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A phenotypically plastic magic trait promoting reproductive isolation in sticklebacks?

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Abstract

This study identifies one possible mechanism whereby gene flow is interrupted in populations undergoing evolutionary divergence in sympatry; this is an important issue in evolutionary biology that remains poorly understood. Variation in trophic morphology was induced in three-spined stickleback by exposing them from an early age either to large benthic or to small pelagic prey. At sexual maturity, females given a choice between two breeding males, showed positive assortative mate choice for males raised on the same diet as themselves. The data indicate that this was mediated through a preference for males with trophic morphology similar to that of fish with which the females were familiar (from their pre-testing holding tanks). This study has shown for the first time that expression of a plastic trait induced at an early age not only results in specialisation for local foraging regimes but can also play a significant role in mate choice. This is equivalent to an environmentally induced, plastic version of the “magic traits” that promote ecologically-driven divergence in sympatry, hence the proposed descriptor “plastic magic trait”.

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Introduction

The process whereby gene flow is interrupted in populations undergoing evolutionary divergence when in sympatry is an important issue in evolutionary biology that remains poorly understood. One possible mechanism involves an ecologically important trait under divergent selection also contributing to reproductive isolation, so-called "magic traits" (Gavrilets 2004; Servedio et al. 2011). Although evidence of such traits in nature is sparse (Servedio et al. 2011), magic traits are usually envisaged as inherited and linked to mate choice through pleiotropy. However, much research interest has focussed on the possible role of phenotypic plasticity in the initiation of evolutionary change through the development of discrete alternative phenotypes (West-Eberhard 1989, 2003; Fitzpatrick 2012). If expressed alternative phenotypic traits induced by the environment through plasticity, also form part of the mate choice system of the diverging organism, assortative mating based on such traits might generate reproductive barriers between individuals presenting different phenotypes (Fitzpatrick 2012) and thus may act as a magic trait without the requirement of pleiotropy.

Discrete alternative phenotypes associated with foraging, or trophic polymorphisms (*sensu* Skúlason & Smith (Skulason and Smith 1995), have been strongly implicated in sympatric speciation events (Dieckmann and Doebeli 1999). The divergent morphological traits are often the result of foraging conditions experienced during development (Day and McPhail 1996; Adams et al. 2003), so can only result in evolutionary change if mechanisms exist that result in gene pool segregation (West-Eberhard 1989; Smith and Skulason 1996; Schluter 2003). Here we explore one possible mechanism, namely morph-specific mate choice by breeding females. Using the three-spined stickleback (*Gasterosteus aculeatus*) as a model system, we present an example of a developmentally-plastic, trophic specialisation acting as a magic trait generating reproductive isolation and suggest a mechanism through which this comes about.

Trophic polymorphism is particularly common among freshwater fishes, including sticklebacks, it often takes the form of co-existing but discrete phenotypes with morphological and behavioural specialisations for feeding on benthic invertebrates in the littoral zone or zooplankton in the pelagic zone (Skulason and Smith 1996; Adams and Huntingford 2002a; Proulx and Magnan 2004). Typically, the benthic form is robust, with a large mouth, small eyes and few short gill rakers, while the pelagic form is lightly built, with a relatively small mouth, large eyes and longer and more numerous gill rakers (Adams et al. 1998; Adams and Huntingford 2002a). Although in some cases the two sympatric forms are fully reproductively isolated, more commonly reproductive isolation is partial, weak or non-existent (Schluter and McPhail 1992; Hendry et al. 2009).

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The main aim of this study was, having induced variable trophic morphology in three-spined sticklebacks from a single population by manipulating early feeding regimes, to determine whether these plastic, diet-induced differences in trophic morphology were associated with different patterns of mate choice. A second aim was to seek possible behavioural mechanisms that might explain the observed patterns of mating.

Methods

Diet Treatments

240 juvenile three-spined sticklebacks fry (5-9 mm length) were collected by dip nets from a small freshwater pond in Scotland (56°3'N; 004°21'W) and transported to rearing facilities at the Scottish Centre for Ecology and the Natural Environment (SCENE), Glasgow University, Loch Lomond. Fish were assigned randomly in groups of 40 to 6 rearing aquaria (21L) and raised in the laboratory for 11 months, during which time they were fed twice daily to satiation on one of two diet treatments known to induce differences in trophic morphology (Day and McPhail 1996). Half of the groups were fed on frozen *Daphnia* spp in a bag hanging at the water surface, simulating pelagic prey; the rest were fed on frozen chironomid prey placed on the bottom of the tank, simulating benthic prey.

Analysis of Induced Morphological Differences

After 10 months, the sticklebacks were anaesthetised with benzocaine and photographed on their left side with a Canon EOS digital 350D camera (8.0 megapixels). The sexually-mature fish used in mate choice experiments were re-photographed at 11 months immediately following the mate choice experiments. Size and shape were quantified and diet groups compared on the basis of 20 landmarks and landmark position was analysed using a Relative Warp Analysis (Rolf, 2006). The second relative warp (analogous to a Principal Component), which explained 13% of the total shape variation, opposed traits typical of pelagic and benthic feeders (Day and McPhail 1996; Figure S1).

Mate Choice Trials

Twenty eight females (21 from the chironomid diet and 7 from the *Daphnia.* diet) and 36 males (21 from the chironomid diet and 15 from the *Daphnia* diet) were used in trials of mate choice (Kraak and Bakker 1998). Female mate choice was examined using a well-tested methodology in which a single gravid female was placed alone in an aquarium (35 x 25 x 20 cms, screened on 3 sides), allowed to settle for 12h and was then presented simultaneously with two breeding males in equally-sized sections (25 x 35 x 20 cms) of an adjacent aquarium. During trials, females could see both males, but the males were separated by an opaque partition and so did not have visual contact with each other. In any trial, the female was presented with one *Daphnia*-fed and one chironomid-fed male. To avoid

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effects of size and familiarity with specific males, the two males in any given trial were size-matched as far as possible and importantly taken from a different rearing tank from the female.

Each trial lasted for 5 minutes, during which, the time the female spent on the side of the tank adjacent to each male was recorded. Three replicates of each pairing trial were conducted, swapping the male position each time. A female was deemed to have chosen a male if she spent at least 60% of the total time of the trial near that male (Kraak and Bakker 1998). Males and females were used maximally in four trials on different days; males were re-used in fresh combinations so that the female was never exposed to the same pair of males. Although male pairs were matched in size as nearly as possible, small discrepancies between pairs remained. No significant difference in size was detected between chosen and rejected males (mean $\pm$ SE size differences between accepted and rejected males =0.04 cm $\pm$ 0.02 one sample T test: T = 1.68, p = 0.10).

Results

Effects of Diet on Morphology

The second relative warp varied markedly both between and within diets. Effects of gender (ANOVA: $F_{1,60}$=3.11; p=0.08) and of gender by diet (ANOVA: $F_{1,60}$=2.86; p= 0.09) were not significant. However, there was a highly significant difference in morphology between the chironomid fed (mean $\pm$ SE = 6.753 $\pm$0.23) and *Daphnia* fed sticklebacks (mean $\pm$ SE = 4.82 + 0.34. ANOVA: $F_{1,60}$= 22.2; p< 0.0001). The higher scores of fish fed on the benthic diet reflected shorter heads, shorter maxillary bones, smaller eyes and deeper bodies. This score was transformed to create only positive values (by adding 6 and multiplying by 100) and hereafter this dimension of shape variation is referred to as the pelagic-benthic (PB) shape score. Lower PB scores indicate shapes tending towards a more typical of a pelagic foraging fish; higher scores tending towards a more benthic foraging fish shape.

Mate Choice In Benthic- And Pelagic-Fed Females

Retrospective examination showed a range of differences in PB score between test males across all trials. In all of the following analyses, tests in which there was effectively no difference in diet-related morphology between the test males (that is where the difference in PB score was <0.50) were omitted. In addition, trials involving one pair of males both of which had an extreme pelagic-like morphology (a low PB shape score) were also omitted. Thus a total of 88 trials was analysed further. Since in nature, female sticklebacks review a number of males before selecting a nest in which to lay her eggs and since the females in this study were always tested with different pairs of (unfamiliar) males, trials on

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the same female were considered as independent. Table 1a shows the frequency with which benthic diet-fed and pelagic diet-fed females chose or rejected the male with the lower PB score (i.e. with a more pelagic-like morphology). Overall there was a significant difference in preference between females reared on benthic (chironomid) and pelagic (*Daphnia*) diets. Pelagic diet-reared females were significantly more likely to accept the male with the lower pelagic-benthic score (i.e. more pelagic-like; 73% of trials χ^2 = 6.53. P = 0.01); benthic diet-reared females showed no preference between males (male with lower score chosen in 47% of trials. χ^2 = 0.27. P = 0.60).

As a more conservative test of non-random mating by trophic morphology, the overall mate choice of individual females was expressed as the proportion of trials in which she took part where the male with the lower PB score was selected. This proportion was significantly higher for females reared on a pelagic diet (Figure 1a. T test: T = 3.59. df = 12. P = 0.004). The preference score was significantly greater than 50% for pelagic diet-reared females (One sample T = 3.67. N=7. P < 0.01), but not for benthic diet-reared females (One sample T = 1.05. N = 17. P =0.31). This analysis confirms that females reared on the pelagic diet tended to prefer the male with the lower PB score (more pelagic-like males), whereas females reared on a benthic diet were non-selective. Our data therefore demonstrate partial assortative mating by diet-induced phenotype, based on the behaviour of pelagic-reared females.

Behavioural Mechanisms Of Mate Choice

To explore possible mechanisms for this difference in female preference by diet, behavioural data were analysed in more detail. Females were reclassified with respect to their own trophic morphology (as opposed to their diet). Females with PB scores greater than 6 were classified as benthic-like and those with scores lower than 6 were classified as pelagic-like. No difference in mate preference were found in females classified in this way (Table 1b), nor was there a difference in the proportion of trials in which the females chose the more pelagic-like male (lower PB score). Mean $\pm$ SD PB score of preferred male =5.2 $\pm$ 2.0 and 5.3 $\pm$ 1.9 for females below and above the mean respectively (T test: T = 0.08. df = 17. P = 0.94). Therefore the effect of rearing diet on mate choice described above is not a simple consequence of the females' own morphology.

Another possible behavioural mechanism by which rearing diet might influence a female stickleback's mate choice is through previous experience of the fish with which she was reared, whose shape will, on average, reflect their common rearing diet. To test this possibility, we took advantage of the variability in PB scores between rearing tanks on a given diet, relating preferences expressed in the mate choice tests to the average trophic morphology of the fish with which the females had been

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raised. The mean morphology of fish in the rearing tank was thus classified as 'pelagic-like' morphology for a tank mean PB score of less than 6 (nominally category P: actual values: 4.2 and 5.5), 'neutral morphology' with a PB score between 6 and 7 (category N: actual values: 6.1 and 6.3) and 'benthic-like' morphology with a PB score of more than 7 (category B: actual values: 7.06, 7.1 and 7.4). Females reared in these three categories of tank were thus accustomed to seeing fish with pelagic-like morphology, neutral morphology and benthic-like morphology, respectively. Table 1c shows the numbers of females from these three tank categories that preferred or rejected the more pelagic-like of the two males in choice experiments, and the percentage preference for the more pelagic-like male. There was a significant effect of tank morphology category on mate choice, with a threshold change above 7, at which the preference shifts from the more pelagic-like to the more benthic-like male. It would seem that the female sticklebacks in this study have a base-line tendency to prefer the more pelagic-like of two males, but that this preference is weakened and to some extent reversed by long-term exposure to benthic-like fish.

To explore further the determinants of mate choice, firstly we looked for an effect of the magnitude of the difference in trophic morphology between the pair of males used in each trial. Figure 1b shows the mean ($\pm$SE) of the difference in magnitude of the PB score between males in trials in which the least pelagic shape male was chosen or rejected, in relation to rearing tank mean shape category. There was no main effect for tank (ANOVA: $F_{1,83} = 0.22$. $P = 0.80$) or outcome (ANOVA: $F_{1,83} = 0.08$. $P = 0.78$). However, there was a significant interaction (ANOVA: $F_{1,83} = 3.44$. $P = 0.04$). For females reared in tanks of category N, used to seeing fish with neutral trophic morphology, there was no relationship between how different males in the pair-wise choice were and whether the male with the higher or lower PB score was chosen. For females reared in tanks of category P (PB score <6), which were accustomed to seeing pelagic-like morphology fish and for trials in which the male with the more pelagic-like phenotype was chosen (the "correct" choice for assortative mating by diet-induced trophic morphology), there was a larger morphological difference between the male pair presented. For females in category B (PB score >7), which were used to seeing benthic-like morphology fish, the magnitude of the morphological difference was larger in trials in which the male with the more benthic phenotype was chosen (the "correct" choice in this case). In other words, females were more likely to choose males with a familiar phenotype when the difference between target males is large and presumably easy to assess.

Although females reared in tanks with an average PB score of >7 were more likely than females from the other tank categories to choose the more benthic-like of two males, they did not always do so, even when the size difference was large enough to be easily perceived. To explore possible causes for

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such "incorrect" choices, we compared the absolute PB score of the rejected male in cases in which the more benthic-like male was chosen or rejected, for females from the tank category B (Figure 1c). In trials in which the females chose the more pelagic-like male (the "incorrect" choice for assortative mating in females from tank category B), the rejected, benthic-like males had significantly higher PB scores than in trials in which the more benthic-like male was preferred (T test: T = 6.67. df = 35.P > 0.001). This indicates that females are avoiding males with extreme PB scores in favour of a mate with a PB score closer to the average.

Discussion

Our results confirm the findings of previous studies demonstrating a plastic response of morphological traits to rearing diet in three-spined sticklebacks (Day and McPhail 1996). More significantly, they have demonstrated for the first time that exposure to different diets during the juvenile phase can influence the mating preferences shown by breeding females. Thus, females reared on the pelagic diet tended to prefer the male with a more pelagic-like morphology; females reared on a benthic diet however mated randomly with respect to trophic morphology. Thus there is partial assortative mating by diet-induced phenotype. Unlike the case of assortative mating on the basis of diet specialisation in the mustard leaf beetle, which appears to use olfactory cues to identify mates (Geiselhardt et al. 2012) the sticklebacks in this experiment only had visual cues are available to them. However, it is quite possible that olfactory cues might also have affected mate choice had they been available. In addition, the effect reported here did not result from female familiarity with specific individual males, as females were never tested with males from the same rearing tank.

We additionally show that mate choice was not dependent directly of the female's own trophic morphology. Arguably, this is not surprising, since it is difficult to see how a female stickleback could know what her own morphology is like. Instead the differences in mate choice must be a consequence (direct or indirect) of the experience of being raised on a pelagic or a benthic diet. Making use of the significant variation in morphology between and within rearing tanks exposed to different and the same diets, we show that the expressed morphology of other fish with which the female is familiar (from the same rearing tank) is a good predictor of mate choice, particularly for fish from tanks expressing a pelagic-type morphology. It is highly likely that in the wild also sticklebacks grow up with fish exploiting a similar diet to themselves and thus with similar diet-induced morphology, as individuals exploiting the same foraging resources are more likely to come into contact with each other, than those that do not share a common diet (Garduño-Paz and Adams 2010).

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Despite a clear tendency for assortative mating by trophic morphology, females quite often made the opposite choices. This was most often the case when the difference between the two males was relatively small, but also occurred when if the morphology of the predicted choice male was an extreme benthic or pelagic-type morphology. One can envisage at least two plausible mechanistic explanations for this, which are not mutually exclusive. It may be that, rather than responding to familiarity *per se*, females have learned about the foraging efficacy of fish with the range of morphologies that she has experienced during development. If this were the case, this could result in the avoidance of males of extreme morphology, even if this morphology is familiar to her. Alternatively, it may be that particularly extreme morphologies may invoke some other behavioural response such as the fear. The data from the experiment reported here do not allow us to distinguish which, if either of these possibilities may be operating here.

Although coexisting trophic morphs are thought to play a part in sympatric speciation (Skulason et al. 1999), this is unlikely to be completed without some mechanism for morph-specific assortative mating (Skulason et al. 1999). Several routes though which this might occur have been suggested. For example specialist morphs might occupy different habitats. Olafsdottir and co-workers (Olafsdóttir et al. 2006) for example, showed that sticklebacks specialising in living in habitats with little vegetation had reduced nest building behaviour and as a result weed-living specialists from the same lake mated assortatively with other weed-living specialists when using nest quality as a mate choice criterion. Disruptive sexual selection is also known to play a significant role in the divergence of recently evolved African cichlid species (Stelkens et al. 2008). Here uniquely we demonstrate assortative mating on the basis of morphological traits that frequently express as discrete forms in the wild, have strong functional significance for resource acquisition (Adams and Huntingford 2002b) are thought to be under strong selection pressure and the expression of which is significantly modulated by plasticity effects. This result indicates that trophic morphology is both a plastic and a magic trait for sticklebacks, thus that pleiotropy may not always be required for traits to operate as magic traits.

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Table 1. The frequency with which the male with the higher pelagic-benthic score (more benthic-like) was chosen and rejected by female sticklebacks classified a) by diet, b) by Pelagic-Benthic (PB) score and c) by tank PB category.

a)

	Male with lower P-B score	
Female diet	Chosen	Rejected
Benthic	27	31
Pelagic	22	8
χ^2 DF P	5.75 2 0.015	

b)

	Male with lower P-B score	
Female PB score	Chosen	Rejected
High	30	26
Low	18	14
χ^2 DF P	0.60 2 0.74	

c)

	Male with lower P-B score		
Tank mean	Chosen	Rejected	% chosen
4-5	10	6	63
6	24	10	71
7+	16	22	42
χ^2 DF P	6.2	2	<0.05

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Figure legends

Figure 1. A) The mean (+SE) proportion of trials in which individual females reared on the pelagic and the benthic diet preferred the more pelagic-like of two males (the male with the lowest PB score); 0.5 represents no preference. B) The mean (+SE) difference in PB score between males in pairwise trials in which the male with the lowest PB morphology score (i.e. more pelagic-like) was chosen or rejected, according to category of tank in which the female was reared (mean tank PB score: <6, 6-7 and >7 (more pelagic-like, neutral and benthic-like in morphology respectively)). For females in tanks with mean PB score <6 and >7, dark grey indicates cases there the "correct" male (i.e. most similar to the tank mean) was chosen. C)The mean (+SE) PB score for the rejected male in pairwise trials on females reared in tanks with a mean PB score of >7 in which the male with the highest PB score was rejected or chosen.

Supplementary Material

Figure S1. The landmark configurations used in the morphometric analysis of trophic morphology in sticklebacks. The landmarks are connected to aid visualisation of fish shape. Arrows represent vectors describing deformations that change the mean shape of sticklebacks fed on benthic prey to the mean shape of those fed on pelagic prey.

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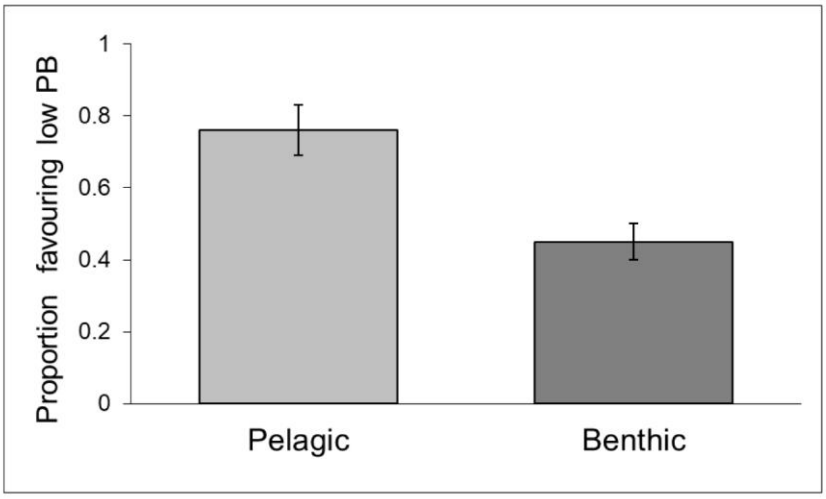

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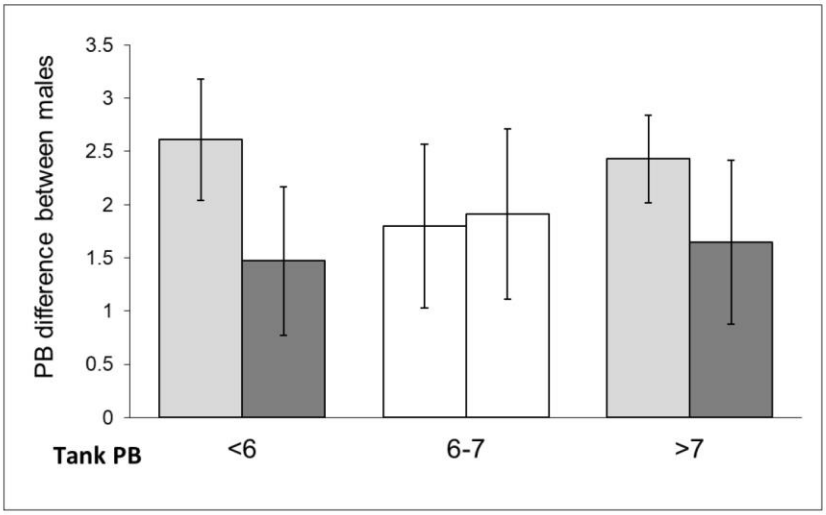

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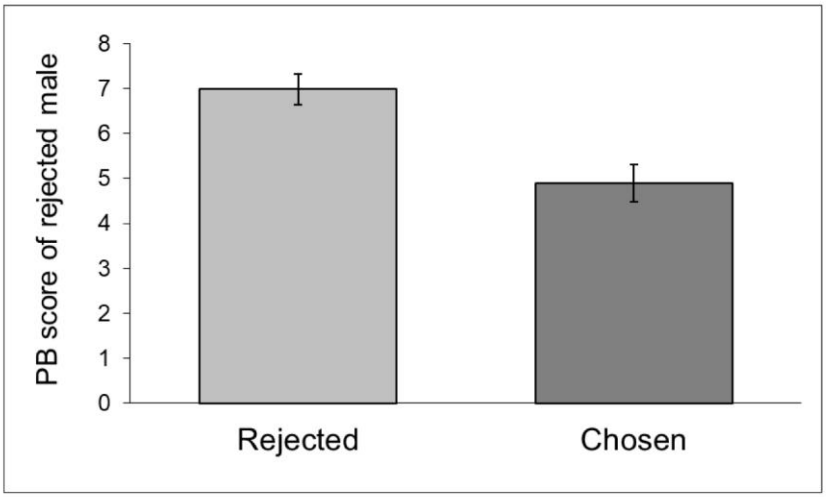

1C

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Supplementary material Fig S1