4-14-2017

Parasitism of free-ranging Neotropical primates: examining parasite-host and parasite-parasite relationships

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A Dissertation Submitted to The Graduate School at the University of Missouri-St. Louis

in partial fulfillment of the requirements for the degree
Doctor of Philosophy in Education
with an emphasis in Ecology, Evolution and Systematics

May
2017

Advisory Committee

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Introduction

The most common types of species interactions include competition, predation, mutualism, commensalism, and parasitism. Two of these, predation and parasitism, appear similar in that one organism ultimately gains while the other loses, however they differ in fundamental ways. First, whereas predators kill their prey, it is not necessarily in the interest of the parasite to kill its host; a host is required to complete all or part of a parasite's life cycle. Secondly, while predators can be slightly larger or smaller than their prey, parasites are magnitudes of scale smaller than their hosts. Parasites are diverse in form, ranging from arthropods that live on a host's external layers to worms or protozoans that occupy deeper tissues, and they are unified by the fact that they all acquire resources at a cost to their hosts.

That parasites are small, microscopic even, has resulted in their frequent exclusion from ecological studies. When included, the fact that parasites steal resources from their hosts has resulted in a generally negative portrayal of their roles. Yet, a number of studies from wildlife systems show that infection with one or more parasites is not only normal in nature (Cox, 2001; Pedersen and Fenton, 2007), but that the occurrence of diverse parasite communities may be indicative of population- or community-wide health (Hudson et al., 2006). For example, food network studies that incorporate parasites-host relationships can double or triple the number of species interactions (Lafferty et al., 2006), revealing strong relationships between organisms at polar ends of a trophic network. This suggests that parasites contribute toward balancing interactions between free-living organisms, and that the removal of native parasite infections could partially destabilize wildlife systems. In a similar vein, studies of parasite concomitant infections demonstrate that the presence of multiple parasites or multiple strains of the same parasite can have variable effects on host health, in some cases improving host health or chances of survival (Balmer et al., 2009; Knowles, 2011).

Increasingly, parasites are being recast into a light that does not ignore their ability to trigger declines in host populations, but also acknowledges there potential to signal community stability, or at the very least, ecological change or disturbance (Chapman et al., 2006; Gillespie and Chapman, 2008). As such, the overarching goal of this dissertation was to lay a foundation for research that explores this duality of parasites in nature and at multiple scales; single hosts, host populations, and host communities. Specifically, here I focus on identifying parasites and describing how they are distributed in a Neotropical nonhuman primate community in Southeastern Perú.

Primates are an excellent model for the study of parasite ecology for a number of reasons. First, Primates encompass upwards of 500 species, distributed throughout tropical and subtropical latitudes around the world. Within this group is variation in size, sexual dimorphism, preferred habitat, diet, reproductive biology and behavior, and especially social organization, all of which are necessary to account for different outcomes of parasite-host relationships that may be influenced by environmental or social factors. Second, nonhuman primates are closely related to humans and have been the focus of large numbers of biomedical research programs and health screenings (Nunn and Altizer, 2005). Consequently, we now have ample data on what parasites infect primates in nature and in captivity, and many associated pathologies. Nevertheless, parasite sampling from wild primate communities remains unevenly distributed, with the largest gaps in the Neotropics, and the majority of available data is devoid of ecological context (Hopkins and Nunn, 2007). Third, the conservation status of primates is critical, and current estimates suggest that over 55% of primate species are threatened with extinction and 75% of species have declining populations (Estrada et al., 2017). Unfortunately, the bulk of this crisis is caused by the loss of habitat as a result of human activity. This in turn has given rise to greater questions surrounding the exchange of parasites and pathogens between human and nonhuman primates. Finally, as threats to nonhuman primates increase, we should not forget that some of the ecosystem services they provide are irreplaceable. For example, primates are the sole or primary distributors of seeds for a large number of tropical tree species (Rosin and Swamy, 2013).

Since seed dispersal is a primary mechanism for the maintenance of plant diversity, declining primate populations will inevitably effect tropical tree diversity (Swamy et al., 2010), and hence, biodiversity overall. Monitoring parasitism, although not a traditional measure for host population status, could be a particularly effective method for shy and elusive primate species, particularly because data can be obtained from noninvasively collected fecal samples.

The primates that were the focus of this dissertation consisted of two sympatric tamarin hosts, *Saguinus imperator* and *Leontocebus weddelli*, that have participated in an annual mark-recapture program since 2009. A mark-recapture program enabled the collection of multiple sample types for screening parasites (i.e. blood and fecal samples) alongside detailed information on every host animal across multiple years. Thus, in addition to asking what parasites are present, I was able to ask how they are distributed across host demographic factors. Tamarins are atypical among primates in that they live in small, often polyandrous, groups where reproduction is monopolized by a single dominant female (Sussman and Kinzey, 1984). To accommodate individual variation in reproductive capability, I developed a reliable model for accurately assigning breeding status to all individuals, regardless of age (Chapter 4). I was then able to systematically screen for parasites from blood (Chapter 1) and feces (Chapter 2) and analyze how they are distributed across host factors (age, sex, and breeding status) within and between the two hosts. Finally, I conducted targeted screening for *Plasmodium*, the causative agent of malaria in humans, because of its wide interest in the scientific and public health communities. In Chapter 3, I report my findings from this effort within a larger context of what is currently known about nonhuman primate malarial parasites in the New World. All together, this dissertation represents one of the most detailed and comprehensive sampling efforts for blood and gastrointestinal parasites from two intact, free-ranging populations of New World monkeys to date. I have provided baseline data that can be used for comparison to parasite studies from different field sites or to future points in time at the same site. Results from this dissertation open several new lines of inquiry that can contribute much more to our understanding of primate parasite

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Chapter 1

Temporal and demographic blood parasite dynamics in two free-ranging Neotropical primates

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Note: Nucleotide sequence data reported in this paper are available in the GenBank™ , EMBL and DDBJ databases under the accession numbers KX932481 – KX932490

Abstract

Parasite-host relationships are influenced by several factors intrinsic to hosts, such as social standing, group membership, sex, and age. However, in wild populations, temporal variation in parasite distributions and concomitant infections can alter these patterns. We used microscropy and molecular methods to screen for naturally occurring haemoparasitic infections in two Neotropical primate host populations, the saddleback (*Leontocebus weddelli*) and emperor (*Saguinus imperator*) tamarin, in the lowland tropical rainforests of southeastern Peru. Repeat sampling was conducted from known individuals over a three-year period to test for parasite-host and parasite-parasite associations. Three parasites were detected in *L. weddelli* including *Trypanosoma minasense*, *Mansonella mariae*, *and Dipetalonema* spp., while *S. imperator* only hosted the latter two. Temporal variation in prevalence was observed in *T. minasense* and *Dipetalonema* spp., confirming the necessity of a multi-year study to evaluate parasite-host relationships in this system. Although callitrichids display a distinct reproductive dominance hierarchy, characterized by single breeding females that typically mate polyandrously and can suppress the reproduction of subdominant females, logistic models did not identify sex or breeding status as determining factors in the presence of these parasites. However, age class had a positive effect on infection with *M. mariae* and *T. minasense*, and adults demonstrated higher parasite species richness than juveniles or sub-adults across both species. Body weight had a positive effect on the presence of *Dipetalonema* spp. The inclusion of co-infection variables in statistical models of parasite presence/absence data improved model fit for two of three parasites. This study verifies the importance and need for broad spectrum and long-term screening of parasite assemblages of natural host populations.

Keywords: co-infection; blood parasites; cooperative breeding; longitudinal sampling; Callitrichidae

1. Introduction

The surveillance of parasites and pathogens in wildlife populations has received international attention, since wildlife conservation outcomes can be affected by parasitic infections (van Riper et al., 1986; Levin et al., 2013), and since wildlife are increasingly found to host pathogens that can infect humans (Guberti et al., 2014). Parasite-host associations are dictated by characteristics intrinsic to the host, the parasite, and the environment. Although associations can vary, parasitism is frequently correlated with host density (Poulin, 2004; Fernandes et al., 2012), age (Sol et al., 2003; Clough et al., 2010; Parr et al., 2013; Leclaire and Faulkner, 2014) sex (Poulin, 1996; Schall et al., 2000; Clough et al., 2010; MacIntosh et al., 2010), and dominance status (Muehlenbein and Watts, 2010). Meta-analyses across species indicate that parasitism positively correlates with group size (Vitone et al., 2004; Rifkin et al., 2012), but this is modulated by the mode of transmission and mobility of the parasites in question (Cote and Poulin, 1995). In addition to these host-specific factors, when longitudinal data are available, studies of parasite prevalence in diverse taxa demonstrate temporal effects of season (Huffman et al., 1997; Raharivololona and Ganzhorn, 2010) and year (Bakuza and Nkwengulila, 2009; Clough et al., 2010; Moreno et al., 2013).

In nature, animals almost always exhibit infections by several different parasites at the same time and in succession (Pedersen and Fenton, 2007; Telfer et al., 2008; 2010). Since parasites can bring about distinct changes to host hematology, body condition and immune investment (Budischak et al., 2012; van Wyk et al., 2014), it follows that even disparate parasites can boost (Monteiro et al., 2007b; Knowles, 2011; Thumbi et al., 2014) or suppress one another (Moreno et al., 2013) via their influences on host immune function (Cox, 2001; Ulrich and Schmid-Hempel, 2012). Although the logistics and economics of collecting long-term, individual-based infection data are challenging, these data are critical to study the effects of age, social structure, life history, time, seasonal variation, and co-infection on disease dynamics (Clutton-Brock and Sheldon, 2010). For example, with repeat sampling we can assess how particular parasites influence host susceptibility to other parasites (Telfer

et al., 2008), and if concomitant parasite infections reduce or increase host fitness overall (Balmer et al., 2009). While long-term studies on human populations are numerous (Weil et al., 1999; Bloch et al., 2011), comparable monitoring of wild animal populations are rare (Telfer et al., 2008; Tompkins et al., 2010); however, such studies are critical in the case of long-lived hosts, such as the primates, with complex social organization.

A minority of primates exhibit social systems in which non-biological parents care for the offspring of dominant individuals in a group in a process known as alloparenting (Riedman, 1982; Sussman and Kinzey, 1984). These cooperative breeders, primarily tamarin and marmoset genera within the Callitrichidae, may exhibit greater amounts of parasitism than solitary or pair-bonded breeders due to elevated levels of sociality (Burkart, 2015), which will influence density- and frequency dependent parasite-host relationships (Anderson and May, 1978; Altizer et al., 2003; Poulin, 2004; Patterson and Ruckstuhl, 2013). However, if sociality affords an overall reduction in group energy expenditure, then cooperative breeding could instead decrease parasitism by allowing improved individual host immune function (Spottiswoode, 2007; Lutermann et al., 2013). Here, we present novel haemoparasite infection data from a longitudinal study of two free-ranging sympatric populations of cooperatively breeding primate species - the saddleback tamarin (*Leontocebus weddelli,* formerly *Saguinus fuscicollis wedelli*) and the emperor tamarin (*S. imperator*) (Matauschek et al., 2011; Buckner et al., 2015). This study explores the potential influences of intrinsic host factors, co-infection, and temporal variation on parasite prevalence via a mark-recapture program that allowed us to track the parasite infection status of individual animals across multiple years. This enabled us to control for biases due to temporal, environmental and protocol-related changes, which have been rarely addressed in studies of these species to date (Lisboa et al., 2000; Phillips et al., 2004; Wenz et al., 2009; West et al., 2013), but see (Monteiro et al., 2007a; 2007b).

We predicted four patterns of parasite prevalence would occur within this study system. Due to our

observations of consistent host social group sizes through an annual mark-recapture program from 2009 to 2015 (Watsa et al., 2015), we assumed stable host populations and that parasite prevalence would exhibit only minor fluctuations between years due to random stochastic variation in the environment (Schall et al., 2000; Knowles et al., 2013). Second, although sex-biased parasitism is a topic of long debate across taxonomic orders (Morales-Montor et al., 2004) with a tendency to assign greater parasite risk to males (Poulin, 1996; Klein, 2004; Muehlenbein, 2005; Muehlenbein and Watts, 2010) we predict the opposite trend in this host system. Callitrichid sociality is characterized by stark competition among females for breeding opportunities, with primary breeding females in return suppressing the reproduction of subdominant females behaviorally or through physiological stress (Ziegler et al., 1987; Beehner and Lu, 2013). Conversely, callitrichine males share mate access with little to no overt antagonism, and do not invest in potentially costly secondary sexual characteristics or extensive mate-guarding rituals (Hamilton and Zuk, 1982; Setchell et al., 2009). Callitrichids exhibit unusually high rates of twin offspring among primates (> 80% of births) with groups usually consisting of a single female that reproduces, while all other adults assist in rearing her offspring (Sussman and Kinzey, 1984; Terborgh and Goldizen, 1985). While absolute male-female sex ratios are not skewed in this population (Watsa, 2013), operational sex ratios are biased towards males, since typically a single female reproduces in each group. Thus, if there is a parasite risk associated with maintaining social status we predict that it should be borne predominantly by the primary breeding female in a group. Third, while immunosuppression in juveniles can lead to a preponderance of infections in younger age classes (Sol et al., 2003), immunosenescence in aging adults intensifies the accumulation of parasites over a lifespan, resulting in high incidences of infection among older age classes (Shanley et al., 2009). However, although callitrichids can live up to 20 years in captivity, higher predation risks in the wild result in lower lifespan maximums of around 9 to 11 years (Goldizen, 1996). We therefore predict a lower parasite prevalence in adults vs. subadults or juveniles in this study system. Finally, we predict significant associations between co-infecting parasites that present ecological overlap in infection sites, host resource use, or arthropod

vectors, since they are more likely to interact directly or indirectly via the host immune response (Cox, 2001).

2. Material and methods

2.1 Study area and sample collection

Sample collection took place at the Estación Biológica Rio Los Amigos (EBLA) in the Madre de Dios Department of southeastern Peru (12°34'07"S, 70°05'57"W)). The 900-hectare tropical rainforest research station is located at the confluence of the Los Amigos and Madre de Dios Rivers, and is contiguous with the much larger Los Amigos Conservation Concession (~1400 km^2) that lies within the buffer zone of Manu National Park (Watsa, 2013). Samples were collected annually during the dry season between May and August from 2012 to 2014. All of the primate social groups in this study inhabit a uniform area of forest with similar access to terra firme and várzea habitat. A safe animal mark-recapture program ongoing since 2009, based on the Peruvian trap model (Savage et al., 1993), was optimized to minimize the risk of harm to animals (Watsa et al., 2015). Animals were given permanent identification tags via subcutaneous microchips (Avid, Home Again©). Blood samples (<300ul) drawn from the femoral vein under ketamine hydrochloride anesthesia (Ketalar, Pfizer Inc., New York, USA) were stored dry on Whatman FTA Micro Elute Cards, and 2 to 3 blood smears were prepared with fresh blood from each animal. All sampling protocols adhere to guidelines outlined by the American Society of Mammalogists (Sikes and Gannon, 2011) and were approved by the Institutional Animal Care and Use Committee at the University of Missouri-St. Louis (317006-2, 733363-2) and the Directorate of Forest and Wildlife Management (DGFFS) of Peru annually.

2.2 Age class and breeding status determination

Among callitrichids, age and sexual maturity can become desynchronized, particularly among females, as a result of reproductive suppression of all but one dominant breeding female per group (Ziegler et al., 1987; Saltzman et al., 1998). Reproductive suppression has not been confirmed among

males; however they do exhibit significant variation in testicular volume and body size within a group, suggestive of a loose dominance hierarchy (Garber et al., 1996; Watsa, 2013). Therefore, we used separate methods to classify age-sex classes and breeding status for both sexes.

We used three broad age classes based on dental eruption patterns (Watsa, 2013). Juveniles were defined as individuals whose adult teeth were absent or not fully erupted (<11 mo). Sub-adults were animals with adult teeth, but that were juveniles in the preceding year. All remaining individuals were assigned to the adult age class. Older adults could not be distinguished based on tooth eruption patterns alone and were thus pooled within the adult age-class.

Breeding status assignments were based on individual weight measurements and reproductive morphology following Watsa et al. (2016, bioRxiv:047969). Briefly, body size and suprapubic gland area for both sexes, testes volume in males, and average nipple length and vulva index in females were combined in a principal component analysis (PCA). Coordinate values from the first two dimensions, accounting for > 80% of variation in the dataset, were applied to a linear discriminant function analysis that assigned all individuals of unconfirmed breeding status to one of three categories: primary breeder, secondary breeder, or non-breeder.

2.3 Parasite detection and identification by microscopy

Immediately after blood draw, blood smears were made on standard microscope slides and air-dried. All smears were fixed for five minutes in 100% methanol within six hours. They were stained in Giemsa's solution following Valkuinas et al. (2008) within three weeks of fixation, and observed at 400x magnification using light microscopy for the presence of parasites. Small extracellular and intracellular blood parasites were recorded while conducting a total leukocyte count estimation (enumeration of leukocytes in 10 non-overlapping fields of view in the smears' monolayer at 400x magnification) and differential (classification of 200 leukocytes in the monolayer at 1000x

magnification); each slide examination took less than 30 minutes. Examinations were carried out in a systematic direction to avoid overlapping fields of view, and damaged sections, where leukocytes and parasites were too distorted to identify, were excluded. To rule out the possibility of false negatives for trypanosome infection, blood smears from individuals that tested negative from two targeted PCR screenings were scanned a second time for 15 minutes at 1000x magnification, including areas of the smear not in the monolayer. Representative micrographs of each parasite were recorded under oil-immersion at 400x and 1000x magnification from the blood smears of at least ten separate individuals per host species. Measurements of length and width and the location of key anatomical features were made using printed photographs, which were compared to reference values from the literature to confirm species identification (Eberhard et al., 1979; Petit et al., 1985; Bain et al., 1986; Sato et al., 2008).

2.4 Molecular detection and sequencing

DNA was isolated from a 3 mm diameter hole punch from the blood stored on Whatman FTA Micro Elute Cards into 30ul of ddH_20 according to manufacturer instructions (GE Health Care Life Sciences, Pittsburgh USA). First and second elutions of DNA were obtained from each hole punch and both effectively amplified parasite DNA from infected animals; we used the first elution for consistency across animals for this study. Two genes were targeted to detect and identify filarial nematodes. The internal transcribed spacer 1 (ITS1) was amplified using forward primer S.r.ITSI-NC5/F1 and reverse primer NC13R following the protocol of Sato et al. (2008). Also, mitochondrial cytochrome oxidase I (COI) was amplified using forward primer COIintF and reverse primer COIintR following the protocol of Casiraghi et al. (2001). Both targets produced a single solid band on agarose gels for infected individuals that did not differentiate the two nematode parasites. The additional information from thin blood smears was used to assign single or concomitant infection status. If parasite infections remained unclear after viewing multiple blood smears for each animal, Sanger Sequencing of COI (rather than ITSI) clearly differentiated the parasites by sequence, or resulted in

chromatograms with double-peaks in the case of co-infection (Supplementary Fig. S4). A universal nested PCR reaction targeting the ssrRNA gene was used to detect and identify *Trypanosoma* spp. in all samples. The outer reaction used forward primer TRY927F and reverse primer TRY927R, and the inner reaction used forward primer SSU561F and reverse primer SSU561R following the protocol of Noyes et. al. (1999), but see primer clarifications in (Noyes *et al.*, 2000). For all parasites we concluded that a sample was positive for an infection if it appeared in a blood smear or if it could be successfully amplified by PCR. Conversely, to be considered negative for a given parasite, a sample had to be blood smear negative and PCR negative across a minimum of three replicate reactions.

For parasite classification, a subset of the positive PCR products were purified using Machery-Nagel PCR Clean-up Kits (Bethlehem USA) and sent to EuroFin Genomics (Louisville USA) for Sanger Sequencing using forward primers S.r.ITSI-NC5/F1 and COIintF for microfilariae, and SSU561F for *Trypanosoma* spp.

2.5 Statistical analyses

To test for significant changes in parasite prevalence across the study period while controlling for repeat measures of individual animals, we implemented randomized Z-tests of proportions. Each individual in our study was selected at random only one time for 1000 iterations of the test, thereby removing concerns of non-independence. If greater than or equal to 95% of the iterations resulted in p-values < 0.05, a difference in prevalence was considered significant. If a significant difference across the entire study period (2012 – 2014) was detected, then similar pairwise tests between all combinations of years were carried out but with p-values adjusted using the Holm-Bonferonni procedure (Holm, 1979).

The presence/absence of each parasite was modeled using generalized linear mixed effect models (GLMMs) with logit link functions and binomial errors (Bates et al., 2015, arXiv:1406.5823v1).

Fixed factor model terms included 'species', 'sex', 'age class', and 'breeding status', while 'group', 'individual identity', and 'year' factors were incorporated as random effects when they showed any impact on model outcomes (Zuur et al., 2007). If convergence errors occurred during model selection, individual identity was excluded from the most saturated fixed-factor models, as there were only ~ 1.7 captures/individual in the study. However, all random effects were reinstated following the first one or two rounds of model selection (Telfer et al., 2008). Model selection was carried out by stepwise term deletion and comparison of nested models with likelihood ratio tests, and we confirmed that the removal of all minimal model terms increased the Akaike Information Criterion (AIC) by at least two units (Akaike, 1974). All statistical analyses were performed using R software v.3.2.2 (R Development Core Team, 2015).

To discern the influences of host intrinsic factors from other concomitant parasite infections, a nested modeling approach was used (Telfer et al., 2008). Concomitant parasite infection data were added to models of each parasite response variable with its optimal host factor structure. This enabled us to determine whether co-infections generally improved model fit or strengthened or weakened associations with host factors. Additionally, we constructed a GLMM with Poisson errors to model parasite species richness. For this analysis, factors were the same as in other models, except 'group size' was inserted as additional fixed factor and 'species' was converted to a random effect since one of the three parasites discovered did not infect *S. imperator*.

3. Results

3.1 Blood parasite detection and identification

In total, we collected 186 blood samples (120 *Leontocebus weddelli*, 66 *Saguinus imperator*) from 111 individuals (74 *L. weddelli*, 37 *S. imperator*) (Table 1). Three blood parasites were identified by microscopy and targeted PCR screening: filarial nematodes *Mansonella mariae* and *Dipetalonema*

spp. and kinetoplastid *Trypanosoma minasense*. 90% of animals infected with a filarial nematode were detected by PCR alone; however, the remaining (8/76 and 10/94 for each nematode, respectively) infections were only detected by microscopy. We relied primarily on PCR to detect infections of *T. minasense* since low numbers of parasites (1-3 parasites/blood smear/infected animal) led to frequent false negatives; however, ~20% (25/127) of our positives came from blood smears for which corresponding PCR screening was repeatedly negative.

Table 1. Host sampling stratification by year, sex, age class, and breeding status

	Year	2012	2013	2014
L. weddelli		35	49	36
Sex	Male	18	30	19
	Female	17	19	17
Age	Juvenile	8	10	4
	Sub-adult	4	6	1
	Adult	23	33	31
Breeding Status	Non-breeder	11	12	4
	Secondary Breeder	5	15	18
	Primary Breeder	19	22	14
S. imperator		21	24	21
Sex	Male	10	15	10
	Female	11	9	11
Age	Juvenile	6	3	4
	Sub-adult	2	3	1
	Adult	13	18	16
Breeding Status	Non-breeder	8	4	5
	Secondary Breeder	2	6	4
	Primary Breeder	11	14	12
Mean captures per individual '12 – '14			*L. weddelli*	1.6 (1 - 3)
			S. imperator	1.8 (1 - 3)
Median captures per individual '12 –' 14			*L. weddelli*	1
			S. imperator	2

Our morphological description and measurements of *Mansonella mariae* and *Trypanosoma minasense* (Supplementary Table 3S) were consistent with previously published references for these parasites (Petit et al., 1985; Sato et al., 2008). Also, our partial sequences of ITS1 (N = 7) for *M. mariae* and ssurRNA gene (N = 10) for *T. minasense*, were both 99% identical (100% coverage) to records already on GenBank. *Dipetalonema* spp. did not sequence cleanly using ITS1; instead, we

amplified CO1. Our sequences (N=8) matched with *Dipetalonema* spp. on GenBank (95% identify, 100% coverage), and did not differentiate between three congeners, *D. gracile*, *D. graciliformis*, and *D. robini*. Morphological measurements from thin blood smears suggested mixed infections of *D. gracile,* and *D. graciliformis* present in both hosts based on reference values from the literature (Table 3S) (Eberhard et al., 1979; Bain et al., 1986). Sequences of all three parasites have been deposited on GenBank (accession nos. for *Dipetalonema* KX932481, KX932482; for *M. mariae* KX932483, KX932484; for *T. minasense* KX932485, KX932486, KX932487, KX932488, KX932489, KX932490).

3.2 Infection prevalence over time

Across the study period the prevalence of *M. mariae* remained stable (0.54 to 0.67) (Fig. 1). Although Figure 1 suggests that there should be significant increases in *Dipetalonema* spp. infection across the study period, after controlling for repeated measures, the upward trend approached significance for *L.weddelli* alone (mean χ^2= 2.41, df = 2, mean *P* = 0.074, but P < 0.05 63.4% of the time) (Supplementary Table 4S). Differences in prevalence for *T. minasense* across the study period were significant considering just *S. imperator* and both host species combined (P < 0.05 96.7% and 100% of the time, respectively), and approached significance for *L. weddelli* (P < 0.05 92% of the time) (Table 4S). By graphing changes in infection status per individual per parasite across the entire study period, the sources of annual variation in prevalence could be tracked (Fig. 2). A large spike in the presence of *T. minasense* in 2014 was the result of previously uninfected individuals acquiring infections, with no previously infected individuals losing infection – this was different from 2012 to 2013 when equal numbers of individuals gained and lost infection. A small number of previously uninfected individuals acquired new infections of *Dipetalonema spp.* each year, and we observed only one instance for which a previously infected individual was not found infected with *Dipetalonema* spp. in the subsequent year.

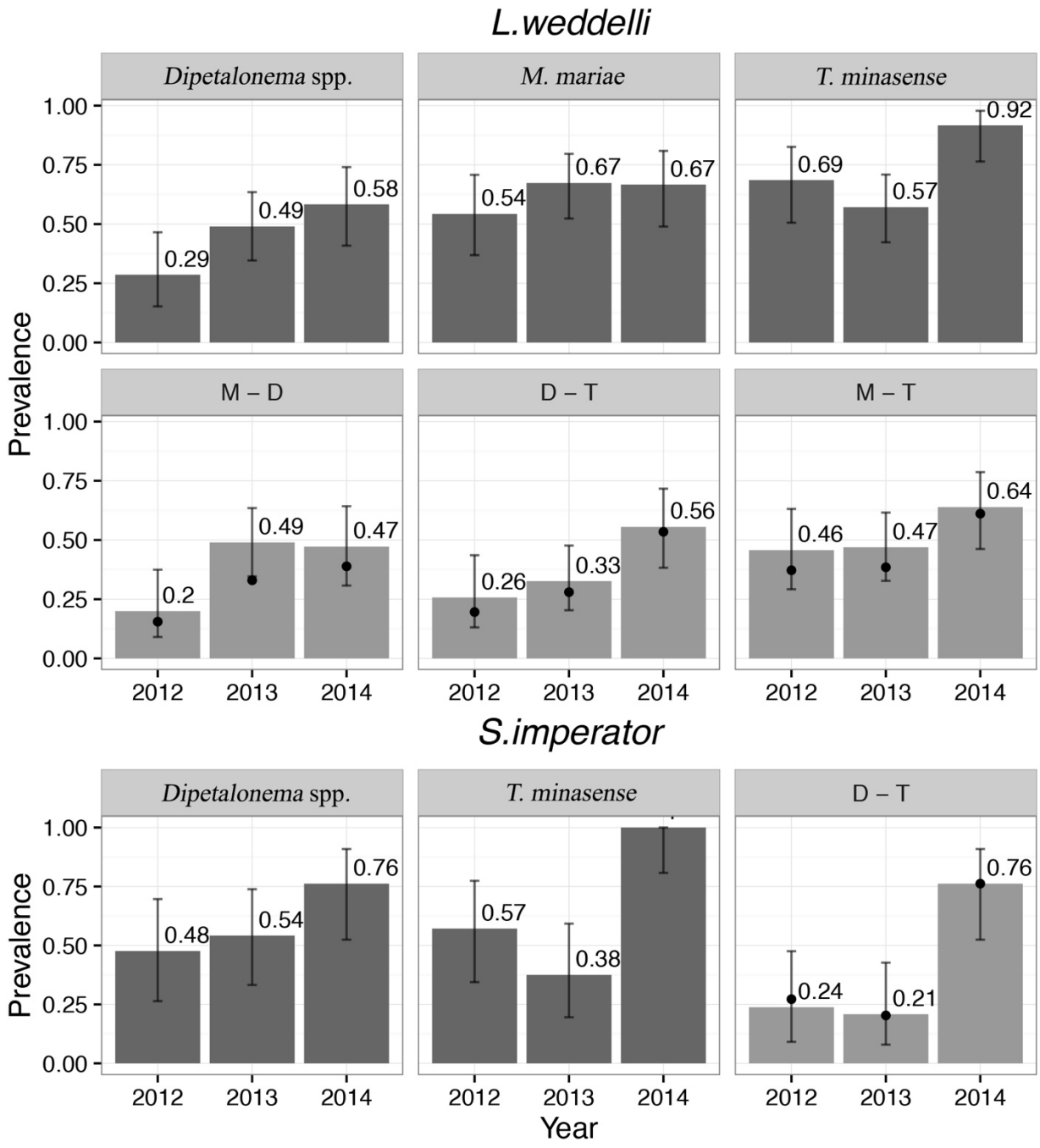

Fig. 1. Annual prevalence of single- and co-infections by species. Prevalence indicated for each parasite (dark gray), and each pairwise combination of parasites (light gray). Numbers near the top of each bar show the exact prevalence; black lines indicate 95% confidence intervals; dots indicate expected levels of co-infection (refer to section 3.2). M-D is co-occurrence of M. mariae and Dipetalonema spp., D-T is Dipetalonema spp. and T. minasense, and M-T is M. mariae and T. minasense.

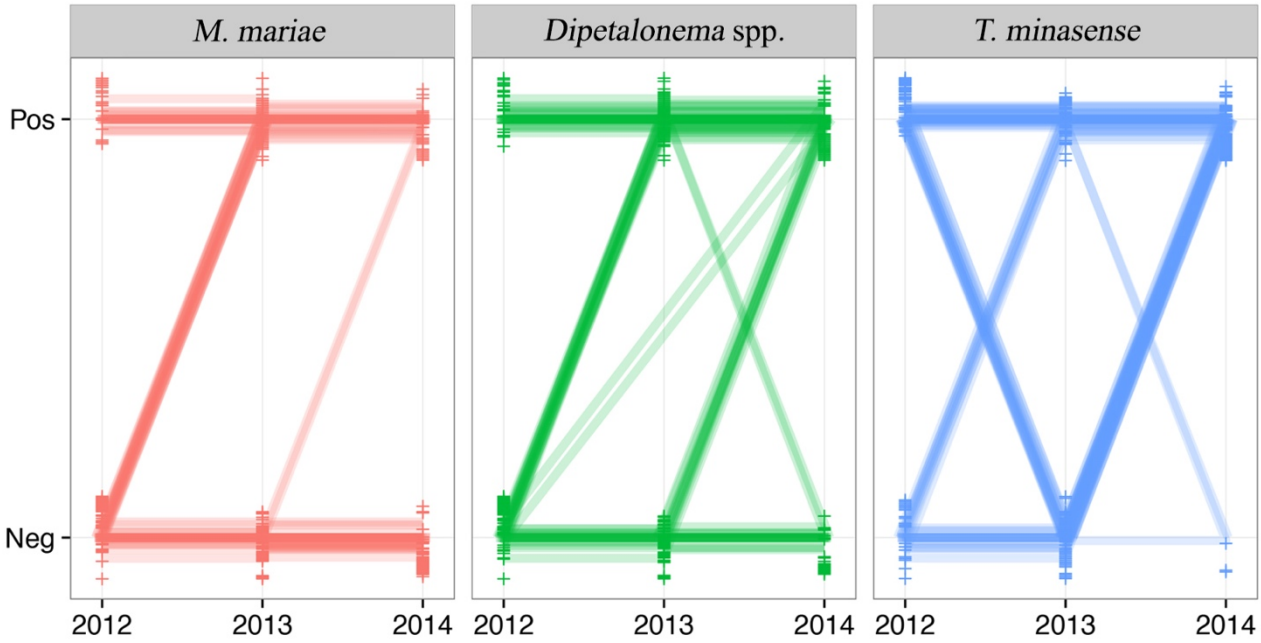

Fig. 2. Individual infection status by parasite by year. Strength and thickness of lines are scaled to the number of individuals that took a given infection trajectory from one year to the next. Two diagonal lines span 2012 – 2014 because those individuals were not sampled in 2013. The + symbols represent every infection or non-infection found across all individuals in the study.

In any given year, assuming independence, a simple expectation for the rate of co-infection can be obtained by multiplying the prevalence of two parasites together. All co-infection rates met these expectations except the rate of co-infection of *Dipetalonema spp.* and *M. mariae*, which was significantly higher in 2013 (χ^2= 10.2, df = 1, $P < 0.001$) (Fig. 1).

3.3 Modeling outcomes

To explore parasite-host and parasite-parasite associations we constructed a series of nested GLMMs. The goal of the first model was to identify those host variables that best explained the presence or absence of each infection, and subsequent models incorporated co-infection explanatory variables to

observe if model fit improved, or whether significant predictors remained the same. Infection by *Dipetalonema spp*. was positively associated with body weight and model fit was improved by adding of co-infection with *M. mariae* ($x^2 = 5.032$, df =1, $P = 0.0249$), although the GLMM estimate for *M. mariae* only approached significance (Table 2). Minimal models of prevalence of *M. mariae* included either age class or breeding status, and not both, as these factors were negatively correlated (Fisher's Exact Test, $p < 0.001$). When co-infection variables were introduced, the presence of *Dipetalonema* spp. also had a significant positive effect on *M. mariae*. *T. minasense* infection was predicted by an animal's age class or breeding status (again, correlation required factor reduction), and the addition of co-infection with *M. mariae* significantly improved model fit (Table 2). Regarding individual parasite species richness (PSR), younger age classes exhibited significantly fewer unique parasite infections (Fig. 3, Table 2)

Table 2: Model outcomes for each parasite response variable and parasite species richness

Dipetalonema spp.					
	estimate	Std. Err	Wald (x^2)	Df	P-value
W/out co-infection: n=186, df=181, AIC = 178.0					
(intercept)	-0.79	1.80			
Body weight	5.66	2.37	5.70	1	0.017
W/ co-infection: n=186, df=181, AIC = 175.0					
(intercept)	-1.81	1.60			
Body weight	5.10	2.23	5.24	1	0.022
M.mariae	2.91	1.66	3.07	1	0.080
Mansonella mariae					
	estimate	Std. Err	Wald (x^2)	Df	P-value
W/out co-infection: n=120, df=116, AIC = 94.5					
(Intercept)	-4.34	2.12			
Br_Primary	7.76	3.51	5.12	2	0.077*
Br_Secondary	5.19	2.63			
W/ co-infection: n=120, df=114, AIC = 88.7					
(Intercept)	-4.89	1.99			
Br_Primary	7.14	2.79	6.59	2	0.037
Br_Secondary	4.42	1.96			
Dipetalonema spp.	2.56	1.13	5.18	1	0.023
Trypanosoma minasense					
	estimate	Std. Err	Wald (x^2)	Df	P-value
W/out co-infection: n=186, df=180, AIC = 205.0					
(Intercept)	1.66	0.81			
Age_Juvenile	-1.72	0.54	10.01	2	0.007
Age_Sub-adult	-0.56	0.63			
W/ co-infection: n=186, df=179, AIC = 202.2					
(Intercept)	1.19	0.88			
Age_Juvenile	-1.16	0.54	4.57	2	0.102*
Age_Sub-adult	-0.45	0.64			
M. mariae	1.07	0.50	4.69	1	0.030

Parasite Species Richness					
	estimate	Std. Err	Wald (x^2)	Df	P-value
	n=186, df=181, AIC = 496.7				
(Intercept)	0.61	0.15			
Age_Juvenile	-1.43	0.26	34.89	2	<0.001
Age_Sub-adult	-0.59	0.25			

Minimal models shown above. Saturated models included fixed factors 'species', 'sex', 'body weight', 'breeding status', and 'age class'. Co-infection models began with terms from minimal host infection models and other parasites as fixed factors. Parasite species richness was modeled using only host factors. All tests included random effects 'animal identity', 'group', and 'year' when they evidenced any discernable effect on model outcomes. The * symbol indicates factors where only one of two levels was significant, and therefore a combined x^2 statistic for all levels of those factors is not significant.

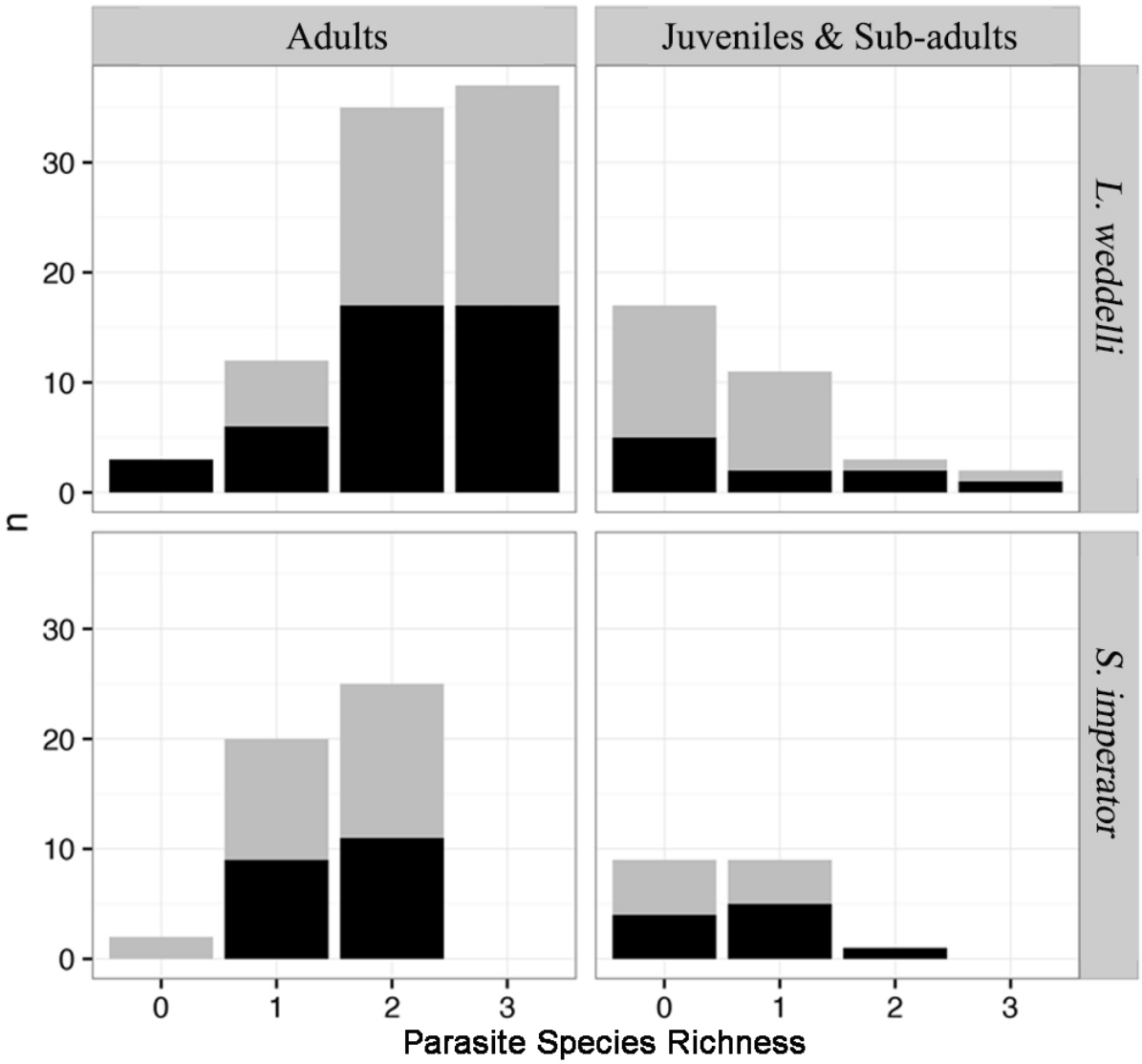

Fig. 3. Parasite species richness by species, age class and sex. Colors represent females (black) and males (grey)

4. Discussion

The majority of studies on parasite distributions from natural populations are constrained by sampling that takes place at a single time point on unknown host individuals. This limits our ability to interpret data due to the normal fluctuations in parasite prevalence and uncertainty regarding an animal's

future or past parasite infection status. In our study, we were able to sample from consecutive years at the same site, thus minimizing confounding variables such as animal disappearances or sampling from different individuals between years. The use of combined microscopy and molecular methods to screen for haemoparasitic infections improved our confidence of the infection status of each individual. We encountered slight discordance in microfilariae infection status, ~10% of blood smear positive samples were PCR negative. We think that this disparity is most likely attributable to parasitemia levels that are below the sensitivity of the PCR assay, poor sample quality, or a combination of both, as both of our assays worked 90% of the time and each primer set has been used on a broad range of nematodes in other studies (Casiraghi et al., 2001; Sato et al., 2006; Merkel et al., 2007; Sato et al., 2008). Discordance for *T. minasense* was greater, ~20% of blood smear positive samples were PCR negative. This level of parasite under-detection is unsurprising, as chronically infected humans and animals with low levels of parasitemia are known to occur with *Trypanosoma* spp. (Piron et al., 2007). Qvarnstrom et al. (2012) observed that different genotypes of *Trypanosoma cruzi* are differentially detected by real-time PCR assays, and therefore recommend the use of multiple protocols that target different genes. It is not known whether similar challenges are associated with detection of *T. minasense*, but if so, we would expect false-negatives to be equally represented across the study period. Instead, we had zero false-negatives in 2014, suggesting again that low parasitemia or poor sample quality were the likely culprits of false negatives. Nested PCR reactions, which we employed in this study, are the current gold standard for detection of active Trypanosome infections (Ndao et al., 2004; Aguiar et al., 2012).

We predicted that haemoparasite prevalences would remain constant over the study period, reflecting stable numbers of host individuals with average group sizes varying from 4.3 – 4.9 individuals over a range of 13 to 15 social groups per year. While prevalence remained stable for *M. mariae*, we cautiously report annual increases for *Dipetalonema* spp.; increases approached significance among *L. weddelli* after controlling for animal repeated measures through randomization tests (significant

63.4% of the time in 1000 iterations). *Mansonella mariae*, like other filarial nematodes such as the human-infecting *Wuchereria bancrofti*, likely exhibits circadian migrations from deeper tissues to peripheral blood where it is picked up by hematophagus vectors (Barrozo et al., 2004). We reliably detected continued *M. mariae* infection in all animals harboring infections the previous year, so temporal sampling bias would not explain differences in prevalence from *Dipetalonema* spp. Given the relatively stable climate between years and that both parasites are vectored by ceratopogonid biting midges (Shelley and Coscarón, 2001; Lefoulon et al., 2015), we suspect that within-host dynamics are responsible for the variation observed between these two filarids, although the physiological consequences of these species on wild hosts remain unknown (Strait et al., 2012). It is worth mentioning that *Dipetalonema spp.* likely represents two species, *D. gracile* and *D. graciliformis*, and our study does not differentiate whether one or both are responsible for the increase in prevalence. Phylogenetic relationships within the *Dipetalonema* clade of the Onchocercidae are an area of active research that is beyond the scope of this study (Lefoulon et al., 2015), and since mixed infections are common in nature (Sato et al., 2008; Strait et al., 2012), we do not know if parasite-host relationships would vary at sub-genus levels. The prevalence of *T. minasense* did change significantly across the study period, and most dramatic was the 2014 spike to 100% of *S. imperator* individuals and close to 100% among *L. weddelli*. Like *M. mariae*, circadian patterns of parasitemia could contribute to varying prevalence when sample collection occurs at different timings (Deane and Da Silva, 1974), however, in this study sample collection consistently took place between 6am and noon. Instead, a new environmental stressor on the host populations, or changes in vector populations, in 2014 might explain the spike in prevalence, but follow-up studies are needed to confirm this.

In addition, multi-host, multi-parasite systems are common in nature but often unacknowledged, particularly in areas of high species density (Hopkins and Nunn, 2007). In Kibale National Park in Uganda, a long-term study of the sympatric black-and-white (*C. guereza)* and red (*Pilocolobus tephrosceles)* colobus monkeys by Chapman et al. (2005) found that forest disturbances precipitated a

population increase in *P. tephrosceles* but a decrease in *C. guereza*. Simultaneously, the authors observed that a shared roundworm parasite, *Trichuris* sp., decreased in prevalence and intensity in *P. tephrosceles* while increasing in *C. guereza*. Although *Trichuris* sp. is typically asymptomatic at low levels, it can cause pathologies at higher intensities (Gillespie and Chapman, 2008; Gachinmath et al., 2014), and may very well have contributed to the decline of *C. guereza* through altered parasite-host dynamics initiated by forest clearings. Our data on haemoparasites establish a baseline that will facilitate similar research in the event of future climate or landscape disturbances. That *L. weddelli* and *S. imperator* form mixed-species associations (Watsa, 2013), possess broadly overlapping diets (*pers. obs*.), and exhibit similar social structures and reproductive strategies (Goldizen, 1996), but only share two of the three parasites is surprising. We did not expect that *M. mariae* would be absent from *S. imperator* given that it was previously documented in a more distantly related primate, the common squirrel monkey (*Saimiri sciureus*) (Sato et al., 2008). Nevertheless, as *M. mariae* evidenced a positive association with *Dipetalonema* spp., which are shared by both hosts, its potential for indirectly regulating parasitism in *S. imperator* should not be discounted.

Contrary to our hypothesis, and to a recent study on parasite distributions from a cooperatively breeding meerkat population (Smyth and Drea, 2015), we found no evidence that dominant individuals (in this case primary breeding females) were more or less parasitized than others across both host species. Our results are consistent with Viljoen et al. (2011) who found no clear relationship between parasitism and female reproductive dominance in the highveld mole rat (*Cryptomys hottentotus pretoriae*). One explanation for this negative finding is methodological. Since effects on host health and fitness are often parasitemia dependent, our reliance on parasite presence-absence, rather than densities, decreases our ability to detect small associations between parasites and host factors. Hence, we can challenge the existence of a strong relationship between blood parasites and sex or breeding status, but not entirely rule it out. Another explanation might be that cooperative breeding behavior produces group-wide energy conservation benefits that offset any extra burden that

would be experienced by dominant breeding individuals (Lutermann et al., 2013). It will also be worth considering the effects of social rank from the perspective of gastrointestinal parasites in this same population. Parasites found in the gastrointestinal tract versus peripheral blood generally differ in their modes of transmission (usually direct and indirect versus arthropod-vectored, respectively) and this can precipitate fundamentally different associations with host populations. For example, the encounter-dilution effect predicts a negative association between parasitism and social group size for arthropod-vectored parasites (Mooring and Hart, 1992; Cote and Poulin, 1995). Our data showed no relationships between group size and parasite species richness; however group size only ranges from 3-8 in this system (Watsa et al., 2015), which may not be sufficiently variable to test this hypothesis (Patterson and Ruckstuhl, 2013).

Across all of our models, breeding status and age class were too tightly correlated to be included as separate explanatory variables, and future studies might avoid this limitation by developing fine scale measures to differentiate the adult population. That parasite species richness was significantly elevated among adult individuals and breeders, as opposed to juveniles or sub-adults, suggests that more time for parasite exposure, and not immune status, is responsible for age-biased parasite distributions. We do not think this result was influenced by parasite prepatent periods, that can last months for filarial nematodes (Wong et al., 1969), since the trend included both juveniles and sub-adults ranging from 4 to 18 months of age. Additionally, positive relationships between prevalence and intensity of filarial infections and host age are well-documented from studies on human populations (Vivas-Martínez et al., 2000; Terhell et al., 2001; Opara and Fagbemi, 2008). We also established a small but significant positive association between body weight and prevalence of *Dipetalonema* spp. Generally, the potential effects of host body size are considered with respect to parasite species richness in interspecific comparisons (Hubbell, 1997; Vitone et al., 2004), and not intraspecific prevalence. In this study, *S. imperator* is about 25% larger than *L. weddelli* (Watsa et al., 2015), and yet our models did not detect an interspecific difference in microfilariae infection, after

controlling for concomitant infections. We suspect that body size covaries with other factors, such as age class, which we have shown to effect prevalence.

Controlled experimental studies on co-infection dynamics (Cox, 2001; Knowles, 2011) and studies from wild populations (Pedersen and Fenton, 2007; Monteiro et al., 2007b; Telfer et al., 2008) consistently show that relationships between parasites should not be ignored when evaluating parasite distributions in a single host (Christensen et al., 1987). This study provides additional evidence for that conclusion; specifically, the nested modeling approach uncovered two instances in which the addition of co-infection variables improved model fit. The patterns of co-infection in this study also raise questions. *Trypanosoma minasense* and *Dipetalonema spp.* (at least marginally) exhibited increases in prevalence, and we might expect an association between the two that is mediated by the immune system. Instead, co-infection between *M. mariae* and *Dipetalonema spp*. exhibited a positive association, and *M. mariae* alone best predicts the presence of *T. minasense*.

These findings demonstrate that longitudinal sampling from known individuals provides valuable insight into limiting confounding variables and unraveling complicated relationships between parasites and wild host populations. They also reemphasize the importance of factoring co-occurring parasites into analyses on parasite distributions. Challenges associated with conducting repeat sampling of blood from wild mammalian hosts have skewed prior research towards noninvasive gastrointestinal parasite monitoring from fecal samples, yet it is important to detect deviations in these patterns due to differences in parasite life cycles. Particularly when attempting to understand parasite distributions and individual risks among hosts with complex social organization, longitudinal sampling protocols that incorporate multiple hosts can be indispensable. Here, we have established that two very similar cooperatively breeding hosts in sympatric association differ in their blood parasite assemblages, and further, that individual differences engendered by callitrichid cooperative breeding dynamics do not appear to influence blood parasite prevalence. We recommend that future

work incorporate measures of individual immune status alongside blood parasite data, and also include analyses of gastrointestinal parasites.

Acknowledgements

We wish to thank researchers from Field Projects International who aided in the collection of biological samples, in particular Frances Humes. We want to thank Cindee Retke for her assistance with wet laboratory analyses. We express our gratitude to the following agencies and organizations that provided non-monetary support to this investigation: Centro de Ecologia y Biodiversidad, Amazon Conservation Association, el Estación Biológica Río Los Amigos, Dirección General Forestal y de Fauna Silvestre (Perú).

Funding: This work was supported by Field Projects International, the American Society of Mammalogists, University of Missouri Trans World Airlines Scholarship Program, Whitney R. Harris World Ecology Center, Sigma Xi Society, IdeaWild, and the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases, Comparative Medicine Branch.

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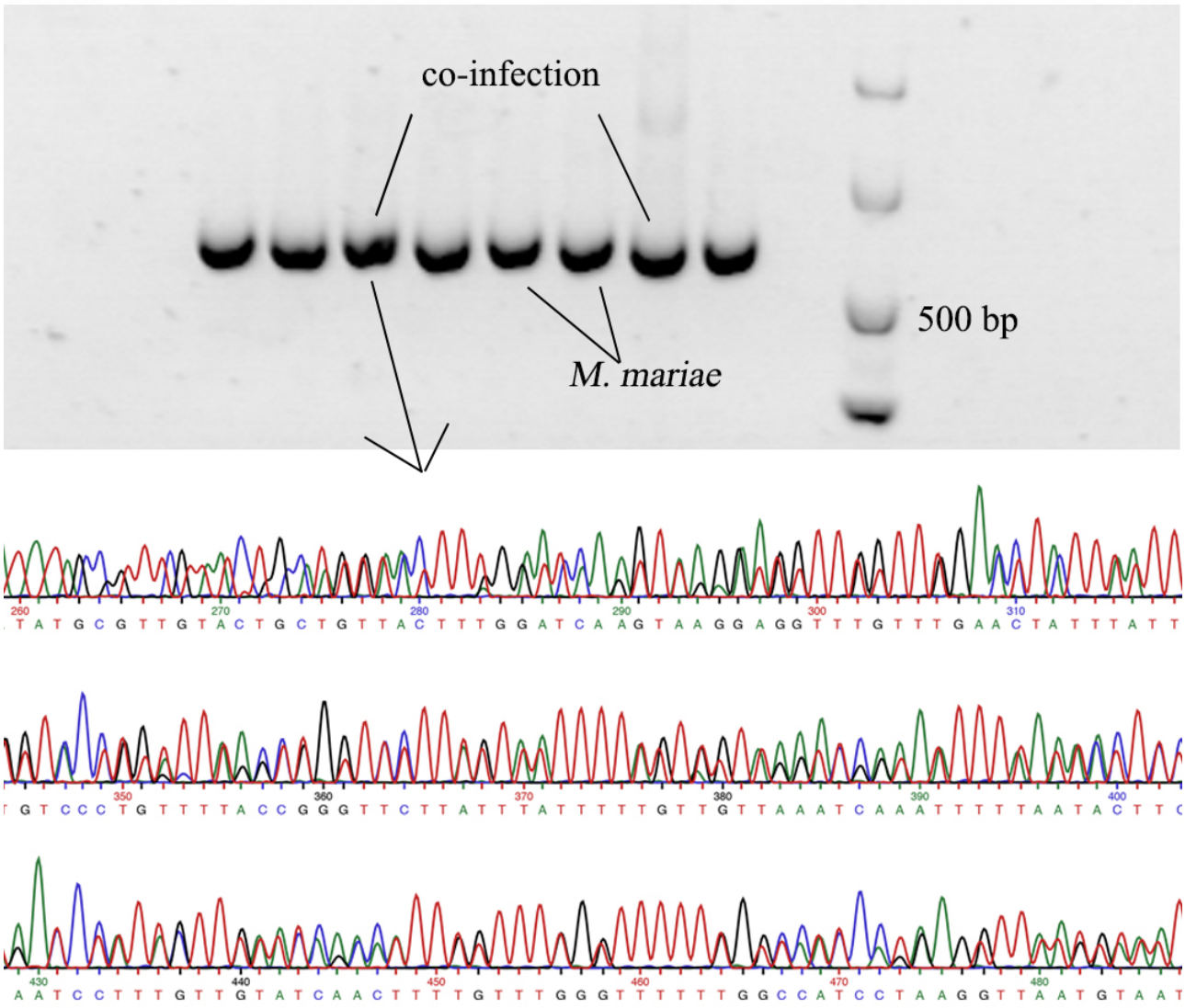
Supplemental Materials



Supplementary Fig. S4. Agarose gel image and chromatogram of COI gene. (a) Gel bands of individuals with infection with either *Dipetalonema spp.* (not indicated), *M. mariae* (indicated), or both (indicated). (b) Co-infection is confirmed on a chromatogram by the presence of double-peaks.

Table 3S. Parasite measurements

Parasite	Length ± sd (min - max)	width	n
Mansonella mariae	313.5 ± 21.0 (290 – 360)	2.25 (2.00-2.50)	12
Dipetalonema gracile	119.0 ± 20.3 (87-150)	5.3 ± 0.5 (4.5 – 6.0)	7
Dipetalonema graciliformis	102.6 ± 7.1 (95-120)	4.8 ± 0.5 (4-6)	27
*Trypanosoma minasense**	41.6 ± 4.8 (20-50)		23

All measurements are in µm

Table 4S. Randomized Z-tests of parasite prevalence across years

Dipetalonema spp.					
Host(s)	**Comparison**	**Mean x^2**	**Df**	**mean P**	**%**
L. weddelli	3 years	2.41	2	0.074	63.4
S. imperator	3 years	1.90	2	0.486	2.8
Both species	3 years	5.14	2	0.138	32.5
T. minasense					
L. weddelli	3 years	6.05	2	0.020	92
L. weddelli	‘12 v ‘13	0.39	1	0.665*	0.1
L. weddelli	‘12 v ‘14	3.61	1	0.245*	3.8
L. weddelli	‘13 v ‘14	6.01	1	0.064*	44.4
S. imperator	**3 years**	**10.05**	**2**	**0.012**	**96.7**
S. imperator	‘12 v ‘13	1.75	1	0.436*	1.9
S. imperator	‘12 v ‘14	5.72	1	0.273*	1.2
S. imperator	‘13 v ‘14	8.97	1	0.043*	43.6
Both species	**3 years**	**16.46**	**2**	**<0.001**	**100**
Both species	‘12 v ‘13	1.88	1	0.312*	3.2
Both species	‘12 v ‘14	10.33	1	0.041*	72.2
Both species	**’13 v ‘14**	**15.41**	**1**	**0.001***	**100**

Each test is the result of 1000 iterations of randomly re-sampling the study population so that each host individual is represented a single time. The % symbol refers to the percentage of iterations with P-values <0.05. Significant differences are highlighted in bold. The * symbol represents adjusted P-values using the Holm-Bonferroni method.

Chapter 2

A multi-year survey of helminths from the gastrointestinal tract of wild saddleback (*Leontocebus weddelli*) and emperor (*Saguinus imperator*) tamarins

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Introduction

Parasitism has a fundamental role to play in the persistence of animal populations in nature, and the richness of parasite communities may serve as effective population and ecosystem level measures of health (Hudson, 1998; Hudson et al., 2006). There is evidence from animal and human populations that natural infections with multiple parasites can have positive effects on host health (Petney and Andrews, 1998). For example, helminth infections have been shown to reduce allergic, autoimmune and inflammatory reactions (Maizels and McSorley, 2016), and helminth-modulated macrophages are now being studied as possible therapies for inflammatory diseases, such as diabetes, multiple sclerosis, and bowel disease (Steinfelder et al., 2016). Although the "hygiene hypothesis" has led to much misunderstanding about the importance of personal hygiene in preventing disease, it has fueled studies that are increasingly linking the incidence of allergies and autoimmune diseases to lack of exposure to diverse microbes during development (Scudellari, 2017). Thus, while "parasitism" connotes the acquisition of resources (space, food, etc.) by one organism at the expense of another, we should be cautious about considering parasites as detrimental in complex environments where they may play a role in maintaining an ecological balance that is necessary for species persistence.

Recent taxonomic revisions have described 504 species in 16 extant families within the Primate Order, of which 60% are threatened with extinction and 75% evidence population declines (Estrada et al., 2017). This pattern is strongly linked to human pressures on nonhuman primate populations through habitat loss via industrial agriculture (including palm oil production), cattle ranching, logging, oil and gas drilling, and gold mining (Lewis et al., 2015; Vijay et al., 2016). The conservation status of many primates is further threatened by the effects of global climate change driven by anthropogenic factors (Gouveia et al., 2016; Malhi et al., 2008; Pearson and Dawson, 2003), as well as disease spillover originating as a byproduct of increased contact between human and wild nonhuman primates (Dashak et al 2008). Hence, increasingly close monitoring of the health of

wild primate populations is critical to global human health.

Gastrointestinal parasites, which can be detected through noninvasive surveying, may be ideally suited for health monitoring efforts of a primate community (Gillespie, 2006; Howells et al., 2011). They are relatively easy to evaluate from fecal samples collected from habituated primate groups, and can also be acquired in the absence of habituation by scat detection dogs or by searching beneath known feeding or resting locations (Arandjelovic et al., 2015; Orkin et al., 2016). Several long-term research programs have successfully used temporal parasite data to examine ecological perturbations of threatened primate populations (Bakuza and Nkwengulila, 2009; Chapman et al., 2005; Gillespie and Chapman, 2008; Gillespie et al., 2005). In contrast, in the absence of temporal data, comparative studies between isolated and more urban primate populations are effective at evaluating impacts of increased contact with humans (Salzer et al., 2010; Wenz et al., 2009). Despite the utility of such studies, parts of the world with the highest primate diversity, such as the Neotropics, remain relatively inadequately sampled for naturally occurring gastrointestinal parasites (reviewed in Hopkins and Nunn (2007).

Except for a few South American primate taxa, notably howler monkeys (*Allouatta* spp.), golden lion tamarins (*Leontopithecus rosalia*), and golden-headed lion tamarins (*Leontopithecus chrysomelas*) (Milton, 1996; Monteiro et al., 2007a; 2007b; Stuart et al., 1998; Valdespino et al., 2010), patterns of parasite-host relationships have been tested primarily among primates in Africa and Asia. These works highlight that parasitism varies with host population demographic variables, including age class and sex (Clough et al., 2010; Gillespie et al., 2013; 2010; MacIntosh et al., 2010), and sexual maturity or dominance (MacIntosh et al., 2012; Muehlenbein and Watts, 2010), although not all studies concur (Gillespie et al., 2010; Setchell et al., 2009; 2006). Host behavior in combination with parasite mode of dispersal can also structure parasite populations (MacIntosh et al., 2010; Nunn and Heymann, 2005). Influences of concomitant parasite infections are not routinely analyzed, but when

they are, their impacts are comparable to those exerted by host or environmental factors {Telfer:2008jw}(Erkenswick et al., 2017; Monteiro et al., 2007b; Nunn et al., 2014). Also, meta-analyses find general support for increasing parasite species richness as social group size increases (Cote and Poulin, 1995; Nunn et al., 2003; Rifkin et al., 2012; Vitone et al., 2004), but the scale (population, community-wide, regional, or global) at which this pattern holds true requires additional consideration. Finally, seasonal and annual variation in parasite communities is also well documented (Clough et al., 2010; Gillespie et al., 2010).

Hence, the range of factors that can explain parasite-host patterns in nature is large, often contextually-dependent on the environment and time, and therefore may be best approached through longitudinal monitoring efforts of host communities at the level of the individual (Clutton-Brock and Sheldon, 2010; Stuart et al., 1998). A primary challenge has been that research on wild primates requires long, careful habituation to observers, which often puts constraints on sample sizes, thereby making it difficult to analyze many of the factors mentioned {Williamson:2003vp}. There are many studies, including those in the Neotropics, that have offered single snapshots of parasite prevalence levels, focused on just one or two parasites of known interest, sampled a single primate host, or have reported data from health inspections, or necropsies, after animal extraction from the wild (Cosgrove et al., 1968; Porter, 1972; Wolff, 1990); collectively they have created a broad foundation of primate parasite data (see Nunn and Altizer, 2005 for a detailed compilation). Emerging patterns can now be examined carefully in the wild to look at influences of host demography and development, mode of transmission, and change over time at the level of a population. As an example, for almost a half-century it has been well known that New World monkeys are broadly infected by *Plasmodium braslianum*, a quartan malarial parasite, that may in fact be the same as the human parasite *Plasmodium malariae* (Collins and Jeffery, 2007; Lalremruata et al., 2015). However, only this year do we have the first evidence that it may persist in a highly aggregated manner among a small number of chronically infected non-human primate hosts (Erkenswick et al. in review). In addition, long-term

studies that incorporate more than one primate host individual will be essential to examine several longstanding hypotheses of how sociality influences parasite prevalence, intensity, and diversity (Altizer et al., 2003; Freeland, 1976; 1979), as will long-term studies of multiple sympatric species to examine species-specificity of infection dynamics.

The Callitrichidae (comprised of tamarins and marmosets) are small arboreal primates that are widely distributed throughout the forests of South America (Sussman and Kinzey, 1984). They are frequently found in sympatry with other New World monkeys and in some cases have proven relatively resilient and flexible in the face of encroachment by human populations (Gordo et al., 2013; Leite et al., 2011; Soto-Calderón et al., 2016). Part of their ecological flexibility may be due to their generalist diets that include fruits, insects, tree exudates, and fungi (Sussman and Kinzey, 1984), a characteristic that also could expose them to a wide array of parasites that are dispersed by intermediate arthropod hosts. Studies of the gastrointestinal parasites of callitrichids have documented overlap with other primate families including in the Ateledae, Cebidae, and Aotidae (Michaud et al., 2003; Phillips et al., 2004; Tantalean et al., 1990; Wolff, 1990). Considering the approximately 60 species and subspecies of Callitrichidae, there have been only a handful of comprehensive evaluations of gastrointestinal parasites from free-ranging populations (Monteiro et al., 2007a; Müller, 2007; Wenz et al., 2009), and only two populations in which parasites have been monitored routinely over time (Monteiro et al., 2007b).

The principle aim of this study was to characterize the gastrointestinal helminth assemblages from two populations of sympatric, individually identifiable, free-ranging callitrichids, the saddleback tamarin *(Leontocebus weddelli,* formerly *Saguinus fuscicollis weddelli*) (Buckner et al., 2015; Matauschek et al., 2011) and emperor tamarin (*Saguinus imperator*), from noninvasively collected fecal samples. Both hosts exhibit group sizes (3 – 8 individuals), mating systems, and reproductive

behaviors characteristic of most Callitrichids (Watsa et al., 2016). Typically, a single, reproductively dominant female will mate with multiple males and give birth to twin offspring once a year, and the remaining adult group members provide alloparental care of the offspring (Sussman and Kinzey, 1984; Wislocki, 1939). By sampling these hosts across three years, we attempted to determine precise estimates of the prevalence of gastrointestinal parasites, parasite species richness, and the extent of parasite overlap between the two host species. We also calculated rates of change in infection status from animals that were screened for helminths in two consecutive years. In doing so, we establish robust baseline data for future comparative studies on changing weather patterns due to climate change, habitat loss/modification, or greater human encroachment. Secondarily, we analyzed how parasite prevalence varies by host demography, age class and sex, and co-infection. As a result of greater social burdens placed on females to compete for dominant breeding opportunities, we predicted that an age-sex interaction will influence prevalence and parasite species richness. Specifically, adult females of both host species will have higher prevalence and richness. Based on prior research of blood parasites from these populations (Erkenswick et al., 2017), we predicted that there will be non-random prevalence of several co-infections, considering all pairwise combinations of parasites. Finally, we test the hypothesis that there will be a relationship between group size and parasite species richness, and predicted that larger groups will harbor greater numbers of parasites, which has not yet been tested within the Callitrichidae. Our findings are additionally discussed in terms of parasite pathogenicity and parasite mode of dispersal.

Methods:

Field site and study subjects:

Sample collection took place annually from 2012 – 2014 in the Madre de Dios Department of

Southeastern Perú at the Estacion Biologica Rio Los Amigos (EBLA) (12°34'07"S, 70°05'57"W), which is managed by the Associación para la Conservación de La Cuenca Amazonica (ACCA). All sampling took place within a forest trail system that covers approximately 900 hectares of tropical evergreen rainforest that is adjacent to the Los Amigos Conservation Concession, which is inside the buffer region of Manu National Park. There are two definite seasons each year at this site – the wet season from October to March, (average monthly precipitation > 250 mm), and the dry season from April to September. Mean total annual rainfall was 2584 ± SD 492 mm, with average precipitation in the dry season of 136 mm
± SD 19 mm (Watsa, 2013). All sampling took place during the dry season, from May – July each year, precluding the study of the effects of seasonality on the parasite community in these primates.

There are three callitrichines at this site: the saddleback and emperor tamarins (Watsa, 2013), as well as the more cryptic Goeldi's monkey (*Callimico* goeldii) (Watsa et al., 2012). They share this habitat with eight other primate species including three species of Cebidae, and two species each of Atelidae and Pithecidae, as well as owl monkeys (*Aotus nigrifrons*) (Watsa, 2013). At EBLA, both *S. imperator* and *L. weddelli* have average group sizes of ~ 5 individuals and group compositions are similar (Watsa et al. 2016, BioRxiv https://doi.org/10.1101/047969). The primary differences between *S. imperator* and *L. weddelli* are adult weight, 515g and 386g, respectively, and nuances in feeding behavior including greater amounts of fungi consumption in *S. imperator* (pers. obs.; Terborgh, 1983).

Each individual sampled was classified in three broad age classes based on dental eruption patterns (Watsa, 2013). Juveniles were defined as individuals whose adult teeth were absent or not fully erupted (<11 months old). Sub-adults were animals with adult teeth, but that were juveniles in the preceding year. All remaining individuals were assigned to the adult age class. Due to small sample sizes from the sub-adult class, the juveniles and sub-adult classes were combined to analyze the

effects of age on parasite prevalence.

Sample collection and storage

Since 2009, an annual mark-recapture program has been implemented on ~ 70 saddleback and emperor tamarins by Field Projects International. During capture, each individual was permanently tagged with a Home Again microchip, and was overtly identifiable by unique patterns of bleached rings around the tail, as well as a tricolor beaded necklace that signified group, sex and individual identity (for the full capture protocol see Watsa et al., 2015). In addition to collecting fecal samples at the time of capture, we used radio telemetry to track tamarins in 14 groups each year via a SOM2190 radio collar {Wildlife Materials, weight ~8g, less than 3% of average adult body weight as per Cuthill:1991cf} placed on the breeding female in each group. We used both full (sleep-site to sleep site, spanning ~ 11 hours) and half-day (minimum 5 hours) follows to opportunistically collect fecal samples from all group members as they were produced.

Upon collection, all fecal samples were transferred using sterile technique into numbered plastic bags stored in a chilled thermos. Within 6 hours of collection, each sample was fixed in 10% neutral buffered formalin (1:2, feces to preservative ratio). For each sample, we recorded species, individual ID, group, date, time of day, and type of collection (follow or trapping event). Only samples produced by identified individuals were included in this study. All samples were exported to the Parker laboratory at the University of Missouri – St. Louis for analyses.

All sampling protocols adhered to guidelines outlined by the American Society of Mammalogists (Sikes and Gannon, 2011) and were approved by the Institutional Animal Care and Use Committee at the University of Missouri-St. Louis and the Directorate of Forest and Wildlife Management (DGFFS) of Perú annually. The DGFFS also granted export permits for the samples, while the CDC

and US Fish and Wildlife Services approved the import of these samples into the USA.

Laboratory analysis:

Isolation of parasite cysts, eggs, and larvae from fecal samples followed a two-step process based on sedimentation procedures as per MacIntosh (2010) and Zajac and Conboy (2006). In Step 1, we used a fecal straining procedure in which fecal samples were 1) diluted in 10% neutral buffered formalin, 2) strained of large debris through cheese cloth into a plastic cup, 3) transferred to a 15ml falcon tube with an empty weight already recorded, 4) centrifuged at 800xg for 5 minutes to form a fecal pellet, 5) removed of the supernatant and weighed, 6) re-suspended and homogenized in 5ml of 10% formalin. In Step 2, we followed the centrifugal sedimentation test outlined by Zajac and Conboy (2006) with 1ml of the homogenized suspension from Step 1. Sedimentations from Step 2 were re-suspended in exactly 1ml of preservative, and 80ul aliquots were placed onto clean slides with a coverslips for evaluation under an Olympus CX31 light microscope. Evaluations of parasites were timed and tabulated using a free online data counter, COUNT (http://erktime.github.io/count/), and each unique infection/sample was documented with multiple micrographs taken with a Leica ICC50 HD camera. Three separate aliquots per sample were evaluated with each evaluation taking an average of 10 minutes.

A minimum of 10 representative micrographs per parasite per species were measured with a calibrated ruler in Image J (https://imagej.nih.gov/ij/) to the nearest 1 µm. Measurements of all parasite forms were compared to known references values in the literature and identified to the lowest taxonomic scale possible.

Statistical analysis

Average prevalence, as well the proportion of individuals that acquired infection, lost infection, or showed no change in infection status, were calculated for each helminth identified by microscopy across the three-year study period. Significant differences in helminth prevalence between host species was tested with Fisher's Exact Test and adjusted p-values following the Holm-Bonferonni method{Holm:1979hl}. To test for variation in the presence of parasitic infections across host variables we used mixed-effect logistic regression models with a binary response variable and binomial errors. Fixed effects included 'sex', 'age class', and 'species' and random effects included 'animal identity' and 'year' to accommodate individual resampling and possible inter-annual variation. We also incorporated the number of samples collected per animal per year as an offset to account for temporal sampling bias (Walther et al., 1995). Parasite species richness, which was a discrete numerical response variable, was analyzed with an identical model formula but using Poisson errors. Model selection for all models was carried out with step-wise term deletion by removing non-significant factors and comparing nested models with a likelihood ratio test.

To test for significant correlations between group size and parasite species richness we calculated rarified parasite community richness estimates per group. The use of species accumulation curve estimates are advocated by Walther et al. (1995), because raw values of parasite community richness are easily biased by uneven sampling. We used Spearman's rank correlations to test if parasite community richness estimates with similar sampling effort were associated with group size.

To identify any nonrandom parasite co-occurrences, we compared the prevalence of all observed pairwise co-infections with expected estimates of co-infection (calculated as prevalence of A * prevalence of B = expected prevalence of AB). We then plotted expected against observed values to identify high or low levels of co-infection, and if applicable, used a two-sample z-test to compare proportions. All statistical analyses were performed in R (R Development Core Team, 2015).

Results

In total, we collected 288 individually identified fecal samples from 105 unique tamarins (71 *L. weddelli*, 34 *S. imperator*) distributed across 13 groups of *L. weddelli* and 7 groups of *S. imperator*. The number of samples collected per individual per year ranged from 1 to 7, with a mean of 1.6 and median of 1. The average fecal sample weight, post Step 1in sample processing (see Methods), was 0.41grams +/- 0.22. Considering all sex and age classes, our sampling included slightly more males than females across years, and sub-adults of both hosts species were the least sampled group (Table 1)

Table 1. Numbers of samples distributed by species, sex, age class, and year

	Year	2012	2013	2014	Total
L. weddelli		36	46	34	116
Sex	Male	19	28	17	64
	Female	17	18	17	52
Age class	Juvenile	8	9	3	20
	Sub-adult	4	6	1	11
	Adult	24	31	30	85
S. imperator		18	23	19	60
Sex	Male	7	14	10	31
	Female	11	9	9	29
Age class	Juvenile	6	3	3	12
	Sub-adult	2	3	1	6
	Adult	10	17	15	42

We were able to differentiate 11 helminth parasites by morphology and identified 5 to the species or genus level, 2 to a genus we suspect, 2 to the appropriate family, and another 2 were placed in the correct phylum (Table 2). Most, if not all, of these parasites have been detected in the Callitrichidae in the past, and representative micrographs and measurements can be found in Supplementary Materials (Fig. 3). All but one rare parasitic infection, *Spirura guianensis*, were found in both host species, although prevalence profiles varied (Table 2). Prevalence for Dicrocoeliidae was significantly higher in *S. imperator* (Fisher's test mean-adjusted P-values = 0.012), and *Hymenolepis* sp. was significantly higher in *L. weddelli* (Fisher's test mean adjusted P-value = 0.008).

Table 2. Mean annual prevalence by host species and parasite

		L. weddelli		*S. imperator*				
Class	**Parasite**	**Prev.mean**	**Prev.sd**	**Prev.mean**	**Prev.sd**	**Diff**	**Mode of dispersal**	**Pathogenic**
Acantho cephala	*Prosthenorchis* sp.	0.85	0.04	0.78	0.08	0.07	Trophic	Yes
Cestoda	*Hymenolepis* sp.	0.44	0.02	0.07	0.02	0.37*	Trophic	Unknown
Nemato da	*Spirura guianensis*	0.06	0.06	0	0	0.06	Trophic	Yes
	P. jacchi	0.04	0.05	0.07	0.08	0.03	Trophic	Unknown
	Gongylonematidae	0.15	0.09	0.13	0.06	0.02	Trophic	No
	Trypanoxyuris-like	0.16	0.08	0.25	0.1	0.09	Trophic	Unknown
	Large larvated ova	0.16	0.08	0.21	0.07	0.05	Unkown	Unknown
	Small larvated ova	0.04	0.04	0.15	0.06	0.11	Unkown	Unknown
	Strongyloides-like	0.43	0.11	0.4	0.16	0.03	Direct	Unknown
	Molineus spp.	0.83	0.07	0.76	0.22	0.07	Direct	No
Tremat oda	Dicrocoeliidae	0.08	0.1	0.4	0.12	0.32*	Trophic	Unknown

The * symbol indicates significant differences (Fisher's exact $p<0.05$). Prev.mean = mean prevalence across the study period, Prev.sd = mean standard deviation across the study period, Diff= difference in in mean prevalence between the two host species

To explore changes in infections status we subset our data to all instances were an individual was sampled across a two-year period, either 2012-2013 or 2013-2014, and calculated the mean proportion of individuals that acquired, lost, or did not change infection status (Fig 1). For most helminths, individual infections remained unchanged with small, but even, proportions of individuals switching status in both directions. This pattern differed among *S. imperator* for *Molinues spp.*, in which ~0.4 individuals either gained or retained the same infection status, while 0.2 had a previous infection that was not re-detected. Also, ~0.45 of Dicroceoliidae infections were either lost or remained unchanged across years, while 0.12 appeared to be new (Fig1, Supplemental Table 5). Among *L. weddelli*, Dicroceoliidae was rare and we only documented individuals missing a prior infection, 0.12, or remaining uninfected, 0.88. We had no cases of *L.weddelli* losing infection of *P. jachhi*, but on average 0.07 individuals acquired new infections annually. Also among *L. weddelli*, 0.5 individuals evidenced no change in infection status for *Hymenolepis sp.*, while 0.3 lost infections, and 0.18 acquired infections on average (Fig 1, Supplemental Table 5).

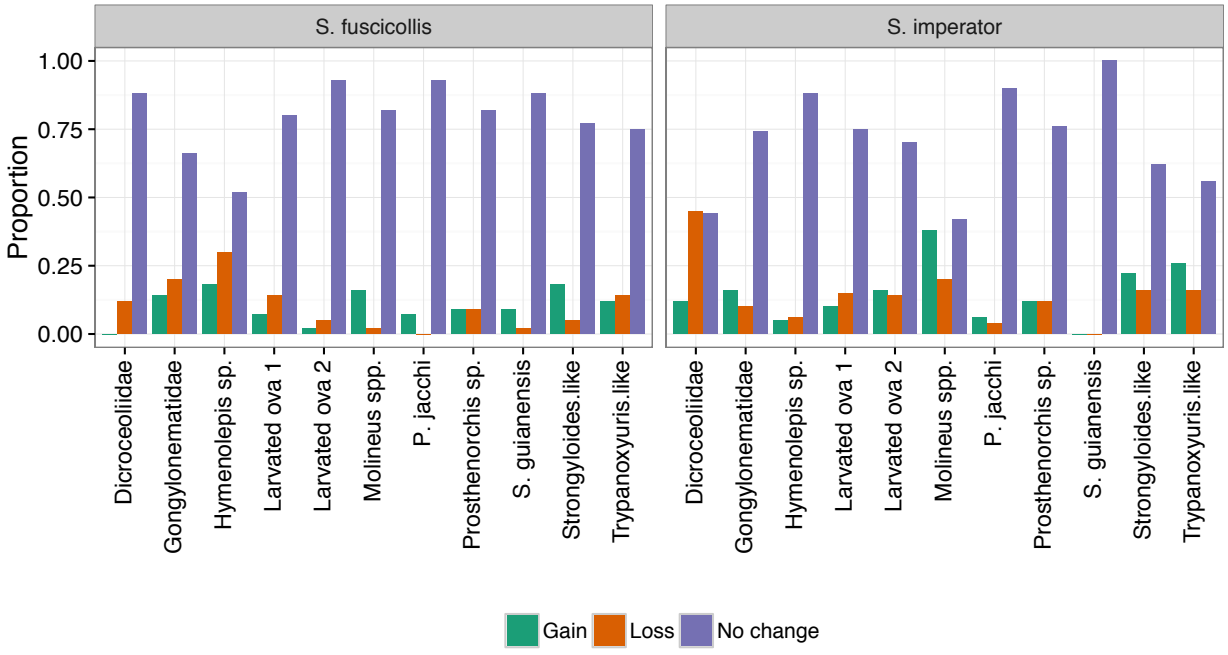

Fig 1. **Average proportion of individual infection status change by host species and parasite.**

Considering each year separately, we did not detect any significant deviations between expected and observed prevalence of co-infection (Fig. 2); the largest absolute difference in prevalence across all parasite combinations throughout the study period was 0.07. We also found no evidence of a relationship between group size (ranged 3 – 8) and estimated parasite species richness within groups after controlling for sampling effort (Spearman's rank correlation = -0.36, P-value = 0.058, n = 28).

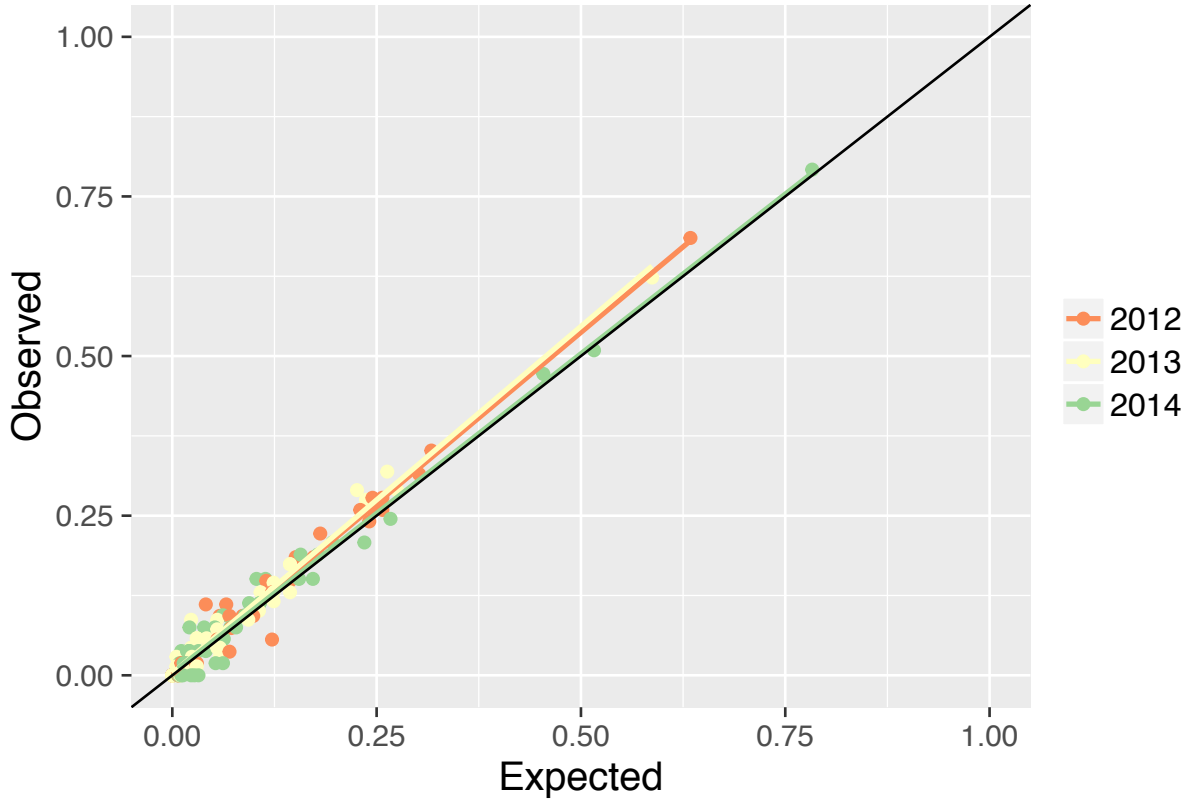

Fig 2. **Observed versus expected prevalence of parasite co-infection.**

Out of the 11 helminths identified, 8 were common enough to evaluate their distributions across host species, sex and age classes; *Spirura guianensis*, Gongylonematidae, and *Primasubulura jachii* were too rare to analyze prevalence patterns using statistical models. No parasitic infection exhibited a significant sex bias, however age and species did significantly predict the presence of 7 parasite species, though not always in the same direction (Table 3). Relative to *L. weddelli*, *S. imperator* was

positively associated with Dicrocoeliidae but negatively associated with *Molineus* and *Hymenolepis*. Relative to adults, juveniles and sub-adults were negatively associated with *Prosthenorchis* and Strongyloides-like larva, but positively associated with *Hymenolepis* and the larvated nematode ova. Our models of parasite species richness identified host species as the only significant predictor considered in this study (Table 3), which had a significantly negative estimate for *S. imperator*.

Table 3. GLMM outcomes for each parasite and parasite species richness

Parasite	Fixed effects	B	Std. Error	Wald(x^2)	DF	P-value
Prosthenorchis sp.	intercept	0.7772	0.4963			
	Age: Sub-adult	-1.4417	0.5459	6.9742	1	0.008
Molineus sp.	intercept	0.4347	0.45			
	Species:*S.imp*.	-0.8282	0.4322	3.6726	1	0.055
Hymenolepis sp.	intercept	-2.4263	0.3764			
	Species:*S.imp*.	-4.1473	0.8134	25.998	1	<0.001
	Age: Sub-adult	1.9867	0.6172	10.36	1	0.001
Large larvated ova	intercept	-4.2509	0.5863			
	Age: Sub-adult	1.3999	0.5733	5.9619	1	0.015
Strongyloides-like larva	intercept	-1.7792	0.3472			
	Age: Sub-adult	-1.511	0.6875	4.8303	1	0.028
Dicrocoeliidae	intercept	-4.4469	0.7193			
	Species:*S.imp.*	2.0681	0.4903	17.794	1	<0.001
PSR	intercept	-0.4743	0.1495			
	Species:*S.imp*.	-0.6413	0.1848	12.041	1	<0.001
Small larvated ova	Could not reject null model					

Minimal, best-fit models for the presence of each parasite and parasite species richness (PSR). Model selection began with fixed factors host 'sex', 'age class', and 'species', while 'individual identity' and 'year' were incorporated as random effects, and the number of fecal samples collected for each individual/year was included as a model offset. Data were insufficient to analyze the distribution of *Spirura guianensis*, Gongylonematidae, and *Primasubulura jachii*.

Discussion

It is customary for wild animals to acquire and maintain multiple parasitic infections during their lifetime (Cox, 2001; Petney and Andrews, 1998). While clinical and/or experimental studies are effective at demonstrating the pathogenicity of parasites, translating such findings to natural systems

is not always straightforward. For example, the thorny-headed worms (Phylum Acanthocephala) are well-known parasites of Central and South American primates (King, 1993; Tantalean et al., 1990).They attach themselves to the intestinal mucosa of their primate hosts and cause inflammatory responses, obstruction of the lumen, and the formation of lesions and ulcers, which may lead to secondary infections or even peritonitis in the worst cases (King, 1993; Strait et al., 2012). In spite of the potential to cause severe pathogenicity, Acanthocephala infections (e.g. *Prosthenorchis* spp.) are extremely common in this study population, routinely found in other surveys of callitrichids (Müller, 2007; Tantalean et al., 1990; Wenz et al., 2009), and are also present in other New World primate families such as the Cebidae and Atelidae (King, 1993; Phillips et al., 2004; Wenz et al., 2009). It has been shown before that in nature, where the host's ability to tolerate infection is most important to its fitness, parasite pathogenicity may be dictated by environmental factors (Cardon et al., 2011), what Walker et al. (2010) discussed as "environmental forcing of pathogenicity". On top of this, it is well-documented that interactions between diverse parasite species modulate pathogenicity (Balmer et al., 2009; Lello et al., 2004; Monteiro et al., 2007b; Petney and Andrews, 1998). Of the 10 helminths documented in this study, seven are of unknown pathogenicity, two are probably non-pathogenic, and two known to be pathogenic (Table 2 and Supplemental Table). That the two most common parasites in this study, across years, consisted of a nonpathogenic (*Molineus*) and pathogenic (*Prosthenorichis*) helminth reinforces that caution is necessary before translating clinical findings to real-world systems and labeling a parasite as harmful. Specifically, observations of particularly pathogenic parasites (e.g. Acanthocephalans) should be viewed in context with the broader parasite community and changes in the environment, which requires detailed longitudinal data collection (Haukisalmi et al., 1988).

Although our analysis did not identify nonrandom associations between co-infecting parasites, it is still possible that within-host parasite interactions are at play. The use of presence-absence infection data is much less sensitive at detecting relationships than accurate measures of parasite intensity or burden (Knowles et al., 2013; Lello et al., 2004), which will be an aim of future studies. Our inability

to detect a relationship between group size and parasite species richness is consistent with our previous work on blood parasites (Erkenswick et al., 2017), and may be a consequence of too little variation in group sizes, which ranged from 3 to 8. Other genera of Callitrichids can occur in slightly larger groups than our hosts species, for example *Callithrix* groups can be as large as 15 members (Pontes and Cruz 1995)(Watsa et al., 2016), but it also may be that generalities about group size and parasite diversity do not apply within the Callitrichidae.

The data we have provided here represent the first description of the intestinal parasite for free-ranging *Saguinus imperator*. Although the IUCN currently lists *S. imperator* of least concern (Rylands and Mittermeier, 2008), the core of its distribution is surrounded by one of the fastest growing gold mining industries in the world (Asner et al., 2013). Moreover, *S. imperator* is currently one of the most valuable Peruvian monkeys in the illegal wildlife trade (Watsa, 2015). Hence, close monitoring of intact populations of *S. imperator* is crucial to elevate its conservation status. We also provide a new comparative dataset of gastrointestinal parasites from *L. weddelli* (formerly *S. fuscicollis weddelli*), which is also a primate of 'least concern,' though recent taxonomic revisions may result in revision of its conservation status (Buckner et al., 2015; Matauschek et al., 2011).

We find some noteworthy similarities and differences between this study and previous studies of gastrointestinal parasites from congeneric callitrichids. Phillips et al. (2004) screened one group of *S. fuscicollis* in the relatively nearby Tambopata National Reserve and identified four parasites (*Trichuris*, *Iodamoeba*, *Entamoeba*, and an unidentified strongyle), none of which were found in our hosts. Although they had a small sample size of 4 individuals, it is surprising that they did not detect *Prosthenorchis* sp. infection, which they did find in 1 of 18 squirrel monkey (*Saimiri sciureus*) fecal samples at the same site. In northern Peru, both Wenz et al. (2009) and Muller (2007) conducted gastrointestinal parasite surveys over a single season from sympatric callitrichine hosts, *S. fuscicollis* and *S. mystax*, and reported a parasite assemblage that mostly overlaps with our findings

(*Prosthenorchis*, *Hymenolepis*, large and small Spirurids, *Primasubulura,* Strongylid larvae). Both recorded higher prevalence than our study for every helminth except *Prosthenorchis*, which was considerably less common. Comparing both of their tamarin hosts, they detected higher prevalence of *Hymenolepis* and PSR for *S. fuscicollis*, as we did. The variation in prevalence between the studies is likely related to ecological differences between the locations. We suspect that greater diversity of helminths observed in this study is associated with a higher diversity of primates (11 compared to 4), but also may be a consequence of prior human activities at the site which altered the densities of primate species at EBLA (Rosin and Swamy, 2013)

Interpreting these findings in light of parasite mode of transmission, direct or trophic, is challenged by the lack of information on the exact intermediate hosts of many trophic parasites, and many could have more than one intermediate host (Supplemental Material). Two helminths in this study, *Molineus* and Strongylid larvae, are transmitted between individuals via direct contact or a contaminated substrate, and yet prevalence differed by ~ 0.40. Although neither host age class nor sex explained prevalence of either of these parasites, there was a significant species difference (increased prevalence of *Molineus* sp. among *L. weddelli*). While a comprehensive study of feeding and foraging behavior has not been conducted on our host populations, it has been conducted on sympatric *S. fuscicollis* and *S. mystax* in Northern Perú. Findings from two studies on tamarin predation agree that *S. fuscicollis* spends significantly more time foraging in the lower strata and on the ground, while the opposite was true of *S. mystax* (Heymann and Knogge, 2000; Smith, 2000). If parasite free-living phases persist longer on the ground or in certain forest strata, then niche specialization might account for this observed difference in prevalence. Smyth (2000) also documented that each host species exercised distinct feeding preferences such as color and size of prey items. This could account for the two observed species differences among trophically transmitted parasites, Dicrocoeliidae and Hymenolepis, since size and coloration of tropical arthropods and small vertebrates are important evolutionary characters across different forest habitats. Consistent with the prevalence of blood

parasites from this population (Erkenswick et al., 2017), age class predicted the presence of four trophically transmitted parasites, though not always in the same direction. We attribute this to differences in diet and foraging efficiency between younger and older individuals which would alter parasite encounter rates.

By tracking prevalence of parasitic infections over time in wild populations it is possible to infer whether natural parasite communities are stable, however, longitudinal data at the level of the individual provides insights into the source, or lack, of population stability {Knowles:2013eb}. In some cases, it could even aid in identification of parasites that have negative health consequences. For example, if parasite prevalence and incidence of new infection is consistently high across years, but there is no evidence of individuals clearing infections, then previously infected hosts must be disappearing regularly. In this study, we encountered few disparities in the rate of acquisition or loss of parasites, and considered in concert with observed prevalence, we see no obvious signs of negative health consequences. If any, further research on potential health effects of *Molineus spp*. may be warranted due to the higher rate of acquiring than losing infection, and given that its elevated prevalence was not accounted for by age-class, or the arrival of new individuals by birth. As our data were amalgamated over three years it is worth addressing the potential for false negatives to influence our esimated rates of change in helminth infection status due to sampling error. Across both of our host species there were 19 animals sampled for all three years. For each parasite, we counted the frequency in which an individual's infection status switched from infected to uninfected and back to infected in 2014, which could be an indication of false negatives. Across 11 helminths this occurred 12 times, on average 1.09+/-1.3 (0:4) instances per parasite, with a median of 1. The highest observed frequency was 4 with *Molineus* spp. infections, and all other frequencies were 2 or less per helminth. Although this seems high for *Molineus* spp., considering all 12 occurrences together, 3 took place for separate helminth infections in one *S. imperator* individual, while the other 9 were isolated occurrences across 9 different host individuals. Hence, it seems unlikely that sampling error could

explain our findings.

The results provided here, in combination with recent works on hemoparasites (Erkenswick et al., 2017; Erkenswick et al., in review), represent a benchmark against which future parasitological surveys can be compared. Represented in these callitrichine host parasite assemblages are parasites that are transmitted directly, trophically, or by arthropod vectors. Given changes in the environment that alter food availability or vector populations, we would expect corresponding deviations from what has been documented here. The near ubiquity across South America rainforests, propensity to be found in sympatry with other New World primates, and relative resilience to human altered landscapes, make Callitrichidae a potential flagship family for the regional detection of ecological changes, or even environmental threats.

Acknowledgements

We wish to thank research assistants from Field Projects International who aided in the collection of tamarin fecal samples. We want to thank Cindee Retke for her assistance with wet laboratory analyses. We express our gratitude to the following agencies and organizations that provided non-monetary support to this investigation: Centro de Ecologia y Biodiversidad, Amazon Conservation Association, el Estación Biológica Río Los Amigos, Dirección General Forestal y de Fauna Silvestre (Perú).

Funding: This work was supported by Field Projects International, the American Society of Mammalogists, University of Missouri Trans World Airlines Scholarship Program, Whitney R. Harris World Ecology Center, Sigma Xi Society, IdeaWild, and the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases, Comparative Medicine Branch.

Supplemental Materials

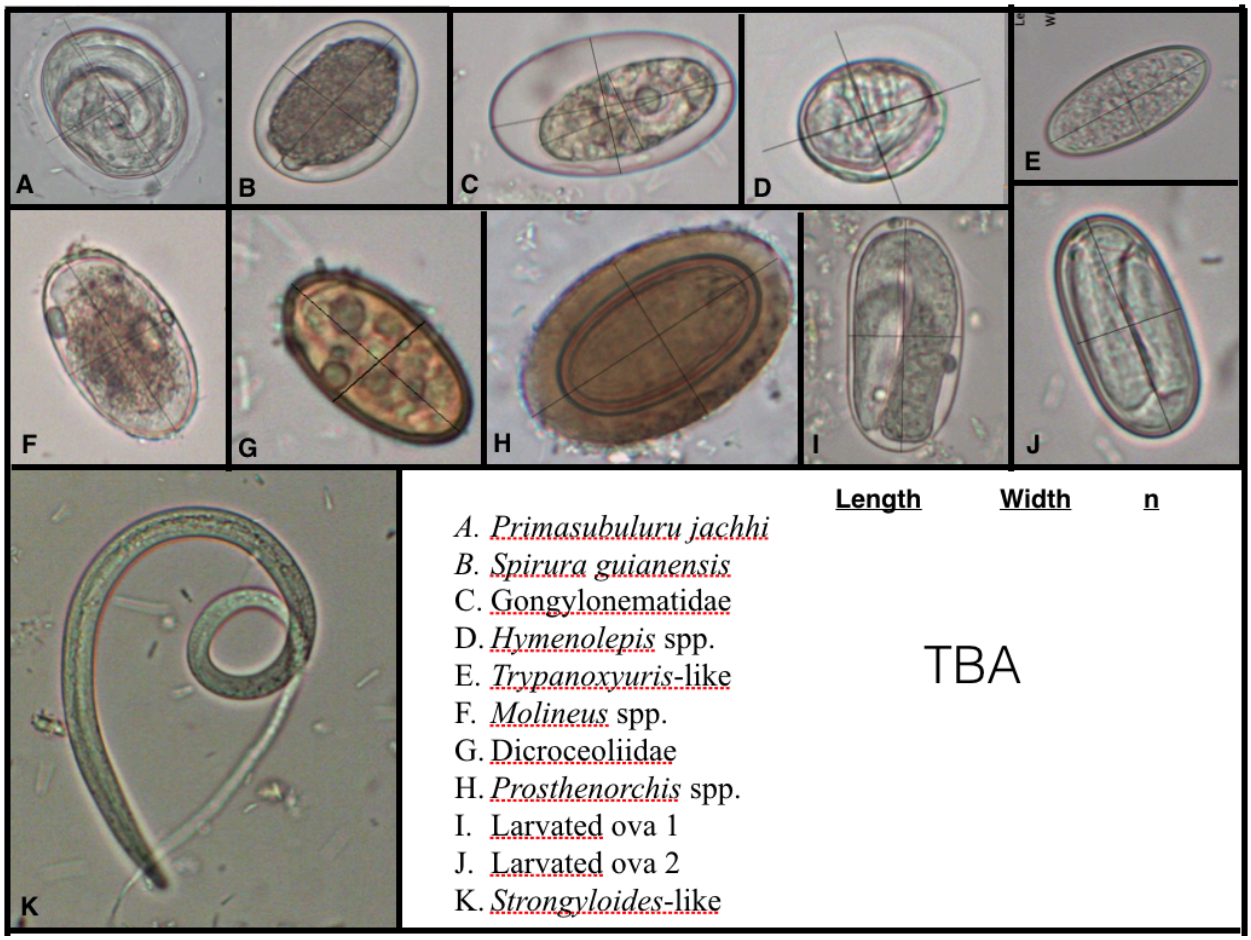

Figure 4. **Representative micrographs and measurements for each helminth parasite**

Table 5. Average proportion of individual infection status change by host species and parasite.

		Mean			Standard Deviation		
Species	Parasite	Gain	Loss	No change	Gain	Loss	No change
S. fuscicollis	Dicroceoliidae	0	0.12	0.88	0	0.09	0.09
	Gongylonematidae	0.14	0.2	0.66	0.06	0.16	0.23
	Hymenolepis sp.	0.18	0.3	0.52	0.06	0.04	0.1
	Larvated ova 1	0.07	0.14	0.8	0.03	0.06	0.09
	Larvated ova 2	0.02	0.05	0.93	0.04	0	0.03
	Molineus spp.	0.16	0.02	0.82	0.1	0.04	0.06
	P. jacchi	0.07	0	0.93	0.03	0	0.03
	Prosthenorchis sp.	0.09	0.09	0.82	0	0	0
	S. guianensis	0.09	0.02	0.88	0.13	0.04	0.09
	Strongyloides.like	0.18	0.05	0.77	0.13	0	0.13

	Trypanoxyuris.like	0.12	0.14	0.75	0.09	0	0.1
S. imperator	Dicroceoliidae	0.12	0.45	0.44	0.02	0.07	0.05
	Gongylonematidae	0.16	0.1	0.74	0.05	0.14	0.09
	Hymenolepis sp.	0.05	0.06	0.88	0.07	0.09	0.02
	Larvated ova 1	0.1	0.15	0.75	0.14	0.21	0.07
	Larvated ova 2	0.16	0.14	0.7	0.05	0.09	0.14
	Molineus spp.	0.38	0.2	0.42	0.12	0.28	0.16
	P. jacchi	0.06	0.04	0.9	0.09	0.05	0.14
	Prosthenorchis sp.	0.12	0.12	0.76	0.02	0.02	0.05
	S. guianensis	0	0	1	0	0	0
	Strongyloides.like	0.22	0.16	0.62	0.16	0.05	0.12
	Trypanoxyuris.like	0.26	0.16	0.56	0.09	0.05	0.05

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Chapter 3

Chronic *Plasmodium brasilianum* infections in wild Peruvian tamarins

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Abstract

There is an increased interest in potential zoonotic malarias. To date, *Plasmodium malariae* that infects humans remains indistinguishable from *P. brasilianum*, which is widespread among New World primates. Distributed throughout tropical South America, the Callitrichidae are small arboreal primates in which detection of natural *Plasmodium* infection has been extremely rare. Most prior screening efforts have been limited to small samples, the use of low-probability detection methods, or both. Rarely have screening efforts implemented a longitudinal sampling design. Through an annual mark-recapture program of two sympatric callitrichids, the emperor (*Saguinus imperator*) and saddleback (*Saguinus fuscicollis*) tamarins, whole blood samples were screened for *Plasmodium* by microscopy and nested PCR of the cytochrome b gene across four consecutive years (2012 – 2015). Following the first field season, approximately 50% of the samples collected each subsequent year were from recaptured individuals. In particular, out of 250 samples from 134 individuals, 11 samples from 6 individuals were positive for *Plasmodium*, and all but one of these infections was found in *S. imperator*. Importantly, the cytochrome b sequences were 100% identical to former isolates of *P. malariae* from humans and *P. brasilianum* from *Saimiri* sp. Chronic infections were detected as evidenced by repeated infections (7) from two individuals across the 4-year study period. Furthermore, 4 of the 5 infected emperor tamarins were part of a single group spanning the entire study period. Overall, the low prevalence reported here is consistent with previous findings. This study identifies two new natural hosts for *P. brasilianum* and provides evidence in support of chronic infections in wildlife populations. Given that Callitrichids are often found in mixed-species associations with other primates and can be resilient to human-disturbed environments, they could contribute to the maintenance of *P. malariae* populations if future work provides entomological and epidemiological evidence indicating human zoonotic infections.

Keywords*: Plasmodium brasilianum*; *Plasmodium malariae*; Callitrichidae; Chronic infection

Introduction

In 2015 malaria was diagnosed in approximately 212 million people, and resulted in the loss of 429,000 lives worldwide (1). In malaria-endemic regions, infections are unevenly distributed among human populations, with the highest prevalence among children and adolescents. Today, malaria control programs remain among the largest public health efforts, costing an estimated $4.75 billion annually (2), despite the fact that the causative agents of malaria (protozoan parasites of the genus *Plasmodium*) were first discovered as early as 1880 (3). A potential challenge faced by ongoing efforts to eliminate malaria in human communities is the possibility of zoonotic infections (4). In particular, there is compelling evidence that some *Plasmodium* species infecting humans are also circulating in nearby simian and ape communities. Whether such non-human primate host can act as a reservoir of human malarias is a matter of great interest.

According to the Global Mammal Parasite Database, 27 species of *Plasmodium* have been documented in nonhuman primates (5), three of which (*P. falciparum*, *P. vivax*, and *P. malariae*) frequently occur in humans. Along with *P.ovale*, *Plasmodium* species that infect humans do not form a monophyletic group (6). The two parasites that cause the greatest morbidity (*P. falciparum* and *P. vivax*) are part of larger clades of species that include many that parasitize nonhuman primates (4,7,8).

In South America, *Plasmodium brasilianum* was first described in monkeys in the beginning of the 20th century and has now been documented in approximately 31 species of New World monkeys (9). This broad host range is unusual among other non-human primate malarias and may indicate a very resilient parasite species. To date, numerous studies have looked for, but not found, any reliable morphological, serological, or genetic differences between *P. brasilianum* and *P. malariae* that infect humans (7,10-13). Lalremruata et al. (12) collected blood samples from several remote populations of the Yanomami people in Venezuela, and isolated 33 infections by nested-PCR screening for the 18S

gene of *P. malariae*. Of these, 12 sequences were 100% identical to *P. brasilianum* strains recovered from howler monkeys in French Guiana, and the remainder were 99-100% identical to *P. malariae* strains from Myanmar and Papua New Guinea. Although the 18S gene does not allow us to accurately discern recent host switches due to its rate and semi-concerted mode of evolution (14), the genetic distance between all 33 strains seems consistent with intraspecific variation observed within other single species of *Plasmodium.* The only factor that has ever been used to differentiate these two parasites is host-identity (human or not), and yet experimental studies have demonstrated that nonhuman primates are susceptible to *P. malariae* (15). These findings suggest that *P. brasilianum* and *P. malariae* could very well be the same organism.

Plasmodium malariae/brasilianum causes quartan malaria, so termed for having 72-hour erythrocytic cycles, unlike *P. falciparum* or *P. vivax* that cycle every 48 hours (16). *Plasmodium malariae* has led to nephrotic syndromes in humans and experimentally infected monkeys (16,17), and has been shown to persist in humans for years, suggesting that a similar pattern may occur in non-human primates like chimpanzees (18,19). Recrudescent infections of *P. malariae/brasilianum* can occur when hosts are subjected to stressful conditions or become immunocompromised (16,20). In malaria-endemic regions, co-infections of *P. malariae/brasilianum* with other *Plasmodium* spp. are common (12,21,22). Since host parasitemia for *P. malariae/brasilianum* is relatively low, co-infections are probably under-detected when screenings are performed only by microscopy (16,17), and yet microscopy remains the most broadly utilized diagnostic technique around the world today.

The ability to reside in non-human primates, induce renal pathology, persist as a chronic infection in humans, and interact with other species of *Plasmodium* qualifies *P. malariae* as an important health concern at the human-wildlife interface given that present research suggests it is the same as *P. brasilianum*. Fundamental to assessing health risks are the development of a clear understanding of host breadth and associated prevalence for these (or this) species. The majority of infections found in

wild New World monkeys are confined to the Atelidae, Pitheciidae, and Cebidae, while detection in the Callitrichidae, perhaps the most speciose and widespread Neotropical primate family, remains rare (9,13,21,23). Callitrichids are always found in sympatry with other New World primate species, frequently in the context of mixed-species, or polyspecific associations (24,25). Unlike the majority of other New World primates they can also persist in disturbed and human occupied areas (26-28). Moreover, continuing removal of large Neotropical primates due to poaching and hunting may be leading to a population expansion of the Callitrichids (29). If proven to be vectors of *Plasmodium* infections, these characteristics of the callitrichids might implicate them as an important sylvatic component for malaria control efforts.

When considering accessible survey data on *Plasmodium* infections from wild populations of callitrichids to date, several biases stand out. First, although five of the seven callitrichid genera have been tested for *Plasmodium brasilianum* (*Saguinus*, *Cebuella*, *Callithrix, Leontopithicus, and Mico*), the majority of species have not been sampled (5,23). Of the ~24 species investigated so far, nine have shown an infection, including *Saguinus midas*, *S. niger*, *S. geoffroyi*, *S. martinsi*, *Mico humeralifer*, *Leontopithicus chrysomelas*, *L. chrysophagus*, *L. rosalia*, and *Callithrix geoffroyi*. However, only the first three of these species (*S. midas*, *S. niger*, and *S. geoffroyi*) are clear cases of natural infection; the other infections were recently detected from captive animals at primate research or rescue centers (23,30). Of the cases representing natural infections, the number of infected individuals and corresponding sample sizes consist of 1/1000 for *S. geoffroyi* (31), 4/109 for *S. niger* (32), 4/178 in another study of *S.niger* (32,33), and 3 in 54 for *S. midas* (13). This is relevant since *P. brasilianum* tends to exhibit low prevalence, averaging 0.045±0.043 for the Callitrichidae and 0.023±0.024 for the Primate Order (34). Third, no studies to date report chronic natural infections by sampling the same individuals across years. If true, this would provide evidence that the Callitrichidae could be suitable hosts for *P. malariae/brasilianum* and may act as a reservoir for human malaria.

Here we screen for natural *Plasmodium* infections longitudinally across four years in two sympatric species of callitrichids, the saddleback tamarin, *Saguinus fuscicollis weddelli* (but see ongoing taxonomic revisions (35,36)), and the emperor tamarin, *Saguinus imperator*. Given the observed host breadth of *P. brasilianum/malariae* and the small sample sizes of prior efforts, we predict that callitrichine species could be competent reservoirs for this parasite. Second, since *P. malariae* infection can be chronic in humans, we predict that the same will be true for these callitrichine hosts. In addition to testing these predictions, our goal is to establish the prevalence of *Plasmodium*, and incidence of new infections, in both species, and to explore any patterns in how infections are distributed across host characteristics such as sex, age class, and group membership.

Materials and methods

Study Subjects and Sampling

Samples were collected from a free-ranging population of saddleback (*Saguinus fuscicollis*) and emperor (*Saguinus imperator*) tamarins at the Estación Biológica Rio Los Amigos (EBLA) in the Madre de Dios Department of southeastern Perú (12°34'07"S, 70°05'57"W). This privately owned field station is managed and protected by the Amazon Conservation Association (ACA) and receives more than 150 visitors each year. The field station is located at the confluence of the Los Amigos and Madre de Dios Rivers, and the 900-ha plot is contiguous with the much larger Los Amigos Conservation Concession that lies within the buffer zone of Manu National Park. The site exhibits lower densities of large-bodied primates than has been recorded from nearby forest in the government protected Tambopata National Reserve, which is attributed to hunting that took place prior to purchase by the ACA (29); however densities of medium- and small-bodied primates are higher. The study groups of both species inhabit both *terra firme* and *várzea* habitat.

Since 2009, we have encountered approximately 70 unique individuals each year, across both species.

Our program is optimized to ensure habituation of primates to subsequent human observation (37). Animals are given permanent identification tags via subcutaneous microchips (Avid, Home Again©) so samples could be collected from the same individual across years. Samples for this study were collected across four years (2012-2015) in June and July (the dry season). During capture, blood samples of < 300 uL were drawn from the femoral vein of each animal while it was anesthetized with ketamine hydrochloride (Ketalar, Pfizer Inc., New York, USA). Each sample was stored dry on Whatman FTA Micro Elute Cards for subsequent DNA extraction and at least two blood smears were prepared with fresh blood. All sampling protocols adhere to guidelines outlined by the American Society of Mammalogists (38) and were approved by the Institutional Animal Care and Use Committee at the University of Missouri-St. Louis (317006-2, 733363-2) and the Directorate of Forest and Wildlife Management (DGFFS) of Perú annually.

Blood parasite microscopy

Immediately after blood draw, blood smears were made on standard microscope slides and air-dried. All smears were fixed for five minutes in 100% methanol within six hours and stained in Giemsa's solution following Valkiunas et al. (39) within three weeks of fixation. Smears were observed at 400x magnification using light microscopy (Olympus CX31) for the presence of parasites. Blood parasites were recorded while conducting a total leukocyte count estimation (enumeration of leukocytes in 10 non-overlapping fields of view in the smears' monolayer at 400x magnification) and differential (classification of 200 leukocytes in the monolayer at 1000x magnification); each slide examination took less than 30 minutes. Examinations were carried out in a systematic direction to avoid overlapping fields of view, excluding damaged sections, where leukocytes and parasites were too distorted to identify.

Molecular detection and sequencing

DNA was isolated from a 3 mm diameter hole punch from the blood stored on Whatman FTA Micro

Elute Cards into 30ul of ddH_20 using standardized protocols recommended by the manufacturer (GE Health Care Life Sciences, Pittsburgh USA). DNA samples from the first three years (2012 – 2014) were screened for haemosporidian parasites by a nested polymerase chain reaction (nPCR) protocol that targets part of the parasite cytochrome b (cytb) gene, 709 base pairs (bp), following Duval et al. (40). To confirm infection status and to obtain the near complete mitochondrial cytb gene (1,131bp) for infected individuals across the entire study period (2012 – 2015), we employed a separate nPCR protocol that amplifies a 1,038 bp fragment with specific forward-TGTAATGCCTAGACGTATTCC and reverse-GTCAAWCAAACATGAATATAGAC primers for the outer PCR and forward-TCTATTAATTTAGYWAAAGCAC and reverse-GCTTGGGAGCTGTAATCATAAT primers for the inner PCR, following Pacheco et al. (41). PCR amplifications were carried out in a 50 µl volume with 8ul of total genomic DNA, 2.5 mM $MgCl_2$, 1X PCR buffer, 1.25 mM of each deoxynucleoside triphosphate, 0.4 mM of each primer, and 0.03 U/µl AmpliTaq polymerase (Applied Biosystems, Roche-USA). The PCR conditions were: a partial denaturation at 94 °C for 4 min, 36 cycles with 1 min at 94 °C, 1 min at 53 °C and 2 min extension at 72 °C, and a final extension of 10 min added to the last cycle. Then, a nested PCR using 1 µl of the first amplification as the template was performed under identical PCR conditions. After electrophoresis, all amplified products were excised from the gels, purified by the QIAmp Gel Extraction Kit (Qiagen), and both strands were sequenced using an Applied Biosystems 3730 capillary sequencer.

Phylogenetic analysis

Complete cytb gene sequence identity for samples positive for *Plasmodium* was confirmed using BLAST against NCBI. Electropherograms were visually examined to rule out mixed infections. In addition to the sequences obtained in this study, we included a total of 26 sequences available in GenBank for *Plasmodium* parasites isolated from mammals in the subsequent phylogenetic analysis. The phylogenetic relationships between sequences were inferred on the *cytb* gene using MrBayes v3.2.6 with the default priors (42). Alignments were made using ClustalX v2.0.12 and Muscle as

implemented in SeaView v4.3.5 (43) with manual editing. The data were fit with a General Time-Reversible model (GTR + I + Γ) that had the lowest Bayesian Information Criterion (BIC) score (44). Bayesian support for the nodes was inferred in MrBayes using 4×10^6 Markov Chain Monte Carlo (MCMC) steps, and after convergence was reached (posterior probability < 0.01, potential scale reduction factor between 1.00 and 1.02), we discarded 25% of the samples as burn-in (42). Then, the sequence divergence between species was calculated using a Kimura two-parameter model of substitution as implemented in MEGA v.6.05 (45).

Results

In total, we collected 250 blood samples (153 from *Saguinus fuscicollis*, 92 from *Saguinus imperator*) spread across 134 individuals (83 and 46, respectively) during the study period. Zero infections were confirmed from examination of thin blood smears; however, 10 samples were successfully amplified by nPCR from emperor tamarins (three each from 2012 and 2013, and two each from 2014 and 2015) and one from a saddleback tamarin in 2014 (Table 1). The single saddleback tamarin infection only amplified once during preliminary screening for *Plasmodium* following the Duval et al. (40) protocol, and because the cytb fragment was of shorter length it was excluded from phylogenetic analysis; however, the sequence was 100% identical to others obtained in this study. The remaining 10 partial cytb sequences (1,038bp) were 100% identical to each other and to reference sequences for human isolates of *Plasmodium malariae* and squirrel monkey (*Saimiri* sp.) isolates of *P. brasilianum* (Fig 1); only one sequence per year is included in the phylogeny. These 10 sequences have been deposited to GenBank (Accessions KY709297– KY709306).

Table 1. Nested-PCR Screening Results

Individuals sampled	Prevalence (*S. imperator* only)	Incidence (*S. imperator* only)	Animal ID	Species	Sex	Age class	Group ID (size)	Sample Collection Date
S.fuscicollis 35 *S. imperator* 21	0.14	NA	81	*S. imperator*	M	Adult	9 (6)	6/13/12
			32	*S. imperator*	M	Adult	9 (6)	6/13/12
			89	*S. imperator*	F	Adult	15 (3)	6/18/12
S. fuscicollis 45 *S. imperator* 24	0.13	0.33	34*	*S. imperator*	F	Sub-adult	9 (7)	7/10/13
			32	*S. imperator*	M	Adult	9 (7)	7/10/13
			36**	*S. imperator*	F	Adult	9 (7)	7/10/13
S.fuscicollis 36 *S. imperator* 21	0.10	0.0	140	*S. fuscicollis*	M	Adult	13 (4)	6/27/14
			32	*S. imperator*	M	Adult	9 (6)	7/6/14
			36	*S. imperator*	F	Adult	9 (6)	7/6/14
S.fuscicollis 37 *S. imperator* 26	0.08	0.0	32	*S. imperator*	M	Adult	9 (8)	6/27/15
			36	*S. imperator*	F	Adult	9 (8)	7/3/15

*Infection was not detected in this individual in 2014.

**This individual is natal to this group, born in 2011. An infection was not detected in 2012 as a sub-adult.

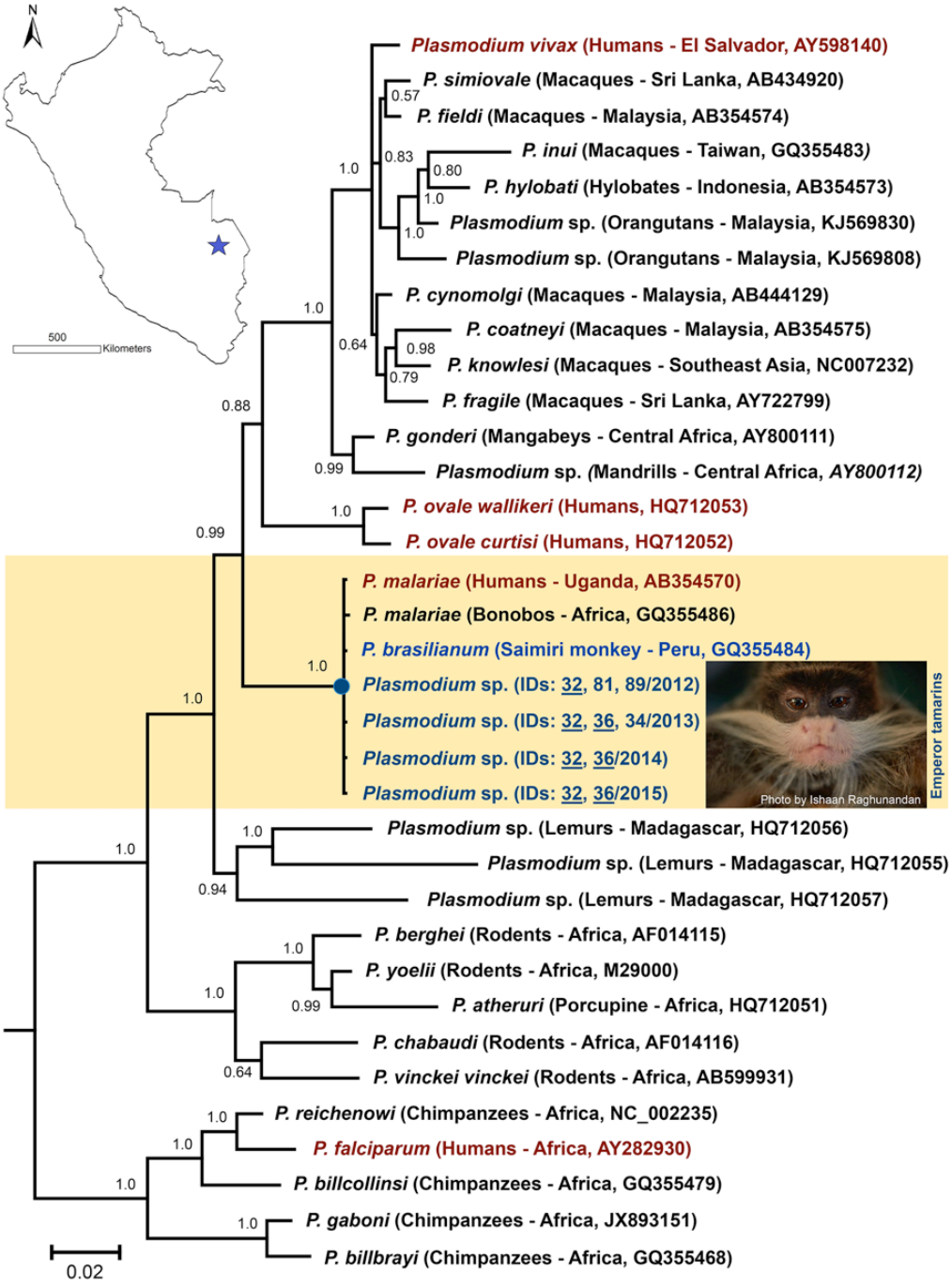

Fig 1. Cytochrome b phylogeny with new *Plasmodium* isolates. One *Plasmodium* isolate per year from this study has been included in the phylogeny with infected animals indicated by their unique animal ID numbers. *Plasmodium* isolates from humans are indicated in red and a squirrel monkey isolate from Perú is in blue. For each sequence, host species, sample locations, and

GenBank accession numbers are provided. The map (upper left) indicates the sampling location for this study.

Prevalence of infection among emperor tamarins was 0.14 in 2012 (n = 21), 0.13 in 2013 (n = 24), and 0.10 in 2014 (n = 21), and 0.08 in 2015 (n=26) with an average across years of 0.11+/- 0.03 (Table 1). While prevalence remained relatively stable across years, incidence decreased from 0.33 in 2013 to 0 in 2014 and 2015. Prevalence was maintained by 1 adult male that remained infected across the entire study period, and 1 adult female, born in this same group in 2011, that acquired and maintained an infection from 2013 to 2015 (see Table 1). Two other emperor tamarin individuals (an adult male and female from 2012) were found infected in the only years they were sampled. An infection in one sub-adult female from 2013 could not be detected in 2014 or 2015. Although this study assessed 7 emperor tamarin groups, 4 of the 5 emperor tamarin infections belonged to the same group. The infected saddleback tamarin was an adult male from 2014 whose home range partially overlapped with the infected emperor tamarin group and also included parts of the basecamp at the field site. An infection was not detected from this individual in 2015.

Discussion

A handful of *Plasmodium* species other than *P. malariae/brasilianum* can infect both human and nonhuman primates. However, in many cases, there is still limited evidence that non-human primates are a reservoir of human malaria. A clear example of zoonotic malaria is *Plasmodium knowlesi*, a simian parasite in southeast Asia that has been repeatedly found in humans (46,47). This parasite appears to have independently infected humans in many areas of Southeast Asia (47-49). In addition, *Plasmodium cynomologi* parasitizes Asian macaques and has at least one documented case in humans (50). Beyond these two cases, other studies have detected human malarias in non-human primates but the epidemiological and genetic data are still insufficient to

implicate non-human primates as reservoirs for human malaria. The human parasite *P. vivax* is suspected to circulate in a subset of west African apes that are positive for the Duffy blood group antigen molecule (4). The normal hosts for *P. simium* are large New World monkeys (Atelidae), but there is at least one case of a human infection (51). *Plasmodium falciparum*, the most virulent human *Plasmodium*, is sometimes detected in New World monkeys (8 species over 5 genera) (5) but there is no evidence indicating that such non-human primates act as malaria reservoirs.

Although host switches are common in non-human primates, not all host switches indicate the presence of a zoonosis (41,52). As an example, *P. falciparum* has been found in apes, particularly chimpanzees (19,53). However, such infections had a human origin because they were all resistant to commonly used antimalarial drugs (41). Thus, it was shown that apes can acquire the parasite from humans; however, whether there could be human infections from a non-human primate host (a true zoonosis) requires additional evidence beyond the detection of identical parasites. In particular, evidence of active gene-flow and the presence of competent vectors that can infect humans from a non-human primate are missing. A case in which zoonoses have been clearly established by these criteria is *Plasmodium knowlesi* from isolated macaque (*Macaca* spp.) populations in Borneo and Peninsular Malaysia (49). A first step in the case of *P. malariae/brasilianum*, however, is to better characterize its host range throughout South America.

Plasmodium brasilianum has been screened for in *S. fuscicollis* on two separate occasions between 1995 and 2013 (n = 19 and 6, respectively) in Brazil (9,21), and only once in *S. imperator* (n = 2) (54), with zero reported infections for both species. Here we confirm for the first time that these two species are susceptible to *P. brasilianum/malariae*. Like past studies of *Plasmodium* from other simian hosts in South and Central America, *P. brasilianum* was genetically identical to *P. malariae* using cytb, reinforcing that they are likely to be a single organism (13). That we were only able to amplify a single saddleback tamarin infection in a

single assay is likely caused by poor sample quality, extremely low parasitemia, or both. As the sample was physically isolated from any other surrounding positives, it would represent a very improbable instance of contamination during laboratory analyses.

Importantly, our data suggest that chronic infections of *P. brasilianum* occur in the wild, consistent with the low, stable prevalence in emperor tamarins despite decreasing incidence of new infection (Table 1). If true, this provides evidence that Callitrichidae might act as reservoirs for human zoonotic malaria; however further investigation should take place to show that a complete parasite lifecycle is taking place, such as the presence of intraerythrocytic development. Our findings also suggest that these non-human primates may naturally clear infection of *P. brasilianum*; however, it will be necessary to differentiate a natural clearance from a sub-microscopic infection with a parasitemia that is below PCR detection thresholds. Since infections appear to be clustered in our study population, additional years of data will allow us to track the rate of transmission to new group members (for example, offspring within infected groups) and to new groups. This also opens possibilities for measuring individual health parameters before and after the onset of *P. brasilianum* infections and whether there exist associations with other natural parasites (20).

The parasite prevalence we observed for emperor tamarins was in the same range that has been published from other wild Neotropical primate populations. Although prevalence was too low to analyze variation between different demographic groups, we observed that 4 of 5 infections occurred in a single group (out of 7). Previous studies on *Plasmodium* from Neotropical primates make little mention of how parasites are distributed within host populations, but potentially uneven distributions would be an important factor for assessing disease risk (49), particularly if some of those non-human primates share competent vectors with humans. Although the available data are limited, there are several explanations for the observed pattern of clustered infections.

First, the mosquito vector might show preference for certain vertebrate hosts. This hypothesis requires additional data, including some evidence of population structure in the parasite that is linked to specific hosts. Indeed, *P. inui*, also a quartan parasite, does not show host population structure in Borneo (55) but *P. knowlesi* does in Malaysia (49). This effect could even be exaggerated by host behavior, if, for example, the infected group utilizes unusually open sleep site locations. Nunn and Heyman (56) found preliminary support for the hypothesis that primates that sleep in closed microhabitats experience lower prevalence of *Plasmodium* infection. Emperor tamarins generally sleep in thick tangles of branches and vines, and sometimes tree holes, and although this is an unlikely explanation, it will be worth ruling out in future years. Second, there could be additional hosts within the home range of the infected group of *S. imperator* that might increase the parasite encounter rate. The latter scenario is not unlikely, as there are 9 other nonhuman primate species present that could host *Plasmodium*, including members of the Atelidae, Cebidae, Pithecidae, and Aotidae, as well as a small but dynamic population of human researchers. However, all of the nonhuman primate species occur concurrently throughout the study area, and thus may not explain clustering within one species and one social group. Regarding the risk from infected humans at EBLA, the home range of this group does not overlap with the stations basecamp. Of two saddleback and two emperor tamarin groups with home ranges that do intersect with the basecamp, only the sole saddleback tamarin that appeared infected for a single year is member to these groups. That these groups closest to basecamp, which are the most well-sampled and exposed to the highest degree of proximity with researchers, accounted for a single *Plasmodium* infection from one year, suggests that transmission from human to nonhuman primates is not the source of *P. brasilianum* infection at EBLA. However, greater efforts to detect *P. malariae* in human populations that are in contact with non-human primates infected with *P. brasilianum* are needed to fully assess whether there are zoonotic infections. Finally, there could be differences in host susceptibility or simply very low parasitemia below the detection of the PCR implemented in this study. As we have only

sampled tamarin groups that occur within an approximate 200-hectare area, it would be worthwhile to expand the study area to see if other clusters are present.

Acknowledgements

We wish to thank the following individuals for their assistance in the field collecting samples or in laboratory running analyses: Frances Humes, Cindee Rettke, Alaa Kuziez, and Kudakwashe Muranda. This work could not have been accomplished without the support of the following institutions and organizations: the Amazon Conservation Association (ACA, USA) and the Asociación para la Conservación de la Cuenca Amazónica (ACCA, Perú), El Centro de Ecología y Biodiversidad (CEBIO, Perú), El Dirección General Forestal y de Fauna Silvestre (Perú). In particular, we wish to thank Field Projects International (FPI: formerly Primates Peru) for the logistical and infrastructural support, with over a hundred personnel including field managers and research assistants who contributed to sample and data collection.

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Chapter 4

The influence of sex-based developmental classes on group reproductive output in wild callitrichids

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Abstract

Prior research on cooperative breeders has considered correlations between group reproductive output (GRO) and the number of individuals in each age-sex class, but without controlling for uneven sampling efforts, the underlying effects of group size, and pseudoreplication at the group and species levels. Among callitrichids, age-sex classes do not provide meaningful categories, as individuals within an age-sex class can demonstrate varying reproductive development due to reproductive dominance of a few individuals per group. This study re-assesses the drivers of GRO in callitrichids by a) conducting a meta-analysis of published studies of callitrichid group composition; b) determining a novel method to assign developmental class based on reproductive morphology; and c) utilizing a multistep modelling approach to assess whether any sex-based developmental class predicts both the presence and the numbers of surviving offspring among free-ranging saddleback (*Leontocebus weddelli*) and emperor tamarins (*Saguinus imperator*) in Peru. The meta-analysis utilizing a historical dataset revealed that adult females and group size, but not the number of adult males is significantly correlated with GRO. Statistical models of the new dataset revealed that only mature males predicted if groups had any infants at all, but that the number of surviving infants was predicted by mature females and group size. Thus mature males appear to be necessary for groups to raise any infants, but mature females and a larger group size increase group reproductive output overall.

Keywords
Callitrichid, cooperative breeding, group composition, reproductive output, development

Introduction

Callitrichids exhibit a cooperative breeding system in which offspring receive care from alloparents, or individuals other than biological parents (Garber 1997; Jennions 1994; Sussman and Garber 1987). Groups typically consist of a single breeding female (although other females may be present) and variable numbers of adult and sub-adult males. Adults and subadults in a group can participate in infant rearing, including infant provisioning and transportation (Bales et al. 2000; Goldizen and Terborgh 1986; Huck et al. 2004). Despite the monopoly of breeding by a single adult female in most cases, callitrichids are rarely monogamous, but rather display a range of flexible mating strategies over time and within groups (Garber 1997; Garber et al. 2015; Goldizen 1988; Goldizen et al. 1996; Sussman and Garber 1987; Terborgh and Goldizen 1985).

One of the primary arguments for the presence of helpers, typically unrelated adult males or natal subadults, is that they alleviate the cost of rearing energetically expensive twin offspring that constitute over 80 % of all births in callitrichids (save *Callimico*) (Tardif 1997; Wislocki 1939). Alloparenting behaviors by helpers benefit offspring survival, and thus increase group reproductive output (GRO) (Bales et al. 2000; 2001; Boulton and Fletcher 2015; Garber 1997; Heymann 2000; Koenig 1995). By investing in the care of offspring, helpers could incur indirect fitness benefits if they are related to the biological parents; they also benefit from and contribute to group benefits, including increased vigilance and protection from predators, as well as access to valuable resources (for reviews see Bales et al. 2000; French 1997; Tardif 1997). Prior research suggests that the

effects of helpers on GRO are not uniform, and can vary based on helper sex and social status (Bales et al. 2000). There may also be species differences in how helpers of different age-sex classes influence GRO due to differing costs of infant-rearing between species (Díaz-Muñoz 2015; Heymann 2000). For example, pygmy marmosets (*Cebuella pygmaea*) have infant-parking strategies (Heymann 2000), while Goeldi's monkeys (*Callimico goeldii*) do not produce twins (Porter 2001), which reduces the cost of infant-care in these species.

Five cases in the published literature have attempted to explain variation in GRO by correlational analyses of group demographics. First, a study by Garber et al. (1984) found that the average number of infant moustached tamarins (*Saguinus mystax*) that survived to become juveniles was significantly positively correlated with the number of adult male helpers in a group. A follow up to this study further indicated that groups with one adult male had one third the number of dependent offspring that groups with three or more adult males did, independent of group size (Garber 1997). Second, a review of research on wild common marmosets (*Callithrix jacchus*) revealed that the number of juveniles was significantly correlated only with the number of adult males, and no other age-sex class (Koenig 1995). Third, using a large dataset on golden lion tamarins (*Leontopithecus rosalia*), Baker et al. (1993) calculated a higher mean number of offspring for two-male groups than in single-male groups, only including adult non-natal males in these analyses. Fourth, Bales et al. (2000) examined the effects of particular alloparents on GRO in the same population of *L. rosalia* by classifying alloparents in two ways, a) as "helpers", defined as all animals over 18 mo of age other than the reproductive pair and

reproductive subordinate females and b) "adult males", both breeding and nonbreeding (Garber 1997; as with Garber et al. 1984; Koenig 1995). They found that among young groups (formed for three years or less) both numbers of helpers and adult males were positively correlated with the number of surviving infants, but that in established groups (formed for over three years), only the number of helpers correlated with GRO (Bales et al. 2000). And finally, infant numbers were found not to correlate with numbers of adult males or numbers of adult and subadult group members in an analysis of 21 group-years of pygmy marmosets (*Cebuella pygmaea*); however, juvenile numbers were strongly positively correlated with the number of adult and subadult group members (irrespective of sex) (Heymann and Soini 1999; Soini 1988).

Other than these correlational analyses, two published studies have attempted to model the predictors of GRO in callitrichids to date. Bales et al. (2001) modelled the effects of several maternal factors on female reproductive output in a population of *L. rosalia* that has two birthing seasons per year. Their analysis accounted for female identity, prior female reproductive success, repeat sampling of females across multiple years, and age. Female body mass predicted female reproductive output for litters in the first birth season, whereas that the number of helpers (as defined in Bales et al. 2000) explained offspring numbers in the second birth season. They concluded that mothers with increased helpers may carry infants less and thus be in better body condition for the subsequent birth season (Bales et al. 2001). A study of a single group of moustached tamarins (*Saguinus mystax*) from 1999 to 2008 used a logistic regression to model the if infants survived to age 3 months (response variable) against the number of male helpers

(Culot et al. 2011). This analysis revealed that the number of male helpers significantly affected infant survival to 3 months, but GRO was not modelled directly. However, they did find that 33.33% fewer infants survived when 2 vs. 3 males were present in the group, and infants died significantly more often with a median of 2.5, rather than 3, males present.

The prior analytical approaches used to explore the role of helpers of both sexes, including adults and subadults, on GRO exhibit several methodological complications that should be addressed. Strict correlational analyses of mean GRO with numbers of individuals in different age-sex classes (Garber 1997; Koenig 1995) do not account for variable group sizes, which are often uneven across a species. For example, an assessment of data from a thirteen-year study on *Leontocebus weddelli* (formerly *Saguinus fuscicollis weddelli* (Buckner et al. 2015; Matauschek et al. 2010)) (Goldizen et al. 1996) with groups containing 1-4 adult males revealed that 25% (12/47) of groups had only one male, 68 % (32/47) of groups had 2 adult males, while only 5 % (2/47) had 3 males, and 2 % (1/47) had four males – disparate sample sizes that preclude using means to test the effect of age-sex class on GRO as per Garber (1997). In another approach, Koenig (1995) attempted to assess the impacts of group size on GRO across multiple studies, but these analyses did not consider uneven sampling or random variation between studies, which can be accounted for by meta-analytical statistics. While Bales et al. (2001) used more powerful statistical methods such as generalised linear mixed modelling, theys examined only the effects of female-factors on GRO in *L. rosalia*,

excluding the potential influences of individuals from other age-sex classes (particularly adult males) from their model.

When considering the Callitrichidae, an important distinction should be made between the set of individuals that copulate within a group (the mating system) and the smaller subset of individuals that contribute towards the gene pool of viable offspring (the breeding system) (Garber 1997). Kappeler and van Schaik (2002) refer to these as the social and the genetic mating systems respectively. Molecular techniques can be used to reliably identify participants in the genetic mating system (i.e. the biological parents) in wild populations (Garber et al. 2015; Huck, Löttker, Böhle, et al. 2005; Huck et al. 2007; Nievergelt et al. 2000), and as expected, this mating system usually contains a single male and female. In rare cases, multiple males can father offspring in the same litter (Díaz-Muñoz 2011; Huck, Löttker, Böhle, et al. 2005; Suárez 2007). The social mating system, however, remains difficult to describe since copulation can be cryptic, occur infrequently, or be of short duration in many arboreal species(Campbell 2006; Watsa 2013) Nevertheless, the potential to contribute to the gene pool rather than an individual's actual contribution, which can be affected by many factors, may be relevant to understanding GRO in cooperatively breeding primates, particularly because biological parents' efforts are not the sole contributors to infant survival. As yet, factors that describe the social mating system of each group, such as variation in individual reproductive development, have not been explored in relation to offspring survival, but they could clarify why all adults do not contribute equally to GRO (Garber 1997).

There are several mechanisms can contribute to variation in the reproductive capabilities, or developmental class, of callitrichids within the same age-sex class. Although further research is warranted to determine its frequency, there is evidence of reproductive suppression of female callitrichids from both wild and captive settings (Barrett et al. 1990; Beehner and Lu 2013; Ziegler et al. 1987). Additionally, there is evidence of differences of up to 174% in testicular volumes of male moustached tamarins (*Saguinus mystax*) within the same group (Garber et al. 1996), which suggests that there may be reproductive skew among these males. A study of 24 male *Leontopithecus rosalia* in 14 groups was able to demonstrate quantifiable reproductive suppression in subordinate males unrelated to the dominant breeding male in a group (Bales et al. 2006). There are also studies documenting delayed dispersal of individuals from natal groups in the wild (Garber et al. 2015; Goldizen and Terborgh 1989), which can have physiological ramifications (Ginther et al. 2001; Ziegler et al. 1987). Thus, in a sampling of wild individuals, it is possible to encounter variations in reproductive morphology pertaining to scent-glands, vulva, nipples, testes and body weight measurements that may not reliably correspond with age.

Reproductive development has been assessed before through endocrine studies of derivatives of testosterone, estradiol, and prolactin among callitrichids in captivity (Ziegler et al. 1993) and in the wild (Bales et al. 2006; French et al. 2003; Löttker et al. 2004). However, wild studies are invariably challenged by the inability to collect blood for peptide hormones or adequate numbers of fecal steroid samples from known individuals across multiple ovarian cycles and breeding seasons (Löttker et al. 2004). One

study that measured testosterone levels among wild moustached tamarins (*Saguinus mystax*) found that concentrations varied too widely during maturation to reliably determine an individual's level of reproductive development, including between twin siblings (Huck, Löttker, Heymann, et al. 2005). In golden lion tamarins (*Leontopithecus rosalia*) however, androgen levels were found to be different only in the case of subordinate males unrelated to the dominant male, and age class had no effect on hormone profiles, indicating that reproductive capability is sensitive to group demography and not reflected by age-class alone (Bales et al. 2006).

Another means to evaluate reproductive development is to assess dominance status in a group, the definitions for which differ by sex. Among females, reproductive dominance can be exerted through endocrine monitoring, interactions with breeding males (marmosets: Sousa et al. 2005), infanticide and aggression (Bezerra et al. 2007; Digby and Saltzman 2009), and through age effects (i.e. the oldest female is the breeding female) (moustached tamarins: Garber 1997). Among males, agonistic interactions have been used by some to identify a dominant male (Baker et al. 1993), because using these criteria is not always feasible (Huck, Löttker, Heymann, et al. 2005),. Nevertheless, given that reproductive skew has been observed in wild and captive callitrichids, it is clear that current metrics can fail to differentiate individuals of varying reproductive potential, particularly in light of species, demographic and site-specific variation. Thus, since all individuals in an age-sex class cannot be assumed to possess similar reproductive potential, it is critical that developmental class, and not only age-classes, be assessed for possible impacts on GRO.

In this study, we compiled two datasets to contrast the utility of using simple age-sex classes or morphology-based developmental classes on GRO. The first is a historical dataset of all published studies on wild callitrichids that provide data on the numbers of individuals in each age-sex class and the numbers of surviving offspring per year. We apply a meta-analytical approach to this historical dataset that builds on past works in estimating the average magnitude of correlations between age-sex classes and GRO across studies (Scheiner and Gurevitch 2001). In captive studies, males with only a single mate for a helper lost significantly more weight during offspring rearing than those with additional helpers (Achenbach and Snowdon 2002). Also, the addition of more sexually active males to a group was found not to threaten group stability, unlike cases of multiple breeding females within a group (Burkart 2015). Based on these findings and prior studies that have examined the effect of age-sex classes on GRO (Baker et al. 1993; Bales et al. 2000; 2001; Culot et al. 2011; Garber 1997; Heymann 2000; Koenig 1995) we predicted that the number of adult males should be significantly positively correlated to GRO across studies. Further, several cases of maternal infanticide have been reported for some tamarin species (Bezerra et al. 2007; Culot et al. 2011; Digby 1995; Tirado Herrera et al. 2000), largely in the case of multiple breeding females reproducing in a single group, such as in *Callithrix jacchus* (Digby and Saltzman 2009). An analysis of *Saguinus mystax* also predicted that infants were four times as likely to die before reaching the age of 3 months in groups with 2 breeding females rather than one (Culot et al. 2011). We therefore also predicted that high numbers of adult females would have a negative effect on GRO across studies.

The second dataset, from a 6-year study on saddleback (*Leontocebus weddelli*) and emperor tamarins (*Saguinus imperator*) in southeastern Peru, consists of group compositions by sex-based developmental classes and the numbers of surviving offspring each year. We developed a method that uses multiple morphological variables collected via a mark-recapture program to reliably assign individuals to one of three developmental classes – infantile, immature, and mature - reflecting their potential to participate in the social mating system of a group. We use these data to answer two questions on the factors that drive GRO. First, which developmental classes have a significant effect on determining if a group has any offspring at all? Second, which developmental classes have a significant effect on predicting the number of surviving offspring (0 to 3)? These are two fundamentally different ways of addressing the question of GRO, however, we predicted (as with the historical dataset), that mature males would have a significant positive effect, while mature females would have the opposite effect. In our analyses, to account for group size variation, we used proportions of individuals belonging to each developmental class instead of raw numbers, as in the past.

Methods

(a) Study Site and Subjects

We studied 21 groups of free-ranging saddleback tamarins (*Leontocebus weddelli*, formerly *Saguinus fuscicollis weddelli* (Buckner et al. 2015; Matauschek et al. 2010)) and emperor tamarins (*S. imperator*) at the Estación Biológica Río Los Amigos (EBLA)

(12°34′S 70°05′W) in the Madre de Dios Department of southeast Perú annually across 6 seasons (2010-2015). We used a mark-recapture program (detailed protocol in (Watsa et al. 2015)) of 166 animals (106 *Leontocebus weddelli* and 60 *Saguinus imperator*) with an average of ~55 captures per year, for a total of 331 capture instances. At capture, infants were 4 to 7 months old, readily identifiable by facial pelage and dentition. The largest suspected breeding female in each group was fitted with a radio collar to facilitate tracking as a part of a larger behavioral study. Groups were followed for an average of 425 hours (range: 116 to 1135 hours) each season (May to August) and instances of mating and dispersal were recorded *ad libitum*. All groups were censused at least twice a month for group composition. In total, we monitored the study groups for a total of 2127 hours across the 6-year period. We recorded a total of 143 instances of mating across 33 males of both species.

(b) Assigning developmental status:

In this paper, we call those individuals that participated in mating, and who have the potential to contribute to the gene pool, as mature females or males. We were able to classify a female as mature if the female displayed a nipple length of > 3 mm for *Leontocebus weddelli* or > 4 mm for *Saguinus imperator* (Soini and de Soini 1990; Watsa 2013), indicating a prior birth record, regardless of whether multiple adult females or infants were present in the group. Mature males were considered to be any males that we observed copulating. We identified immature individuals in groups as those who were between 1 and 2 years of age i.e. were known to have been born in the prior census year. Thus, during a census, groups could consist of mature or immature members of both

sexes, as well as any offspring born in that same year who would belong to the infantile class. Based on these criteria, we identified a subset of individuals of known reproductive developmental status that could be used to validate our models to predict reproductive developmental status in other animals.

During capture, we recorded body mass and length and width of genitalia and suprapubic glands to formulate indices of developmental status as follows: vulvar index (length + width), suprapubic gland area (length * width), average nipple length, and testicular volume (a semi-spherical estimate) (Garber et al. 1996; Soini and de Soini 1990). In 2.4 % (8/331) of captures, a measurement (not always the same one) was not recorded by accident. We included these 8 instances by replacing the missing values with the mean value for the measurement in that developmental class (if known, N=4), or in that age class instead (N=4). We mean-centred and scaled all measurements and indices by standard deviation for use in a principal components analysis by species-sex groups (Principal Components Analysis: FactoMiner package in R (Beehner and Lu 2013)). We used individual coordinate values from the first two principal components in a linear discriminant function analysis (LDA) to model assign individuals of unknown developmental status to three categories: mature, immature, and infantile. Resampling of individuals occurred 1 to 4 times per animal, with 51.8 % captured at least twice. To avoid pseudoreplication, we used mean individual component scores across years for animals with known developmental status to train the LDA functions. We checked each species-sex class for normality (q-q normal plots), linear relationships (linear regression), and homoscedasticity between developmental categories (Bartlett's test of homogeneity

of variance, $p > 0.05$). We omitted infant males of both species from the LDA due to limited variance causing heteroscedasticity; but since they were < 7 months old, this exclusion had no impact on adult and sub-adult male classifications. We calculated the percentage of known individuals that were correctly classified by this PCA-LDA model (Table 1), and used a MANOVA (manova: MASS package in R (Venables and Ripley 2002)) to test the null hypothesis that all predicted developmental status groups were indistinguishable based on individual component scores. All statistical analyses were performed in R v.3.2.2 (R Development Core Team 2015).

Table 1: Sample sizes of developmental classes before and after the LDA model

	LEONTOCEBUS WEDDELLI			**SAGUINUS IMPERATOR**		
	Known developmental class	% correctly classified by LDA	Full dataset	Known developmental class	Full dataset	% correctly classified by LDA
MATURE FEMALES	24	100 %	36	17	26	100 %
IMMATURE FEMALES	17	94 %	41	12	15	100 %
INFANTILE FEMALES	18	100 %	19	17	17	100 %
MATURE MALES	21	81 %	55	12	42	83 %
IMMATURE MALES	9	67 %	26	5	15	100 %
INFANTILE MALES	28	NA	28	11	11	NA

(c) Group reproductive output in the historical dataset

To assess the reliability of previous findings that numbers of adult males is strongly associated with GRO, we performed a meta-analysis of all available historical data. We utilized Google Scholar and Scopus to conduct a literature review for published information demographics and group reproductive output in wild callitrichid populations. We compiled a historical dataset from 15 studies published from 1976 to 2015 on wild populations of *Saguinus* spp. (including *S. geoffroyi, S. mystax, S. weddelli* (now *Leontocebus weddelli* (Buckner et al. 2015; Matauschek et al. 2010))*, S. tripartitus,* and *S. oedipus*)*, Leontopithecus caissara,* and *Callithrix jacchus*. Studies were included in our analysis only if they reported raw numbers of individuals per age-sex class and GRO across a minimum of 5 group-years (Appendix 2 in Electronic Supplementary Material). For the meta-analysis of numbers of adult males to GRO, we included an additional study (now N=16) on *L. rosalia* by Bales et al. (2000) by calculating the effect size from the sample size and Spearman's rank correlations presented in the study. To combine data from multiple studies, we used a Spearman's rank correlation weighted by the number of group-years in the study as a standardized effect size. In this dataset, groups (within a study) and species (across multiple studies) were subject to repeated sampling over time, which could render certain data points non-independent. To control for interspecific differences, we added species as a moderator variable in a mixed effect meta-analysis of the historical dataset. Species did not have a significant effect and was subsequently removed; we proceeded with a random effects meta-analysis that does not assume equal effect sizes across studies. Regarding repeated sampling of a subset of groups in long-term studies, we feel that their inclusion does not bias our study more than their

exclusion, which would drastically shrink the dataset. However, we use a more conservative significance level of $p < 0.01$ for the meta-analyses (see Gurevitch et al. 1992; Poulin 1994 for detailed explanation of this reasoning).

(d) Group reproductive output in the Los Amigos dataset

Correlations are pair-wise, not predictive, and cannot control for group identity or species (Bolker et al. 2009). Further, group size can be controlled for by using proportions of individuals in each age-sex or developmental class. With this in mind, we first constructed a mixed-effect logistic regression model with a binomial error structure and a logit link function to predict a binary response variable - offspring presence or absence based on proportions of individuals of each developmental class as fixed factors. As per Bales et al. (2001) we also built generalised linear mixed models (GLMMs: lme4 in R (Bates et al. 2014)) with a Poisson error structure, response variable GRO (ranging from 0-3), and proportions of individuals per developmental class as fixed factors. We used saturated fixed-effect models to optimise random structures, and incorporated group identity, species, and year as needed to ensure independence of data points across all models. Correlation analyses were conducted on all pairwise combinations of explanatory variables and any fixed factor redundancies were removed. Each explanatory variable was plotted against the response variable to ensure that there were no nonlinear relationships. We established minimal models using Akaike Information Criterion (Akaike 1994) by backwards non-significant term deletion, retaining terms only if they reduced criteria by two units (Moreno et al. 2013). Minimal models were confirmed by performing a likelihood ratio test, which compares the difference in log-likelihoods of

nested models with a Chi-square distribution. The residuals of best fit models were plotted to ensure that they were randomly distributed around zero.

(d) Ethical Note

This study follows the Animal Behaviour Society Guidelines (Rollin and Kessel 2006) and American Society of Mammalogists' Guidelines on wild mammals in research (Sikes and Gannon 2011). The study is part of an ongoing, long-term annual capture-and-release program that began at this site in 2009. In brief, we captured entire groups at baited compartment traps to which they are habituated, and processed and released them on the same day to minimize disruption and discomfort to the animals. Although based on previous capture protocols established for callitrichids (Savage et al. 1993), our study utilizes a novel two-step chemical restraint method that has improved recapture rates, virtually eliminated capture-related injuries, and has no visible effect on habituation for subsequent behavioral research (see Watsa et al. 2015 for protocol comparisons).

The Peruvian Ministry of the Environment (SERFOR) granted annual research and collection permits, and the Animal Studies Committees of Washington University in St. Louis and the University of Missouri - St. Louis approved protocols.

Results

(a) Mean Group Reproductive Output per Age-Sex Class

As observed in one of the best longitudinal datasets on wild callitrichids (Cocha Cashu, *S. fuscicollis*, now *Leontocebus weddelli*) (Goldizen et al. 1996), the pattern of using average GRO that does not account for uneven sample sizes held true for the historical

dataset. Confidence in the average number of offspring per number of adult males in a group is directly related to the frequency with which groups of variable numbers of males have been observed. Even the largest datasets are biased towards groups with 1 to 2 males, and much less frequently collect data on groups with 3 or more males. Although previous publications have suggested an increase in the average number of offspring with increasing numbers of males, disparate sample sizes result in hugely overlapping confidence intervals that confound the use of mean GRO as an effective method for comparison. For example, mean offspring =1.10 ± SD 0.87 (CI: 0.94-1.27) in groups with two adult males while mean offspring = 0.93 ± SD 0.77 (CI: 0.72-1.14) in groups with one adult male in data from Cocha Cashu (Figure 1). This precluded the use of mean GRO to evaluate the effect of age-sex class on GRO as per Garber (1997).

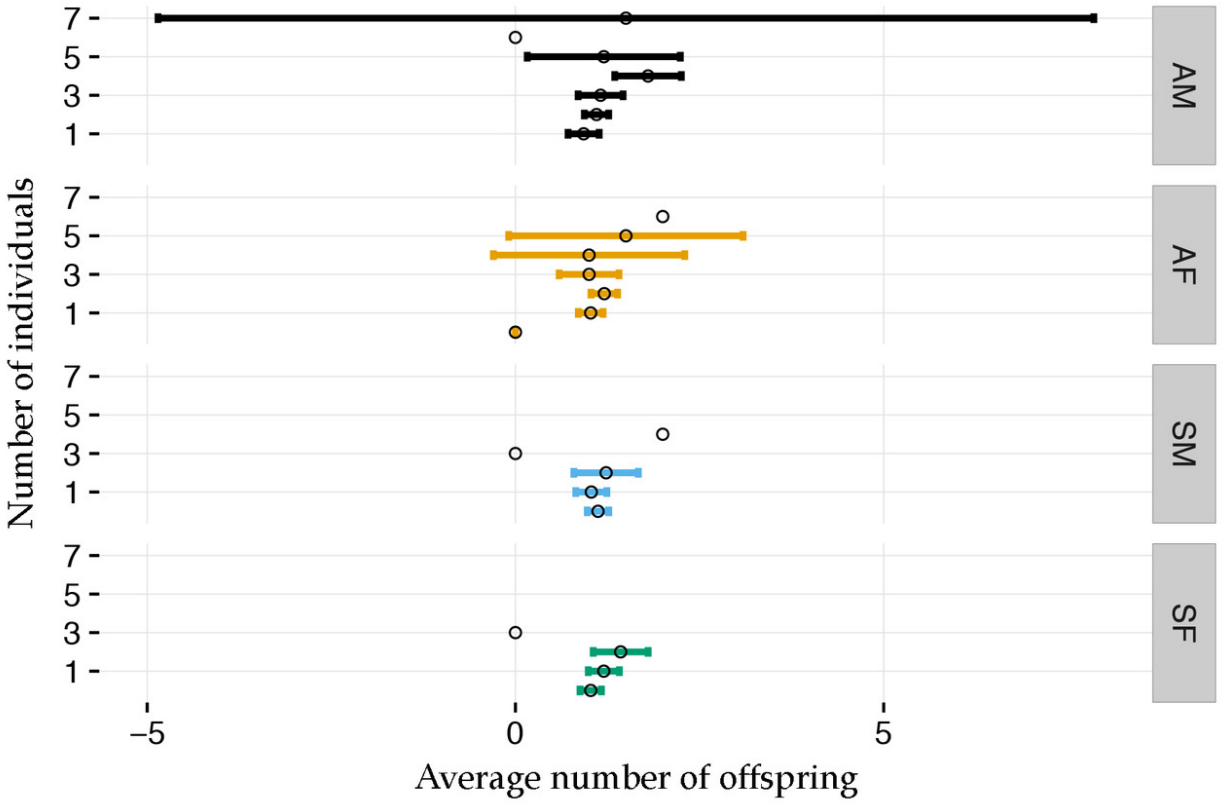
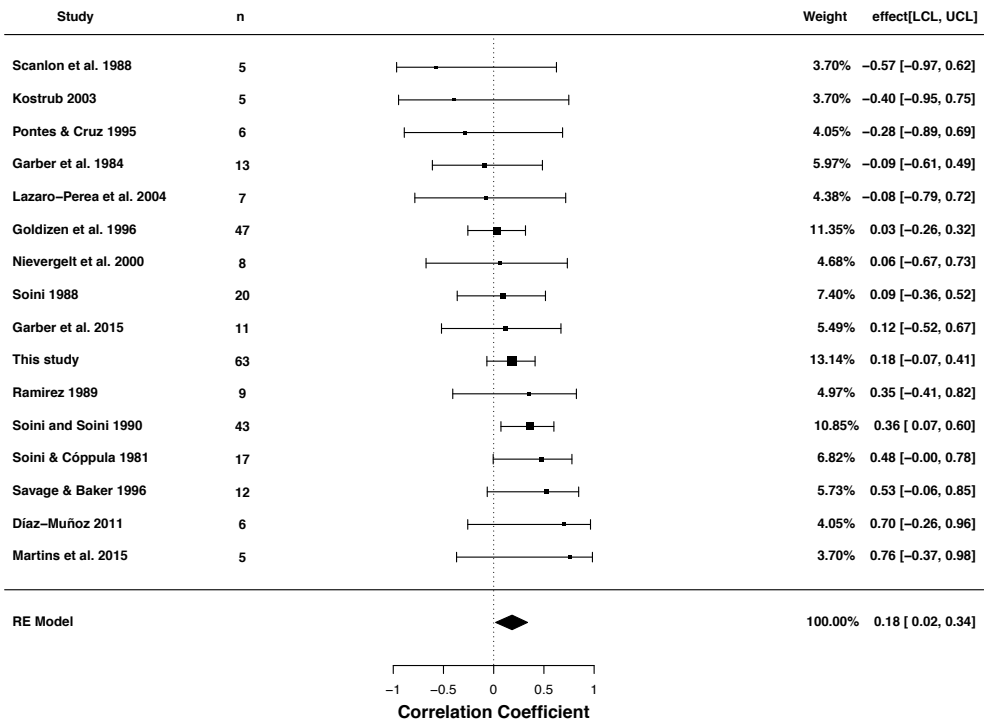


Fig. 1 Average number of infants (circles), with 95% C.I. (lines) depending on the number individuals from each age-sex class in the complete historical data set; adult males (AM), adult females (AF), sub-adult males (SM), sub-adult females (SF)

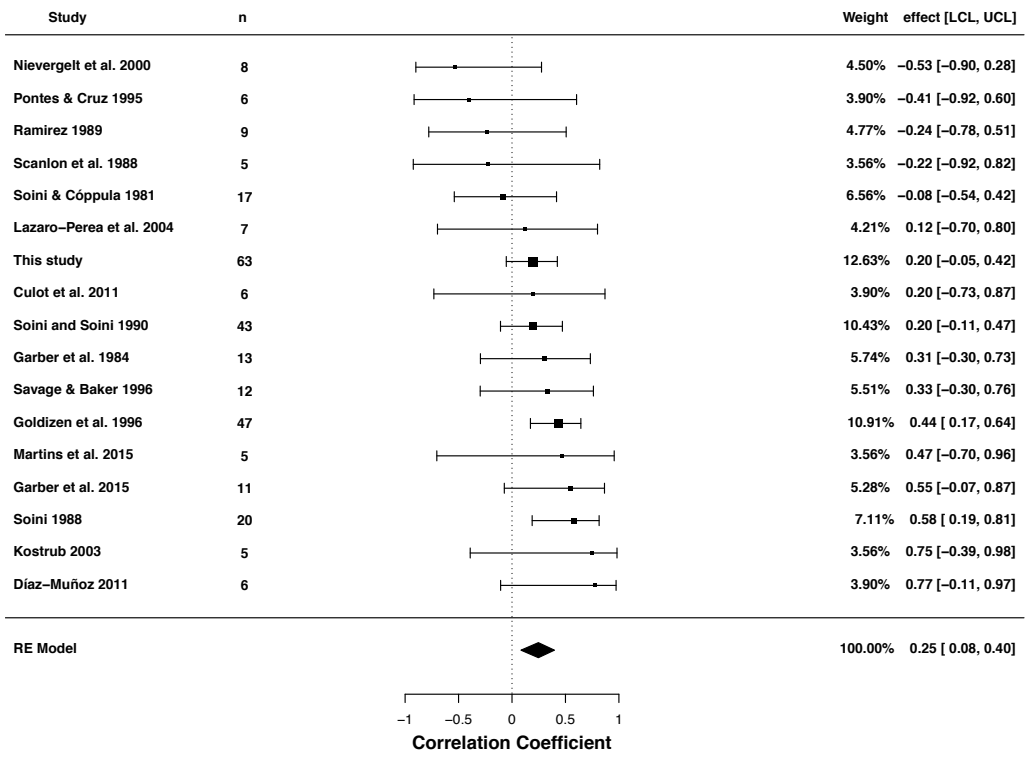
(b) Meta-analyses of GRO in the Historical Dataset

A random-effects meta-analysis combining data from prior studies and the present study revealed significant Spearman's rank correlations between adult females and GRO (weighted average r_s (16) = 0.185, P < 0.028), as well as group size and GRO (weighted average r_s (17) = 0.252, P < 0.003) (Figure 2). Adult males and subadults of either sex were not significantly correlated with GRO across studies (P > 0.05). These results remain unchanged when our study was excluded from the analysis.

A



B


Study
n
Weight
effect [LCL, UCL]
Nievergelt et al. 2000 8 4.50% −0.53 [−0.90, 0.28]
Pontes & Cruz 1995 6 3.90% −0.41 [−0.92, 0.60]
Ramirez 1989 9 4.77% −0.24 [−0.78, 0.51]
Scanlon et al. 1988 5 3.56% −0.22 [−0.92, 0.82]
Soini & Cóppula 1981 17 6.56% −0.08 [−0.54, 0.42]
Lazaro−Perea et al. 2004 7 4.21% 0.12 [−0.70, 0.80]
This study 63 12.63% 0.20 [−0.05, 0.42]
Culot et al. 2011 6 3.90% 0.20 [−0.73, 0.87]
Soini and Soini 1990 43 10.43% 0.20 [−0.11, 0.47]
Garber et al. 1984 13 5.74% 0.31 [−0.30, 0.73]
Savage & Baker 1996 12 5.51% 0.33 [−0.30, 0.76]
Goldizen et al. 1996 47 10.91% 0.44 [0.17, 0.64]
Martins et al. 2015 5 3.56% 0.47 [−0.70, 0.96]
Garber et al. 2015 11 5.28% 0.55 [−0.07, 0.87]
Soini 1988 20 7.11% 0.58 [0.19, 0.81]
Kostrub 2003 5 3.56% 0.75 [−0.39, 0.98]
Díaz−Muñoz 2011 6 3.90% 0.77 [−0.11, 0.97]
RE Model 100.00% 0.25 [0.08, 0.40]
−1
−0.5
0
0.5
1
Correlation Coefficient

C

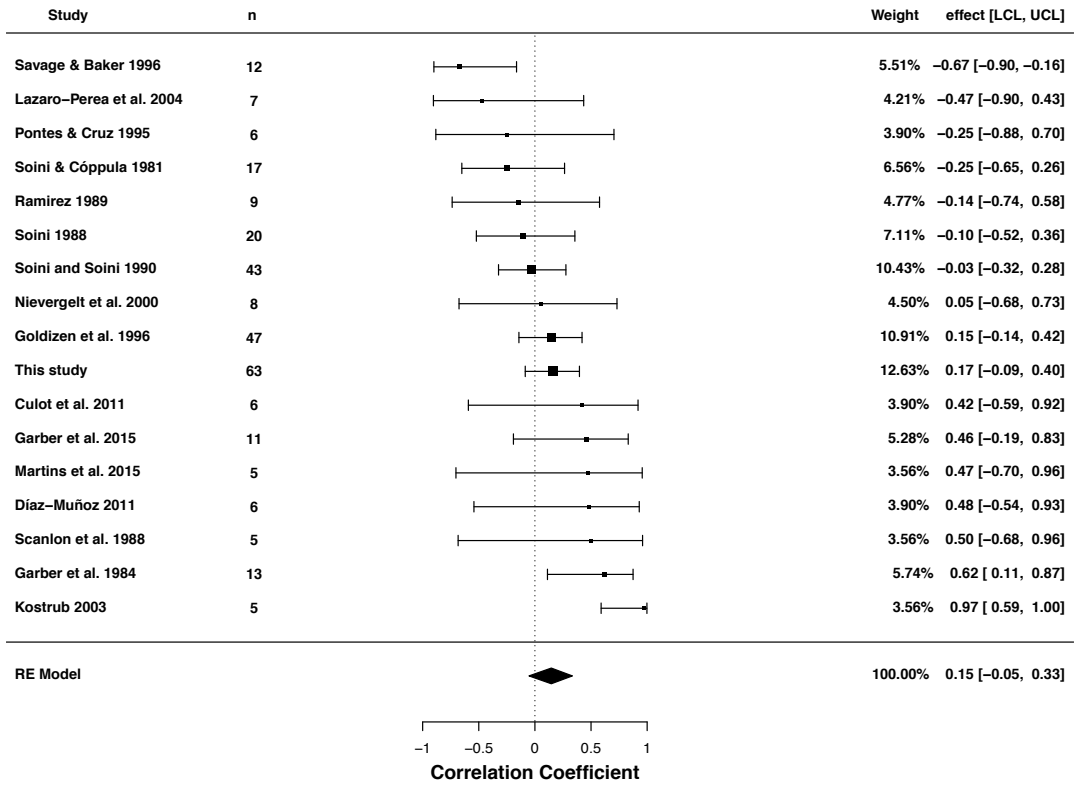

Study
n
Weight
effect [LCL, UCL]
Savage & Baker 1996 12 5.51% −0.67 [−0.90, −0.16]
Lazaro−Perea et al. 2004 7 4.21% −0.47 [−0.90, 0.43]
Pontes & Cruz 1995 6 3.90% −0.25 [−0.88, 0.70]
Soini & Cóppula 1981 17 6.56% −0.25 [−0.65, 0.26]
Ramirez 1989 9 4.77% −0.14 [−0.74, 0.58]
Soini 1988 20 7.11% −0.10 [−0.52, 0.36]
Soini and Soini 1990 43 10.43% −0.03 [−0.32, 0.28]
Nievergelt et al. 2000 8 4.50% 0.05 [−0.68, 0.73]
Goldizen et al. 1996 47 10.91% 0.15 [−0.14, 0.42]
This study 63 12.63% 0.17 [−0.09, 0.40]
Culot et al. 2011 6 3.90% 0.42 [−0.59, 0.92]
Garber et al. 2015 11 5.28% 0.46 [−0.19, 0.83]
Martins et al. 2015 5 3.56% 0.47 [−0.70, 0.96]
Díaz−Muñoz 2011 6 3.90% 0.48 [−0.54, 0.93]
Scanlon et al. 1988 5 3.56% 0.50 [−0.68, 0.96]
Garber et al. 1984 13 5.74% 0.62 [0.11, 0.87]
Kostrub 2003 5 3.56% 0.97 [0.59, 1.00]
RE Model 100.00% 0.15 [−0.05, 0.33]
−1
−0.5
0
0.5
1
Correlation Coefficient

Fig. 2. Forest plot showing the meta-analysis of a correlation of group reproductive output (GRO) with A) number of adult females ($P < 0.001$, N= 15); B) group size ($P < 0.001$, N=15) and C) number of adult males ($P > 0.05$, N=16). Confidence intervals that do not overlap zero are generally not considered to be significant. Adult females (A) and group size (B) are significantly positively correlated with GRO across studies, while adult males (B) are not. These results are not altered when this study is removed from the dataset.

(c) Group Demographics from Our Study Population

Over 6 years we observed 21 groups across 63 group-years during which they could have reproduced, including 14 groups of *Leontocebus weddelli* sampled for a mean of 2.86 ± SD 1.35 years and 7 groups of *Saguinus imperator* sampled for a mean of 3.43 ± SD 1.27 years. Mean group sizes (Table 2), adult group sex ratios (males:females) (*L. weddelli*: 1.23 ± SD 0.63; *S. imperator*: 1.65 ± SD 1.34), and GROs (*L. weddelli*: 1.03 ± SD 0.87; *S. imperator*: 0.92 ± SD 0.88) were not significantly different between species (Welch's Two Sample t-test, $p > 0.05$). Across the study, 8.7% of all captured animals were infants, with 1-2 offspring per group and only one instance of three offspring. We also observed 7 instances of two mature females present in a single group – four cases in *L. weddelli* and three in *S. imperator*.

Table 2: Group compositions based on developmental class status. All figures are provided as mean number of individuals ± standard deviation (range).

SPECIES	*N*	*GROUP SIZE*	*MATURE FEMALES*	*IMMATURE FEMALES*	*MATURE MALES*	*IMMATURE MALES*	*ALL FEMALES*	*ALL MALES*	*ALL INFANTS*
LEONTOCEBUS WEDDELLI	14	4.95 ± 1.63 (3-8)	0.95 ± 0.50 (0-2)	0.90 ± 0.78 (0-3)	1.40 ± 0.98 (0-3)	0.65 ± 0.74 (0-2)	1.88 ± 0.69 (1-4)	2.05 ± 0.90 (0-5)	1.03 ± 0.86 (0-3)
SAGUINUS IMPERATOR	7	5.21 ± 1.41 (3-8)	1.08 ± 0.41 (0-2)	0.67 ± 0.96 (0-3)	1.71 ± 1.23 (0-4)	0.63 ± 0.77 (0-2)	1.96 ± 1.00 (1-4)	2.33 ± 1.20 (0-6)	0.92 ± 0.88 (0-2)

Note: N = Number of unique groups.

(d) The Developmental Class Model

In our model, the minimum requirements for factor analyses were satisfied, with an average of 19 and 23 samples per variable for the females and males, respectively. The first two dimensions represented an average of 86 % (range: 82 – 90 %) of total group variation. For all species-sex classes, Principal Components Analysis dimension 1 was determined by all morphological variables and Principal Components Analysis dimension 2 was determined primarily by nipple length in females and suprapubic area and body mass in males (Tables 1 and 2 in Appendix 1 of Electronic Supplementary Materials).

For animals with known developmental class (57.1 % of *Leontocebus weddelli* and 59.5 % of *Saguinus imperator*), the LDA correctly assigned 98.3 % of female *L. weddelli*, 100 % of female *S. imperator*, 76.7 % of male *L. weddelli*, and 88.2 % of male *S. imperator* (Figure 3, Table 1). The LDA classification mismatched one immature female to the infantile class (*L. weddelli*); four mature males were reclassified as immature males, three immature males as mature males (*L. weddelli*); and two mature males as immature males (*S. imperator*). The LDA successfully distinguished between developmental classes for females and males of both species (MANOVA, P <0.0001, Table 3), and we calculated mean values and ranges of morphological variables per species-sex group (Table 4). For both species, we observed variation in developmental classes in all age-classes except among infants (Figure 4).

Table 3: MANOVA results distinguishing if developmental classes are significantly differentiated within all species-sex classes. Female assessment included three developmental classes (df=2), but males used only two (df=1).

SPECIES-SEX CLASS	***WILKS' Λ***	***F***	***DF***	***P-VALUE***
***FEMALE* LEONTOCEBUS WEDDELLI**	0.0461	82.35	2	<0.0001
***FEMALE* SAGUINUS IMPERATOR**	00348	56.67	2	<0.0001
***MALE* LEONTOCEBUS WEDDELLI**	0.3722	43.29	1	<0.0001
***MALE* SAGUINUS IMPERATOR**	0.4756	19.48	1	<0.0001

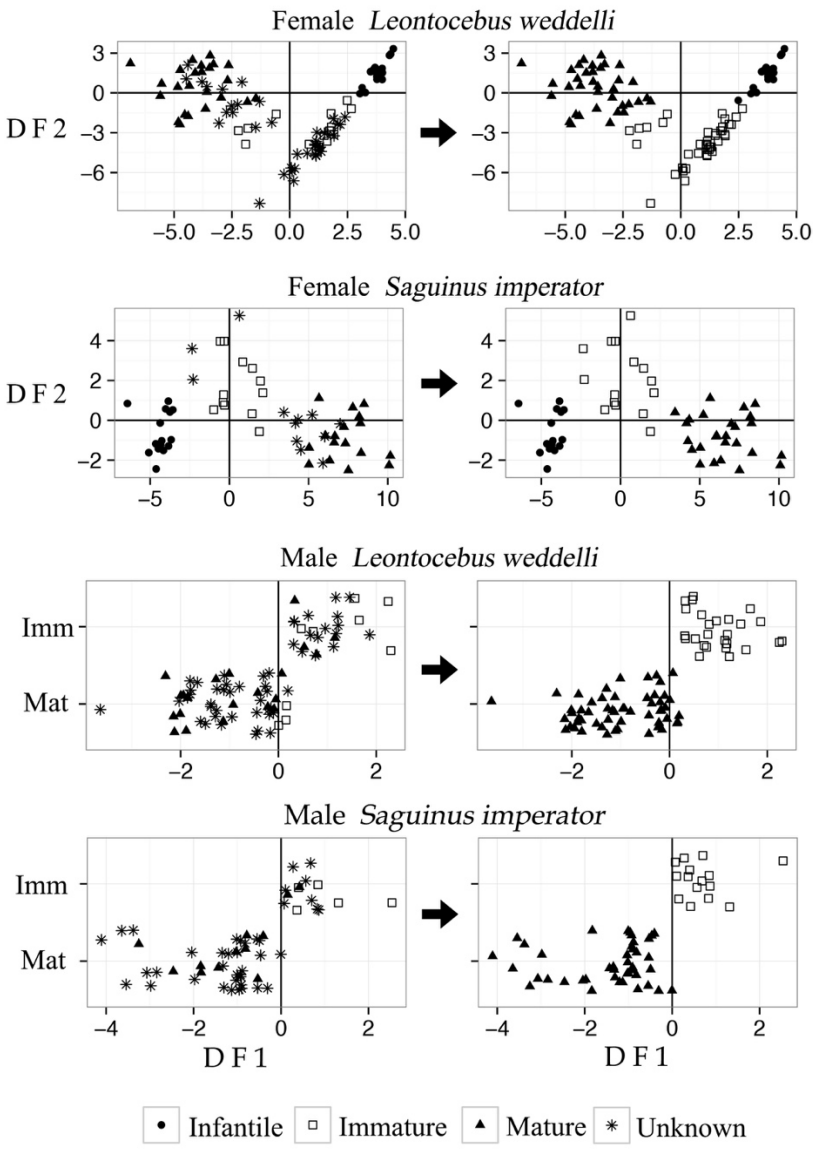


Fig. 3 Developmental status by species and sex before (left) and after (right) implementing the PCA-DFA assignment model and classifying all individuals of uncertain status (star symbol) to a category based on reproductive morphology and mass. Female categories are differentiated by discriminant functions 1 and 2 (DF1 & DF2), while mature (Mat) and immature males (Imm) are differentiated by DF1 only; males in the infantile developmental class were removed from the DFA.

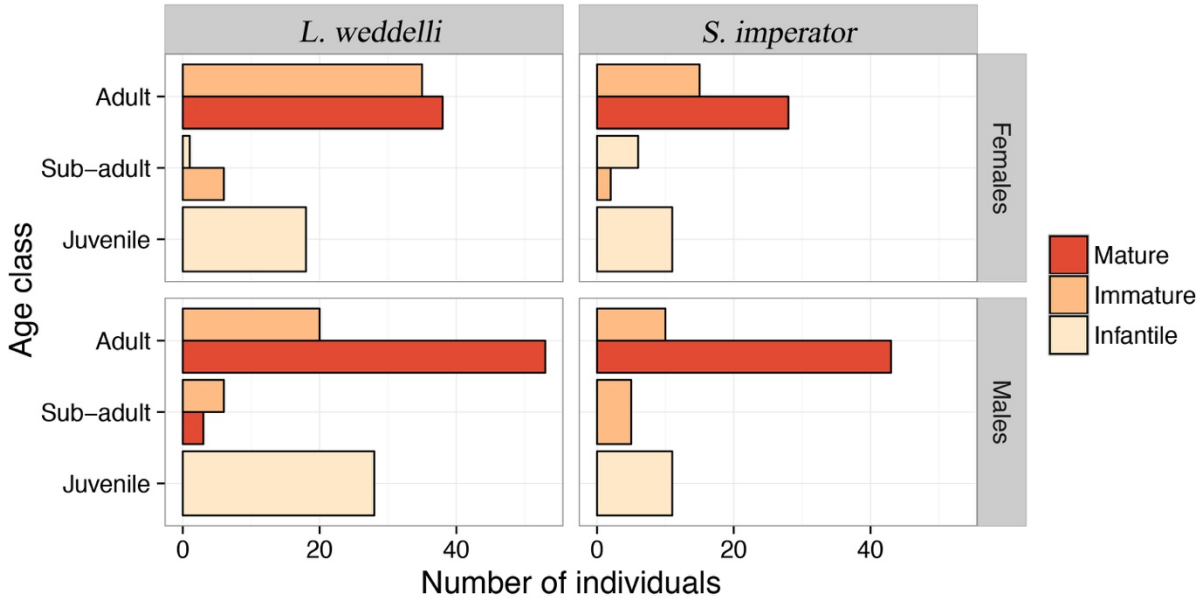


Fig. 4. The distribution of developmental classes (mature, immature, and infantile) between age-classes (adult, subadult and juvenile) for males and females of both callitrichine species at Los Amigos.

Table 4: Morphological variables by developmental class. All values expressed as mean ± s.d. (range)

SPECIES-SEX CLASS	DEVELOPMENTAL CLASS	NIPPLE LENGTH (MM)	SUPRAPUBIC AREA (MM^2)	VULVA INDEX (MM)	MASS (G)	TESTES VOLUME (MM^3)	N	IND.
FEMALE *LEONTOCEBUS WEDDELLI*	Infantile	0.00	22.30 ± 32.52 (0.00-104.28)	10.98 ± 2.05 (7.90-16.15)	230 ± 47.55 (135-330)	NA	19	14
	Immature	0.24 ± 0.58 (0.00-1.91)	251.27 ± 101.29 (75.36-483.48)	19.11 ± 3.26 (13.50-28.10)	394.59 ± 34.08 (305-475)	NA	41	29
	Mature	3.41 ± 0.79 (2.15-5.60)	276.32 ± 86.21 (103.14-522.21)	21.74 ± 2.52 (17.40-29.40)	401.11 ± 31.96 (340-490)	NA	36	21
MALE *LEONTOCEBUS WEDDELLI*	Infantile	NA	8.09 ± 22.05 (0.00-101.99)	NA	219.82 ± 34.52 (160-285)	106.37 ± 45.48 (42.60-243.39)	28	27
	Immature	NA	83.25 ± 49.00 (0.00-174.03)	NA	363.15 ± 24.94 (310-430)	671.33 ± 146.03 (321.88-953.17)	26	21
	Mature	NA	148.54 ± 69.11	NA	396.91	1029.13 ±	55	30

			(0.00-323.22)		± 23.52 (350-460)	248.34 (579.83-1986.27)		
FEMALE *SAGUINUS IMPERATOR*	Infantile	0	0.79 ± 3.25 (0.00-13.41)	12.64 ± 3.72 (0.00-16.55)	318.24 ± 73.06 (200-460)	NA	17	11
	Immature	1.21 ± 1.00 (0.00-3.000)	151.51 ± 88.09 (0.00-295.22)	20.67 ± 2.95 (13.38-24.70)	518.33 ± 43.33 (455-595)	NA	15	12
	Mature	5.15 ± 0.94 (3.65-7.45)	232.02 ± 67.37 (91.08-364.00)	25.59 ± 3.55 (19.88-32.65)	572.50 ± 52.20 (465-645)	NA	26	10
MALE *SAGUINUS IMPERATOR*	Infantile	NA	0	NA	258.18 ± 51.73 (150-320)	122.06 ± 36.82 (56.16-175.11)	11	11
	Immature	NA	15.43 ± 35.45 (0.00-122.20)	NA	453.93 ± 42.46 (360-520)	518.24 ± 104.06 (298.30-722.06)	15	13
	Mature	NA	69.51 ± 90.96 (0.00-300.83)	NA	517.02 ± 52.66 (420-645)	832.76 ± 189.08 (417.14-1150.34)	42	18

Note: N = total number of samples; Ind. = number of unique individuals in this class

(e) Group Reproductive Output in the Los Amigos Dataset

Our logistic regression model indicated that the proportion of mature males (B = 3.877, s.e. = 1.961, $\chi^2 = 3.91$, $P < 0.05$) was the sole significant factor in predicting the presence of offspring (Figure 5). The mean proportion of mature males in groups with no offspring (0.27 ± SD 0.23, N=22) was significantly lower than in groups with one or more offspring (0.41 ± SD 0.23, N=41; t=-2.32, df=43, P = 0.025) (Figure 5). The proportions of mature females and immature males or females were not significant predictors of the presence of offspring in this analysis. However, a GLMM with offspring number as a discrete numerical response variable revealed that the proportion of mature females relative to group size (B = 3.559, s.e. = 0.962, $\chi^2 = 13.69$, $P < 0.001$) and group size (B = 0.343, s.e. = 0.128, $\chi^2 = 7.15$, P = 0.008) were the only two significant factors. Removing group-years in which there were multiple mature females in a group (7 instances) did not alter the outcome of this GLMM. Greater proportions of mature females and larger group sizes were significantly associated with GRO. The proportions of mature males and immature males or females were not significant predictors of GRO in the GLMM.

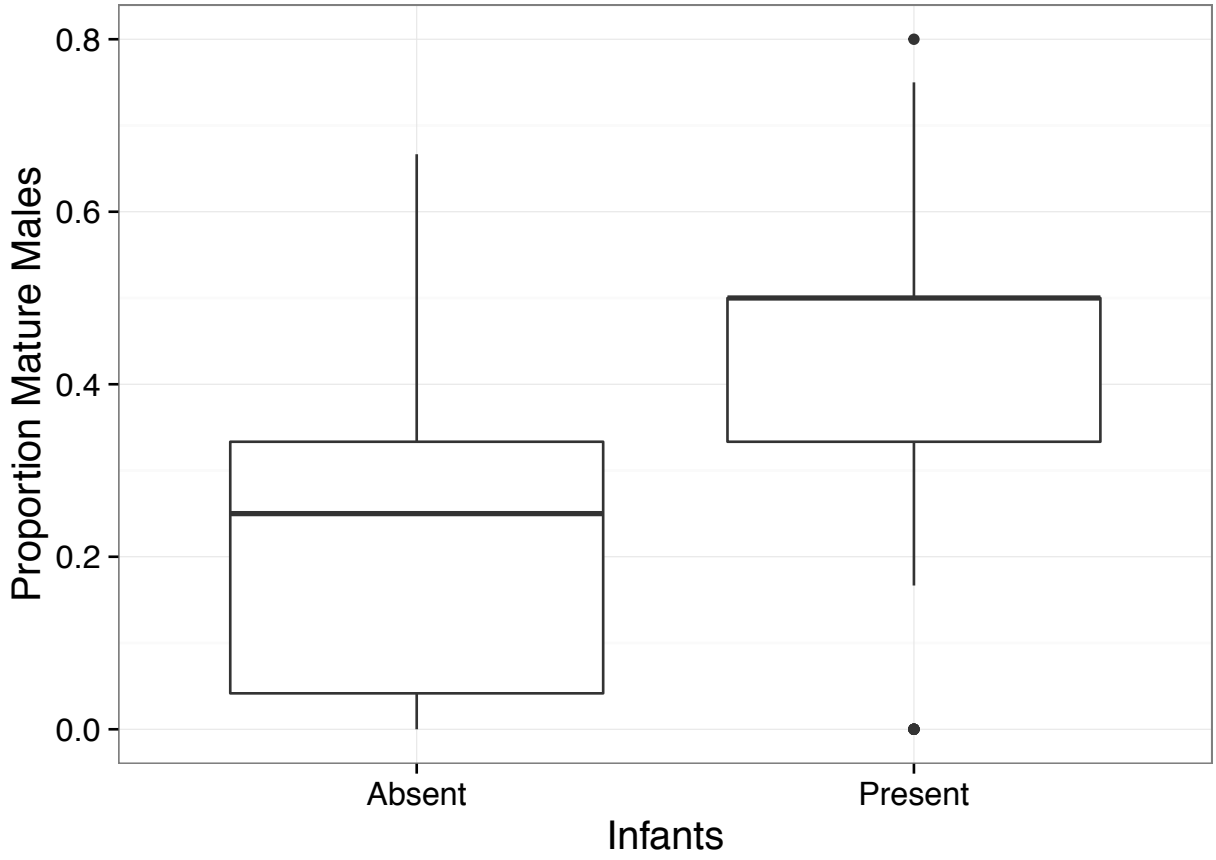


Fig. 5. A box plot of the proportion of mature males in groups where infants are either present or absent. The two proportions are significantly different as revealed by a logistic regression model.

Discussion

Like other callitrichids, both study species at Los Amigos twinned frequently and formed groups with multiple breeding females (Garber et al. 2015; Watsa 2013). Though these species diverged ~9.10-10.07 mya and are now placed in separate genera (Buckner et al. 2015; Matauschek et al. 2010), we noted no significant differences between them in mean

group size, adult group sex ratios, or mean GRO. Thus, we pooled data from both species for the purposes of this study.

Reproductive status has previously been evaluated in callitrichids through measurements of their genitalia (Soini and de Soini 1990). In addition, scent-gland morphology is known to signal estrus, changes around parturition (*Callithrix jacchus* (Moreira et al. 2015)), and differs by sex (French and Cleveland 1984; Watsa 2013; Zeller et al. 1988); suggesting that variation in scent gland morphology is representative of an animal's ability to reproduce (Watsa 2013). Here we created a reliable model that is based on genitalia and scent gland morphology, as well as body mass, to assign animals into developmental classes. That females were more reliably assigned to the correct class than males is likely due to the availability of better external indicators of developmental status, such as observed nursing and nipple lengths (Soini and de Soini 1990). Higher resolution of male developmental class would require the inclusion of all or most copulation records, which is not feasible as copulation is cryptic among arboreal primates (Campbell 2006) and of short duration (1-12 s) in tamarins (Watsa 2013). Nevertheless, our model successfully discriminated between developmental categories for all species-sex classes, and revealed that all animals of a particular age-sex class do not have equal reproductive capabilities. This method allowed us to re-examine how group composition influences GRO, by discriminating based on developmental status, in addition to age-sex classes.

More than any other factor, the number of adult males in a group have long been considered the key to increased callitrichine GRO (Heymann 2000; Koenig 1995). However, there are many reasons unrelated to offspring survival for why a group might vary in the number of males it contains (Carnes et al. 2011; Heymann 2000; Kappeler 2000; Ridley 1986). For

instance, the number of adult males in a group have been proposed to increase with shorter breeding seasons (Ridley 1986), since a single male probably cannot successfully monopolize multiple reproductively synchronised females (Carnes et al. 2011; Dunbar 2000). However, it has also been suggested that primate males simply "go where females are" (Altmann 1990). Cross-species analyses that control for phylogeny show that these theories are not necessarily exclusive - the number of males is tightly positively correlated with the number of females in primate groups across species (Mitani et al. 1996), but either overlap or synchrony of female breeding can predict adult male numbers after female numbers are controlled for (Nunn 1999). Other theories for larger numbers of males in groups include heightened predation risk (Savage et al. 1996; van Schaik and Hörstermann 1994) or, as with callitrichids, the necessity for alloparents due to the high costs of caring for twin infants (Heymann 2000; Tardif 1994; 1997). With the exception of *Callimico*, Heymann (2000) found the number of adult males to be positively correlated with litter mass gain and daily path length among callitrichids, implying that adult males are necessary to counter increased costs of infant care. This conclusion was recently supported by an extensive cross-genera analysis of the effect of infant care costs on variation in reproductive behaviors (Díaz-Muñoz 2015), which identified genus *Saguinus* (which now includes genus *Leontocebus*) as facing the highest infant care costs among all callitrichine genera. Thus we expected that the number of males would positively influence GRO across the Callitrichidae, including our study species.

Surprisingly, our meta-analysis, which utilized a robust methodology commonly used in the field of medicine for summarizing outcomes across a range of studies (Gurevitch et al. 1992; Scheiner and Gurevitch 2001; Vetter et al. 2013), did not meet this expectation. Instead,

group size and the number of adult females were positively correlated with GRO across five callitrichine genera for which data were available. This does not necessarily imply that adult males have a negligible effect on infant survival. Our meta-analysis was restricted to using a common quantitative metric that could be applied to all studies across the historical dataset on GRO in wild callitrichids, which was a correlation with the number of infants in a group, and not estimates of whether infants were present or absent (a categorical variable) given the increased numbers of males. Our dataset from Los Amigos that comprised 63 group years of a population of free-ranging *Saguinus imperator* and *Leontocebus weddelli*, did lend itself to a logistic regression model that tested this alternative perspective on male contributions to infant survival. Our finding that groups with high proportions of mature adult males are most likely to have one or more infants present concurs with those of Culot et al. (2011), who analysed factors explaining infant survival to three months of age in *S. mystax* using similar methodology. These results demonstrate that how we define reproductive success in callitrichids, as the simple presence of any offspring at all or the actual number of offspring, has a bearing on whether males are the key to GRO or not. Additionally, it suggests that the developmental class of adult males is also a significant factor to be considered.

Although females are not usually identified as playing a significant role in determining GRO, a study of the most comprehensive dataset on a wild callitrichine population to date (*L. rosalia* from Poço das Antas Reserve in Brazil) did highlight the importance of many female factors to reproductive success (Bales et al. 2001). Our analyses indicate that, after controlling for group size, the proportion of mature adult females in a group is the primary determinant of GRO. This finding was not due to the seven cases of multiple mature females in a single group that are a part of our dataset, as our results remained the same even when those instances were excluded from the analysis. This behooves us to consider the natural

circumstances under which the proportion of mature adult females could contribute significantly to increased GRO, given numerous observations of adults and subadults of both sexes actively participating in alloparenting (reviewed in Erb and Porter 2017). We posit three possible scenarios for high proportions of mature adult females in a group: Case 1) a single mature female present in a relatively small group, Case 2) multiple mature females present in a relatively small group, or Case 3) multiple mature females present in an average sized group.

In the first case, a single mature female will form a large proportion of group membership if group size is small, and by being the only mature female she experiences reproductive dominance without challengers. The proportion of mature females would be maximized at 0.5 if there were only one mature adult male that comprised the rest of the group. A recent review of a range of wild tamarin studies (N= 183 groups and an additional 66 resampled cases) reported this group composition in only 9.4% of cases, and these single breeding pairs invariably failed to raise infants in the wild (Garber et al. 2015). In fact, prior to our study, there was only one reported case exception to this trend, in *Saguinus imperator* at Cocha Cashu (Windfelder 2000). In our study, however, we report mixed reproductive success from our smallest groups i.e. those that contained a single pair of mature adults and one immature subadult. In three cases of *L. weddelli* and one of *S. imperator* there were no living infants at the time of evaluation, either because the female did not give birth to them or the male-female pair was unable to raise offspring to the age of weaning. In contrast, we observed two groups of *L. weddelli* where one infant survived successfully to weaning age. In one of these groups, this occurred in two consecutive years, and the infant from the first year was still present in the second year as an immature subadult. Thus, of 7 group-years of a single pair of

mature adults, we observed a ~43% success rate in raising infants to weaning, which is more common in this dataset than the remaining callitrichine data.

In cases 2 and 3, multiple mature adult females coexist in a single group of small or average size and could enhance infant survival in several ways. If only one female breeds successfully (i.e. there is high female reproductive skew), then the second female can enhance infant survival indirectly by increasing vigilance and foraging efficiency, or directly by alloparenting. This in turn could be beneficial to this non-breeding female in a variety of ways (parenting experience, future reproductive opportunities, or via kin-selection if she is the breeder's sibling or close relative) (reviewed in Erb and Porter 2017). In a study of 12 groups of *L. weddelli* in Bolivia (Garber et al. 2015), 25% of the groups contained two parous females (determined by nipple length), and the majority of these pairs were genetically verified to be likely mother-daughter pairs. Unfortunately, none of the groups contained infants at the time of assessment, so whether both females bred simultaneously is unknown. In the longest running study to date on *L. weddelli* at Cocha Cashu spanning 13 years, female reproductive skew was high, with a suspected 50% of females never breeding (Goldizen et al. 1996), although how the reproduction of these females was limited is not known precisely. However, a broader review of all callitrichine studies to date presented in by Garber et al. (2015) reveals that groups with multiparous females see minimal evidence (6.3% or 18 of 287 group-years) of both females breeding in tamarins (genera *Saguinus* and *Leontopithecus*) but a higher propensity for this (41.7% or 25 of 60 group-years) in the marmoset genus *Callithrix* (Garber et al. 2015). Due to marmoset propensities to carry multiple litters in a year, there are more breeding opportunities available to mature females in

groups, groups are themselves larger, and in several of these cases females gave birth several months apart (reviewed in Garber et al. 2015).

The danger inherent to multiple females breeding in a single group is that of one female losing its offspring to infanticide, over ten cases of which have been reported in the wild for both marmosets and tamarins together, including some involving cannibalism (Arruda et al. 2005; Bezerra et al. 2007; Ferrari and Digby 1996). Despite the risk of infanticide that could reduce GRO in groups with multiple mature females that have offspring, not all outcomes result in increased infant-care costs to the group and reduced GRO relative to the rest of the population. For example, we observed a case of allonursing of infants by a mature female *L. weddelli* who most likely lost her own infants at birth either to predation, infanticide, or other injury. This permitted a pair of twin offspring to nurse until close to six months of age, whereas they would normally be weaned around three months (full account in Watsa 2013). This pair survived for over three years in their natal group before dispersing. Allomaternal care such as this can be greatly beneficial to the survival of offspring across primate species (Fedigan and Jack 2011; Isler and van Schaik 2012; Smith et al. 2001) and occurs commonly in cooperatively breeding meerkats (*Suricata suricatta*) as well (MacLeod 2013). Multiple females breeding in a group a few months apart might also enhance GRO, if separately timed births reduce conflicts related to infant care. We observed multiple breeding females in a group of *L. weddelli* in which a pair of infants differed in age by approximately two months based on timings of tooth eruption, indicating that only one infant from each female survived. Offspring survival from both females has been observed in at least two cases in the genus *Saguinus* (Calegaro-Marques and Bicca-Marques 1995; Garber et al. 1993) and in multiple cases of *Callithrix* (Digby 1994; Digby and Ferrari 1994; Ferrari and Digby 1996; Roda 1989). We also report a case in which a group of two adult male and two adult female *L.*

weddelli raised three offspring of approximately the same age, implying that the females had produced offspring simultaneously. This group composition of adults is a common minimum among callitrichines; for example, 32% of groups assessed in the longest study of *L. weddelli* (Goldizen et al. 1996) and 66.7% of groups of *L. weddelli* assessed recently in Bolivia (Garber et al. 2015) had at least two adult males and females, although the precise developmental classes of the adults is too data deficient to be assessed.

Our data emphasize the value of long-term, individual-based field studies in which morphology can be evaluated via mark-recapture programs in evaluating overall patterns of reproductive output, a view shared by others in the field of primatology (Clutton-Brock 2012; Clutton-Brock and Sheldon 2010; Robbins 2010). Many other valuable characteristics of this study population are currently being evaluated to further inform these analyses. First, analyses of dental ecomorphology will allow us to determine more fine-tuned age-classes or even predict chronological age based on an ever expanding dataset of identified individuals. With chronological age for each individual in this study population, we can explore how reproductive status changes over an individual's lifetime, monitor for shifting population demographics in developmental status, and more carefully look for interspecific differences in development and reproductive behavior between *Saguinus* and *Leontocebus*. We can also use genetic sampling to determine paternity and relatedness to directly address the impacts of developmental class on the identities of actual biological parents in groups. Additional behavioral observations of actual infant care in this population could elaborate on the role of individuals of different developmental class on group reproductive output. This study highlights the differences in the impacts of mature adult male and female presence on group reproductive output, which allows us to further understand the composition of groups capable

of reproducing and contributing to population viability, which is an important consideration for the conservation of these primates. A recent assessment of the conservation status of the Callitrichidae revealed that of the 48 identified species, six remain data deficient and ~36% (15 species) of the remaining species are classified as threatened by the International Union for the Conservation of Nature (Estrada et al. 2017). Thus, these data will form an important benchmark for this study population against which future data can be compared in for the monitoring of long-term viability of these primates.

Electronic Supplementary Material

Supporting Information on Principal Components Analysis (Appendix 1) and the historical dataset (Appendix 2) are available online.

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

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Electronic Supplementary Materials (Appendix 1)

Table 1: Loading (L) and correlation (C) matrix for PCA

Species-Sex Class	*Morphological Variable*	*Dimension 1*		*Dimension 2*		*Dimension 3*		*Dimension 4*	
		L	C	L	C	L	C	L	C
Female *Leontocebus weddelli*	Weight	0.517	0.884	-0.307	-0.255	0.778	0.386	0.182	0.070
	Vulvar Index	0.553	0.944	-0.058	-0.048	-0.202	-0.100	-0.807	-0.310
	Suprapubic Area	0.527	0.900	-0.298	-0.247	-0.593	-0.294	0.531	0.204
	Nipple Length	0.386	0.660	0.902	0.747	0.056	0.028	0.185	0.071
Male *Leontocebus weddelli*	Weight	0.461	0.547	0.737	0.753	0.494	0.366	N/A	N/A
	Testicular Volume	0.725	0.860	0.009	0.009	-0.689	-0.510	N/A	N/A
	Suprapubic Area	0.512	0.608	-0.676	-0.690	0.530	0.393	N/A	N/A
Female *Saguinus imperator*	Weight	0.494	0.897	-0.535	-0.278	0.677	0.341	0.105	0.045
	Vulvar Index	0.510	0.927	-0.072	-0.037	-0.305	-0.154	-0.801	-0.341
	Suprapubic Area	0.503	0.913	-0.202	-0.105	-0.615	-0.310	0.573	0.244
	Nipple Length	0.492	0.893	0.817	0.425	0.265	0.133	0.140	0.060
Male *Saguinus imperator*	Weight	0.421	0.502	0.774	0.794	0.473	0.343	N/A	N/A
	Testicular Volume	0.727	0.868	0.024	0.025	-0.686	-0.497	N/A	N/A

Suprapubic Area	0.542	0.647	-0.633	-0.649	0.553	0.400	N/A	N/A

Table 2: Eigenvalues for the PCA

Species-Sex Class	*Dimension*	*Eigenvalue*	*% Variance*	*Cum. % variance*
Female *Leontocebus weddelli*	Dimension 1	2.9194	72.98	72.98
	Dimension 2	0.6865	17.16	90.15
	Dimension 3	0.2461	6.15	96.30
	Dimension 4	0.1480	3.70	100.00
Male *Leontocebus weddelli*	Dimension 1	1.4083	46.94	46.94
	Dimension 2	1.0431	34.77	81.71
	Dimension 3	0.5486	18.29	100.00
Female *Saguinus imperator*	Dimension 1	3.2948	82.37	82.37
	Dimension 2	0.2699	6.75	89.12
	Dimension 3	0.2535	6.34	95.46
	Dimension 4	0.1817	4.54	100.00
Male *Saguinus imperator*	Dimension 1	1.4227	47.42	47.42
	Dimension 2	1.0529	35.10	82.52
	Dimension 3	0.5244	17.48	100.00

Conclusion

In this dissertation I screened broadly for blood and gastrointestinal parasites from two free-ranging, sympatric nonhuman primate hosts found in the lowland Amazon rainforest of Southeast Perú. Blood and gastrointestinal parasites are fundamentally different in how they are distributed: the former acquired actively through contact with conspecifics, contaminated substrates, or through ingestion of a parasite's intermediate host, and the latter is acquired passively from haematophagus arthropod vectors (e.g. mosquitos). As the two primate hosts considered in this dissertation are closely related, exhibit a high degree of ecological overlap including diet, habitat use, social organization, and are frequently found together in polyspecific association, I expected that most parasites would be shared. In addition, the successful implementation of a primate mark-recapture program spanning multiple years allowed me to give each host a unique identity, as well as determine its sex, age class, and breeding status. Thus, beyond merely determining if parasites are shared by both host species, I explored if parasites were distributed across demographic groups in the same way.

While detailed discussions regarding the blood and gastrointestinal parasites discovered in this dissertation can be found at the end of each chapter, it is worth pointing out the overarching findings of this work. Across the entire study period, I collected blood and fecal samples from 134 animals, 83 *Leontocebus weddelli* and 46 *Saguinus imperator*, spread across 21 groups. Regarding blood parasite infections, I found that parasite prevalence varied significantly across years, demonstrating the necessity of a longitudinal sampling design to assess parasitism in natural primate populations. Despite that blood parasites are distributed by arthropod vectors that likely feed from both hosts evenly, I documented 2 potential cases of host specificity. Of the four haemoparasites identified, 1 filarial nematode was absent from *S. imperator*, and except for a single positive PCR, which could not be replicated, *Plasmodium brasilianum* was absent from *L. weddelli*. The two remaining blood parasites were identically distributed across both primate hosts, with older or mature animals more

likely to have an infection than younger and immature animals. Finally, a series of mixed-effect logistic models suggested that the inclusion of co-infection variables was always important for predicting the presence or absence of parasite infections.

My survey of gastrointestinal parasites from the same population also highlighted that parasitism varies across years and that longitudinal data was needed to determine accurate baseline levels of parasite prevalence. Unlike for blood parasites, I documented no cases of parasite-host specificity, and I observed that prevalence for a subset of trophically transmitted helminths was significantly different between hosts. This could be attributed to different rates of parasite exposure as a consequence of subtle differences in arthropod feeding behaviors or preferences. Also, while older or reproductively mature individuals were more likely to harbor blood parasites, the same trend was not observed among gastrointestinal helminths. There was even one trophically transmitted gastrointestinal helminth that was significantly more prevalent among younger and less mature individuals. Finally, these data on gastrointestinal helminths showed no significant associations of parasite co-occurrence.

As a result of this dissertation a number of new avenues of research are possible, some of which are already in progress. First, there are nine other sympatric primate hosts on site from which fecal samples have been collected and are being examined for parasites by both microscopy and DNA metabarcoding techniques. Second, an additional primate host, *Callicebus brunneus*, or the dusky titi monkey, has been incorporated into the annual mark-recapture program, and hence, its blood can be screened for similar filarial, trypanosome, and *Plasmodium* parasites. Third, I can now combine data on blood and gastrointestinal helminth infections to assess within-host species interactions that can only be mediated by the host immune response, since these parasites occur in different tissues. Also with respect to co-infection, it would be beneficial to re-examine these samples and estimate levels of parasitemia, instead of relying on only presence or absence data. Four, I am eager to pursue new

dental cast imaging methods that would allow me to differentiate adult animals into finer classes (i.e. young, middle, and old adults) based on tooth wear. This would likely disassociate age and breeding status further and enable the construction of more accurate models to explain variation in individual infection status.

This work would not have been possible without the guidance and support of a number of people, in particular my dissertation advisor, Dr. Patricia Parker, and my primary research collaborator in Peru, Dr. Mrinalini Watsa. It also would not have been possible without the assistance of several agencies and organizations including Field Projects International, the Amazon Conservation Association, the Whitney R. Harris World Ecology Center, and the Centro de Ecología y Biodiversidad. This work was conducted in full compliance with the IACUC of the Unversity of Missouri-St. Louis and the Servicio Nacional Forestal y de Fauna Silvestre of Perú.