Article

Cryptic Triploids and Leaky Premating Isolation in an *Odontophrynus* Hybrid Zone

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Abstract: The diploid *Odontophrynus cordobae* and its autopolyploid counterpart *O. americanus* (4n) co-occur in a small-sized contact zone in Central Argentina, together with numerous specimens of a cryptic triploid taxon. Additionally, we monitor another five localities inhabited by taxa of this species complex within a radius of 76 km north and south of the contact zone to look for possible co-occurrence of taxa. In this study, we analyze the reproductive interactions in this breeding assemblage covering three levels of ploidy. We focus on the advertisement call structure as a powerful mechanism of premating isolation and on the mating preferences of males and females in the natural habitat. Advertisement calls of triploids were indistinguishable from those of tetraploids, and both differed significantly in pulse rate from diploids. Analyses of 21 amplexi demonstrated that heterospecific matings dominated breeding: one between a diploid female and tetraploid male, and ten between triploids and tetraploids. At three localities with syntopic diploids and tetraploids, premating isolation was almost perfect, preventing the formation of triploids. Therefore, we question the putative origin of triploids from hybridization and discuss alternatives. This unique system, including three bisexual taxa of distinct ploidy, which interact reproductively, remains to be investigated in more detail to fully understand the mechanism stabilizing its persistence.

Keywords: *Odontophrynus americanus*; *Odontophrynus cordobae*; polyploidy; vocal communication; mating preference; natural triploids; Central Argentina

1. Introduction

In Amphibia, 56 out of the currently known 8426 species are polyploid [1,2]. The number of chromosome sets usually rises in even number, as exemplified in the anuran genus *Xenopus* with diploid, tetraploid, octoploid, and dodecaploid species [3]. Genome duplication may result from autopolyploidy, like in tetraploid *Odontophrynus americanus*, or from allopolyploidy, like in many tetraploid toads *Bufotes* spp. [4–10]. Polyploidy can lead to post-reproductive isolation from the diploid parental species because hybrids are often inviable or infertile [11]. *Bufotes* toads are the exception from the rule, being presently the only known amphibian species complex that includes diploid, triploid, and tetraploid bisexually reproducing taxa [8,9]. Triploids coexist sympatrically with diploid or tetraploid taxa in Kyrgyzstan, where all three ploidy levels interact reproductively [8]. The tetraploid taxon involved is the allopolyploid *B. pewzowi* (*B. lastastii* ♂ × *B. perrini* ♀), the diploid one *B. perrini* (taxon names according to [2,12]). Triploid males—females are absent—are most probably hybrids from 2n mothers and 4n fathers, i.e., their chromosome sets combine two from *B. lastastii* and one from *B. perrini*, but if they are fertile, an origin through backcrosses with 2n females may be possible as well [8,12]. The advertisement calls of triploids were significantly distinct from the diploid parental species but indistinguishable from those of

tetraploids, which may facilitate backcrosses with 4n females [13]. An increase in genome size altered the temporal structure of the advertisement call of experimentally produced autotriploids originating from diploid *Hyla chrysoscelis* [14]. The main effect of polyploidy is on the pulse rate of advertisement calls, which is considerably lower in tetraploids than in diploids [15–17]. Consequently, distinct advertisement calls of diploids and tetraploids may promote assortative mating based on phonotactic selectivity of gravid females.

This uniqueness of the *B. pewzowi*-breeding system is challenged by the recent discovery that triploid *Odontophrynus* are abundant in a narrow contact zone with diploid *O. cordobae* and tetraploid *O. americanus* [18–20]. In contrast to the *B. pewzowi*-breeding system, numerous triploid males and females are present, and they use the same breeding ponds as *O. cordobae* and *O. americanus*. Before, triploid *Odontophrynus* have been produced artificially by experimental crosses in the laboratory or were observed in a single specimen within a purely diploid population [21–24]. Since the karyologically proven detection of numerous triploid specimens in the breeding season of 2014/15, we continued to explore the features of this peculiar multi-ploidy breeding assemblage. Recently, we showed that triploid specimens enjoy the same longevity as *O. cordobae* and *O. americanus* and that they share similar growth characteristics [25]. It is currently unknown if triploids originate from direct hybridization from the 2n and 4n parental species and if they are fertile. In this study, we focus on the behavioral aspects of reproductive interactions at the breeding pond, namely advertisement call features and mating preferences.

We complemented the intensive surveys at the area with three levels of ploidy with a transect, including two populations with syntopic diploid and tetraploid individuals, one with exclusively diploid *O. cordobae* in the north, and two with exclusively tetraploid *O. americanus* in the south. Data collected were advertisement call records, pair formation, posterior ploidy determination, and morphometric features. We test the hypothesis that reproductive interactions among syntopic specimens are promoted by advertisement call similarity and constrained by dissimilarity reflecting their respective ploidy level. Three testable predictions follow from this hypothesis:

Prediction 1. *Hybridization due to natural mating between the diploid O. cordobae and tetraploid O. americanus is rare to absent. Rationale: we expect that the significant difference in advertisement call structure allows the females to distinguish between conspecific and heterospecific males. Effective premating isolation based on female choice may be undermined by males that clasp coincidentally heterospecific females in the three localities with syntopic populations [18]. Character displacement in pulse rate may increase the interspecific differences in these localities [26].*

Prediction 2. *Advertisement call structure of triploids resembles more O. americanus than O. cordobae. Rationale: analogous to Bufotes and Hyla taxon groups of three ploidy levels, we expect polyploids irrespective 3n or 4n to call with lower pulse rates as diploids [8,9,16].*

Prediction 3. *Frequent heterospecific matings occur between O. americanus and triploid specimens, but not with O. cordobae. Rationale: we expect that premating isolation among specimens >2n ploidy levels is leaky due to the assumed similarity of advertisement calls in polyploid taxa.*

2. Materials and Methods
2.1. Study Localities and Sampling Design

We studied ploidy, external morphology, and advertisement call features of *Odontophrynus* specimens (americanus-group) in six localities in the province of Cordoba, Argentina (Figure 1, Table 1). The populations were situated along a 125 km transect beginning at the type locality of diploid *Odontophrynus cordobae* (Villa General Belgrano) in the north and ending at a population of tetraploid *O. americanus* in Rio Cuarto [18]. Between these localities marking the geographical extremes of the transect was the area of contact between *O. cordobae* and *O. americanus*, where recently triploid specimens were discovered [20]. Study sites were visited at least once per year during the breeding period of *Odontophrynus* spp (November to March), starting in 1998 at Villa General Belgrano and Rio Cuarto, in 2004 at Rio de los Sauces, El Cano, and Coronel Baigorria, and in 2014 at La Escondida.
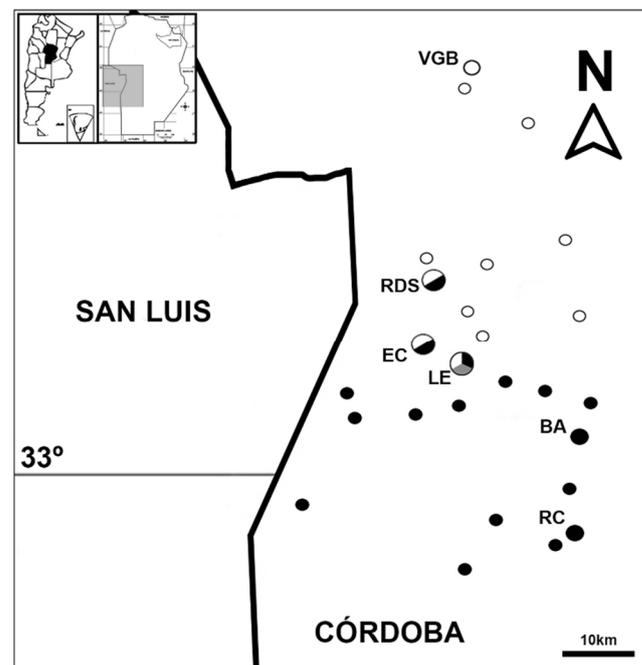
Figure 1. Map of the study localities. White dots indicate localities with exclusively *O. cordobae*, black dots localities with exclusively *O. americanus*, dots with 2–3 three color codes mixed assemblages (gray color indicates the presence of triploids). VGB = Villa General Belgrano, RDS = Rio de los Sauces, EC = El Cano, LE = La Escondida, BA = Coronel Baigorria, RC = Rio Cuarto. The broad line marks the western border between the provinces of Cordoba and St. Luis. Inserts give the location of the study site within Argentina.

Table 1. Geographical features of the six *Odontophrynus* populations sampled in the province of Cordoba, Argentina. Distance gives the line-of-sight distance between a locality and the La Escondida locality. The local *Odontophrynus* taxa are listed.

Locality	Latitude Longitude	Altitude (m a.s.l.)	Distance (km)	*Odontophrynus* Taxa
Villa General Belgrano	31°58′ S 64°32′ W	730	76	*O. cordobae* (2n)
Rio de los Sauces	32°32′ S 64°36′ W	734	15	*O. cordobae* (2n) *O. americanus* (4n)
El Cano	32°37′ S 64°35′ W	724	6	*O. cordobae* (2n) *O. americanus* (4n)
Escuela La Escondida	32°40′ S 64°32′ W	690–740	0	*O. cordobae* (2n) *O. americanus* (4n) *Odontophrynus* (3n)
Coronel Baigorria	32°51′ S 64°20′ W	509	32	*O. americanus* (4n)
Rio Cuarto	33°06′ S 64°20′ W	458	57	*O. americanus* (4n)

The sampling protocol at each locality began with recording the advertisement calls of the males, followed by the collection of the recorded male for ploidy determination and morphometric measurements. Ploidy was determined by erythrometry based on a blood smear; individuals were released again in situ following measurements. In these surveys, we obtained the complete data set on reproductive males.

There were some complementary surveys, too, in which we recorded exclusively advertisement calls or captured *Odontophrynus* present at a breeding site independent of

gender or calling activity, yielding either bioacoustic data or morphometric and ploidy data. The analyses of mating preferences at the La Escondida locality were based on two surveys, in which we encountered several pairs in amplexus: $N = 13$ on 6 November 2019, and $N = 8$ on 5 February 2020. Pairs were collected, placed separately in plastic containers, and finally processed by taking a blood sample and measuring morphometric features. All pairs were released again in situ and were observed to continue reproduction. Our field study was authorized by the Environmental Secretary of the Córdoba Government (A01/2013).

2.2. Recording and Analysis of Advertisement Calls

Advertisement call series were recorded in situ using a Walkman Digital AudioTape (DAT) SonyTM TCD-100 with stereo microphone ECM-MS907 SonyTM and a High Resolution Flash Recorder MarantzTM PMD671 with microphone SennheiserTM K6/ME66. Throughout each recording, the microphone was held at around 1 m distance from the focal calling male. Immediately after recording, water and soil temperatures (to the nearest 0.1 °C) were registered at or near the calling site. Please note that not all recorded males could be captured.

Stereo recordings were converted to mono at a sampling rate of 44.1 kHz and resolution of 16 bits, and subsequently, sonograms and oscillograms were prepared using Raven Pro 1.6 (Cornell University, Ithaca, NY, USA) and ADOBE Audition 1.0. Sonograms (Adobe Systems Inc., München, Germany), and frequency analyses were obtained by applying Blackman–Harris Fast Fourier transformation with an FFT size of 1024 Hz. Call structure was characterized by measuring six variables: (1) call duration (ms), (2) pulses per call, (3) pulse rate (pulses/s), (4) pulse duration (ms), (5) interpulse interval (ms), and (6) dominant frequency (Hz) [18,27]. We measured 3–6 single calls per advertisement call series emitted by the same frog. To avoid the introduction of intra-individual call variation, we used the average of all single measurements per call series as the representative of an individual male for further data analysis.

2.3. Erythrometric Ploidy Determination and Morphometry

Blood samples were collected from the vena angularis to prepare microscope slides with air-dried smears of fresh blood [28]. Photographs of blood cells were obtained by using an Axiophot Microscope (Carl Zeiss, Jena, Germany) at a magnification of 1000× with a Canon Powershot G6 Digital Camera (Canon, Krefeld, Germany) and ZoomBrowser EX (Canon, Krefeld, Germany)and saved in TIFF files. We measured the length (L) and width (W) of 40 randomly chosen erythrocytes and their respective nuclei for all individuals using ImageJ software (Version 1.0, Wayne Rasband, NIH, freeware) [29]. Cell and nuclear area were calculated assuming an ellipsoid shape (L × W × π/4). Ploidy levels were estimated based on the size of the nuclear area of the erythrocytes [29,30]. In 63 randomly chosen individuals, erythrometric ploidy determination was corroborated using chromosome set analyses. These individuals were transported to the laboratory and euthanized for karyological ploidy determination [31].

The external morphology of the specimens was described by measuring 15 morphometric distances to the nearest 0.1 mm using a digital caliper Mahr 16 ES [18,32,33]: (1) snout-vent length (SVL); (2) maximal head width; (3) head length; (4) snout-eye distance; (5) internarinal distance; (6) interocular distance; (7) eye-narinal distance; (8) rostronarinal distance; (9) eye diameter; (10) arm length; (11) length of 3rd finger; (12) femur length; (13) tibia length; (14) foot length; (15) length of 4th toe.

2.4. Statistical Analyses

All variables were first tested for normality. If data were skewed, they were normalized using log10-transformation. Descriptive statistics included arithmetic means, corresponding standard errors, and 95% confidence intervals.

Snout-vent length was tested for sexual dimorphism and taxon-specific difference in an analysis of variance (two-factor ANOVA, type III sums of squares) using sex and taxon as

categorical factors. The complete morphometric data set is given in the supplementary Table S1. A principal component analysis (PCA) was used to detect potential differences in shape among taxa without a priori delimitation of groups. The extraction criterion for principal components was an eigenvalue >1. In morphometric data sets, PC1 represents size, and PC2 and PC3 represent size-independent aspects of shape. A discriminant analysis was used to test for morphometric differentiation among the taxa, which were a priori delimited. Variables were standardized by dividing the difference between value and arithmetic mean by the standard deviation. We considered a rate of correct classification of at least 80% as an indicator of significant morphological differentiation.

Advertisement call variation without a priori delimitation of groups was explored using a factor analysis on the standardized call and temperature variables. The complete bioacoustic data set is given in the supplementary Table S2. Extracted principal components were submitted to an orthogonal VARIMAX-rotation to yield factor loading of original variables close to 1 (strong association) or 0 (no association). A discriminant analysis was used to test for bioacoustic differentiation among the taxa, which were a priori delimited. Again, we considered the criterion of 80% correct classification as an indicator of significant call differentiation. The six call variables (see Section 2.2) recorded in allopatric and sympatric populations of each taxon were compared using an ANCOVA with population type as a fixed categorical factor and soil and water temperature as continuous co-variables to control for the impact of temperature on call structure.

Significance level was set at alpha = 0.05. All statistical procedures were performed using the program package Statgraphics Centurion version 18.1.01 (64-bit; Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results

3.1. Odontophrynus Species Composition at the Study Localities

The taxon composition of *Odontophrynus* specimens collected or recorded during advertisement at the six study localities varied considerably along the transect (Table 2). The northernmost breeding assemblage at Villa General Belgrano included exclusively *O. cordobae*, the two southernmost ones at Coronel Baigorria and Rio Cuarto exclusively *O. americanus*. Species identification was based on advertisement call features, ploidy assessment using erythrocyte features, and occasionally karyology. At the three central localities of the transect, the two species coexisted in varying proportions. Exclusively at the La Escondida locality, we detected additional triploid specimens. We did not attempt to quantitatively assess the abundance of taxa at mixed sites.

Table 2. Frequency of *Odontophrynus* taxa at the study localities. The number of males and females collected for ploidy determination (erythrocyte/nuclear size) and that of males recorded during advertisement is given. Note that not all males recorded calling were captured for ploidy determination and vice versa.

Locality	*O. cordobae* (2n)		*O. americanus* (4n)		*Odontophrynus* (3n)	
	Erythrocytes	Advertisement Calls	Erythrocytes	Advertisement Calls	Erythrocytes	Advertisement Calls
Villa General Belgrano	17	38	-	-	-	-
Rio de los Sauces	12	15	-	2	-	-
El Cano	6	7	13	-	-	-
Escuela La Escondida	53	28	57	51	77	38
Coronel Baigorria	-	-	14	13	-	-
Rio Cuarto	-	-	28	45	-	-

3.2. Morphometric Features of Odontophrynus Taxa at the La Escondida Study Site

Adult *O. cordobae*, *O. americanus*, and triploids did not differ significantly in size (two-factor ANOVA of log10-normalized SVL, $F_{2,236} = 0.98$, $p = 0.3767$). Sexual size dimorphism was absent as well (two-factor ANOVA of log10-normalized SVL, $F_{1,236} = 0.26$, $p = 0.6133$). Interactions between ploidy and sex were not significant (two-factor ANOVA of log10-normalized SVL, $F_{2,236} = 0.71$, $p = 0.4923$).

Principal component analysis of the standardized morphometric variables yielded three PCs with an eigenvalue >1 that explained 54.6% of total variance (Figure 2A). The size-related PC1 (eigenvalue = 4.46) accounted for 29.8% of total variance, the shape-related PC2 (eigenvalue = 2.70) and PC3 (eigenvalue = 1.02) for 18.0% and 6.8%, respectively. There was no detectable segregation of taxon groups with respect to PC2 and PC3.
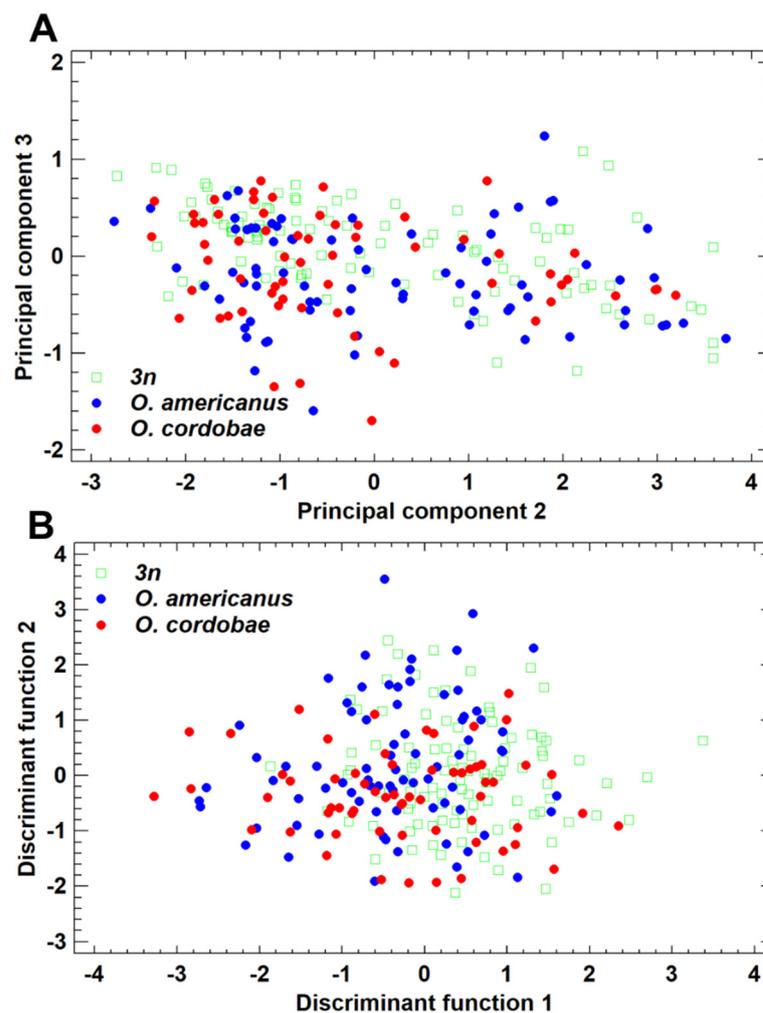

Figure 2. Morphometric differentiation of di-, tri-, and tetraploid *Odontophrynus* taxa at the La Escondida contact zone. (**A**) Scatter plot of PC scores without *a priori* delimitation of groups. (**B**) Scatter plot of discriminant function scores with a priori delimitation of groups.

Discriminant analysis of the same data set yielded a single marginally significant discriminant function (eigenvalue = 0.17, canonical correlation = 0.3783, $X^2 = 45.7$, df = 30, $p = 0.0329$; Figure 2B). The second function did not contribute significantly to group separation function (eigenvalue = 0.05, canonical correlation = 0.2144, $X^2 = 10.7$, df = 14, $p = 0.7112$). The rate of correct classification was low in all taxa: 40.3% in *O. cordobae*, 41.6% in *O. americanus*, and 56.1% in the triploids. The misidentified specimens were equally distributed over the other two taxa. The three syntopic *Odontophrynus* taxa were indistinguishable using size- and shape-related morphometric characters.

3.3. Advertisement Call Features of Odontophrynus Taxa

Advertisement calls of diploid *O. americanus* and tetraploid *O. cordobae* consisted of a single pulse group, with pulses located at the beginning of the call having low intensity, followed by increasing intensity pulses, and a decrease in pulse intensity at the end of call (Figure 3A,B). Pulse duration was shorter in *O. cordobae* and the number of pulses per call greater than in *O. americanus*, resulting in a higher pulse rate as a distinguishing call feature at any ambient temperature. The calls of triploid males resembled in all aspects the call of *O. americanus* (Figure 3C).
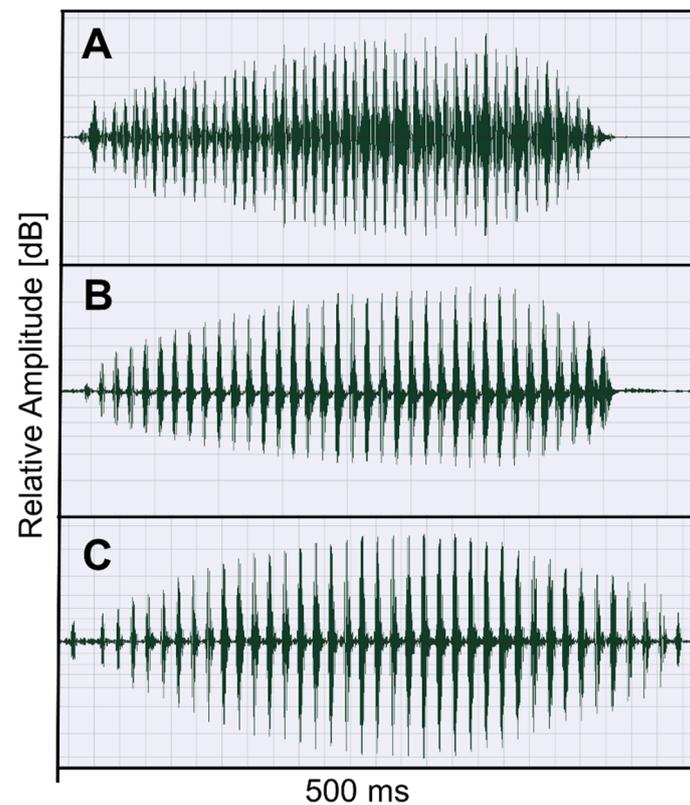
Figure 3. Representative oscillograms of advertisement calls given by males of (**A**) diploid *O. cordobae*, (**B**) tetraploid *O. americanus*, and (**C**) triploid specimens. Recording details: (**A**) 15 March 2021, La Escondida, temperatures 20.6 °C (water), 18.4 °C (soil); (**B**) 5 February 2020, La Escondida, temperatures 19.1 °C (water), 23.0 °C (soil); (**C**) 5 February, 2020, La Escondida, temperatures 18.1 °C (water), 22.7 °C (soil).

To explore the range of variation in the advertisement calls without a priori delimitation of taxon groups, we applied a factorial analysis to the data set, including six call parameters and two environmental temperature variables. There were three statistically independent factors with an eigenvalue >1, which explained 79.5% of total variance. Factor 1 (eigenvalue = 1.71, accounting for 21.4%) was significantly loaded by soil and water temperature, call duration, interpulse interval, and pulse rate, i.e., representing the temperature-sensitive structure of the call. Factor 2 (eigenvalue = 3.44, 43.0%) was significantly loaded by the number of pulses per call, pulse duration, and again pulse rate, i.e., representing the temperature-independent structure of the call. Factor 3 (eigenvalue = 1.21, 15.1%) was loaded exclusively by the dominant frequency. The factor scores of diploid *O. cordobae* individuals formed one homogeneous group, whereas those of the tetraploid *O. americanus*, together with those of the triploid individuals, formed an independent second one (Figure 4A).
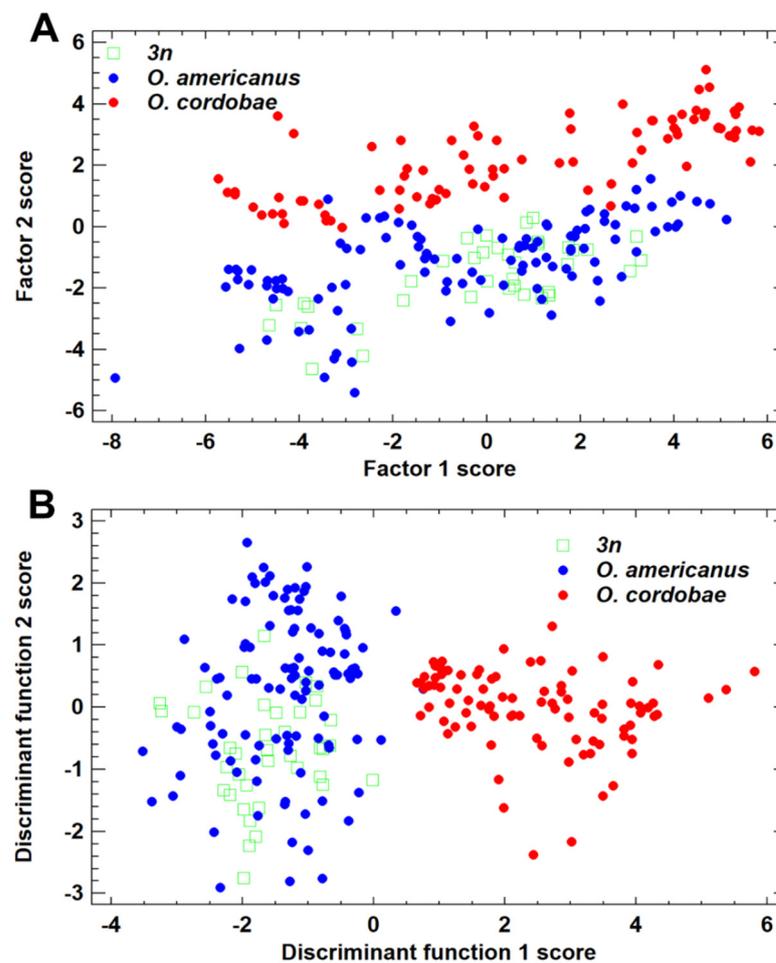
Figure 4. Bioacoustic differentiation of di-, tri-, and tetraploid *Odontophrynus* taxa. (**A**) Scatter plot of factor scores without a priori delimitation of groups. (**B**) Scatter plot of discriminant function scores with a priori delimitation of groups. Each symbol represents one individual.

Based on the data set including the studentized residuals of the six temperature-adjusted call parameters, a discriminant analysis was performed to maximize differences among the three a priori delimited *Odontophrynus* taxon groups. We obtained two significant discriminant functions (DF1: eigenvalue = 3.59, canonical correlation = 0.8846, X^2 = 377.8, df = 12, p < 0.0001; DF2: eigenvalue = 0.1, canonical correlation = 0.3186, X^2 = 24.8, df = 5, p = 0.0002). The rate of correct classification was 100% in *O. cordobae*, but considerably lower in *O. americanus* (64.0%) and in the triploids (73.7%; Figure 4B). The misidentified specimens were *O. americanus* confused with triploids and vice versa.

3.4. Advertisement Call Features of Allopatric and Sympatric Odontophrynus Populations

Independent of the syntopic presence of other *Odontophrynus* taxa and of ambient temperature (ANCOVA: soil temperature, $F_{1,236}$ = 0.45, p = 0.5012; water temperature, $F_{1,236}$ = 0.01, p = 0.9647), the number of pulses per call was species-specific and distinguished reliably populations of *O. cordobae* (51.0 ± 0.5) and *O. americanus* (40.0 ± 0.5) (ANCOVA: $F_{6,236}$ = 48.07, p < 0.0001). In contrast, triploids produced the same number of pulses per call (40.5 ± 0.8) as *O. americanus* (Multiple average comparison, p > 0.05).

The temperature-adjusted call variables pulse rate and pulse duration were sensitive to the syntopic presence of other taxa (Figure 5). In *O. cordobae*, syntopy with *O. americanus* alone or with additionally triploid increased significantly pulse duration compared to allopatric populations (ANCOVA with multiple average comparisons: p < 0.05). Pulse rate increased exclusively in the presence of triploids over the level of allopatric populations

(ANCOVA with multiple average comparisons: $p < 0.05$). In *O. americanus*, the described response pattern of *O. cordobae* was reversed. The parameter level of triploids did not differ significantly from those of syntopic *O. americanus* but did so compared to allopatric populations of the tetraploid (ANCOVA with multiple average comparisons: $p < 0.05$).
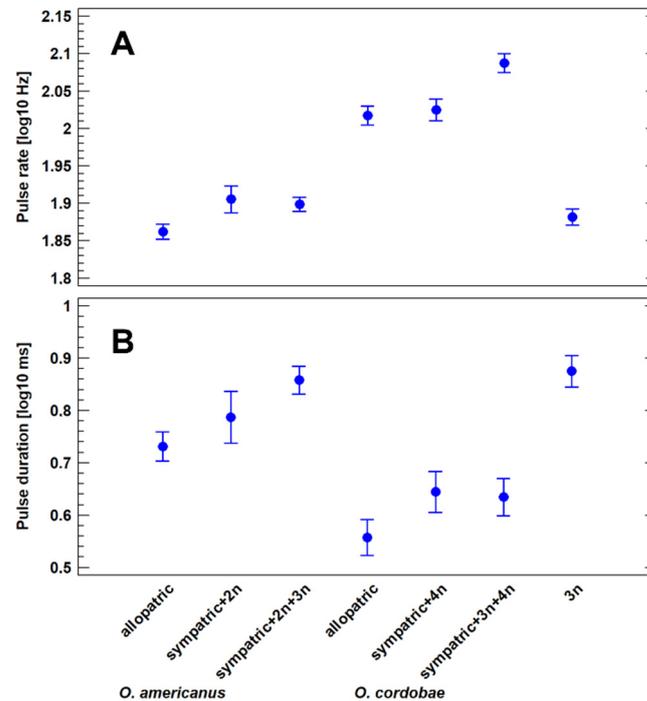

Figure 5. Sensitivity to the presence of one or two syntopic *Odontophrynus* taxa in temperature-adjusted call parameters pulse rate (**A**) and pulse duration (**B**). Each dot represents the least square mean and its corresponding 95% Bonferroni confidence interval, calculated using an ANCOVA on the log10-normalized response variables.

Local variations of the temperature-adjusted call parameters call duration and pulse interval did not show effects of sympatry with other *Odontophrynus* taxa.

3.5. Mating Preferences at the La Escondida Contact Zone

Homotypic pairing constituted 10 out 21 amplexi analyzed (Table 3). Yet, almost perfect premating isolation among taxa was limited to *O. cordobae* and *O. americanus*, and *O. cordobae* and triploids, respectively. We observed a single mismating of an *O. americanus* male with an *O. cordobae* female. In contrast, triploid specimens were more often found in amplexus with *O. americanus* than each taxon in homotypic pairings.

Table 3. Taxonomic composition of amplecting *Odontophrynus* pairs (N = 21) collected at the La Escondida locality. Ploidy of males was assessed by erythrocyte size and advertisement call features, that of females exclusively by erythrocyte size.

	O. cordobae ♂	*O. americanus* ♂	*O.* **3n** ♂
O. cordobae ♀	4 (19.0%)	1 (4.7%)	-
O. americanus ♀	-	1 (4.7%)	4 (19.0%)
O. **3n** ♀	-	6 (28.7%)	5 (23.8%)

4. Discussion

We provide evidence that *Odontophrynus* toads of three ploidy levels interact reproductively in their natural habitat, the La Escondida locality in Central Argentina. This is the first example in an autopolyploid anuran species complex, and the second one for

Amphibia [8]. While in the *Bufotes pewzowi*-breeding system exclusively triploid males are involved, the *Odontophrynus*-breeding system reported in this study is more complex, with triploid males and females mating with each other and with the tetraploid *O. americanus*. Heterospecific amplexi of triploids with the syntopic diploid *O. cordobae* were never observed. Consequently, our findings support the hypothesis that ploidy-dependent advertisement call structure shapes the reproductive interactions at the contact area of the three taxa. We highlight support in detail by contrasting bioacoustic analyses and field observations with the three predictions.

4.1. Prediction 1: Hybridization Due to Natural Mating between the Diploid O. cordobae and Tetraploid O. americanus Is Rare to Absent

As previously reported, the temporal structure of the advertisement calls differs mainly in the pulse rate because the pulse duration is significantly shorter in the diploids than in the tetraploids at a similar call duration [18,26,27]. The contrast between these features remained stable in syntopy, allowing for a highly effective discriminatory mate choice of phonotactically approaching females. Consequently, mismatings between diploids and tetraploids were very rare. In fact, the only heterospecific amplexus including a diploid and a tetraploid was between an *O. cordobae* ♀and an *O. americanus* ♂. As reproductive males counteract female choice by clasping actively any individual of appropriate size and shape that they notice in their vicinity, the heterospecific amplexus was most probably the result of rape rather than of female choice. As the *Odontophrynus* adults of all ploidy levels do not differ from each other in external morphology, the chance of erroneous mate choice of males increases. Mismatings between the morphologically similar diploid-tetraploid treefrogs *Hyla chrysoscelis* and *Hyla versicolor* support the case [34].

4.2. Prediction 2: Advertisement Call Structure of Triploids Resembles More O. americanus than O. cordobae

In agreement with call analyses in triploids of the *Bufotes* species group and of *Hyla*, triploid *Odontophrynus* produced advertisement calls that were indistinguishable from that of *O. americanus* [13,15,17,35,36]. A trend for larger cells in the midbrain auditory centers and larger spinal motor neurons of polyploids has been proposed to account at least partly for the different call structures of diploids, but current evidence is not unequivocal [14]. Whatever the proximate cause for the alteration of call structure from diploids to polyploids may be, the amazing similarity of advertisement calls of triploid and tetraploid *Odontophrynus* undermines effective premating isolation by female phonotaxis between these two ploidy levels. As the viability of triploids is like that of the parental species [25], unavoidable heterospecific matings may result in a significant genetic sink for the *O. americanus* population.

4.3. Prediction 3: Frequent Mismatings Occur between O. americanus and Triploid Specimens, but Not with O. cordobae

In fact, heterospecific amplexi between triploids and tetraploids constituted almost half of all amplexi analyzed, corroborating their incapacity to identify and choose a mate of the same ploidy. The high frequency of heterospecific amplexi and of homotypic triploid pairings raises the question of whether triploids are fertile. In the *Bufotes* species group, there are three triploid bisexual species (*B. baturae*, *B. pseudoraddei* and *B. zugmayeri*) that are fully fertile [6,12,37]. Presently, we do not have direct evidence for the fertility of the *Odontophrynus* triploids, but ongoing research focuses on this issue.

4.4. Putative Origin of Triploids and Their Local Long-Term Persistence

In this study, we identified three localities with syntopic occurrence of *O. cordobae* and *O. americanus*, but in only one we detected triploids. Hybridization of the paternal species occurs but is most probably too rare to account for the long-term persistence of numerous triploids at the La Escondida locality. Thus, hybridization is one possible explanation of the origin. Alternatively, triploidization may have been a spontaneous event, resulting from

problems in the disjunction of chromosomes in gamete formation or in the first zygotic division [24]. A third possibility is pollution-induced effects on meiosis, which could result from the intensive local application of agrochemicals at the La Escondida area [38].

Whatever has caused the initial emergence of triploid *Odontophrynus*, the only plausible explanations for their continued presence in large numbers are unaffected viability and full fertility. Viability comparable to that of the paternal species has already been proven [25]. The second prerequisite for persistence remains to be investigated. If we postulate full fertility of triploids, gametogenesis is expected to produce haploid and diploid gametes. Offspring generated by backcrosses with *O. americanus* would yield partly the paternal species and partly triploids. Offspring generated from pure triploid pairing would additionally produce diploids, i.e., *O. cordobae*. In fact, the pulse rate of the advertisement calls given by the La Escondida diploids was significantly higher than that recorded at any other locality with *O. cordobae*. A possible cause could be character displacement, but alternatively may reflect differences from the gene pool of *O. cordobae* from the type locality, if specimens result from triploid mating rather than from dispersal of roaming individuals. Ongoing research will focus on this aspect as well.

5. Conclusions

The occurrence of numerous triploid specimens inhabiting an area of a few square kilometers together with diploid *O. cordobae* and tetraploid *O. americanus* is unique for an autopolyploid species complex. Reproductively interacting anurans of three ploidy levels were reported before exclusively in allopolyploid *B. pewzowi*-species complex [8]. While there is no doubt about the phenomenon itself, many open questions remain to be resolved before reaching a rudimentary understanding of interactions. Among the most urgent topics of future research are the process(es) leading to triploid formation, the assessment of fertility, and the mechanisms of gametogenesis. Another issue is the surprising geographic restriction of triploids to a single small-sized area, though the area of contact between *O. cordobae* and *O. americanus* is much larger. In conclusion, much remains to be learned from this peculiar species complex.

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