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Polyploidy alters advertisement call structure in gray treefrogs

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Whole-genome duplication is believed to have played a significant role in the early evolution and diversification of vertebrate animals. The establishment of newly arisen polyploid lineages of sexually reproducing animals requires assortative mating between polyploids. Here, we show that genome duplication can directly alter a phenotypic trait mediating mate choice in the absence of genotypic change. Our results suggest that the direct effect of polyploidy on behaviour is a consequence of increased cell size.

Keywords: polyploidy; genome duplication; evolution; speciation; behaviour

1. INTRODUCTION

Genome duplication is an important evolutionary mechanism in generating organismal diversity and increased organic complexity. Duplicate genes can evolve to take on novel functions, giving rise to new gene families and facilitating rapid taxonomic radiations. Increasing evidence suggests that two genome duplications occurred early in the origin of vertebrates, with a third duplication occurring in the actinopterigian lineage giving rise to modern fishes (Meyer & Schartl 1999). More recent duplications have played a significant evolutionary role within some groups of sexually reproducing fish and anuran amphibians (Bogart 1980; Schultz 1980). Thus, the formation of polyploid species by genome duplication is a recurrent phenomenon in organic evolution and is associated with the origin of highly successful, rapidly radiating taxa (Meyer & Schartl 1999).

The formation of polyploid individuals within diploid populations is likely to occur infrequently and the conditions that favour the establishment of a polyploid species from such individuals are restrictive (Thompson & Lumaret 1992). Newly arisen polyploids face the daunting task of surviving in direct competition with their diploid parental species. Sexually reproducing polyploids must have an even number of sets of chromosomes (=orthoploidy, i.e. tetraploid, hexaploid, etc.) so that identical 'haploid' gametes can be produced at high frequency during meiosis (Haldane 1930). In order to become established as new species, polyploid individuals must avoid back-crossing with diploids (Muller 1925). Diploid–polyploid (intercytotypic) matings produce asexual, infertile or inviable odd-ploidy (i.e. triploid) offspring (Muller 1925; Haldane 1930; Bogart & Wasserman 1972). In plants, selfing is an important component of polyploid speciation because it increases the frequency of intracytotypic (i.e. same ploidy) mating and prevents rare cytotype extinction as a result of intercytotypic hybridization (Levin 1975). The frequency of intracytotypic matings in plants can also be enhanced by ploidy-specific changes in phenotype that result in spatial/ecological separation or pollinator biases (Rodríguez 1996; Husband 2000). In animals that are incapable of self-fertilization, polyploid speciation requires preferential mating of like cytotypes. The probability of successful establishment of a polyploid species would therefore be greatly enhanced if genome duplication had direct effects on traits that are important for mate choice.

Genome duplication can significantly alter the developmental pattern and physiology of cells. Increased genome size is correlated with increased cell size, decreased cell number, longer cell cycle duration and a slower developmental rate (Cavalier-Smith 1978). In animals, increased genome size is correlated with decreased complexity in the central nervous system (Roth *et al*. 1993). These effects may occur in direct response to ploidy-specific changes in gene dosage (Guo *et al*. 1996), transcriptional regulation (Galitski *et al*. 1999) and/or 'nucleotypic' effects of increased nuclear volume (Bennett 1972). If these ploidy-specific changes affect alterations of phenotypic traits that are important for mate choice, they could promote assortative mating of cytotypes when accompanied by correlated changes in female preference or by selection against females that do not mate with males of like ploidy. Even if insufficient to achieve complete behavioural isolation, direct effects of polyploidy on assortative mating could allow polyploids to persist long enough for reproductive character displacement to drive divergence to the point of complete pre-zygotic isolation (Liou & Price 1994).

Polyploid speciation is generally a rare phenomenon in animals, but there are a number of cases among anuran amphibians (frogs and toads), including members of six families within two suborders. Most polyploid anurans are of autopolyploid origin rather than resulting from genome duplication following hybridization, as is the case in *Xenopus* (Bogart & Wasserman 1972; Tymowska 1991). Anurans use acoustic signals for mate choice. Females approach and usually initiate contact with calling males, and differences in call structure are necessary and sufficient for promoting homospecific pairing (Gerhardt 1991). Ploidy-specific structural changes in the calls of males could therefore provide a basis for assortative mating among cytotypes (Bogart & Wasserman 1972). To date, only Ueda (1993) has directly tested the effect of polyploidy on call structure using artificially produced autopolyploid frogs. Although confirming that the values of some call properties are correlated with ploidy level, the subject of that study, *Hyla japonica*, has no polyploid

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relatives against which the magnitude of the effect could be scaled (Ueda 1993). Here we examine the effect of polyploidy on advertisement call structure using a pair of diploid–polyploid North American treefrogs with a clearly determined progenitor–offspring relationship (Ptacek *et al*. 1994). Males of the tetraploid gray treefrog *Hyla versicolor* produce calls with pulse repetition rates (pulse rate) that are 30–60% slower than those of the diploid *Hyla chrysoscelis*, depending on temperature and the populations being compared (Gerhardt 1982). Pulse rate is a key call property used by females in selecting conspecific mates (Gerhardt 1994). Because mismating results in the production of triploid offspring, which have reduced viability and are effectively sterile (Johnson 1959, 1963), there is strong selection against hybridization. Divergence in response to this selection, direct effects of polyploidy on the tetraploid species or a combination of both processes could have contributed to the present day differences in pulse rate between the two species.

We compared the pulse rates of the advertisement calls of hybrid allotriploid and artificially produced autotriploid gray treefrogs with the pulse rates of *H. chrysoscelis* and *H. versicolor*. This comparison provided an estimate of the relative contributions of the direct effects of polyploidy (cytotypic effects) and of post-speciation divergence (genotypic effects) on contemporary call differences between the species. Because hybrid triploids have a recombinant genotype consisting of both *H. chrysoscelis* and *H. versicolor* alleles, call structure will depend on both cytotypic and genotypic effects. In contrast, autotriploids have only *H. chrysoscelis* alleles; any changes in call structure can therefore be attributed solely to cytotypic effects.

2. METHODS

We recorded *H. chrysoscelis*, *H. versicolor* and hybrid males at a syntopic breeding assemblage in Phelps County, MO, USA. Autotriploid offspring of *H. chrysoscelis* from the same locality were generated by cold shock (Fankhauser & Griffiths 1939) at 1–4 °C for 1 h starting 5 min after oviposition. The autotriploids were raised in a common laboratory environment with diploid (*H. chrysoscelis* individuals from cold-shock treatments which failed to become polyploid) and tetraploid (*H. versicolor*) controls. Mature males of one to two years' post-metamorphosis were recorded in acoustically transparent screen cages. We measured the body surface temperature between the thigh and abdomen, thus allowing us to standardize pulse rate for variation in this variable (Gayou 1984). A few males called spontaneously, but most were stimulated to call by exposure to 45 min of artificial rain at dusk followed by playbacks (call period of 6 s) of synthetic advertisement calls of *H. chrysoscelis*. Pulse rate was not expected to be affected by acoustic experience because individuals raised in acoustic isolation (Doherty & Gerhardt 1983) or only exposed to heterospecific calls (Burger 1980) produced calls with pulse rates that were nearly the same as those of wild-type males. After the recordings had been analysed, we determined the ploidy of each putative hybrid found in the field and each male that had been raised in the laboratory using flow cytometry (Ptacek *et al*. 1994) or direct chromosome counts from cultured lymphocytes (Wiley & Meisner 1984). Frogs of different ploidy could not be distinguished by external morphology.

Recordings were digitized and analysed using commercial sound analysis software (Soundedit 16; Macromedia, San Francisco, CA, USA). The mean pulse repetition rate was calculated from at least six calls for each male prior to ploidy determination; thus, call analyses were performed 'blind' with respect to cytotypic identity. Temperature correction was performed using the least-mean-square (LMS) regression coefficient for each group of males. The significance of the regression coefficients was tested using ANOVA (Zar 1984). When there was insufficient temperature variation and/or sample size for accurate regression analysis, we made comparisons at the mean temperatures for those groups. The means of two variables in a LMS regression specify a constant point for all possible slopes (Glass & Hopkins 1984). Statistical comparisons of temperature-corrected pulse rates were made with the non-parametric Mann–Whitney *U*-test (Zar 1984). All statistical comparisons were made at a critical significance level of $\alpha = 0.05$.

Erythrocyte nuclear areas were calculated from length and width measurements made with an ocular micrometer at ×1000 magnification using the equation for the area of an ellipse. Erythrocyte nuclear area was used as a general index of relative cell size for comparisons between diploid, hybrid triploid, autotriploid and tetraploid individuals. Nuclear area and cell volume are highly correlated (Kuramoto 1981), but nuclear area exhibits substantially less variability than cell volume, which is affected by osmotic damage during preparation for microscopic observation. Increased genome size also has a direct effect on the size of the nucleus (Kuramoto 1981), while the increase in cell volume is more likely to be a secondary response to the increase in nuclear volume (Cavalier-Smith 1978). Changes in nuclear area are therefore indicative of a 'nucleotypic' response to genome duplication.

3. RESULTS

As expected, wild-type *H. versicolor* males produced calls with pulse rates that were more than 50% lower than those of the wild-type *H. chrysoscelis* males over a wide range of temperatures in both the field-recorded and laboratory-reared samples (figure 1*a*,*b*). There was a significant ($p < 0.05$) correlation between pulse rate and temperature in each case (table 1). The mean temperature-corrected pulse rates of *H. versicolor* were lower than those of *H. chrysoscelis* by 59.8% among the field-recorded males and 60.6% among the laboratory-reared males.

All of the field-recorded hybrid males ($n = 4$) produced calls with mean pulse rates lower than the predicted mid-parent value for the field-recorded *H. chrysoscelis* ($n = 22$) and *H. versicolor* ($n = 11$) controls (figure 1*a*). There was insufficient temperature variation among recordings of the hybrid triploid males for meaningful regression. The hybrid males had a mean pulse rate that was 38.6% lower than the temperature-corrected mean pulse rate of the field-recorded *H. chrysoscelis* controls (table 1).

All of the laboratory-reared autotriploid males produced calls with mean pulse rates that were substantially higher than the predicted mid-parent value for the laboratory-reared control *H. chrysoscelis* ($n = 10$) and *H. versicolor* ($n = 5$) males (figure 1*b*). Preliminary analysis of temperature-corrected data indicated that the pulse rates of the autotriploid *H. chrysoscelis* males were bimodally distributed with individuals belonging to two distinct groups (37–41 or 45–48 pulses s^{-1} at 20 °C); therefore, the data were divided between 'slow' ($n = 8$) and 'fast' ($n = 4$) autotriploid groups in subsequent analyses (figure 1*b*).

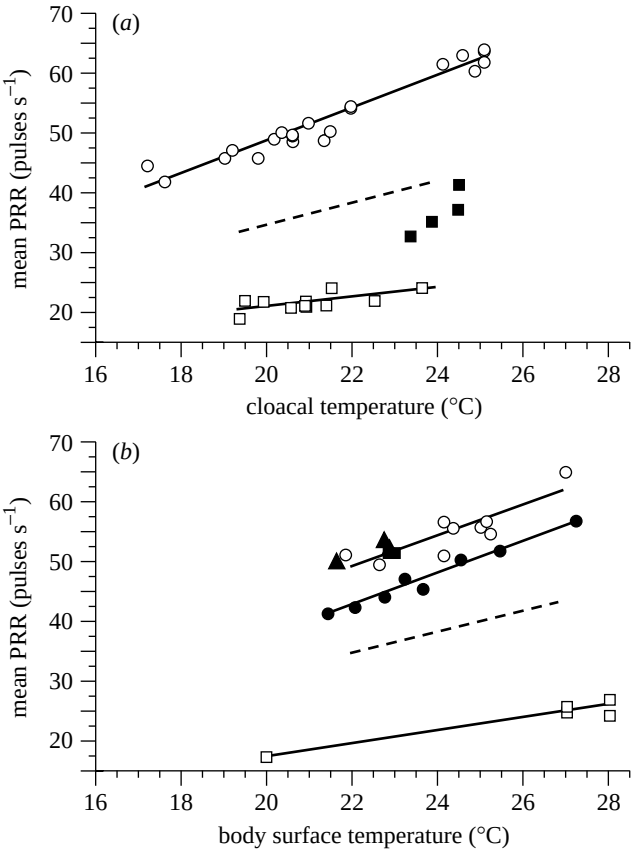

Figure 1. Mean pulse rate as a function of temperature. (*a*) Individual means for field-recorded diploids (*H. chrysoscelis*) (open circles), tetraploids (*H. versicolor*) (open squares) and triploid hybrids (filled squares). (*b*) Individual means for laboratory-reared diploids, tetraploids and fast (filled triangles) and slow (filled circles) autotriploids. Lines of best fit from LMS linear regression are indicated by solid lines and dashed lines indicate mid-parent values based on the means of predicted values from the diploid and tetraploid regressions over a range of temperatures.

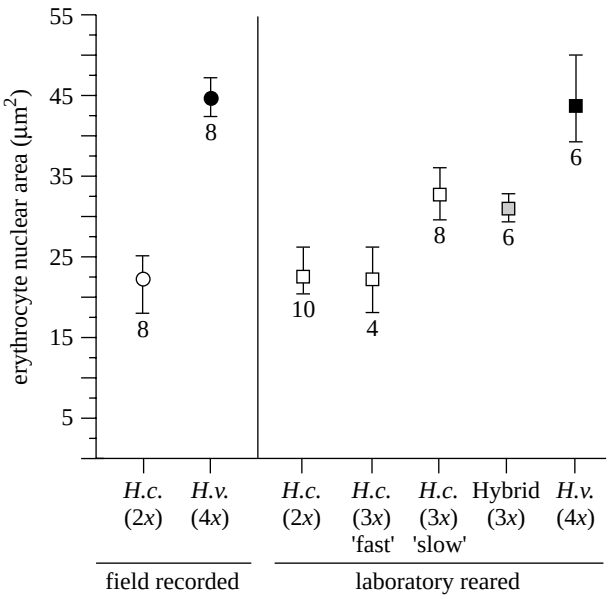

Figure 2. Variation in mean erythrocyte nuclear area between *H. chrysoscelis* (open symbols), *H. versicolor* (filled symbols) and hybrids (shaded symbol) in relation to ploidy level. Field-collected (circles) and laboratory-reared (squares) groups are presented separately. Autotriploid *H. chrysoscelis* are divided between fast and slow groups on the basis of pulse rate. The bars indicate the range of nuclear areas and sample sizes are given for each group.

There was a significant correlation between temperature and pulse rate in the slow autotriploid group (table 1); however, there was insufficient temperature variation between recordings for estimating a regression coefficient for the fast autotriploids. The mean pulse rate of the fast autotriploids was slightly (4.2%) but not significantly ($U_{4,10}=32$ and $p=0.09$) higher than the temperature-corrected pulse rate of the diploid controls (table 1). However, the slow autotriploids had a significantly ($U_{8,10}=80$ and $p<0.05$) lower mean temperature-corrected pulse rate than the diploid controls by 13% (table 1).

The erythrocyte nuclei of *H. versicolor* males had approximately twice the mean area of those of *H. chrysoscelis* males and the ranges of variation within species were similar for field-collected and laboratory-reared individuals (figure 2). The laboratory-reared hybrids (which failed to reach maturity) had intermediate erythrocyte nuclear areas relative to those of the parental species (figure 2). As was the case with pulse rate, the distribution of erythrocyte nuclear area in the autotriploid *H. chrysoscelis* was bimodal. The slow autotriploids all had erythrocyte nuclear areas similar to those of the hybrid triploids, whereas the fast autotriploids all had erythrocyte nuclear areas similar to those of the diploid controls (figure 2).

4. DISCUSSION

The mean pulse rate of the hybrid triploid males was 15% lower than the predicted mid-parent value (figure 1*a*). Gerhardt *et al*. (1994) reported a qualitatively similar relationship, with triploid hybrids from West Virginia having a mean pulse rate that was 9% lower than the mid-parent value for diploids and tetraploids from the same area. The intermediacy of the hybrid triploid calls might be explained as a direct effect of polyploidy on call structure as a result of increased gene dosage, ploidy-specific alterations in gene expression, increased nuclear and cell volume or a combination of these factors. Alternatively, the intermediacy of the hybrid calls might have nothing to do with polyploidy *per se*, but instead be the result of hybrids having a recombinant genotype that is heterozygous for divergent alleles at loci contributing to call differences. Gerhardt (1974) reported that putative natural hybrids between the diploid treefrog species *H. chrysoscelis*×*Hyla femoralis* and *H. chrysoscelis*×*Hyla avivoca* produced calls with pulse rates that were intermediate relative to those of the parental species. Doherty & Gerhardt (1983) also found that laboratory-reared diploid hybrids between *H. chrysoscelis* and *H. femoralis* had intermediate pulse rates. This suggests that genotypic differences between species alone might be sufficient for explaining the intermediate call structure of hybrid triploid gray treefrogs.

We can infer the relative contributions of the direct effects of polyploidy and of subsequent genetic divergence

Table 1. *LMS intercept* (pulses s^{-1}), *regression* (pulses $s^{-1}\,°C^{-1}$) *and correlation* (*r*) *coefficients and mean temperature-corrected pulse rates* (*PRR*) (pulses s^{-1}) *of wild-type, hybrid triploid and autotriploid male gray treefrogs*

(An asterisk indicates that regression was not possible for hybrids; the pulse rates of field-recorded males were corrected to the mean temperature of the hybrid sample. A double asterisk indicates that regression was not possible for fast autotriploids; the pulse rates of laboratory-reared males were corrected to the mean temperature of the fast autotriploid sample.)

	field-recorded males				laboratory-reared males			
	LMS intercept	regression coefficient	correlation coefficient	PRR (24.2 °C)	LMS intercept	regression coefficient	correlation coefficient	PRR (22.4 °C)
H. chrysoscelis	−5.6	2.71	0.97	59.9	−6.4	2.52	0.87	50.0
H. versicolor	5.8	0.75	0.69	24.1	−4.7	1.09	0.92	19.7
hybrid triploids	*	*	n.a.	36.7	—	—	—	—
'slow' autotriploids	—	—	—	—	−17.0	2.71	0.99	43.5
'fast' autotriploids	—	—	—	—	**	**	n.a.	52.1

on contemporary differences between the diploid and tetraploid species by comparing the pulse rates of autotriploids with those of the hybrid triploid males. The slow autotriploid *H. chrysoscelis* males, which had nuclear areas that were expected for triploids, produced calls with pulse rates that were significantly lower (by 13%) than those of diploid controls (figure 1*b*). A similar difference in pulse rate between slow autotriploids and diploid controls (12%) was reported for *H. japonica* (Ueda 1993). Because the difference between triploid hybrids and diploids was greater (38.6%), we concluded that both a direct effect of polyploidy and genotypic divergence, probably as a result of selection, contributed to the contemporary differences in pulse rate between *H. chrysoscelis* and *H. versicolor*. Furthermore, Ueda (1993) reported that the pulse rate of autotetraploid males of *H. japonica* was *ca.* 10% slower than that of the autotriploids, suggesting that the decrease in pulse rate is proportional to ploidy level. Given that the slow autotriploid *H. chrysoscelis* and autotriploid *H. japonica* showed the same reduction in pulse rate, we estimate that autotetraploids of *H. chrysoscelis* would produce calls with a mean pulse rate *ca.* 23% slower than the diploid controls. On the one hand, this reduction in pulse rate, expected to result from the direct effects of polyploidy, is obviously far less than the average difference between wild-types (60% in Missouri and 40% in some eastern populations) (Gerhardt 1999). On the other hand, females of *H. chrysoscelis* from throughout the range of distribution preferred synthetic calls with pulse rates typical of males in their population to alternative calls with pulse rates that are 20% slower (Gerhardt 1999). Thus, this difference in pulse rate would be sufficient for rejection of autotetraploid calls by females of the diploid species. Preferential mating by newly formed tetraploids could be promoted by parallel changes in their sensory system as suggested by Bogart & Wasserman (1972). However, this mechanism would not be required as long as some variation in female preference for pulse rate existed because intense selection would favour individuals that prefer pulse rates typical of male tetraploids.

The occurrence of the fast autotriploids, which had nuclear areas in the range expected for diploids and did not have pulse rates significantly different from the diploid controls (figure 2), was unexpected. These four individuals came from treatments involving three different pairs of parents and each had full-sibs in the slow autotriploid group. There are two possible explanations for this result. First, the fast autotriploids could have actually been diploid–triploid mosaics, which occur at low frequency in cold-shock treatments (Nishioka & Ueda 1983). Thus, it is possible that the lymphocytes from which mitotic figures were obtained were triploid, whereas the erythrocytes were actually diploid. This explanation is unlikely because there were also no significant differences in the nuclear areas of non-mitotic lymphocytes from the diploids and fast autotriploids. Alternatively, there may have been a failure of nucleotypic induction of increased nuclear volume and, therefore, cell volume in response to increased genome size in the fast autotriploids (Cavalier-Smith 1978). It is possible that variations in the cold-shock treatments or larval rearing environments resulted in phenotypic diploidization of some triploid individuals. Data such as treatment group membership, location of rearing tank or larval density were not tracked within sib-groups and, thus, could not be assessed for correlation with adult cell morphology. Although there are few data available concerning environmental effects on cell size in general, it has been shown, for example, that temperature variation can alter nuclear and cell volumes by as much as a factor of two in diploid frog embryos (Valouch *et al.* 1971). However, regardless of the conditions that produced autotriploids with unchanged nuclear volumes, the occurrence of 'diploid-like' autotriploids suggests that the direct effect of polyploidy on call structure results from altered cell dimensions. That is, because triploids with diploid-like nuclear sizes produced diploid-like calls, cell size rather than gene dosage must be the major contributor to the cytotypic effect of polyploidy (i.e. reduced pulse rate) on call structure, as originally hypothesized by Bogart & Wasserman (1972).

Ploidy-specific alterations in phenotypic traits mediating mate choice are critical for the successful establishment of new polyploid lineages that arise through genome duplication. Our results indicate that polyploidy can have a direct and biologically significant effect on phenotypic traits that promote assortative mating among cytotypes. However, the magnitude of this effect is not necessarily sufficient for explaining present day differences between diploids and polyploids,

indicating that subsequent genotypic evolution can drive further divergence between species. Furthermore, it appears that ploidy-specific changes in phenotype are a consequence of altered cell dimensions rather than increased gene dosage. Thus, changes in cell size that occur in direct response to genome duplication may provide a general mechanism for phenotypic change in the absence of genotypic change.

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