte E. Causton; Methodology: Courtney L. Pike, Ismael E. Ramirez, David J. Anchundia, Birgit Fessl, George E. Heimpel, Charlotte E. Causton; Formal analysis and investigation: Courtney L. Pike, Ismael E. Ramirez, David J. Anchundia; Writing - original draft preparation: Courtney L. Pike; Writing - review and editing: Courtney L. Pike, Ismael E. Ramirez, David J. Anchundia, Birgit Fessl, George E. Heimpel, Charlotte E. Causton; Funding acquisition: Birgit Fessl, Charlotte E. Causton, George E. Heimpel; Resources: Birgit Fessl, George E. Heimpel, Charlotte E. Causton; Supervision: David J. Anchundia, Birgit Fessl, George E. Heimpel, Charlotte E. Causton.

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Data Availability Data can be shared if requested.

Code Availability Not applicable.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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CHAPTER 2

Nest climate and blood quality of two host species of the ectoparasitic *Philornis downsi* in the Galapagos Islands as factors of larval development

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Nest climate and blood quality of two host species of the ectoparasitic *Philornis downsi* in the Galapagos Islands as factors of larval development

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ABSTRACT

The avian vampire fly is an invasive species on the Galapagos Islands, where its hematophagous larvae utilize nestling birds as hosts. It poses a serious threat to populations and species of Darwin finches and other avian species. Prior studies suggest that the avian vampire fly larvae develop more rapidly in nests of small tree finches compared to sympatric green warbler finch nests. Here we investigate factors that may differentially influence larval growth in the two host species, including nest microclimate and nestling diet. We collected nest base temperature and humidity data as proxies for nest microclimate. As indicators of dietary composition and quality, we measured the nestling blood, stable isotope $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, the C:N ratios therein as an indicator of relative protein content, plus the amino acid composition of the blood. The hematophagous larvae living off nestling blood were analyzed for the same parameters excluding amino acid composition. Nest base temperature

was higher in small tree finch nests at lower humidity levels and declined in both species as nest base humidity increased. Nest base humidity was higher in small tree finch nests before the predicted onset of nestling thermoregulation and decreased thereafter, yet increased in green warbler finch nests. Blood of small tree finch nestlings has significantly higher $\delta^{13}\text{C}$ values than those of green warbler finch nestlings, suggesting a difference in nestling food composition and source. However, $\delta^{15}\text{N}$ values did not differ significantly between species. Additionally, the C:N ratios and amino acid composition of nestling blood of both species were not significantly different, suggesting similar dietary quality. Differences in nest base climate suggest that small tree finch nests may have a higher stable heat capacity than green warbler finch nests and that other factors may cause the observed difference in larval development between the two host species, rather than host blood quality parameters measured, i.e. C:N ratios and amino acid composition.

KEYWORDS

Nest parasite, stable isotopes, amino acids, thermal conditions

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INTRODUCTION

Host suitability and host quality are important factors in parasite life history and development. Parasites may choose hosts based on many factors, such as habitat, host distribution, size, and abundance, host defense, and/or nutritional status (e.g., Seppälä and Leicht, 2015; Stokke et al., 2018). For generalist polyphagous parasites, fitness may also vary depending on the quality of each host species (e.g., Manzoli et al., 2021) and this variation could be particularly pronounced in newly introduced parasites on remote archipelagos, where many of the potential host species may be suboptimal. Further understanding on how host quality and suitability affects the development and success of introduced parasites in non-native ranges is important in invasion biology research.

The Galapagos Islands is a UNESCO World Heritage Site that hosts numerous endemic species. Both human-introduced and invasive species are threatening the wildlife and disrupting ecosystems in the Galapagos. For birds on the Galapagos, the invasive avian vampire fly, *Philornis downsi*, parasitizes at least 21 host species, including passerines and doves (Fessl et al., 2018; Coloma et al., 2020; Anchundia and Fessl, 2021). The avian vampire flies oviposit in host nests during both incubation and nestling phases, with greater parasitism loads in the nestling phase (Cimadam et al., 2014; Pike et al., 2021; McNew and Clayton, 2018; Common et al., 2020). In the incubation phase, the avian vampire fly larvae feed on the brooding adult female and it is assumed they live in the nest material. However, during the nestling phase, larvae initially develop in the nasal cavities and ear canals of nestlings, while the 2nd and 3rd instar larval stages generally reside in the nest base during daylight hours and nocturnally feed on nestling hosts' blood and tissue (Fessl et al., 2006). Blood feeding nest ectoparasites, such as the avian vampire fly, rely solely on the hosts for optimal conditions and sufficient nutrients for growth. Though important in the context of conservation strategies to protect the emblematic Darwin finches of the Galapagos, little is known about larval growth requirements under natural conditions and the trade-off between optimal larval development and nestling survival (Kleindorfer et al., 2014).

Variation in parasite growth, development and numbers among the different host species suggest that hosts vary in profitability for the parasite. For example, Common et al. (2021) suggested a shift towards host specialization of the avian vampire fly that favors their choice for the medium tree finch, when compared with other Darwin's finch species in a study on Floreana Island, Galapagos. On Santa Cruz Island, Cimadam et al. (2014) reported differences in avian vampire fly parasitism between the small tree finch and the green warbler finch. More recently, Cimadam and Tebbich (2021) noted a change in parasitism levels in host nests. Mean avian vampire fly parasitism decreased in green warbler finch nests but increased in small tree finch nests during 2012-2017 compared to data from the early 2000's. This may suggest a potential change in host preference of the avian vampire fly for the small tree finch compared to the green warbler finch. Additionally, Cimadam and Tebbich (2021) reported that the proportion of 3rd instar larvae and pupae increased at a faster rate in small tree finch nests compared to those in the green warbler finch nests suggesting faster larval growth, maturation and completion of larval stages. It suggests that the small tree finch may be a higher quality host and/or provides a more suitable habitat for the avian vampire fly than the green warbler finch. A variety of natural, biotic conditions/factors related to hosts may influence larval growth and maturation in insects, depending on their life history. In the case of nest parasites, such as the avian vampire fly, these factors may include microclimate of the nest base and overall nutritional quality of the hosts' blood.

Nest microclimate, including nest temperature and humidity, are influenced by ambient weather conditions, parent and nestling mass, brood size, brooding rate of female parents, among other factors. Some studies have investigated the effects of ambient temperature on nest ectoparasite abundance (e.g., Mennerat et al., 2021; Castaño-Vasquez and Merino, 2021), but field studies on the effects of direct nest temperature on larval development are limited. Prior studies have experimentally shown that black soldier fly, *Hermetia illucens*, larvae and *Protocalliphora* blowfly larvae may develop faster at higher temperatures (Bennett and Whitworth, 1991; Palma et al., 2019). Nest microclimate can be influenced by nest composition (e.g., Michielsen et al., 2019). As green warbler finch and small tree finch nests differ in size, material, and mean nest height (Kleindorfer and Dudaniec, 2009; ST pers. obs.; CP unpublished data), they potentially have different nest microclimates that may influence larval growth. Nest humidity has also been shown to be an important factor in parasitism in a study where nest humidity influenced the prevalence of a hematophagous bird parasites, and vice versa, parasite prevalence affected nest humidity (Heeb et al., 2000).

Nutritional levels in the diet are essential for adequate larval development in insects. Natural food sources of hematophagous larvae may vary in nutritional composition and measuring blood protein composition and quality can shed light on these differences. The stable isotopes of carbon can be informative for investigating the source and quality of the diet. Stable isotope analysis is a technique commonly used in ecological/ entomological research (Hood-Nowotny and Knols, 2007; Quinby et al., 2020) to investigate food sources and food uptake. The stable isotopes composition $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ may vary depending on the diet per se and the trophic position of hosts and their ectoparasites. $\delta^{15}\text{N}$ is dependent on the trophic level of the study organism, with higher tissue $\delta^{15}\text{N}$ values indicating consumption of organisms higher up the trophic ladder. In the case of insectivorous or omnivorous hosts, higher tissue $\delta^{15}\text{N}$ may be driven by more arthropods compared to seeds or arthropods further up the carnivorous food chain, such as spiders, in the host diet (Meyhoff et al., 2023, Hood-Nowotny et al., 2023). Additionally, lower C:N ratio of the host species blood or parasite indicates more protein-N per molecule of carbon. Prior research indicates C:N ratios, i.e. protein concentrations in food sources have profound impacts on larval growth of insects, including mosquitoes, black soldier flies and fruit flies (Balagawi et al., 2014; Hood-Nowotny et al., 2012; Palma et al., 2019). More specifically, larval growth can be positively influenced by lower C:N ratios (i.e. higher protein contents) of larval diet alone (Hood-Nowotny et al., 2012; Ul Haq et al., 2010), or can act in conjunction with higher temperatures to improve growth (Palma et al., 2019). However, in a prior experimental study on *Aedes aegypti*, faster larval development was reported for larvae fed a diet of 61-77% carbohydrates to 20-33% proteins (Van Schoor et al., 2020) when compared with lower and higher protein to carbohydrate diets. Therefore, this suggests there may potentially be optimal levels of carbohydrates for larval growth, which may help account for differences in sizes of larvae. However, experimental protein levels used by Van Schoor et al. (2020) may not reflect those common in natural conditions. Previous studies of parasite-host feeding systems demonstrated that stable isotope analysis is an effective method for determining dietary sources. Schmidt et al. (2011) reported carbon and nitrogen stable isotope ratios in blood-feeding larvae of the tick, *Ixodes ricinus*, were reflective of the differing diets of the two host species. Ticks that fed on rabbit hosts with lower $\delta^{13}\text{C}$ blood values than gerbil hosts also had significantly lower $\delta^{13}\text{C}$ values than ticks that fed on gerbil hosts (Schmidt et al., 2011). Green warbler finch nestlings are fed a more insectivorous diet than small tree finch nestlings (Filek et al., 2018) and we thus predict a more protein-rich

blood composition in green warbler finch nestlings, demonstrated by both a lower C:N ratio and higher $\delta^{15}\text{N}$ levels.

Amino acids are equally essential factors in the growth and development of insects. Prior studies on dietary amino acids in insects have reported specific amino acids that are essential for the completion of larval developmental stages (Singh and Brown, 1957; Davis, 1975; Chang, 2004). Insect larvae lacking specific non-essential amino acids in their diet may survive, but larval growth may be delayed (e.g., Davis, 1975; Chang, 2004). Additionally, an excess of specific amino acids may also slow or hinder larval growth (Hinton et al., 1951). Thus, investigating amino acid composition in natural blood diets of avian vampire fly larvae may provide insight into the nutritional requirements of avian vampire fly larval development and additionally host blood quality.

Our aims for this study were to identify if (1) there are significant differences in temperature or humidity levels in the nest base of small tree finch and green warbler finch, (2) using host blood, determine if there are dietary differences between nestlings of the two bird species, and (3) if this difference also appears in the hematophagous avian vampire fly larvae living off nestling blood. We thus monitored climatic variables in nests of both the green warbler finch and small tree finch and measured carbon and nitrogen ratios and the stable isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in the nestling blood and larvae from nests of both species. Furthermore, we quantified amino acid composition in the blood of both focal host species.

METHODS

Study site and focal species

This study took place in Los Gemelos (0°37'50" S, 90°23'25" W, 569 - 634 m a.s.l.), a protected area within the Galapagos National Park on Santa Cruz Island in the Galapagos Islands, Ecuador. This study site is a humid forest mainly characterized by the endemic daisy tree, *Scalesia pedunculata*, along with other trees and shrubs, such as the endemic guayabillo tree, *Psidium galapageium*. Additionally, the field site includes introduced plant species, such as the guava tree, *P. guajava*, and the highly invasive raspberry, *Rubus niveus*, among others (Rentería et al., 2012; Jäger et al., 2017; Walentowitz et al., 2021); however, in our study area, there has been long-term management of *R. niveus* by the Galapagos National Park (Cimadam et al., 2019).

Samples were collected from two avian host species, the green warbler finch, *Certhidia olivacea*, and the small tree finch, *Camarhynchus parvulus*. Nests of these two host species were monitored in 2021-2022 during the incubation and nestling/feeding phases. Nests were visited every 2-3 days to confirm activity and hatching date. Nestlings were considered zero days old on hatch date, when the first egg hatched. All nests were monitored until activity ceased, which was confirmed by visual observations and filming with an endoscopic camera. Once a nest was no longer active, it was collected and avian vampire fly larvae and pupae were quantified.

Nest climate data collection

We used data loggers to measure temperature and humidity in the nest base of green warbler finch and small tree finch nests between 6 February - 2 May 2021 and 16 February -

21 April 2022. Hygrometers (iButton® DS1923, Maxim Integrated), recording both temperature and humidity, were placed in the nest base of eight green warbler finch nests and 11 small tree finch nests in 2021 and four green warbler finch and five small tree finch nests in 2022. Additionally, thermochrons (iButton® DS1925, Maxim Integrated), measuring temperature only, were placed in two green warbler finch nests and one small tree finch nest in 2021 and in four green warbler finch and two small tree finch nests in 2022, due to a limited number of hygrometers. All data loggers were placed in the nest base under the top base layer where avian vampire fly larvae commonly develop and pupate. Climate data was recorded every 30 min. during the nestling phase. Data loggers were removed from each nest after termination of breeding activity. All data was downloaded via the OneWireViewer software. Percent relative humidity (RH) data were automatically compensated for temperature and saturation drift within each data logger. We only used data points from data loggers when we could confirm at least one nestling was still alive in the nest.

Blood and larvae field collections

Nestlings

Blood samples were collected between 12 February to 3 May 2021 from 36 green warbler finch nestlings of 21 nests and 16 small tree finch nestlings of 9 nests at the age of 5-6 days. Nestlings were removed from their nest for a maximum of 15 minutes. Simultaneously standard body measurements and weight of each nestling were recorded. Each nestling was also checked for effects of avian vampire fly larval parasitism. One to two drops of blood (<30 µL) were collected from the brachial vein in the left wing of each nestling using a new non-heparinized microcapillary tube. Immediately, the blood from each nestling was transferred onto separate fiber glass filter discs (the size of a hole punch circle) and stored in 1.5 mL Eppendorf tubes. Each fiber glass filter disc with blood was dried at approximately 40°C for one hour. After drying, the Eppendorf tubes were stored in a cool, dry place until exportation.

Larvae from natural nests

From 15 February - 3 May 2021, we collected avian vampire fly larvae from green warbler finch and small tree finch nests once nest activity ceased. All larvae were collected from nests in which nestling blood samples were taken for stable isotope analysis. One to five whole larvae per nest were collected and stored in separate 1.5 mL Eppendorf tubes with 70% ethanol. In total, 32 avian vampire fly larvae from eight green warbler finch nests and 32 larvae from seven small tree finch nests were collected. All larvae were 2nd and 3rd instar stages. These samples were stored at room temperature until stable isotope analysis was conducted in Austria.

Stable isotope analysis

Stable isotope analyses of all samples (nestling blood, avian vampire fly larvae, and larval diet) were conducted at BOKU, Tulln, Austria. Total C and N analysis and bulk ¹³C and ¹⁵N isotope analysis, were conducted by elemental isotope ratio mass spectrometry. For samples stored in alcohol samples were repeatedly (3 times) washed and dried at 40°C to remove traces of alcohol. These and other samples were then dried (40°C for 72 h), either accurately weighed (3-5 mg) or just put into (in the case of the fiberglass filter discs) 9 by 5 mm tin cups. Samples were analyzed using a Thermo Flash 2000 Organic Elemental analyzer, linked to a Thermo Delta V Advantage automated isotope ratio mass spectrometer (IRMS) (Thermo, Bremen, DE). A full complement of internal and external standards was run with the samples to calculate isotopic ratios and %C and %N values. The isotope ratios were expressed as parts

per thousand per mille (‰) or δ deviation from the internationally recognized standards Vienna Pee Dee Belemnite (VPDB) and ambient air.

Amino Acid analysis

Stock amino acids

Amino acid stock solutions for standards were made from purchased pure amino acids and dissolved in Millipore water. Standards included 0.1M concentrations of l-alanine, l-leucine, l-arginine, l-proline, l-cysteine, l-asparagine, d-serine, l-glycine, l-threonine, l-lysine, l-phenylalanine and l-aspartic acid, 0.05M concentration of l-valine, 0.02M concentration of l-isoleucine and l-methionine, and a 0.005M concentration of l-glutamic acid and l-tyrosine.

Sample preparation - bird blood digestion for amino acid determination

Half of a disc of quartz filter containing a drop of the host blood was used for analysis. Blood samples from 18 green warbler finch and 10 small tree finch nestlings were analyzed to quantify amino acid levels. To each disc sample, 500 μ L 6M HCl solution was added and samples incubated at 110 °C for 24 hours in sealed glass vials. After incubation, once samples reached room temperature, they were exposed to nitrogen (N₂) gas for up to 24 hours to evaporate the HCl, and then diluted in 500 μ L of deionized water.

Derivatization of amino acids

Derivatization of samples was conducted following protocols from Bertrand et al. (2008). In brief, 300 μ L of either amino acid standard stock solution or bird blood sample was added to a 1 ml glass vial. Then, 300 μ L alcohol EtOH was added to each tube, and vortexed 10 s. Then, 75 μ L pyridine was added to each tube, followed by 165 μ L NaOH, and vortexed for 10s after each addition. After, 100 μ L ethyl chloroformate was added to each tube, followed by 60 μ L ethanol-stable chloroform. This solution was then left for 5 min. for the CO₂ to evaporate. Finally, 750 μ L of ethanol-stable chloroform was added and each tube was vortexed. All reagents used were of gas chromatography quality and all necessary health and safety precautions adhered to (e.g., all activities were conducted under the fume hood).

The organic chloroform layer was extracted from each sample, placed in a new tube and evaporated using N₂ gas for generally 2-3 hours until dry and 200 μ L of iso-octane was added to each sample to resuspend it. For each sample, 100 μ L of the resuspension was decanted into a 1.5 mL glass vial containing a ~150 μ L glass insert for gas chromatography.

Gas chromatography

Amino acid samples were run on an HP 5890 Series II Plus gas chromatograph. Samples were analyzed by injecting 1 μ L of the organic layer. A non-chiral RTX1701 column (60 m long x 0.25 mm diameter, 1 μ m thickness; Restek) was used. Helium was used as the carrier gas with a flow rate of 1.2 mL min⁻¹. The oven program consisted of an initial temperature set for 120 °C for 10 min, followed by a ramp of 20.0 °C/min, with a final temperature of 275 °C for 10 min. Total run time was 27.75 min. Using the ChemStation program (Agilent Technologies, USA), peaks of amino acids were identified by comparing with retention times from reference amino acid peaks in the chromatograms (Supplementary Table 1).

Statistical Analysis

Nest climate

For this study, 13,045 data points were included in the analysis for temperature and 9,407 data points included for percent humidity. Descriptive statistics were obtained using

describe.by function in the *psych* package (Revelle, 2022). Daily mean values for nest base temperature and humidity (% RH) were calculated for days with complete data (24 hrs). Daily mean ambient temperature and humidity (% RH) values from the “El Carmen” weather station near the study site were also used in the analysis (CDF, unpublished data). To account for the estimated age at which nestlings begin to thermoregulate (Dunn, 1976, 1979; Ricklefs and Hainsworth, 1968), each data point was classified into one of two age groups, based on the age of nestlings when the data was collected: 0-6 days old and 7-14 days old.

Initially, individual linear mixed models were run using *lme4* package (Bates et al., 2015) and included temperature and humidity as dependent variables. Overall, 194 daily mean values for nest base temperature and 130 daily mean values for nest base humidity were used in the models. Fixed effects included species, age group, year, number of nestlings alive each day, nest height (m), number of parasites, and the ambient temperature or humidity. Additionally, interaction terms in each model included: 1) species*ambient temperature in the temperature model / species*ambient humidity in the humidity model, 2) species*age group, and 3) species*number of parasites. In the temperature model, the interaction term nest humidity*species was also included. Each model also included iButtonID/NestID as a nested random factor. All continuous variables were mean centered and scaled before running models and the log of the number of parasites was taken and used in the analyses. Robust linear mixed models were run using the *robustlmm* package (Koller, 2016) for both nest base temperature and humidity to account for non-normality and/or heteroscedasticity.

Stable isotopes

Six separate linear mixed models were run using the *lme4* package to analyze the effect of species on $\delta^{13}\text{C}$, mean $\delta^{15}\text{N}$, and mean C:N ratios of nestling blood and larval values, with $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and C:N ratio of nestling blood and larvae as the dependent variables. Species, blood sampling date, and number of days the nestlings lived after sampling (as an indicator of nestling starvation) were included as fixed effects in all models. Nest ID was included as a random factor to control for relatedness between nestlings or larvae in the same nest. Due to non-normal residuals and heteroscedasticity, robust linear mixed models for nestling blood $\delta^{15}\text{N}$ and C:N values were run using the *robustlmm* package. Due to non-normal residuals, a generalized linear mixed model with a gamma family with log link was run for $\delta^{15}\text{N}$ larval values.

Mean $\delta^{13}\text{C}$, mean $\delta^{15}\text{N}$, and mean C:N were calculated for nestlings and larvae separately for each nest. Separate correlation tests were run in R Studio to test for correlations of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and C:N values between nestlings and larvae from each nest using the *corr.test* function. For testing all values of both species together, Spearman correlation tests were run due to non-normally distributed nestling values. For testing correlations using data from each species separately, Pearson correlation tests were run for green warbler finch $\delta^{13}\text{C}$ and C:N values and small tree finch $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. For green warbler finch $\delta^{15}\text{N}$ and small tree finch C:N, Spearman correlation tests were run due to non-normally distributed nestling values.

Amino acids

Based on the chromatograms from gas chromatography, we quantified the area for each identified amino acid peak per sample using the ChemStation program. We then summed up a group of defined pre-selected amino acid peak areas per sample for a total area. Peak areas of amino acids that had two peaks close together (methionine, phenylalanine, cysteine, asparagine) within the expected range of the amino acid were added together. For glutamic acid, the peak area of the expected peak was added with the area of its secondary peaks. Next,

for each amino acid, individual peak area was divided by total area per sample to obtain a percent composition value or relative abundance measure, a method routinely used for analyzing amino acid composition. All calculations were performed in Microsoft Excel.

Generalized linear mixed models were conducted with percent composition of each amino acid as the dependent variable. Species and sample collection date were included as fixed effects and nest ID as a random effect. All models were run using the *glmmTMB* function from the *glmmTMB* package (Brooks et al., 2017) using the beta family and logit link function.

All analyses were conducted in R Studio (R Core Team, 2021). A variance inflation test was done for all models using the *vif* function in the *car* package (Fox and Weisberg, 2019) to confirm the fixed effects were not highly correlated (all *vif* values were <2.98). For each linear mixed model, the residuals were inspected for normality using QQ plots, histograms, and a Pearson standardized versus residual plot. Each model was also inspected for heteroskedasticity using scaled residual plots. All beta models were assessed for overdispersion using the *DHARMA* package (Hartig, 2022). All dispersion values were < 1.0. The *lmerTest* package (Kuznetsova et al., 2017) was used to obtain p-values for the linear mixed models and generalized linear mixed model with the gamma family. The *summary* function and *tab_model* function in the *sjPlot* package (Lüdtke, 2021) were used to obtain p-values for the robust linear mixed models. For all statistical analyses, $p \leq 0.05$ was considered significant. All figures were made using the *ggplot2* package (Wickham, 2016) in R Studio.

RESULTS

Nest climate

Small tree finch and green warbler finch nest base temperatures differed significantly in relation to nest base humidity. At lower nest base humidity levels (e.g., 60%), nest base temperature was higher in the small tree finch nests than in the green warbler finch nests. Small tree finch nest temperature declined more at higher nest humidity levels than green warbler finch nest base temperature (significant nest base humidity*species interaction, $p = 0.02$; Table 2, Figure 1). As nest base humidity reached 100%, nest base temperatures for both species were the most similar (Figure 1). The difference between species became accentuated when accounting for thermoregulation (marginally significant age group*species interaction, $p < 0.07$, Table 2): in the age group 0-6 days, representing pre-thermoregulation, nest base temperatures were higher in the small tree finch than in the green warbler finch. However, in the age group 7-14 days, the small tree finch nest base temperature decreased, while it increased in green warbler finch nests (Figure 2a). There was no significant effect of year or nest height on nest temperature (Table 2).

Table 1: Descriptive statistics for nest climate by species.

	Mean $\pm$ SE	n	Min	Max
Temperature (°C)				
<i>Small tree finch</i>	28.77 $\pm$ 0.05	6930	18.50	38.89
<i>Green warbler finch</i>	28.75 $\pm$ 0.04	6115	18.11	38.1
Humidity (%)				
<i>Small tree finch</i>	79.19 $\pm$ 0.20	5335	18.27	100
<i>Green warbler finch</i>	80.33 $\pm$ 0.22	4072	40.75	100

Small tree finch nests showed a lower minimum humidity (% RH) and a greater humidity range than green warbler finch nests (Table 1). Nest base humidity (% RH) also differed between age groups, but this difference varied between species. Nest base humidity declined from 0-6 days to 7-14 days in green warbler finch nests, but increased in small tree finch nests (marginally significant age group*species interaction, $p < 0.07$; Table 3; Figure 2b).

Table 2: Model output of the robust linear mixed model for nest base temperature as the dependent variable. All continuous, numeric variables were first mean-centered and scaled. p-values ≤ 0.05 are denoted in bold. GWF = green warbler finch.

Predictors	Estimate $\pm$ SE	t-value	p-value
Intercept	30.10 $\pm$ 0.80	37.81	<0.001
Species (GWF)	-2.37 $\pm$ 0.94	-2.51	0.012
Age group (7-14 days)	0.32 $\pm$ 0.18	1.81	0.070
Year (2022)	1.14 $\pm$ 0.93	1.23	0.220
Number nestlings	-0.21 $\pm$ 0.20	-1.07	0.286
Number of parasites	-0.01 $\pm$ 0.11	-0.11	0.914
Nest height (m)	0.12 $\pm$ 0.49	0.25	0.803
Ambient temperature	-0.02 $\pm$ 0.09	-0.25	0.799
Nest base humidity	-2.15 $\pm$ 0.13	-16.32	<0.001
Ambient temperature*species (GWF)	0.17 $\pm$ 0.19	0.92	0.360
Age group (7-14 days)*species (GWF)	-0.49 $\pm$ 0.31	-1.54	0.123
Number parasites*species (GWF)	0.19 $\pm$ 0.18	1.06	0.287
Nest base humidity*species (GWF)	0.43 $\pm$ 0.19	2.32	0.020

Table 3: Model output of the robust linear mixed model for percent humidity as the dependent variable. All continuous, numeric variables were first mean-centered and scaled. p-values ≤ 0.05 are denoted in bold. GWF = green warbler finch.

Predictors	Estimate $\pm$ SE	t-value	p-value
Intercept	76.41 $\pm$ 7.75	9.86	<0.001
Species (GWF)	1.98 $\pm$ 7.53	0.09	0.793
Age group (7-14 days)	1.83 $\pm$ 2.32	0.78	0.432
Year (2022)	0.69 $\pm$ 7.51	0.09	0.927
Number nestlings	0.15 $\pm$ 2.52	0.06	0.953
Number of parasites	1.04 $\pm$ 0.89	1.17	0.244
Nest height (m)	-5.69 $\pm$ 4.05	-1.41	0.160
Ambient humidity	1.43 $\pm$ 1.80	0.80	0.425
Ambient humidity*species (GWF)	4.03 $\pm$ 2.48	1.62	0.105
Age group (7-14 days)*species (GWF)	-7.54 $\pm$ 4.17	-1.81	0.070
Number parasites*species (GWF)	-1.44 $\pm$ 1.49	-0.97	0.332

Stable isotopes in nestling blood

Small tree finch nestlings showed significantly higher $\delta^{13}\text{C}$ levels than green warbler finch nestling ($p = 0.0013$; Table 4 & 5, Figure 3a). In contrast, $\delta^{15}\text{N}$ levels were only marginally lower in small tree finch nestlings compared to green warbler finch nestlings

($p = 0.058$; Table 5, Figure 3b). The C:N ratio of nestling blood was not significantly different between the two species (Table 6, Figure 3c). Both the $\delta^{15}\text{N}$ levels and the C:N ratios were lower in nestlings that survived longer.

Table 4: Values of stable isotopes for both hosts and parasites including nestling and adult host life stages and larval stage of the avian vampire fly. Values are reported as mean $\pm$ standard error (SE). GWF = green warbler finch, STF = small tree finch.

Species	N	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	C:N
<i>Green Warbler Finch</i>				
Nestlings (2021)	36	-27.92 ± 0.12	8.89 ± 0.51	3.56 ± 0.04
<i>Small Tree Finch</i>				
Nestlings (2021)	16	-26.37 ± 0.46	7.52 ± 0.51	3.83 ± 0.15
<i>Avian Vampire Fly</i>				
Larvae	32	-27.62 ± 0.22	11.17 ± 0.29	4.45 ± 0.09
(Host: GWF nests)				
Larvae	32	-26.39 ± 0.23	11.66 ± 0.53	4.38 ± 0.12
(Host: STF nests)				

Table 5: Linear and robust linear mixed models for stable isotope values of nestling blood. p -values ≤ 0.05 are denoted in bold. GWF = green warbler finch

Dependent variable	Fixed effects	Estimate $\pm$ SE	t-value	p-value
$\delta^{13}\text{C}$ nestling blood	Intercept	-26.51 ± 1.072	-24.72	<0.0001
	Species (GWF)	-1.60 ± 0.447	-3.57	0.0013
	Day of year	-0.002 ± 0.010	-0.27	0.790
	Number days active after sampled	0.06 ± 0.061	0.90	0.378
$\delta^{15}\text{N}$ nestling blood	Intercept	8.96 ± 1.447	6.20	<0.001
	Species (GWF)	1.15 ± 0.604	1.90	0.058
	Day of year	-0.01 ± 0.014	-0.46	0.645
	Number days active after sampled	-0.18 ± 0.827	-2.18	0.029
C:N ratio nestling blood	Intercept	3.87 ± 0.202	19.17	<0.001
	Species (GWF)	-0.10 ± 0.084	-1.24	0.214
	Day of year	0.001 ± 0.002	0.37	0.714
	Number days active after sampled	-0.040 ± 0.011	-3.44	0.001

Amino acids

Amino acid percent composition did not significantly differ between the two host species (Table 7, Figure 5). The day of year each sample was collected, included in the models as a covariate, had a significant effect on percent composition for alanine ($p = 0.04$), serine ($p = 0.023$), and glutamic acid ($p = 0.007$; Table 7).

Table 7: Results of a generalized linear mixed model with beta family and logit link, with each amino acid as a separate dependent variable, species and day of year as fixed effects and NestID as a random factor. The dependent variable for each model was percent composition of each amino acid in nestling blood samples from both the small tree finch and the green warbler finch. p -values ≤ 0.05 are denoted in bold. GWF = green warbler finch.

Dependent variable	Predictors *	Estimates $\pm$ SE	z-value	p-value
<i>Alanine</i>	Intercept	-2.70 $\pm$ 0.14	-19.62	<0.001
	Species (GWF)	0.06 $\pm$ 0.07	0.76	0.447
	Day of year	0.004 $\pm$ 0.002	2.05	0.040
<i>Glycine</i>	Intercept	-3.08 $\pm$ 0.15	-20.68	< 0.001
	Species (GWF)	0.04 $\pm$ 0.08	0.52	0.603
	Day of year	0.001 $\pm$ 0.00	0.46	0.645
<i>Valine</i>	Intercept	-2.26 $\pm$ 0.13	-17.96	< 0.001
	Species (GWF)	0.07 $\pm$ 0.07	1.09	0.277
	Day of year	0.001 $\pm$ 0.002	0.83	0.408
<i>Aspartic Acid</i>	Intercept	-2.61 $\pm$ 0.21	-12.59	< 0.001
	Species (GWF)	0.03 $\pm$ 0.11	0.27	0.785
	Day of year	-0.004 $\pm$ 0.003	-1.48	0.140
<i>Leucine</i>	Intercept	-1.67 $\pm$ 0.11	-15.42	< 0.001
	Species (GWF)	-0.03 $\pm$ 0.06	-0.57	0.570
	Day of year	0.001 $\pm$ 0.001	0.50	0.617
<i>Isoleucine</i>	Intercept	-3.04 $\pm$ 0.09	-33.50	< 0.001
	Species (GWF)	0.02 $\pm$ 0.05	0.36	0.717
	Day of year	0.001 $\pm$ 0.001	0.60	0.551
<i>Proline</i>	Intercept	-2.68 $\pm$ 0.16	-16.79	< 0.001
	Species (GWF)	0.03 $\pm$ 0.09	0.31	0.754
	Day of year	0.003 $\pm$ 0.002	1.53	0.126

<i>Asparagine</i>	Intercept	-2.89 ± 0.20	-14.13	< 0.001
	Species (GWF)	-0.11 ± 0.11	-1.01	0.312
	Day of year	0.003 ± 0.003	1.19	0.232
<i>Lysine</i>	Intercept	-2.89 ± 0.23	-12.80	< 0.001
	Species (GWF)	0.10 ± 0.12	0.84	0.399
	Day of year	-0.002 ± 0.002	-0.73	0.469
<i>Glutamic Acid</i>	Intercept	-1.27 ± 0.16	-7.85	< 0.001
	Species (GWF)	0.03 ± 0.09	0.384	0.701
	Day of year	-0.005 ± 0.002	-2.70	0.007
<i>Serine</i>	Intercept	-7.01 ± 0.57	-12.41	< 0.001
	Species (GWF)	-0.02 ± 0.30	-0.07	0.942
	Day of year	0.02 ± 0.01	2.28	0.023
<i>Arginine</i>	Intercept	-4.13 ± 0.53	-7.78	< 0.001
	Species (GWF)	0.07 ± 0.29	0.24	0.814
	Day of year	-0.003 ± 0.01	-0.51	0.611
<i>Methionine</i>	Intercept	-4.62 ± 0.40	-11.71	< 0.001
	Species (GWF)	-0.12 ± 0.18	0.68	0.499
	Day of year	0.01 ± 0.01	1.50	0.135
<i>Phenylalanine</i>	Intercept	-2.96 ± 0.22	-13.35	< 0.001
	Species (GWF)	-0.13 ± 0.12	-1.09	0.275
	Day of year	0.003 ± 0.003	1.08	0.28
<i>Cysteine</i>	Intercept	-4.93 ± 0.44	-11.14	< 0.001
	Species (GWF)	-0.22 ± 0.23	-0.96	0.336
	Day of year	-0.01 ± 0.01	-0.81	0.418

Stable isotopes in avian vampire fly larvae

$\delta^{13}\text{C}$ levels were significantly higher in larvae collected from small tree finch nests, compared to larvae from green warbler finch nests ($p = 0.032$; Table 6, Figure 3a). $\delta^{13}\text{C}$ levels increased with day of year ($p < 0.028$) and were lower in nests where nestlings survived longer ($p < 0.024$) (Table 6). There were no significant differences of larval $\delta^{15}\text{N}$ nor C:N ratios in larvae collected from the two host species (Table 6, Figure 3).

Table 6: Linear mixed models for $\delta^{13}\text{C}$ and C:N ratio larval values as dependent variables and of a robust linear mixed model for $\delta^{15}\text{N}$ larval values as dependent variable. Fixed effects in each model included species, day of year, and number of days a nest was active after the sampling date. NestID was included as a random factor to control for pseudoreplication. All p-values ≤ 0.05 are denoted in bold. GWF = green warbler finch

Dependent variable	Fixed effects	Estimate $\pm$ SE	t-value	p-value
$\delta^{13}\text{C}$ larvae (lmer)	Intercept	-27.92 $\pm$ 0.87	-32.23	<0.001
	Species (GWF)	-0.86 $\pm$ 0.34	-2.50	0.032
	Day of year	0.02 $\pm$ 0.01	2.61	0.028
	Number days active after sampled	-0.12 $\pm$ 0.05	-2.69	0.024
$\delta^{15}\text{N}$ larvae (glmer, gamma family)	Intercept	8.29 $\pm$ 2.84	2.92	0.004
	Species (GWF)	0.26 $\pm$ 1.11	0.23	0.817
	Day of year	0.04 $\pm$ 0.03	1.38	0.168
	Number days active after sampled	-0.10 $\pm$ 0.15	-0.68	0.494
C:N ratio larvae (lmer)	Intercept	4.75 $\pm$ 0.58	8.18	<0.001
	Species (GWF)	-0.04 $\pm$ 0.23	-0.18	0.86
	Day of year	-0.01 $\pm$ 0.01	-1.05	0.32
	Number days active after sampled	0.04 $\pm$ 0.03	1.36	0.20

Stable isotope values showed no significant correlation between nestling blood and larvae (pooled values for both species, Spearman correlation test, $\delta^{13}\text{C}$: $S = 308$, $p = 0.094$; $\delta^{15}\text{N}$: $S = 334$, $p = 0.137$; C:N: $S = 751.51$, $p = 0.212$, $n = 15$; Figure 4). When testing values for small tree finches and green warbler finches separately, $\delta^{15}\text{N}$ for green warbler finches and larvae were significantly correlated ($S = 16$, $p = 0.022$, $n = 7$) and C:N values for small tree finches and larvae marginally ($S = 95.716$, $p = 0.074$, $n = 8$) correlated.

DISCUSSION

Nest climate

Nest base daily mean temperatures were significantly different between species in relation to daily mean nest base humidity. At lower nest base humidity levels, the small tree finch nests had higher temperatures than the green warbler finch nests; however, at higher nest base humidity levels, the nest base temperature of small tree finches decreased more than that of green warbler finches. At higher humidity levels, it seems neither species can maintain a higher temperature. One possible explanation for this is that at higher humidity levels, water molecules evaporate and cool down the nests (i.e., evaporative cooling). Additionally, our findings suggest that small tree finch nests may be better at maintaining temperatures at lower

humidity levels, and thus, their nests may be better insulated and have a lower thermal conductance than green warbler finch nests. This may be potentially due to differences in nest material and/or nest structure, such as nest wall thickness and/or nest hole diameter (Skowron and Kern, 1980; Windsor et al., 2013; Heenan and Seymour, 2011), as the small tree finch nests are larger than green warbler finch nests (ST, CP pers. obs.). Additionally, other factors, such as exposure to wind, female brooding patterns, at least early in the nesting phase, and nestlings' influence on nest microclimate, might differ between species and explain these differences in variation.

Nest climate in relation to nestling age

Nest base temperature and humidity levels varied between age groups and species. In the younger age group, small tree finch nest base temperatures were higher than for green warbler finch nests. In the older age group, when nestlings are expected to thermoregulate, nest base temperature decreased in the small tree finch, but increased in green warbler finch nests, compared to the younger age group. Nest base humidity contrastingly increased in small tree finch nest, but decreased in green warbler finch nests. Factors affecting these observed differences in nest climate between species may include female brooding behavior, nestling thermoregulation, resting metabolic rates of nestlings, and nestling size. During the incubation phase, small tree finch females have longer bout durations than green warbler finch females (BK, unpublished data); however, it is unknown if this difference between these species is similar for brooding in the nestling phase. Additionally, female small tree finches are larger than green warbler finch females, so when small tree finch females brood nestlings, the females may cover the nest base more than green warbler finch females. However, when nesting females terminate brooding when nestlings are approximately six days old, the nestlings may then be subject to self-thermoregulation and nestlings of the two species may differ in their huddling behaviors (e.g., Dunn 1979), potentially accounting for a greater range of temperatures in small tree finch nests than green warbler finch nests at older nestlings ages. Although we accounted for nestling thermoregulation by considering age groups at the estimated age it takes place, songbird/passerine nestlings may also thermoregulate at earlier ages when more than one nestling is present in the nest (Dunn, 1976, 1979). However, in our study, we did not find a significant effect of number of nestlings in the nests on nest base climate. Furthermore, body mass and BMR are positively correlated in tropical passerines, with heavier birds having higher metabolic rates (BMR) (Bushuev et al. 2018). In our study in 2021, however, body mass did not significantly differ between 5-6 day old small tree finch and green warbler finch nestlings ($U=266$, $p=0.267$, $n=59$). Therefore, it would be expected that nestlings of these two host species would have similar BMRs, at least based on body mass.

Temperature and humidity levels in the nest base may affect larval growth and development of nest parasites like the avian vampire fly. The higher temperature in small tree finch nests at an early age and/or the greater range in temperature at a later age may positively influence larval development, accounting for the observed differences in larval stage proportions in Cimadom et al. (2021). Currently, at the CDRS laboratory, 2nd instar avian vampire fly larvae are reared at 30-31 °C and 50-70% RH (P. Lahuatte, pers. comm.) after adjustments from 36 °C and 80-95% RH (Lahuatte et al. 2016) improved larval production. Other studies on insects have reported faster larval growth and development in lab conditions ranging from 32-35 °C and at 75% RH compared to lower temperatures and 55% RH (e.g., lesser mealworm: Kotsou et al. 2021; mealworm: Machona et al. 2023; nest fly: Bennett and Whitworth 1991; black soldier fly: Palma et al. 2019). Additionally, lower temperatures (e.g. 20 °C) may in fact stunt larval development, as reported for the mealworm

Tenebrio molitor (Machona et al., 2023). Our two focal species had mean percent RH values within or near the range that the avian vampire fly lab currently uses for rearing the avian vampire fly. However, while humidity is also an important factor in larval growth and development of insects (Machona et al., 2023), these humidity levels may not be the most beneficial for larval development of the avian vampire fly, as our focal host species could be suboptimal hosts.

To date there is no systematic study on the optimal temperature and humidity levels required for optimal growth of the other larval stages of the avian vampire fly in the laboratory. Further studies should be conducted on how temperature and humidity affect larval development and growth in the avian vampire, these are necessary to determine optimal developmental conditions. Additionally, further information on how nest climate varies between other host species in similar and different habitats in Galapagos would assist us in understanding the avian vampire fly's thermal thresholds and the ranges for larval development in the wild.

Stable isotope trophic signatures and C:N

In line with our hypothesis, dietary differences between small tree finch and green warbler finch were detectable in nestling blood composition and consequently in the hematophagous parasitic larvae. The $\delta^{13}\text{C}$ values were higher both in the blood of the more omnivorous small tree finch nestlings and in their *Philornis* larvae. Other factors, such as the proportion of lipids in the blood, can also affect isotopic $\delta^{13}\text{C}$ values (Cherel et al., 2005), as lipids have a more negative $\delta^{13}\text{C}$ than proteins (Deniro and Epstein 1977). We did not defat blood samples of either host species; however, variations of the amount of fat in avian diets may affect lipid levels in the blood of certain avian species (Bavelaar and Beynen, 2003; Regar et al., 2019; although see Baighi and Nobakht, 2017). Therefore, the observed differences in $\delta^{13}\text{C}$ in our host species may, in fact, be partially attributed to blood lipid levels. Furthermore, C isotope ratios ($\delta^{13}\text{C}$) are also relatively conserved across trophic levels, but vary depending on the photosynthetic pathways and the degree of water stress experienced at the base of the food web (Peterson and Fry, 1987; Still and Powell, 2010; Basu et al., 2015).

$\delta^{15}\text{N}$ generally increases 3-4‰ from diet to consumer, as ^{15}N is more highly retained than ^{14}N (Deniro and Epstein 1981). In our study, green warbler finch nestling blood $\delta^{15}\text{N}$ signatures were on average 1.5‰ higher compared to small tree finch nestling blood, suggesting dietary sources of the green warbler finch are half a level up on the trophic ladder (assuming an average diet to consumer enrichment of 3‰) and therefore more protein rich. The higher $\delta^{15}\text{N}$ of the green warbler finch is in line with the observation of a more insectivorous diet, compared to the small tree finch who partly also consume seeds and plant materials (Loo et al., 2019).

Despite the suggested difference in trophic levels of small tree finch and green warbler finch nestlings, the hematophagous *Philornis* larvae in the nests of the two species did not differ significantly in $\delta^{15}\text{N}$. Our results indicate a characteristic trophic shift from host to larvae of $\sim 2 - 4\text{‰}$ $\delta^{15}\text{N}$ and $\sim 0.02 - 0.3\text{‰}$ $\delta^{13}\text{C}$ isotopic enrichment, similar to other studies on host-parasite enrichment (Post, 2002; Gómez-Díaz and González-Solís, 2010). However, the $\delta^{13}\text{C}$ isotopic enrichment for small tree finch nestlings and their larvae was quite low in our study. The absence of differences in $\delta^{15}\text{N}$ between larvae from nests of

both species could be possibly explained by the fact that the larvae are probably under protein stress or starvation conditions which often increases the delta $\delta^{15}\text{N}$ signature (Olive et al., 2003), potentially overriding the trophic signature. However, when testing for correlations between nestlings and larvae from their nests, by species separately, we did find green warbler finch and larval $\delta^{15}\text{N}$ were highly correlated; however, this must be interpreted cautiously due to low sample size ($n=7$).

Blood C:N ratios were not significantly different between the two bird species, but most interestingly, nestlings with lower C:N ratios survived longer. Nestlings with more larval parasites may also have had depleted protein levels (and therefore higher blood C:N) due to parasitism, weakening them and causing these nestlings to die at an earlier age than those with lower C:N blood ratios. Alternatively, or additionally, diet composition may interact with other biotic factors such as the larval microbiota that influence larval development. Bacterial communities in avian vampire fly larvae collected from green warbler finch nests versus tree finch nests including the small tree finch, were significantly different in a prior study (Ben-Yosef et al., 2017). In this study, larvae from green warbler finch nests had microbial communities commonly with *Serratia* and *Acinetobacter* bacteria, while larvae from small tree finches had differing genera of bacteria (Ben-Yosef et al., 2017). These bacterial communities may interact with diet composition and inhibit larval development, as reported for tephritid flies with *Serratia* bacteria (Fitt and O'Brien, 1985).

Amino acid composition

Despite the differences in the dietary composition of the small tree finch and the green warbler finch nestlings observed in previous studies (e.g., Filek et al., 2018) and herein confirmed by the isotopic signatures, there were no significant differences in the amino acid percent composition between species. Additionally, due to limitations in our methodology, some amino acids, including tyrosine, tryptophan, and histidine were not detected/quantified for the analysis of blood amino acid composition. However, we did include three unidentified peaks (probable amino acids) in our percent composition calculations, which may have accounted for some of the missing amino acids. Given C:N in nestling blood was not significantly different between species, it is not surprising that the amino acid composition was also similar between species. Protein deficiencies or increases in protein can influence amino acid concentrations in bird blood (Hebert et al., 2002); however, in a prior study on herring gulls, these differences were only detected between gulls in different habitats, not ones occupying the same habitat. This suggests blood quality of avian vampire fly hosts may also be more influenced by differences in habitat and location than by different hosts occupying the same habitat.

Host quality between the small tree finch and the green warbler finch does not appear to vary significantly in our study, based on the parameters/characteristics investigated. However, these species may still differ in quality for avian vampire fly, in other factors that we did not measure but that directly influence larval development, or other life history factors that may influence oviposition success and/or larval survival. One limitation in our study was that we only measured blood values when nestlings were 5-6 days old. It may be that prey composition differs between species earlier or later in the nestling phase, potentially influencing C:N blood values. Additionally, while we investigated host blood quality, we did not investigate the hosts' tissue quality. As avian vampire fly larvae also feed on nestlings' tissue (Fessl et al., 2006), the C:N of host tissue may influence larval development/growth.

We also only measured relative abundance of each amino acid and not total amounts or concentrations of each amino acid in the blood as this would have been logistically very difficult. Thus, the small tree finch nestlings may have higher concentrations of amino acids that could influence avian vampire fly larval development, but we could not detect this. Further investigation into C:N and amino acid composition in tissue and host blood and tissue of younger and older nestlings and brooding females may provide additional insight into diet quality for avian vampire fly larval growth and/or development.

CONCLUSIONS

This study is the first to investigate directly the differences in quality between hosts of the avian vampire fly and the trophic impacts of diet quality on larval values of C and N stable isotopes. This data not only informs about the quality of diet of the larvae of avian vampire fly but also contributes general knowledge on the diet of endemic Darwin's finches. The avian vampire fly has a wide range of host species; thus, future studies could include investigating the factors related to the avian vampire fly's host preferences. These studies could include investigating nest conditions, microbiomes and optimal levels of protein in the diet of many other host species of the avian vampire fly larvae. Additionally, immune responses of the hosts may influence larval development, so it may also be beneficial to investigate nestlings' immune responses. Furthermore, there is currently limited information on the factors controlling larval development and growth in the field, which is important information for parasite control. Therefore, our study provides insights into host quality for an invasive parasite in the Galapagos useful for planning future studies and control initiatives. Additionally, these data will provide insight into natural conditions for larval growth and development that may help in optimizing the laboratory conditions for mass rearing of the avian vampire fly for the purpose of developing other control methods such as the sterile male technique. We therefore suggest building off our study by examining the effects of temperature variation in larval growth and development in experimental conditions, which may inform optimal conditions for mass rearing of avian vampire fly larvae.

Ethics statement: All applicable institutional and/or national guidelines for the care and use of animals were followed.

FIGURES

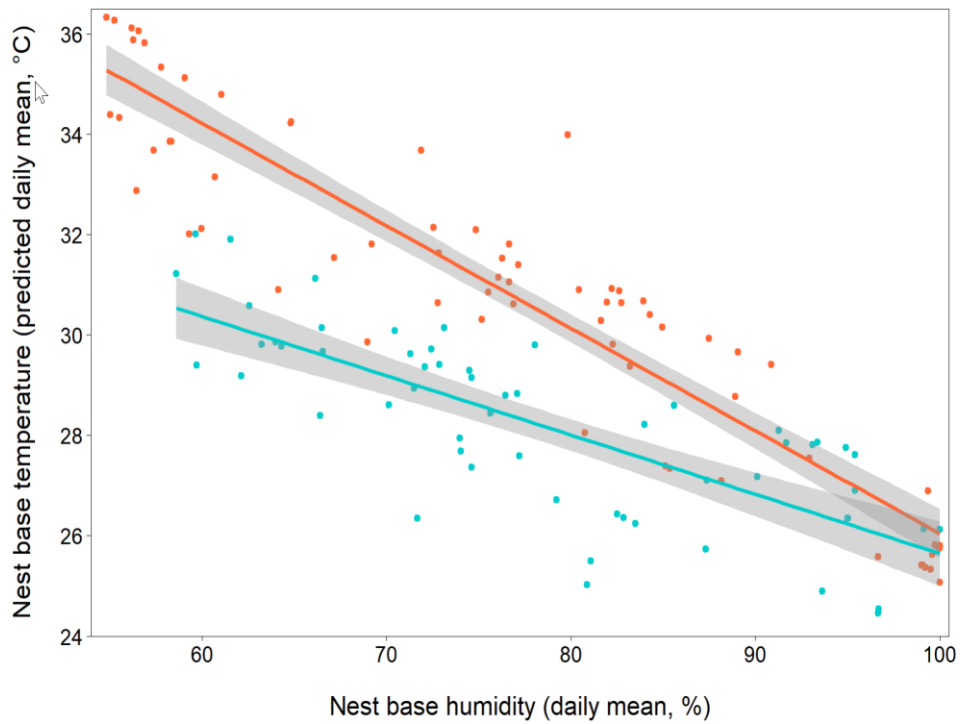


Figure 1: Nest base temperature relative to nest base humidity, for the small tree finch (orange) and green warbler finch (teal). Temperatures (dots) are displayed as daily means (°C). Lines shown are model predicted values for daily mean nest base temperature.

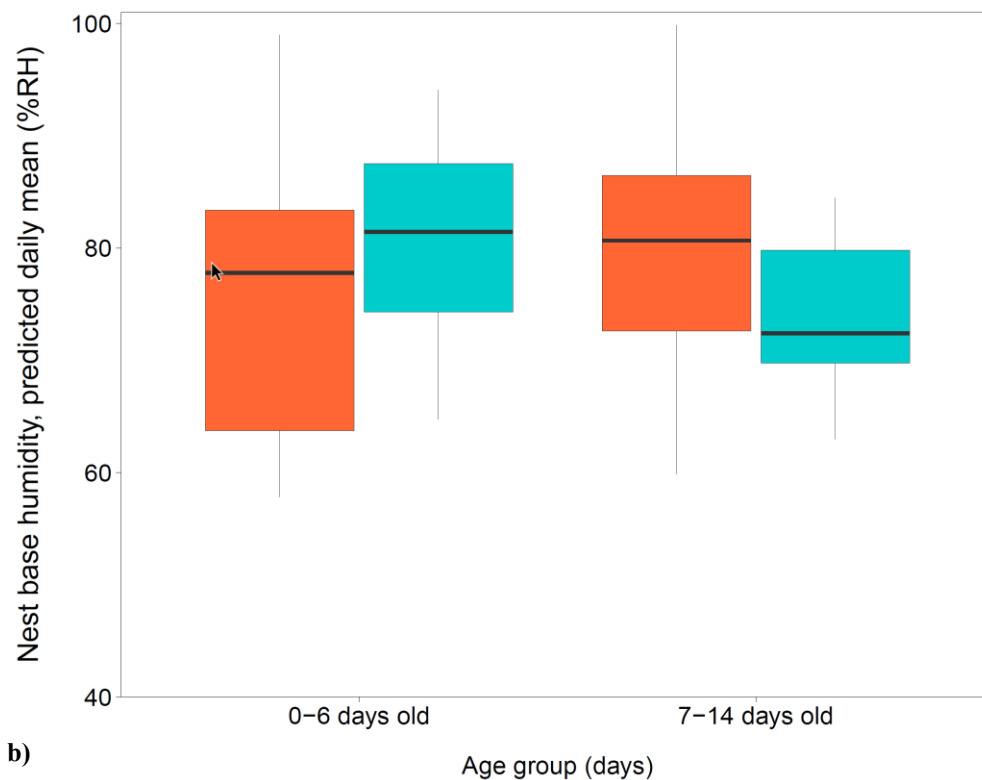
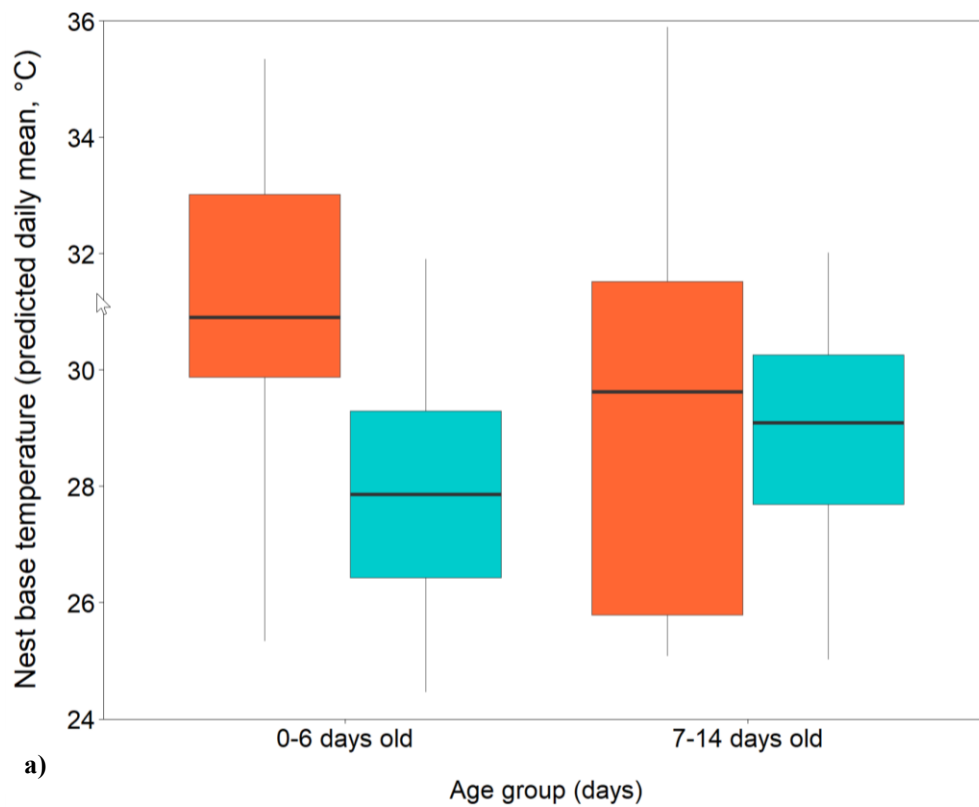


Figure 2: Nest base climate relative to age group (days), per species, for a) nest base temperature (°C) and b) nest base humidity (% RH). Species include the small tree finch (orange) and the green warbler finch (teal). Both nest base temperature and humidity are displayed as daily means. Lines shown are model predicted values for daily mean nest base temperature and humidity.

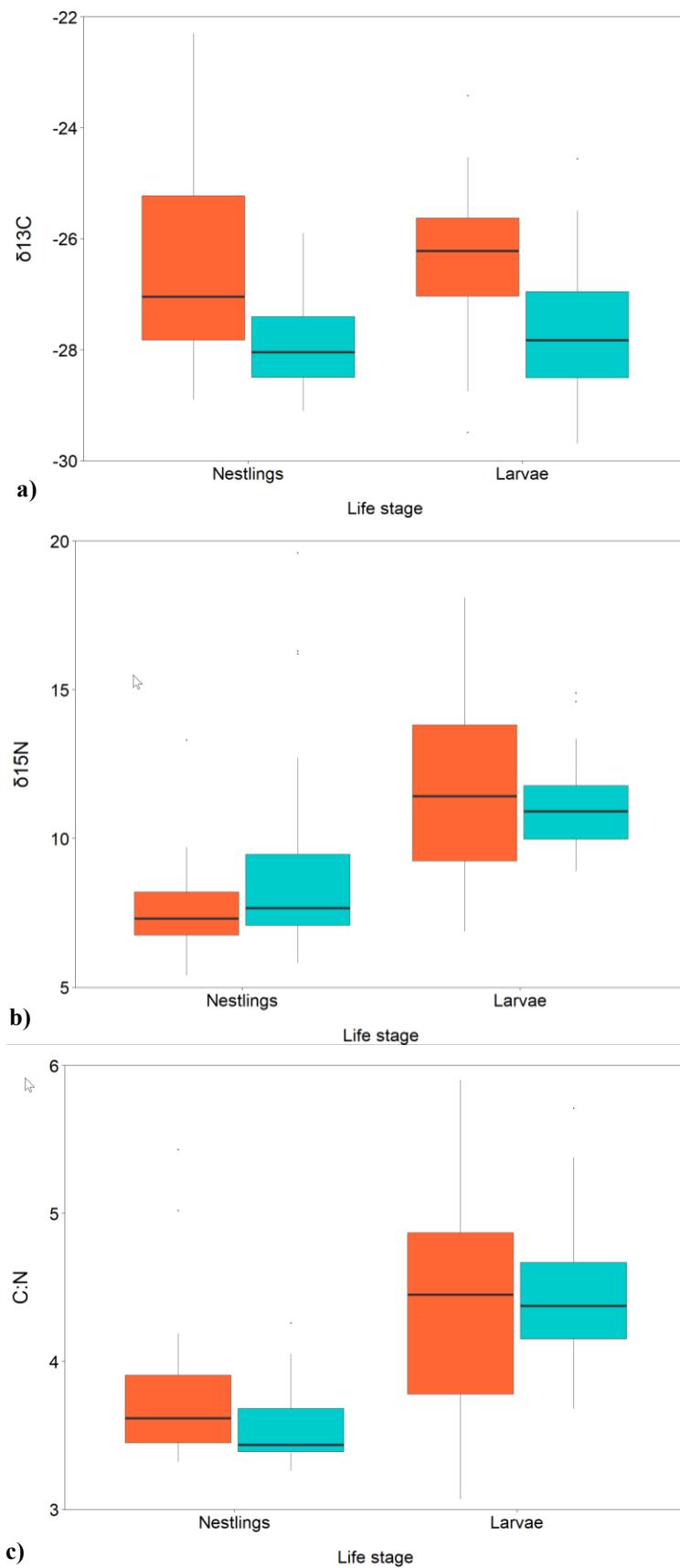


Figure 3: Boxplot of nestling blood and avian vampire fly larvae stable isotope values for a) $\delta^{13}\text{C}$, b) $\delta^{15}\text{N}$, and c) C:N for the small tree finch (orange) and the green warbler finch (teal).

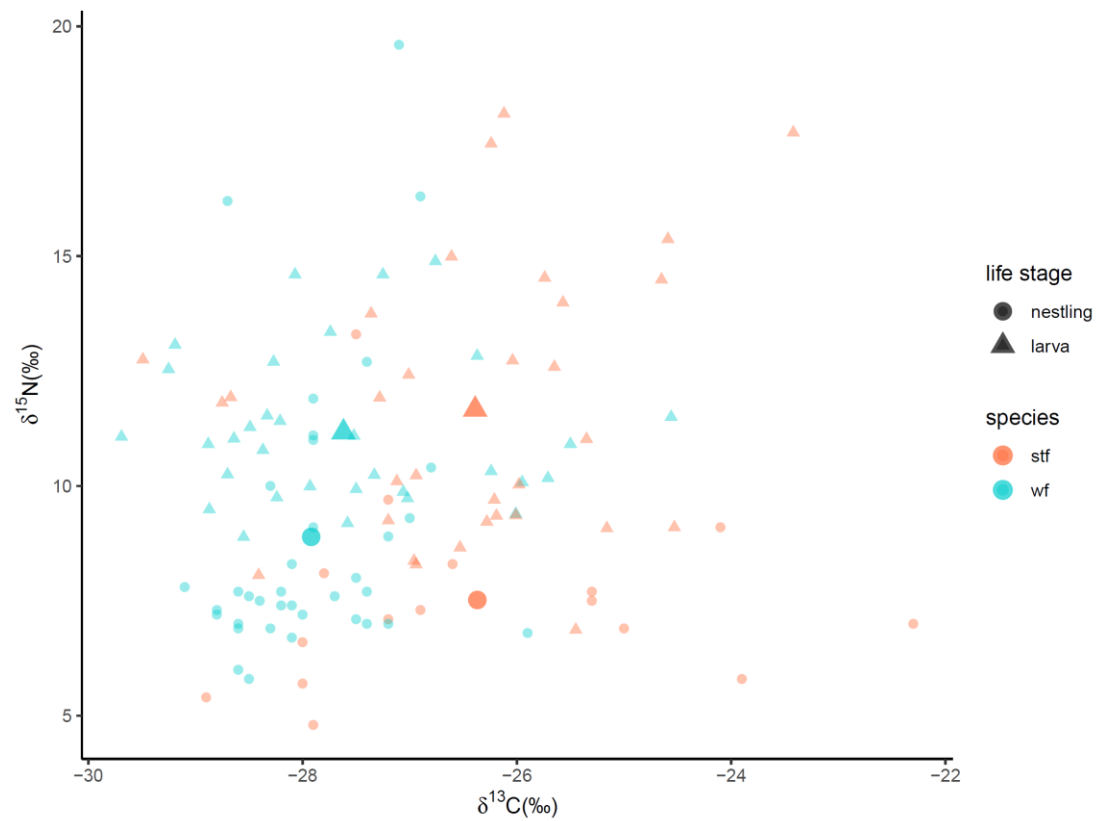


Figure 4: Nestling blood and larval $\delta^{13}\text{C}$ vs $\delta^{15}\text{N}$ values. Data are displayed as small diamonds (nestlings) and triangles (larvae) with mean values displayed as large diamonds and triangles.

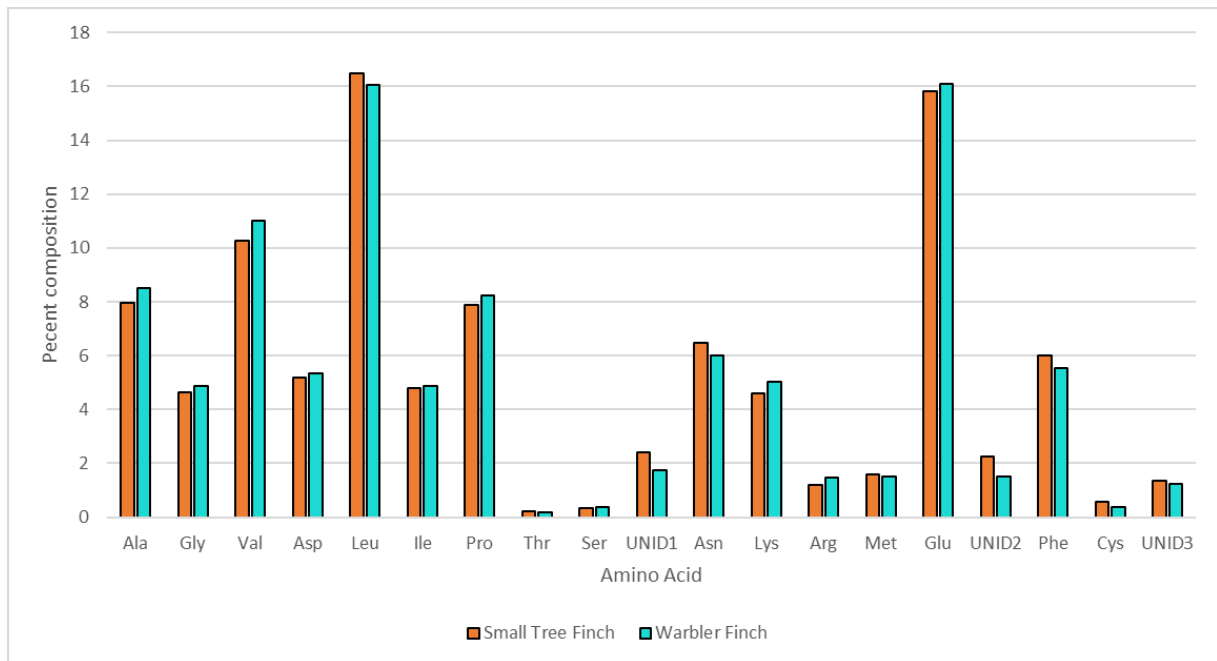


Figure 5: Amino acid composition (%) in nestling blood for the green warbler finch and small tree finch. Ala: alanine, Gly: glycine, Val: valine, Asp: aspartic acid, Leu: leucine, Ile: isoleucine, Pro: proline, Thr: threonine, Ser: serine, UNID1: first unidentified peak, Asn: asparagine, Lys: lysine, Arg: arginine, Met: methionine, Glu: glutamic acid, UNID2: second unidentified peak, Phe: phenylalanine, Cys: cysteine, UNID3: third unidentified peak.

Supplementary Table 1:

Retention times for amino acid standards run on GC for identification in bird blood.

Amino Acid	Retention time RT (min.)
L-alanine	18.676 – 18.706
L-glycine	19.967 – 19.003
L-valine	19.694 – 19.731
L-aspartic acid	19.963 – 20.002
L-leucine	20.362 – 20.401
L-isoleucine	20.495 – 20.534
L-glutamic acid (2 nd peak #1)	21.112 – 21.157
L-proline	21.293 – 21.388
L-threonine	21.383 – 21.452
D-serine	21.459 – 21.589
UNID1	21.760 – 21.872
L-glutamic acid (2 nd peak #2)	22.157 – 22.209
L-asparagine	22.390 – 22.896
L-lysine	23.131 – 23.185
L-arginine	23.503 – 23.560
L-methionine	24.060 – 24.636
L-glutamic acid	24.924 – 24.992
UNID2	25.546 – 25.617
L-phenylalanine	25.833 – 26.146
L-cysteine	26.322 – 27.104
UNID3	27.236 – 27.316

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CHAPTER 3

Parental food provisioning and nestling growth under *Philornis downsi* parasitism in the Galapagos Green Warbler-Finch, classified as ‘vulnerable’ by the IUCN

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Parental food provisioning and nestling growth under *Philornis downsi* parasitism in the Galapagos Green Warbler-Finch, classified as 'vulnerable' by the IUCN

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Abstract

In the Galapagos Islands, many endemic landbird populations are declining due to habitat degradation, food availability, introduced species and other factors. Given nestlings typically lack efficient defense mechanisms against parasites, hematophagous ectoparasites such as the larvae of the introduced Avian Vampire Fly, *Philornis downsi*, can impose high brood mortality and cause threatening population declines in Darwin finches and other landbirds. Here, we assess whether the food compensation hypothesis (i.e., the parents' potential to compensate for deleterious parasite effects via increased food provisioning) applies to the Green Warbler-Finch. We differentiated nests with low or high infestation levels by *P. downsi* and quantified food provisioning rates of male and female parents, time females spent brooding nestlings, and nestling growth. Male provisioning rates, total provisioning rates and female brooding time did not significantly vary in relation to infestation levels, nor by the number of nestlings. Opposed to the predictions of the food compensation hypothesis, females showed significantly reduced provisioning rates at high infestation levels. Nestling body mass was significantly lower and there was a reduction of skeletal growth, although not significantly, in highly infested nests. The females' response to high infestation may be due to parasites directly attacking and weakening brooding females, or else that females actively reduce current reproductive effort in favor of future reproduction. This life-history trade-off may be typical for Darwin finches and many tropical birds with long lifespans and therefore high residual reproductive value. Conservation strategies may not build on the potential for parental food compensation by this species.

Keywords Nest parasite · Parental care · Nestling growth · Host defense · Galapagos Islands · *Philornis downsi*

Zusammenfassung

Elterliche Nahrungsversorgung und Nestlingswachstum beim durch die IUCN als „gefährdet“ eingestuftem Waldsänger-Darwinfink bei Befall durch die parasitische invasive Vampirfliege *Philornis downsi*

Auf den Galapagos-Inseln sind auf Grund von Habitatstörungen, eingeschleppten und invasiven Organismen und anderen Faktoren viele Vogelarten und Populationen in ihrer Existenz bedroht. Beispielsweise führen die hämatophagen Larven der eingeführten Vampirfliege *Philornis downsi* zu einer hohen Brutsterblichkeit unter den endemischen Darwinfinken, insbesondere da bei Nestlingen die Immunabwehr noch schwach und unvollständig ausgeprägt ist. Hier wird untersucht, ob brütende Waldsänger-Darwinfinken *Certhidea olivacea* in der Lage sind, die für das Nestlingswachstum schädlichen

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Parasiteneffekte durch eine erhöhte Fütterungsrate zu kompensieren (Nahrungskompensations-Hypothese). Entgegen dieser Hypothese zeigte sich zwischen Nestern mit schwachem und starkem Parasitenbefall kein signifikanter Unterschied sowohl in der Gesamtfütterungsrate der Nestlinge als auch in der Anwesenheitsdauer der Weibchen im Nest. Entsprechend waren Nestlinge in stark befallenen Nestern leichter und im Skelettwachstum eingeschränkt. Weibchen in stark befallenen Nestern zeigten sogar signifikant geringere Fütterungsraten, weil möglicherweise Parasitenlarven auch brütende Weibchen befallen und schwächen. Möglich ist auch, dass Weibchen ihre derzeitigen Reproduktionsanstrengungen zugunsten einer zukünftigen Reproduktion aktiv reduzieren, wie durch die Life-history-Theorie vorausgesagt wird. Dieser Life-history Trade-off kann insbesondere bei Darwinfinken und tropischen Vogelarten, auf Grund ihrer relativ langen Lebensdauer und demzufolge einem relativ hohen zukünftigen Reproduktionswert, erwartet werden. Maßnahmen zum Schutz von Darwinfinken sollten deshalb ihr beschränktes Potenzial zur Nahrungskompensation miteinbeziehen.

Introduction

Birds are hosts of many types of parasites, including nest ectoparasites (for reviews, see e.g., Loye and Zuk 1991; Crompton 1997; Janovy 1997; López-Rull and Macías García 2015). Nestlings of altricial birds are ideal hosts for ectoparasites since they are left unattended in the nest for many hours each day and have limited defenses against parasites, due to their reduced mobility and their still naïve immune functioning. Selection will thus favor parental control of parasite numbers or compensation of parasite effects on nestlings (e.g., Tschirren et al. 2009; López-Rull and Macías García 2015). However, parental compensation via a higher food supply to nestlings, or other behaviors, will increase current reproductive effort, but at the expense of reducing residual reproductive value (Perrin et al. 1996), a life-history trade-off experimentally demonstrated in many studies (e.g., Richner and Tripet 1999). Higher food provisioning allows nestlings to allocate more resources to blood and tissue replacement and growth (Mason 1944; Johnson and Albrecht 1993; Morrison and Johnson 2002). The extent of parental compensation may be influenced by environmental conditions and the amount or quality of available food (Tremblay et al. 2005), among other factors. However, ectoparasites may also exploit and weaken the brooding female, which may then decrease food provisioning rates and presence and direct care of nestlings (Koop et al. 2013; Knutie et al. 2016).

In the Galapagos Islands, Ecuador, one of the major threats to many landbird species including most Darwin finches is the invasive Avian Vampire Fly, *Philornis downsi* (Diptera: Muscidae). Female flies lay eggs in bird nests where subsequently the hematophagous larvae feed on blood and tissue of nestlings (Fessl et al. 2006b). First reports of the fly date back to the 1960's (Causton et al. 2006) and thus the fly is assumed to have recently invaded the Galapagos archipelago. It currently exploits at least 21 native and endemic Galapagos landbird species (Fessl et al. 2018; Anchundia and Fessl 2020; Coloma et al. 2020), several of which are currently classified by the IUCN as vulnerable or critically endangered (IUCN 2022). *Philornis downsi* can cause detrimental effects including

blood loss, poor growth, deformed nasal cavities that interfere with sexual selection (Kleindorfer et al. 2019), and up to 100% brood mortality (reviewed in Kleindorfer and Dudaniec 2016). Lower breeding success caused by *P. downsi* parasitism has been documented for the majority of Darwin's finch species and the Little Vermilion Flycatcher (*Pyrocephalus nanus*) (as reviewed in Fessl et al. 2018; Cimadom and Tebbich 2021; Mosquera et al. 2022). Evidence for parental compensation via an increase in care and food provisioning to nestlings in Galapagos bird species is mixed: Knutie et al. (2016) showed that Galapagos Mockingbird (*Mimus parvulus*) parents can compensate for *P. downsi* parasitism, but this compensation was not found in another study (McNew et al. 2019), suggesting that parental compensation is a flexible response in this species. No compensation via increased parental provisioning rate was found in the Small Ground Finch (*Geospiza fuliginosa*), Medium Ground Finch (*Geospiza fortis*) (O'Connor et al. 2014; Knutie et al. 2016) or the Small Tree Finch (*Camarhynchus parvulus*) (Heyer et al. 2021). In a comparative study, Green Warbler-Finches (*Certhidea olivacea*) showed both a significantly lower infestation intensity and higher breeding success than sympatric Small Tree Finches (*Camarhynchus parvulus*) (Cimadom et al. 2014), which could be due either to lower parasite numbers or parental compensation, or both.

Many tree finch species in Galapagos, including two critically endangered species, the Mangrove Finch (*Camarhynchus heliobates*) and the Medium Tree Finch (*Camarhynchus pauper*), are in decline in part due to *P. downsi* parasitism (Fessl et al. 2010, 2017; Dvorak et al. 2012, 2017; Cimadom et al. 2014). Populations of the Green Warbler-Finch (*Certhidia olivacea*) have continued to decline, including in its small habitat range in Santa Cruz Island, as shown by close monitoring of populations since 1997 (Dvorak et al. 2012; Cimadom et al. 2014; Fessl et al. 2017). Understanding behavioral adaptations of avian hosts to *P. downsi* parasites, including the parents' ability to compensate for effects of parasites, is critical for the choice and allocation of conservation efforts.

In this study, we evaluated whether male and female Green Warbler-Finches compensate for *P. downsi* parasitism

via higher provisioning rates and feeding efforts. We assessed infestation levels in Warbler-Finch nests and for data analysis grouped nests in low and high infestation categories, respectively. Our working hypothesis of parental compensation predicts that one or both parents increase the rate of food provisioning under high parasite infestation intensity, and that consequently nestlings of the high and low infestation categories should show similar growth and development. Knowledge of parental compensation behavior and efficiency is relevant for informing conservation efforts and strategies.

Methods

Study site

The study took place within the Galapagos National Park protected areas at Los Gemelos (0° 37' 50" S, 90° 23' 25" W, altitude 569–625 m a.s.l.) on Santa Cruz Island in the Galapagos Islands, Ecuador. The site consists of a 0.33 km² area in a cloud forest habitat dominated by the endemic *Scalesia pedunculata* and includes other characteristic plant species such as the cat's claw (*Zanthoxylum fagara*), ferns, and the endemic Galapagos guava tree (*Psidium galapagium*) (CLP, pers. obs.; McMullen 1999). Since 2012, this area has also undergone long-term management for the invasive Mysore raspberry (*Rubus niveus*) (Cimadam et al. 2019).

Nest monitoring and treatment

From February to mid-April 2021, Green Warbler-Finch nests ($n = 26$) were monitored for activity every two to three days during incubation. Incubating nests close to hatching were monitored every one to two days for recording the precise hatch date. Nests located in the canopy of *Scalesia* trees at a height of 1.5 to 5.4 m were monitored for parental activity during incubation and using an endoscopic camera attached to a long carbon fiber pole to visually inspect nestlings in the nest after hatching. Eight nests were treated against parasites by manual injection with 5 mL of a 0.5% permethrin solution (Permacap CS) (for details of this method see Cimadam et al. 2019), applied with a 5 mL plastic syringe inserted into the base of the nest from the outside. Nests were treated within three days before or after the nestlings hatched. Hatching date was recorded as the date when the first egg hatched and was confirmed by visual observation via the camera monitor. Nestlings on hatch date were recorded as zero days old.

Feeding rate observations

Each nest was observed for a duration of 60 min between 6:30 a.m. and 10:00 a.m. when the nestlings were five to six days old. The number of feeding visits by the male and female were recorded, along with the duration the female spent inside the nest. Observations were not conducted in rainy weather. Observers kept a minimum distance of four meters from the nest as to not disturb the parents, a distance also used in other studies (e.g. Heyer et al. 2021).

Data recorded at each nest were standardized following the methodology in Heyer et al. (2021). Briefly, a *feeding visit* was defined as a 'visually confirmed insertion of food into a nestling's beak, and in cases the insertion could not be seen, a parent coming to the nest with food and leaning inside the nest in a feeding-like behavior'. For data analysis, *total provisioning rate* per nest was defined as the summed number of feeding visits per hour by both parents plus the male passing food to the female. Since males sometimes give the food to the brooding female who may then feed the nestlings, we defined *male provisioning rate* as the number of feeding visits per hour to nestlings by the male plus the feedings delivered to the female. Thus, male provisioning rates reflect total male parental effort. In principle, it is irrelevant whether food brought by the male is consumed by the female or given to the nestlings, since in the first case the female can bring more of the food items caught by herself to nestlings instead of consuming them for her own maintenance. *Female provisioning rate* was defined as the number of direct feeding visits per hour to nestlings by the female parent. *Female nest attention* was defined as the minutes per hour a female spent sitting in the nest during the observation period. Nests that were no longer active were collected, dismantled in the laboratory, and the number of *P. downsi* larvae and pupae were counted.

Nestling measurements

We collected measurements on 5 to 6-day-old nestlings. To limit disturbance time to under 15 min, maximally two nestlings per nest were briefly removed from the nest. We recorded tarsus length (tarso-metatarsus in mm) of the right leg and nestling mass to the nearest 0.01 g using an electronic scale. We additionally examined each nestling for signs of *P. downsi* parasitism, such as larvae clinging to the body and nares or typical body wounds caused by blood-sucking larvae. All nestling manipulation was done either directly after the feeding observation, or a day before or after observation as to not disturb the natural behavior of the parents and nestlings on observation day.

Statistical analysis

For the analysis, we grouped nests by infestation level, based on the number of *P. downsi* larvae or pupae found after nest collection. The “low infestation” group included nests with 0–3 parasites and the “high infestation” group included nests with 5–65 parasites. Three nests not treated with permethrin had naturally very few parasites (0, 1, and 2, respectively) and hence were added to the “low infestation group”. Two nests that were treated still had 7 and 10 parasites and thus were added to the “high infestation group”. Infestation level with *P. downsi* was significantly higher ($W=144$, $p<0.001$) in nests in the high infestation group than in nests in the low infestation group. Mean infestation intensity per nestling was more than 15-fold higher in the “high infestation” group (low infestation: mean = $0.59 \pm \text{SD } 0.533$, median = 0.50, $n=9$; high infestation: mean = $9.73 \pm \text{SD } 7.696$, median = 7.33, $n=16$). For provisioning rates, one nest observation from the high infestation group was excluded due to absence of parents during the entire observation. Brood sizes were on average $1.93 \pm \text{SE } 0.17$ (or $\text{SD } 0.68$) for the high infestation intensity nest group ($n=16$) and $2.33 \pm \text{SE } 0.24$ (or $\text{SD } 0.70$) for the low infestation intensity nest group ($n=9$). Brood size did not depend significantly on infestation level (glm (quasipoisson family), $t=0.298$, $p=0.768$). Additionally, sample size for nestling measurements differed from the sample size for provisioning rates due to limited access to higher nests to measure nestlings.

All analyses were conducted in R Studio (R Core Team 2021) using the *lme4* and *cars* packages (Bates et al. 2015; Fox and Weisberg 2019). We conducted separate generalized linear models (GLM) of the Poisson family with provisioning rates as the response variables. A separate GLM of the quasipoisson family was done for female provisioning rates with significant ($p=0.024$) underdispersion. Predictors for each model included infestation group (high and low infestation), the number of nestlings per nest on observation day, and the time each observation started between 6:30 and 9:00 a.m. For the duration of female nest attention (min per hour), we conducted a linear model (LM) with infestation group, number of nestlings on observation day and time of day at start of observation as predictors. Tarsus length (mm) and body mass (g) of nestlings were analyzed using linear mixed models (LMM) with the infestation group and number of nestlings per nest on the date of measurements as predictors. Nest ID was included in each LMM as a random factor, to control for pseudoreplication since multiple nestlings were measured from a nest. Each model was checked for normal distribution of residuals using tests for normality and by visually inspecting the QQ plots. Each GLM was additionally tested for overdispersion and heteroscedasticity, while LMMs were checked for heteroscedasticity only. No problems with dispersion or heteroscedasticity were detected,

except for the underdispersion in the female provisioning rates model, addressed using the quasipoisson family. For all tests, statistical significance was considered as $p \leq 0.05$.

Results

Food provisioning and breeding success

The total food provisioning rate per nest was not significantly affected by infestation level with *P. downsi* (Tables 1, 2), nor the number of nestlings or time of observation (Table 2). There was no significant difference in male provisioning rate between nests with low or high infestation level or the number of nestlings (Table 2). Male provisioning rates were close to significantly higher earlier in the mornings ($p=0.056$; Table 2). Female provisioning rate was significantly lower at nests with higher infestation, but not significantly affected by the number of nestlings or time of observation (Table 2). Although females spent on average more time attending nestlings of the high infestation group, the difference was not significant, nor affected by brood size (Table 2). Female nest attendance was significantly higher earlier in the morning than later (Table 2).

At low infestation levels, parents were slightly more successful in fledging at least one offspring (78% of nests) compared to high infestation levels (68% of nests), yet the difference was not significant (Mann Whitney *U* test, $W=65$, $p=0.66$, $n=25$).

Nestling size

Mean nestling mass (g) at age 5 to 6 days old was significantly higher in nestlings with low infestation, compared to nestlings with high infestation (Tables 3, 4). Mean tarsus length (mm) was longer at low infestation levels; however, not significantly so (Tables 3, 4).

Table 1 Food provisioning data for both high and low infestation groups of Green Warbler-Finch nests. Provisioning data are reported as mean values with standard error

	High infestation (<i>n</i>)	Low infestation (<i>n</i>)
Total provisioning rate	4.88 ± 0.50 (16)	6.22 ± 0.92 (9)
Male provisioning rate	2.13 ± 0.39 (16)	2.11 ± 0.65 (9)
Female provisioning rate	2.75 ± 0.25 (16)	4.11 ± 0.51 (9)
Female nest attention	19.50 ± 3.3 (16)	12.11 ± 4.67 (9)

Number of nests are reported in parentheses after each value. Total per nest, male, and female provisioning rates are reported as mean visits per hour; while the female nest attention is reported as total number of min. per hour the female was sitting in the nest

Table 2 Results from mixed models for each dependent variable

Dependent variable	Fixed effect	Model estimate $\pm$ SE	z-score or <i>t</i> value	<i>p</i> value
Total provisioning rate	Intercept	2.80 $\pm$ 1.192	2.35	0.019
	Infestation level (low)	0.18 $\pm$ 0.182	0.97	0.330
	Number nestlings	0.09 $\pm$ 0.131	0.70	0.482
	Time of day	-0.19 $\pm$ 0.151	-1.25	0.210
Male provisioning rate	Intercept	4.29 $\pm$ 2.001	2.15	0.032
	Infestation level (low)	-0.10 $\pm$ 0.296	-0.33	0.739
	Number nestlings	0.04 $\pm$ 0.205	0.18	0.854
	Time of day	-0.49 $\pm$ 0.257	-1.91	0.056
Female provisioning rate	Intercept	0.85 $\pm$ 0.993	0.86	0.400
	Infestation level (low)	0.35 $\pm$ 0.156	2.26	0.035
	Number nestlings	0.12 $\pm$ 0.115	1.08	0.294
	Time of day	-0.01 $\pm$ 0.124	-0.09	0.928
Female nest attention	Intercept	98.61 $\pm$ 32.924	3.00 (<i>t</i>)	0.007
	Infestation level (low)	-6.60 $\pm$ 5.386	-1.23 (<i>t</i>)	0.234
	Number nestlings	-5.79 $\pm$ 3.799	-1.53 (<i>t</i>)	0.142
	Time of day	-9.12 $\pm$ 4.127	-2.21 (<i>t</i>)	0.038

Values are the model (LM or GLM) estimate with the standard error, z-score or *t*-statistic (specified in parentheses) and *p* value

Table 3 Nestling growth measurements by infestation level, reported as mean per nest with standard error and number of nests in parentheses

	High infestation (<i>n</i>)	Low infestation (<i>n</i>)
Number of parasites		
Mean per nest	14.36 $\pm$ 1.515 (11)	1.7 $\pm$ 0.448 (10)
Measurements		
Body mass (g)	5.14 $\pm$ 0.236 (11)	6.26 $\pm$ 0.183 (10)
Tarsus (mm)	13.20 $\pm$ 0.407 (11)	14.50 $\pm$ 0.354 (10)

Discussion

Overall, we found no significant difference between parental provisioning rate in nests with high versus low infestation intensity, similar to other studies on parental food compensation in Darwin finches with *P. downsi* nest parasitism, including the Medium Ground Finch (Knutie et al. 2016), Small Tree Finch (Heyer et al. 2021), and

Small Ground Finch (O'Connor et al. 2014). Contrastingly, in one study, Galapagos Mockingbirds responded to *P. downsi* parasitism with increased provisioning rates (Knutie et al. 2016). Studies on similar systems in North America involving hematophagous larvae of *Protocalliphora* spp. also failed to show a significant difference in parental food provisioning in relation to infestation, as for example, Eastern Bluebirds (*Sialia sialis*) infested by the blowfly *Protocalliphora sialis* (Grab et al. 2019), or House Wrens (*Troglodytes aedon*) infested by *Protocalliphora parorum* (Morrison and Johnson 2002).

Many factors may influence parental food provisioning behavior in Green Warbler-Finches. Proximate factors include nestling begging rates, as demonstrated in Galapagos Mockingbirds (Knutie et al. 2016), food availability, food quality and others. The soil of the typical breeding habitats of the Green Warbler-Finch on Galapagos is often densely covered by invasive plant species (e.g., invasive blackberry) that prevent foraging on or near the ground, thus causing limited access to food that may prevent higher food provisioning rates to nestlings. It is unlikely that parents of highly

Table 4 Results from a linear mixed model with tarsus length and mass as the dependent variables, infestation level as the predictor and nest ID as a random factor to control for pseudoreplication

Dependent variable	Fixed effect	Model estimate $\pm$ SE	<i>t</i> value	<i>p</i> value
Mass	Intercept	4.97 $\pm$ 0.552	8.99	<0.001
	Infestation level (low)	0.95 $\pm$ 0.376	2.51	0.023
	Number nestlings	0.12 $\pm$ 0.246	0.50	0.622
Tarsus length	Intercept	12.60 $\pm$ 0.97	12.99	<0.001
	Infestation level (low)	1.14 $\pm$ 0.654	1.75	0.099
	Number nestlings	0.31 $\pm$ 0.430	0.72	0.478

infested nests brought higher quality food to the nestlings to compensate for parasites, given that nestlings from highly infested nests showed significantly lower body mass than nestlings from nests with low infestation intensity, despite similar food provisioning rates in the two groups of high and low infestation. As a proximate factor, it may be that nestlings in nests with high infestation intensity were too weak to beg, as reported in the Small Ground Finch (O'Connor et al. 2014). However, begging rates of nestlings are rarely assessed in relation to *Philornis* parasitism on Galapagos and elsewhere. Overall, infestation intensity and prevalence were lower in the year of this study than in previous years (Cimadom et al. 2014; Cimadom and Tebbich 2021), potentially due to a weak La Niña event during the breeding season in 2021 (NOAA 2021), which may have led to a lower abundance of insects, including *P. downsi*. Although unlikely, infestation rates even in the 'high parasitism' nests may have been too low for detecting a significant increase in parental provisioning rates.

Ultimate factors are typically based on life-history trade-offs that will determine the evolution and expression of parental compensation strategies. That is, an increase in food provisioning rates, and thus parental effort, will entail a decrease in residual reproductive value. Thus, the cost of an increase in parental provisioning rates under low food availability, lower food quality, higher predation exposure and many other factors is predicted to ultimately affect future reproduction. It will influence the decision of parents to increase parental effort in a currently infested nest. In an experimental study on Great Tits (*Parus major*), a forced increase in male and female current reproductive effort led to an increase in malaria infections (Richner et al. 1995; Oppliger et al. 1996) and affected return rates of breeders the following year (Richner et al. 1995), i.e., future reproduction. In Blue Tits (*Parus caeruleus*), parents of nests experimentally parasitized with the Hen Flea (*Ceratophyllus gallinae*) had lower return rates and breeding success the year following infestation (Richner and Tripet 1999). Similarly, Alpine Swifts (*Apus melba*) experimentally infested with louse flies (*Crataerina melbae*) experienced significantly reduced breeding success the year following infestation (Bize et al. 2004).

Females spent more time brooding the nestlings earlier in the morning while males provisioned food at a close to significantly higher rate earlier in the morning than later on. Nestling thermoregulation at an age of 5–6 days is not yet well developed, and at our study site in the highlands of Santa Cruz, the mornings can be cooler, between ~ 19 and 21 °C, with the temperature typically increasing between sunrise and midday. Thus, earlier in the morning it was likely more profitable for the female to keep nestlings warm and at an optimal temperature for metabolizing food, rather than spending time foraging. Males then partly compensated

by higher food provisioning rates earlier in the morning. Similarly, Small Tree Finch females of infested nests spent more time at the nest than females of nests with low parasite numbers (Heyer et al. 2021). It is unknown whether the additional time was used for active brood care or simply due to the weakening of the female, given that parasites also use brooding females as a food source.

The parental food compensation hypothesis predicts that parents will increase investment in nestlings when parasitized, and in turn parasitized nestlings will benefit and show similar growth rates as uninfested ones. Our results lend no support to this hypothesis: nestlings of highly infested nests had poorer growth and female parents, opposed to the prediction, lowered provisioning rates when highly infested. Lower nestling mass as a consequence of *P. downsi* infestation was also reported in the Galapagos Mockingbird, Medium Ground Finch and Small Ground Finch (Fessl et al. 2006a; Koop et al. 2011; Knutie et al. 2016), but interestingly not in another study on the Small Ground Finch (O'Connor et al. 2014) where nestlings of infested and uninfested broods were of similar body size and mass. However, there, nestlings died sequentially in infested nests but survived in nests treated against parasites (O'Connor et al. 2014), which suggests that body size and mass could be maintained due to smaller brood sizes in infested nests. In another similar host-parasite system with *Protocalliphora* parasites, Blue Tit nestlings had higher body mass in nests with lower infestation intensities (Bañbura et al. 2004). In an experimental system with Great Tits infested by Hen Fleas, parents increased provisioning rates, yet nestlings still had lower body mass (Christe et al. 1996). This actually supports theoretical models that predict partial, but not full compensation due to the life-history trade-off in current versus future investment (Perrin et al. 1996). The observed difference in compensation behaviors between non-tropical Passerines and Galapagos landbirds may be explained by the difference in longevity: Galapagos landbirds experience typically long average life span, most likely selected by low predation risk combined with a fluctuating food supply among breeding seasons (Grant and Grant 2011). Thus, their life-history trade-offs are strongly in favor of residual reproductive value and will thus disfavor a significant increase in current reproductive effort and hence the evolution of parental compensation strategies. It may well explain why essentially all studies on Darwin finches so far did not find parental compensation for the effects of nest parasitism by *P. downsi*. Thus, future conservation efforts should rather not build on expectations of parental compensation behaviors for the effects of *P. downsi* parasites.

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Data availability Datasets generated and analyzed in this study can be provided by the corresponding author upon request.

Declarations

Conflict of interest The authors declare no competing interests relevant to this article.

Ethics approval The study was conducted under permission by the Galapagos National Park, under accordance of approved research permits (Project permit numbers: PC 35-19, PC 24-20, PC 08-21, Anexo de Muestras MAE-DPNG-IA-2020-08, Anexo de Muestras MAE-DPNG-IA-2021-05) for our experimental protocols. All procedures were conducted with the utmost care for the welfare of birds included in this study.

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General Discussion

Host-parasite interactions and parasite adaptations

The Avian Vampire Fly's successful establishment in the Galapagos Islands, along with the correlation of its behaviors with host behaviors, demonstrate its highly flexible nature in adopting novel hosts in new ranges. **Chapter 1** results have shed light/identified the avian vampire fly's behavior at hosts nests, specifically of an endemic cavity-nester, the Galapagos Flycatcher. In this chapter, I have provided evidence of behavioral adaptations and predator avoidance by this invasive Dipteran parasite. This includes host-seeking behaviors of the adult flies to locate host nests and time their entrance and exit of the nest to host behavior, which may vary between individuals or species. Similar to prior observations of Avian Vampire Fly behavior at host nests in Galapagos (O'Connor et al., 2010; Lincango et al., 2015), I found flies visiting in late-afternoon hours and when adult birds were absent. Similarly, we observed flies entering Green Warbler Finch nests four times when birds were absent (unpublished data). Kleindorfer et al. (2021) recently found that the number of Avian Vampire Fly parasites per nest was higher in nests with lower female brooding times and less male feedings during the nestling phase. Thus, these prior studies, combined with my results provide evidence of host behavior influencing Avian Vampire Fly behavior at nests.

Similar to observations by Lincango et al. (2015), we observed that avian vampire flies entered nest cavities by walking into them. I also observed flies walking on and into the nests of Green Warbler Finch nests four times over two different days (unpublished data). Avian vampire flies walking into nests or on the outside surface of nests may be a common behavior of the *Philornis* genus. Adult *Philornis* were caught outside nests of oropendolas (*Zarhynchus wagleri* & *Psarocolius decumanus*) and the cacique (*Cacicus cela*) using sticky paper (Smith, 1968). However, evidence of flies walking into nests has only been observed in Galapagos (O'Connor et al., 2010; Lincango et al., 2015) and may be due to a lack of observational studies at nests with *Philornis* parasitism in native ranges.

Additionally, my study provided limited evidence of anti-parasitic behaviors by nestlings and their parents. Adult and nestling preening and nest sanitation were observed in in-nest video recordings of Medium and Small Ground Finch nests (O'Connor et al., 2010), but other host species in Galapagos may not react to Avian Vampire Fly parasitism with similar behavioral adaptations. In a prior study on hosts of other *Philornis* sp. flies with subcutaneous

larvae, brood parasitic cowbird nestlings (*Scaphidura oryzivora*) were observed preening *Philornis* larvae from their nest mates and stomach content analysis of nestlings revealed all life stages of *Philornis* (Smith, 1968). This suggests these nestlings can fly-catch and eat adult *Philornis* flies within an enclosed nest. However, nestlings of host species (of the genera *Cacicus* and *Zarhynchus*) in the same nests did not exhibit preening or other anti-parasitic behaviors against *Philornis* sp., similar to my observations on the Galapagos Flycatcher nestlings. Thus, there may be an innate predisposition for anti-parasitic behavior by nestlings that is species dependent. Given this variation in response to parasitism, not all Avian Vampire Fly hosts in Galapagos may exhibit this behavior.

Combined, my findings have high relevance for short and long-term measures to control the Avian Vampire Fly. Firstly, given higher fly visitation to host nests in afternoon hours, future studies on fly activity at host nests in field conditions, including further visual observations, identification, and trapping, can be timed better to fly activity. Secondly, future studies can also build off the information acquired in **Chapter 1** by conducting video recording and/or observations at nests of other host species for fly-host interactions, including determining both fly and host adaptations. Thirdly, my findings of flies walking into nests (**Chapter 1**) have been utilized in short-term methods for control. The recent spraying application of insecticides outside nests where the flies would walk to enter has successfully reduced the number of Avian Vampire Fly parasites in nests (Barbara Kofler, in prep). Finally, my observations of flies mating outside the filmed nest cavity provide insight into courtship and mating behavior. Every instance of mating took place during evening hours near sunset. The *P. downsi* breeding center at the Charles Darwin Research Station in Galapagos is trying to rear avian vampire flies for long-term control methods, such as sterile insect technique; however, mating success rates have been low. Therefore, it is important to understand the optimal conditions for success in mass rearing flies. Thus, my observations of mating behavior are valuable for improving laboratory conditions to mass rear flies for long-term control initiatives (Causton et al., 2013). In fact, improvements in timing and lighting (during late afternoon with visual cues of natural sunset lighting) for mating experiments has increased the rate of successful mating in the *P. downsi* breeding center in Galapagos (P. Lahuatte, pers comm). In my study, all observations of mating involved one fly present on the outside of the nest cavity and a second fly landing before courtship and mating took place. It may be that Avian Vampire Fly mate recognition occurs using visual cues, such as male coloration (Rutowski, 1992) or UV reflectance off the wings at a specific frequency, as

reported in other flying insects (e.g., Green Bottle Fly, Eichorn et al., 2017). A detailed study focused on these would be beneficial for a better understanding of mate recognition for the Avian Vampire Fly. Furthermore, evidence of mating outside host nests suggests resource based mating, with males courting females (Wilkinson and Johns, 2005). Given the evidence of flies mating outside nests and potential visual cues including light reflectance, future studies could examine whether Avian Vampire Fly adults have differing wing beat frequencies and light reflectance between sexes. Additionally, further observations at host nests for fly mating, coupled with fly trapping of mating flies would also be useful to determine whether host nests are commonly utilized as a mating resource.

Host quality and larval development

In a study by Cimadom and Tebbich (2021), Small Tree Finch nests had a greater proportion of mature larvae in their nests and this proportion increased at a faster rate than in Green Warbler Finch nests. Based on these findings, Cimadom and Tebbich (2021) suggested that Avian Vampire Fly larvae are developing faster in Small Tree Finch nests than in Green Warbler Finch nests. Therefore, in **Chapter 2**, I aimed to understand if host quality differs between these two host species by examining factors that could influence larval development. Specifically, I aimed to determine whether nest base climate (the location of larval development) and/or nestling blood quality differed between the two host species. Overall, I found: 1) predicted nest base temperature decreased in both species as nest base humidity increased, but the decline in temperature was more pronounced in Small Tree Finch nests; however, Small Tree Finch nests maintained higher nest base temperatures than Green Warbler Finch nests at a range of nest humidity levels, 2) $\delta^{13}\text{C}$ blood values were significantly different between the two host species, reflecting the trophic levels of their prey, and 3) host blood quality, measured as C/N, did not significantly differ between nestling blood of both species. Furthermore, analyzing blood of both species, I also found no evidence of significant differences in amino acid composition for the amino acids analyzed. These results provide a better understanding of how host quality varies between species and of biotic factors that may influence development and/or growth of nest parasites. Results from this study suggest that nests of the Small Tree Finch may have a lower thermal conductance and better insulation. These factors may play a role in influencing larval development as nests provide the habitat for Avian Vampire Fly larvae to develop. Contrastingly, similar C:N and amino acid composition between host species suggests that either the two species have

different diets but still acquire similar nutrients, and/or that in the study season, nestling diet was similar between species, contrasting prior observations of different nestling diet (Filek et al., 2018). My results also suggest that blood quality may not vary significantly between host species and that it may not be the cause of potentially faster larval development in Small Tree Finch nests. Nest insulation and/or nestling thermoregulation may differentially affect nest climate and, in turn, affect larval development; however, other factors may also influence larval development, such as pH, nestling immune response or tissue quality and/or female immune response, among others (Otranto, 2001; Koop et al., 2013; Meneguz et al., 2018; Torres et al., 2022).

Results from this thesis provide important information for applying to management and control of this invasive parasite. Data on nest conditions in a natural environment may be utilized in the Avian Vampire Fly breeding center for optimizing climatic conditions for larval development and pupation (Causton et al., 2013). Furthermore, given our results on stable isotope ratios of blood from nestlings of two host species, the lab may also utilize this information to help optimize the blood diet for rearing larvae. In a prior study, increasing C:N ratios had a greater positive effect on Black Soldier Fly (*Hermetia illucens* L.) larval growth at 34°C than at 28°C (Palma et al., 2019). Furthermore, larvae of the Yellow Fever Mosquito, *Aedes aegypti*, developed faster with diets of mid-high C:N ratios (van Schoor et al., 2020). In addition, Hood-Nowotny et al. (2012) suggest that the size of adult malaria mosquitoes, *Anopheles arabiensis*, is determined by total nitrogen availability in the larval diet. These studies suggest dietary requirements for larval growth and development vary between species and may be mediated by temperature. Therefore, the results from my host quality study (**Chapter 2**) can be utilized to conduct trials in the lab on optimizing the rearing conditions of the Avian Vampire Fly and to better understand its growth and developmental ecological requirements. I suggest that future studies should include larval development/growth trials involving further alterations in diet in a controlled environment, with varying temperatures and humidity levels, based on our climatic ranges found in **Chapter 2**, and by varying C:N in the larval diet to determine how larval development is influenced. These trials are pertinent for understanding optimal rearing conditions for control initiatives of the Avian Vampire Fly. Additionally, given larvae from different host species nests vary in their microbiome (Ben-Yosef et al., 2017), it would be worthwhile to investigate if there is an effect of microbial community composition on larval development. Future studies involving diet composition and the role of microbes in larvae growth and development may also provide a better insight

into potential interactions between factors that could influence growth or development of Avian Vampire Fly larvae in natural conditions.

Moreover, immune response may also be a contributing factor in different proportions of mature larvae in reported in Cimadam and Tebbich (2021) for the Small Tree Finch and Green Warbler Finch. So far, however, there is no evidence that nestlings can develop a proper immune response (via antibody production) against Avian Vampire Fly parasitism (Koop et al., 2013; Knutie et al., 2016). A recent study by Knutie et al. (2023, Preprint) found gene expression for specific immune functions differed between urban and non-urban Small Ground Finch nestlings with Avian Vampire Fly parasitism. Given this, Green Warbler Finch and Small Tree Finch nestlings may also have differential gene expression in response to parasitism. Therefore, I suggest examining the immune response for both species using similar techniques.

Host adaptations and defenses

Cimadam et al. (2014) found green warbler finches had higher breeding success than small tree finches and suggested this may be attributed to parental compensation in the Green Warbler Finch. Considering this, I investigated whether Green Warbler Finch parents can compensate for Avian Vampire Fly parasitism (**Chapter 3**). I found no significant difference in Green Warbler Finch parental feeding rates in response to higher and lower parasite loads, suggesting they do not compensate for nest parasitism with higher visitation rates. Additionally, I found that nestlings from more highly parasitized nests had significantly lower weight/mass than less parasitized nestlings, suggesting that parents were also not compensating by bringing larger or higher quality prey items to the nestlings. Lack of evidence for parental compensation by the Green Warbler Finch in response to Avian Vampire Fly nest parasitism may be attributed to numerous factors. During the year of the study, there could have been limited food availability preventing the Green Warbler Finch from compensating for parasitism with increased provisioning trips or higher quality food, as there was a La Niña and limited precipitation during my field study in 2021 (NOAA, 2023; CDF, unpublished data), which can affect insect abundance. Comparatively, during study years in Cimadam et al. (2014), there was an El Niño in 2010, but a La Niña in 2012 (NOAA, 2023); however, Cimadam et al. (2014) also noted high humidity levels during 2012, so La Niña may not have affected the field site as strongly during the months of data collection. These climatic events may have influenced precipitation levels in the field site and provided

conditions for higher food abundance than in 2021. Additionally, despite studies on numerous host species in Galapagos, there is still little evidence for host adaptations to Avian Vampire Fly parasitism, specifically via parental compensation (O'Connor et al., 2014; Knutie et al., 2016; Heyer et al., 2021). Only one prior study reported parental compensation in the Galapagos Mockingbird (Knutie et al., 2016); however, contrastingly, in a study by McNew et al. (2019), no evidence for parental compensation behavior by the Galapagos Mockingbird was found. Given the lack of evidence for parental compensation for the Green Warbler Finch (**Chapter 3**) and many other avian hosts in Galapagos (O'Connor et al., 2014; Knutie et al., 2016; McNew et al., 2019; Heyer et al., 2021), these species may not have been predisposed to adapt to the Avian Vampire Fly parasitism or nest parasitism at these levels. Parent birds may not be adapting their behavior to higher nest parasitism if their nestlings are weakened by the hematophagous larval feeding and cannot beg at optimal levels (O'Connor et al., 2014). This may also be a life history characteristic to save their energy for future reproduction (Richner and Tripet, 1999; as discussed in **Chapter 3**). This may also apply to other avian host-nest parasite systems with birds that have a long life span.

While hosts may not compensate for parasitism and have limited host defenses, including nest sanitation, preening, and fly-catching (**Chapters 1 & 3**), hosts may develop alternative defense mechanisms against avian vampire fly parasitism. Aromatic plants, such as the aromatic endemic Guayabillo tree, *Psidium galapageium*, could have a repellent effect on the Avian Vampire Fly from laying eggs in host nests (Cimadam et al., 2016). Adult Darwin's finches have been observed rubbing Guayabillo leaves on their feathers (Tebbich et al., 2021); however, so far there is no indication that hosts use the aromatic Guayabillo in their nests, given extensive nest examinations in many studies at the same study site (e.g., Cimadam et al., 2014, 2016, 2019; Cimadam and Tebbich, 2021; **Chapter 3**). Additionally, as results from **Chapter 3** suggest Green Warbler Finch nestlings have limited tolerance to Avian Vampire Fly parasitism, other factors influencing higher breeding success of the Green Warbler Finch than the Small Tree Finch should be investigated. As mentioned above, investigations into immune responses of both the Green Warbler Finch and Small Tree Finch to Avian Vampire Fly parasitism may be informative for understanding both higher breeding success in the Green Warbler Finch and slower larval development in its nests.

Lack of evidence for parental compensation by Darwin's finches in response to Avian Vampire Fly parasitism also indicates that conservation efforts may not rely on natural compensation nor via supplemental food for these endemic species. Our research group was

planning to do supplementary feeding trials for endemic birds in Galapagos, but given a lack of evidence for parental compensation in prior studies and my recent study (**Chapter 3**), this may require reconsideration. On one hand, it may not be ideal to put in effort for supplementary feeding for species that are not adapted to increasing their feeding rate to parasitized nestlings. However, on the other hand, it may be worth conducting trials on supplementary feeding in the future over multiple years, as parental compensation may not be possible in years with low food availability (McNew et al., 2019) and/or inadequate species composition (Heyer et al., 2021).

Limitations

In **Chapter 1**, I only filmed three nests of the Galapagos Flycatcher due to time intensiveness of filming and limited resources. Additionally, as discussed in the introduction, the Galapagos Flycatcher was the only focal host in this study due to its adaptability to nest in artificial structures and comfortability with human interactions. Furthermore, for my PhD, I originally aimed to examine fly visitation to tree finch nests in Los Gemelos, a field site in the highlands of Santa Cruz, in 2020 and 2021. I attempted to film outside nests of the Green Warbler Finch and Small Tree Finch. However, these species were more sensitive to filming and responded with nest abandonment, so I discontinued filming. Despite this, I believe that the results of my study on Galapagos Flycatchers can be applied to understand co-adaptations between the Avian Vampire Fly and other host species. However, we acknowledge that we only focused on one host species and suggest observing fly-bird interactions at nests of other host species in Galapagos as well. Furthermore, I also initially aimed to investigate differences in immune response to Avian Vampire Fly parasitism in Green Warbler Finch and Small Tree Finch nestlings as a potential factor influencing larval growth and development. I sampled nestling blood and made blood smears in the Galapagos for examining innate immune response. However, upon examining these blood smears in Austria, the slides were unfortunately degraded and it was not possible to quantify white blood cell differentials properly. Furthermore, I acknowledge that the small sample sizes for **Chapter 3** could be a limitation. Ideally, we would have observed and measured nestlings from a large sample size of nests. However, due to the size of the study site, limited months during the breeding season, accessibility of nests for observations, and nest failure, we had a limited sample size. Additionally, further studies on parental compensation and food availability over multiple years and with higher sample sizes would be highly informative to

know whether my results from **Chapter 3** are potentially due to a year with low food abundance/availability (McNew et al., 2019).

Conclusions

Understanding a specific host-parasite system can be useful for control /management in the future (Davis, 2006). Thus, this PhD thesis provides critical information on the Avian Vampire Fly and interactions with its novel hosts in the Galapagos Islands to inform management planning and control of this invasive parasite. My research also demonstrates the importance of integrating research techniques from various fields/aspects of biology, in order to gain a better understanding of invasions and host-parasite interactions. Behavioral data on both the parasite and its hosts is highly important to further document and understand co-evolutionary processes that may occur in the novel range of the parasite. I found adaptations in the invasive parasite (a very flexible parasite), including behaviors related to host seeking and avoidance of predation, in which flies generally waited for the adult birds to exit before entering the nest cavity themselves. However, I did not find evidence of adaptations in the novel hosts I studied, perhaps an implication of a generalist parasite. The invasive Avian Vampire Fly has coevolved with various avian host species in its native environment; therefore, this may facilitate its success to parasitize novel hosts in Galapagos, its invaded range. However, these novel avian hosts in Galapagos are not known to have had exposure to hematophagous nest parasites like the Avian Vampire Fly. Therefore, they may not have adaptive behaviors or physiological responses to prevent this nest parasitism or mount defenses against it. Even though Darwin's finches are known to be innovative and flexible (Tebich et al., 2010), they cannot seem to cope with this novel parasite. This is reflected by the fly's increased virulence and lower breeding success for these species, compared to other Galapagos landbird host species, including the Galapagos Mockingbird, which is more tolerant to Avian Vampire Fly parasitism (reviewed in Fessl et al., 2018; McNew and Clayton, 2018). This situation poses a serious threat to the Galapagos avifauna and involves the risk of extinction for particularly affected species. Therefore, effective control measures are urgently needed in order to protect Darwin's finches and other landbirds in Galapagos. This PhD thesis provides further insights into the life history and adaptations of the Avian Vampire Fly that may help contribute to the control of this detrimental parasite before unique, endemic species of the Galapagos are lost.

Additionally, changes in climatic conditions can impact host-parasite interactions, including having significant effects on food availability, which in turn can affect the ability of breeding parents to compensate for nest parasitism (Grant, 1999; Cimadam et al., 2019; McNew et al., 2019). As Galapagos has a highly variable climate year to year, especially due to ENSO events (Paltán et al., 2021; NOAA, 2023), it is of great importance to conduct long-term monitoring and studies to have an enhanced understanding of this overall dynamic. Furthermore, given the plasticity of invasive species and their capacity to adapt to new environments (reviewed in Davidson et al., 2011), as environmental conditions change, these species may adapt/fare better than their hosts. Thus, behavioral plasticity and adaptations of the targeted invasive species must also be considered for successful control and future management initiatives to minimize the negative impacts on native and endemic flora and fauna.

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Summary

Larvae of the invasive Avian Vampire Fly, *Philornis downsi*, attack nestling birds in the Galapagos Islands, posing a major threat. Utilizing behavioral observations and laboratory analyses, I studied interactions between the Avian Vampire Fly and novel host species and factors related to host quality and larval development. In Chapter 1, I filmed nests in field conditions to record behavioral interactions between the Avian Vampire Fly and the Galapagos Flycatcher. I found that flies visit nests more in early evening hours, adult fly visitation to host nests is correlated with bird visitation, and flies may mate outside the nest cavity (Chapter 1). In Chapter 2, I investigated factors influencing host quality of the Green Warbler-Finch and the Small Tree Finch and larval development in their nests by measuring nest climate and host blood quality. Our results suggest Small Tree Finch nests have a lower thermal conductance and may better buffer against external environmental factors. However, there was no difference in nestling blood quality between species (Chapter 2). In Chapter 3, I compared parental feeding rates at Green Warbler-Finch nests with low and high parasitism to determine whether this host can compensate for larval parasitism. I found no evidence of the Green Warbler-Finch compensating for high Avian Vampire Fly parasite loads with higher feeding rates (Chapter 3). Overall, results from this thesis provide valuable behavioral information on Avian Vampire Fly nest visitation and timing of activity with hosts, however, no evidence for host adaptation. These findings are relevant for conservation measures.

Zusammenfassung

Larven der invasiven Vampirfliege *Philornis downsi* greifen junge Vögel auf den Galapagos-Inseln an und stellen eine große Bedrohung dar. Mithilfe von Verhaltensbeobachtungen und Laboranalysen untersuchte ich die Wechselwirkungen zwischen der Vogelvampirfliege und neuartigen Wirtsarten sowie Faktoren im Zusammenhang mit der Wirtsqualität und der Larvenentwicklung. In Kapitel 1 habe ich Nester unter Feldbedingungen gefilmt, um Verhaltensinteraktionen zwischen der Vogelvampirfliege und dem Galapagos-Fliegenschnäpper aufzuzeichnen. Ich habe herausgefunden, dass Fliegen in den frühen Abendstunden häufiger Nester besuchen, dass der Besuch erwachsener Fliegen in Wirtsnestern mit dem Besuch von Vögeln korreliert und dass Fliegen sich außerhalb der Nesthöhle paaren können (Kapitel 1). In Kapitel 2 habe ich Faktoren untersucht, die die Wirtsqualität des Grünen Waldsängers und des Kleinen Baumfinkens sowie die Larvenentwicklung in ihren Nestern beeinflussen, indem ich das Nestklima und die Blutqualität des Wirts gemessen habe. Unsere Ergebnisse deuten darauf hin, dass Nester kleiner Baumfinken eine geringere Wärmeleitfähigkeit haben und möglicherweise einen besseren Schutz gegen äußere Umweltfaktoren bieten. Es gab jedoch keinen Unterschied in der Blutqualität zwischen Nestlingen beider Arten. In Kapitel 3 habe ich die Fütterungsraten der Eltern in Nestern von Waldsängern mit niedrigem und hohem Parasitismus verglichen, um festzustellen, ob dieser Wirt den Larvenparasitismus kompensieren kann. Ich habe keine Hinweise darauf gefunden, dass der Waldsänger die hohe Parasitenlast der Vogelvampirfliege durch höhere Fütterungsraten kompensiert (Kapitel 3). Insgesamt liefern die Ergebnisse dieser Arbeit wertvolle Verhaltensinformationen zum Nestbesuch von Vogelvampirfliegen und zum Zeitpunkt der Aktivität mit Wirten, jedoch keine Hinweise auf eine Anpassung des Wirts. Diese Erkenntnisse sind für Erhaltungsmaßnahmen relevant.