

**Team colours matter when playing away from home: aggression biases in geographically isolated  
Mbuna cichlid populations**

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The rocky shore habitats of the African Great Lakes support high densities of cichlid fishes, including many closely-related/ecologically similar species. Aggressive behaviours between conspecifics, and perhaps heterospecifics, influences this unusually high level of species coexistence. In dichotomous choice aggression trials, male *Maylandia thapsinogen* were presented simultaneously with two heterospecific intruders (*Maylandia emmiltos* and *Maylandia zebra*). *M. thapsinogen* were significantly more aggressive towards intruders from an allopatric species (similar orange dorsal fin colour - *M. emmiltos*), than towards a different allopatric species (blue dorsal fin - *M. zebra*). Aggression biases disappeared when colour differences were masked using monochromatic lighting. A second experiment compared female aggression biases between *M. emmiltos* with *M. thapsinogen*, species similarly coloured to one another, the former possessing a yellow, as opposed to a black throat as the latter does. *M. thapsinogen* preferentially attacked females of their own species in full but not monochromatic light, while female *M. emmiltos* showed no significant bias in aggression under any lighting. Responses were not affected by olfactory cues provided by the stimulus fish. These results indicate that divergence in colour might facilitate species co-existence in some cases, but not all, which could be important should populations re-join through lake level drops.

**Keywords**

**Fish; heterospecific aggression; coexistence; diversity; cichlids**

**Introduction**

Many sympatric species and allopatric populations of haplochromine cichlid fish differ strikingly in male colour (Allender *et al.*, 2003) and this is particularly true for the rock-dwelling ‘mbuna’ cichlids of Lake Malawi (Konings, 2007). Closely related sympatric mbuna species may occupy very similar trophic niches, differ little in morphology (Genner and Turner, 2005), show extremely conserved behaviour (Myers, 1960) and differ in colour traits associated with secondary sexual characteristics

(Konings, 2007). Furthermore, geographical variants of fish considered the same species may differ slightly in traits such as dorsal fin or throat colour (e.g. *Labeotropheus fuelleborni*, Konings 2007; p30). Divergence in signals associated with sexual selection is thought to play a key role in speciation in cichlid fishes (Seehausen, 1996; Maan *et al.*, 2004), as well as in other taxa such as the Hawaiian *Drosophila* spp. complex (Boake *et al.*, 2000; Coyne *et al.*, 2002).

Male mbuna cichlids compete aggressively for territories and those males that are unable to secure a territory are unlikely to breed successfully (Genner and Turner, 2005). Territorial males are generally more aggressive to conspecific intruders, with which they compete for mates as well as other resources such as food and shelter (Genner *et al.*, 1999). Differences in male signal traits are associated with reduced interspecific aggression in sympatric cichlid fish (Pauers *et al.*, 2007; Dijkstra *et al.*, 2006), birds (Alatalo *et al.*, 1994), damselflies (Anderson *et al.*, 2010; Tynkkynen *et al.*, 2004) and other taxa (Grether *et al.*, 2009). Aggression biases in some cases are stronger in sympatric populations, leading to suggestions that agonistic character displacement may be a significant process in evolutionary processes (Grether *et al.*, 2009), facilitating co-existence of recently diverged species (Seehausen and Schluter, 2004). Field studies have suggested that there is reduced competition between differently coloured cichlid fishes in Lakes Victoria (Seehausen and Schluter, 2004) and Malawi (Young *et al.*, 2009).

Reduced interspecific aggression need not only apply to males; females too can be territorial (Dijkstra *et al.*, 2008; Genner and Turner, 2005). Signal traits that are thought to influence aggressive responses may be divergent in females as well as males. Thus, where pairs of competing species co-occur, if signal divergence reduces aggression towards females, the resulting fitness benefit could augment selection for differentiation in traits normally associated with male signalling.

Laboratory experiments indicate suggests that mbuna dorsal fin colour can influence biasing of aggressive responses (Pauers *et al.*, 2008), but other colour elements may diverge among closely-related allopatric populations. In Lake Malawi, several populations of “red top zebra cichlids” are characterised by striking blue and black striped males with bright orange dorsal fins. Among these, the fish at Chimwalana or Eccles Reef (classed as *M. thapsinogen*) are unique in having yellow rather than black throats. Aquarium observations suggest that these additional patches may serve some purpose as they are expanded and thus made more visible during threat displays. A phylogenetic analysis (Allender *et al.*, 2003) suggests that the nearby, but dissimilar, colour forms like *M. zebra* (Chiofu Bay)

and *M. thapsinogen*, are more closely related than are geographically distant but phenotypically similar colour forms, such as *M. thapsinogen* and *M. emmiltos*. Blais *et al.*, (2009) investigated the strength of reproductive isolation in these three populations and found some assortative mating between *M. emmiltos* and *M. thapsinogen*, suggesting that cues other than dorsal fin colour, such as throat pigmentation, may influence mate choice.

Aggression between presently allopatric populations is of interest as water levels in Lake Malawi have been shown to rise and fall over geological time (*e.g.* Scholz *et al.*, 2007). This could force allopatric populations previously confined to separate rocky headlands or islands to mix. It has been suggested that coexistence of near-identical fish species may be promoted by subtle colour differences alone (Seehausen and Schluter, 2004), but does the appearance of visible differences in colour necessarily indicate a significant biasing of aggression towards members of the same population? To test this, we employed laboratory trials to investigate the role of visual and non-visual (olfactory) cues in aggression by fish from allopatric populations of Malawian rock dwelling cichlids.

## Materials and Methods

### *Experimental animals*

The study populations used were members of the *Maylandia zebra* species complex (also known as *Metriaclima* - Stauffer *et al.*, 1997), endemic to Lake Malawi (Figure 1). All were formerly known as *M. zebra*, but several populations have been given formal species names based on differences in colour pattern and subtle differences in morphology (Smith and Kornfield, 2002). *Maylandia zebra* males are powder blue, with contrasting black heads and vertical bars on the flanks. Male *Maylandia emmiltos* are similar, but have a bright orange dorsal fin. *Maylandia thapsinogen* males not only have the orange dorsal fin, but also have bright yellow throat (branchiostegal or gular) membranes and the black flank bars extend into lower part of the dorsal fin. All fish were collected by professional aquarium fish collectors (Stuart M. Grant Ltd) in 2008. Adult *Maylandia thapsinogen* were obtained from Eccles (Chimwalana) Reef and *M. zebra* from Chiofu Bay, both in the southeastern part of the lake, while *M. emmiltos* were collected from Mphanga Rocks in the northwestern part of the lake.

### *Experiment 1*

Twelve *M. thapsinogen* males used as resident (focal) fish for this experiment were isolated within stock tanks (approximately 1:1 M: F ratio) before experiments began. To allow the fish to become

dominant and territorial, they were separated off by ‘partial partitions’ that allowed visual and olfactory contact with conspecifics but prevented intrasexual aggressive contact with other fish in the stock tanks for up to a week before experimentation. After experiments, males were placed in separate tanks to ensure fish were used only once as focal fish. In each trial, each resident fish was presented simultaneously with a size-matched pair of ‘intruders’ from allopatric populations differing in dorsal fin colour: one *M. zebra* ‘Chiofu’ (mean standard length *M. zebra* = 101.6 mm, S.D =  $\pm$  5.1mm) and one *M. emmiltos* (100.9 mm  $\pm$  7.1mm). Size-matching was intended to minimise the effect of individual fish size on aggression: the size asymmetry between the stimulus fish, calculated as  $(L-S)100/S$ , where L is the standard length of the larger fish, and S is the standard length of the smaller fish, was less than 5.5% (mean 0.7%) which is less than asymmetries found in published research using pairwise aggression trials in Lake Victoria cichlids (*e.g.* Dijkstra *et al.*, 2006).

The first experiment comprised of each focal fish being presented with its own intruder pair in a 1.5-meter tank containing a central refuge. Two transparent jars equidistant from the central refuge area held intruder males during the experiment. Transparent jars were un-perforated, preventing chemical communication between focal and stimulus fish. A focal male fish was added to the set up and allowed to acclimatize for 48 hours. This gave the resident male time to become territorial over the refuge provided which was essential for optimising male response towards intruders. One male intruder was randomly assigned a left or right side transparent jar and given 1 minute after being added to acclimate. Trials lasted 10 minutes, as in previous experiments using haplochromine cichlid aggression trials (Dijkstra *et al.*, 2006).

The second half of experiment 1 used the same transparent unperforated jars but colour differences were masked by wrapping the jars in orange/red optical filter acetate (Lee filters 026). This filter was chosen because it only transmits light at the wavelengths between 500–700 nm, effectively eliminating the difference between the orange and blue dorsal fin colour. Each focal fish was again tested with its own pair which was again randomly assigned to either the left or right jar.

All fish were kept on a 12L:12D light cycles and fed aquarium flake fish food. Tanks were maintained at 23 – 26 degrees C.

#### *Experiment 2*

Female *M. thapsinogen* and *M. emmiltos* were tested to determine whether subtle differences in pigmentation may lead to aggression biases among these two very similar allopatric members of the *M.*

zebra complex. Females came from mixed-sex stock tanks containing only their own species. Eight females of each species were used as focal fish. The 16 focal fish were size matched in pairs (one of each species) and three stimulus pairs were randomly assigned to each focal fish. Therefore, many of the focal fish were also used as stimulus fish. Each stimulus female pair were size matched for standard length (mean *M. thapsinogen* = 82.3 mm; S.D. = 4.4mm; mean *M. emmiltos* = 86.25 mm; S.D = 3.1mm).

Each focal fish was presented with three pairs of intruders in all three treatments. In all trials for each of the three treatments (below) focal fish were housed in a 1.5 metre tank with a central refuge. Two jars were placed equidistant from this central refuge. One intruder was randomly assigned one end of the tank whilst the other occupied the remaining jar. During initial trials of the setup, it was found that 24 hours was sufficient for females to become aggressive towards intruders. The time of each trial was also reduced to 5 minutes, following previous studies on haplochromine female aggression (Dijkstra *et al.*, 2008). In the first treatment, visual and olfactory cues were made available by placing the intruders in translucent, perforated transparent plastic jars. The trial began when the two intruders were added simultaneously to the jars. All 16 focal fish (eight of each species) were tested this way. The second treatment used unperforated jars preventing potential olfactory communication. The same three pairs of intruders, used in the first treatment, were used again for the same focal fish but they were randomised with respect to the order they were presented and side of intrusion with respect to the first treatment. Again, all 16 focal fish were tested this way before the third and final treatment. The third treatment replicated the two preceding experiments but jars were wrapped in red/orange acetate (same filter as experiment 1 – again without perforation) in an attempt to remove colour differences between the two intruder fish species (*i.e.* throat colour). Again, the focal fish were presented with the same triplicate of intruder pairs, which were again randomised with respect to order and side of central refuge. Trials were sequential elements within a replicate, however at least 72 hours were given between.

The results for each focal fish in any given treatment were pooled and a mean number of aggressive behaviours given to each type of intruder was calculated. This attempted to mitigate against the smaller sample size ( $n = 8$ ) for each species tested. Experiments were carried out over a six-week period in 2009. All fish were kept on a 12L:12D light cycles and fed aquarium flake, tanks were maintained between 23 and 26 degrees C, and filtered with biological filters, including activated charcoal.

In both male and female experiments, stimulus fish behaviour was not quantified due to the difficulty of recording both focal and stimulus fish behaviour simultaneously. However, it was noted than stimulus fish always initially explored the jar in which they were enclosed and would react aggressively to aggressive behaviours given by the focal fish.

#### **Ethical statement**

All experiments were approved by the Bangor University Ethical Review Committee.

#### **Data collection and analysis**

Data sets were analysed with respect to distribution and equality of variances. If assumptions for parametric testing were not met, transformation attempts were made. All data was analysed in SPSS v20.

#### **Results**

##### *Experiment 1*

Male *M. thapsinogen* (orange dorsal) performed significantly more attack and threat behaviour towards intruding male *M. emmiltos* (orange dorsal) rather than male *M. zebra* (blue dorsal) (paired t-test,  $t = 2.2$ , d.f. = 11,  $p = 0.02$ ; figure 2, table 1). There was no significant bias when the experiment was repeated under monochromatic light (paired t-test,  $t = -1.9$ , d.f. = 11,  $p = 0.83$ ; figure 2, table 1).

## Experiment 2

Yellow-throated female *Maylandia thapsinogen* performed significantly more aggressive behaviour towards females from their own population than to female *M. emmiltos*, under full sensory contact (paired t-test,  $t = 5.4$ , d.f. = 7,  $p < 0.001$ ; figure 3, table 2) and when intruders were held behind solid partitions preventing olfactory contact (paired t-test,  $t = 8.2$ , d.f. = 7,  $p < 0.001$ ), but again the bias was lost under monochromatic light (paired t-test,  $t = 8.3$ , d.f. = 7,  $p = 0.21$ ; figure 4, table 2). By contrast, female *M. emmiltos* showed no significant bias towards either intruder population under any of the experimental treatments: whether full sensory contact (paired t-test,  $t = 0.55$ , d.f. = 7,  $p = 0.29$ ), olfactory contact blocked ( $t = 1.497$ , d.f. = 7,  $p = 0.089$ ) or monochromatic light ( $t = 0.69$ , d.f. = 7,  $p = 0.183$ ) (fig.4, table 2).

## Discussion

The results of this study suggest that colour cues influence targeting of aggressive responses by some male mbuna cichlid fish: resident male *M. thapsinogen* were more aggressive towards the intruder which was most similar to itself. This bias was lost when intruders were presented under monochromatic light, suggesting that colour differences, presumably in the dorsal fin, played a role in the targeting of male territorial aggression. Similar findings have been reported for other cichlid fish species, including *Labeotropheus* from Lake Malawi (Pauers *et al.*, 2008) and *Pundamilia* from Lake Victoria (Dijkstra *et al.*, 2010). However, our study was novel in indicating a role for colour (but not olfaction) in targeting of aggression by female cichlid fishes, and in demonstrating that closely-related species may differ in the significance of differences in particular pigmentation elements.

Many animals rely on colour vision and visual cues are much more effective over the distances that concern territorial and non-territorial mbuna fish. Territories are typically no smaller than 1 meter in diameter (*e.g.* Holzberg, 1978) at which distance colour differences are probably very clear to these fish. This is in contrast with olfactory cues which by their nature are likely to be diluted or completely lost at anything other than very small distances in a still-water aquatic environment. However, Bayani *et al.*, (2017) found evidence that another African Great Lake cichlid *Neolamprologus pulcher*, from Lake Tanganyika, uses olfactory cues to actively communicate aggressive intentions, but species specificity of these olfactory cues were not investigated nor the distance at which they are detectable.

Cichlid fish have been studied extensively with respect to the visual pigments they use and the likely range of colours they can see (Dalton *et al.*, 2011). Haplochromine cichlids possess 7 cone opsin genes (Dalton *et al.*, 2011) with minor variation in opsin sequences among the rock-dwelling species of Lake Malawi (Carleton and Kocher, 2001; Spady *et al.*, 2005). They are believed to have colour vision including sensitivity to UV wavelengths (at least in *M. zebra* – Carleton *et al.*, 2001). Habitat type, water depth, bottom depth, available sunlight and proximity to water bottom all affect irradiance and radiance of the light field in aquatic environments, but the background water column will generally appear blue or blue-green (Sabbah *et al.*, 2011). Yellow pigments (roughly middle of the colour spectrum, > 550nm) are very common in Lake Malawi cichlids (see Konings, 2007) and are believed to have evolved numerous times in haplochromine cichlids. This is probably because in clear lake waters, yellow contrasts most with blue in cichlid vision (Boughman, 2001; Deutsch, 1997).

In our experiments, female *Maylandia thapsinogen* showed a significant tendency to threaten or attack conspecifics rather than intruders of a very similar species. As the stimulus females differed in throat pigmentation, and often expand this throat membrane during threat displays, it seems possible that this might be used as a species-specific cue in aggression. In contrast, *Maylandia emmiltos* females do not appear to be more aggressive towards their own species. Perhaps the yellow throat of *M. thapsinogen* is a derived state, actively selected as a distinct signal, and therefore it would not be surprising if fish from populations lacking this signal do not differentiate among individuals with different throat colours.

It has been proposed that disruptive selection on nuptial colours may reduce interspecific aggression in sympatric cichlids and field studies have provided support for this idea (Seehausen and Schluter, 2004). Studies of birds and damselflies have suggested that sympatric co-occurrence of competing species has led to selection for divergent signals to reduce competition (Alatalo *et al.*, 1994; Anderson and Grether, 2010; Tynkkynen *et al.*, 2005). However, a study of North American salamanders showed markedly higher levels of aggression to heterospecifics when they co-occurred in sympatry, suggesting selection for enhanced aggression in the presence of resource competitors (Deitloff *et al.*, 2009). Both responses suggest evolutionary change if in sympatry in response to heterospecific competition. If disruptive selection is acting to reduce interspecific aggression in rock-dwelling cichlids, then the evolution of



additional yellow pigment elements would probably be most effective in clear water habitats. In fact, at several locations around Lake Malawi the placing of a patch of yellow/orange pigmentation does differ between sympatric species. However, allopatric populations of the same species also vary colour, including in yellow/orange markings (e.g. *Labeotropheus fuelleborni*, Konings 2007; p30). This could be the result of intraspecific selection for sexually selected signal traits, but it seems possible that there could also be an influence of heterospecific interactions, suggesting that further investigation of the local community structure and behavioural interactions could be useful.

Within a complex species-rich group such as the haplochromine cichlids, taxa may vary in the role played by visual signals in species co-existence. *Maylandia* spp. are broadly considered trophic generalists in their environment and may tolerate other heterospecific generalists (Genner *et al.*, 1999). By contrast, more specialised feeders, such as *Tropheops* spp. in Lake Malawi and *Tropheus* spp. from Lake Tanganyika appear intolerant of heterospecifics with similarly specialised feeding behaviour (Kohda and Mboko 1994; Genner *et al.*, 1999). Thus, selection for signal divergence among sympatric heterospecifics might be expected to vary among taxa, depending on the ecological context.

Divergence in male signals may sometimes be facilitated by a synergistic interaction between divergent female choice and disruptive selection for the reduction in heterospecific aggression among males: this may lead to increased reproductive isolation as well as reduced competition. If divergent allopatric populations are partially reproductively isolated before secondary contact occurs (e.g. Knight & Turner, 2004) the result, through interbreeding between geographical variants, may be polymorphism of male nuptial colouration in sympatry. This has been proposed as a pre-requisite in models describing how Lake Victoria haplochromine cichlids may have diverged sympatrically (Dijkstra *et al.*, 2005).

In our experiments, *M. thapsinogen* females showed much higher levels of aggression than those of *M. emmiltos*, particularly when all sensory cues were available. This difference may reflect differences in selection pressures among locations, for example in terms of resource competition. This may be difficult to test in short-term field studies, as it has been shown that food abundance varies seasonally in mbuna communities (Marsh *et al.*, 1986).

In conclusion, our investigations have shown that pigment elements such as dorsal fin and throat membrane colour may be important stimuli in triggering aggressive responses by haplochromine cichlid fishes of both sexes, but that the significance of these signals varies among species. It seems possible that such divergence in signal and response may contribute to species co-existence in sympatry.

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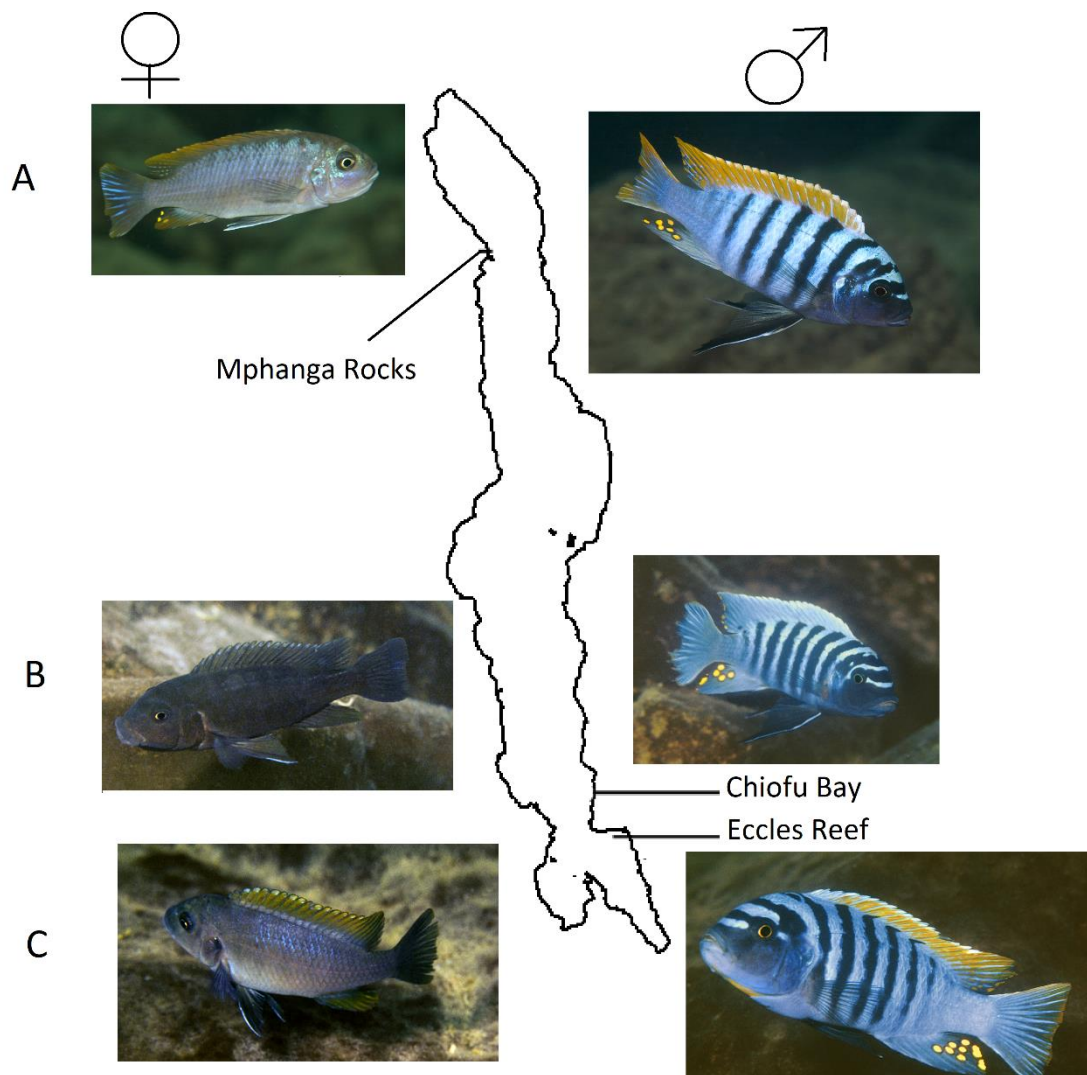
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378 Figure1. Location of study species wild caught from Lake Malawi in 2008 used in this study. A)

379 *Maylandia emmiltos*, B) *M. zebra*, and C) *M. thapsinogen*. Photographs by Ad Konings

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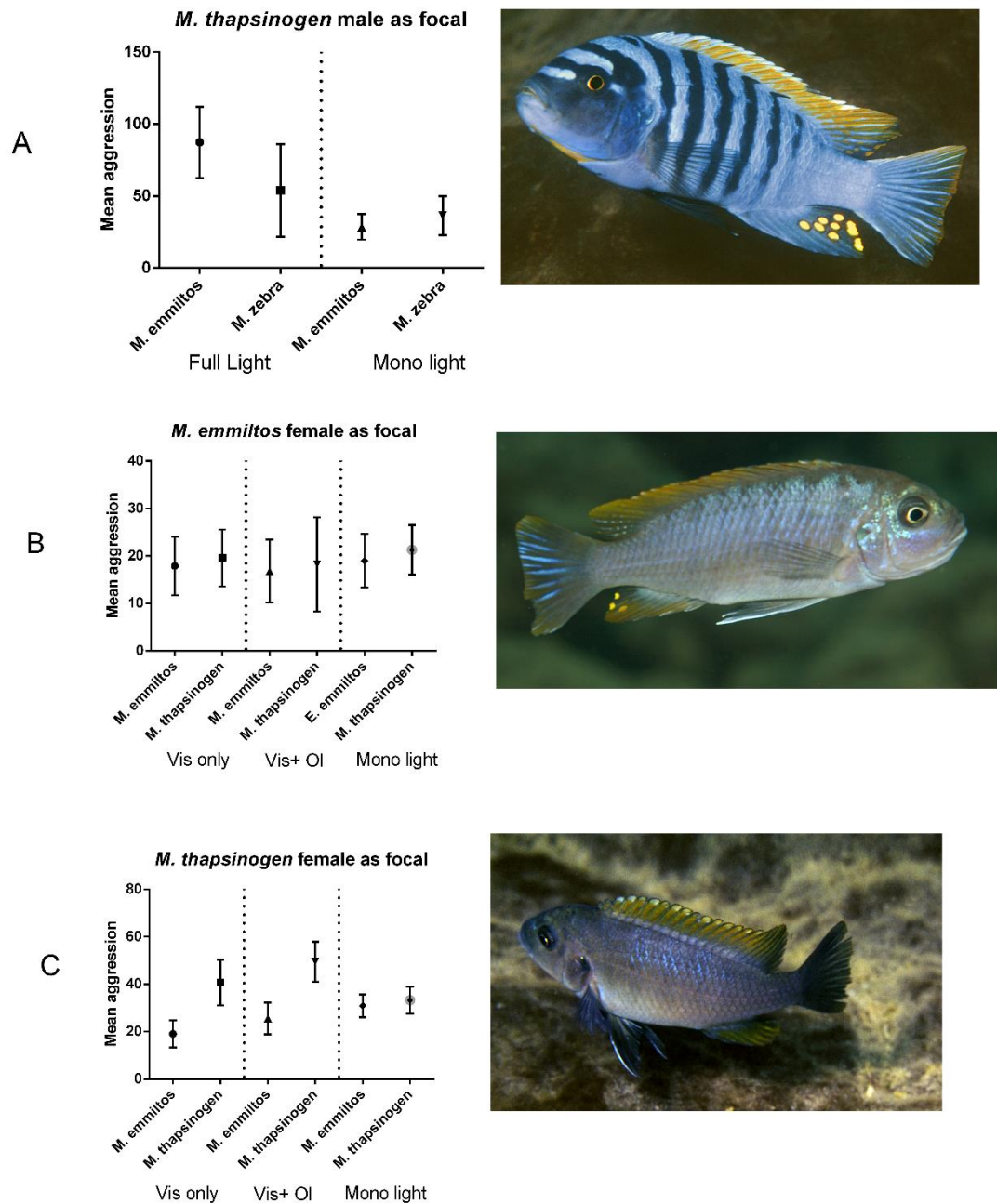


Figure 2. A) Resident male *Maylandia thapsinogen* showed significantly more aggression towards *M. emmiltos* than *M. zebra* intruders when full-spectrum visual cues were available, but not under monochromatic light treatment, N = 12; B) *Maylandia emmiltos* females showed no significant differences in levels of aggression towards conspecifics or females of the yellow-throated *M. thapsinogen* under any of the experimental treatments, N = 8 focal fish tested against three intruder pairs; C) *Maylandia thapsinogen* females directed significantly more aggressive behaviour towards conspecifics than to female *M. emmiltos* under full-spectrum lighting conditions (Vis only/Vis+ Ol), irrespective of whether olfactory information was potentially available, but no significant difference

390 was apparent under monochromatic light (Mono light),  $N = 8$  focal fish tested against three intruder  
391 pairs. All plots show means and 95% confidence intervals.  
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**Table 1** All aggressive behaviours given during the two treatments for *M. thapsinogen* male focal fish. Non-contact displays (lateral display, frontal display and quiver) were pooled into the ‘display’ category, while overt aggression (lunge and bite) were pooled into the ‘attack’ category. S.E = standard error

Experiment	Behaviour	Mean	Mean	t	p
		Agg.(SE) <i>M. emmiltos</i>	Agg.(SE) <i>M. zebra</i>		
Full light	Display	64 (6)	39 (7)	2.05	0.03
	Attacks	23 (3)	16 (2)	1.97	0.04
	<b>Total</b>	<b>87 (7)</b>	<b>54 (9)</b>	<b>2.25</b>	<b>0.02</b>
Monochromatic light	Display	20 (2)	26 (3)	1.51	0.88
	Attacks	8 (1)	10 (1)	0.83	0.21
	<b>Total</b>	<b>29 (2)</b>	<b>42 (4)</b>	<b>1.40</b>	<b>0.83</b>

**Table 2** Aggressive behaviours performed during the three treatments (visual and olfactory cues, visual only, monochromatic light) for resident female *M. emmiltos* and *M. thapsinogen* presented simultaneously with a pair of intruders of each species. Non-contact displays (lateral display, frontal display and quiver) are pooled into ‘display’ category, and overt aggression (lunge and bite) pooled into ‘attack’ category.

Experiment	Focal	Behaviour	Mean	Mean	t	p
			Agg.(SE) Conspecific	Agg.(SE) Heterospecific		
Visual and olfactory	<i>M. thapsinogen</i>	Display	24 (2)	12 (2)	4.60	0.001
		Attacks	25 (2)	14 (2)	4.51	0.001
		<b>Total</b>	<b>49 (3)</b>	<b>26 (2)</b>	<b>5.95</b>	<b>&lt;0.001</b>
	<i>M. emmiltos</i>	Display	11 (2)	12 (2)	0.55	0.30
		Attacks	6 (1)	6 (2)	0.40	0.34
		<b>Total</b>	<b>17 (2)</b>	<b>18 (4)</b>	<b>0.58</b>	<b>0.29</b>
Visual only	<i>M. thapsinogen</i>	Display	25 (2)	11 (2)	8.67	< 0.001
		Attacks	16 (3)	8 (2)	5.61	< 0.001
		<b>Total</b>	<b>41 (3)</b>	<b>19 (2)</b>	<b>8.23</b>	<b>&lt;0.001</b>
	<i>M. emmiltos</i>	Display	14 (2)	15 (1)	-0.68	0.25
		Attacks	4 (1)	5 (1)	-1.27	0.12
		<b>Total</b>	<b>18 (2)</b>	<b>20 (2)</b>	<b>-1.50</b>	<b>0.089</b>
Monochro matic	<i>M. thapsinogen</i>	Display	15 (2)	14 (1)	0.49	0.32
		Attacks	18 (1)	17 (2)	0.87	0.20
		<b>Total</b>	<b>33 (2)</b>	<b>31 (2)</b>	<b>0.83</b>	<b>0.21</b>
	<i>M. emmiltos</i>	Display	11 (1)	11 (1)	-0.38	0.357
		Attacks	8 (1)	10 (2)	-1.613	0.07
		<b>Total</b>	<b>19 (2)</b>	<b>21 (1)</b>	<b>-0.97</b>	<b>0.183</b>