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Heterozygosity, sexual ornament and body size in the crested newt

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Abstract

Both genome-wide heterozygosity and heterozygosity at major histocompatibility complex (MHC) genes are often associated with higher fitness. Recent theoretical work indicates that sexual ornaments may reveal information about individual heterozygosity, and that preference for such ornaments may benefit females via the increased heterozygosity of their progeny. Here, we used path analysis to investigate the direct and indirect (via body size used as an index of condition) effects of heterozygosity at six microsatellite loci and the MHC class II *DAB* gene on the size of a sexual ornament, the crest, in the crested newt *Triturus cristatus*. We found that microsatellite heterozygosity, but not MHC heterozygosity, significantly predicted male body size, and that male body size significantly predicted crest height. However, there was no direct effect of MHC or microsatellite heterozygosity on crest height. Furthermore, microsatellite heterozygosity significantly increased with age, indicating that it had a positive effect on survival. Overall, our results are consistent with the hypothesis that heterozygosity determines condition, and that variation in condition is expressed as variation in sexual ornamentation.

Introduction

Heterozygosity-fitness correlations (HFC) have been reported for numerous species. Although the average effects are rather small, there is considerable variation in the strength of such associations (Chapman *et al*., 2009), likely because of the particularities of different mating systems and population structures (Reid & Keller, 2010; Szulkin, Bierne & David, 2010; Jourdan-Pineau *et al*., 2012). One of the more interesting HFC types is the association between heterozygosity and the elaboration of sexual ornaments. The evolution of such ornaments, which occurs more often in males than in females, was attributed by Darwin to the fact that elaborate ornaments resulted in increased sexual appeal, and this notion has been confirmed by numerous studies documenting female preferences for highly ornamented males (reviewed in Andersson, 1994). More recently, researchers have directed their efforts toward understanding the evolution of this preference (reviewed in Andersson & Simmons, 2006). In species in which males contribute nothing but gametes to reproduction, the maintenance of sexual preference has been explained in terms of indirect genetic benefits: male progeny may inherit genetic variants underlying sexual attractiveness (Fisher, 1930) or those associated with general health and vigor (Zahavi, 1975). Several mechanisms have been proposed for the maintenance of genetic variation necessary for these genetic benefits to be realized, including balancing selection, gene flow and the continuous influx of deleterious mutations (reviewed in Radwan, 2008). Male genetic quality (defined as the breeding value for total fitness; Hunt *et al*., 2004; Tomkins *et al*., 2004) may be revealed by sexual ornaments if ornament expression depends on male condition. Condition-dependence implies that the state of most of the genes affecting an individual's condition will be reflected in sexual ornament size (Andersson, 1986; Rowe & Houle, 1996). Because heterozygosity may also affect an individual's condition, it should concurrently be reflected by condition-dependent ornaments. Sexually selected traits have been shown to be sensitive to the increased homozygosity that has been achieved via experimental inbreeding in several studies, although the magnitude of the effect varies among species (reviewed in Prokop *et al.* 2010).

Because of the apparent prevalence of HFCs, Brown (1997) proposed that females should choose their mates so as to maximize offspring heterozygosity. This can be achieved by choosing genetically distant, compatible mates rather than mates with elaborate ornaments. However, if the

heterozygosity of parents correlates with that of their offspring, which can occur under some circumstances (Mitton *et al*., 1993), preferences for ornaments that reveal heterozygosity will result in the increased fitness of progeny (reviewed in Kempenaers, 2007). Indeed, in the song sparrow population of Mandarte Island, the inbreeding coefficient (a measure of expected homozygosity resulting from identity by descent) was correlated with male song repertoire (Reid *et al*., 2005), and in the same system, a positive correlation between the inbreeding coefficients of parents and offspring was found (Reid, Arcese & Keller, 2006). Similarly, in Antarctic fur seals, heterozygous males sired more offspring, and sire heterozygosity predicted offspring heterozygosity (Hoffman *et al*., 2007). Recent models have shown that such parent-offspring heterozygosity correlations are likely to arise in structured populations, within which subpopulations exchange a limited number of migrants (Fromhage, Kokko & Reid, 2009; Reid & Keller, 2010). Under such conditions, mate choice for ornaments revealing male heterozygosity evolves easily, and despite directional selection on ornaments, genetic variation is not easily depleted (Fromhage *et al.* 2009; but see Aparicio, 2011).

Associations between heterozygosity and fitness can arise in two ways (reviewed in Kempenaers, 2007). Homozygosity may reveal the deleterious effects of recessive and partially recessive mutations, which are currently considered the major cause of inbreeding depression (Charlesworth & Willis, 2009). Alternatively, heterozygosity may be intrinsically associated with higher fitness, for example, because it allows an organism to deal with a wider range of environmental conditions (Lerner, 1954). Heterozygote advantage has been demonstrated for major histocompatibility complex (MHC) genes that code for proteins involved in the recognition of antigens derived from pathogenic organisms and that incite adaptive immune responses (reviewed in Piertney & Oliver, 2006; Spurgin & Richardson, 2010). For example, MHC-heterozygous mice were better able to clear infections caused by multiple strains of salmonella than MHC-homozygous mice (Penn, Damjanovich & Potts, 2002). Furthermore, some studies have demonstrated that male MHC heterozygosity is associated with higher reproductive success (Sauermann *et al*., 2001; Bonneaud *et al*., 2006).

While estimates of genome-wide heterozygosity based on a small panel of microsatellite markers have been shown to correlate only weakly with the inbreeding coefficient *F* calculated from pedigrees (Balloux, Amos & Coulson, 2004; Pemberton, 2004; Slate *et al*., 2004), a recent study by Forstmeier *et al*. (2012) demonstrated that a small panel of polymorphic microsatellites may reflect the identity by descent (and thus inbreeding) of a genome with an accuracy similar to that shown by a large panel of single nucleotide polymorphisms and better than that shown by pedigree-based *F*. HFCs are particularly likely to arise if there is considerable variation in *F* within populations (Szulkin *et al*., 2010), as occurs, for example, when local populations with small to moderate *Ne* are substantially inbred, but immigration from other populations produces some highly heterozygous individuals (Reid *et al.*, 2006; Fromhage *et al.*, 2009). Amphibians are characterized by a relatively low level of mobility, and many temperate species depend on water bodies that are patchily distributed throughout the habitat for reproduction. Metapopulations are consequently formed (Marsh & Trenham, 2001), and considerable variation in heterozygosity within local populations may occur.

Here, we investigated the effect of heterozygosity at microsatellite loci and the MHC class II *DAB* locus on crest size in the crested newt *Triturus cristatus*. The *DAB* shows a landmark of historical positive selection in terms of elevated d_n/d_s ratio (Babik *et al*., 2009), but current selection acting on this locus has not been investigated. The crest, which is expressed in males during the breeding season, was used by Darwin (1871) as an example of a sexually selected trait. Further research has since confirmed that females prefer males with tall crests in this species (Hedlund, 1990; Malmgren & Enghag, 2008). As crest height is condition dependent and correlated with male size (Hedlund, 1990; Green, 1991), in this study, we used path analysis to infer possible direct and indirect (via body size) effects of heterozygosity on crest height. If crest-size is condition-dependent, both MHC and microsatellite heterozygosity should be associated with taller crests, but the effect should be mostly indirect, via body size, which we used as an index of condition.

Methods

Samples

During the 2007 breeding season, 128 adult male crested newts were sampled from a pond in Inwald, a village in southern Poland (49°51′N, 19°23′E). Newts were collected using funnel traps and anesthetized in a ~0.2% solution of MS-222 (tricaine methanosulfonate). A toe was sampled from each individual and stored in 95% ethanol for molecular analyses. Animals were photographed and released. Permission for the sampling was given by the Local Ethical Committee in Krakow (2006/16) and the Ministry of the Environment (DLOPiK/ogiz-4200/II-06/3139/06/aj).

Molecular methods

Genomic DNA was extracted from each toe using a Genomic Minikit (A&A Biotechnology, Gdynia, Poland). The bones were retained and later analysed using skeletochronology. All individuals were screened for allelic variation in six previously described microsatellite loci: *Tcri13, Tcri43, Tcri35, Tcri29, Tcri27* and *Tcri36* (Krupa *et al*., 2002). Loci were amplified with Multiplex PCR Master Mix (Qiagen, Hilden, Germany), using one fluorescently labeled primer in each primer pair. The 10-μl reaction mixture included 5 μL of Master Mix, 0.2–0.4 μM of each primer, and 20–100 ng of genomic DNA. The reaction conditions were as follows: 15 min of denaturation at 95°C, 36 cycles of 30 s at 94°C, 1 min at 55°C, 1 min at 72°C, and 10 min of final extension at 72°C. PCR products were electrophoresed using an ABI 3130xl instrument; 0.3 μL of Gene Scan 500 LIZ internal size standard was added to each

reaction mixture. Genotypes were obtained by analysing the results with GeneMapper 4.0 (ABI, Paisley, UK). Mean heterozygosity was calculated as a multi-locus heterozygosity (i.e. the sum of heterozygous loci divided by the number of loci). MHC was typed on the ABI 3130xl using single strand conformation polymorphism as described in Babik *et al*. (2009).

Age estimation

Skeletochronology has been successfully used to assess the ages of urodeles specimens for decades (Smirina, Klevezal & Berger, 1986; Castanet & Smirina, 1990). In the past, sections of humerus or femur were used, but currently phalanges are preferred as they give the same results without the need of sacrificing animals (Gittins, Steed & Williams, 1982; Rozenblut & Ogielska, 2005). In the temperate zone, amphibian long bones grow during the spring and summer, but growth slows during hibernation. As a result, the structure of compact bone layers produced during active and inactive periods differs considerably. During active periods, more loose tissue is deposited and manifests itself as thick, light rings called annuli lines that are visible in transverse sections. During hibernation, in contrast, osteogenesis stops, and highly mineralized tissue is formed; in transverse sections, this tissue is visible as very thin, well-stained rings (± 1 μm) called lines of arrested growth. An experimental study has shown that the number of LAGs corresponds to the number of hibernations in the crested newt (Francillon, 1979). Individual phalanges cleared of soft tissue were obtained as the samples were prepared for DNA extraction. For skeletochronology purposes, the longest phalanges were used. Skeletochronological samples were prepared according to the protocol described by Skierska and Ogielska (unpublished, see Supporting Information for details).

Morphometric measurements

Using digital images, the snout-vent length (SVL) and crest height of each male were measured twice, using the software ImageJ version 1.44p (developed at the US National Institutes of Health and available at http://developer.imagej.net/). We also measured twice the crests areas of a randomly selected subset of 20 males. Crest area proved highly correlated with crest height (r = 0.9), but measurements of the latter were more repeatable (repeatability = 99 for crest height vs. 0.89 for crest area); therefore, we only measured crest height for the full dataset. The SVL was measured from the most distal point of the snout to the center of the cloaca. The height of the crest was measured from its highest point to its base and at a perpendicular angle to the body's axis of symmetry. For both traits, the average of two measurements was calculated.

Statistical analyses

We used the program Genepop 4.1.2 (Rousset, 2008) to evaluate the conformance of actual genotype frequencies at each microsatellite and MHC *DAB* locus with the Hardy–Weinberg expectation, as well as to test for linkage disequilibrium between each pair of loci. Null allele frequencies were estimated with FreeNA (Chapuis & Estoup, 2007). The distributions of SVL and crest height were not significantly different from normal (Kolomogorov–Smirnov test, $P > 0.05$), and age was log-transformed to improve the normality of its distribution. Because of the complex interrelationships between variables analysed (Fig. 1), we used path analysis, which allows modeling directed dependencies among a set of variables and simultaneous assessment of direct and indirect relationships between them. A path analysis was performed using the package sem in R (R Development Team, 2008). The initial model included the relationships depicted in Fig. 1. Non-significant paths were individually removed from the model, and the fit of each reduced model was compared to that of other reduced models and the full model based on Bayesian Information Criterion (BIC). Deletion testing was performed until no further improvement of the model fit could be achieved. The final model (Table 1) did not contain MHC heterozygosity and retained only the paths marked by solid lines in Fig. 1.

Results

Molecular markers and phenotypic measurements

We found two previously described MHC class II *DAB* alleles in the Inwald population of crested newts: *Trcr*-DAB*01 and *Trcr*-DAB*02. Among the 112 animals for which the MHC locus was successfully genotyped, there were 18 heterozygotes and 94 *Trcr*-DAB*01 homozygotes. Genotype frequencies did not depart from the Hardy–Weinberg expectation ($P = 0.999$).

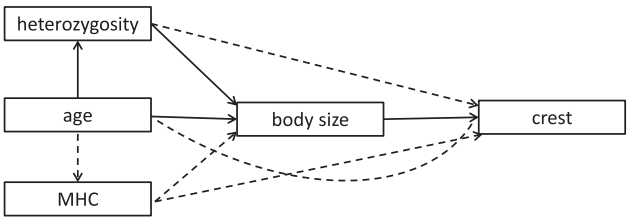

Figure 1 Path diagram showing the relationships included in the initial model. Solid bars represent the significant paths retained in the final model. MHC, major histocompatibility complex.

Table 1 Final model testing the direct and indirect effects of microsatellite heterozygosity, age and body size on crest height. Major histocompatibility complex heterozygosity was removed from the model during deletion-testing (see Methods). Final model: $\chi^2 = 2.239$, d.f. = 2, $P = 0.326$, Comparative Fit Index = 0.99

Path	Estimate	SE	Z	*P*
Comb ← body size	0.194	0.003	58.04	<0.001
Body size ← age	2.353	0.197	11.91	<0.001
Body size ← heterozygosity	3.168	0.462	6.82	<0.001
Heterozygosity ← age	0.407	0.012	34.66	<0.001

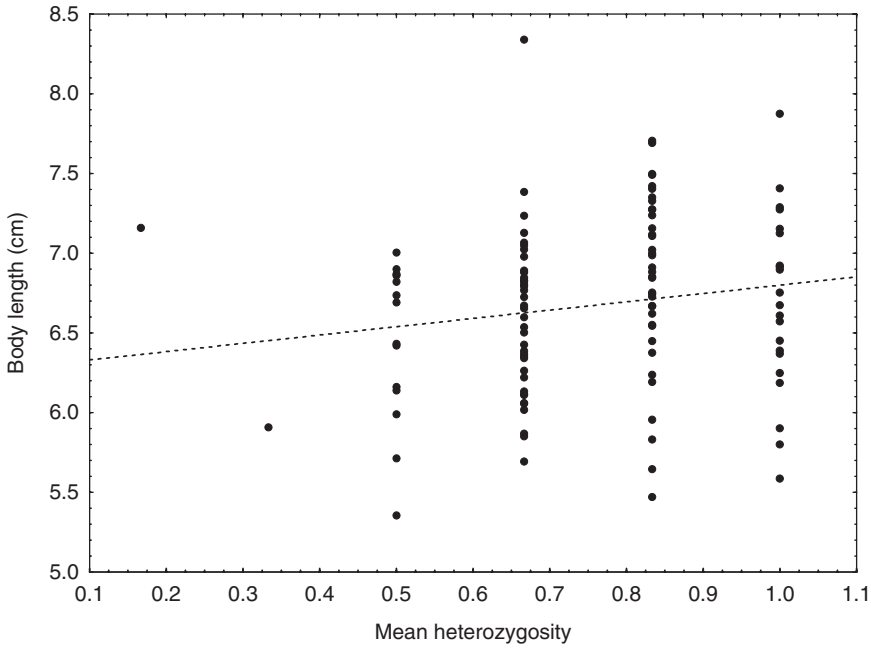

Figure 2 Relationship between mean heterozygosity at six microsatellite loci and body size in the crested newt.

We found 7, 9, 9, 10, 11 and 15 alleles for the *Tri13*, *Tri43*, *Tri35*, *Tri29*, *Tri27* and *Tri36* microsatellite loci, respectively. One out of the six loci (*Tri29*) showed, after applying the Bonferroni correction (i.e. at $P < 0.008$), a significantly greater number of homozygotes than would be expected based on Hardy–Weinberg, probably due to the presence of null alleles. The estimated frequency of null alleles at this locus was nevertheless low (0.057), so it was not excluded from further analyses (an analysis excluding locus *Tri29* gave identical results, Supporting Information Table S1). None of the tests for linkage disequilibrium were significant after applying the Bonferroni correction. MHC heterozygosity was not correlated with microsatellite heterozygosity ($r = -0.02$, $P = 0.848$).

Means ($\pm$ standard deviation) for SVL, crest height and age (back-transformed) were 8.33 ± 0.53, 2.01 ± 0.27 and 5.92 ± 1.36, respectively.

Path analysis

Path analysis indicated that crest size depended on genome-wide heterozygosity only indirectly (Table 1). Heterozygosity affected body size (Fig. 2), and body size, in turn, determined crest size, (Fig. 3). The direct effect of heterozygosity on crest size was removed during deletion testing (Δ BIC = 3.22; $P = 0.91$ for this effect in the unreduced model). Body size was also dependent on age. Age was associated with genome-wide heterozygosity, with individuals with higher heterozygosity being older (Table 2). MHC heterozygosity, on the other hand, was not significantly associated with any of the variables measured. Analysis with the same set of variables, but with body size removed from the model, yielded the same conclusions, except that the direct effect of heterozygosity on crest size has become significant (Estimate = 0.41, SE = 0.14, $Z = 2.96$, $P = 0.036$).

Discussion

Condition-dependent sexual ornaments are predicted by the 'good genes' theory to reflect the genetic quality of their bearers. In the case of interpopulation variation in genome-wide heterozygosity, sexual ornament elaboration may be predicted to increase as the individual heterozygosity index increases (Brown, 1997; Kempenaers, 2007). In particular, it may be predicted that heterozygosity determines condition, and that variation in condition will be expressed as variation in sexual ornamentation. In such a case, condition should explain most of the variation in ornament size, and an indirect, rather than a direct, effect of heterozygosity (acting via condition) on ornament size would be expected. There is considerable debate over the precise meaning of the term condition. It is generally understood to be efficiency in acquiring resources and converting them into fitness (Rowe & Houle, 1996), but body size is often assumed to be a reasonable index of condition, as good foragers with efficient physiologies will tend to grow to bigger sizes (see Tomkins *et al.*, 2004). Our results appear consistent with this prediction, as we found a significant effect of heterozygosity on male body size and of body size on crest height, but no direct effect of heterozygosity on crest height when body size was included in the model. However, when body size was removed from the model, heterozygosity significantly affected crest height, suggesting that crest size can be used by females as a cue to this aspect of male

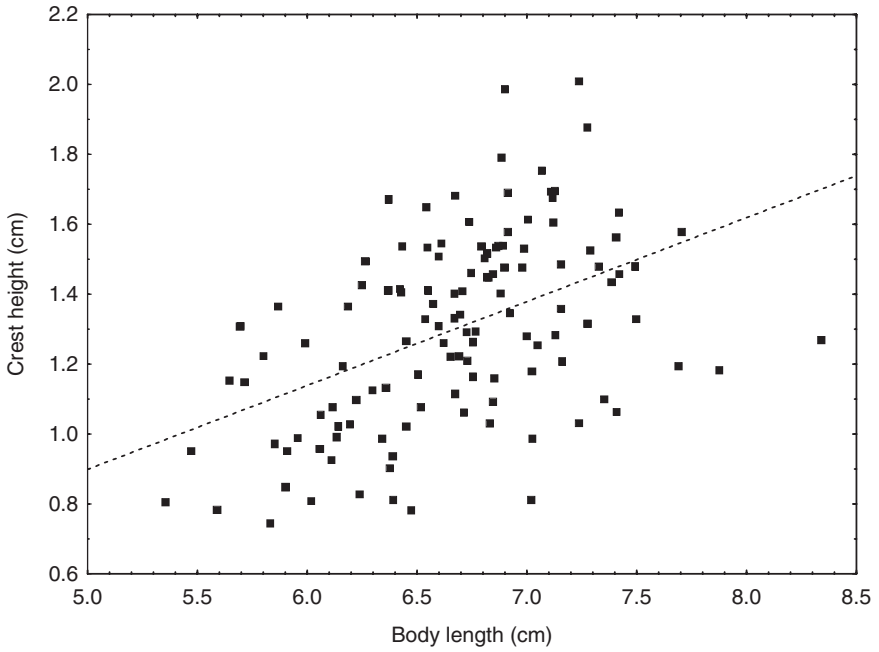

Figure 3 Relationship between crest height and body size in the crested newt.

Table 2 Microsatellite and major histocompatibility complex (MHC) heterozygosities in different age classes in male crested newts

Age class (years)	*n*	Microsatellites		MHC	
		Mean	SD	Mean	SD
3	6	4.33	1.02	0.21	0.41
4	11	4.54	1.03	0.16	0.41
5	25	4.52	0.93	0.18	0.40
6	24	4.29	1.22	0.26	0.45
7	13	4.46	0.90	0.23	0.43
8	10	4.60	1.12	0.15	0.37
9	6	4.66	1.07	0.16	0.39
>9	8	4.87	0.81	0.33	0.52

genetic quality. Previous research has shown that although male size affects mating success in crested newts, the maximal height of the male's crest is more important (Hedlund, 1990; Malmgren & Enghag, 2008). A possible reason for this preference is that crest size is easier for females to assess than body size. Similarly, in stalk-eyed flies, females prefer to lek with males with large eye-span, but inbreeding depression in eye-span is statistically accounted for by the effect of inbreeding on body size (Prokop *et al.*, 2010).

While several studies have shown the effect of controlled inbreeding on sexual ornament size (reviewed in Prokop *et al.*, 2010), only studies utilizing natural variation can reveal whether ornament size reflects individual heterozygosity in natural populations, and thus whether mate choice possibly yields benefits in terms of an increased heterozygosity of progeny. In song sparrows, an individual's level of inbreeding was correlated with the complexity of its song (Reid *et al.*, 2005), and more heterozygous wire-tailed manakins were more likely to be chosen as mates and were characterized by brighter, longer feather traits and longer tarsi (Ryder *et al.*, 2010). Some studies have documented an association between male heterozygosity and reproductive success (Hoglund *et al.*, 2002; Garant, Dodson & Bernatchez, 2005; Hoffman *et al.*, 2007), but others have not found a significant association (e.g. Richardson *et al.*, 2005; Bonneaud *et al.*, 2006; Lampert *et al.*, 2006). Our results indicate that a trait that is positively associated with reproductive success in crested newts, body size (Hedlund, 1990), is positively influenced by male heterozygosity, although we detected no significant direct effect of heterozygosity on the size of the sexual ornament, the crest.

Furthermore, we found that older individuals demonstrated increased heterozygosity, indicating that genome-wide heterozygosity is associated with better survival. Alternatively, genetic variation in this population may be declining, resulting in older individuals being more heterozygous. It seems unlikely, however, that such a decline could occur over the few years analysed in the context of this study. Thus, the better survival of more heterozygous individuals seems to be the most likely explanation for our results, adding to a large body of evidence showing that heterozygosity is associated with various correlates of fitness (reviewed in Kempenaers, 2007).

In contrast to genome-wide heterozygosity, MHC heterozygosity was not significantly associated with any of the variables we measured. Associations between MHC heterozygosity or multi-locus MHC diversity and male reproductive success have been reported for rhesus monkeys (Sauermann *et al.*, 2001) and house sparrows (Bonneaud *et al.*, 2006), but other studies have failed to detect significant relationships (e.g. Paterson & Pemberton, 1997; Landry *et al.*, 2001; Ekblom *et al.*, 2004; Westerdahl, 2004; Sommer, 2005). As

MHC genes tend to be very polymorphic in most species, many studies may have limited statistical power to detect significant MHC heterozygosity effects, as homozygotes are rare. However, in crested newts, there are only two MHC *DAB* alleles in post-glacial expansion areas (i.e. the habitat in which our population is located), and so any effects of MHC type or heterozygosity should be relatively easy to detect statistically. Apart from MHC heterozygosity, resistance to infection is often associated with particular alleles. Indeed, Eizaguirre *et al.* (2009) recently reported that, in sticklebacks, an allele associated with resistance to infection by *Gyrodactylus* was also associated with male mating success (but not with the intensity of sexual coloration). In the case of our population, there were only two genotypes (81% of common homozygotes and 19% of heterozygotes), so that the effects of being a heterozygote and possessing a rare allele were confounded. The lack of an effect of heterozygosity thus implies that possessing a rare allele also has no effect. Coupled with the lack of deviation of MHC *DAB* from H-W equilibrium, our results thus indicate very weak (or non-existent) contemporary selection acting on MHC genes in the crested newt.

In conclusion, we have found that microsatellite, but not MHC heterozygosity has positive effects on male body size and survival. These results indicate that genome-wide heterozygosity is associated with higher fitness in *T. cristatus*. Crest height was associated with heterozygosity only indirectly (via body size), in agreement with the hypothesis that sexual ornamentation captures genetic variation in condition.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. The path analysis model for loci in Hardy–Weinberg equilibrium.

Table S1. The model equivalent to that reported in Table 1, but using only five loci which did not deviate from Hardy–Weinberg equilibrium. Model $\chi^2 = 1.75$, d.f. = 2, $P = 0.42$, Comparative Fit Index (CFI) = 1.0.