Genome size rather than content might affect call properties in toads of three ploidy levels (Anura: Bufonidae: *Bufo viridis* subgroup)

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In vertebrates, genome size has been shown to correlate with nuclear and cell sizes, and influences phenotypic features, such as brain complexity. In three different anuran families, advertisement calls of polyploids exhibit longer notes and intervals than diploids, and difference in cellular dimensions have been hypothesized to cause these modifications. We investigated this phenomenon in green toads (*Bufo viridis* subgroup) of three ploidy levels, in a different call type (release calls) that may evolve independently from advertisement calls, examining 1205 calls, from ten species, subspecies, and hybrid forms. Significant differences between pulse rates of six diploid and four polyploid ($3n$, $4n$) green toad forms across a range of temperatures from 7 to 27 °C were found. Laboratory data supported differences in pulse rates of triploids vs. tetraploids, but failed to reach significance when including field recordings. This study supports the idea that genome size, irrespective of call type, phylogenetic context, and geographical background, might affect call properties in anurans and suggests a common principle governing this relationship. The nuclear-cell size ratio, affected by genome size, seems the most plausible explanation. However, we cannot rule out hypotheses under which call-influencing genes from an unexamined diploid ancestral species might also affect call properties in the hybrid-origin polyploids.

ADDITIONAL KEYWORDS: advertisement calls – cell size – evolution – release calls.

INTRODUCTION

Although 'next-generation sequencing transforms today's biology' (Schuster, 2008) we have still only relatively vague ideas about how genome size rather than informational content might influence evolution (e.g. Bennetzen & Kellogg, 1997; Zuckerkandl, 2002). In vertebrates, genome size shows correlation with nuclear and cell sizes in several tissues and taxonomic groups (Olmo, 1983; Gregory, 2003), including an inverse relationship with brain complexity (Roth, Blanke & Wake, 1994; Wake, Wake & Specht, 2011). Recently evolved polyploids might help to better test the influence of genome size on various evolutionary features. With the probable exception of mammals (Svartman, Stone & Stanyon, 2005) and some single birds (e.g. Tiersch, Beck & Douglass, 1991), natural polyploid vertebrates occur rarely but are phylogenetically widespread among teleosts (Le Comber & Smith, 2004), amphibians (Bogart, 1980; Schmid, 1980; Kawamura, 1984; Mable, Alexandrou & Taylor, 2011), and reptiles (Kearney, Fujita & Ridenour, 2009). Polyploids evolved in urodelan and particularly frequently among anuran amphibians, with the latter reaching as much as do-decaploidy in Pipidae ($12n$; e.g. Evans, 2008). Intriguingly, polyploids in at least three different anuran families produce advertisement calls with longer notes and intervals than their diploid counterparts. Such lower 'pulse rates' (call-notes per unit of time) in polyploids have been found in Hylidae (Bogart & Wasserman, 1972; Ralin, 1977;

Mable & Bogart, 1991; McLister, Stevens & Bogart, 1995; Keller & Gerhardt, 2001), Leptodactylidae (Bogart & Wasserman, 1972; Martino & Sinsch, 2002), and Bufonidae (Tandy *et al*., 1982; Castellano *et al*., 1998, 2002; Stöck, 1998).

Bogart & Wasserman (1972) hypothesized that polyploidy affects calls through changes in cellular dimensions or tissue mass or density, as later emphasized by Ralin (1977). Artificially produced autopolyploid tree frogs (*Hyla japonica*) supported this hypothesis, as mean pulse rate in triploids was about 10% lower than in diploids, and in tetraploids was about 20% lower than in diploids (Ueda, 1993). Accordingly, reviewing anuran vocalization, Gerhardt (1994) discussed Bogart & Wasserman's (1972) idea as plausible. For hylid frogs, Keller & Gerhardt (2001) found the cell-size hypothesis confirmed in several independently arisen polyploid lineages (Ptacek, Gerhardt & Sage, 1994; Holloway *et al*., 2006), as well as in some artificially produced autotriploids, and 'concluded that both a direct effect of polyploidy and genotypic divergence (. . .) contributed to the contemporary differences'. Artificially produced autotriploid females show a shift in pulse-rate preference in the direction of the pulse rate produced by males of the tetraploid species (Tucker & Gerhardt, in press).

To test whether this pulse rate – ploidy relationship also occurs in release calls that are supposedly less constrained by sexual selection and may evolve independently from advertisement calls (Castellano *et al*., 2002), we examined release calls across the radiation of Palearctic green toads (*Bufo viridis* subgroup) involving three natural ploidy levels.

MATERIAL AND METHODS

STUDY ANIMALS AND CALL RECORDINGS

We examined calls from 66 males of eight green toad forms and species representing most phylogenetic clades of the *B. viridis* subgroup (Stöck *et al*., 2006), several of which represent polyploids of known or hypothetical hybrid origin (Stöck *et al*., 2010). Ploidy of Asian and most European toads was determined by flow cytometry of blood samples (Stöck *et al*., 2010) or karyotyping (Stöck *et al*., 2005), or was inferred from microsatellite data (Colliard *et al*., 2010). A total of 1205 release calls (Table 1 and Supporting Information, S1) were recorded in the field (450 calls; mostly between 20 and 28 °C; Central Asia, Europe) or in the laboratory (755 calls; at 7, 12, 17, 22, or 27 °C; constant air temperature of 22.5 °C. The toads were given 30 min to adjust to each temperature in a series of water baths. We stimulated the elicitation of release calls by holding the toad behind its forelimbs between human thumb and finger as described

Table 1. Taxa, locality of origin, and ploidy levels of toads (geographical information follows Stöck *et al*., 2001, 2006, 2010)

| Taxon	No. of individuals recorded in the field	No. of individuals recorded in the laboratory	No. of calls	Ploidy	Locality
B. balearicus × *B. siculus*	–	2	87	2*n*	Laboratory F_1-hybrid
B. shaartusiensis	4	3	252	2*n*	Tajikistan (Shaartuz)
B. siculus	–	1	73	2*n*	Italy, Sicily (Palermo)
B. turanensis	3	–	53	2*n*	Iran (Gholaman, Kapkan, NE of Gonbad-e-Kavus)
B. variabilis	5	1	105	2*n*	Albania (Dürrosh), Iran (Kerman)
B. viridis viridis	28	–	165	2*n*	Germany (Halle)
B. baturae	2	3	129	3*n*	Pakistan (Gilgit, Pasu)
B. turanensis × *B. pewzowi*	–	1	51	3*n*	Kyrgyzstan (S of Bishkek)
B. oblongus	1	–	28	4*n*	Iran (Baghestan, Birjand)
B. pewzowi	9	3	262	4*n*	China (Kashgar), Kyrgyzstan (Naryn), Uzbekistan (Nuratau)
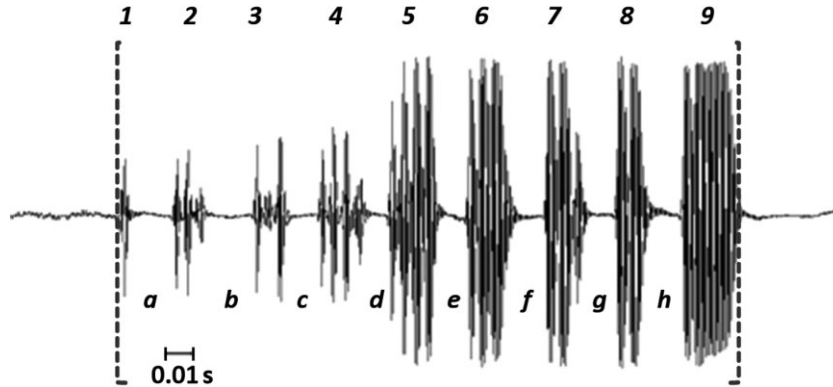
Figure 1. Example of a release call of Palearctic green toads of the *Bufo viridis* subgroup. *Bufo baturae* (Gilgit, Pakistan, at 22 °C): numbers *1–9* indicate notes, where *1* and *9* are the first and last note, respectively. Letters *a–h* represent internote intervals; interval *c* in this call measures 10 ms. The start and end of the release call are indicated by the dashed lines.

(Brown & Littlejohn, 1972; Leary, 2001), 1–2 cm in front of a microphone (ME 66, module K6, Sennheiser), and recorded calls on a Dell Latitude laptop. Measurements were performed using Avisoft Bioacoustics SASLab software (Specht, Berlin, Germany; http://www.avisoft.com/).

For each call with at least four pulses, we measured two parameters in the oscillogram: the call duration (beginning of first to end of the last pulse) and the number of pulses (Fig. 1). Pulse rate was obtained by dividing the number of pulses by the call duration.

STATISTICAL ANALYSIS

Statistical analyses was performed using R 2.10.1 (R Development Core Team, 2009). Median values of parameters were computed for each temperature and individual, as some emitted more calls than others, resulting in a total of 115 values (63 from the laboratory, 52 from the field). As laboratory temperature data appeared more reliable than measurements obtained under field conditions, statistical analyses were performed first with laboratory data only, and then for the entire data set, which also served to analyse potential relationships of call duration and number of pulses to ploidy.

To test how ploidy affects pulse rate, call duration, and number of pulses, we first had to remove the effect of temperature, on which temporal call parameters depend in poikilothermic anurans. To do this, we performed for each parameter (pulse rate, call duration, number of pulses) a linear regression as a function of body temperature and computed the residuals. Then, to obtain only a single value per individual, regardless of temperature, we calculated individual average residuals. Finally, to test if ploidy affects pulse rate, a one-way ANOVA was computed for the mean pulse rate residuals of each individual,

with ploidy as a fixed factor. To understand whether differences not only might exist between diploids ($2n$) and polyploids ($3n$, $4n$), which could result from absence vs. presence of call-affecting genes in a second genome of hybrid-origin allopolyploids (Stöck *et al*., 2010), but also between triploids ($3n$) and tetraploids ($4n$), two comparisons were performed using *t*-tests: diploids vs. polyploids and triploids vs. tetraploids.

RESULTS

Laboratory data revealed an inverse relationship between pulse rate and ploidy (Table 2, Fig. 2), characterized by a significant effect of ploidy on the mean residual pulse rate ($F = 16.130$, $P < 0.001$; Fig. 2B). In addition, *t*-tests supported significant effects of ploidy on both differences between diploids vs. polyploids ($t = 5.14$, d.f. = 12, $P < 0.001$), and triploids vs. tetraploids ($t = 3.69$, d.f. = 5, $P = 0.014$).

Differences were also obtained between diploids and polyploids for the whole data set, although we failed to find significant differences between triploids and tetraploids (Table 2, supporting Fig. S1). Similar results were observed for the analysis of call duration (Table 2; supporting Fig. S2) and number of pulses (Table 2; supporting Fig. S3).

DISCUSSION

While anuran advertisement calls evolve under sexual selection through female choice (e.g. Ryan, 1985), release calls are closer to aggressive calls (Gerhardt, 1994), and serve as sex identification signals, which warn males to dismount erroneously clasped conspecific or interspecific males (Brown & Littlejohn, 1972). No correlation between pulse rates of advertisement and release calls was found within *Bufo*

Table 2. Results of statistical comparisons according to main text, Figure 1 and supporting information, Figs S1–S3

| Data | Parameter | Tests performed for: | | |
		Ploidy	$2n$ vs. $3n + 4n$	$3n$ vs. $4n$
Laboratory only	Pulse rate	< 0.001	< 0.001	0.014
Laboratory and field	Pulse rate	0.008	0.002	0.436
	Call duration	< 0.001	< 0.001	0.214
	No. of pulses	0.008	0.003	0.270
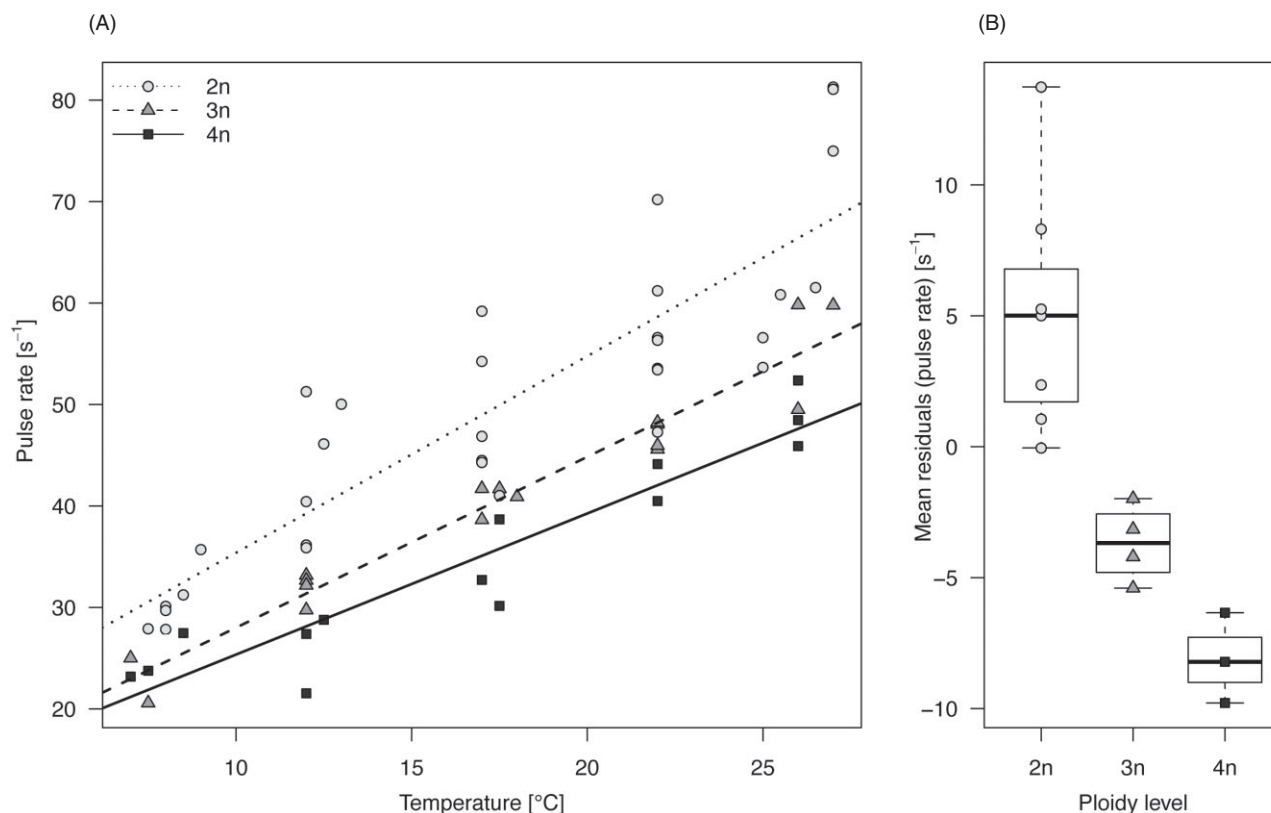
Figure 2. Pulse rate of release calls in relation to temperature and ploidy level. A, pulse rate as a function of temperature for the laboratory measurements. Lines are regressions of pulse rate on temperature for each ploidy level. B, box-plots of mean pulse rate residuals as a function of ploidy level.

americanus (Sullivan, 1992), *Bufo valliceps* (Sullivan & Wagner, 1988), and green toads (Castellano *et al.*, 2002), suggesting independent evolution of both call types. Therefore, the relationship between ploidy and pulse rate found by us might support the idea that genome size, irrespective of call type, phylogenetic context, and geographical background, affects call properties in anurans. Indeed, the six diploid forms, including the interspecies F_1-hybrids (*B. balearicus* × *B. siculus*), showed significantly higher pulse rates than the four polyploid (3n, 4n) forms examined. This and previous data on advertisement calls from three anuran families (Introduction) suggest a common

principle to govern this relationship. A proximate explanation provides the hypothesis on cell sizes, which in green toads also have been shown to be larger in polyploid than in diploid forms (Stöck & Grosse, 1997; Stöck *et al.*, 2001).

Castellano *et al.* (2002) found that 'release calls vary congruently with the phylogeny' while examining three (two diploid, one tetraploid, but no triploid) species of green toads. However, their allozyme phylogram did not consider the hybrid origin of polyploids, as shown by Mezhzherin & Pisanets (1995), and as later confirmed with nuclear sequence data (Stöck *et al.*, 2010).

We studied only one of two (possibly three?; Stöck *et al.*, 2006) existing tetraploid green toad species under laboratory conditions, and our combined analysis of field and laboratory data failed to show significant differences between calls of triploids and tetraploids. Therefore, we cannot rule out hypotheses under which call-influencing genes from an unexamined diploid ancestral species (e.g. with lower pulse rate) might *also* influence call parameters in allopolyploids. This might be the case in the microhylid genus *Neobatrachus*, where four tetraploid species with at least two independent origins (Mable & Roberts, 1997) 'have calls with (. . .) low pulse rates: broadly similar to calls of diploid *N. fulvus*' (Roberts, 1997). Similarly, within the hylid *Phyllomedusa*, where tetraploids have recently been suggested to be of autotetraploid origin (Brunes *et al.*, 2010), calls of diploids and tetraploids have very similar pulse rates (Haddad, Pombal & Batistic, 1994), but cell sizes have not been reported. Finally, despite previous bioacoustic data of Vigny (1979) pointing to another inverse ploidy to pulse rate relationship (e.g. *Xenopus wittei* $4n = 72$ and *X. ruwenzoriensis* $12n = 108$ have lower pulse rates than *X. fraseri* $2n = 36$), Tobias, Evans & Kelly (2011) concluded that 'no call character was significantly correlated with ploidy' in *Xenopus* and *Silurana*. However, despite many fascinating evolutionary and phylogenetic insights of this paper, the lack of temperature data might miss the 'fine-tuning' required to detect the correlation with pulse rate discussed here.

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SUPPORTING INFORMATION

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

Figure S1. Pulse rate as a function of temperature for the laboratory and field measurements. Lines are regressions of pulse rate on temperature for each ploidy level.
Figure S2. Call duration as a function of temperature for the laboratory and field measurements. Lines are regressions of call duration on temperature for each ploidy level.
Figure S3. Number of pulses as a function of temperature for the laboratory and field measurements. Lines are regressions of the number of pulses on temperature for each ploidy level.
Table S1. Primary dataset used for this study.