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A comparative study of an innate immune response in Lamprologine cichlid fishes

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Abstract Social interactions facilitate pathogen transmission and increase virulence. Therefore, species that live in social groups are predicted to suffer a higher pathogen burden, to invest more heavily in immune defence against pathogens, or both. However, there are few empirical tests of whether social species indeed invest more heavily in immune defence than non-social species. In the current study, we conducted a phylogenetically controlled comparison of innate immune response in Lamprologine cichlid fishes. We focused on three species of highly social cichlids that live in permanent groups and exhibit cooperative breeding (*Julidochromis ornatus*, *Neolamprologus pulcher* and *Neolamprologus savoryi*) and three species of non-social cichlids that exhibit neither grouping nor cooperative behaviour (*Telmatochromis temporalis*, *Neolamprologus tetracanthus* and *Neolamprologus modestus*). We quantified the innate immune response by injecting wild fishes with phytohaemagglutinin (PHA), a

lectin that causes a cell-mediated immune response. We predicted that the three highly social species would show a greater immune reaction to the PHA treatment, indicating higher investment in immune defence against parasites relative to the three non-social species. We found significant species-level variation in immune response, but contrary to our prediction, this variation did not correspond to social system. However, we found that immune response was correlated with territory size across the six species. Our results indicate that the common assumption of a positive relationship between social system and investment in immune function may be overly simplistic. We suggest that factors such as rates of both in-group and out-group social interactions are likely to be important mediators of the relationship between sociality and immune function.

Keywords Sociality · Group living · Cooperation · Inflammatory response · Parasite · Pathogen · Phytohaemagglutinin · Teleost · Lake Tanganyika

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Introduction

Sociality, or the tendency to live in a group, is both taxonomically widespread and highly variable across species (Krause and Ruxton 2002). Individuals range from those that spend almost all of their time in solitude to those that spend their entire life in close proximity to conspecifics (Danchin et al. 2008; Krause and Ruxton 2002, 2010). From differences across species in grouping and social behaviour to individual differences in the tendency to affiliate with other conspecifics, variation in sociality has led behavioural biologists to question why some species or individuals are so much more social than others. In order to understand the evolution of social living, it is crucial that we understand the costs and benefits of sociality. There are many benefits to grouping behaviour, including the

dilution of predation risk, increased foraging efficiency and the mutual defence of a shared territory (Hamilton 1971; Alexander 1974; Brown 1986; Elgar 1989; Wrona and Dixon 1991; Uetz et al. 2002). However, there are also costs to social life (e.g. Lindström 1989; Cresswell 1994; Janson and Goldsmith 1995). One of the commonly cited costs of group living is increased pathogen and parasite transmission (Alexander 1974; Altizer et al. 2003; Fincher et al. 2008; Fincher and Thornhill 2012). The higher density associated with living in a group increases the risk of transmitting pathogens and parasites (Anderson and May 1979; Hoogland 1979; Brown and Brown 1986; Coté and Poulin 1995; McCallum et al. 2001). Furthermore, greater horizontal transmission of pathogens and parasites (i.e. transmission between conspecifics) is associated with increased virulence (Anderson and May 1982; Ewald 1983, 1987; Frank 1992; Clayton and Tompkins 1994; Lipsitch et al. 1995, 1996), which further increases the costs of pathogens in social animals. Social animals are therefore predicted to have both higher rates of pathogen infection and an immune system that is primed for defence against these challenges (Alexander 1974).

While group-living and non-grouping animals have been compared in terms of their rates of infection (e.g. Davies et al. 1991; Poulin 1991a, 1991b; Ranta 1992; Coté and Poulin 1995; Watve and Sukumar 1995; Bordes et al. 2007), there are far fewer empirical tests of the degree to which investment in immune function covaries with social system. One exception is Hochberg (1991), who used comparative data on virus resistance to support the notion that gregarious butterfly and moth species (Lepidoptera) invest more in immune defence than do solitary species. Later, Møller et al. (2001) demonstrated that the inflammatory immune response, which is a measure of investment in innate immune function, was positively correlated with nesting colony size across 13 species of swallows and martins (Hirundinidae). However, two further studies examining a different measure of immune investment, circulating leukocytes, found no relationship with the degree of gregariousness across primate species (Nunn et al. 2000; Semple et al. 2002). Finally, contrary to the original Hochberg (1991) results, solitary Lepidopteran species displayed a more robust immune response than did gregarious species when a phylogenetically controlled approach was used (Wilson et al. 2003). Thus, empirical support for the notion that social species have enhanced immune function remains equivocal.

Cooperative breeding represents a special case of group living (Gaston 1978; Hatchwell and Komdeur 2000; Bergmüller et al. 2007; Hatchwell 2009; Riehl 2013). Cooperative breeding is a social system where individuals live in groups and some group members provide care for the offspring of other more dominant group members (Gaston 1978; Hatchwell and Komdeur 2000; Clutton-Brock 2002; Bergmüller et al. 2007; Hatchwell 2009; Riehl 2013).

Cooperatively breeding groups are often composed of highly related individuals, and these individuals spend a great deal of time in contact with one another (Clutton-Brock 2002; Koenig and Dickinson 2004). Cooperatively breeding groups are also typically characterized by strong membership restrictions and limited dispersal opportunities for one or both sexes (Stiver et al. 2004, 2006, 2007; Russell and Lummaa 2009; Jordan et al. 2010a), and individuals of cooperatively breeding species prefer to group with familiar or closely related social partners (Jordan et al. 2010b; Fincher and Thornhill 2012). The tendency for cooperatively breeding species to frequently associate with close relatives may lead to particularly high rates of parasite and pathogen transmission for two reasons. First, close relatives share resistance alleles and so will be susceptible to similar pathogen strains (Frank 1996). Further, high relatedness between interacting individuals accelerates co-adaptation between hosts and pathogens, which is thought to lead to increased infectiousness of pathogens (Ebert and Hamilton 1996). Therefore, cooperatively breeding species are expected to face particularly high parasite and pathogen loads, invest more heavily in immune function to defend against parasites relative to non-social species, or both. However, this hypothesis has rarely been tested, and never in fish. Poiani (1992) examined rates of parasitism in cooperative and non-cooperative Australian passerines and found no relationship between affliction with ectoparasites and social system. However, Spottiswoode (2008) found that among African birds, cooperatively breeding species exhibit a higher inflammatory response to an immune challenge when compared to non-cooperative species, which provides support for the notion that cooperatively breeding species invest heavily in immune defence.

In this current study, we explored the relationship between social system and immune function by measuring the innate immune response of six closely related species of Lamprologine cichlid fishes endemic to Lake Tanganyika, East Africa. Lamprologine cichlids are an excellent model for questions related to social behaviour. Within this tribe, closely related species (Day et al. 2007; Sturmbauer et al. 2010) display a remarkable diversity of social systems (Brichard 1989; Konings 2005; Heg and Bachar 2006), yet are similar in their ecological and habitat requirements (Brichard 1989; Konings 1998, 2005). All Lamprologines are substrate spawners and provide biparental care (Brichard 1989). The six species examined in the current study were *Telmatochromis temporalis*, *Neolamprologus pulcher*, *Julidochromis ornatus*, *Neolamprologus modestus*, *Neolamprologus tetracanthus* and *Neolamprologus savoryi* (Fig. 1). *N. pulcher*, *J. ornatus* and *N. savoryi* are highly social species that display group-living, cooperatively breeding social systems (Heg et al. 2005; Heg and Bachar 2006; Wong and Balshine 2011). Conversely, *T. temporalis*, *N. modestus* and *N. tetracanthus* are all non-social species that exhibit no

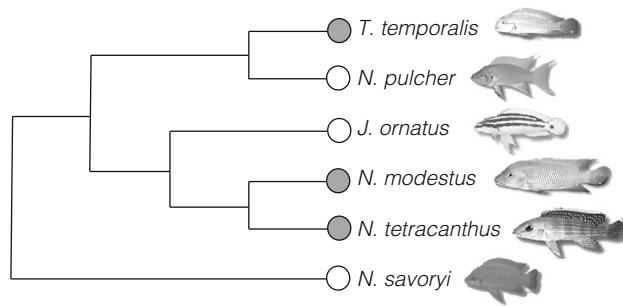


Fig. 1 The phylogenetic relationships among the six species of Lamprologine fish included in the current study. *White labels* represent highly social species, while *grey labels* represent non-social species. These relationships are based on the ND2, cytochrome B and control region sequences and created using Bayesian Markov chain Monte Carlo analysis with *A. leptura*, *B. fasciatus*, *B. microlepis*, and *X. flavipinnis* as the out-groups (see **Methods** for full details; see **Table 2** for sequence NCBI accession numbers)

grouping or cooperative behaviour (Brichard 1989; Heg et al. 2005). All six species are otherwise similar in their mating systems and ecology (Brichard 1989; Maréchal and Poll 1991).

To quantify the relative investment in immune function in these six species, we used an injection of phytohaemagglutinin (PHA) to measure the innate immune response. PHA is a lectin derived from red kidney beans that simulates a cell-mediated immune response characterized by localized swelling (Caspi et al. 1982). Measurement of the inflammatory response in response to PHA exposure has been used as an effective measure of investment in immune function across a variety of taxa (e.g. Smits and Williams 1999; Smits et al. 1999; Ardia and Clotfelter 2006; Martin et al. 2006), including studies of immune function relative to social system in birds (Møller et al. 2001; Spottiswoode 2008). A robust inflammatory response following PHA injection is indicative of a strong innate immune system and therefore indicates high investment in immune defence. In the current study, we predicted that group-living and cooperatively breeding cichlid species would invest more heavily in immune defence and therefore display a higher inflammatory response to PHA injection, relative to the non-grouping, non-cooperative species.

Methods

Study site and study species The study site was located in southern Lake Tanganyika, in Kasakalawe Bay, Zambia (8°46'52" S, 31°5'18" E). The site is characterized by a mix of sand and cobble substrate and a gentle descent to depth (for detailed descriptions of the study site, see Balshine-Earn et al. 1998; Balshine et al. 2001; Bergmüller et al. 2005; Dierkes et al. 2005; Heg et al. 2005; Stiver et al. 2005; Wong et al. 2012). In February–March, 2013, the six species of cichlids

were located and captured using SCUBA between depths of 6–12 m. Fish were collected using fence and hand nets and transferred to trolling-style bait buckets (i.e. opaque plastic containers that are designed to hold live fish underwater, and outfitted with a series of small holes that provide a constant flow of fresh lake water). Bait buckets had a volume of approximately 6 L, and fish were held at densities of no more than $n=6$ fish per bucket. Once in the bait buckets, fish were brought slowly to the surface. At the surface, fish were held in two concrete raceways, each measuring 200×100 cm, and filled to a depth of approximately 60 cm with fresh lake water (approximately 1,200 L each). The lake water in the raceways was aerated using battery-operated pumps and refreshed daily. Raceways were fully shaded and covered by netting to prevent predation. Fish were held at densities of no more than $n=20$ individuals per raceway. In total, we used $n=140$ fish, with between 19 and 26 individuals per species. We used individuals of both sexes, and all individuals were sexually mature (Table 1).

Study treatments We measured the inflammatory response to PHA injections following protocols previously found to be effective in a small teleost fish, *Betta splendens* (Ardia and Clotfelter 2006; Clotfelter et al. 2007) and slightly modified for the field setting. Fish were held in the raceways for 18–24 h, and then captured using hand nets. The thickness of the caudal peduncle was measured three times with a Shimano digital micrometer (Digital Measurement Metrology, Brampton, ON). Each individual fish (between 14 and 21 individuals per species, see Table 1) was then injected at the top of the caudal peduncle with 4 µg of PHA (61764, Sigma-Aldrich, St. Louis, MO) in 2 µL of phosphate-buffered saline using a 10-µL 26-gauge Hamilton syringe (Hamilton Company, Reno, NV). To verify the methodology, a small number of fish from each species (between five and six individuals per species, see Table 1) was injected with 2 µL of phosphate-buffered saline as a vehicle control. To reduce variation in the measurements, the same investigator performed all pre- and post-injection measurements on all individuals. Fish were uniquely fin-clipped and returned to the raceways following injections. After 24 h, fish were recaptured, and the thickness of the tissue at the location of injection was again measured three times. Fish were then weighed on a portable battery-operated scale, measured (standard length, SL) and sexed by examination of the external genitalia. A subset of fish ($n=10$ per species) was euthanized for another study, and sex was confirmed by dissection in these individuals. The rest of the fishes were returned to the site of capture following the experiment.

Phylogenetic analysis Nucleotide sequences for the ND2, cytochrome B and control genes for our six study species, as well as for out-group species *Asprotilapia leptura*, *Bathybates*

Table 1 Measured characteristics (sex, standard length and mass) and sample sizes of the fishes used in the current study. Values are presented as mean± standard error of the mean

Social system	Species	Treatment	Sex	Standard length (mm)	Mass (g)	Sample size		
Social	<i>N. pulcher</i>	Saline	Male	57.6±2.3	4.6±0.4	3		
			Female	52.9±0.7	3.7±0.0	2		
		PHA	Male	50.4±2.3	3.4±0.4	7		
			Female	47.8±1.9	2.9±0.4	7		
		<i>J. ornatus</i>	Saline	Male	61.2±0.7	5.0±0.6	2	
				Female	66.2±1.0	6.2±0.3	4	
	PHA		Male	59.1±1.2	4.4±0.3	10		
			Female	57.2±2.1	4.1±0.5	10		
	<i>N. savoryi</i>	Saline	Male	50.7±3.5	3.6±0.7	4		
			Female	38.8	1.4	1		
		PHA	Male	51.0±0.8	3.6±0.1	13		
			Female	43.2±1.2	2.3±0.2	6		
Non-social			<i>T. temporalis</i>	Saline	Male	57.6±3.1	4.8±0.5	5
					Female			0
	PHA	Male		53.9±1.7	4.0±0.3	17		
		Female		50.5±6.2	3.6±1.5	4		
	<i>N. modestus</i>	Saline	Male	73.2±3.2	10.6±1.3	4		
			Female	69.1	8.8	1		
PHA		Male	73.4±1.8	10.2±0.7	9			
		Female	69.1±1.0	8.5±0.2	6			
<i>N. tetracanthus</i>	Saline	Male	77.2±0.3	9.2±0.3	4			
		Female	62.7±4.7	3.4±1.8	2			
	PHA	Male	78.1±0.7	9.5±0.3	12			
		Female	61.0±1.0	4.6±0.2	7			

fasciatus, *Boulengerochromis microlepis* and *Xenotilapia flavipinnis* (Sturmbauer et al. 2010), were retrieved from the National Center for Biotechnology Information (NCBI) nucleotide data (<http://www.ncbi.nlm.nih.gov/nucleotide/>) (Table 2). Nucleotide sequences were aligned using MUSCLE (Edgar 2004). Following the alignment, we used the software programme BEAST with BEAUTi version 1.8 (Drummond and Rambaut 2007; Drummond et al. 2012) to complete 10,000,000 runs of a Bayesian Markov chain Monte Carlo model with a relaxed uncorrelated lognormal molecular clock (Drummond et al. 2006). The software programme Tracer 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>) was used to investigate parameter estimates, and the maximum clade credibility tree was determined using TreeAnnotator version 1.8 (<http://beast.bio.ed.ac.uk/TreeAnnotator>). Out-groups were trimmed using Mesquite (Maddison and Maddison 2011) and the final tree visualized using the programme FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) (Fig. 1).

Statistical analyses The variation in micrometre measurement was calculated for both the pre-injection and post-injection measurements using percent coefficient of variation (% COV). The inflammatory response of each individual to the saline or PHA treatment was calculated as the percent change in caudal peduncle thickness following injection (Ardia and

Clotfelter 2006) based on the average of the three measures taken at each of the two time points.

First, the efficacy of the PHA treatment was validated for each species by comparing the response of saline-treated individuals to the response of PHA-treated individuals using Welch's *t* tests. Following validation of the PHA technique, the variation in individual PHA response among species was compared among the six species using a one-way Welch's analysis of variance (ANOVA) test. A Shapiro-Wilk goodness-of-fit test confirmed that the distribution of residuals was normal, but a Levene's test revealed unequal variances among the species, and therefore, a Games-Howell post hoc test for unequal variances was used to determine which species differed (Games and Howell 1976).

To compare PHA response between the social and non-social species, the mean PHA response was calculated for each species, and the mean inflammatory response was then compared between the social and non-social species while accounting for phylogenetic relationships (Harvey and Pagel 1991) using a generalized least square (pGLS) model using the package "ape" (Paradis et al. 2004). All analyses were performed in R version 3.0.2 (R Core Team 2013), and the level of significance for all tests was assessed at $\alpha=0.05$.

Table 2 Accession numbers for the NDS, cytochrome B and control region sequences used to construct the phylogenetic tree of the Lamprologine cichlids fishes used in the current study (see [Methods](#) for full details and [Fig. 1](#) for the final trimmed phylogenetic tree)

Social system	Species	NCBI accession numbers		
		ND2	Cytochrome B	Control region
Social	<i>N. pulcher</i>	HM623795	AF438813	EF462306
	<i>J. ornatus</i>	EF191082	FJ706632	EF462291
	<i>N. savoryi</i>	HM623806	JQ950370	EF462309
Non-social	<i>T. temporalis</i>	EF462234	EF679293	EF462296
	<i>N. modestus</i>	HM623821	HM049954	FJ706203
	<i>N. tetracanthus</i>	HM623822	FJ706680	EF462282
Out-groups	<i>A. leptura</i>	EF679238	EF679270	AF400701
	<i>B. fasciatus</i>	AY663733	EF679268	AY663779
	<i>B. microlepis</i>	AF317229	AF370632	AY929939
	<i>X. flavipinnis</i>	AY337794	AY337811	AY339030

Post hoc analyses of territory size and PHA response Modelling by Watve and Jog (1997) and Wilson et al. (2003) suggest that in some social species, increased disease transmission within social groups may be balanced by decreased disease transmission among groups. All of the species examined in the current study hold permanent year-round territories (Brichard 1989; Maréchal and Poll 1991), and territory size can be used as an approximation of the potential for out-group interactions. Increased territory size changes both the rate and type of social interactions (Watve and Jog 1997; Wilson et al. 2003). With increased territory size, the interactions that occur with unfamiliar individuals is higher relative to the frequency of interactions with familiar individuals because of the following: (1) there is a greater boundary length on a large territory along which to encounter unfamiliar individuals moving through the environment and (2) a large territory is more difficult to defend, resulting in more intrusions. Thus, with larger territories, social interactions that occur are more likely to occur between individuals that are unfamiliar with one another (Watve and Jog 1997; Wilson et al. 2003). Therefore, following our investigation of PHA response between social and non-social species, we examined territory size in relation to the inflammatory response to PHA injection in the six species of cichlids in the current study as a preliminary exercise to determine whether out-group interaction rates may be an important factor influencing immune function in cichlid fishes. We collected

data on territory sizes for *N. pulcher*, *N. savoryi* and *N. modestus* during the same field season and at the same field site for the purpose of a different study. We searched the literature for information on territory sizes in *J. ornatus*, *T. temporalis* and *N. tetracanthus* (see [Table 3](#)). For *N. pulcher*, *N. savoryi* and *N. modestus*, territory size was defined as the area defended by a social group or breeding pair, observed during two separate 10-min behavioural observations. Territory size for *J. ornatus* was calculated from [Fig. 3b](#) presented in [Heg and Bachar \(2006\)](#), using the data for breeding males, and was observed in fish from southern Lake Tanganyika, at Kasakalwe Bay. Territory size for *T. temporalis* was calculated by combining all of the values reported for breeding males by [Mboko and Kohda \(1995\)](#) and was observed in fish from northwestern Lake Tanganyika, near Uvira, Democratic Republic of the Congo. Finally, territory size for *N. tetracanthus* was calculated by combining all of the values reported for breeding males observed in southern Lake Tanganyika, at Wonzye Point, near Mpulungu, Zambia, by [Matsumoto and Kohda \(1998\)](#). Territory size for *T. temporalis* and *N. tetracanthus* was defined as the area defended by the individual ([Mboko and Kohda 1995](#); [Matsumoto and Kohda 1998](#)), while territory size for *J. ornatus* was defined as the maximum distance the fish travelled from the breeding shelter ([Heg and Bachar 2006](#)). Using the combination of field-collected and literature data, we ran a Spearman's correlation between mean inflammation

Table 3 Average territory size of the fishes used in the current study. Values are presented as mean±standard error of the mean

Social system	Species	Territory size (m ²)	Sample size	Reference
Social	<i>N. pulcher</i>	0.37±0.06	13	Field observations
	<i>J. ornatus</i>	10.80±2.73	9	Heg and Bachar 2006
	<i>N. savoryi</i>	0.52±0.05	9	Field observations
Non-social	<i>T. temporalis</i>	0.97±0.08	13	Mboko and Kohda 1995
	<i>N. modestus</i>	0.67±0.16	12	Field observations
	<i>N. tetracanthus</i>	9.45±0.06	8	Matsumoto and Kohda 1998

following PHA injection and mean territory size for each species.

Ethical note During all procedures, we took care to minimize handling time and stress as much as possible for the study animals. The methods described for animal capture, housing, marking and treatments were assessed and approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol No. 10-11-71) and adhered to both Canadian and Zambian laws as well as the guidelines of the Canadian Council for Animal Care and the Animal Behaviour Society/Association for the Study of Animal Behaviour.

Results

Validation of PHA injection technique Intra-individual variation (% COV) in measurements was 1.5 % for the pre-injection measurements and 1.2 % for the post-injection measurements. The PHA injection caused a significant increase in local inflammation relative to the saline control in all species (Welch's *t* tests: *N. pulcher* $t_{17}=-3.6, p=0.002$; *J. ornatus* $t_{24}=-7.1, p<0.001$; *N. savoryi* $t_{22}=-2.1, p=0.04$; *T. temporalis* $t_{24}=-4.4, p<0.001$; *N. modestus* $t_{18}=-4.5, p<0.001$; *N. tetraodon* $t_{23}=-4.0, p<0.001$).

Variation in PHA response among species There were significant differences in the inflammatory response to the PHA injection among the six species (Welch's ANOVA: $F_{5,102}=33.2, p<0.001$; Fig. 2). The highly social species *J. ornatus* displayed the highest inflammatory response, followed by the

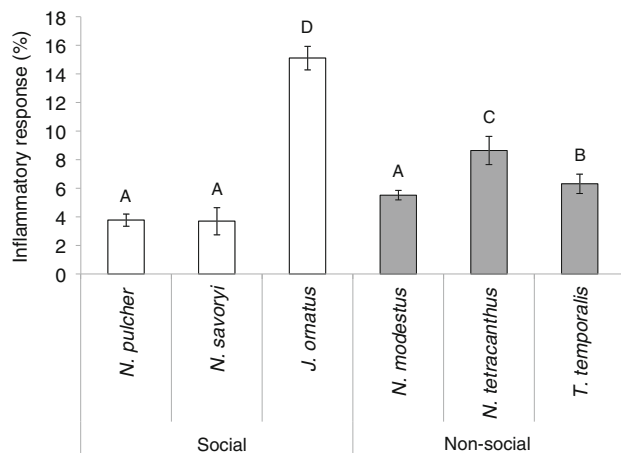


Fig. 2 The inflammatory response to an injection of phytohaemagglutinin (PHA) in six species of Lamprologine cichlids. Different letters indicate statistically significant differences among species (one-way Welch's analysis of variance model followed by Games-Howell post hoc test for unequal variances, $\alpha=0.05$; see Methods for full details). Values are presented as mean±standard error of the mean

non-social species *T. tetraodon* and then the non-social *T. temporalis*. The non-social species *N. modestus* and the social species *N. pulcher* and *N. savoryi* displayed the lowest inflammatory responses (Fig. 2).

Variation in PHA response between social and non-social species We found no difference in the average inflammatory response following PHA injection between social and non-social species (pGLS model: $\lambda=0.18, t_{1,5}=-0.35, p=0.74$; Fig. 3).

Post hoc analyses of territory size and PHA response We found a significant relationship between average territory size and average PHA response across our six species of social and non-social species (Spearman's $\rho=0.97, p=0.001$; Fig. 4).

Discussion

Social species are predicted to suffer from high rates of parasites and pathogens because frequent interactions with conspecifics can enhance pathogen transmission. In the current study, we predicted that highly social and cooperatively breeding cichlids would invest more heavily in immune defence against pathogens than non-social and non-cooperative cichlids, and used the inflammatory response to an injection of PHA as a measure of innate immune function. Although we

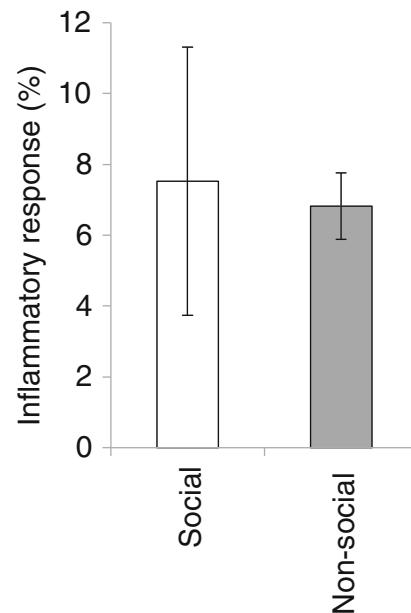


Fig. 3 There is no difference in the inflammatory response to an injection of phytohaemagglutinin (PHA) between social and non-social Lamprologine cichlids (phylogenetically correlated generalized least square model, $\alpha=0.05$; see Methods for full details). Values are presented as mean±standard error of the mean

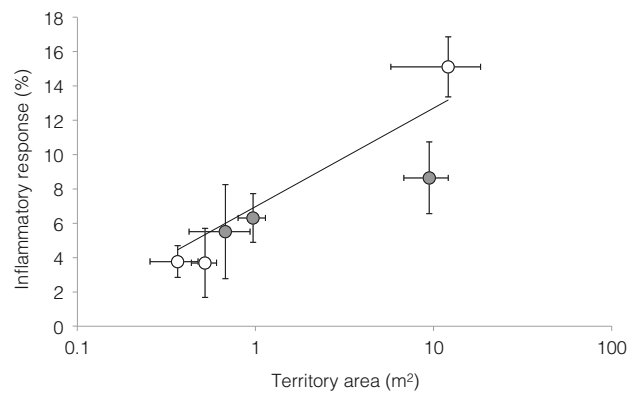


Fig. 4 The significant correlation (Spearman's correlation, $\alpha=0.05$; see [Methods](#) for full details) between territory size and the inflammatory response to an injection of phytohaemagglutinin (PHA) in six species of Lamprologine cichlids. White labels represent highly social species, while grey labels represent non-social species. Values are presented as mean \pm 95 % confidence intervals

found species-level variation in PHA response, we did not find differences in PHA response between social and non-social cichlids.

Our phylogenetic tree was based on three mitochondrial genes, and was consistent with previous analysis of phylogenetic relationships among Lamprologine cichlids using mitochondrial genes (Sturmbauer et al. 1994, 2010; Day et al. 2007). However, these mitochondrial phylogenies show some differences from a phylogeny built using nuclear genes (Sturmbauer et al. 2010). Namely, the nuclear phylogeny places *N. savoryi* as a sister species to *N. pulcher*. Sturmbauer et al. (2010) suggest that historic introgressive hybridization may explain the discrepancies between the nuclear and mitochondrial phylogenies of the Lamprologine cichlids, and inferring speciation history from the mitochondrial phylogeny alone can therefore be misleading. For the purposes of the current study, however, the placement of *N. savoryi* within the phylogeny does not have an effect on the overall results. Excluding *N. savoryi* from the dataset, and doing pairwise comparisons of species that differ in the degree of sociality (Felsenstein 1985) with both the mitochondrial and nuclear phylogenies, all yield the same result that social and non-social species do not differ in their inflammatory response to PHA injection with the current dataset and sample size.

We found that the highly social cichlid *J. ornatus* exhibited the highest inflammatory response to an injection of PHA, followed by the non-social cichlids *N. tetracanthus* and *T. temporalis*. The non-social cichlid *N. modestus*, and the highly social *N. pulcher* and *N. savoryi* had the lowest inflammatory response to the injection of PHA. Seeing this pattern in the data, we hypothesized that rates of interactions with in-group versus out-group conspecifics may be an important factor driving immune function in these cichlids. In terms of interaction rates, *N. pulcher* and *N. savoryi* live in social

groups that hold small territories within large colonies. Individuals of both species interact frequently with members of their own group and colony, but very rarely with fish from outside the colony (Heg et al. 2005; Wong and Balshine 2011). Conversely, *J. ornatus* are facultative rather than obligate cooperative breeders (Awata et al. 2005), do not live in large colonies and range more extensively than other species from their territories when they are not breeding (Brichard 1989). Further, subordinates are recruited from outside the group in *J. ornatus* (Heg and Bachar 2006), suggesting high rates of out-group interactions. Therefore, we predicted that the difference among the species in the degree of typical out-group interactions might account for some of the observed variation in inflammatory response. There is less information available on interaction rates for the non-social species, but all species live in permanent territories. As a preliminary exercise, we examined territory size as an approximation of the potential for out-group interactions in relation to the inflammatory response to PHA injection in the six species of cichlids in the current study. For simplicity, we assumed that with larger territories, social interactions would be more likely to occur with unfamiliar out-group individuals. With this preliminary post hoc analysis, we found that territory size is strongly positively correlated with innate immune function in our six species of social and non-social cichlids. Territory size for *J. ornatus* was calculated slightly differently than territory size for the other five species. However, we also ran the correlation excluding *J. ornatus* and found that the correlation between PHA inflammatory response and territory size was still significant in the remaining five species. Thus, we feel that this finding is robust even with some variations in the definitions and calculations of territory size. While the scope of these results is limited by the post hoc nature of the analysis and our small sample size, the significant positive correlation between territory size and inflammatory immune response suggests that the notion that group living or cooperative breeding will inherently lead to increased costs of parasitism may be an oversimplification. Rather, a variety of factors likely influence exposure to parasites and immune function in fishes and other vertebrates, potentially including relative rates of within- and between-group social interactions.

The relationship between territory size and immune function could be mediated by the endocrine responses that underlie territorial behaviour, particularly by androgens, nonapeptides or both. Androgens are elevated during aggressive territorial defence in a variety of taxa (Wingfield et al. 1990), including in Lamprologine cichlids (Hirschenhauser et al. 2004; Desjardins et al. 2006). Elevated androgens also have a suppressive effect on immune function (Hau 2007). Similarly, increased levels of the nonapeptide hormone vasopressin (arginine vasotocin in fishes) are associated with increased aggressive territorial behaviour in birds and fishes (Goodson and Evans 2004; Santangelo and Bass 2006), and

also have a suppressive effect on immune function (Shibasaki et al. 1998). However, more detailed information on interaction rates, and information on the rates and the intensity of territorial defence behaviour, is necessary in order to fully elucidate these relationships. Alternatively, the relationship that we found between territory size and immune function could arise because holding a larger territory simply increases the number of pathogens that an individual encounters within their environment, independent of pathogens transmitted by social interactions. For example, risk-taking behaviour is related to pathogen exposure and therefore immune investment, with more bold exploratory animals being exposed to more pathogens (Kortet et al. 2010; García-Longoria et al. 2014). In summary, our preliminary post hoc results highlight the value in future studies that explicitly investigate immune function in relation to territory size and associated factors such as conspecific interaction rates, territorial behaviour and exploratory behaviour.

In the current study, we used injections of PHA as a measure of the innate inflammatory immune response. This has proven a useful measure of immune investment in many ecological studies across a wide variety of taxa (e.g. Smits and Williams 1999; Smits et al. 1999; Møller et al. 2001; Ardia and Clotfelter 2006; Martin et al. 2006; Spottiswoode 2008). However, injections of PHA only provide a single measure of the innate cell-mediated response to an immune challenge. The immune response is complex, and using a panel of assays that measure multiple aspects of both innate and acquired immune responses provides a more comprehensive measure of immunocompetence and investment in immune defence than any single metric (Norris and Evans 2000). We encourage future studies to incorporate multiple measures of immune function, in order to obtain a clearer picture of immune investment. It is worth noting that immune responses are also influenced by stress (Padgett and Glaser 2003; Tort 2011). Stressors such as the capture and holding that our fish experienced are known to influence multiple aspects of immune function in fishes (e.g. Yin et al. 1995; Montero et al. 1999; Ortuno et al. 2001). Thus, it is possible that differences we saw among species reflect, at least in part, differences in the ability of the fish to maintain a robust inflammatory response following a stressor, rather than differences in baseline immune response. However, as all individuals were treated equally, this measure still reflects an ecologically relevant aspect of immune functioning.

It is possible that the fish species used in this study differed in parasite or pathogen infection burdens, rather than differentially investing in immune defence against parasites and pathogens. To our knowledge, no studies to date have examined both of these parameters in the same set of species. Research investigating both parasite and pathogen loads as well as investment in immune defence would be useful and provide insight into how the two aspects of pathogen risk and

defence co-vary with social system. Finally, environmental factors play a role in both the prevalence of pathogens and the mode of transmission (Agnew and Koella 1999). Preliminary studies have begun to document the fish parasite communities of Lake Tanganyika (see Vanhove et al. 2010, 2011; Gillardin et al. 2011; Bukinga et al. 2012; Raeymaekers et al. 2013). Parasites that transfer directly from fish to fish (e.g. monogeneans, Vanhove et al. 2010, 2011; Gillardin et al. 2011; Bukinga et al. 2012; Raeymaekers et al. 2013) as well as parasites that require an intermediate host (e.g. acanthocephalans and digeneans, Raeymaekers et al. 2013) have been described and found in Tanganyikan cichlids of Lake Tanganyika. However, there has been no investigation to date on the common parasites of the Lamprologine cichlids or their host specificity. More detailed investigation of the parasite and pathogen communities of the Lamprologine cichlid tribe would be useful and help clarify the relationship between social interactions and parasites and pathogens.

Our results contribute to an emerging and complex picture of the relationship between immunology and sociality. We suggest that the straightforward view that social species face greater costs than non-social species defending against parasites and pathogens is overly simplistic and must be refined to fit with new empirical results. In the current study, we collected data from only six species of fish, and we are therefore limited in our extrapolation of this data. However, this study is one of only a very few that examine sociality in relation to immune function. Despite the small sample size, our preliminary result that territory size is positively correlated to the innate immune response is intriguing and points out a clear direction for future research. Why are these species so variable in their immune function? Would patterns of social interactions, or other aspects of behaviour such as territorial or exploratory behaviour, help to explain the variation in immune function that we recorded? And finally, what are the fitness consequences of such wide variation in immune response? Parasites and disease are an extremely important ecological and evolutionary force (Hamilton et al. 1990; Ridley 1993; Fincher and Thornhill 2012; Diepeveen et al. 2013). There is strong selection on traits that are relevant to immune function, and understanding variation in immune function is important for understanding phenotypic diversity among species (Diepeveen et al. 2013). Higher immune costs also sequester resources from other functions, including reproduction and potentially even lifespan (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Norris and Evans 2000). Thus, the large variation in immune response we have documented among these cichlid fishes will have implications for fitness trade-offs among these species. Understanding the relationship between parasite and pathogen pressure and ecological traits in cichlids is an essential aspect of understanding the evolution of these fishes.

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