

## Discrimination of mates and intruders: visual and olfactory cues for a monogamous territorial coral reef butterflyfish



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Recognition of conspecifics is essential for territorial and monogamous animals in order to maintain pair bonds, mate-guard and defend territories. However, cues required for mate discrimination are essentially unknown in monogamous fishes, despite the importance of recognition behaviour that promotes this mating system. This field study tested the role of visual and olfactory cues in the discrimination of mates and unfamiliar conspecifics in the territorial, socially monogamous pebbled butterflyfish, *Chaetodon multinctus*. A series of model bottle field experiments presented cues from a mate and an unfamiliar nonmate to a focal resident fish within its feeding territory. When both visual and olfactory cues were first matched, the resident spent more time and engaged in more agonistic displays near the bottled intruder. In contrast, when olfactory cues of bottled fish were first mismatched, the resident fish spent equivalent time at each bottle stimulus. When scent cues were then reversed to a matched odour condition (odour released next to bottled fish), the resident again spent more time with the nonmate. Resident fish spent equivalent time with the mate and nonmate in the absence of any associated odour stimuli. In addition, resident fish did not show a differential response when the mate's odour was associated with only one of two intruder fish. Thus both visual and olfactory cues are necessary for butterflyfish to discriminate their mate from intruders within feeding territories. These results indicate that mate recognition in animals with long-term pair bonds may require multimodal stimuli and future studies on mate recognition should address multiple sensory channels.

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Individual recognition of mates is of profound importance for animals that maintain long-term social bonds and is especially important for territorial species. Recognition of specific individuals is important for social interactions (Mateo, 2004; Tibbetts & Dale, 2007; Wiley, 2013) and is well documented for many invertebrates (Gherardi & Tiedemann, 2004; Karavanich & Atema, 1998; Müller, Eggert, & Elsner, 2003), fish (Griffiths, 2003; Griffiths & Ward, 2011), amphibians (Bee & Gerhardt, 2002), reptiles (Carazo, Font, & Desfilis, 2008; Husak & Fox, 2003), birds (Clark, Boersma, & Olmsted, 2006; Robertson, 1996; Wanker, Apcin, Jennerjahn, & Waibel, 1998; Wascher, Szipl, Boeckle, & Wilkinson, 2012) and mammals (Charrier & Harcourt, 2006; Koren & Geffen, 2011; Miller & Thomas, 2012). Cues and signals used for recognition of mates in monogamous species are important to maintain pair bonds, yet only a few studies have rigorously investigated

multimodal cues used to distinguish mates from unfamiliar conspecifics (Tibbetts & Dale, 2007). When monogamous individuals encounter conspecifics after a period of separation from their mate, rapid discrimination of the mate from a territorial conspecific intruder can be favoured. Failure to distinguish a mate from another territorial conspecific could weaken the pair bond and result in an injurious attack or loss of mate and territory.

The evidence for discrimination of familiar from unfamiliar conspecifics by fishes comes primarily from laboratory studies on shoaling behaviour (Griffiths, 2003; Griffiths & Ward, 2011) in nonreproductive contexts. In addition, several freshwater or estuarine fish species recognize and discriminate familiar from unfamiliar nonkin (Brown & Colgan, 1986; Brown & Smith, 1994; Croft, Arrowsmith, Webster, & Krause, 2004; Edenbrow & Croft, 2012; Ward, Duff, Horsfall, & Currie, 2008). Discrimination and individual recognition is associated with visual (Fricke, 1973), acoustic (Myrberg & Riggio, 1985), electrosensory (Painter & Kramer, 2003), olfactory or possible multiple cues and signals (Hojesjo, Johnsson, Petersson, & Järvi, 1998; Noble & Curtis, 1939; Sogabe, 2011). However, the relevant stimuli used for mate discrimination are not

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well defined and may be especially important for the maintenance of monogamous and harem social systems.

Monogamy is relatively rare among teleost fishes (Barlow, 1984; Whiteman & Côté, 2004); however, individuals must discriminate mates from nonmates after periods of separation in order to maintain the pair bond. In addition, mate recognition may be of value for harem species in which males defend several females in a social group (Aldenhoven, 1986; Gaisner, 2005; Hourigan, Stanton, Motta, Kelley, & Carlson, 1989; Neudecker & Lobel, 1982). Few studies have examined the proximate cues required for discrimination of mates from nonmates in monogamous fishes in natural settings. The butterflyfishes (family Chaetodontidae) are speciose (>120 spp.) and well represented on Indo-Pacific coral reefs (Nelson, 2006). Members of this family exhibit no parental care, lack obvious sexual dimorphisms, and many are socially monogamous and territorial (Hourigan, 1989; Reese, 1975; Roberts & Ormond, 1992; Whiteman & Côté, 2004). Some species maintain long (up to 10 years) socially monogamous pairings (Hourigan, 1989) and thus are able to discriminate between mates and nonmates that enter feeding territories (Driscoll & Driscoll, 1988; Sutton, 1985; Tricas, 1989a; Yabuta, 2000). The efficient discrimination of familiar mates from unfamiliar intruder conspecifics could reduce territory incursions, enhance the efficiency of aggressive behaviours and eliminate misdirected aggression towards mates. Thus, because of their long-term monogamous pairing and site-attached territorial behaviours, butterflyfish are an excellent taxon to examine the in situ proximate cues used for discrimination of familiar and unfamiliar conspecifics.

Butterflyfish may employ multiple sensory cues (vision, olfaction, acoustic, mechanosensory) to facilitate rapid recognition of conspecifics and heterospecifics. Previous experimental observations on territorial behaviour in monogamous species show that aggressive territorial behaviour is initiated towards captive conspecifics and model fish placed within feeding territories (Fricke, 1986; Strang, 2005; Tricas, 1989a; Yabuta, 2008). These studies indicate that visual signals and cues are important in butterflyfish social interactions, but sound production during territorial behaviour is also involved (Tricas, Kajiura, & Kosaki, 2006). In addition, the close spatial interactions associated with agonistic behaviour and pairing may facilitate chemical communication. In this study we tested the hypothesis that both visual and olfactory cues are used for the discrimination of mate from nonmate conspecifics in a territorial and monogamous fish. A major prediction of this multimodal cue recognition hypothesis is that removal of one or more relevant stimuli should reduce the ability of a fish to distinguish its mate from a nonmate. We tested this prediction with a series of five two-choice experiments on a wild population of pebbled (multiband) butterflyfish, *Chaetodon multicinctus*, a monogamous and territorial species endemic to the Hawaiian archipelago. We assessed the responses of a free-swimming resident mate in its natural territory to presentations of olfactory and visual stimuli from a partner (its mate) and an intruder (an unfamiliar nonmate).

## METHODS

### *Experimental Pairs and Nonmate Fish*

Mate recognition experiments were conducted by scuba divers on adult pebbled butterflyfish (standard length, SL,  $\geq 72$  mm) that occur in pairs on shallow reefs along the northwest shore of the island of Hawaii. Experimental fish pairs were confirmed by direct observations for 10–15 min of pair swimming/following behaviour, feeding and common defence of territorial borders (Tricas, 1989a, 1989b). One member of the resident pair (the mate stimulus) was

randomly selected, captured by hand net and held away from the territory for approximately 10–20 min during set-up of the experimental materials. The remaining individual of the pair was used as the focal individual in the experiment. A third fish (the nonmate stimulus) was collected from a distant, nonbordering territory far away from the experiment area and selected to be a similar size to the mate stimulus individual. After each experiment (except in three trials of experiment 5), the mate and nonmate fish were measured (SL to the nearest mm) and sex determined underwater by gonad catheterization (Ross, 1984). The sex of the focal resident fish (which was not catheterized) was inferred after sex determination of its mate as territorial fish form heterosexual pairs (Hourigan, 1989; Tricas, 1989a).

### *Stimulus Presentations*

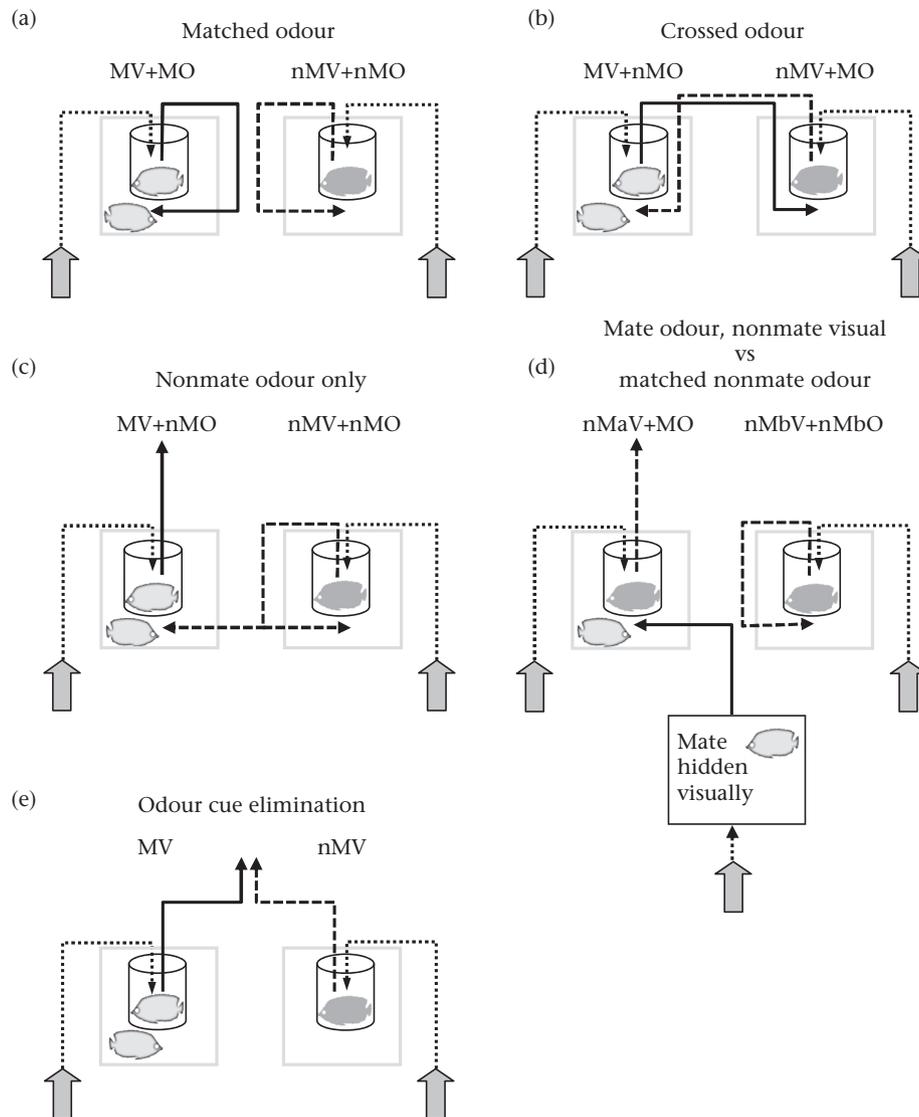
Experiments were designed to test the ability of one resident fish to discriminate between its mate and a nonmate conspecific based on associations with visual and chemical cues. The two stimulus fish (mate and nonmate) were transferred to separate glass bottles (3.8 litres, 22 cm wide) which were sealed with a metal lid fitted with two water flow tubes made of 13 mm clear vinyl tubing. Bottles with the two stimulus fish were then positioned approximately 2 m apart within the experimental arena, which consisted of two adjacent  $1.5 \times 1.5$  m ( $2.25$  m<sup>2</sup>) plots that were separated by an interbottle neutral zone distance of approximately 66 cm (Fig. 1). The experimental arena was outlined with marked nails for observation and positioned within the territory of the resident fish. Chemical cues from stimulus fish were produced by a flow of fresh sea water through the bottle via Atwood Water Buster submersible bilge pumps (750 litres/h) attached to the distal end of the incurrent tube positioned 2–3 m from the arena (Fig. 1a). The excurrent tube (3 m long) collected water flow from the bottom of the bottle and released the collected water and odour stimulants at either of the two fish bottles (see experiments below). After set-up, divers ascended approximately 5–10 m above the experimental arena and recorded the time that the focal individual (the free-swimming mate) spent within or outside the square plot around each bottle. Each 15 min observation period began when the resident fish first entered one of the two square plots in the experimental arena. Divers recorded with a water-resistant stopwatch (and logged on waterproof paper) the time that the resident fish entered and exited each of the two stimulus arenas (Fig. 1). From these data we determined the total time spent at, and the number of visits to, each stimulus.

### *Multimodal Mate Recognition Hypothesis Predictions*

A general prediction of the hypothesis that butterflyfish can discriminate mates from unfamiliar conspecifics is that fish will associate differentially with simultaneous presentations of a mate and nonmate. Several previous studies (Strang, 2005; Tricas, 1989a; Tricas et al., 2006) and pilot data showed that resident pairs were highly motivated to approach and interact with captive bottled fish that presented visual cues. Thus when necessary cues for discrimination were present, the test individual was predicted to associate differentially with nonmates and mates. The contribution of visual and olfactory cues was tested with a series of five experiments which are outlined with specific predictions below.

### *Experimental Trials*

We conducted five experiments (detailed below) of which the first four involved two phases. Phase 1 involved approximately 7.5 min of initial observations of the behaviour of the focal fish in



**Figure 1.** Diagrammatic representation of the experimental arenas used in field studies on mate and nonmate discrimination by the monogamous territorial pebbled butterflyfish, *Chaetodon multicinctus*. Mate and nonmate fish were placed into bottles and positioned 2 m apart within 2.25 m<sup>2</sup> plots (grey line, 1.5 m per side) that were separated by a distance of 0.66 m. Divers recorded the time spent in each plot by the free-swimming resident fish in association with its mate and nonmate. (a) Matched odour condition: fresh sea water was perfused by pump (grey block arrows) through each bottle to create a fish odourant that was released at the source fish bottle. (b) Crossed odour condition: fresh sea water was perfused by pump through each bottle to create a fish odourant that was released at the adjacent fish bottle. Submersible pumps perfused water through 3 m of tubing to the opposite bottle location: from mate to intruder and intruder to mate. (c) Nonmate odour only condition: fresh sea water was perfused through the bottle with a nonmate fish (right), which was then split and released at both the nonmate and mate bottles. Odorant from the bottled mate (left) was transported away from the experimental arena. (d) Mate odour, nonmate visual versus matched nonmate odour condition: two nonmates (nonmate A and B) were placed in bottles with one fish matched with its odour stimulus (left side) and the other paired with a nonmatched odorant from the captured mate held in a bottle covered with an opaque bag (right side). Perfused water from the second nonmate was transported away from the experimental arena. (e) Odour cue elimination: both mate and nonmate bottles were ventilated with pumped water, but all excurrent flow was carried away from the experimental arena. (a–e) Black dotted line arrows indicate 3 m tubing from pumps to bottles, black dashed line arrows indicate 3 m tubing carrying water from nonmate bottles, and solid black line arrows indicate 3 m tubing carrying water from mate bottles. MV: mate visual cue; MO: mate olfactory cue; nMO = nonmate olfactory cue; nMV: nonmate visual cue; nMaV: nonmate A visual cue; nMbV: nonmate B visual cue.

response to the treatment. After completion of the phase 1 observations, divers descended and changed the visual, olfactory or spatial configuration of stimuli, depending on the experiment. Divers then reascended and began the phase 2 observations to record the behaviour of the free-swimming mate in response to the modified stimulus for an additional 7.5 min. Experiment 5 (odour cue elimination) involved only one phase with an observation period of approximately 15 min.

#### Experiment 1: matched odour first

During phase 1, we tested the association of the free-swimming mate with its mate and a nonmate associated with the presentation

of both normal visual and olfactory cues, in which the excurrent tube was placed at the bottle of the odour source (matched odour stimulus condition, Fig. 1a). For phase 2, the excurrent tubes were switched (without change to the bottle positions) to the opposite bottle and fish, so that visual and odour cues of mates and nonmates were now mismatched (crossed odour stimulus condition, Fig. 1b). We expected that if fish are able to discriminate mates from nonmates, the resident fish would spend more time associated with one of the two stimuli. In the second phase of the experiment, if olfactory cues are necessary for discrimination, then resident fish would reverse any association observed in the first phase of the experiment.

### Experiment 2: crossed odour first

We recognized that habituation and precedence of the matched odour experiment described above may mitigate a reversal of association even if olfactory cues were recognized as important factors for consideration. Thus, we tested for an order effect of mate versus nonmate odour presentation to focal fish. In this experiment, phase 1 tested the crossed odour stimulus condition (Fig. 1b), with visual and odour cues of mates and nonmates mismatched. For phase 2, the configuration was reversed to the matched odour condition (Fig. 1a). We expected that if fish are able to discriminate mates from nonmates, two possible outcomes could occur during the first phase of the experiment. (1) If olfactory cues are the most important, then resident fish would show the opposite association observed in the first phase of matched odour first experiments. (2) However, if visual cues alone are sufficient for discrimination, resident fish would show the same association observed in the first phase of the matched odour first experiments. In the second phase of the experiment, a reversal of either outcome was expected.

### Experiment 3: nonmate odour only

We conducted nonmate odour only experiments to test whether the time spent in association by experimental fish was dependent solely on cues from the nonmate odour. In phase 1 of this experiment, the odour stimulus from the nonmate fish was first released at both the nonmate and mate bottles by a single pump that split and directed the excurrent flow to both bottles (nonmate odour condition, Fig. 1c). Excurrent flow from the mate bottle was directed 3 m away from the arena and within the interstices of the reef substrate. For phase 2, the nonmate odour condition was maintained, but the positions of the mate and nonmate bottles were reversed. If fish are able to discriminate mates from nonmates, then again two possible outcomes could occur during phase 1 of the experiment. (1) If olfactory cues are the most important, then resident fish would spend an equivocal amount of time associated with the mate and nonmate fish (with equivalent and nonmate odours) during the first phase of the experiment. (2) However, if visual cues alone are sufficient for discrimination, resident fish would show a similar pattern of association to that observed in the matched odour first experiment. In phase 2 of this experiment, reversal of the bottle location without changing the nonmate olfactory cue present at each bottle could have three possible outcomes. (1) If olfactory cues are important, the effect of bottle reversal should be equivocal. (2) If the resident fish is incapable of discrimination, but a location bias exists, then there should be an experimental phase effect in which resident fish reverse their association with either stimulus from the first phase of the experiment. (3) If resident fish are able to discriminate mates from nonmates with visual cues alone, then resident fish should show the same association pattern observed in the matched odour first experiment and the first phase of the nonmate odour only experiment after reversal of the bottled-fish positions.

### Experiment 4: mate odour, nonmate visual versus matched nonmate odour

This set of paired experiments tested the response of resident fish to visual stimuli from two nonmate fish in model bottles, nonmate A and nonmate B, in association with olfactory cues from the mate. Two nonmate fish A and B were captured from different and distant territories. The two nonmates were placed in bottles and positioned within the experimental arena, while the captured mate was bottled and placed within a black plastic bag that obscured it from view away from the experimental arena (Fig. 1d). In phase 1 (nonmate A visual + mate odour, nonmate B visual - + nonmate B odour, Fig. 1d), the hidden mate odour stimulus was released at the nonmate A bottle (the nonmate A odour was

released away from the arena), and the nonmate B odour was released at the nonmate B bottle. In phase 2 (nonmate A visual - + nonmate A odour, nonmate B visual + mate odour), the nonmate A odour was released at the nonmate A bottle and the mate odour was released at the nonmate B bottle (nonmate B odour was released away from the arena). In phase 1, it was predicted that if fish are able to discriminate mates from nonmates based on olfactory cues from the mate alone, then the resident fish would associate with the mate and nonmate B odour stimuli in a manner similar to that in the matched odour first experiment. After reversal of the mate odour stimulus in phase 2, the resident fish would be expected to reverse the association observed in the first phase.

### Experiment 5: odour cue eliminated

A set of control experiments was performed to confirm the efficacy of odorant stimuli on the behaviour of the free-swimming mate. The captured mate and a nonmate were presented in the experimental arena without an odour stimulus. Pumps perfused fish in each bottle and the excurrent tubing was directed to a common location distant to the arena (Fig. 1e). In a total of six trials, three nonmate fish were used. If fish are able to discriminate mates from nonmates with vision alone, then resident fish are predicted to show an association pattern similar to that in the matched odour first experiment.

### Statistical Analyses

For each experimental phase, the visit rate (visits/min) was calculated based on the number of entries by resident fish within each experimental square. The time spent by resident fish within each square was determined from the entry and exit times into each stimulus arena and expressed as a proportion of the total time for each experimental phase. Only experimental trials in which the resident fish visited both bottles were included in the analysis, as in the few cases in which the resident visited only one bottle it appeared that the second bottle was not seen by the focal resident. One crossed odour first, one nonmate odour only, and two mate odour, nonmate visual versus matched nonmate odour trials were not included in the analysis for this reason. One odour cue eliminated experimental trial was not completed because the resident fish did not visit the model bottle arena.

Time proportions were arcsine square-root transformed and parametric statistics performed after testing for normality and homogeneity of variance. For two phase experiments (matched odour first, crossed odour first, nonmate odour only, and mate odour, nonmate visual versus matched nonmate odour) differences in visit rates and proportion of time spent at either stimulus for each phase and interaction terms were each tested with two-way repeated measures analyses of variance (RM ANOVAs), with a factor for each phase of the experiment, and a stimulus factor (mate or nonmate bottle except in the case of mate odour, nonmate visual versus matched nonmate odour experiments which used nonmate A or nonmate B). The nonmate odour only experiments were used, in part, to test for spatial bias of the resident fish to bottled stimuli (location affect). The bottle location in which the resident fish spent the greatest proportion of time in the first phase of the experiment was identified and included as a factor along with experiment phase in a two-way RM ANOVA to determine whether a location bias was present and preserved across both phases of the experiment. Differences between groups within each RM ANOVA were assessed with Student Newman Keuls (SNK) post hoc tests. Difference in visit rates and proportion of time spent at either bottle in odour cue eliminated experiments were each tested with paired *t* tests. Statistical tests were two tailed with an alpha level of  $P < 0.05$ . Results from the first phase of matched odour first

experiments indicated that resident fish associate more with nonmates (see [Results](#) below). Thus when main effects were observed from statistical tests, in addition to the two-tailed post hoc probabilities, we present *P* values based on the one-tailed probabilities of the alternative hypothesis of resident fish association with nonmate stimuli. Statistical tests were conducted with Sigma Plot 11.0 software (SPSS Inc., Chicago, IL, U.S.A.).

#### Ethical Note

All experiments were conducted under an approved IACUC protocol at the University of Hawai'i at Mānoa. Intruder fish were collected from nonbordering territories on the same reef tract where experiments took place. Fish were collected with hand nets, placed within a 1-litre bottle and transported by divers to the territory approximately 10–20 m away. Mate fish used as stimuli were captured from the experimental territory and treated in a similar way. After completion of each experimental trial, captured fish were transported in the bottle back to the original territories and released next to the remaining mate after sex determination (see below). Fish were captive for periods of less than 90 min. Bottles were approximately 22 cm tall and 15 cm in diameter, which allowed room for the fish (<10 cm total length) to swim actively within the bottle volume, which was ventilated with the fresh sea water pump system. Bottled fish were observed for signs of stress: rapid ventilation, changes in colour, listing and erratic swimming. Captive fish were monitored closely before the experiment, during the experiment and before release. Signs of severe stress (listing, erratic swimming, colour change) were not observed for any fish. For sex determination, bottled fish were transferred into a hand net for approximately 1–2 min. Catheterization for sex determination involved insertion of a 1.1 mm diameter (19 g) catheter into the vent and urogenital tract of the fish, while applying light syringe suction to biopsy a small sample (approximately 2–5  $\mu$ l) of ovary or testis tissue (Ross, 1984). Anaesthetics were not used because the procedure was of minimal invasiveness, brief and performed underwater in order to avoid barotrauma to the swimbladder from taking the fish to the surface. This procedure allows sex determination of sexually monomorphic fish species without lethal methods and a previous study (Ross, 1984) indicated very low mortality in a variety of reef fish families, including Chaetodontidae. After sex determination, fish were released next to their mates within their home territory. All fish were observed swimming with their mates after release. In all experiments, only one member of a territorial pair was captured in order to minimize disruption of feeding territory defence. Experimental fish were confirmed by divers to be reunited with their mate and original territory often for several days after an experiment, and we found no evidence of mortality.

## RESULTS

Resident fish were highly motivated to approach and interact with the visual stimulus produced by the model bottle fish in all experiments (see [Supplementary Video](#)). Once resident fish entered a square within the experimental arena, the percentage of time spent near at least one stimulus bottle was very high for at least the first phase of the experiment (between 23% and nearly 100%, median of all 32 experimental trials 81%). Fish were typically observed in close proximity to each of the stimulus bottles engaged in agonistic-like behaviours that include tail slap displays, tail-up displays, exaggerated feeding and sometimes darkening of coloration. Catheterization made it possible to confirm the sex of at least some female mate and nonmate stimulus fish ([Table 1](#)). In each

experiment, no evidence of variation based on the sex of the stimulus fish or presumed sex of resident fish was observed.

#### Experiment 1: Matched Odour First

During matched odour first experiments, resident fish ( $N = 6$ ) visited both mate and nonmate bottles with high frequency ([Table 1](#)). Visit rates to the nonmate stimulus were higher in the first experimental phase (RM ANOVA:  $F_{5,1} = 6.628$ ; SNK post hoc test:  $P = 0.027$ ), but not the second phase (SNK:  $P = 0.146$ ). Overall visit rates did not differ between experimental phases (RM ANOVA:  $F_{5,1} = 0.723$ ) and there was no stimulus\*phase interaction ( $F_{5,1} = 0.891$ ). Sizes of fish used in these experiments were similar (mean SL of mate 80 mm, nonmate 78 mm). Relative size differences (mate SL–nonmate SL) ranged from –1 to 5 mm.

In these experiments, the responses of residents to mate and nonmate stimuli differed ([Table 1](#), [Fig. 2](#)). There was a statistical effect of mate or nonmate (RM ANOVA:  $F_{5,1} = 9.324$ ), but not experiment phase ( $F_{5,1} = 1.349$ ) or stimulus\*phase interaction ( $F_{5,1} = 0.134$ ). Post hoc tests revealed that resident fish spent more time at the nonmate bottle in the first phase of experiments when odour was matched (SNK:  $P = 0.029$ ), but not in the second phase after odour stimuli were reversed ( $P = 0.065$ ; [Fig. 2](#)). Thus fish displayed a differential response consistent with mate and nonmate discrimination with matched visual and odour stimuli, but not after crossed odour stimuli were presented.

#### Experiment 2: Crossed Odour First

During crossed odour first experiments, resident fish ( $N = 5$ ) visited mate and nonmate bottles with moderate frequency ([Table 1](#)). Visit rates did not differ between mate and nonmate bottles (RM ANOVA:  $F_{4,1} = 0.967$ ) nor experimental phase ( $F_{4,1} = 0.626$ ), and there was no stimulus\*phase interaction effect ( $F_{4,1} = 0.047$ ). Sizes of fish used in these experiments were similar (mate mean = 86 mm SL, nonmate = 85 mm SL). Relative size differences (mate SL–nonmate SL) ranged from 0 to 4 mm.

In these experiments, resident fish responded differentially in terms of proportion of time spent within mate and nonmate stimulus arenas only after switching from crossed odour stimuli to matched odour stimuli ([Table 1](#), [Fig. 3](#)). There was no statistical effect of mate or nonmate bottle (RM ANOVA:  $F_{4,1} = 4.834$ ) or experiment phase (RM ANOVA:  $F_{4,1} < 0.001$ ), but there was a statistical interaction (bottle\*phase; RM ANOVA:  $F_{4,1} = 13.399$ ). Post hoc tests revealed that after the switch from crossed odour stimuli to matched odour stimuli, resident fish spent less time at the mate bottle (SNK:  $P = 0.017$ , which, in the context of results from the intact stimuli of matched odour first, corresponds to a one-tailed  $P = 0.009$  in agreement with the alternative hypothesis of nonmate attraction) and more time at the nonmate bottle ( $P = 0.018$ ; [Fig. 3](#)). In the second, matched odour phase of the experiment, resident fish spent more time at the nonmate bottle than the mate bottle (SNK:  $P = 0.018$ , one-tailed  $P = 0.009$ ; [Fig. 3](#)). Thus fish displayed a differential response consistent with mate and nonmate discrimination only after visual stimuli were matched with odour stimuli.

#### Experiment 3: Nonmate Odour Only

During nonmate odour only experiments, resident fish ( $N = 7$ ) visited mate and nonmate bottles with moderate frequency ([Table 1](#)). Visit rates did not differ between mate and nonmate bottles (RM ANOVA:  $F_{6,1} = 0.659$ ) nor experimental phase ( $F_{6,1} = 2.907$ ), and there was no stimulus\*phase interaction effect ( $F_{6,1} = 0.024$ ). Sizes of fish used in these experiments were similar

**Table 1**  
Visit rates and proportion of time spent by resident butterflyfish, *Chaetodon multicinctus*, with mate and nonmate fish presented in bottles in association with different odour stimuli

Experiment 1–5	Phase	Stimuli	N [n females]	Visit rate (visits/min)				Proportion of time			
				Mean±SD	P stimulus	P trials	P TPI	Mean±SD	P stimulus	P trials	P TPI
Matched odour first	1st	MV+MO	6 [1]	0.58±0.36	<b>0.050</b>	0.434	0.389	0.22±0.16	<b>0.028</b>	0.298	0.729
		nMV+nMO	[1]	0.91±0.26				0.59±0.09			
	2nd	MV+nMO	[1]	0.82±0.36				0.29±0.17			
		nMV+MO	[1]	1.02±0.49				0.57±0.21			
Crossed odour first	1st	MV+nMO	5 [0]	0.34±0.11	0.381	0.473	0.840	0.38±0.23	0.093	0.976	<b>0.022</b>
		nMV+MO	[4]	0.31±0.20				0.54±0.20			
	2nd	MV+MO	[0]	0.26±0.26				0.13±0.15			
		nMV+nMO	[4]	0.26±0.15				0.78±0.30			
Nonmate odour only	1st	MV+nMO	7 [4]	0.65±0.49	0.448	0.139	0.881	0.43±0.22	0.194	0.809	0.947
		nMV+nMO	[0]	0.70±0.51				0.26±0.16			
	2nd	nMV+nMO	[0]	0.54±0.60				0.44±0.23			
		MV+nMO	[4]	0.50±0.28				0.28±0.25			
Mate odour, nonmate visual versus matched nonmate odour	1st	nMaV+MO	8 [1+2]	0.67±0.57	0.826	0.402	0.615	0.37±0.15	0.094	0.477	0.408
		nMbV+nMbO	[3]	0.71±0.49				0.43±0.21			
	2nd	nMaV+nMaO	[1]	0.67±0.58				0.25±0.27			
		nMbV+MO	[3+2]	0.55±0.32				0.54±0.33			
Odour cue eliminated		MV	6 [0*]	0.80±0.45	0.706	—	—	0.29±0.19	0.800	—	—
		nMV	[2]	0.69±0.50				0.33±0.23			

Stimulus column indicates the type of stimulus in each position during each phase of the experiment. *N* refers to the number of experimental trials, *n* females refers to the number of confirmed females used for each stimulus. Resident focal fish are the opposite sex of MO and MV stimuli. *P* values ≤ 0.05 are shown in bold. TPI: trial × phase interaction; MV: mate visual cue; MO: mate odour cue; nMV: nonmate visual cue; nMO: nonmate odour cue; nMaV: nonmate A visual cue; nMbV: nonmate B visual cue; nMbO: nonmate B odour cue; nMaO: nonmate A odour cue.

\* Sex determination by catheterization was not attempted in three individuals.

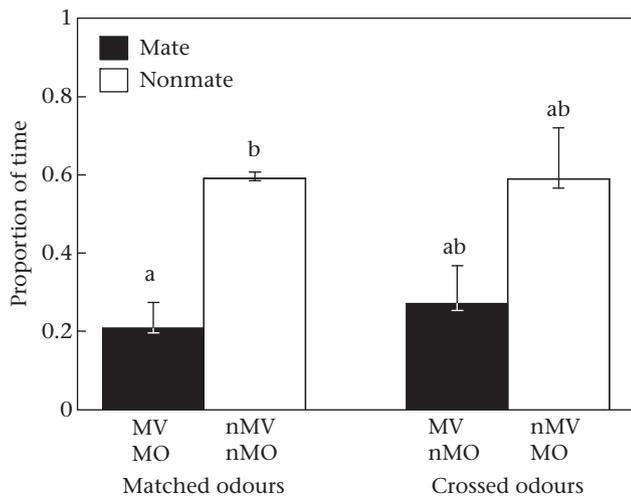
(mate mean 85 mm SL, nonmate 84 mm SL). Relative size differences (mate–nonmate SL) ranged from –2 to 8 mm.

In these experiments, resident fish did not respond differentially to mate and nonmate stimuli in either phase of the experiment (Table 1, Fig. 4). There was no statistical effect of mate or nonmate bottle (RM ANOVA:  $F_{6,1} = 2.143$ ), experiment phase ( $F_{6,1} = 0.064$ ) or statistical interaction (bottle\*phase:  $F_{4,1} = 0.005$ ). Thus, when only nonmate olfactory cues were present, resident fish did not display a differential response and this is consistent with the nondiscrimination of mate from nonmate without an additional mate odour cue. In addition, no evidence of a spatial response to the position of either model bottle was found, as resident fish did not associate

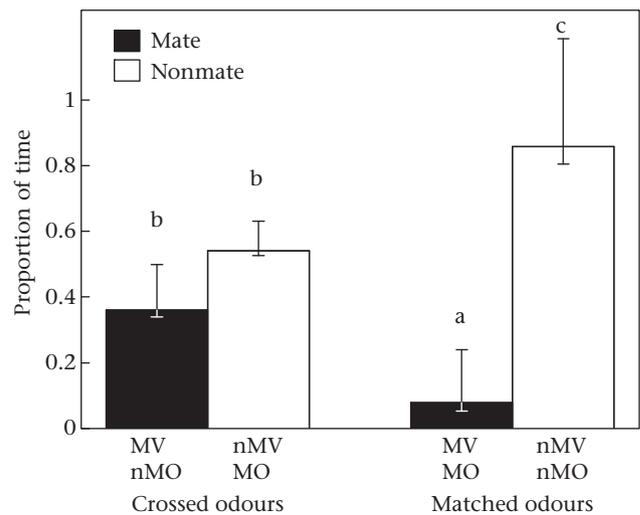
with one location with greater frequency across both phases of the experiment (RM ANOVA:  $F_{6,1} = 0.859$ ,  $P = 0.390$ ).

#### Experiment 4: Mate Odour, Nonmate Visual vs Matched Nonmate Odour

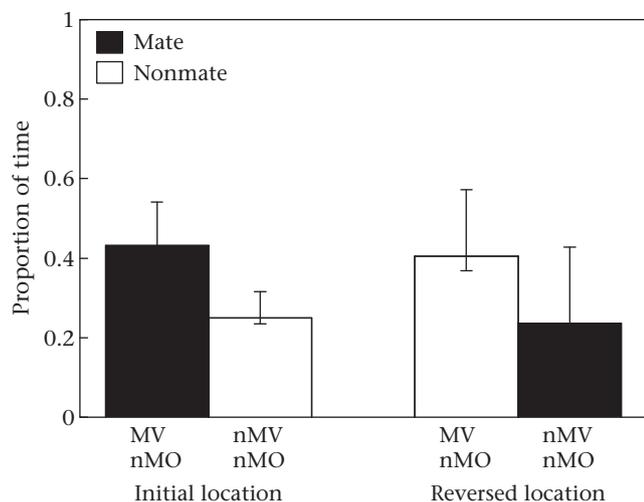
During the mate odour, nonmate visual versus matched nonmate odour trials, resident fish ( $N = 8$ ) visited mate and nonmate bottles with moderate frequency (Table 1). Visit rates did not differ between nonmate A and nonmate B bottles (RM ANOVA:  $F_{7,1} = 0.052$ ) nor experimental phase ( $F_{7,1} = 0.796$ ) and there was no



**Figure 2.** Proportion of time spent by the resident pebbled butterflyfish near the visually conspicuous mate and nonmate bottled fish in the matched odour first experiment. Matched odours: the phase 1 matched odour condition, in which the visual stimulus (mate or nonmate) was paired with the respective odour stimulus. Crossed odours: the subsequent phase 2 crossed odour condition, in which the visual stimulus remained in the same position, but was paired with the opposite odour stimulus. Bars and error bars are back-transformed from means and standard error of arcsine square-root transformed data. Statistically different groups (two-way RM ANOVA, after SNK post hoc tests) are shown by letter groups. MV: mate visual cue; MO: mate odour cue; nMV: nonmate visual cue; nMO: nonmate odour cue.



**Figure 3.** Proportion of time spent by the resident pebbled butterflyfish near the visually conspicuous mate and nonmate bottled fish in the crossed odour first experiment. Crossed odours: the initial phase 1 crossed odour condition, in which the visual stimuli (mate and nonmate) were paired with the opposite odour stimuli. Matched odours: the subsequent phase 2 matched odour condition, in which the visual stimuli were paired with the respective odour stimulus. Bars and error bars are back-transformed (as in Fig. 2). Statistically different groups (two-way RM ANOVA, after SNK post hoc tests) shown by letter groups. MV: mate visual cue; nMV: nonmate visual cue; MO: mate odour cue.



**Figure 4.** Proportion of time spent by the resident pebbled butterflyfish near the visually conspicuous mate and nonmate bottled fish in the nonmate odour only experiment. Initial location: the initial phase 1 nonmate scent only condition, in which both visual stimuli (mate and nonmate) were paired with the nonmate odour stimulus. Reversed location: the subsequent phase 2 of the experiment, in which the position of the visual stimuli were reversed but still paired with the nonmate odour stimulus. Bars and error bars are back-transformed (as in Fig. 2). No statistical difference was found between groups (two-way RM ANOVA, Table 1). MV: mate visual cue; nMO: nonmate odour cue; nMV: nonmate visual cue.

stimulus\*phase interaction effect ( $F_{7,1} = 0.278$ ). Hidden bottled mates, nonmates A and nonmates B were all of similar size (81, 80 and 79 mm SL, respectively). Relative size differences between fish tested were similar (mate–nonmate A SL, range –3 to 5 mm; mate–nonmate B SL, range –4 to 5 mm; nonmate A–nonmate B SL, range –1 to 4 mm).

In these experiments, resident fish did not respond differentially to either nonmate regardless of the odour cue (mate or matched odour) present (Table 1, Fig. 5). There was no statistical effect of the nonmate A bottle or nonmate B bottle (RM ANOVA:  $F_{7,1} = 3.759$ ), experiment phase ( $F_{7,1} = 0.565$ ) or statistical interaction (bottle\*phase;  $F_{7,1} = 0.774$ ). Thus resident fish did not display a differential response consistent with discrimination of nonmates based on olfactory cues (mate or nonmate odours).

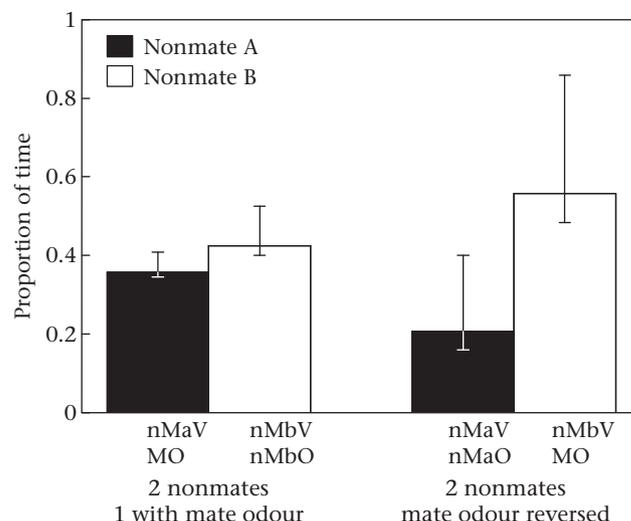
#### Experiment 5: Odour Cue Eliminated

During the odour cue eliminated experiments, resident fish ( $N = 6$ ) visited mate and nonmate bottles with high frequency (Table 1). Visit rates did not differ between mate and nonmate bottles (paired  $t$  test:  $t_5 = 0.400$ ). Sizes of fish used in these experiments were similar (mate mean SL 85 mm, nonmate 84 mm, length of one mate was not measured when the fish escaped). Relative size differences (mate–nonmate SL) of the five measured instances were minimal (range 0–3 mm).

Resident fish did not respond differentially to the mate or nonmate fish in the absence of all odour cues (Table 1, Fig. 6). There was no statistical effect of mate bottle or nonmate (paired  $t$  test:  $t_5 = -0.267$ ). Thus these results are consistent with the requirement of olfactory cues for discrimination of mates versus nonmates.

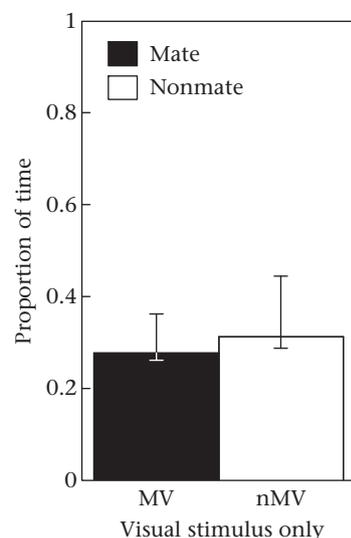
## DISCUSSION

The results of this study are consistent with the hypothesis that monogamous butterflyfish discriminate between their mate and a nonmate by both visual and olfactory cues. Our results further indicate that multiple stimuli may be necessary for butterflyfish to



**Figure 5.** Proportion of time spent by the resident pebbled butterflyfish near two visually conspicuous nonmates in the mate odour, nonmate visual versus matched nonmate odour experiment. Two nonmates, one with mate odour: phase 1 in which the visual stimulus from nonmate A was paired with the mate odour and individual B was paired with its own odour source. Two nonmates, mate odour reversed: phase 2 of the experiment in which the visual stimulus of nonmate A was paired with its own odour and the visual stimulus of nonmate B was paired with the mate's odour. Bars and error bars are back-transformed (as in Fig. 2). No statistical differences were found between groups (two-way RM ANOVA, Table 1). nMaV: nonmate A visual cue; MO: mate odour cue; nMbV: nonmate B visual cue; nMbO: nonmate B odour cue; nMaO: nonmate A odour cue.

discriminate between mates and potential competitors that attempt to forage within their feeding territories. When visual and olfactory stimuli were both present, resident fish spent more time with the unfamiliar fish and engaged in agonistic displays. While there appeared to be considerable variation in the level of aggression displayed by resident fish, there were two instances of strong agonistic behaviour (tail slap and head stand displays and erection of dorsal fin spines) towards the nonmate stimulus under matched



**Figure 6.** Proportion of time spent by the resident pebbled butterflyfish near mate and nonmate bottled fish in the odour cue eliminated experiment. Bars and error bars are back-transformed (as in Fig. 2) with proportion of time spent at mate and nonmate visual stimuli. No statistical differences between groups were found (paired  $t$  test, Table 1). MV: mate visual cue; nMV: nonmate visual cue.

odour conditions. When visual cues of fish were first mismatched with the olfactory stimulus, the free-swimming mate spent equal time with the mate and nonmate, and when the normal olfactory cues were restored the free-swimming mate spent more time with the nonmate. Experiments without both odour cues resulted in equal time spent by residents near mates and nonmates. Aggressive displays towards both mate and nonmate visual stimuli by the resident were observed in one nonmate odour only experiment and one odour-cue eliminated experiment. Focal fish did not show associations with either the mate or the nonmate when only a non-mate odour cue was present. Evidence for discrimination of fish by odour alone was not found, as resident fish did not associate with intruders differentially when mate odour was substituted for matched odour at one of the intruder bottles. We acknowledge that small sample sizes and variability of territorial aggression by resident fish reduce statistical power to resolve potential discrimination of mates and nonmates with incomplete or mismatched odour and visual cues. None the less, these results are consistent with a multimodal recognition hypothesis. As a whole, to our knowledge these results demonstrate for the first time that multimodal olfactory and visual cues are required to identify mates from non-mates in a fish.

The ability to recognize familiar individuals with visual cues alone is known from a variety of animals (Schell, Rieck, Schell, Hammerschmidt, & Fischer, 2011; Tibbetts, 2002; Van Dyk & Evans, 2007; Wilkinson, Specht, & Huber, 2010). Few fish species, however, are known to discriminate individuals from visual cues alone. Visual discrimination of familiar conspecifics was shown in three-spined sticklebacks, *Gasterosteus aculeatus* (Waaas & Colgan, 1994) and threespot damselfish, *Stegastes planifrons* (Thresher, 1979). Discrimination of mates from visual cues was observed in an anemonefish, *Amphiprion bicinctus* (Fricke, 1973) and two species of cichlids: *Hemichromis bimaculatus* (Noble & Curtis, 1939) and *Neolamprologus brichardi* (Balshine-Earn & Lotem, 1998). These studies, however, did not examine the potential role of chemical cues. In our study, fish showed no evidence of discrimination of mates from nonmates by visual cues alone. Stimulus fish in this study were selected to be of similar size, and it is possible that visual discrimination ability in butterflyfish would be improved in situations with a larger discrepancy between mate and intruder size.

It is clear that *C. multicinctus* and other butterflyfish species are visually attracted to conspecifics (Ehrlich, 1977; Fricke, 1986; Reese, 1975), and vision may be used to recognize conspecifics. Visual cues are important for species recognition in many coral reef fishes including butterflyfish (Ehrlich, 1977; Ehrlich & Ehrlich, 1982; Shashar, Rosenthal, Caras, Manor, & Katzir, 2005; Siebeck, 2004; Siebeck, Parker, Sprenger, Mathger, & Wallis, 2010; Thresher, 1976). Butterflyfish in this study showed a strong visually mediated reaction to model bottle stimuli, evidenced in some cases by a cessation of feeding activity and rapid swimming approaches to bottled fish from distances of 5–10 m. This behaviour is similar to reactions from fish after a period of visual separation from mates and provides evidence of at least species recognition, as individual fish do not react in a similar manner to other reef species (Tricas, 1985). Visual discrimination of closely related fish species occurs in freshwater guppies, *Poecilia reticulata* (Warburton & Lees, 1996) and two marine damselfish (Shashar et al., 2005; Thresher, 1976). The bright coloration and conspicuous colour patterns of many butterflyfishes may facilitate visual recognition, as first proposed by Lorenz (1962). Territorial butterflyfish live in an environment that is suited for visual species recognition over considerable distances and such a mechanism could alert fish to the presence of a territorial competitor within a feeding territory or the location of a mate after a period of separation.

Our results show that additional olfactory cues are required to discriminate mates from nonmates. Scent cues are used to attract or signal conspecifics (Cole & Smith, 1992; Lastein, Hamdani, & Døving, 2008), for species discrimination (Fabian, Albright, Gerlach, Fisher, & Rosenthal, 2007; Fisher, Wong, & Rosenthal, 2005; Hankison & Morris, 2003; McLennan & Ryan, 1997, 2007; Ward, Webster, & Hart, 2007; Wong, Fisher, & Rosenthal, 2005), kin recognition (Behrmann-Godel, Gerlach, & Eckmann, 2006; Mann, Turnell, Atema, & Gerlach, 2003; McKaye & Barlow, 1976; Mehlis, Bakker, & Frommen, 2008; Noble & Curtis, 1939; Olsén, Grahn, & Lohm, 2002, 2003; Olsén, Grahn, Lohm, & Langefors, 1997), self-recognition (Thünken, Waltschyk, Bakker, & Kullman, 2009; Ward et al., 2007) and recognition of individuals (Carr & Carr, 1985; Todd, Atema, & Bardach, 1967). However, the function of odour cues for mate recognition in monogamous fishes is largely unexplored. In this study, an odour cue was necessary to elicit a differential response to the visual stimulus of a nonmate and mate. However, resident fish did not respond as would be expected if odour alone were sufficient to discriminate between a mate and nonmate. When mate visual stimuli were hidden and mate odour was released at a nonmate visual stimulus, no difference was observed in time spent near the nonmate visual stimulus paired with mate odour versus a nonmate visual stimulus matched with its own odour. Thus these results further support the hypothesis that both odour and visual cues are required for mate and nonmate discrimination.

Communication in fishes often employs multiple sensory modalities. Mate attraction and courtship in another group of reef fishes (Pomacentridae) is known to include sound and vision, in which male fish produce a strong visual signal (termed the 'signal jump') in association with a courtship sound (Mann & Lobel, 1998). Males of at least some mormyrid fishes produce electric organ discharges with acoustic displays simultaneously during social communication (Crawford & Huang, 1999). Socially mediated sex change in a protogynous goby, *Coryphopterus glaucofraenum*, is mediated by chemical and visual cues (Cole & Shapiro, 1995). In a poeciliid, *Xiphophorus pygmaeus*, multimodal stimuli of vision and olfaction are required to overcome a visual bias in female fish that results in a preference of heterospecific (*Xiphophorus cortezi*) males when olfactory cues are not present (Hankison & Morris, 2003). The role of multimodal stimuli in monogamous fishes is largely unknown. The ability to discriminate mates from unfamiliar fishes was recently observed in a monogamous pipefish, *Corythoichthys haematopterus*, and variation in colour pattern may facilitate visual modes of mate recognition (Sogabe, 2011). However, the close proximity of encounters between fish during greeting rituals could permit additional sensory modalities (chemosensory, mechanosensory and acoustic) to be involved. In the current study, integration of visual and olfactory cues provided fish with the ability to discriminate between species, mates and nonmate individuals.

This study did not directly test the importance of acoustic communication between butterflyfish in mate recognition. However, pebbled butterflyfish are known to produce sounds during agonistic interactions with conspecifics and during distress (Tricas et al., 2006). Individual recognition based on sounds is known from a variety of animals, such as penguins (Aubin & Jouventin, 2002; Lengagne, Aubin, Lauga, & Jouventin, 1999), sea lions (Charrier & Harcourt, 2006), manatees (Sousa-Lima, Paglia, & Da Fonseca, 2002) and possibly bats (Melendez & Feng, 2010). Very few fish are known to discriminate between individuals on auditory cues alone. Myrberg and Riggio (1985) demonstrated that male bicolor damselfish, *Stegastes partitus*, produce more sounds when sounds of unfamiliar males are played from a familiar male's territory. There is evidence of sufficient interindividual variability of acoustic

signals to permit individual recognition in two species of mor-myrids (Crawford, Cook, & Heberlein, 1997). The sounds of pebbled butterflyfish include low-frequency pulses with highly variable and long interpulse intervals (Tricas et al., 2006), unlike bicolor damselfish sounds which consist of rapidly produced pulses with a short interpulse interval but temporal patterning that is hypothesized to allow for individual recognition (Myrberg & Riggio, 1985). Pebbled butterflyfish also communicate with strong tail slap behaviours that transmit both sound pressure stimuli and shorter range hydrodynamic stimuli (Tricas et al., 2006). The bottles used in this study would not permit hydrodynamic stimuli from the tail slap to or from captive fish, and the importance of near-field acoustic stimuli in the identification of individuals or mates remains to be tested.

An additional influence on the behaviour of resident fish is the behaviour of the bottled stimulus fish. Butterflyfish are capable of orientation in a complex spatial environment (Reese, 1989). It is plausible that nonmate stimulus fish might react differently and exhibit more signs of stress than bottled mates if reef landmarks were recognized. However, this seems unlikely given that stimulus fish were confined to a bottle. It is also possible that the olfactory cue of the bottled mate is influenced by visual contact with the resident. If the yet unidentified odour cues are released under control by bottled fish (e.g. through urine, skin or gills; Rosenthal, Fitzsimmons, Woods, Gerlach, & Fisher, 2011), it may be beneficial for fish to advertise their odour identity to avoid misrecognition by their mates. When fish are separated from visual contact with mates, as was the case during mate odour, nonmate visual versus matched nonmate odour experiments, it may be beneficial to minimize odour release which could be a cue for potential predators. The hypothesis of modulation of odour release by mates and nonmates warrants further investigation.

The chemical cue(s) used to discriminate mates from nonmates in pebbled butterflyfish are not known at this time. Potential candidates include major histocompatibility complex (MHC) proteins, prostaglandins, bile and amino acids (Hansen & Reutter, 2004; Sorensen & Caprio, 1998; Zielinski & Hara, 2006). Genetic variation may provide olfactory diversity in terms of MHC, while physiological state may provide cues in terms of prostaglandins, bile salts and amino acids. Multiple chemical cues may be involved in discrimination of a mate from nonmate individuals. Furthermore, differences in territory quality and food resources may affect the physiological state and suite of chemicals released by individual fish. In fact, mate recognition is mediated by odours in burying beetles because of odour cues from the breeding environment (animal carcasses), rather than a cue from the mate itself (Müller et al., 2003). Further study is needed to determine whether characteristics of the feeding territory may also affect the olfactory distinctiveness in monogamous butterflyfishes.

Sex was not controlled for in this study and may account for variability in resident behaviour, as well as affect the stimulation properties of the bottled fish. Both male and female resident fish engage in territorial behaviour and chase conspecifics out of feeding territories, but males more frequently chase intruders (Tricas, 1989a). Sex-specific visual stimuli based on morphology or chromatic cues seem unlikely, at least within the biases of human observers who have not identified sexual dimorphism in *C. multicinctus*. It is possible, however, that sex-specific behaviours of bottled fish (e.g. activity level) would affect the behaviour of resident fish. Sex-specific olfactory cues are used by some fishes to initiate courtship behaviour and attract mates (Cole & Smith, 1987, 1992; Sorensen, 1992; Stacey & Sorensen, 2005). A recent study showed that three-spined sticklebacks respond to a male-specific MHC signal that may be released conditionally in association with reproductive state (Milinski, Griffiths, Reusch, & Boehm, 2010).

Biochemical analysis of sex-specific odours and the neurophysiological response by the olfactory system remain to be studied.

A recent experiment on pebbled butterflyfish, which display strong size assortative mating, demonstrated that females that were experimentally separated from their mates showed no difference in time spent near larger or smaller males, whereas males under the same conditions spent more time with larger females (Strang, 2005). It is not known whether visual, acoustic or olfactory stimuli were responsible for this behaviour of male fish. In light of the results of this study and recent studies on acoustic communication in butterflyfishes (Boyle & Tricas, 2010, 2011; Parmentier, Boyle, Berten, Brié, & Lecchini, 2011; Tricas et al., 2006), multimodal communication may provide cues for mate assessment, choice and recognition. Yabuta (1999, 2002, 2008) observed stereotyped lateral displays in a wild oval butterflyfish, *Chaetodon lunulatus*, in both territorial agonistic contexts and between mates after a period of visual separation. Reciprocal displays by mates are similar in form to threat displays made towards intruders. Thus it was hypothesized that such displays would function to increase the time required for mate recognition and reduce the likelihood of a misdirected attack. Furthermore, the observed frequency of different agonistic behaviours was consistent with this hypothesis (Yabuta, 2002). Individual recognition experienced by social butterflyfishes is a complex task that is undoubtedly affected by multiple relevant cues and the behaviour of both members after pair separation. Future research is needed to determine the roles of both olfactory cues and visually mediated locomotor action patterns that might facilitate the discrimination of mates from nonmates.

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## Supplementary Material

Supplementary material associated with this article is available, in the online version, at <http://dx.doi.org/10.1016/j.anbehav.2014.03.022>.

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