Parasites & Pundamilia: testing the role of parasites in host speciation

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Summary

The relationship between parasites and their hosts is highly intimate and can result in strong evolutionary interactions. Parasites are increasingly recognized as potential drivers of host speciation. Due to their extreme diversification and rapid adaptive radiation cichlid fish form a good model system for studying mechanisms of speciation. The closely related *Pundamilia pundamilia* and *Pundamilia nyererei* inhabit the Mwanza gulf of Lake Victoria, Tanzania. They live along the islands of Luanso, Kissenda, Python and Makobe, where their ecological and genetic divergence increases from Luanso to Makobe. In this study, I aimed to investigate if parasites have contributed to the divergence in their hosts *P. pundamilia* and *P. nyererei*. If this would be the case, I expected differentiated parasite infection profiles in *P. pundamilia* and *P. nyererei*. Moreover, I expected this difference to increase as the genetic divergence of the hosts increased. This difference in infection should precede the genetic divergence. However, I found no such increase in differences in parasite abundances, for none of the observed parasites. Moreover, comparison of parasite abundances with previous years showed that infections are not stable over time, indicating that selection pressure changes over the years. Therefore, this study suggests that parasites have not contributed to speciation in *P. pundamilia* and *P. nyererei*.

Introduction

The relationship between parasites and their hosts is highly intimate and can result in strong evolutionary interactions (Decaestecker *et al.*, 2013; Lively & Dybdahl, 2000). Parasites are increasingly recognized as potential drivers of host speciation (Thompson, 1999), between allopatric as well as sympatric populations (Karvonen & Seehausen, 2012). In a heterogeneous environment, subpopulations can, by the use of different spatial niches or food sources, experience differences in the nature and magnitude of parasite infections (Karvonen & Seehausen, 2012). Adaptation to locally abundant parasites can promote reproductive isolation when individuals are less adapted to parasites occurring outside their niche and therefore acquire a lower fitness in these other niches. Chances of individuals surviving and reproducing outside their own niche could be reduced. Thus, individuals mate more within their niche and could therefore be reproductively isolated from individuals in other niches. Similarly, hybrids can show lower resistance against parasites in either of the parental niches and therefore gain a lower fitness than non-hybrids. Thus, mating with individuals from other niches would be disadvantageous.

This reproductive isolation could be accelerated by the effects of parasites on mate choice, when parasites do not only put a direct fitness cost on males, but also serve as an honest signal for fitness (Hamilton & Zuk, 1982). For example, when brightly coloured males attract more females than dull coloured individuals, and at the same time harbour fewer parasites (Maan *et al.*, 2008). Brightly coloured males will thus obtain more offspring. Assuming that variation in parasite infection is due to genetic variation in resistance, their offspring would in their turn be well-adapted to parasites. Female preferences for the sexual signal of colour can thus promote selection for resistance against parasites. Furthermore, parasites target aspects of the host immune system that influence mate choice, the Major Histocompatibility Complex (MHC) (Milinski, 2006). Females are attracted by males with a certain MHC complex, which could be identical (Eizaguirre *et al.*, 2010) or distinct (Landry *et al.*, 2001) from their own. Either way, females select males for mating on basis of their MHC complex, which is influenced by parasites. In this way, parasites could indirectly increase reproductive isolation.

Cichlids as a model system

Due to their extreme diversification cichlid fish form a good model system for studying mechanisms of speciation (Kocher, 2004). Previous studies, in particular in Lake Tanganyika, have shown that cichlid fish are hosts to a great variety of parasites (Paperna, 1996; Kmentová *et al.*, 2016; Pariselle *et al.*, 2015; Gregoir *et al.*, 2014). Moreover, some of these parasites are host-specific and species and abundances differ between closely related cichlid species and even between allopatric populations of the same species (Vanhove *et al.*, 2015; Raeymaekers *et al.,* 2013). Furthermore, the infections remain stable over time (Raeymaekers

et al., 2013). Since reproductive isolation is derived when populations experience different selection pressures that are maintaining the same direction for each population, these two conditions – differences in parasite infections among populations and stability of infections – are prerequisites for parasite-mediated speciation (Karvonen & Seehausen, 2012). Moreover, observations by Taylor *et al.* (1998) suggest that parasites affect the expression of sexually selected characters in bower-building cichlids in lake Malawi. Thus, these examples indicate the relevance of cichlids as a model system for studying parasite-mediated speciation.

Study objectives

The haplochromine cichlids used in this study - the closely related species *Pundamilia pundamilia* (Fig. 1a) and *Pundamilia nyererei* (Fig. 1b) - are rockdwelling mouthbrooders endemic to Lake Victoria (Seehausen *et al.*, 1998). Within the Mwanza Gulf in Tanzania, they live along a gradient of water transparency, from the turbid waters near the island of Luanso in the south to the clearer waters around Makobe in the north (Fig. 1c). The two species have a different depth distribution, where *P. pundamilia* is most abundant in the upper water layer and *P. nyererei* occupies the deeper water layer (Seehausen & Bouton, 1997; Seehausen *et al.* 2008). At Makobe, where the water transparency is relatively high (250 cm), *P. pundamilia* males display a metallic blue colour, whereas *P. nyererei* males display a bright red and yellow colour. Here, the phenotypic and genetic differentiation between the species is high and they are reproductively isolated (Seehausen *et al.* 1997, 2008). Females of both species have species-assortative mate preferences, choosing males with their species-specific coloration (Seehausen *et al.*, 2008). In turbid areas, such as Luanso (50 cm), in addition to the blue and red phenotype, males of intermediate phenotype occur (Seehausen, 1997). Here, because most females lack preferences (van der Sluijs *et al.*, 2008) the species still interbreed and are therefore not reproductively isolated. Along the transect from turbid to clear water (Luanso – Kissenda – Python – Makobe), the two species show increasing phenotypic and genetic differentiation. Therefore, this system is suited to study divergence along the entire continuum of speciation.

Figure 1 | a) Pundamilia pundamilia. **b)** Pundamilia nyererei. **c)** *Location of the islands in the Mwanza Gulf where fish were caught for this study (excluding Marumbi). Water transparencies are given with the Secchi disk reading, and give the centimetres of transparency in the waters. FromMaan & Seehausen (2010).*

At Makobe, where the *Pundamilia's* are most diverged (Seehausen *et al.*, 2008), a difference in parasite infection patterns has been observed between the two species, with *P. pundamilia* having significantly higher loads of intestinal nematode larvae but *P. nyererei* having significantly more copepods in the gills (Maan *et al.*, 2008). This difference in parasite infections implies that they differ in aspects of immune defence or parasite exposure, which can be related to differences in habitat and diet. *P. pundamilia* primarily feeds on benthic insect larvae whereas *P. nyererei* feeds on zooplankton (Bouton *et al.*, 1997). Moreover, at Kissenda, where species are slightly less diverged, the same difference in copepod abundance has been found, but to a lesser extent (Desêtres, 2010). This indicates that differences in parasite infections might co-vary with phenotypic and genetic host divergence. In order to understand where in the speciation process the parasite assemblages become sufficiently divergent to reduce gene flow between host populations, it is important to study parasite infections along the entire continuum of host speciation (Karvonen & Seehausen, 2012). Therefore Karvonen *et al.* (in prep.) have analysed the parasite load in the gills of *P. pundamilia* and *P. nyererei* along the Mwanza gulf, at the islands Luanso, Kissenda, Python, and Makobe, where they display an increasing divergence. They found a positive correlation between genetic host divergence (F_{st}) and differences in parasite infection between the two species. However, their sample size only amounts to approximately 10 individuals per species per island. Hence, my first objective is to expand on their study by increasing their sample size, and add an intermediate phenotype at Luanso. Secondly, I will compare my data with data from 3 previous studies. This way I will check for temporal stability of the infections, which is considered a prerequisite for parasite-mediated selection (Karvonen & Seehausen, 2012). I will focus on the gills only, since this body part has been used in previous studies as well (Karvonen, unpublished data; Maan *et al.*, 2008; Desetres, 2010). Moreover, the parasite numbers in the gills are a significant proportion of the whole macroparasite community in the fish (Maan *et al.*, 2008).

If parasites have contributed to the divergence of the *Pundamilia* species, I expect different parasite infection profiles in *P. pundamilia* and *P. nyererei*, at all islands. Moreover, I expect the difference in parasite composition to increase as the divergence between the hosts increases (Fig. 2). To make sure the difference in parasite assemblages is not a consequence of the genetic differentiation between the hosts, but a cause, this difference should precede the genetic host differentiation. That implies that infection rates should already differ at Luanso, where the *Pundamilia* species are genetically not yet diverged, and this difference

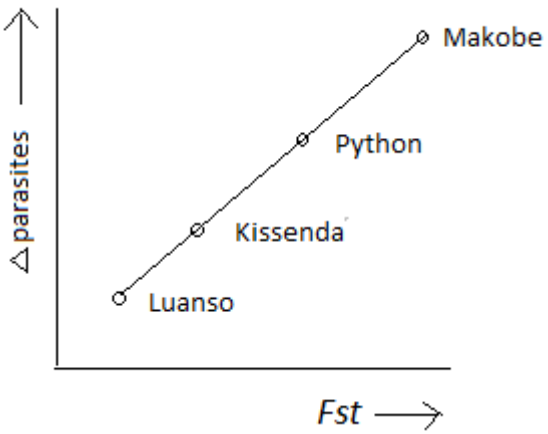


Figure 2 | *Hypothesis. As the divergence between the host increases (higher* Fst*), the differences in parasite abundances should increases as well.*

should increase towards Makobe. Finally, if hybrids are indeed less well adapted to parasites and a genetic origin is assumed, I would expect the intermediate phenotype at Luanso to carry more parasites than *P. pundamilia* and *P. nyererei* at this island.

Material & Methods

Species sampling

Between July and November of 2014 adult males of *P. pundamilia*, *P. nyererei* and intermediates (only at Luanso) were caught at the islands Luanso, Kissenda, Python and Makobe in the Mwanza Gulf of Lake Victoria, Tanzania. The number of fish caught and used for this study are shown in Table 1. Since the individuals at Luanso all interbreed, there is no clear division between *P. pundamilia* and *P. nyererei* here, but they can better be considered as a 'blue', 'red' and 'intermediate' phenotype. Fish at Luanso were assigned a phenotype according to their colour, which was recorded on pictures taken of the individuals right after they had been sacrificed. Each individual got assigned a colour score, 0 for blue (typical *P. pundamilia*), 4 for red (typical *P. nyererei*) and 1, 2 and 3 for the phenotypes in between (Seehausen *et al.,* 2008). Their final colour score was the average of the assigned scores of 5 different *Pundamilia* experts. Of all the 33 individuals caught at Luanso, the highest scores were considered the red phenotype, the lowest the blue phenotype, and the scores in between the intermediate phenotypes.

Table 1 | *The number of fish caught per island.*

Island	*P. nyererei*	*P. pundamilia*	*P. intermediate*
Makobe	41	27	
Kissenda	23	21	
Python	27	30	
Luanso	10	12	11

Fish were caught by angling or gillnets at depths between 0.75 and 18 metres. They were sacrificed on ice immediately after capture. Subsequently the fish were preserved in 4% formalin and later transferred to 70% ethanol. To avoid dehydration of the tissue, transfer to ethanol was done in steps of increasing ethanol concentration. During sampling, capture depth of the fish was recorded.

Gill removal

The standard length (SL, to the nearest 0.1 mm), body depth (BD, to the nearest 0.1 mm) and weight of the fish with both gills (to the nearest 0.1 or 0.01 gram) was measured, from which the condition factor (CF, $100*(weight/(SL/10)^3)$) was calculated. The 4 arches of the right gill were removed one by one with a scalpel (with surgical blades n.10) and placed on a Petri dish (small, diameter 5.5 cm) with 70% EtOH. To identify the 1st (most lateral), 2nd, 3rd and 4th

(most medial) gill arch for later analysis, the Petri dish was placed under a dissection stereoscope (60x) and a small cut with the scalpel was incised on the gill limb at the ventral end of every arch (1st arch = 1 cut, 2nd arch = 2 cuts, 3rd arch = 3 cuts, 4th arch = 0 cuts).

Parasite screening

The species of macroparasites encountered in this study were *Cichlidogyrus* spp., *Lamproglena monodi, Ergasilus lamellifer* and a mollusc larva known as glochidium. *Cichlidogyrus* is a small (0.3-2 mm) flatworm with a ventral attachment organ and two pairs of dorsal, pigmented eyes (Fig. 3a). Copepods *Lamproglena monodi* (3-4 mm) and *Ergasilus lamellifer* (0.8-1 mm) are more easily detectable and can even be seen with the naked eye. They can both possess a chain of clustered eggs attached to the ventral part of their body (Fig. 3b & c). Glochidia are mollusc larvae nested mostly in the end parts of the gill lamellae (Fig. 3d).

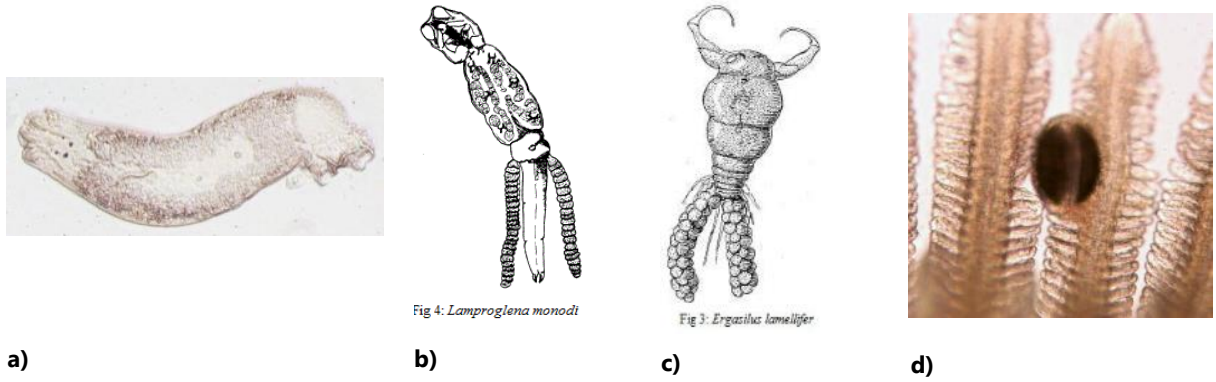


Figure 3 | *Gill parasites encountered in the* Pundamilias. **a)** Cichlidogyrus *spp*. **b)** Lamproglena monodi. **c)** Ergasilus lamellifer. **d)** *Glochidia (from Paperna, 1996)*

The gill arches were screened under a dissection stereoscope (120-250x) ventrally to dorsally by holding the gill limb with a pair of tweezers (Forceps Dumont #5/45, FST cat.nr. 11251-35) and moving the gill filaments with a pin holder (17 cm, FST cat.nr. 26018-17) with a pin (5 cm, FST cat.nr. 26007-02). When a parasite was found, the species name, gill arch number and location (dorsal/median/ventral and distal/central/proximal) on the arch was noted (Fig. 4). For *Lamproglena monodi* and *Ergasilus lamellifer* the occurrence of eggs was noted as well. Although this information was not used in this study, it will be analysed in upcoming research.

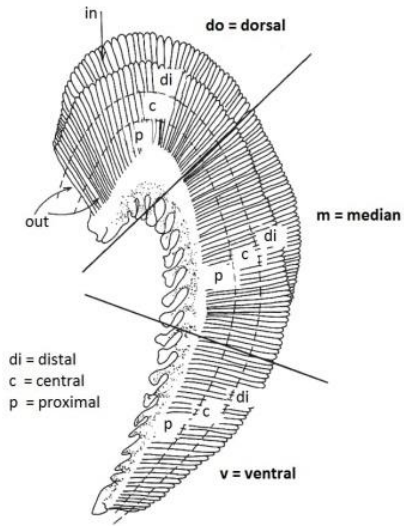


Figure 4| *Parasite location names on a gill arch*

Parasites were not removed from the gills, except for *Cichlidogyrus*, which was removed in order to be determined up to species level in later research. *Cichlidogyrus* was removed with a pair of tweezers (Forceps Dumont #5, FST cat.nr.11251-30) and put in a vial (0.75 ml) filled with 70% EtOH to preserve it. After screening of the 4 arches, the gills were put in a 15 ml vial with 70% EtOH to be preserved.

Statistical analysis

To test whether there were differences in parasite infections between *P. pundamilia* and *P. nyererei* at the 4 islands, I fitted models for each parasite. Since this analysis is hard to perform with conventional statistics, the Bayesian method was used.

Statistical analysis was performed in Rstudio version 1.0.136 requiring R version 3.4.0 (R Core Team, 2014), using Bayesian statistics with packages 'rStan' version 2.15.1 and 'rethinking' version 1.59. The response variables in the dataset were counts and contained high numbers of zero's. Therefore, zero-inflated models were used. These models exist of two parts, a binomial model and a count model. The binomial model calculates the chance that a zero is a false zero, which means that there actually is a parasite, which has not been observed. The count model concerns the raw count data and has a Poisson distribution. The models were run with the Markov Chain Monte Carlo (MCMC) method using the function map2stan(). To compare infection patterns of the species per island the data was divided in 9 populations, where each species at each island is a population, plus the intermediate phenotype at Luanso. For each parasite a model without intercept was fitted. The coefficients of the populations were measured, so that they could be compared to each other. To make sure the outcome of the analysis was not predetermined by the model, priors for the population coefficients were chosen to be flat and normally distributed with a mean of 0 and a standard deviation of 2. These numbers were chosen since the real number of parasites are expected to be approximately in this range. Since the count model has a Poisson distribution, one should take the logarithm of the expected number of parasites to get to the preferred prior numbers. Priors for the binomial part of the model were normally distributed too, with a mean of 0 and standard deviation of 1. The models included SL (Standard Length), which was standardized per species, and depth, to see if these were better predictors of parasite abundance than species identity. However, since depth is an ecological feature of the species it was taken out when analyzing differences in parasite abundances between species. Priors for SL and depth were also normally distributed with a mean of 0 and a standard deviation of 2. Differences in parasite abundances between species within islands were calculated by creating difference posteriors for each island by subtracting the posterior of *P. nyererei* from the posterior of *P. pundamilia,* meaning that with a difference above zero, *P. pundamilia* harbors more parasites, while with a difference below zero, *P. nyererei* carried more parasites. When the 89% confidence interval of the difference does not cross zero, this can be considered a significant difference in parasite infection between the species.

To see if parasite abundances remain stable over time I compared my dataset with data from Makobe in 2003 (Maan *et al.,* 2008), Kissenda in 2005 (Desêtres, 2010), and all the 4 islands in 2010 (Karvonen *et al.*, in prep.). Again Bayesian zero-inflated models were used. For both species, in both years, the mean abundance of parasites was measured. When the 89% confidence intervals of the means do not overlap, this can be considered a significant difference in parasite infection between the populations.

Finally, I performed Spearman-Rank correlations between parasite species to test whether they can be associated with one another.

Results

Comparison between species within islands

For each island and each parasite the difference between *P. pundamilia* and *P. nyererei* estimated by the Bayesian models are shown in Fig. 5. For descriptive statistics see table A1 in the Appendix. Parasite abundances per population are shown in figure A1 in the Appendix.

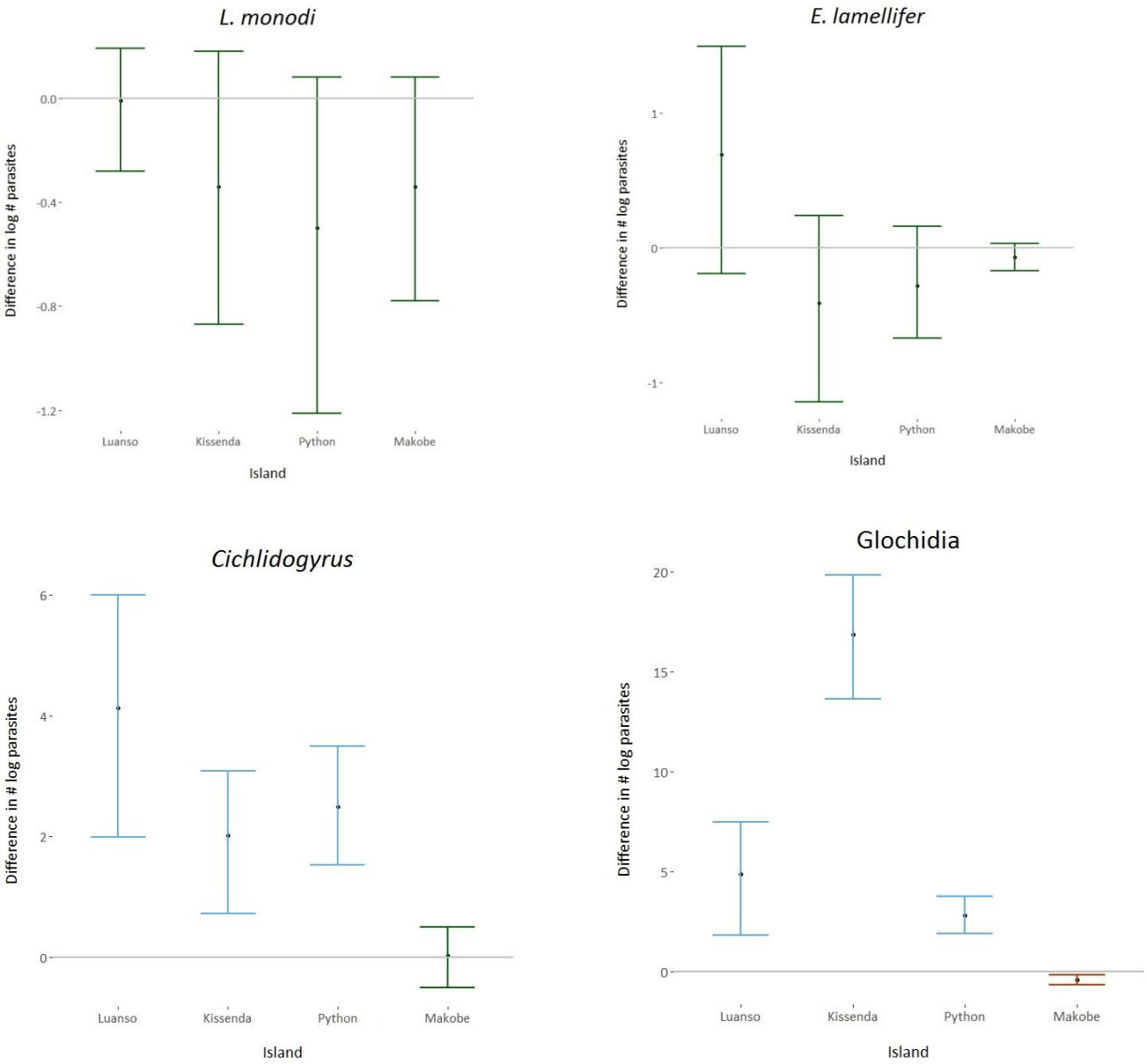


Figure 5 | *The mean and standard deviation of the difference in parasites between* Pundamilia pundamilia *and* Pundamilia nyererei*. The difference is shown as the log of the number of parasites. When* P. pundamilia *harbors more parasites, the results are shown in blue, when* P. nyererei *has more parasites the results are shown in red. When error bars cross the zero-line, differences between the species are not significant, and results are shown in green. From left to right the islands are shown in order of increasing host divergence.*

The Bayesian models show that no significant differences in *L. monodi* and *E. lamellifer* abundance between *P. pundamilia* and *P. nyererei* were found. Although differences in both copepods were not significant, *P. nyererei* was on average more heavily infected than *P. pundamilia*, with exception of *E. lamellifer* infection at Luanso, where *P. pundamilia* was slightly more infected (table A1). For both copepods, infection differences did not increase from Luanso to Makobe. Interestingly, differences in *E. lamellifer* abundance decreased as the host divergence increased.

For *Cichlidogyrus* differences were found at Luanso (mean ± SD of log-transformed differences: 3.74 ± 1.09), Python (2.32 ± 0.57) and Kissenda (2.03 ± 0.08), where *P. pundamilia* was infected more heavily. However, differences in abundance did not increase from Luanso towards Makobe. Contrarily, differences were smallest and insignificant at Makobe (-0.16 ± 0.34).

The largest difference in Glochidia infection was found at Kissenda (17.70 ± 2.08), followed by Luanso (5.41 ± 1.79) and Python (2.55 ± 0.59). At all these three islands, *P. pundamilia* was more heavily infected than *P. nyererei*. At Makobe the differences was smallest (-0.42 ± 0.15), were *P. nyererei* had slightly more parasites than *P. pundamilia*.

At Luanso, not only differences between *P. pundamilia* and *P. nyererei* were measured, but also their difference to an intermediate phenotype. These differences can be found in table A2 in the Appendix. For both the copepod abundances, *P. intermediate* did not differ from *P. pundamilia* or *P. nyererei*. For *Cichlidogyrus* however, the infection pattern of *P. intermediate* resembled *P. pundamilia* and differed from *P. nyererei* (-3.60 ± 1.24). Contrarily, for Glochidia *P. intermediate* was similarly infected as *P. nyererei*, and differed from *P. pundamilia* (4.18 ± 1.69).

Overall, *P. nyererei* was slightly more infected with copepods, although these differences were not significant, whereas *P. pundamilia* carried more *Cichlidogyrus* and Glochidia, although not at Makobe. However, differences were not consistent, meaning that none of the parasites was always higher in abundances in one of the two species and the extent of differentiation in parasite load between *P. pundamilia* and *P. nyererei* was not consistent across parasites (e.g. large differences in infection within *Cichlidogyrus* coincided with small species differences in infection with *L. monodi*) and across islands (e.g. *P. pundamilia* had higher Glochidia infection rates than *P. nyererei* at Luanso, Kissenda and Python, but *P. nyrerei* had higher Glochidia abundances at Makobe). Moreover, differences in parasite abundance have not increased towards increasing host divergence. On the contrary: differences were mostly smallest at Makobe.

Length and Depth

In the models I included depth and Standard Length (SL), to see if they are a better predictor of parasite abundance than species identity. These effects were measured in the complete sample of individuals, meaning both species at all 4 islands, plus intermediates at Luanso. Results are shown in table 2. A significant correlation between depth and parasite abundance was found for *L. monodi*, *Cichlidogyrus* and Glochidia, where deeper living fish carried fewer *Cichlidogyrus* (Mean ± SD of effect sizes: -0.064 ± 0.032) and more *L. monodi* (0.063 ± 0.020) and Glochidia (0.063 ± 0.021). Furthermore, larger fish (SL) obtained more *Cichlidogyrus* (0.020 ± 0.004), *L. monodi* (0.027 ± 0.003) and Glochidia (0.027 ± 0.004). For all parasites the mean effect of depth on parasite abundance was higher than the mean effect of SL (Table 2), suggesting that depth was a stronger predictor of parasite abundance than SL. We don't know the direction of causality of SL or depth on *E. lamellifer* abundance.

Table 2 | *Effect sizes of SL and Depth, both standardized. When the difference between the lower 89% and upper 89% does not cross zero, this is interpreted as a significant effect of this predictor, here printed in bold.*

Response variable	SL				Depth			
	Mean	Standard Deviation	Lower 89%	Upper 89%	Mean	Standard Deviation	Lower 89%	Upper 89%
L. monodi	0.027	0.003	**0.022**	**0.032**	0.063	0.020	**0.031**	**0.095**
E. lamellifer	0.000	0.007	-0.011	0.013	0.053	0.064	-0.041	0.159
Cichlidogyrus	0.020	0.004	**0.013**	**0.026**	-0.064	0.032	**-0.117**	**-0.015**
Glochidia	0.027	0.004	**0.022**	**0.034**	0.063	0.021	**0.029**	**0.095**

To see the effects of depth and SL per population, I correlated these predictors with parasite abundance for every population separately. Results are shown in the Appendix. The effect of depth on parasite abundance varies per population and per parasite species (Figures A3-A6). For example, deeper living *P. pundamilia* at Python carried fewer *L. monodi* (R = -0.463, p = 0.012), whereas deeper living *P. nyererei* at the same island carried more *L. monodi* (R = 0.383, p = 0.048). However, altogether, parasite abundance in *P. nyererei* seem to be less affected by depth than parasite abundances in *P. pundamilia* (Table A3). Although in almost all populations larger fish carried more parasites, the strength of the effect of SL on parasite abundance varies per population (Figures A9-A12) and was mostly not significant (Table A4). The strongest correlations were found at Python, where bigger *P. pundamilia* carried more Glochidia (R = 0.615, p < 0.001) and bigger *P. nyererei* had more *Cichlidogyrus* (R = 0.572, p = 0.002).

Parasite correlations

Finally, I also tested for correlations between parasite species. The results are shown in figure A13 and table A5. Fish that carried more *Cichlidogyrus* also carried more Glochidia (R = 0.277, p < 0.001) and *E. lamellifer* (R = 0.270, p < 0.001), but fewer *L. monodi* (R = -0.151, p = 0.032). Moreover, a higher abundance of Glochidia was correlated with a higher abundance of *E. lamellifer* (R = 0.182, p = 0.009). No correlation between Glochidia and *L. monodi* or the two copepods was found. Graphs of the correlations can be found in figure A13 in the Appendix.

Comparison with previous years

Figure 6 shows the comparison of data from this study of *L. monodi*, *E. lamellifer* and *Cichlidogyrus* with data from 2003 at Makobe (Maan et al., 2008). Figure 7 shows the comparison of these 3 parasites with data from 2005 at Kissenda (Desêtres, 2010). Figure 8 untill 11 show the comparison of these 3 parasites with data from 2010 at all islands (Karvonen et al., in prep.). Because of missing data about Glochidia abundances in the aforementioned studies this species has not been compared with previous years.

Comparison with 2003 at Makobe

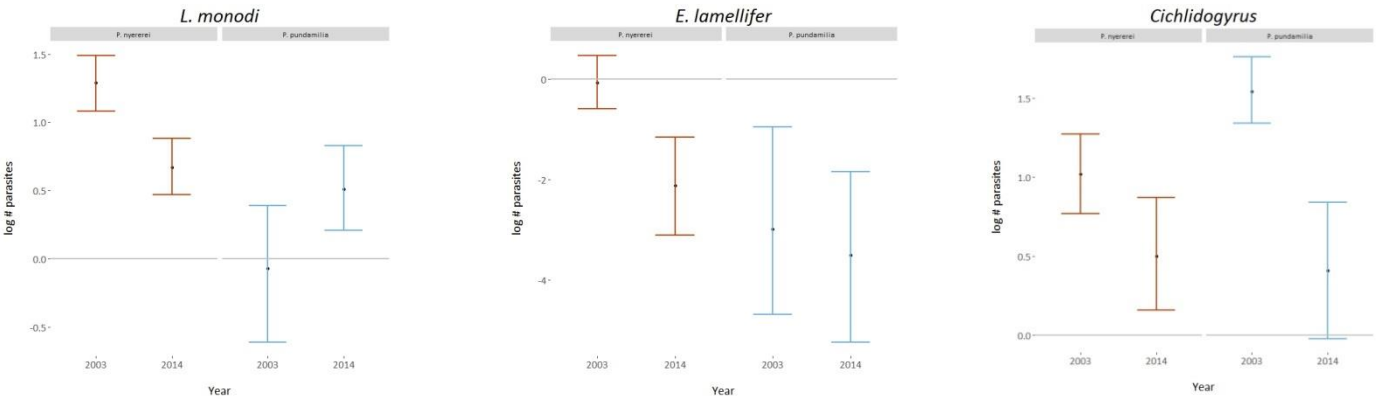


Figure 6 | *The mean and standard deviation of the log of the number of L. monodi, E. lamellifer and* Cichlidogyrus *at Makobe in 2003and 2014.* Pundamilia pundamilia *is shown in blue,* Pundamilia nyererei *in red. When error bars of populations do not overlap, they can be considered significantly different.*

For both *L. monodi* and *E. lamellifer* differences between *P. nyererei* and *P. pundamilia* decreased in 2014. Infection rates in *P. pundamilia* did not significantly change, whereas they declined in *P. nyererei* (Mean ± SD of log-transformed differences: 2.38 ± 0.58), thereby eliminating a significant difference between the species. For *Cichlidogyrus* infection rates were lower for *P. pundamilia* in 2014 than in 2003 (3.12 ± 0.75), whereas for *P. nyererei* there was not a significant difference between 2014 and 2003. However, the direction of the difference did not change in 2014, *P. nyererei* still carries more copepods and less *Cichlidogyrus* than *P. pundamilia*, although differences are not significant anymore.

Comparison with 2005 at Kissenda

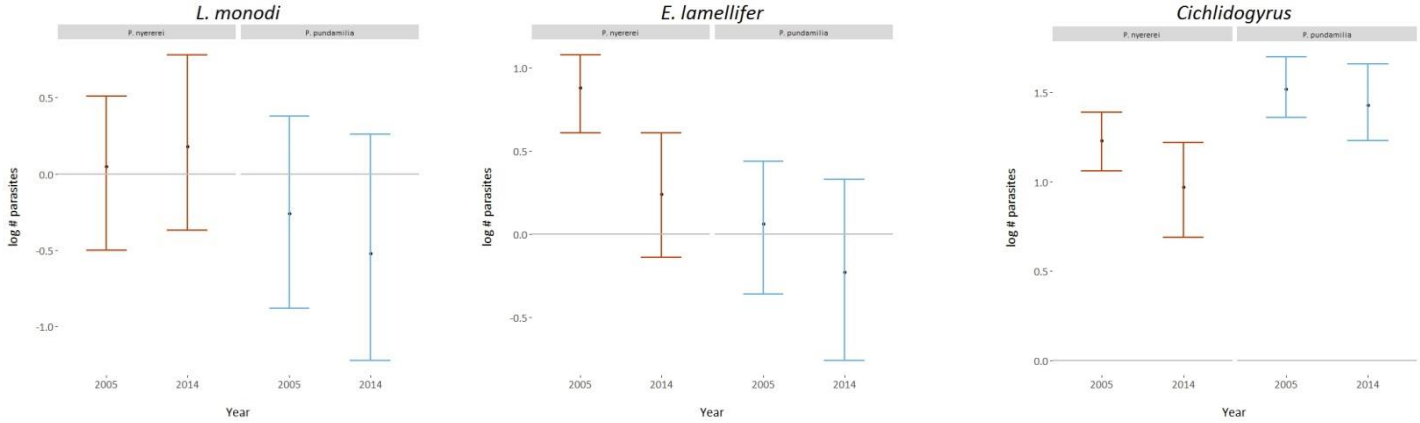
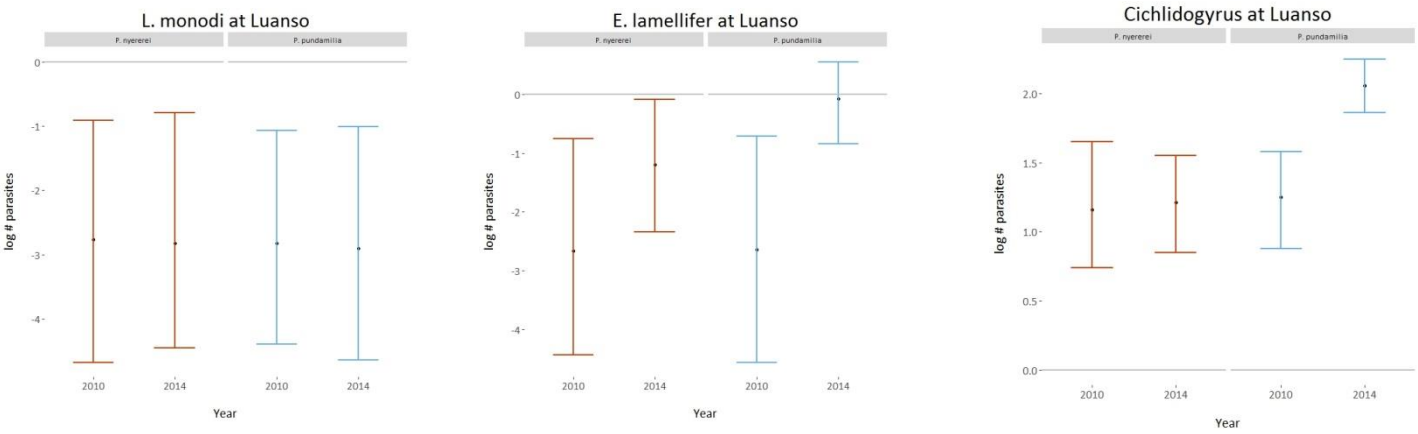


Figure 7 | *The mean and standard deviation of the log of the number of L. monodi, E. lamellifer and* Cichlidogyrus *at Kissenda in 2005 and 2014.* Pundamilia pundamilia *is shown in blue,* Pundamilia nyererei *in red. When error bars of populations do not overlap, they can be considered significantly different.*

For *L. monodi* infection rates in 2014 did not differ significantly from 2003. In both years there was a low infection rate and no difference between the species. For *E. lamellifer* abundances were already low in *P. pundamilia* in 2003, and stayed low in 2014, but decreased in *P. nyererei* (0.96 ± 0.56) in 2014. Therefore, the difference between the species decreased as well. For *Cichlidogyrus* however, infection rates decreased slightly, although not significant, in both species. However, the direction of the difference did not change in 2014, *P. nyererei* still carries more copepods and less *Cichlidogyrus* than *P. pundamilia*, although the difference is not significant.

Comparison with 2010 at all islands

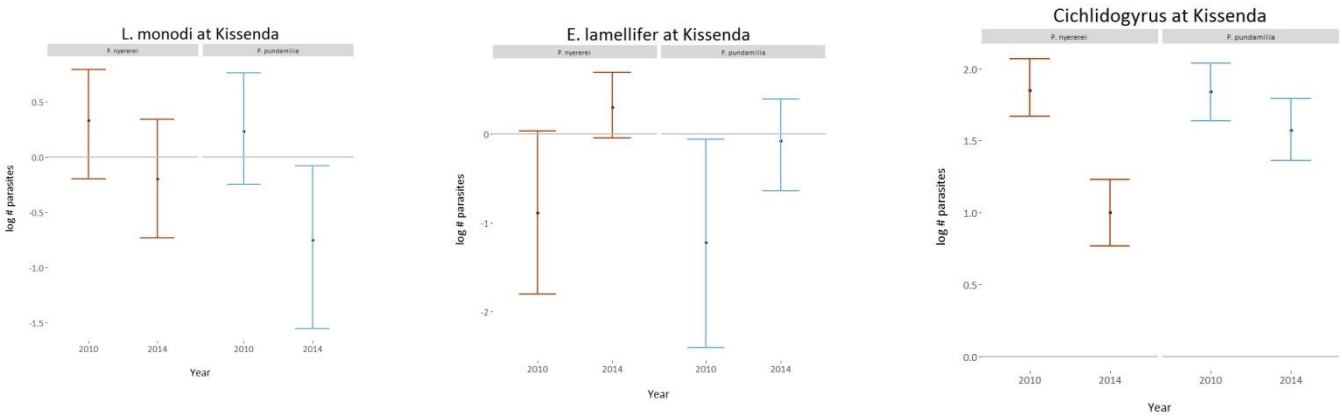
Luanso



Figuur 8 | *The mean and standard deviation of the log of the number of L. monodi, E. lamellifer and* Cichlidogyrus *at Luanso in 2010 and 2014.* Pundamilia pundamilia *is shown in blue,* Pundamilia nyererei *in red. When error bars of populations do not overlap, they can be considered significantly different.*

Infection rates of *L. monodi* stayed the same at Luanso in 2014, while they increased slightly, although not significantly for *E. lamellifer*. For *Cichlidogyrus* infection rates increased for *P. pundamilia* in 2014. Therefore, in contrast to 2003, there was a significant difference in *Cichlidogyrus* abundance in 2014, with *P. pundamilia* carrying more parasites than *P. nyrerei*.

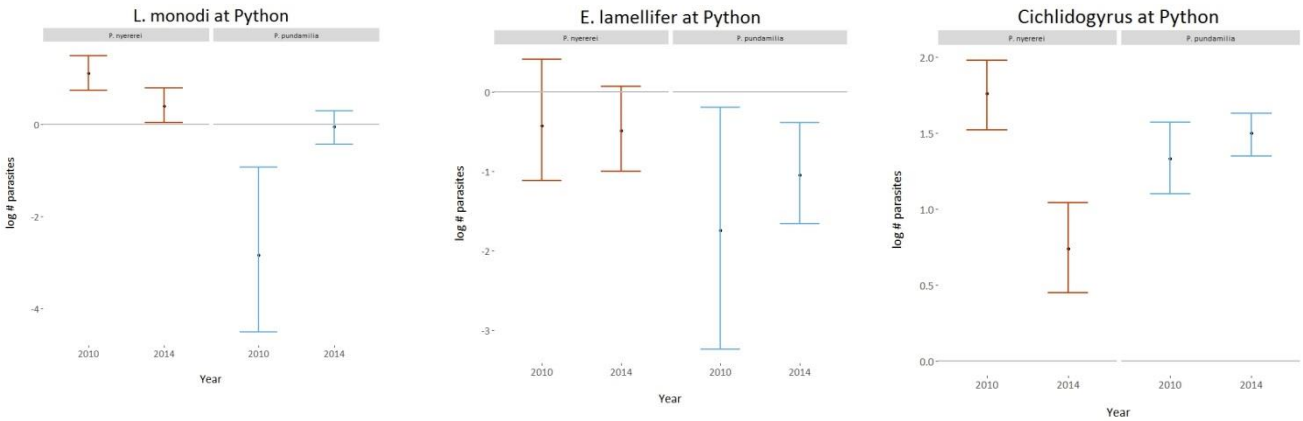
Kissenda



Figuur 9 | *The mean and standard deviation of the log of the number of L. monodi, E. lamellifer and* Cichlidogyrus *at Kissenda in 2010 and 2014.* Pundamilia pundamilia *is shown in blue,* Pundamilia nyererei *in red. When error bars of populations do not overlap, they can be considered significantly different.*

For both *P. pundamilia* and *P. nyererei* infection rates of *L. monodi* and *Cichlidogyrus* were lower in 2014 than in 2003, whereas they were higher for *E. lamellifer*. However, the only significant difference between 2010 and 2014 is the *Cichlidogyrus* abundance for *P. nyererei*.

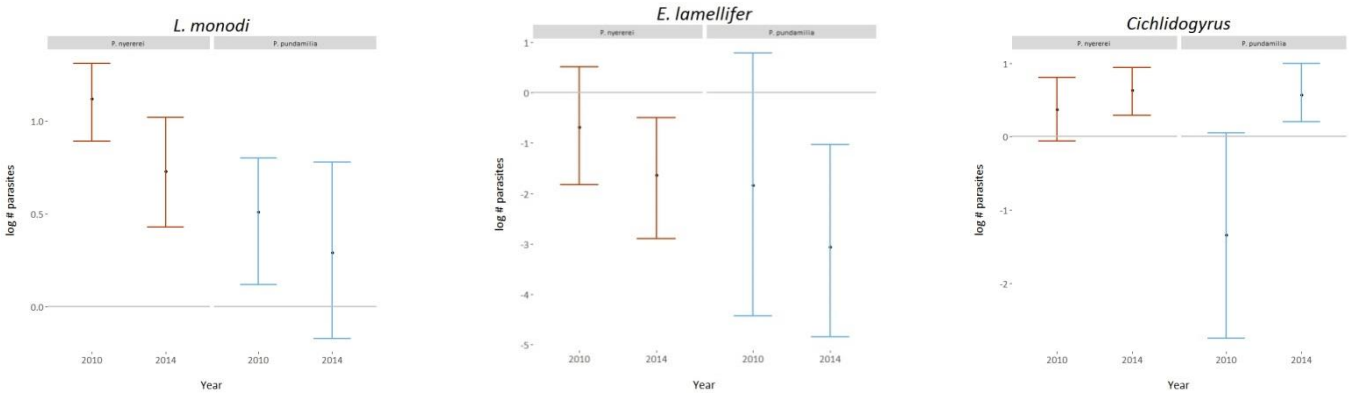
Python



Figuur 10 | *The mean and standard deviation of the log of the number of L. monodi, E. lamellifer and* Cichlidogyrus *atPython in 2010 and 2014.* Pundamilia pundamilia *is shown in blue,* Pundamilia nyererei *in red. When error bars of*

At Python, *L. monodi* and *Cichlidogyrus* abundances increased for *P. pundamilia* whereas they decreased for *P. nyererei*. No significant difference between the years was observed for *E. lamellifer*. Therefore, differences in parasite abundances that were observed in 2010 for *L. monodi* and *Cichlidogyrus*, did not exist anymore in 2014.

Makobe



Figuur 11 | *The mean and standard deviation of the log of the number of L. monodi, E. lamellifer and* Cichlidogyrus *at Makobe in 2010 and 2014.* Pundamilia pundamilia *is shown in blue,* Pundamilia nyererei *in red. When error bars of*

At Makobe there was a slight, albeit nonsignificant, decrease in copepod infection rates in 2014, whereas *Cichlidogyrus* abundances increased in both species. Differences in parasite abundances between the species did not change significantly from 2010 to 2014.

Even though these results show some significant changes in parasite abundance between 2014 and previous years, in most populations parasite infections did not change significantly. Moreover, the observed increases and decreases are not consistent, meaning that for none of the parasites a trend in increase or decrease of abundance in 2014 was observed. Neither in one of the *Pundamilia* species was there a trend in increase or decrease of infection rates from 2003 to 2014.

Discussion

Comparison between species within islands

In this study I aimed to investigate if parasites have contributed to the divergence of their hosts *P. pundamilia* and *P. nyererei*. If this would be the case, I expected different parasite infection profiles for *P. pundamilia* and *P. nyererei* at all islands. Moreover, I expected an increase in the difference in parasite infection as the genetic divergence of the hosts increased. This difference in parasite infection should precede the genetic divergence. In other words, at Luanso, where hosts are ecologically but not genetically diverged, there should be a minor difference in parasite infection, and this difference should increase towards Kissenda, Python and Makobe. However, for none of the parasites, I found such a pattern.

Maan *et al.* (2008) found differences in parasite infection between *P. pundamilia* and *P. nyererei* at Makobe, where *P. nyererei* was infected more heavily by copepods *L. monodi* and *E. lamellifer*, and *P. pundamilia* carried more nematode larvae *Contracaecum.* Moreover, Desêtres (2010) studied gill parasite abundances of *P. pundamilia* and *P. nyererei* at Kissenda, and found a higher infection of *E. lamellifer* in *P. nyererei*. Maan *et al.* (2008) suggested that this difference in parasite infection could be due to a difference in immune defense and/or exposure, which in turn could be related to a difference in habitat or diet, as *P. nyererei* feeds more on zooplankton in deeper waters and *P. pundamilia* primarily feeds on benthic insect larvae in shallower waters (Bouton, Seehausen & van Alphen, 1997). Therefore, *P. nyererei* could have obtained more copepods, which occur in the deep water during their free-living larval stage, whilst trying to get hold onto a host (Paperna, 1996). Although results in this study were not significant, there was indeed a trend of higher infection rates of copepods in *P. nyererei* than in *P. pundamilia*, with exception of *E. lamellifer* at Luanso, where *P. pundamilia* carried slightly albeit non-significantly more *E. lamellifer*.

In contrast to the copepods, significant differences in parasite abundance between *P. pundamilia* and *P. nyererei* were found for *Cichlidogyrus* and Glochidia. However, contradictory to my hypothesis, these differences were absent or minimal at Makobe. Thus, differences were smallest where host divergence was highest. Potentially, parasite abundances at Makobe could be lower in general, and detecting differences between such small numbers of parasites would be less likely.

At Luanso, I expected that *P. intermediate* would be less adapted to parasites than *P. pundamilia* and *P. nyererei* and would carry more parasites. However, *P. intermediate* did not differ from *P. nyererei* nor *P. pundamilia* in copepod infection. *P. nyererei* carried fewer *Cichlidogyrus* than *P. intermediate*, but *P. pundamilia* carried more Glochidia than *P. intermediate*. These results indicate that intermediates do not have a lower resistance against

parasites and therefore a lower fitness. That could imply that parasites do not promote the reproductive isolation of *P. nyererei* and *P. pundamilia* in a sympatric population.

Length and Depth

The models showed that in general, deeper living fish carried fewer *Cichlidogyrus* and more Glochidia and *L. monodi*. However, this effect was measured in the complete sample of individuals, meaning both species at all 4 islands, plus intermediates at Luanso. Therefore, this correlation cannot be an explanation for the differences between hosts within the islands. This is illustrated by the example of *Cichlidogyrus*, where the differences in depth between hosts in strongest at Makobe, while there is no difference in infection observed at that island. Hence, correlations between depth and parasite abundance per population were performed. These results show that parasite infection rates in *P. nyererei* are mostly indifferent to depth, whereas populations of *P. pundamilia* show correlations of depth with parasite abundances albeit in different directions between islands and between parasite species. For example, abundances of *Cichlidogyrus* are higher for deeper living *P. pundamilia*, although the strength of this correlation differs between islands, and abundances of Glochidia can either be higher or lower in *P. pundamilia* at greater depths. The inconsistency of the these results suggest that depth is not a strong predictor of parasite abundance.

I also found that larger fish carried more parasites, with the exception of *E. lamellifer*, where there was no correlation between SL and parasite abundance. Again, this effect was measured in the complete sample of individuals. Thus, length cannot explain the difference in infection levels of *P. pundamilia* and *P. nyererei* within the islands, since not at every island *P. pundamilia* is significantly larger (Fig. A5). Therefore, correlations between length and parasite abundance at each population were measured. In every population, with the exception of intermediates at Luanso, larger fish carried more parasites. Even though only a few of these correlations were statistically significant, this suggests that length is a predictor of parasite abundance, where larger fish carry more parasites.

Comparison to previous years

At Makobe, compared to data from 2003, differences in parasite abundances between *P. nyererei* and *P. pundamilia* had decreased in 2014. This was mostly due to a decline in all parasite species infections in both *P. pundamilia* and *P. nyererei*, with the exception of *L. monodi* in *P. pundamilia*. In 2005 at Kissenda, differences were already minimal for *L. monodi* and *Cichlidogyrus*, and stayed the same in 2014. However, abundances of *E. lamellifer* in *P. nyererei* decreased, thereby deleting the difference between the two species. Compared to 2010, differences in infection rates between the species had not changed much in 2014. However, overall parasite abundances fluctuated over the 3 years. For example at Kissenda,

where *L. monodi* and *Cichlidogyrus* abundances have decreased. Instead, *E. lamellifer* abundance went up. Moreover, *Cichlidogyrus* infections went down strongly for *P. nyererei* at Python, but went up for *P. pundamilia* at Luanso and Makobe. Moreover, the fluctuations over the years do not always go in the same direction for both *P. pundamilia* and *P. nyererei*, therefore they overwhelm the infection differences observed at some islands. Although not all differences in parasite abundances between the years are significant, these results suggest that parasite infections in a population do not stay stable over time, which is a prerequisite for parasite-mediated speciation. Perhaps environmental conditions for parasites to thrive have decreased in this period. Unfortunately, to date, not much is known about the optimal living conditions for these parasites.

Both *Cichlidogyrus* and the copepods do not depend on other, intermediate hosts and their abundance is therefore not limited by other organisms than the cichlid host. *Cichlidogyrus* lay eggs in the open water, where they will hatch. The free-swimming larvae have 4-6 hours to find a host, after which their capacity to attach to one has declined drastically (Paperna, 1996). *E. lamellifer* and *L. monodi* occur in free-living stages as well, before attaching to fish gills permanently (Paperna, 1996). When host density is low, it is therefore harder for free-living parasites to attach themselves to a host than when host density is high. Subsequently, it will be harder to find a partner to mate and to reproduce (Pariselle *et al.*, 2015; Kmentová *et al.*, 2016). Hence, host densities could be a predictor for parasite abundance. However, *Pundamilia* densities at these sites have not been investigated. Moreover, copepods are known not be host-specific (Paperna, 1996), so overall fish density at the studied islands could also have influenced copepod abundance. With high host densities it could be easier for copepods to survive and reproduce and therefore they could be higher in abundance. Fish densities are highest at Makobe, and lowest at Luanso. For *L. monodi* the highest abundances were indeed found at Makobe and the lowest at Luanso. However, this pattern was not observed for *E. lamellifer*.

Possibly, parasite infections rates fluctuate per season. This could have played a role in observed difference between infections in this study, where fish were caught in October, and Maan *et al.* (2008), where fish were caught in winter. However, fish from 2005 at Kissenda (Desêtres, 2010) were also caught in October, so differences in parasite abundances with this study cannot be explained by sampling season. Unfortunately, no sampling dates from Karvonen *et al.* (in prep.), is known. In all studies, fish where caught by angling or gillnets, except for Maan *et al.* (2008), where only angling was used. Therefore, a difference in parasite abundances due to differences in sampling methods seems unlikely.

Parasite correlations

Cichlidogyrus abundances increased as Glochidia and *E. lamellifer* abundances increased, suggesting that resistance against one of these parasites caused resistance against the others as well, or exposure to Cichlidogyrus coincides with exposure to Glochidia and *E. lamellifer* (Figure A13). However, a higher abundance of *Cichlidogyrus,* correlated with a lower *L. monodi* abundance. No significant correlation between the copepods has been measured, suggesting that they don't experience strong competition from each other.

When copepod abundances in other cichlid species of 2014 are compared with 2010 (Karvonen *et al*., in prep.), an interesting pattern is observed (Gobbin, in prep.). While in 2010 *E. lamellifer* dominated the fish gills, in 2014 *L. monodi* was by far the most abundant copepod, suggesting that competition between parasite species plays a role in their occurrence. However, my results do not provide evidence for competition between copepods.

Conclusion

Overall, no increase in differences in parasite abundances between *P. pundamilia* and *P. nyererei* was observed as the host divergence increased. Furthermore, there was no stability in parasite infection over time, which is a prerequisite of parasite-mediated speciation. Therefore, this study does not provide evidence for parasite-mediated speciation in *P. pundamilia* and *P. nyererei*. However, in this study *Cichlidogyrus* was not determined to the species level. Studies in lake Tanganyika showed that the different *Chiclidogyrus* species can be host-specific (Raeymaekers *et al.,* 2013; Kmentová *et al*., 2016), and closely related host species harbor different kind of *Cichlidogyrus* species (Vanhove *et al*., 2015). Currently it is not known if *P. pundamilia* and *P. nyererei* are infected by different *Cichlidogyrus* species. Moreover, parasite differences at Makobe in 2003 were not only determined by ectoparasites, but also by the endoparasite *Contracaceum*. Here, endoparasites were not included in the analysis, and differences could therefore potentially change when they will be included as well.

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Appendix

Parasite abundances per population

Parasite abundances for *P. pundamila* and *P. nyererei* at each island are shown in Fig. A1. At Luanso parasite abundances of the intermediate phenotype are shown as well.

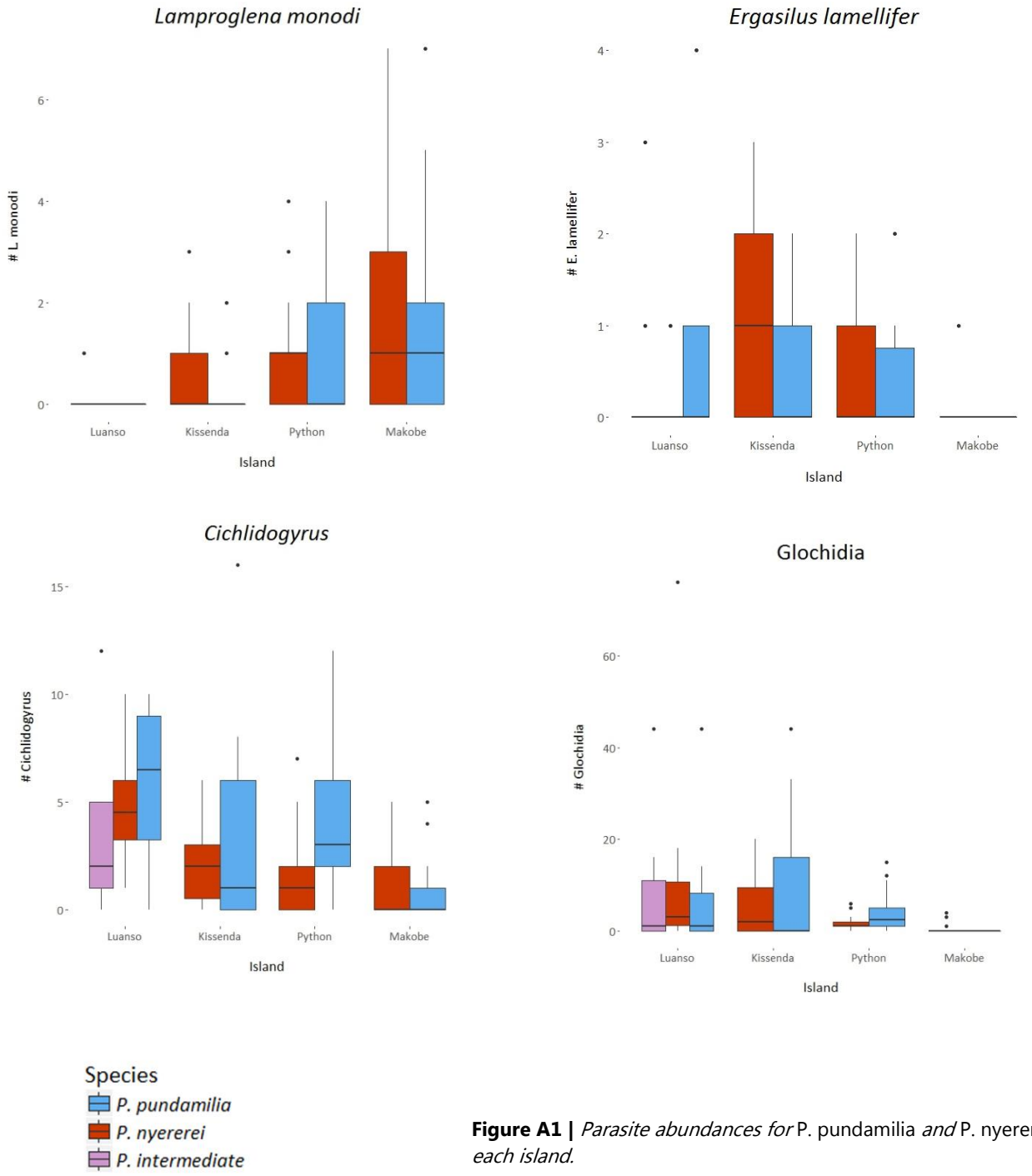


Figure A1 | *Parasite abundances for* P. pundamilia *and* P. nyererei *at each island.*

Descriptive statistics Bayesian models

Descriptive statistics of the Bayesian models for calculating differences within islands, performed in this analysis. The mean is the mean difference in parasite abundance between *P. pundamilia* and *P. nyererei* at that certain island. This difference was calculated by subtracting the posterior of *P. nyererei* from the posterior of *P. pundamilia*. Hence, a mean with a value above zero means that *P. pundamilia* carried more parasites while a value below zero means *P. nyererei* harbored more parasites. When the difference between the lower and the upper value of the 89% interval does not cross zero, a significant difference is observed, printed here in bold.

Table A2 | *Descriptive statistics of the differences in parasite abundances at all 4 islands. The mean of the differences between* P. pundamilia *and* P. nyererei *is shown, with the Standard Deviation of the mean and the lower and upper 89% interval. When the values of the lower and upper 89% do not cross zero, a significant difference is observed.* ***a)*** *L.* monodi. ***b)*** E. lamellifer ***c)*** Cichlidogyrus ***d)*** *Glochidia*

a) ***Lamproglena monodi***

	Mean difference (P-N)	Standard Deviation	Lower 89%	Upper 89%
Luanso	-0.01	0.16	-0.27	0.23
Kissenda	-0.34	0.38	-0.89	0.29
Python	-0.55	0.42	-1.18	0.19
Makobe	-0.33	0.27	-0.79	0.07

b) ***Ergasilus lamellifer***

	Mean difference (P-N)	Standard Deviation	Lower 89%	Upper 89%
Luanso	0.63	0.52	-0.20	1.41
Kissenda	-0.41	0.42	-1.10	0.20
Python	-0.27	0.26	-0.65	0.14
Makobe	-0.06	0.06	-0.17	0.02

c) ***Cichlidogyrus***

	Mean difference (P-N)	Standard Deviation	Lower 89%	Upper 89%
Luanso	**3.74**	**1.09**	**2.11**	**5.57**
Kissenda	**2.03**	**0.80**	**0.83**	**3.34**
Python	**2.32**	**0.57**	**1.43**	**3.25**
Makobe	-0.16	0.34	-0.73	0.37

d)	**Glochidia**			
	Mean difference (P-N)	Standard Deviation	Lower 89%	Upper 89%
Luanso	**5.41**	**1.79**	**2.03**	**7.81**
Kissenda	**17.70**	**2.08**	**14.17**	**20.70**
Python	**2.55**	**0.59**	**1.69**	**3.48**
Makobe	**-0.42**	**0.15**	**-0.63**	**-0.18**

Intermediates

Differences of P. intermediate with *P. pundamilia* and *P. nyererei* at Luanso, for all parasites. Mean differences are calculated by subtracting the posterior of *P. pundamilia* or *P. nyererei* from the posterior of *P. intermediate*. Therefore, positive Means show a lower parasite abundance in intermediates compared to the other phenotypes, whereas an negative Mean shows a higher parasite abundance in *P. intermediate* compared to *P. nyererei* or *P. pundamilia.* When the difference between the lower and the upper value of the 89% interval does not cross zero, a significant difference is observed, printed here in bold.

Table A3 | *Differences of* P. intermediate *with* P. nyererei *and* P. pundamilia *at Luanso*

Luanso intermediates

Parasite	Difference from species	Mean (P/N-I)	Standard Deviation	Lower 89%	Upper 89%
L. monodi	*P. nyererei*	0.06	0.16	-0.18	0.28
	P. pundamilia	0.05	0.18	-0.19	0.30
E. lamellifer	*P. nyererei*	-0.44	0.55	-1.26	0.39
	P. pundamilia	0.21	0.46	-0.44	0.85
Cichlidogyrus	*P. nyererei*	**-3.60**	**1.24**	**-5.61**	**-1.71**
	P. pundamilia	0.58	0.94	-0.87	2.07
Glochidia	*P. nyererei*	-0.73	1.89	-3.74	2.20
	P. pundamilia	**4.18**	**1.69**	**1.39**	**6.76**

Depth

Figure A2 shows the depth distribution of *P. pundamilia* and *P. nyererei* per island.

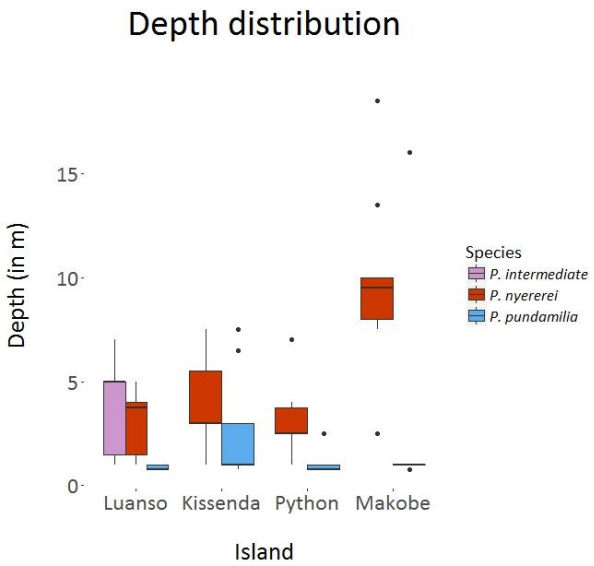


Figure A2 | *Depth distribution of both species per island.*

Figures A3 until A6 show the relationship between depth and parasite abundance for all populations, for all parasites.

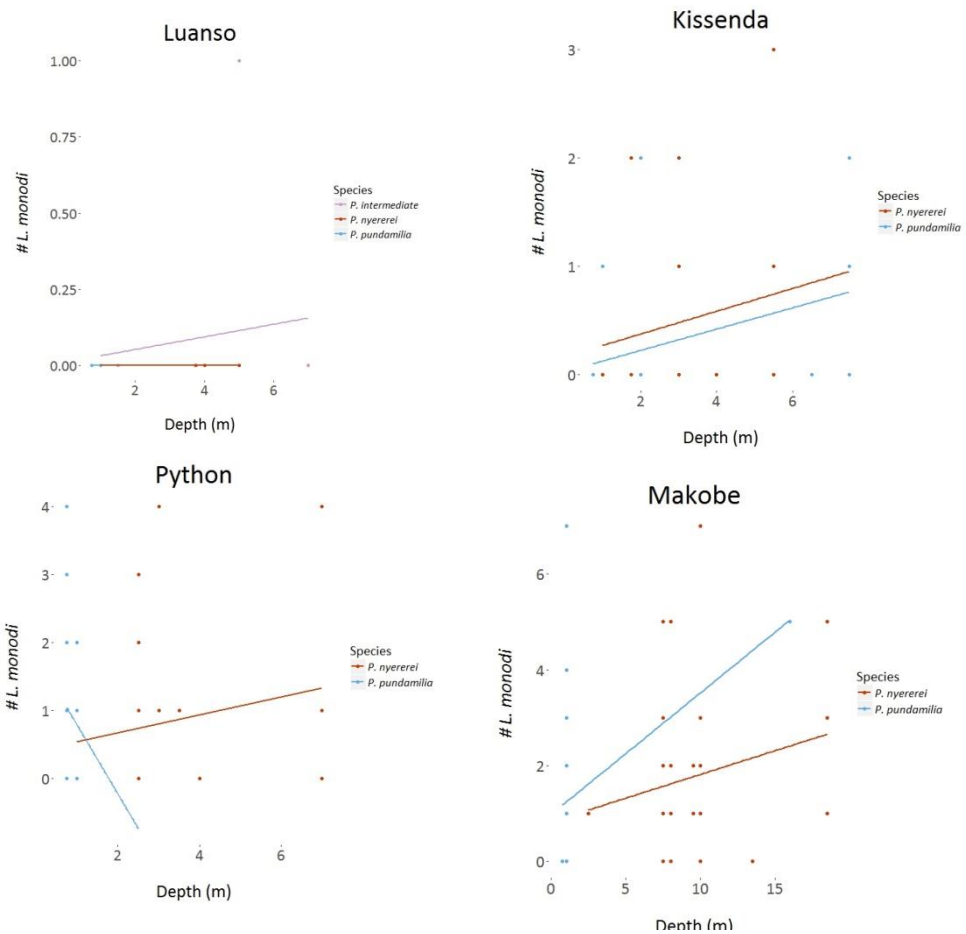


Figure A3 | *Relation between depth and abundance of* L. monodi *in* P. nyererei*,* P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*

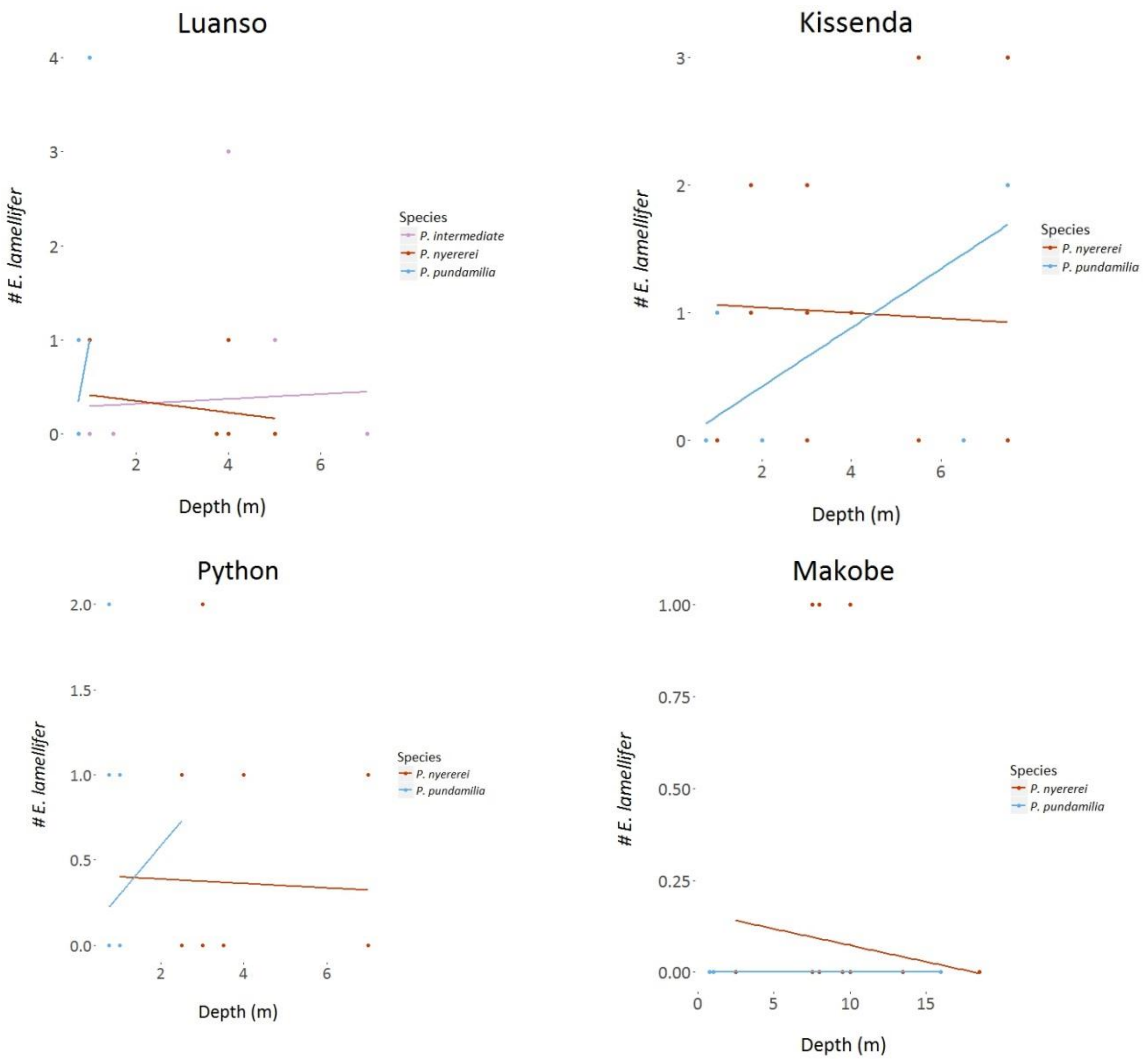
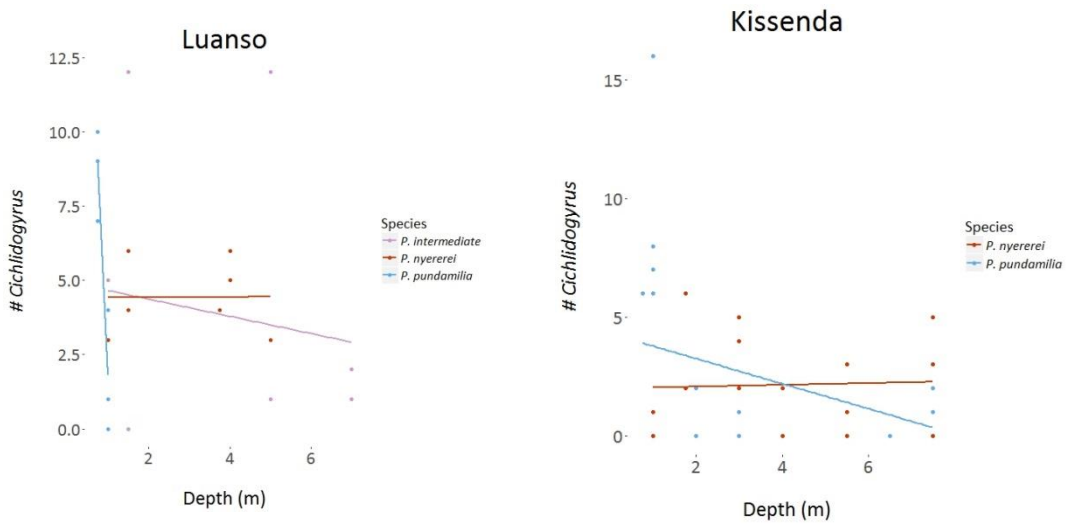


Figure A4 | *Relation between depth and abundance of* E. lamellifer *in* P. nyererei*,* P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*



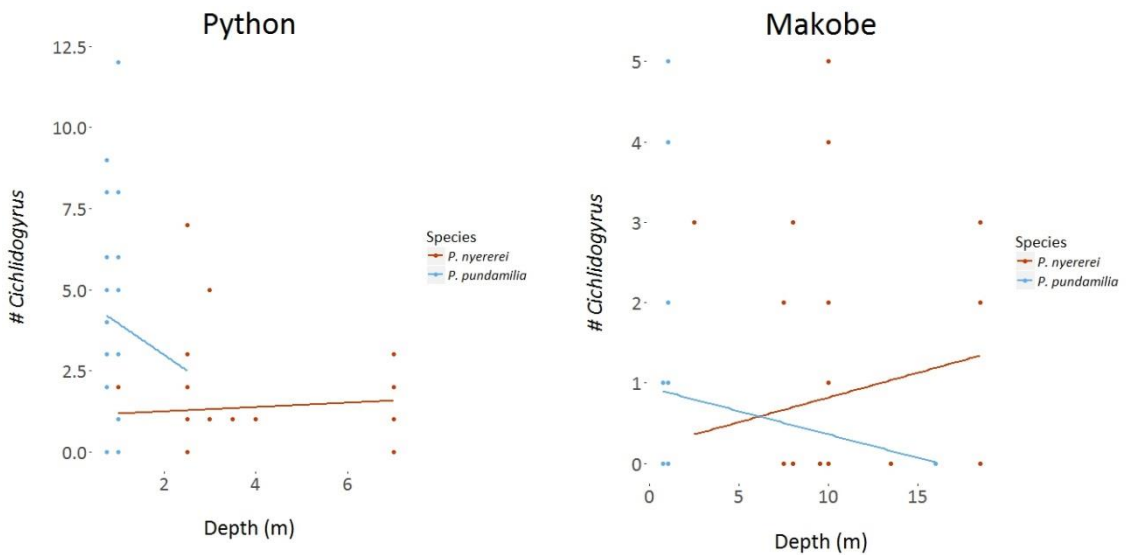


Figure A5 | *Relation between depth and abundance of* Cichlidogyrus *in* P. nyererei, P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*

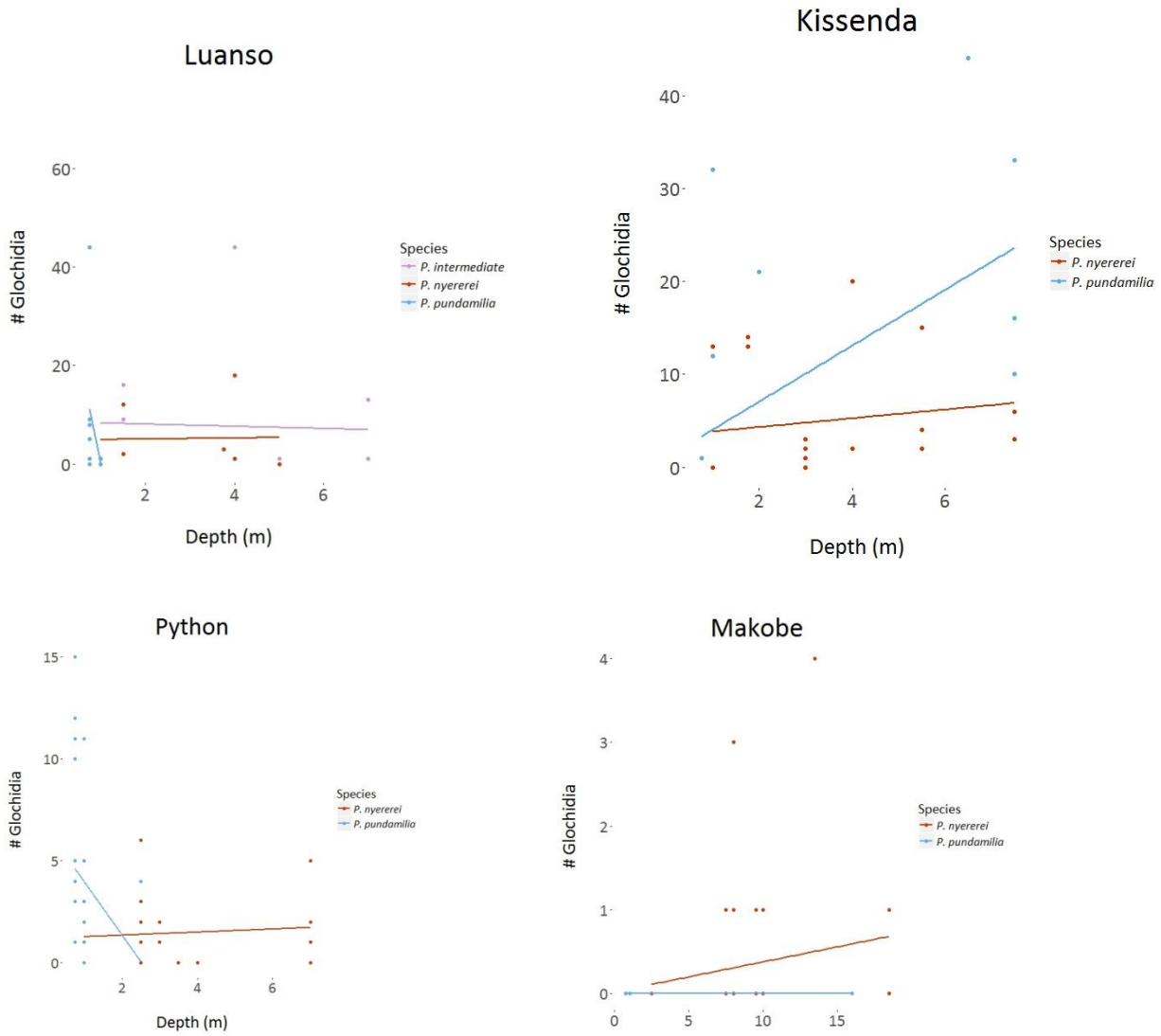


Figure A6 | *Relation between depth and abundance of Glochidia in* P. nyererei, P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*

Table A3 | *Descriptive statistics of the relation between depth and parasite abundance for* ***a)*** L. monodi ***b)*** E. lamellifer ***c)*** Cichlidogyrus ***d)*** *Glochidia*

a) ***L. monodi***

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	NA	NA
	P. nyererei	NA	NA
	P. intermediate	0.180	0.576
Kissenda	*P. pundamilia*	0.330	0.144
	P. nyererei	0.233	0.284
Python	*P. pundamilia*	**-0.463**	**0.012**
	P. nyererei	**0.383**	**0.048**
Makobe	*P. pundamilia*	0.433	0.024
	P. nyererei	0.084	0.613

b) ***E. lamellifer***

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	0.135	0.691
	P. nyererei	-0.315	0.543
	P. intermediate	0.086	0.791
Kissenda	*P. pundamilia*	**0.569**	**0.007**
	P. nyererei	-0.111	0.614
Python	*P. pundamilia*	-0.018	0.924
	P. nyererei	0.087	0.666
Makobe	*P. pundamilia*	NA	NA
	P. nyererei	-0.129	0.432

c) ***Cichlidogyrus***

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	**-0.880**	**<0.001**
	P. nyererei	-0.015	0.978
	P. intermediate	0.835	0.067
Kissenda	*P. pundamilia*	-0.298	0.189
	P. nyererei	0.031	0.889
Python	*P. pundamilia*	-0.141	0.465
	P. nyererei	0.321	0.102
Makobe	*P. pundamilia*	0.627	0.098
	P. nyererei	0.292	0.071

d) **Glochidia**

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	**-0.698**	**0.017**
	P. nyererei	-0.029	0.956
	P. intermediate	0.056	0.863
Kissenda	*P. pundamilia*	0.382	0.087
	P. nyererei	0.343	0.109
Python	*P. pundamilia*	**-0.491**	**0.007**
	P. nyererei	0.073	0.717
Makobe	*P. pundamilia*	NA	NA
	P. nyererei	-0.041	0.805

Standard Length

Figure A7 shows the length of *P. pundamilia* and *P. nyererei* per island. Figure A8 illustrates the correlation of SL with total parasite load, per species.

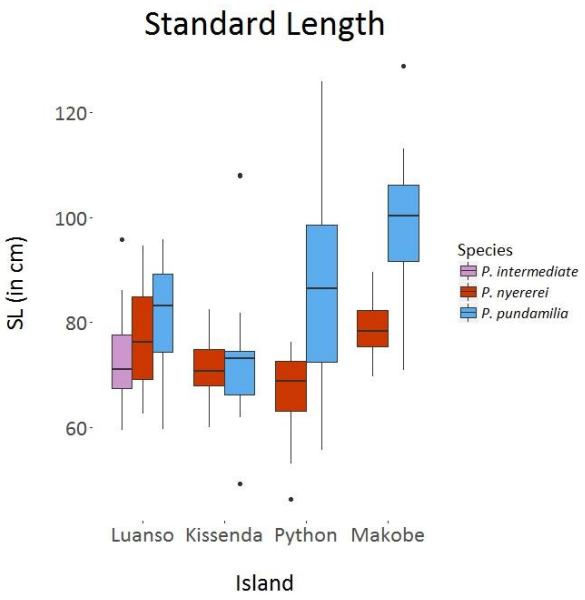


Figure A7 | *Length of* Pundamilias *per island*

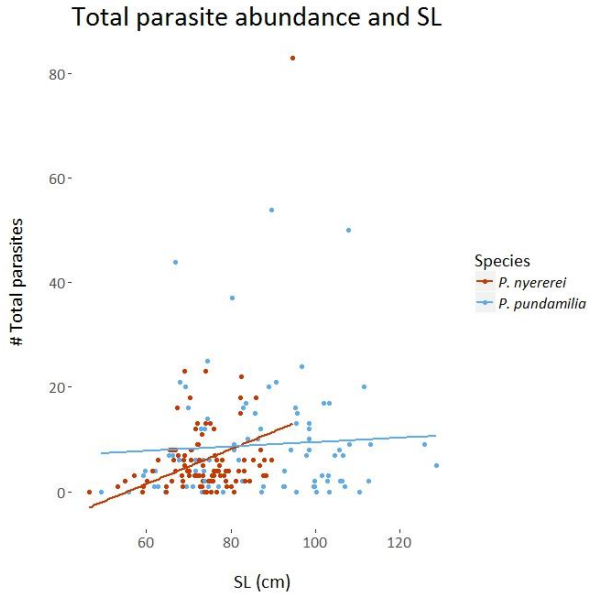


Figure A8 | *Correlation SL with total parasite load, per species*

Figures A9 until A12 show the relationship between depth and parasite abundance for all populations, for all parasites.

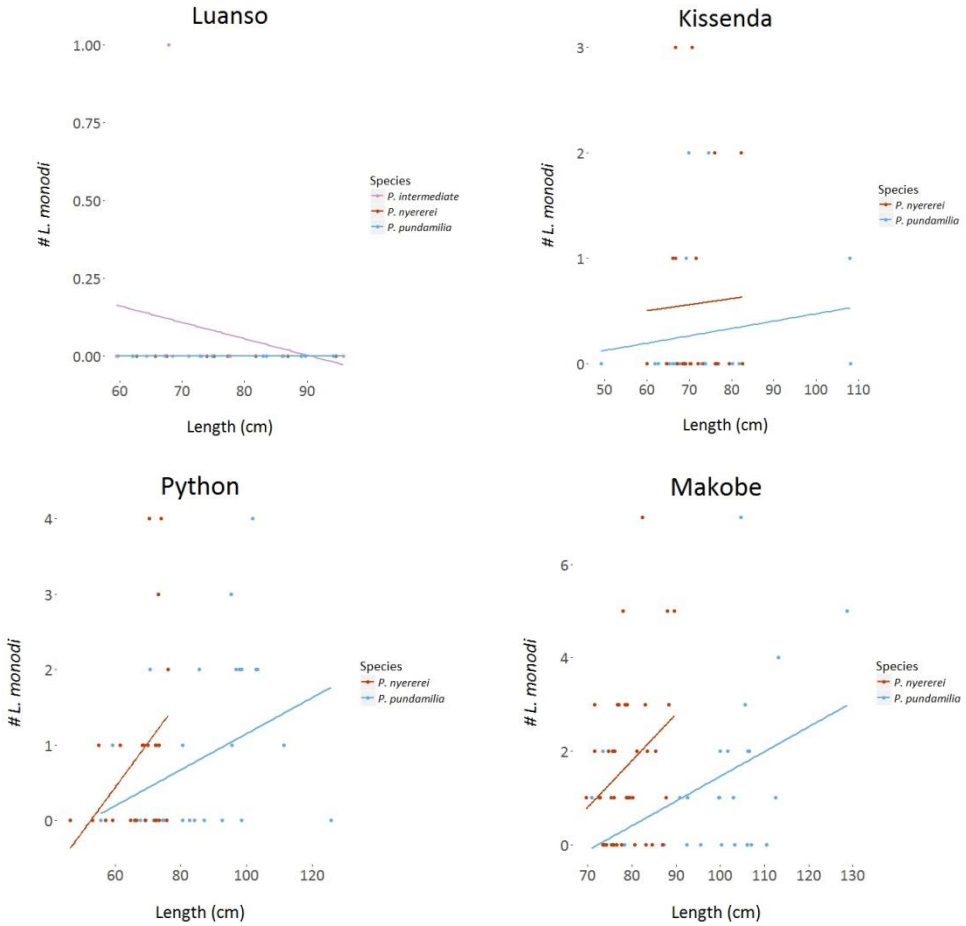


Figure A9 | *Relation between SL and abundance of* L. monodi *in* P. nyererei, P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*

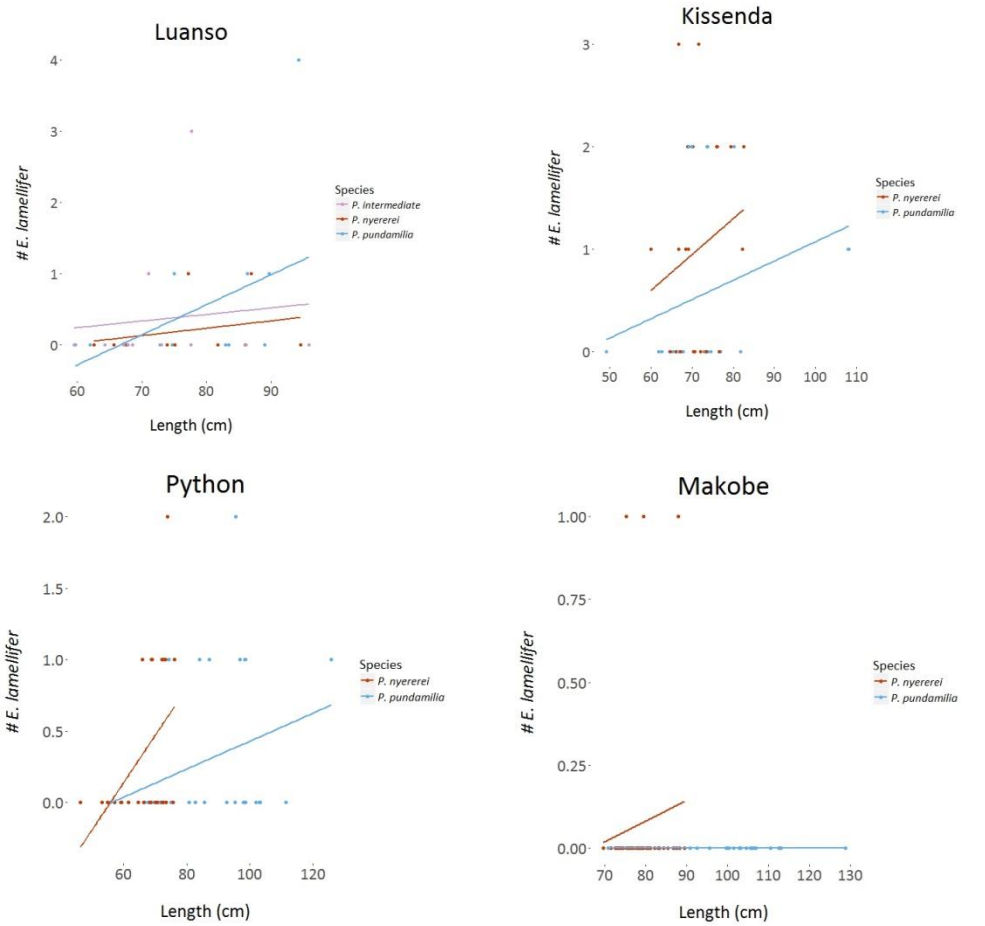


Figure A10 | *Relation between SL and abundance of* E. lamellifer *in* P. nyererei, P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*

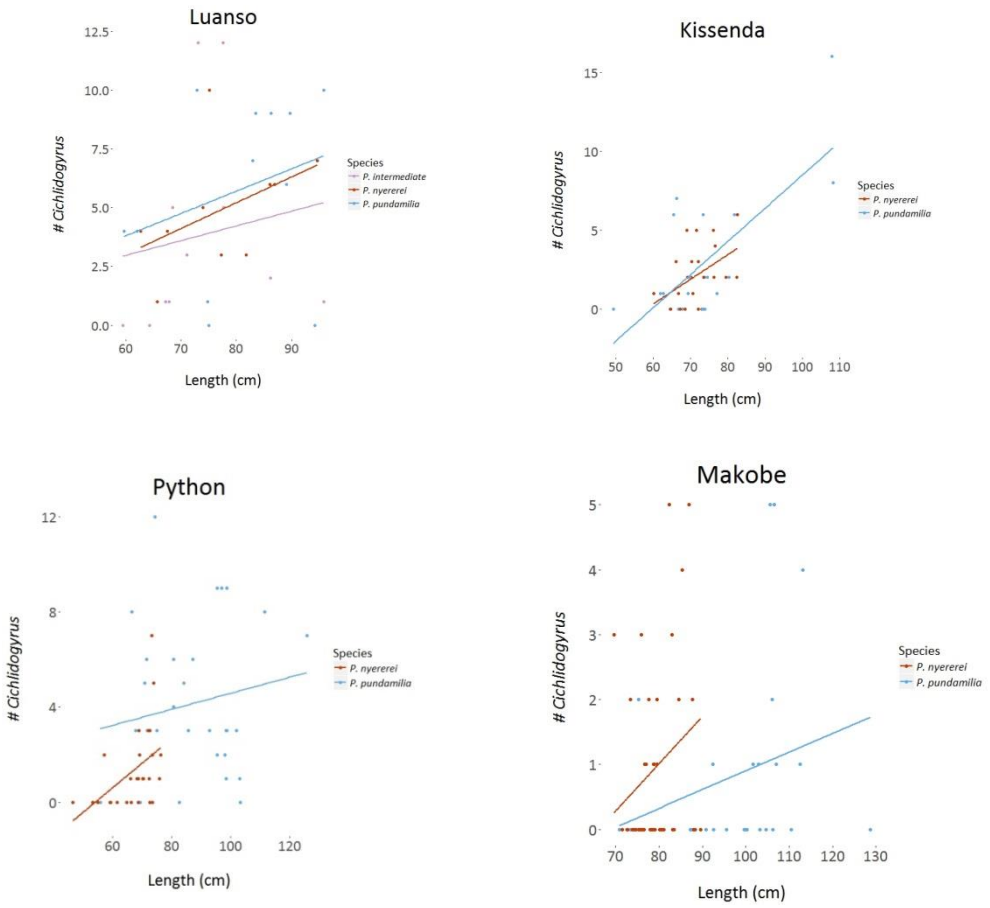


Figure A11 | *Relation between SL and abundance of* Cichlidogyrus *in* P. nyererei, P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*

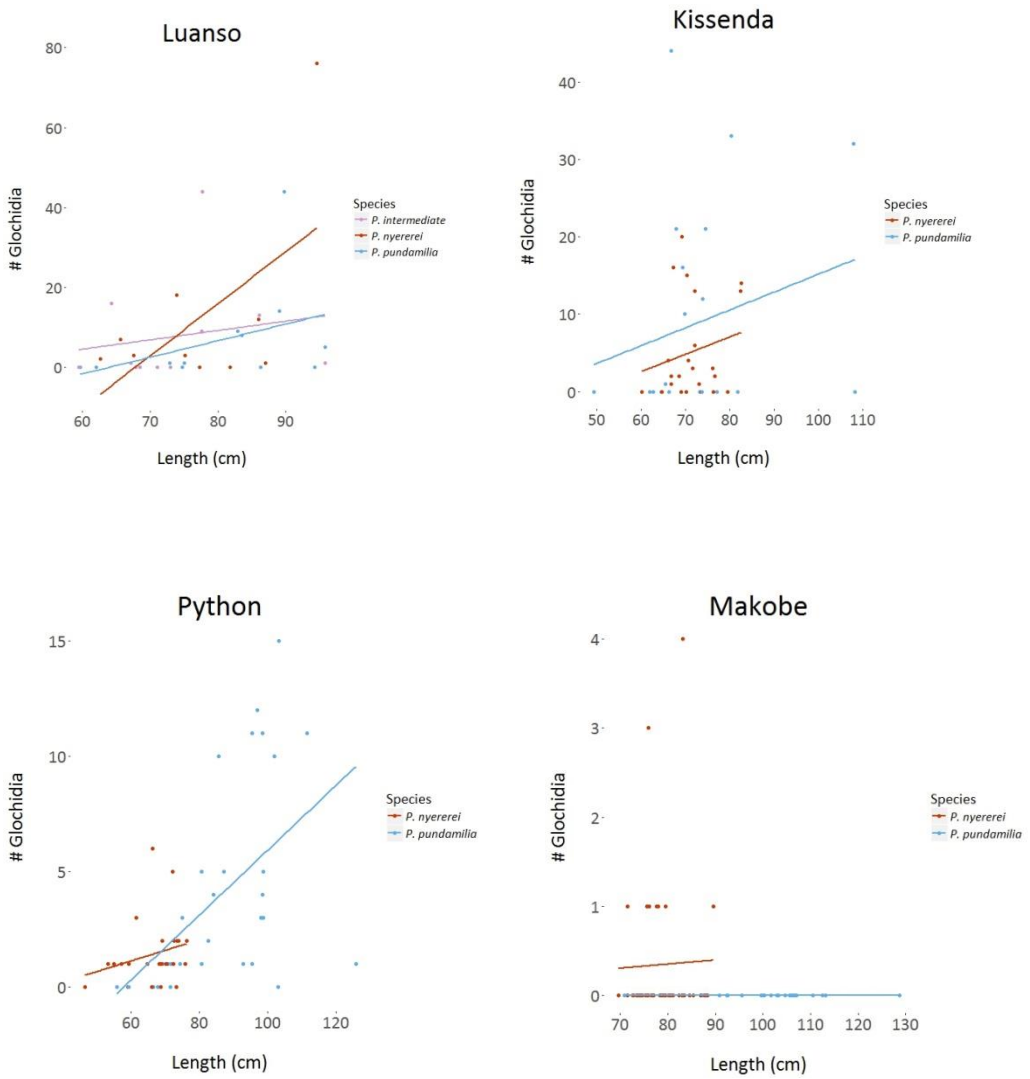


Figure A12 | *Relation between SL and abundance of Glochidia in* P. nyererei*,* P. pundamilia *and* P. intermediate *at* ***a)*** *Luanso* ***b)*** *Kissenda* ***c)*** *Python* ***d)*** *Makobe*

Table A4 | *Descriptive statistics of the relation between SL and parasite abundance for* ***a)*** L. monodi ***b)*** E. lamellifer ***c)*** Cichlidogyrus ***d)*** *Glochidia*

a) *L. monodi*

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	NA	NA
	P. nyererei	NA	NA
	P. intermediate	-0.131	0.685
Kissenda	*P. pundamilia*	0.213	0.353
	P. nyererei	-0.076	0.731
Python	*P. pundamilia*	**0.395**	**0.031**
	P. nyererei	0.418	0.030

Makobe	*P. pundamilia*	0.319	0.105
	P. nyererei	0.222	0.162

b) ***E. lamellifer***

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	0.445	0.147
	P. nyererei	0.311	0.416
	P. intermediate	0.274	0.389
Kissenda	*P. pundamilia*	0.399	0.073
	P. nyererei	0.117	0.594
Python	*P. pundamilia*	0.259	0.166
	P. nyererei	0.499	0.008
Makobe	*P. pundamilia*	NA	NA
	P. nyererei	0.103	0.522

c) ***Cichlidogyrus***

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	0.255	0.424
	P. nyererei	0.538	0.135
	P. intermediate	0.435	0.157
Kissenda	*P. pundamilia*	**0.439**	**0.047**
	P. nyererei	0.440	0.036
Python	*P. pundamilia*	0.106	0.578
	P. nyererei	**0.579**	**0.002**
Makobe	*P. pundamilia*	*0.377*	*0.052*
	P. nyererei	0.222	0.163

d) **Glochidia**

Island	Species	Rho	p-value
Luanso	*P. pundamilia*	0.443	0.149
	P. nyererei	0.042	0.915
	P. intermediate	0.276	0.385
Kissenda	*P. pundamilia*	0.201	0.383
	P. nyererei	0.124	0.575
Python	*P. pundamilia*	**0.615**	**<0.001**
	P. nyererei	0.388	0.045
Makobe	*P. pundamilia*	NA	NA
	P. nyererei	-0.023	0.885

Parasite correlations

Figure A13 shows the correlations between the different parasite species that were observed.

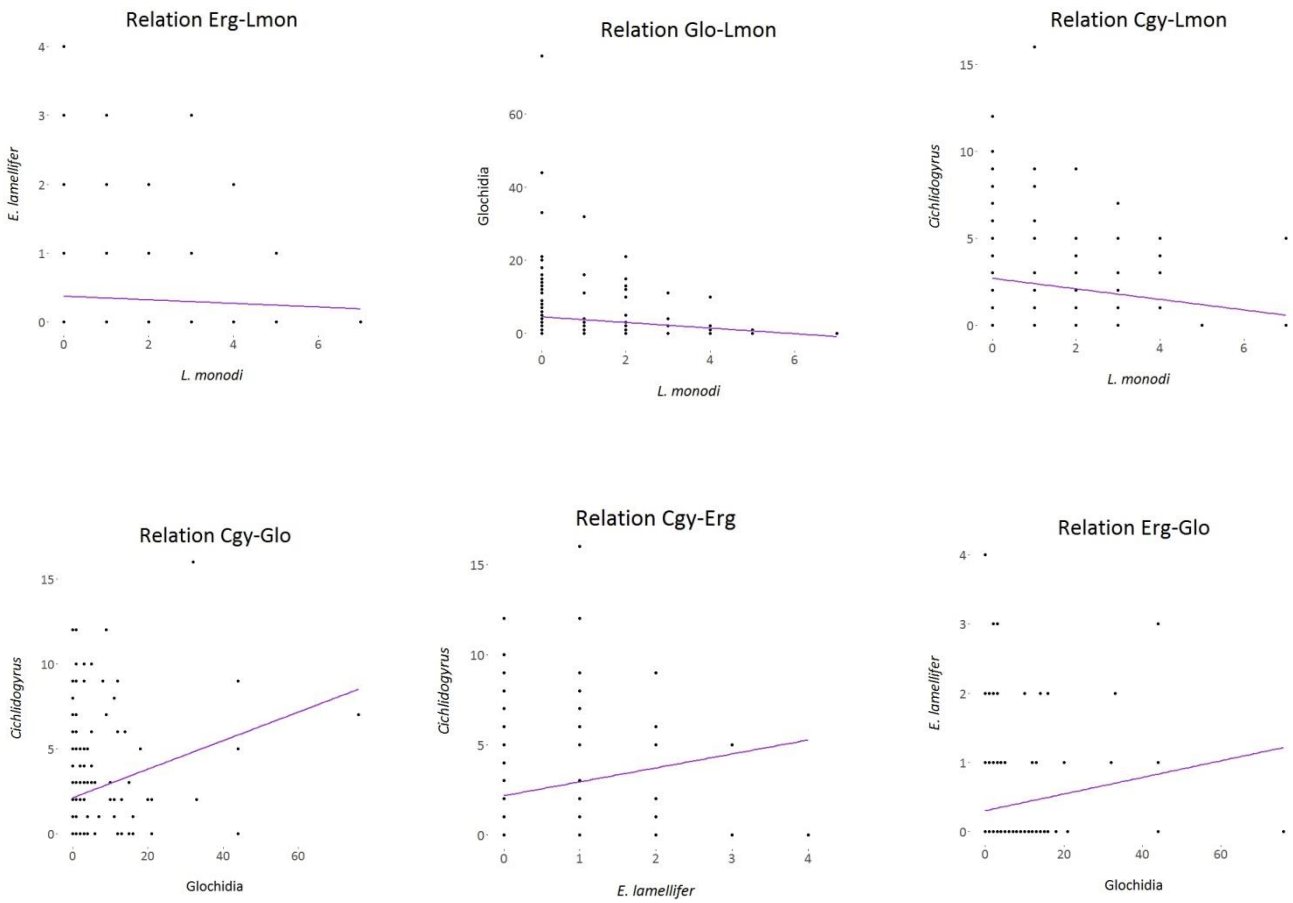


Figure A13 | Correlations between the different kind of parasites observed in this study

Table A5 | Descriptive statistics of correlations between parasites. Significant correlations are printed in bold.

Correlations between parasites	Rho	p-value
Cichidogyrus – E. lamellifer	**0.270**	**<0.001**
Cichidogyrus – L. monodi	**-0.150**	**0.032**
Cichidogyrus - Glochidia	**0.277**	**<0.001**
L. monodi - E. lamellifer	-0.073	0.304
E. lamellifer - Glochidia	**0.181**	**0.009**
L. monodi - Glochidia	-0.096	0.172