Mating call differences between diploid and tetraploid green toads (*Bufo viridis* complex) in Middle Asia

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Abstract. Mating calls of diploid and tetraploid green toads (*Bufo viridis* complex) from Iran, Turkmenistan, Uzbekistan and Kyrgyzstan were investigated during the breeding periods in 1994 and 1995 across a range of temperatures from 9 °C to 22 °C. Interpulse interval duration, pulse duration and call duration were negatively correlated with water temperature. At any given temperature tetraploid toads exhibited longer pulses and interpulse intervals resulting in lower pulse rates than diploid toads. These differences reveal a potential premating isolation barrier and provide an additional argument to consider diploid and tetraploid green toads as separate species. Call duration did not differ significantly between diploid and tetraploid green toads. Fundamental frequencies lay in the same range in both diploid and tetraploid toads.

Introduction

Within the genus *Bufo*, chromosome analyses of more than 77 species have revealed polyploids in only two species groups (King, 1990). *Bufo asmarae* (Tandy et al., 1982) with 4n = 40 chromosomes is a tetraploid species of the 20-chromosomes toad species from the *Bufo regularis* group. Polyploid representatives of the 22-chromosomes toad species which have 4n = 44 chromosomes were found in the Eurasian *Bufo viridis* group (Inger, 1972) and are the subject of the present study.

Mazik et al. (1976) and Bachmann et al. (1978) discovered tetraploid forms in the northern Tian Shan. Pisanets (1978) described similar tetraploid green toads from western Turkmenistan as a separate species *Bufo danatensis*. Meżżerin and Pisanets (1995a, b) demonstrated the allopolyploid origin of tetraploid toads and found three groups of related forms represented (I) in the Pamiro-Alai, northern Tian-Shan and southern Kazakhstan, (II) in high mountains of the Pamir (to 3500 m), and (III) in western and southern Turkmenistan. The large sized diploid subspecies, *B. viridis turanensis* (Hemmer et al., 1978), living in the lowlands of Middle Asia (for definition see: Walter and Breckle, 1996: 276), is generally accepted (Pisanets and Ščerbak, 1979; Roth, 1986; Borkin et al., 1986a; Kuzmin, 1995; Meżżerin and Pisanets, 1995a, b). *Bufo viridis asiomontanus* from

southern Kyrgyzstan is said to be diploid (Pisanets and Ščerbak, 1979), but there is no demonstration of this in the literature. The taxonomy of green toads from Middle and Central Asia was reviewed by Roth (1986) and Kuzmin et al. (1988), but still lacks final clarification (Kuzmin, 1995; Stöck, 1997). Therefore, in the present study — following a suggestion of Roth (1986) — I distinguished only between diploid and tetraploid toads and took the locality of their habitat into account.

Tetraploids live partly in sympatry with their diploid relatives. Until now there has been only little evidence for reproductive isolation between diploid and tetraploid forms in nature. Pisanets (1978) found two triploid females in Danata (fig. 1a), which seemed to be infertile, and electrophoretic results referred to rare hybridizations (F_1) at this locality (Meżžerin and Pisanets, 1995a). Roth (1986) wrote realistically that "...the question of ... interspecific barriers is quite unknown". In artificial experimental crossings (Orlova and Uteshev, 1986; Pisanets, 1992) the F_1 ploidy degree and its importance for fertility were not analysed. Hemmer (1976) mentioned the resemblance between mating calls of green toads from Kazakhstan and Kyrgyzstan (unknown ploidy) and those of European *B. viridis*. Meyer (1991) stressed this similarity concerning tetraploid toads from western Mongolia and considered sonagraphic analyses necessary. Kuzmin (1995) wrote that the voice of "tetraploid *B. danatensis*" is similar to the mating call of *B. viridis*.

Materials and methods

Localities (see fig. 1) of the populations and periods of investigations were as follows: a — Bami (38°37′N, 56°38′E, Turkmenistan, Kyzyl-Arvatskii Rayon, Kopet-Dag-Mountains, valley about 25 km SE the station, south the pass, 750 m NN, March 1994), b — near Bacharden (38°14′N, 57°31′E, Turkmenistan, Ashchabadskaya Oblast', about 10 km W Kelyata, 500 m, April 1994), c — NE-Iran (37°38′N, 55°29′E, frontier district near Turkmenistan, about 50 km NE Gonbad-e-Kāvūs, 250 m, April 1994), d — Danata village (39°06′N, 55°06′E, Turkmenistan, Ashchabadskaya Oblast', stream 2-4 km SE and warm spring about 4 km SE, 200 m, April 1994), e — Bol'shoi Balkhan mountains (39°43′N, 54°29′E, Turkmenistan, Nebit-Dagskii Rayon, N-slope, about 15 km S Oglanly village, 500 m, April 1994), f — Nuratau mountains (40°35′N, 66°30′E, Uzbekistan, Dzhizakhskaya Oblast', Rayon Farish, Nuratau Nature Reserve, N-slope, 1600 m, May 1995), g — Tashkent (41°16′N, 69°13′E, Uzbekistan, 450 m, April 1995), h — Chatkal (41°35′N, 70°07′E, Uzbekistan, 80 km E Tashkent, Chatkal Nature Reserve, 5 km E Burchmulla, 900 m, April 1995), i — Issyk-Kul' lake (42°29′N, 76°20′E, Kyrgyzstan, N-bank near village Sary-Kamysh, 1670 m, April 1995).

Mating calls were recorded with a walkman (WM 6 DC; Sony) and a stereo microphone (Vivanco, dm58) from males of three diploid and six tetraploid populations (see table 1; fig. 1) in spring of 1994 and 1995. The recordings were made during dusk or night from a distance of 0.3 to 2.0 m in the habitats (in one case in the laboratory, table 1). Most of

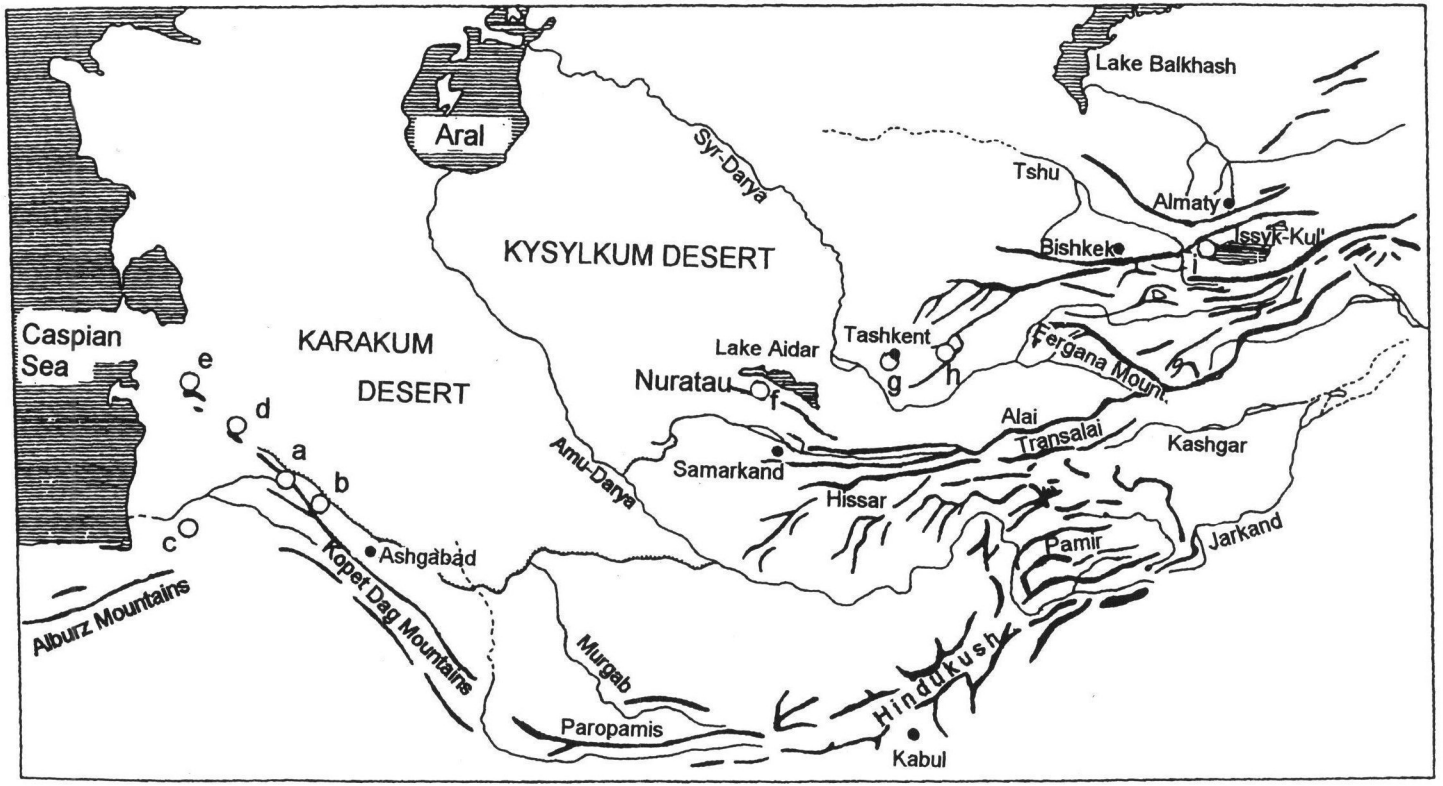

Figure 1. Map of the recording localities in Middle Asia. For letter codes of sample sites, see table 1 and text. (Map by Katrin Schneider and Matthias Stöck using Walter and Breckle, 1991, Vol. **3**: 275).

Table 1. Populations, localities, ploidy of specimens recorded, temperatures and call variables.

Locality (Abbr. in fig. 1)	Toad	Ploidy (K, E, M) [a]	SVL (mm)	Mass (g)	AirT (°C)	WaterT (°C)	Pulse duration (ms)	Interpulse interval duration (ms)	Pulse rate (s^{-1})	Fund. frequency (Hz)
Bami (a)	1	2n (E, K)	76.0	50.0	9.0	10.0	35.9	29.6	15.26	1408
Bami (a)	2	2n (E)	82.8	54.0	10.5	10.5	29.8	35.9	15.22	–
Bami (a)	3	2n (E)	76.4	50.0	9.0	11.0	30.6	36.9	14.81	–
Bami (a)	4	2n (E)	77.3	48.5	9.0	11.0	28.0	41.4	14.40	–
Bami (a)	5	2n (E)	77.6	53.0	9.0	11.0	36.4	32.8	13.81	1270
Bami (a)	6	2n (E)	78.3	49.5	9.0	11.0	35.0	30.3	15.31	1358
Bacharden (b)	7	2n (E, M)	72.8	48.0	16.0	12.0	24.8	28.0	18.93	1333
Bacharden (b)	8 [d]	2n?	–	–	16.0	12.0	24.5	27.8	19.12	1331
NE-Iran (c)	9	2n (E, K)	74.0	40.0	12.5	16.0	24.0	22.0	21.71	1349
NE-Iran (c)	10	2n (E)	68.7	36.0	12.5	16.0	28.4	20.5	20.44	–
Danata (d)	11	4n (E, K)	61.8	22.0	8.0	14.0	39.5	53.8	10.71	–
Danata (d)	12	4n (E, K)	59.0	18.5	8.0	14.0	42.3	64.6	9.35	1320
					lab.: [d]	lab.: [d]				
					12.0	12.0	43.1	68.0	9.09	1320
					22.0	22.0	– [b]	– [b]	– [b]	1320
B. Balkhan (e)	13	4n (E, K)	71.5	39.0	6.5	10.0	41.7	91.1	7.53	–
B. Balkhan (e)	14	4n (E, K)	66.0	30.0	6.5	10.0	50.3	66.0	8.59	1320
B. Balkhan (e)	15	4n? (E)	72.8	48.0	6.5	10.0	48.4	69.7	8.46	1290
B. Balkhan (e)	16 [d]	4n?	–	–	6.5	10.0	47.3	69.3	8.57	1314
B. Balkhan (e)	17	4n (E)	76.0	39.5	10.0	15.0	42.6	43.1	11.65	–
Nuratau (f)	18	4n (E, K)	66.8	30.0	12.0	11.5	43.1	50.9	10.63	1374
Nuratau (f)	19	4n (E)	64.6	26.0	12.0	11.5	45.3	53.4	10.13	1479
Nuratau (f)	20	4n (E)	68.8	32.5	12.0	11.5	51.1	51.2	9.77	–
Tashkent (g)	21	4n (E, K)	63.1	21.0	23.0	22.0	32.0	31.7	15.69	1454
Tashkent (g)	22	4n (E)	73.0	39.5	23.0	22.0	33.3	33.0	15.08	–
Tashkent (g)	23	4n (E)	76.8	49.5	23.0	22.0	34.7	29.7	15.52	–
Chatkal (h)	24	4n (E, K)	74.0	40.0	15.0	12.5	40.1	41.5	12.47	1395
Chatkal (h)	25	4n (E)	69.5	38.0	15.0	12.5	39.4	42.3	12.23	–
Issyk-Kul' (i)	26	4n [c]	–	–	10	12.0	51.2	51.7	9.71	1358
							45.6	51.8	10.26	1358
Issyk-Kul' (i)	27	4n [c]	–	–	9.0	12.0	42.9	68.0	9.01	–
Issyk-Kul' (i)	28	4n [c]	–	–	9.0	12.0	56.4	70.5	7.88	1324
							47.3	80.0	7.85	1320
Issyk-Kul' (i)	29	4n [c]	–	–	12.0	12.0	44.7	70.9	8.65	–

[a] Methods of ploidy determinations: K — karyogram. E — erythrocyte measurements. M — microdensitometry.

[b] because of echos only call duration and frequency determinable.

[c] no individual ploidy determination since only tetraploid populations in the Issyk-Kul' valley are known (Borkin, 1989; Kuzmin et al., 1988; Stöck, 1995, 1997).

[d] not included in statistics.

the males were caught for mass and ploidy determination and other investigations (Stöck, 1995, 1997). Water and air temperature at the individual's calling site were taken to the nearest 0.5 °C immediately after recording the call. Mass was measured 6 to 8 hours after the toads were caught, with an accuracy of 0.5 g using a spiral dynamometer. Different methods of exact individual ploidy determination of nearly each male were

used (see table 1): karyograms, microdensitometrical DNA-measurements and/or size determination of erythrocytes in blood samples (Stöck, 1995; Stöck and Grosse, 1997). Call duration was measured using a stop-watch. Other voice analyses were partly made using LSI Workstation Spectrogramm software in the Tierstimmenarchiv (Animals Voice Archive) at Humboldt University Berlin. Sonagrams (bandwidth 160 Hz; 0 to 4 kHz) and measurements of fundamental frequencies of pulses in the middle section of a call were made for some animals (see table 1). Further sonagraphic examinations were carried out in the Institute of Zoology at Martin-Luther-University Halle-Wittenberg. For each calling male, at least ten successive pulses and interpulse intervals were measured with Avisoft-SonagraphPro software (Version 2.0; Fa. Specht, Berlin) with a sampling rate of 16 kHz (Hemming-window, FFT-length 512, frame 50%, Tchebysheff 8th order; if necessary filtered with a limiting frequency of 0.8 kHz high pass). After determination of the average value of the ten pulses and ten interpulse intervals, pulse rate (pulses per second) was calculated arithmetically (table 1; figs 3, 4). I used regression analyses for statistics.

Results

Breeding season and calling behaviour

Recording dates in the populations Bami (31-3-94 to 3-4-94), Bol'shoi Balkhan (6-4-94 to 9-4-94), Nuratau (13-05-95) and Issyk-Kul' (20-4-95 to 23-4-95) represented the beginning of the spawning period (few clutch strings, few small larvae). In the other populations calls were recorded in the middle or late phase of the breeding period (tadpoles of more advanced developmental stages). No differences in daily periods of calling activity between diploid and tetraploid toads were noticed. Toads showed high calling activity at air temperatures higher than 9 °C (up to 22 °C). Usually sitting half immersed in shallow water, males started calling at dusk and continued till about 3 or 4 o'clock in the morning. Occasionally quickly falling temperatures caused mating calls to finish completely one hour after starting.

The structure of mating calls of diploid and tetraploid green toads

Mating calls of both diploid and tetraploid toads are perceived by the human ear as monotonous trills consisting of series of pulses with a constant duration separated by constant interpulse intervals. Sonagrams reveal deviations in pulse structure at the beginning of the call (fig. 2). The first pulses and interpulse intervals were either shorter or longer than those reached after about the 10th pulse which are characterised by high regularity. The fundamental frequency of the first pulse was the lowest and increased until the individual frequency of the toad was reached.

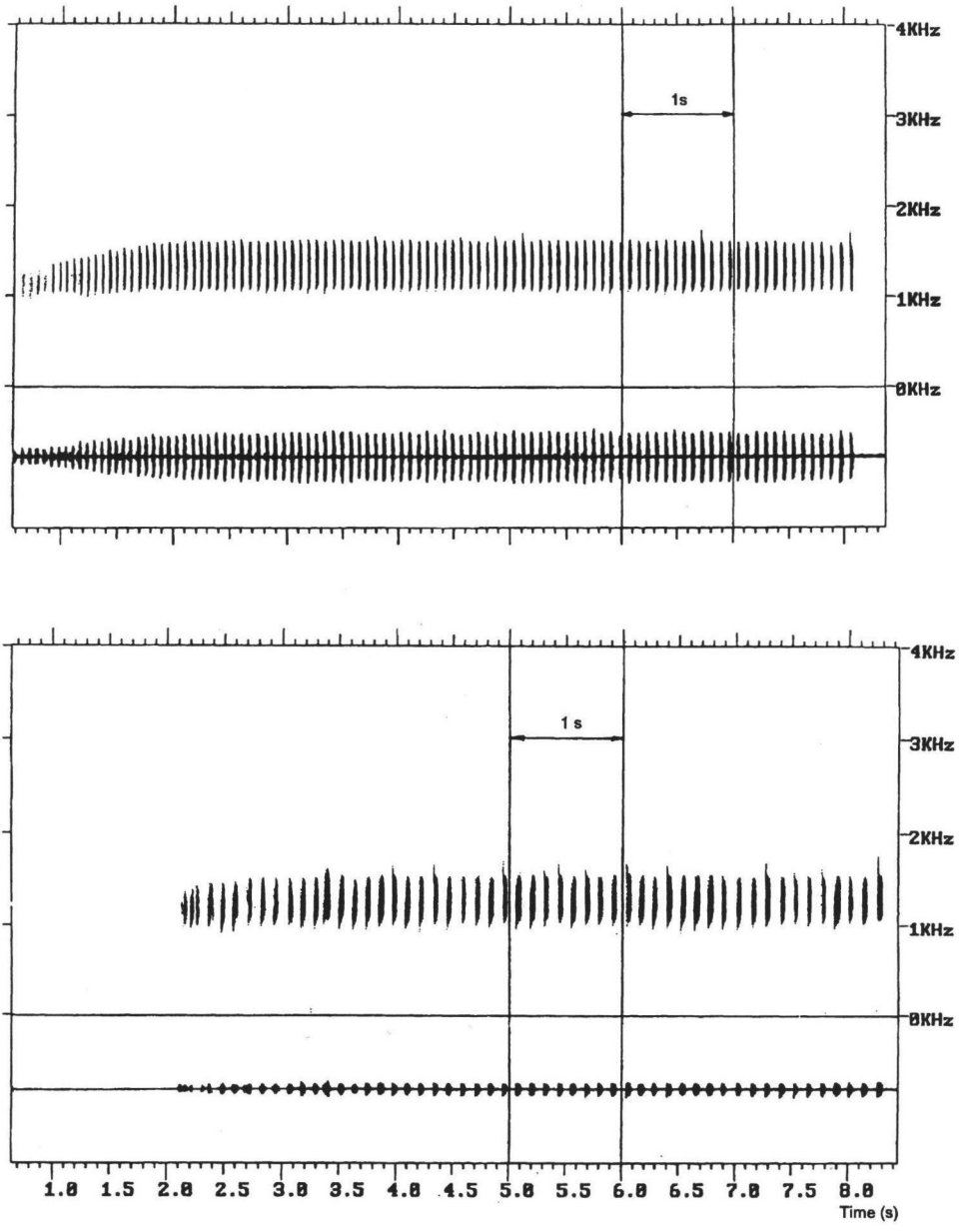

Figure 2. Sonagrams and oscillograms of mating calls of a diploid and a tetraploid male recorded at nearly the same water temperature. Above: diploid male from Bacharden at 12 °C water temperature, fundamental frequency 1333 Hz. Below: tetraploid male from Issyk-Kul' at 11.5 °C water temperature, fundamental frequency 1320 Hz.

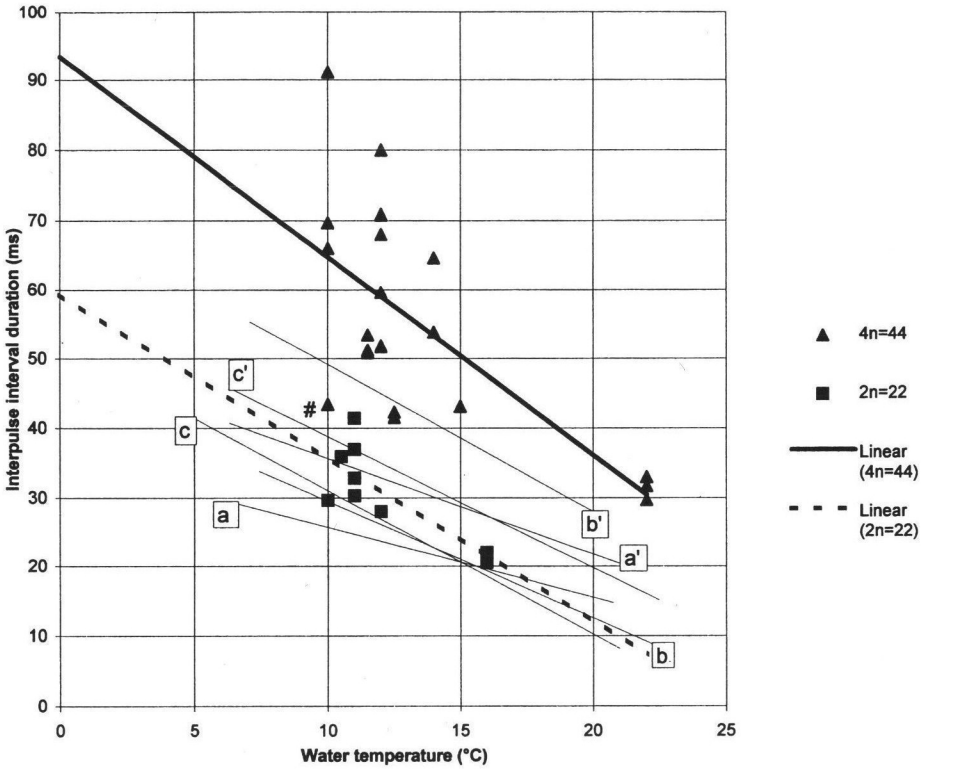

Figure 3. Differences in interpulse interval duration and comparison with literature data. 4n = 44: Tetraploid toads; regression: $y = -2.86x + 93.37$; $r^2 = 0.502$; number (n) of calls included in regression analyses = 21. 2n = 22: Diploid toads; regression: $y = -2.36x + 59.33$; $r^2 = 0.634$; $n = 9$.
a, b, c = Lines of minima. a′, b′, c′ = Lines of maxima. a, a′: *B. viridis* (Israel); Nevo and Schneider, 1976. b, b′: *B. viridis* (Austria); Lörcher and Schneider, 1973. c, c′: *B. viridis* (Kazakhstan); Schneider and Egiasaryan, 1995. # toad B. Balkhan.

Differences in interpulse interval duration. Interpulse intervals of diploid males were clearly shorter than those of tetraploid males (fig. 3). Increasing water temperature resulted in reducing interpulse intervals. Linear regressions of diploid and tetraploid toads had similar slopes ($t = 0.53$, $P > 0.05$) but differed substantially from each other in their intercepts ($t = 2.80$, $P < 0.01$).

Differences in pulse duration. Pulse duration decreased with rising water temperature (fig. 4). The values of diploid males lay in all cases distinctly below those of the tetraploid males. Linear regressions are nearly parallel. Their intercepts differ by about 15 ms. A Student test of parameters of the linear regression of pulse duration on temperature revealed no differences for slopes ($t = 0.06$), while differences for intercepts were nearly significant ($t = 1.83$, $0.05 < P < 0.1$).

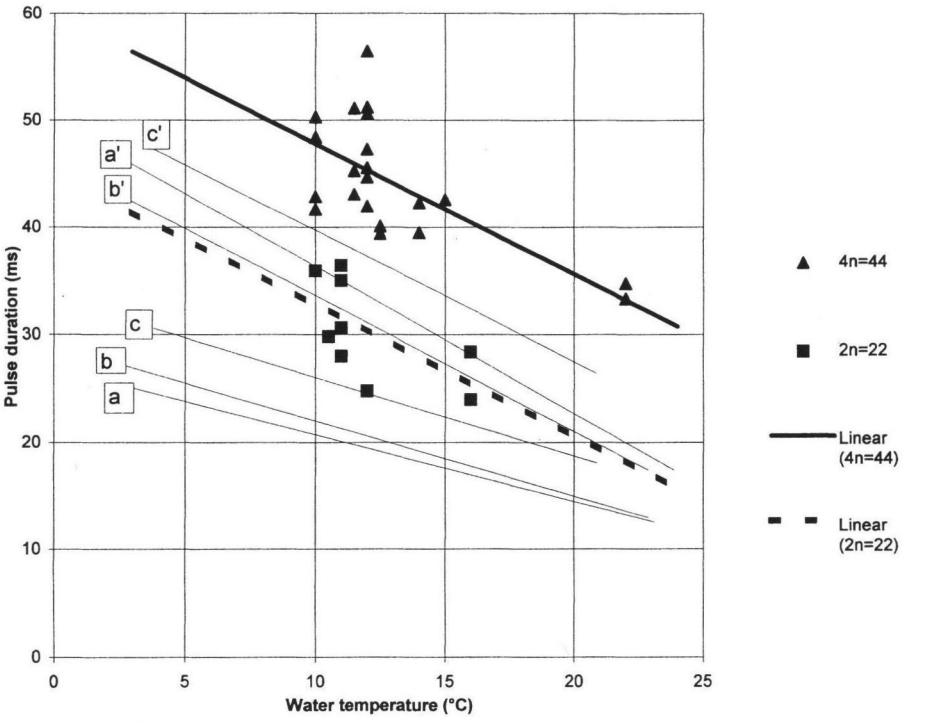

Figure 4. Differences in pulse duration and comparison with literature data. 4n = 44: Tetraploid toads; regression: $y = -1.22x + 60.01$; $r^2 = 0.529$; $n = 21$. 2n = 22: Diploid toads; regression: $y = -1.23x + 45.18$; $r^2 = 0.378$; $n = 9$.
a, b, c = Lines of minima. a′, b′, c′ = Lines of maxima. a, a′: *B. viridis* (Israel); Nevo and Schneider (1976). b, b′: *B. viridis* (Austria); Lörcher and Schneider (1973). c, c′: *B. viridis* (Kazakhstan); Schneider and Egiasaryan (1995).

Differences in pulse rates. Pulse rates of diploid and tetraploid green toads depend on water temperature (regression for tetraploid toads: $y = 0.531x + 3.50$; $r^2 = 0.725$; diploid toads: $y = 1.146x + 3.00$; $r^2 = 0.806$). The ratio of pulse rates of diploid to tetraploid toads at regression lines varies between about 1.6 (at 10 °C), 1.7 (at 15 °C) and 1.8 (at 20 °C). There is no overlapping between pulse rates of diploid and tetraploid toads at any given temperature — even if extrema are included (e.g. # B. Balkhan at 10 °C; see fig. 3). A Student test of parameters of linear regressions of pulse rates on temperature for diploid and tetraploid toads showed the slopes to be significantly different ($t = 2.93$, $P < 0.01$) while their intercepts are similar ($t = 0.19$, $P \gg 0.05$).

Dependence of fundamental frequency on snout-vent-length and mass. For diploid and tetraploid green toads fundamental frequencies between 1200 and 1500 Hz were measured. As expected, they tend (in tetraploids: $r^2 = 0.17$, $n = 7$, in diploids: $r^2 = 0.1$, $n = 5$) to show an inverse relationship with body size (snout-vent-length, mass; table 1),

but sample sizes were very small. Fundamental frequency did not change as a function of water temperature.

Dependence of call duration on water temperature. Call duration decreased with increasing temperature and varied from 3 s to 9 s in diploid and tetraploid green toads at temperatures between 10°C and 15°C. The variability was high and no difference between both forms of toads was found.

Discussion

Ploidy was determined a posteriori in the laboratory and the number of recorded toads that were found to be diploid was relatively low. Data by Lörcher and Schneider (1973), Nevo and Schneider (1976) and Schneider and Egiasaryan (1995) exhibit an impressive similarity of the call patterns within the extremely large distribution range of *B. viridis*, which "illustrates clearly the conservatism of the call system of this species" (Schneider and Egiasaryan, 1995). The call data of diploid Middle Asian toads strongly support this statement. Lines of maxima and minima (figs 3, 4) are bounding values characteristic of diploid green toads from Austria, Israel and Kazakhstan in which all the values of the diploid green toads are also situated. The results of regression analyses clearly show a functional relation of these parameters to water temperature. On the contrary, all tetraploid toads exhibited higher pulse and interpulse interval duration values (few exceptions for the interpulse interval duration). The results of regression analyses and r^2 values in tetraploid toads demonstrate the functional relation distinctly and verify a convincing connection between call structure and ploidy in Middle Asian green toads.

Diploid and tetraploid cryptic species pairs of anurans (Ralin, 1968; Bogart and Wasserman, 1972) show phenomena very similar to those presented. *Hyla chrysoscelis* and *H. versicolor* were first described as two mating call types and later identified as diploid and tetraploid species (Wasserman, 1970). Tetraploid *H. versicolor* is characterised by lower pulse rates than diploid *H. chrysoscelis* at the same temperature. Bogart and Wasserman (1972) demonstrated the same for the tetraploid *Odontophrynus americanus* whose pulse rate is slower than that of its diploid relative (considered for a separate species by Beçak and Beçak, 1974). The results of the present paper show an impressive parallel to those mentioned.

Tetraploid *Phyllomedusa burmeisteri* is acoustically isolated from three diploid species (Barrio, 1976). Its lowest pulse rate could support the thesis "tetraploids exhibit lower pulse rates than their diploid relatives", but three diploid *Phyllomedusa*-species were recorded at higher temperatures (Barrio, 1976). The genus *Xenopus* represents the species complex with the greatest variation in ploidy known among vertebrates ranging from 2n = 20 to 12n = 108 (Schmid and Steinlein, 1991). Twelve species and subspecies in this genus exhibited striking differences in mating call variables (Vigny, 1979). Some species of higher ploidy level (*X. wittei*: 4n = 72; *X. ruwenzoriensis*: 12n = 108) exhibit lower

pulse rates than one of their ancestors (*X. fraseri*, 2n = 36) according to Tymowska (1991). Different calls are considered the main barrier between the diploid/tetraploid *Neobatrachtus centralis/N. sutor* which live sympatrically in large parts of their distribution area (Main, 1957; Mahony and Robinson, 1980). Tandy et al. (1982) found only 40% difference in pulse rate between tetraploid *B. asmarae* and diploid *B. regularis*. Therefore other call parameters and the calling site were discussed. Calling site and time of activity did not differ between diploid and tetraploid green toads. References by Golubev (1990) to differences in their activity contain no information about ploidy determination and seem therefore doubtful.

Differences in pulse rates are considered the main premating isolation mechanism between *H. chrysoscelis/H. versicolor* (Ralin, 1977; Bogart, 1980) whose females chose their own call type (= species) (Littlejohn et al., 1960; Gerhardt, 1994). In these species the average ratio of pulse rates, which overlap at different but do not at the same temperatures, is about 1.8 (Ralin, 1977), but absolute values differed only by a factor of 1.5. In the present study the average pulse rate in diploid toads at 10 °C is about the same as in tetraploid toads at 20 °C.

Since the pulse rate, aside from the fundamental frequency, is the main call parameter significant for female toads to discriminate conspecific males (Blair, 1964; Brauer, 1990), the call differences represent an effective premating isolation mechanism between diploid and tetraploid green toads. Adequate experiments demonstrating this hypothesis are necessary. Bogart (1980) even supposed that the ability of discrimination in sympatry could be the major prerequisite for the existence of even-ploid polyploid bisexual populations in anurans.

If adult males originate from interspecific crosses their calls are usually intermediate (Zweifel, 1968; Green, 1982; Mable and Bogart, 1991; Oliveira et al., 1991; Schlyter et al., 1991). It is possible that the call by a male (which was not caught) from B. Balkhan (# in fig. 3) is such a hybrid call by a (triploid) hybrid, but another reason, e.g. a microclimatic temperature difference between calling site and site where temperature was measured, seems possible, too.

Because of higher DNA-amounts, polyploid amphibians have higher cell volumes and reduced total cell numbers compared with diploid specimens. In tetraploid green toads larger erythrocytes are known (Bachmann et al., 1978; Kudrjavtsev et al., 1988; Stöck, 1995; Stöck and Grosse, 1997) and therefore their total cell number must be smaller too. Ralin (1977) supposed "the cell number ratio (between diploid and tetraploid *H. chrysoscelis/H. versicolor*) in the neural centers, neural pathways or musculature involved in mating call production" causes a divergence in pulse rate as "a purely mechanistic consequence of tetraploidy", which was supported by Bogart (1980) and Mable and Bogart (1991). On the other hand, the allopolyploid origin of tetraploid green toads (Meżžerin and Pisanets, 1990, 1995a, b) would result directly in call differences if the (unknown) parental forms were differentiated acoustically.

Hemmer et al. (1978) noticed in their small sized form (including tetraploid taxa) also longer intervals between pulses of release calls than in those of diploid *B. viridis turanensis*. This could refer to longer pulses and interpulse intervals in tetraploid toads which also Herrmann (1993) presented, but without temperature data and ploidy determination.

Littlejohn (1977) pointed out: carrier frequencies "of closely-related, sympatric, synchronically-breeding taxa ... are usually similar, with ranges of variation overlapping extensively". The present results are an additional example. The range of fundamental frequencies (< 1250 Hz, > 1550 Hz) in diploid and tetraploid toads corresponds with the values given by Lörcher and Schneider (1973), Nevo and Schneider (1976) and Schneider and Egiasaryan (1995). The higher part is in accordance to accumulations of characteristic frequencies in acoustic high sound neurons (around 1400 Hz) in the torus semicircularis (mesencephalon) of *B. viridis* (Mohneke, 1982). The tendency that the fundamental frequency is independent of the water temperature but depends on animal size is in accordance with data for *B. viridis* in Austria and Israel (Lörcher and Schneider, 1973; Nevo and Schneider, 1976) and corresponds to findings in many species of the genus *Bufo* (Martin, 1972; Sullivan and Wagner, 1988; Krupa, 1990).

Mating call analyses without temperature data from Cashmere and Ladakh (N-Pakistan/N-India) by Dubois and Martens (1977) in a *Bufo* considered to be *B. latastii* are of special interest to compare. The fundamental frequency (1000-1300 Hz), range of the pulse duration (30-60 ms) and of the pulse rate (8-13 s^{-1}) correspond to the results presented. Since call samples and morphological data were later combined (Martens, pers. com., 1996) it is possible that calls were produced by tetraploid toads, whose occurrence in the Himalayas is suspected (Pisanets and Ščerbak, 1979; Roth, 1986). Convergence of calls found in some allopatric anurans (Littlejohn, 1977) would not be probable in the case of sympatry of tetraploid green toads with *B. latastii*. Additional investigations are necessary.

Mating calls of *B. kavirensis* (Andrén and Nilson, 1979), recorded in the NW part of Central Iranian Plateau at 30°C, exhibit a pattern rather similar to *B. viridis* if its call parameters would be extrapolated to 30°C. *Bufo kavirensis* displays a pulse rate of about 35 s^{-1} at this temperature. If data of diploid Middle Asian toads are adjusted to 30°C, a pulse rate of approximately 37 s^{-1} results compared to only about 19 s^{-1} in the case of tetraploid toads. Therefore it is very unlikely that *B. kavirensis* represents the same taxon as the Middle Asian tetraploids.

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