Colour pattern as a single trait driving speciation in *Hypoplectrus* coral reef fishes?

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Theory shows that speciation in the presence of gene flow occurs only under narrow conditions. One of the most favourable scenarios for speciation with gene flow is established when a single trait is both under disruptive natural selection and used to cue assortative mating. Here, we demonstrate the potential for a single trait, colour pattern, to drive incipient speciation in the genus *Hypoplectrus* (Serranidae), coral reef fishes known for their striking colour polymorphism. We provide data demonstrating that sympatric *Hypoplectrus* colour morphs mate assortatively and are genetically distinct. Furthermore, we identify ecological conditions conducive to disruptive selection on colour pattern by presenting behavioural evidence of aggressive mimicry, whereby predatory *Hypoplectrus* colour morphs mimic the colour patterns of non-predatory reef fish species to increase their success approaching and attacking prey. We propose that colour-based assortative mating, combined with disruptive selection on colour pattern, is driving speciation in *Hypoplectrus* coral reef fishes.

**Keywords:** speciation; coral reef fishes; colour pattern; population genetics; assortative mating; aggressive mimicry

1. INTRODUCTION

The colours displayed by coral reef fishes are among the most visually stunning phenotypic traits in animals. Coral reef fish colour patterns have been claimed to serve such diverse functions as crypsis (Cott 1940), mimicry (Randall & Randall 1960) or poster coloration to conspicuously identify conspecifics (Lorenz 1966), but the role played by colour pattern in the process of coral reef fish speciation remains poorly understood (McMillan et al. 1999).

Caribbean coral reef fishes in the genus *Hypoplectrus* present an unparalleled opportunity to investigate colour polymorphism and its role in marine speciation. These fish, commonly called hamlets, include at least 11 distinct colour morphs (figure 1), with as many as seven morphs recorded from the same reef (Fischer 1980a). Colour pattern is genetically determined (Domeier 1994) and have been shown to pair assortatively by colour pattern (Fischer 1980a). Nonetheless, spawning events between different colour morphs as well as individuals with intermediate colour patterns have been observed in the wild (present study; Fischer 1980a; Domeier 1994), suggesting the potential for gene flow between colour morphs. There are no intrinsic post-zygotic barriers between colour morphs (Domeier 1994) and to date no ecological, behavioural or morphological trait other than colour pattern has been shown to clearly distinguish the different hamlet morphs.

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Figure 1. Nine Hypoplectrus colour morphs and densities (number of adults per 100 m², mean ± s.e.) in Belize, Bocas del Toro (Panama), Kuna Yala (Panama) and Barbados as assessed from 29, 40, 90 and 21 transects, respectively, covering a total of 72 000 m² of reef. (a), H. puella; (b), H. nigricans; (c), H. unicolor; (d), H. guttavarius; (e), H. aberrans; (f), H. indigo; (g), H. chlorurus; (h), H. gummigutta; (i), tan hamlet. Photographs with permission from Reef Fish Identification, New World Publications, © 2002, Paul Humann.

Slatkin 1982; Dieckmann & Doebeli 1999; Kirkpatrick 2006; Gavrilets 2004; Schneider & Burger 2006). We provide the following three lines of evidence demonstrating the potential for colour pattern to drive speciation in the genus Hypoplectrus: (i) significant genetic differences between sympatric barred (Hypoplectrus puella), black (Hypoplectrus nigricans) and butter (Hypoplectrus unicolor) hamlets in Belize, Panama and Barbados, (ii) behavioural
observations of colour-associated assortative mating in Belize, Panama and Barbados, and (iii) behavioural evidence of aggressive mimicry of *H. unicolor* in association with its putative model, the foureye butterflyfish (*Chaetodon capistratus*) in Panama.

2. MATERIAL AND METHODS
(a) Sampling
In order to investigate the relationship between assortative pairing and genetic differentiation between hamlet colour morphs, we collected large population samples of barred (*H. puella*), black (*H. nigricans*) and butter (*H. unicolor*) hamlets from Belize, Panama and Barbados. These morphs are ubiquitous throughout the Caribbean, thus permitting comparative analysis at a regional scale (figure 1).

Collecting, export and import permits were obtained prior to fieldwork. Sampling was undertaken in the vicinity of Carrie Bow Cay (Belize) in 2004, in Bocas del Toro (Panama) in 2004 and 2005, and along the west coast of Barbados in 2005. Hamlets were collected with microspears, while SCUBA diving over coral reefs at depths between 8 and 100 feet. Behavioural observations and fish collected for genetic analyses were carried out sequentially on the same reefs. Fish were killed in order to use specimens for an array of analyses, including meristic counts and stomach content analysis. The microspare method was efficient and highly selective, causing no damage to the reef or other fish species. The majority of the fish sampled were photographed underwater before and/or immediately after collection and all were preserved on ice until identified, labelled and photographed in the laboratory within a few hours after collecting. Gill tissue samples for genetic analyses were preserved in salt-saturated DMSO buffer. Entire fish were preserved in 10% formalin until accessioned and stored in 70–75% ethanol as voucher specimens in the Neotropical Fish Collection (Bermingham et al. 1997) at the Smithsonian Tropical Research Institute in Panama. Specimens, photographs, and DNA samples of all fish considered in this study are available upon request.

(b) Genetic analyses
An enriched *Hypoplectrus* microsatellite library was constructed and five microsatellite markers were developed (electronic supplementary material). We used these markers as well as five previously developed loci (McCartney et al. 2003) to analyse a total of 371 *Hypoplectrus* samples, with an average sample size of 46 individuals per colour morph per location (electronic supplementary material). *Fst* between sympatric colour morphs was estimated following Weir & Cockerham (1984) and genetic differentiation was tested with 10 000 allele permutations using the G log-likelihood statistic (Goudet et al. 1996).

(c) Hamlet densities and spawning observations
Hamlet densities were assessed during the day on the same reefs where spawning observations were made later in the early evening. Two SCUBA divers surveyed non-overlapping 100 × 4 m transects between 8 and 80 feet, with each diver counting all hamlets observed 2 m on each side of a 100 m transect tape.

Spawning observations were performed using SCUBA in water 10–60 feet deep, during a period beginning roughly 1 h before sunset and ending roughly 15–30 min after sunset. Only hamlets observed in the ritualistic head-to-tail spawning position (Fischer 1980b) were counted as a spawning event. In order to avoid recording the same spawning pair twice, each reef section was surveyed only once.

(d) Behavioural observations to test for aggressive mimicry
Two SCUBA divers observed 12 butter hamlets (*H. unicolor*), the putative aggressive mimic, in the wild during observation intervals lasting 45–60 min in Bocas del Toro in February and March 2006. The same observation methods and times were also employed to independently track 12 barred hamlets (*H. puella*), which we used as the experimental control given that this colour morph does not resemble the foureye butterflyfish (*C. capistratus*), the putative model. Observations were performed between 08.40 and 14.10 at depths ranging from 11 to 43 feet. Time spent by *H. unicolor* tracking *C. capistratus* was recorded and the predatory strikes performed by *H. unicolor* while tracking *C. capistratus* and while swimming alone were counted. ‘Tracking’ was defined as *H. unicolor* actively staying within 30 cm of *C. capistratus*, with sharp changes in speed and/or direction in order to stay close to the putative model (video sequence 1 in electronic supplementary material). ‘Predatory strikes’ were defined as sharp and long accelerations performed by *H. unicolor* (video sequence 2 in electronic supplementary material). Moreover, the time spent by *H. unicolor* in association with all other coral reef fishes and the number of predatory strikes performed during such associations was recorded.

A two-sided Mann-Whitney test was performed to test whether *H. unicolor* spent significantly more time tracking *C. capistratus* than did the control. A two-sided Wilcoxon signed ranks test was performed to test whether both *H. unicolor* and the control, *H. puella*, displayed significantly higher predatory activity while tracking *C. capistratus* versus when swimming alone. We tested whether the proportion of predatory strikes while tracking the putative model was significantly greater than the proportion of time spent tracking the putative model. Bonferroni corrections for multiple tests were applied to test the significance of *p*-values.

3. RESULTS AND DISCUSSION
Microsatellite analysis demonstrates highly significant genetic differences between all pairs of sympatric colour morphs (table 1). The absence of significant genetic differentiation between two samples of *H. puella* collected in Bocas del Toro in 2004 and 2005 empirically confirms that our methodology does not detect significant genetic differences in cases where none are anticipated. This constitutes the first evidence of consistent genetic differentiation between sympatric *Hypoplectrus* colour morphs.

Spawning observations carried out on the same populations analysed genetically show that the assortative pairing of hamlets observed by Fischer (1980a) translates to assortative mating, with 247 out of 251 (98.4%) independent spawning events occurring between two individuals of the same colour morph (table 2 and §2). Different colour morphs were recurrently seen in close proximity during spawning observations, thereby discounting habitat selection as a possible cause of assortative mating. A total of 180 transects covering 72 000 m² of reef revealed considerable variation between sites in relative densities of hamlet colour morphs (figure 1), and in conjunction with the spawning observations indicate that...
hamlets mate assortatively even when rare (table 2 and §2). For example, yellowbelly hamlets (*Hypoplectrus aberrans*) from Bocas del Toro were observed spawning assortatively, despite a density estimated to one individual per 16 000 m² of reef in the area.

Spawning observations show that colour pattern and mate choice are tightly associated. This suggests that colour pattern is the cue for assortative mating, especially in this system where no ecological, behavioural or morphological trait other than colour pattern has been shown to clearly distinguish the different hamlet morphs (Randall 1983; Bohlke & Chaplin 1993). Formal demonstration that colour pattern is the cue for assortative mating is beyond the scope of this study and is not intended to imply that a single gene linked to H24 is responsible for speciation in hamlets, and additional genetic data are required to investigate the genetic architecture in *Hypoplectrus*. Complexes of multiple genes rather than a single gene are actually expected to underlie speciation in hamlets as colour pattern could result from the combination of several characters, and is shown here to be associated with complex foraging and spawning behaviours.

Altogether, spawning observations and microsatellite data show that although extremely closely related, sympatric *Hypoplectrus* colour morphs are reproducively isolated and can therefore be considered incipient species. Furthermore, the data on assortative mating provide the first line of evidence that colour pattern might be a phenotypic trait of the sort envisioned by Maynard Smith (1978), suggested that hamlet colour morphs evolved as aggressive mimics. According to this hypothesis, predatory

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Table 1. Pairwise F_{st} estimates and test of genetic differentiation between *Hypoplectrus puella*, *H. nigricans* and *H. unicolor* from Belize, Bocas del Toro (Panama) and Barbados genotyped at 10 microsatellite loci. (Highly significant genetic differences were observed between all pairs of sympatric colour morphs tested except the control, *H. puella* collected in consecutive years in Panama. *n* sample size; CI, confidence interval. *F_{st}* estimated following Weir & Cockerham (1984). Genetic differentiation tested with 10 000 permutations using G log-likelihood statistic (Goudet et al. 1996). ***, ***, **' significant *p*-value at the 0.05, 0.01 and 0.001 levels, respectively.)

<table>
<thead>
<tr>
<th>locus</th>
<th><em>H. puella</em>/ <em>H. nigricans</em></th>
<th><em>H. puella</em>/ <em>H. nigricans</em></th>
<th><em>H. puella</em>/ <em>H. nigricans</em></th>
<th><em>H. unicolor</em>/ <em>H. nigricans</em></th>
<th><em>H. unicolor</em>/ <em>H. nigricans</em></th>
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hamlets that match the colour pattern of non-predatory coral reef fishes have a fitness advantage resulting from increased success approaching and attacking their prey. Although behavioural confirmation of aggressive mimicry has been lacking, Randall & Randall (1960) and Thresher (1978) identified seven putative mimic–model pairs suggesting, in principle, the possibility that disruptive selection on colour pattern to match models established the colour differences between hamlets.

Although disruptive selection on hamlet colour pattern may result from other ecological mechanisms such as crypsis (notably in H. puella and Hypoplectrus indigo, Thresher 1978; Fischer 1980a), the sheer number of putative mimic–model pairs called for an empirical test of the hypothesis of aggressive mimicry. Aggressive mimicry requires that: (i) the colour pattern of mimic and model match (Randall & Randall 1960; Thresher 1978), (ii) the mimic is rare relative to its model (Bates 1862; Randall & Randall 1960; Thresher 1978; Cheney & Côté 2005), (iii) the mimic spends more time with its model than expected by chance, and (iv) the mimic shows increased predatory activity when associated with its model (Côté & Cheney 2004). Considering that aggressive mimicry is a density-dependent mechanism which can be efficient exclusively when the mimic is rare compared with the model, as well as the substantial variation in colour morphs densities both within and between locations (figure 1), it seems that only a fraction of all putative mimics can actually benefit from aggressive mimicry at each location.

We focused our behavioural observations on H. unicolor because it closely resembles the non-predatory four-eye butterflyfish, C. capistratus (figure 2, inset, and video sequences in electronic supplementary material), thus satisfying expectation one. Furthermore, although sufficiently abundant to permit efficient field observations at our principal study site in Bocas del Toro, it was 20 times less frequent in our transects than its putative model (mean density ± s.e. = 2.4 ± 0.3 and 0.1 ± 0.03 individuals/100 m² for C. capistratus and H. unicolor, respectively), thus matching expectation two. Underwater observation for 19 h using SCUBA showed that H. unicolor spent a significantly higher proportion of its time tracking C. capistratus than did the experimental control H. puella (Mann–Whitney test, p-value < 0.001, figure 2), and was more active as a predator in association with C. capistratus (Wilcoxon signed ranks test, p-value = 0.005, figure 2). In contrast, the control H. puella showed no significant difference in predatory activity in or out of association with C. capistratus (Wilcoxon signed ranks test, p-value = 0.385). Not counting time spent with C. capistratus, H. unicolor spent less than 1% of its time in association with all other coral reef fish species pooled together, and performed 1.2% of its predatory strikes during these intervals. In sum, H. unicolor spent 10% of its time tracking C. capistratus and executed 50% of all predatory strikes within that time (figure 2), thus satisfying the third and fourth conditions required for aggressive mimicry.

Our study permits strong inference that colour pattern is both the cue for assortative mating and subject to natural disruptive selection. Maynard Smith (1966) pointed out 40 years ago that such traits can trigger the evolution of reproductive isolation, but considered their occurrence ‘very unlikely’. Since then, such phenotypic traits have been documented in a limited number of cases
Figure 2. (a) Proportion of time spent by H. unicolor tracking its putative model, the foureye butterflyfish (C. capistratus). (b) Proportion of predatory strikes performed by H. unicolor while tracking C. capistratus versus while swimming alone. Hypoplectrus puella served as the control for both analyses. Means ± s.e., dashed lines: medians, n = 12 observations of 45–60 min for each species. Inset: H. unicolor (above) and C. capistratus (below).

(e.g. Nagel & Schluter 1998; Jiggins et al. 2001; Podos 2001), but both theoretical (Moore 1981; Sladkin 1982; Dieckmann & Doebeli 1999; Kirkpatrick 2000; Gavrilets 2004; Schneider & Bürger 2006) and experimental (Rice & Salt 1988, 1990) studies have shown that they offer one of the most plausible paths for speciation in the presence of gene flow. A question raised more recently by single trait models is whether speciation in the presence of gene flow is still possible when the costs of assortative mating are taken into account (Gavrilets 2004; Schneider & Bürger 2006).

Our data show that incipient speciation is occurring, despite the fact that assortative mating is expected to be costly for rare colour morphs due to the difficulty of finding mates (e.g. H. aberrans in Bocas del Toro, figure 1). This study demonstrates that sympatric colour morphs of the genus Hypoplectrus are incipient species, and establishes a mechanism by which colour pattern can play an important role in marine speciation.

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