

Stable isotope analysis of the *Hypoplectrus* species complex reveals no evidence for dietary niche divergence

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ABSTRACT: The polymorphic coral reef fish genus *Hypoplectrus* (hamlets) provides an excellent system for examining the initial stages of natural biological divergence in the expansive marine environment. Despite close genetic similarities, hamlets occur in assortatively mating colour morphotypes. In this study, we determined whether ecological differences exist between morphs that could reinforce the assortative mating pattern within morphs. We compared the carbon and nitrogen stable isotope dietary signatures of 6 hamlet morphotypes from 5 geographically distant locations. Across 364 individuals, and with the exception of fish sampled in the Gulf of Mexico, no significant isotopic associations with morphotype were detected. Our results therefore provide no evidence that different hamlet morphs are associated with distinct dietary niches, despite finding highly significant geographical differentiation for both isotopes. We argue that tight assortative mating without ecological divergence could be maintained through the demands of reciprocal cooperation within a reproductive pattern of simultaneous hermaphroditism which characterises all hamlets.

KEY WORDS: Caribbean · Dietary niche · Hamlets · Marine adaptive radiation · Colour polymorphism

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INTRODUCTION

Certain natural systems, such as the African lake cichlids (Kornfield & Smith 2000), have provided evolutionary biologists with excellent opportunities to study speciation. These systems are often situated in relatively enclosed environments and it is not yet clear how the inferences derived from such studies apply to organisms in the marine environment, where planktonic larvae can facilitate long distance dispersal (e.g. Bierne et al. 2003). Coral reef fish of the genus *Hypoplectrus* (hamlets), which have attracted increased attention in recent years (McCartney et al. 2003, Ramon et al. 2003, Puebla et al. 2007), provide an ideal model for studying the speciation mechanisms of a polymorphic group with high geographic genetic connectivity within the expansive marine environment.

The *Hypoplectrus* species complex is composed of brightly coloured predatory fishes inhabiting coral reefs throughout the Caribbean and tropical western Atlantic (Heemstra et al. 2002). The group includes 10 described 'species,' and many undescribed morphs (i.e. colour forms), all of which are distinguished by their distinct colouration and pattern. Up to 8 different morphotypes can be found co-existing on a single reef (Domeier 1994). Hamlets show very strong assortative mating preferences for their own colour morph (Domeier 1994, Puebla et al. 2007). Despite this fact, there are few clear genetic distinctions between morphs (McCartney et al. 2003), which suggests recent morph diversification.

While sexual selection is almost certainly involved in the maintenance of *Hypoplectrus* colour morphs (Fischer 1980, Puebla et al. 2007), ecological diver-

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gence is another important selective force that could lead to the evolution and/or maintenance of the morphs. Ecological variation is well recognised as an agent of natural selection from which reproductive isolation can develop (e.g. Seehausen 2006); however, the explicit identification of ecological mechanisms of speciation has been challenging. Ecological divergence may result in reproductive incompatibility, but a number of other potential isolating barriers may also have arisen simultaneously or subsequently. As a result, the initial forces responsible for divergence may no longer be important for maintaining genetic isolation between populations. Species in the initial stages of divergence, such as hamlets, may therefore be particularly good candidates for detecting primary ecological mechanisms of isolation.

Ecological factors that have been implicated in divergence within animal species include diet (Knudsen et al. 2006), predation (Nosil & Crespi 2006), habitat (Langerhans et al. 2003), competition (Grant & Grant 2006), and temporal separation in behaviour (Denoël et al. 2006). Many of these factors may act concurrently, and are often inter-related as they underpin the acquisition of food. Dietary analyses may therefore be useful indicators of general ecological differences. Several studies on the diet of closely related sympatric fish taxa have shown significantly different dietary niches and have implicated a role for foraging ecology in the initial stages of phenotypic divergence involving both prey type (e.g. Adams et al. 2003, Lecomte & Dodson 2004, Horstkotte & Strecker 2005) and habitat use (e.g. Lecomte & Dodson 2004).

For hamlets, divergence based on either diet or habitat, or both, may be the most likely ecological basis to the phenotypic polymorphism of the genus. There is no current indication of differences between morphs in risk of predation or behaviour. By contrast, differences relating to diet have been noted (Randall 1967, Whiteman et al. 2007) or suggested. Thresher (1978), for example, proposed that hamlets are aggressive mimics with different morphs evolving to resemble different non-predatory model species which resulted in more effective capture of crustacean prey. Differences in depth distribution have also been anecdotally documented between some morphs (Fischer 1980, Domeier 1994). Both aggressive mimicry of different models and depth differences could generate variation in diet, involving either differences in food type, feeding habitat or both.

Analyses of carbon and nitrogen stable isotopes have been successfully used to measure differences between individuals, populations, and species with regard to food sources and trophic levels associated with different dietary and/or ecological niches (Gerner et al. 1999, Guiguer et al. 2002, Adams et al. 2003).

Carbon and nitrogen stable isotope ratios obtained from body tissues provide long-term, integrated dietary information (Tieszen et al. 1983) that cannot be obtained from gut content analyses alone. Nitrogen and carbon isotope ratios, $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ (expressed as $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ respectively), are directly related to diet. The 'heavy' nitrogen isotope (^{15}N) tends to be enriched by about 3‰ between predator and prey (Minagawa & Wada 1984, Post 2002) and thus the $\delta^{15}\text{N}$ of predator tissue is often used to provide information on its trophic position. The heavy carbon isotope (^{13}C) is only weakly enriched between trophic levels, and is instead useful as an indicator of the identity of producers at the base of the food web (Tieszen et al. 1983). Since sources of production vary geographically, carbon isotopes are often used as indicators of spatial feeding dynamics (Vizzini et al. 2005), environmental factors that influence primary producers and members of lower trophic levels that feed on them (Sammarco et al. 1999). Other stable isotopes used in ecological studies (e.g. oxygen) have limited or no relevance for understanding diet.

In the present study, we used both carbon and nitrogen isotope analyses to measure dietary variation among hamlets. We sampled fish from a range of geographic locations to determine the extent of regional variation in isotope signatures for carbon (as described above) and for nitrogen due to variation in prey availability and/or feeding preferences. By sampling up to 5 sympatric morphs at each of 5 different locations across the Caribbean, we analysed inter-morph variation, whilst controlling for regional variation for the whole sample, and expected that the isotope signatures served as indicators of whether different hamlet morphs generally differ in diet and/or feeding niche.

MATERIALS AND METHODS

Fish collections. Sampling was undertaken according to the laws and restrictions of each country, with relevant collection and export permits obtained beforehand. Using SCUBA, we collected hamlets from coral reef sites at 5 Caribbean locations: off La Parguera in Puerto Rico (5 sites, sampled in November 2004), Curaçao (9 sites, in June 2005), La Caleta, Dominican Republic (2 sites, in July 2005), Veracruz in Mexico (2 sites, in May 2006) and Bermuda (13 sites, in June 2006) (Fig. 1). Morphotypes were identified using widely available field guides (e.g. Humann 2002). The number of individuals collected varied among locations, depending on local abundance (Table 1). Fish were captured using a baited hook and line or, if necessary and if local laws allowed, micro-spears. We recorded the depth of capture for each individual and

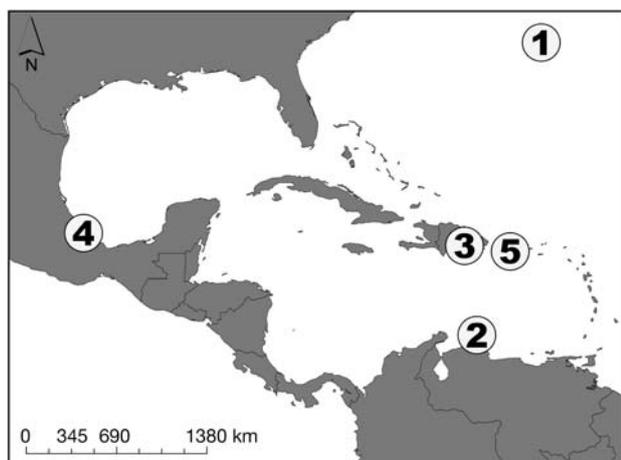


Fig. 1. Sampling sites included in stable isotope study of the *Hypoplectrus* species complex. 1 = Bermuda, 2 = Curaçao, 3 = La Caleta (Dominican Republic), 4 = Veracruz (Mexico) and 5 = La Parguera (Puerto Rico)

the overall habitat type (i.e. rubble field, fringing coral reef, or offshore reef) of each sampling site. After capture, fish were placed in bags, and put on ice. With the exception of Puerto Rico (due to fieldwork constraints), sampling was concentrated in late spring/early summer to minimize possible seasonal effects.

All fish were measured (total length in cm). Samples of white muscle were removed from the posterior region of the body; this is considered the most suitable tissue for teleost stable isotopic analysis (Pinnegar & Polunin 1999). Samples were dried at constant temperature (60°C) for 24 h in a drying oven. Since no drying oven was available in the Dominican Republic, samples were dried at room temperature for 48 h. All dried samples were stored with silica gel until analysis.

Stable isotope analysis. Dried samples were ground to a fine homogeneous powder using a Qiagen Tissue-Lyser. Powdered samples weighing 0.7 mg were placed into 0.3 × 0.5 mm tin capsules, then analysed for carbon and nitrogen stable isotope ratios. The analysis

was carried out by continuous-flow isotope ratio mass spectrometry (IRMS) using a Costech ECS4010 analyser connected to a Thermo Electron Delta XP Plus. Fourteen gelatine standards of unequal mass were run daily to give typical SD of 0.1 ‰ ($\delta^{13}\text{C}$) and 0.2 ‰ ($\delta^{15}\text{N}$). Stable isotope ratios are expressed as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ relative to primary international standards PDB and air (the international standards for carbon and nitrogen, respectively). Fourteen gelatine standards of unequal mass were run daily to give typical SD of 0.1 ‰ ($\delta^{13}\text{C}$) and 0.2 ‰ ($\delta^{15}\text{N}$). All isotopic analyses were undertaken at the Natural Environment Research Council Life Sciences Mass Spectrometry Facility (East Kilbride, UK).

Statistical analysis. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were analysed separately using 3-factor ANOVAs, with morphotype (6 levels) and location (i.e. country, 5 levels) as fixed factors. Site (i.e. where an individual was captured), also a fixed factor, was nested within location. Pairwise differences between levels of each factor were examined with Bonferroni post-hoc tests. We included capture depth and fish total length (cm) as covariates in these ANOVAs, as previous studies have shown that these factors may influence fish stable isotope ratios (Darnaude 2005). Where one or both covariates explained a significant amount of variance in isotope ratios, they were included in the final model. We verified that all data met the assumptions of parametric analysis, using Levene's test for homogeneity of variance and the Kolmogorov-Smirnov test for normality.

As sampling was dictated by the availability of morphs, which varied between locations, the experimental design was unbalanced and sample sizes were at times relatively small (see Table 1). Both could have affected the robustness of our results. To evaluate the extent of this problem, we carried out separate ANOVAs for those locations with the largest and most balanced samples across morphs (Mexico, Curaçao and Dominican Republic). The results of these location-specific analyses generally supported the results of the overall analyses. In addition, we examined the

Table 1. *Hypoplectrus* spp. Number of different hamlet morphotypes analysed for stable isotope variation at 5 locations, from 3 different habitat types (rubble field, fringing coral reef, offshore reef), within the tropical western Atlantic. Dom. Rep.: Dominican Republic

Location	Habitats	<i>H. aberrans</i>	<i>H. chlorurus</i>	<i>H. nigricans</i>	<i>H. puella</i>	<i>H. unicolor</i>	Veracruz white	Total by location
Bermuda	All 3				99			99
Curaçao	Offshore & Fringing reef		33	1	13	25		72
Dom. Rep.	Fringing reef only	10	4	23	15	25		77
Mexico	Fringing reef only			41			41	82
Puerto Rico	Fringing reef only	2	14	4	8	6		34
Total by morph		12	51	69	135	56	41	364

potential effect of having used a different sample drying method in the Dominican Republic. The coefficients of variation for both C and N of Dominican Republic samples were not larger than those of other locations (see Fig. 2), and exclusion of these samples from the overall analyses did not alter our conclusions. We therefore retained all samples in the analyses presented below.

RESULTS

A total of 364 individuals were collected (Table 1) and could be easily identified as belonging to described hamlet morphotypes, with the exception of 41 fish from the Veracruz region of Mexico. According to local sources, this morph is abundant and endemic in the region. We refer to it here as the 'Veracruz white' hamlet.

Stable isotope differences among morphs

Overall, $\delta^{13}\text{C}$ ratios did not differ among morphs (3-way ANOVA, $F_{5,320} = 0.22$, $p = 0.56$) (Fig. 2), but varied significantly with capture depth (3-way ANOVA, $F_{1,320} = 4.52$, $p < 0.001$), with fish captured deeper showing lower $\delta^{13}\text{C}$ (slope ± 1 SE = -0.033 ± 0.008). Fish length was not a significant covariate and therefore was omitted from the final model.

There was significant variation in $\delta^{15}\text{N}$, across morphs (3-way ANOVA, $p = 0.001$) (Fig. 2); however, only 3 pairwise comparisons, all of which involved the Veracruz whites, were significant (Bonferroni post-hoc tests, *Hypoplectrus nigricans*, $p = 0.001$; *H. puella*, $p = 0.045$ and *H. unicolor*, $p = 0.043$). In all 3 cases, Veracruz whites were more enriched in ^{15}N . All other pairwise comparisons were not significant (Bonferroni post-hoc tests, $p > 0.05$ in all cases). When hamlets from Mexico were removed from the analysis, there was no significant effect of morph on $\delta^{15}\text{N}$ (3-way ANOVA, $F_{4,249} = 0.260$, $p = 0.60$). In general, $\delta^{15}\text{N}$ increased significantly with fish size (3-way ANOVA, $F_{1,316} = 9.12$, $p = 0.003$; slope ± 1 SE = 0.077 ± 0.028), but capture depth was not a significant covariate.

Stable isotope differences among locations

There were highly significant differences in $\delta^{13}\text{C}$ among locations (3-way

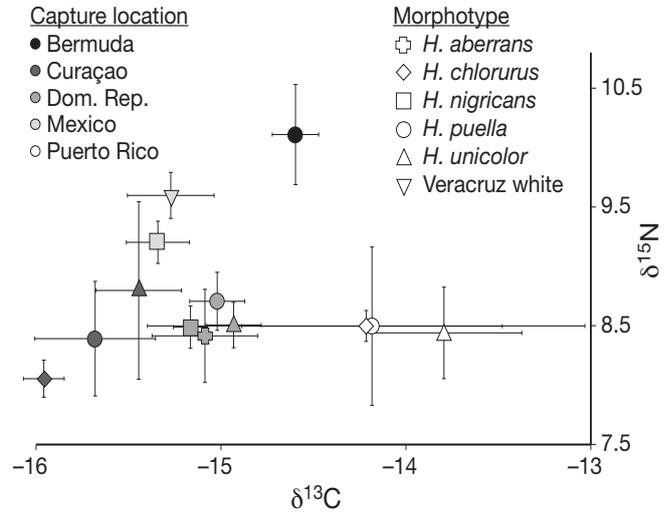


Fig. 2. *Hypoplectrus* spp. Mean stable isotope ratios of 6 morphotypes and from 5 different locations. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values represent the ratio of heavy to light isotopes for each sample, relative to established international standards, for carbon and nitrogen, respectively. Populations with small sample sizes (i.e. <5) not shown. Error bars shown represent 95 % confidence intervals

ANOVA, $F_{4,324} = 27.61$, $p < 0.001$) (Fig. 2). Seven of the 10 pairwise differences in $\delta^{13}\text{C}$ between locations were significant (Table 2). $\delta^{15}\text{N}$ values also differed significantly among locations (3-way ANOVA, $F_{4,324} = 51.25$, $p < 0.001$) (Table 2). Hamlets from Bermuda and the Gulf of Mexico showed significantly greater enrichment in ^{15}N than hamlets from Caribbean Sea locations ($p < 0.001$ in all cases) (Fig. 2, Table 2), with $\delta^{15}\text{N}$ in fish from Bermuda being significantly higher than in those from Mexico ($p = 0.003$) (Fig. 2, Table 2). $\delta^{15}\text{N}$ did not differ significantly between other locations (Fig. 2, Table 2).

Table 2. *Hypoplectrus* spp. Pairwise comparisons of carbon (above the diagonal), and nitrogen (below the diagonal) stable isotope ratios in all hamlets from 5 locations. Values represent differences in estimated marginal means of stable isotope ratios (isotopic ratio of column location minus isotopic ratio of row location). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant. All significant differences remained significant after Bonferroni adjustment for multiple comparisons

	Bermuda	Curaçao	Dom. Rep.	Mexico	Puerto Rico
Bermuda		1.05***	0.45*	0.70**	-0.53*
Curaçao	1.92***		-0.60***	-0.35 ns	-1.58***
Dom. Rep.	1.74***	-0.17 ns		0.25 ns	-0.98***
Mexico	0.75**	-1.17***	-1.00***		1.23***
Puerto Rico	1.81***	-0.10 ns	0.07 ns	1.07***	

Stable isotope differences among sites

Stable isotopic ratios varied significantly among sites within locations (3-way ANOVAs, $\delta^{13}\text{C}$: $F_{26,320} = 4.16$, $p < 0.001$; $\delta^{15}\text{N}$: $F_{26,324} = 51.25$, $p < 0.001$). Post-hoc tests revealed that for $\delta^{13}\text{C}$, significant inter-site differences occurred at only 2 locations, Bermuda and Puerto Rico. In Bermuda, only 3 (of 78) pairwise comparisons among sites were significant. In Puerto Rico, only 2 (of 10) pairwise comparisons among sites were significant. A greater number of significant inter-site differences in $\delta^{15}\text{N}$ were found: 46 (of 78) in Bermuda, 8 (of 36) in Curaçao and 1 (out of 1) in Mexico. The small sample sizes at a few of the sites will undoubtedly have decreased our ability to detect significant differences.

Many of the significant $\delta^{15}\text{N}$ differences between sites in Bermuda and Curaçao involved sites with different habitats (the 2 Mexican sites were both fringing reefs). When $\delta^{15}\text{N}$ comparisons were restricted to pairs of sites with the same habitat type, only 6 (of 23) and 1 (of 22) inter-site comparisons were significant in Bermuda and Curaçao, respectively.

DISCUSSION

Significant variation existed in isotope signatures of hamlets between sites and between habitats, but there was no clear evidence that individual hamlet colour morphs differed in diet. While hamlet morphs varied significantly in $\delta^{15}\text{N}$, which indicates trophic position, the variation was driven solely by individuals from Mexico. Our results showed high levels of variation and large overlap in diet between morphotype, across and within locations. These results do not suggest a high degree of dietary niche specialisation within the hamlet species complex.

The lack of difference in isotope signatures among hamlet morphs in $\delta^{13}\text{C}$ is likely to be the result of morphs incorporating similar carbon sources, suggesting that there is little or no distinction among morphs in the food webs on which they feed. There were also no significant differences between morphs in $\delta^{15}\text{N}$ once the Mexican fish were removed from the analysis. Veracruz whites have higher $\delta^{15}\text{N}$ values than 3 of the other morphotypes, suggesting a higher trophic position, which may be due either to prey availability or dietary preferences. The former is suggested by the fact that the Mexican sample as a whole (i.e. both black and Veracruz white) was more enriched in heavy nitrogen than samples from all other locations other than Bermuda. The presence of a new hamlet morph in Mexico deserves further study. Marine populations of other taxa from the Gulf of Mexico have been shown to

be isolated from those in the Caribbean and Atlantic (Tringali & Bert 1996), and other endemic taxa have been discovered in the region (Felder & Staton 2000).

The present study represents the most extensive stable isotope survey in the Caribbean Sea to date, and we demonstrated significant variation in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ among sampling locations. Gut content analyses by Whiteman et al. (2007), which included some of the fish sampled in this study, have shown that while significant dietary differences exist between some morphs within some locations, general morphotype diet is not highly specific and variation exists within morphs across locations. Our results revealed little overall differences in long-term dietary niche and that differences are not consistent across the distribution range of hamlets. Taken together, these studies suggest that ecological divergence through food partitioning does not play an important role in the maintenance of hamlet morphs.

When compared with previous Caribbean coral reef fauna isotope studies, hamlets seem to feed at a relatively high trophic level typical of predatory fishes (de la Moriniere et al. 2003). The relatively high overall $\delta^{15}\text{N}$ variation shown in our samples is unusual for fish stable isotope studies, indicating that hamlet diet is generalized. This is consistent with published gut content analyses (e.g. Randall 1967, Whiteman et al. 2007), which found a variety of crustaceans, including mysids, mantis shrimp, crabs and fishes, in *Hypoplectrus* stomachs. It remains to be determined whether the highly significant spatial variation among morphs found here and by Whiteman et al. (2007) is caused by differences in food availability or foraging preferences.

While other factors, such as predation, remain untested, our stable isotope study adds to the results of a number of studies which have failed to show distinct hamlet colour morph ecological preferences (Fischer 1980, Domeier 1994, E. A. Whiteman et al. unpubl. data). Consequently, the role of ecological factors in the maintenance of this polymorphism is becoming increasingly unlikely, and further investigation may benefit from pursuing alternative explanations. One unexplored possibility is that sexual selection itself may reinforce the colour differences, which have perhaps arisen or are at least maintained through assortative mating. Hamlets are simultaneous hermaphrodites that spawn using 'egg-trading' behaviour, whereby individuals pair up and alternate sexual roles, sequentially releasing either eggs or sperm (Fischer & Petersen 1987). Such behaviours are thought to have evolved to reduce the opportunities for 'cheating,' which would occur if individuals predominantly played the less-costly 'male' role in spawning. The requirements for reciprocal cooperation within simul-

taneous hermaphroditism may generate strong sexually selected signals (such as colour pattern) even when ecological differences are small. Therefore, in the absence of clear ecological differences between morphs, we suggest that a future area of investigation should be to determine whether divergent colour polymorphism in hamlets is maintained by the costs of 'hybrids' failing to find a reciprocally cooperating mate, rather than ecological fitness costs of poorly adapted hybrids.

Our results do not exclude the possibility that feeding ecology initially drove hamlet colour morph divergence but is no longer required to maintain the polymorphism. Further molecular work is also required in order to resolve the current status of colour morphs and the overall nature of the species complex. Our study demonstrates the importance of sampling hamlet populations throughout their overall distribution to allow for more general conclusions to be reached regarding their ecology.

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