

## SHORT COMMUNICATION

**Heterozygosity and orange coloration are associated in the guppy (*Poecilia reticulata*)**

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ornament;  
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**Abstract**

The good-genes-as-heterozygosity hypothesis predicts that more elaborate male sexual ornaments are associated with higher levels of heterozygosity. Recent theoretical work suggests that such associations are likely to arise in finite, structured populations. We investigated the correlation between multilocus heterozygosity (MLH), which was estimated using 13 microsatellite loci, and male coloration in a wild population of guppies (*Poecilia reticulata*), a model species in sexual selection research. We found that MLH was a significant predictor of the relative area of orange spots, a trait that is subject to strong female preference in this species. Neither the relative area of black spots nor the number of black or orange spots was significantly correlated with MLH. We found no statistical support for local effects (i.e. strong effects of heterozygosity at specific markers), which suggests that relative orange spots area reflects genome-wide heterozygosity.

**Introduction**

Indirect genetic benefits are thought to maintain female preferences for male sexual ornaments in species in which males contribute nothing but gametes to reproduction. They may arise because preferred males pass on their sexual attractiveness to their male progeny (Fisher, 1930; Prokop *et al.*, 2012) or confer higher nonsexual fitness to progeny of both sexes (Zahavi, 1975). However, the latter mechanism requires that sexual ornaments reveal genetic quality. Sexual ornaments are expected to reflect genetic quality if ornament elaboration is condition-dependent and thus captures variation in fitness-related traits that affect condition, including optimal resource allocation, metabolic efficiency, health and the ability to cope with stress (Andersson, 1986; Rowe & Houle, 1996; Tomkins *et al.*, 2004). Because the values of fitness-related traits are often positively associated with heterozygosity, an organism's degree of heterozygosity should be reflected

by condition-dependent ornaments (reviewed in: Brown, 1997; Kempnaers, 2007).

Heterozygosity may affect fitness-related traits in two ways. First, in homozygotes, the deleterious effects of recessive mutations are exposed (Charlesworth & Willis, 2009). Second, heterozygosity may be intrinsically associated with higher fitness due to overdominance (Lerner, 1954). Homozygosity substantially increases when mating occurs between close relatives, and the resulting decline in trait values, termed inbreeding depression, has been shown to strongly affect most fitness-related traits (DeRose & Roff, 1999; Charlesworth & Willis, 2009). However, the association between multilocus heterozygosity (MLH) and higher fitness in natural populations will depend on interindividual variation in inbreeding, which will, in turn, depend on the mating system, population size and population structure (reviewed in Szulkin *et al.*, 2010). These factors will differ among species and populations. As a result, it is perhaps not surprising that correlations between heterozygosity and fitness are significantly positive on average but differ in magnitude across studies (reviewed in: Coltman & Slate, 2003; Chapman *et al.*, 2009). Furthermore, the association between MLH, which is typically assessed using neutral markers, and fitness may arise either because MLH reflects genome-wide inbreeding (referred to as general effects by David,

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1997) or because homozygosity at certain specific marker loci reflects homozygosity at linked fitness loci (local effects). In theory, variation in the association between heterozygosity and fitness among populations may reflect random variation in the probability that some of the markers used are linked to fitness loci. However, as a large number of slightly deleterious mutations account for most observed inbreeding depression (Charlesworth & Willis, 2009), general effects seem more likely (Szulkin *et al.*, 2010).

Several studies have demonstrated that experimental inbreeding has strong negative effect on sexual ornaments (e.g. sexual coloration in the guppy (Sheridan & Pomiankowski, 1997; van Oosterhout *et al.*, 2003) and beak colour in the zebra finch (Bolund *et al.*, 2010)), a finding that is consistent with the hypothesis that sexual ornaments reveal genome-wide heterozygosity. However, in other studies, inbreeding depression was found to be moderate (e.g. song frequency in *Drosophila montana* (Aspi, 2000) or eye span in the stalk-eyed fly (Prokop *et al.*, 2010)) or not significant (e.g. calling effort in field cricket (Drayton *et al.*, 2007) or nuptial coloration in the threespine stickleback (Frommen *et al.*, 2008)). Nevertheless, laboratory studies may underestimate the effects of inbreeding, which tend to increase considerably in stressful environments (Fox & Reed, 2011), and thus, further research on this topic is needed, especially under natural conditions.

Associations between heterozygosity and sexual ornament elaboration in natural populations have rarely been documented, and many studies focus on bird species. For example, heterozygosity is positively correlated with male ornament elaboration in spotless starlings (Aparicio *et al.*, 2001) and blue tits (Foerster *et al.*, 2003), but not in common yellowthroats (Dunn *et al.*, 2013). Inbreeding estimates calculated from pedigrees were strongly related to male song repertoire size in the song sparrow (Reid *et al.*, 2005). In teleost fish species, a positive correlation between male heterozygosity and breeding coloration was found in the European minnow (Muller & Ward, 1995), but not in the Arctic charr (Janhunen *et al.*, 2011). In the crested newt, heterozygosity affected body size, which is in turn strongly correlated with sexually selected crest size (Herdegen *et al.*, 2013).

The guppy (*Poecilia reticulata*), a tropical freshwater fish that inhabits northern South America, is a model species in sexual selection research (reviewed in: Houde, 1997; Magurran, 2005; Evans *et al.*, 2011). Male guppies have conspicuous coloration; their bodies are covered by a mixture of spots that occur in a wide variety of colour combinations. Yellow, orange and red spots are carotenoid-based, whereas black spots are melanin-based; spots can also be iridescent. Females across many populations show colour preferences, with orange coloration being the most consistently preferred (reviewed in: Houde, 1997; Magurran, 2005). However,

it is unclear what maintains female preferences (Magurran, 2005).

Several laboratory studies have shown that both the number of and the total area covered by orange and black spots diminish in response to inbreeding (Sheridan & Pomiankowski, 1997; van Oosterhout *et al.*, 2003; Mariette *et al.*, 2006). However, it is unknown if variation in inbreeding in natural populations is high enough for sexual coloration to be associated with genome-wide heterozygosity.

Here, we tested whether MLH is revealed by traits known to be preferred by females (reviewed in: Houde, 1997; Magurran, 2005) using a wild guppy population that inhabits a stream, called Casupo, in northern Venezuela. Guppy populations inhabiting different streams show strong population structure, although non-negligible migration takes place between populations (Crispo *et al.*, 2006). When coupled with relatively small population sizes (Barson *et al.*, 2009), these factors may result in considerable variation in genome-wide heterozygosity (Reid, 2007; Fromhage *et al.*, 2009; Szulkin *et al.*, 2010). Under such conditions, a positive correlation between MLH and sexual coloration would be expected to arise.

## Materials and methods

### Samples

One hundred and forty-seven male guppies were sampled from the Casupo River (10°15'N, 66°25'W) in northern Venezuela in 2011. The stream is one of the several tributaries of the Guatire River which drains into Caribbean Sea. The Casupo River is a relatively shallow (20–40 cm in depth) stream, about 4–5 m wide, with gravel substrate and covered by sparse canopy (about 20%). Water flow is about 5 m s<sup>-1</sup> in the centre of the stream, but most of the guppies aggregate in slower-flowing areas near the edges. The fish were collected from an approximately 800-metre-long stretch of the stream using a deep net. Sampled fish were euthanized using a ~0.03% solution of MS-222 (tricaine methanesulfonate), photographed on their left sides and fixed in 95% ethanol until molecular analyses could be performed.

### Molecular methods

We used 13 polymorphic microsatellite loci to assess MLH (multilocus heterozygosity; i.e. the proportion of heterozygous loci present within an individual). A recent study has shown that, in the zebra finch, the power of a set of 11 microsatellite loci to detect heterozygosity and fitness correlation (HFC) is similar to that of 500–1500 SNPs (for populations whose inbreeding coefficients show large or small variances, respectively), and is higher than that of the inbreeding

coefficient  $F$  calculated from pedigrees (Forstmeier *et al.*, 2012). The authors argue that this power stems from the fact that highly polymorphic markers carry more information about identity by descent than biallelic SNPs or than  $F$ , which estimates the expected, rather than the actual, proportion of the genome that is identical by descent.

Total genomic DNA was extracted from all males using the Wizard<sup>®</sup> Genomic DNA Purification Kit (Promega). All males were screened for allelic variation at thirteen previously described microsatellite loci: AG1 and AG9 (Olendorf *et al.*, 2004); G75, G183, G255 and G325 (Shen *et al.*, 2007); Pret-52 and Pret-48 (Watanabe *et al.*, 2003); TACA033 (GenBank Acc. No. AY258896); CA061 (GenBank Acc. No. 30 AY258683); TAGA033 (GenBank Acc. No. 258667); Pre15 (GenBank Acc. No. AY830943); and Pre26 (GenBank Acc. No. AY830946). Loci were amplified using Multiplex PCR Master Mix (Qiagen) in five multiplex reactions. Each of them contained three to four primer pairs; one of the primers in each primer pair was fluorescently labelled (Table S1). The 10- $\mu$ L PCR mixture contained 5  $\mu$ L of master mix, 0.2–0.4  $\mu$ M of each primer and 20–100 ng of genomic DNA. The reaction conditions were as follows. A 15-min denaturation step at 95 °C was followed by 36 cycles of 30 s at 94 °C, 1 min at 52 °C and 1 min at 72 °C, before undergoing 10 min of final extension at 72 °C. The PCR products were mixed with a GeneScan LIZ500 size standard and electrophoresed on an ABI 3130xl Genetic Analyzer. Genotyping was performed using the ABI software GeneMapper 4.0. The procedure was repeated for 4% of the samples to estimate genotyping error.

### Phenotypic traits

We measured the body area covered by orange and black spots, body length (tail fin excluded) and caudal peduncle height by analysing the photographs of the males using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, <http://imagej.nih.gov/ij/>, 1997–2012). The numbers of orange and black spots were visually determined from the photographs. Body length multiplied by body height at the caudal peduncle was used to estimate body size and was thus measured in the same units as spots area. Relative orange and black spots areas were calculated by dividing an individual's total spots area of a given colour by its body size.

### Statistics

A test for the presence of null alleles was performed using FreeNA (Chapuis & Estoup, 2007). We tested for departures from Hardy–Weinberg equilibrium at all loci and linkage disequilibrium at each pair of loci using

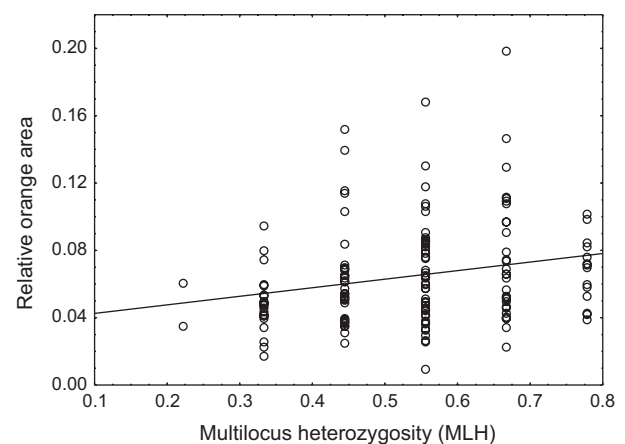
GENEPOP 4.1.2 (Rousset, 2008). A Bonferroni correction was used to control for the multiple tests performed.

The relationships between MLH and relative orange and black spots areas were tested using linear regression. The ability of heterozygosity to predict numbers of orange and black spots was analysed using generalized linear models with Poisson error distributions. All analyses were performed using STATISTICA v.10.

We also tested for local effects using the procedure developed by David (1997), in which the amount of variance explained by regressing a trait value on MLH (model  $m_1$  – general effects) is compared with the amount explained by a multiple regression model including the effect of each locus (model  $m_2$  – local effects). We used normalized heterozygosities as recommended by Szulkin *et al.* (2010). We compared the deviances of the  $m_1$  and  $m_2$  generalized linear models (Szulkin *et al.*, 2010).

## Results and discussion

All 13 microsatellite loci were polymorphic, with the number of alleles ranging from 3 to 45 (mean = 16.8; Table S2). Following Bonferroni correction, no linkage disequilibrium was found between any pair of loci. Four loci (G255, G325, Pret-48 and TACA033) were significantly more homozygous than expected given the assumption of Hardy–Weinberg equilibrium (Table S2). Null allele frequencies at those loci were estimated to be 0.14, 0.11, 0.05 and 0.04, respectively. Analyses performed on a restricted subset of nine loci that showed no significant departures from Hardy–Weinberg equilibrium yielded results that were qualitatively identical to those obtained using the full set of loci. Below, we report only the results of the analyses performed on the restricted subset.



**Fig. 1** Regression of the relative orange spots area on multilocus heterozygosity estimated from nine microsatellite loci.

We found a highly significant positive correlation between MLH and relative orange spots area (model  $m_1$ :  $\beta = 0.22$ ,  $F_{1,145} = 7.68$ ,  $P = 0.006$ , Fig. 1). There was no support for local effects, as the amount of variance explained by the  $m_2$  (local effects) model was not significantly greater than that explained by the  $m_1$  (general effects) model (model comparison:  $F_{8,143} = 0.82$ ,  $P = 0.598$ ).

In contrast, there was no significant relationship between MLH and relative black spots area (model  $m_1$ :  $\beta = -0.03$ ,  $F_{1,146} = 0.15$ ,  $P = 0.696$ ). The  $m_2$  model did not explain more variance than the  $m_1$  model ( $F_{8,143} = 1.30$ ,  $P = 0.247$ ). Finally, we found no significant relationship between MLH and the number of orange or black spots (orange spots number: estimate =  $-0.05$ ,  $\chi^2 = 0.904$ ,  $P = 0.342$ ,  $SE \pm 0.05$ ; black spots number: estimate =  $-0.02$ ,  $\chi^2 = 0.26$ ,  $P = 0.609$ ;  $SE \pm 0.05$ ). The  $m_1$  and  $m_2$  models did not differ in their explanatory power ( $P > 0.2$  in both cases).

The strong association between MLH and relative orange spots area in the absence of significant local effects lends support to the hypothesis that sexual ornaments reveal genome-wide heterozygosity as they reflect much of the variance in their bearer's condition (Brown, 1997; Kempnaers, 2007). Previous laboratory studies (Sheridan & Pomiankowski, 1997; van Oosterhout *et al.*, 2003; Mariette *et al.*, 2006) have shown that experimental inbreeding can affect orange spots, although no significant relationship was found by Zajitschek & Brooks (2010). However, this last study used guppies that originated from a feral Australian population, which could have been less prone to inbreeding depression as recessive deleterious alleles may have been purged during past bottlenecks (Zajitschek & Brooks, 2010). It thus appears that male orange coloration is particularly reflective of genome-wide heterozygosity because of its dependence on multiple autosomal loci, even if several genes known to determine orange spots occurrence are Y-linked (Winge & Ditlevsen, 1947; Tripathi *et al.*, 2009) and thus insensitive to inbreeding. Our results show that the potential of orange coloration to signal genome-wide heterozygosity is realized in a natural guppy population.

No other measure of male coloration we analysed was significantly associated with heterozygosity. Although several studies have reported that female guppies pay attention to black spots (Brooks & Caithness, 1995; Brooks & Endler, 2001), orange spots are more frequently reported to be the object of female preference (Endler, 1983; Kodric-Brown, 1985, 1993; Houde, 1987). The fact that a preference for black spots is not present in all populations and/or is weaker than the preference for orange spots suggests that melanin-based coloration may not be associated with indirect genetic benefits to females. Indeed, both orange ornament intensity (Kodric-Brown, 1989) and area (Nicoletto, 1993) are known to be more condition-dependent.

Orange ornaments utilize carotenoids obtained from the diet and, as a result, likely signal the ability to acquire resources. In contrast, black spots are melanin-based, and they may not be directly linked to an individual's condition. These mechanisms could explain why heterozygosity was strongly associated with orange spots area, but not black spots area.

The effect size of the MLH–orange spots area correlation ( $r = 0.22$ ,  $P = 0.006$ ) exceeds those typically found for correlations between heterozygosity and life-history traits, indicating that sexual ornaments may be reliable indicators of genome-wide heterozygosity under natural conditions. Preference for ornaments revealing heterozygosity may result in indirect genetic benefits if there is a correlation between the heterozygosity of parents and fitness of their offspring. Recent models have shown that mate choice for ornaments revealing heterozygosity is indeed likely to bring genetic benefits in finite, structured populations, which can lead to the evolution of preferences for condition-dependent sexual ornaments (Fromhage *et al.*, 2009; Reid & Keller, 2010; but see Aparicio, 2011). Guppy populations, which are characterized by low effective sizes and limited but detectable gene flow (Barson *et al.*, 2009), are likely to have a similar internal genetic structure. Whether offspring fitness correlates with parental heterozygosity remains to be examined in future research.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1** List of primers for microsatellite amplification.

**Table S2** Observed and expected heterozygosities for each microsatellite locus; loci in red showed significant deviations from Hardy-Weinberg equilibrium.

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