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Supplementary material

Molecular phylogenetics and phenotypic reassessment of the *Ramphotrigon* flycatchers: deep paraphyly in the context of an intriguing biogeographic scenario

Supplementary material Appendix 1

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Extended Materials and methods

Species tree and divergence times estimation

We used *BEAST (Heled and Drummond 2010) to estimate a time-calibrated species tree under the multispecies coalescent model as implemented in BEAST 1.8 (Drummond et al. 2012). All loci were placed in separate partitions under the models of nucleotide substitution selected with jModelTest or the closest one available in BEAST (see main text). To date divergence events among taxa we implemented a relaxed uncorrelated lognormal clock and four different substitution rates, one for each locus. For cyt *b* we used the rate of 2.1% sequence divergence between lineages per Myr (1.05×10^{-2} substitutions/site/lineage/Myr; Weir and Schluter 2008), while for the other two mitochondrial genes we specified the rates reported by Lavinia et al. (2019): 1.04×10^{-2} s/s/l/Myr for COI and 1.38×10^{-2} s/s/l/Myr for ND2. For the autosomal nuclear intron FIB5, the rate specified was 1.35×10^{-3} s/s/l/Myr. For the unknown rate of the nuclear indels we used the CTMC rate reference prior (Ferreira and Suchard 2008). We ran the analysis for 200 million generations sampling every 1000 generations and checked for stationarity and adequate posterior sampling using Tracer 1.5 (Rambaut and Drummond 2007). We discarded the first 10% of the sampled trees as burn-in and then used the remaining 180000 topologies to estimate the maximum clade credibility species tree using mean node heights, and calculate the 95% highest posterior density (HPD) intervals of divergence dates with TreeAnnotator 1.8 (Drummond et al. 2012).

Morphology

We measured the beak size and the length of the wing, tail and tarsus on museum skins representative of *D. flammulatus* and the three species of *Ramphotrigon*. For the bill, we measured beak length (measured from the point at which the feathers of the forehead cease to hide the culmen to the tip of the culmen in a straight line), and beak width and depth (i.e., height) at the distal edge of the nostrils (Baldwin et al. 1931, Eck et al. 2011). These variables were summarized into a single one (PCbeak) representing beak size through a principal component analysis (PCA). Tarsus length was taken from the middle point of the joint between tibia and tarsus to the lower edge of the lowest undivided scute of the junction between tarsus and toes. Wing

length was measured in a straight line from the farthest anterior point on the anterior edge of the carpal joint to the tip of the longest primary in the naturally folded wing (Baldwin et al. 1931, Eck et al. 2011). Both left and right wings were measured and then averaged to obtain a single wing length per individual. Tail length was taken from the pygostyle felt from below to the tip of the longest tail feather in the naturally folded tail (Eck et al. 2011). All morphological characters were measured three times with digital calipers with a resolution of 0.01 mm (except for the length of the tail that was measured using a thin ruler with a zero starting point), and then averaged to obtain a single value for each variable and individual.

Plumage coloration

We measured plumage reflectance spectra on five plumage patches (throat, chest, belly, head and back) three times replacing the probe each time. We employed an Ocean Optics USB 2000 spectrophotometer, with a PX-2 xenon light source (effective range of emission from 220 to 750 nm) calibrated against a WS-1 diffuse reflectance white standard (Ocean Optics, Inc., Dunedin, Florida, USA). Plumage was illuminated and the reflected light was collected with a bifurcated probe located perpendicularly and 16 mm away from the museum skin surface. The probe was placed on a rubber holder that kept it at a fixed distance from the surface and isolated from ambient light. The spectrophotometer resolution was 0.35 nm, each spectrum was the average of 10 readings with an integration time of 28–37 msec (determined by the "automatic" option of the software OceanView 1.6.7), and we used a boxcar smoothing function of 10. We re-calibrated the equipment against the white standard before measuring plumage reflectance on each new individual.

The reflectance spectra obtained were analyzed with the package *pavo* 2.0 (Maia et al. 2019) in R 3.5.2 (R Core Team 2016). We averaged the three readings obtained for each plumage patch and individual in order to obtain a single reflectance spectrum with one reflectance value per nm between 300 and 700 nm. We used the Vorobyiev and Osorio (1998) visual model implemented in *pavo* to estimate the perceptual distance (ΔS) in the chromatic component of plumage color for each plumage patch between all individuals. These distances are expressed in units of just noticeable differences (*jnd*) and values larger than 1 *jnd* are considered to represent color differences discernible by birds under the conditions considered for the

analysis (Maia and White 2018). For the calculation of these noise-corrected perceptual distances, we selected the "average V" avian visual sensitivity to determine the absorbance at each wavelength for each cone type. There are no data about the specific sensitivity of each cone type in the *Ramphotrigon* flycatchers, but it is likely that they belong to the V sensitive group (short-wavelength sensitivity biased toward violet) since Tyrannidae has been shown to be one of the passeriform families lacking ultraviolet sensitive (UVS) type of color vision (Ödeen et al. 2011). For our calculations, we specified a forest shade illuminant, an idealized background, the Eurasian Blue Tit (*Cyanistes caeruleus*) relative photoreceptor densities (1:2:2:4), and a Weber fraction of 0.1. Following Maia and White (2018), pairwise *ΔS* values were used to estimate an average distance in color space between the geometric means of within-species distances for each plumage patch (rather than simply averaging the pairwise distances estimated between all individuals of different species). These averages values where then compared to the theoretical discrimination threshold of biological significance (1 *jnd*) to assess whether differences among species were perceptually discriminable by the birds. For each average *ΔS* value we estimated a 95% confidence interval based on 1,000 pseudoreplicates. Average *ΔS* values among species and bootstrap values were obtained with the function *bootcoldist* in *pavo*.

In addition to the calculation of *ΔS* values, we measured three spectral variables typically used to describe plumage color (Montgomerie 2006). We calculated plumage mean brightness (B2 in *pavo*) following the formula $\Sigma R_{(300–700)}/401$ where 401 is the total number of data points (i.e. wavelengths) in each spectrum We also estimated the violet chroma (S1V in *pavo*) as the ratio between the light reflected between 300–415 nm and the light reflected over all wavelengths ($\Sigma R_{(300-415)}/\Sigma R_{(300-700)}$). Similarly, green chroma (S1G in *pavo*) was calculated as the ration between the light reflected between 510–605 nm and the total reflectance ($\Sigma R_{(510-605)}/\Sigma R_{(300-700)}$) for all plumage patches except for the yellow belly, for which we obtained the long wavelength chroma (carotenoid chroma or S9 in *pavo*) as the ratio between reflectance between 450-700 nm and reflectance at 700 nm ($\Sigma R_{(700-450)}/ R_{700}$). Differences among taxa in these variables were assessed

through one-way ANOVAs followed by Tukey's post hoc comparisons (or Welch's *F*-tests followed by Dunnett's T3 post hoc tests when the assumption of homoscedasticity was not met).

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Supporting Figures

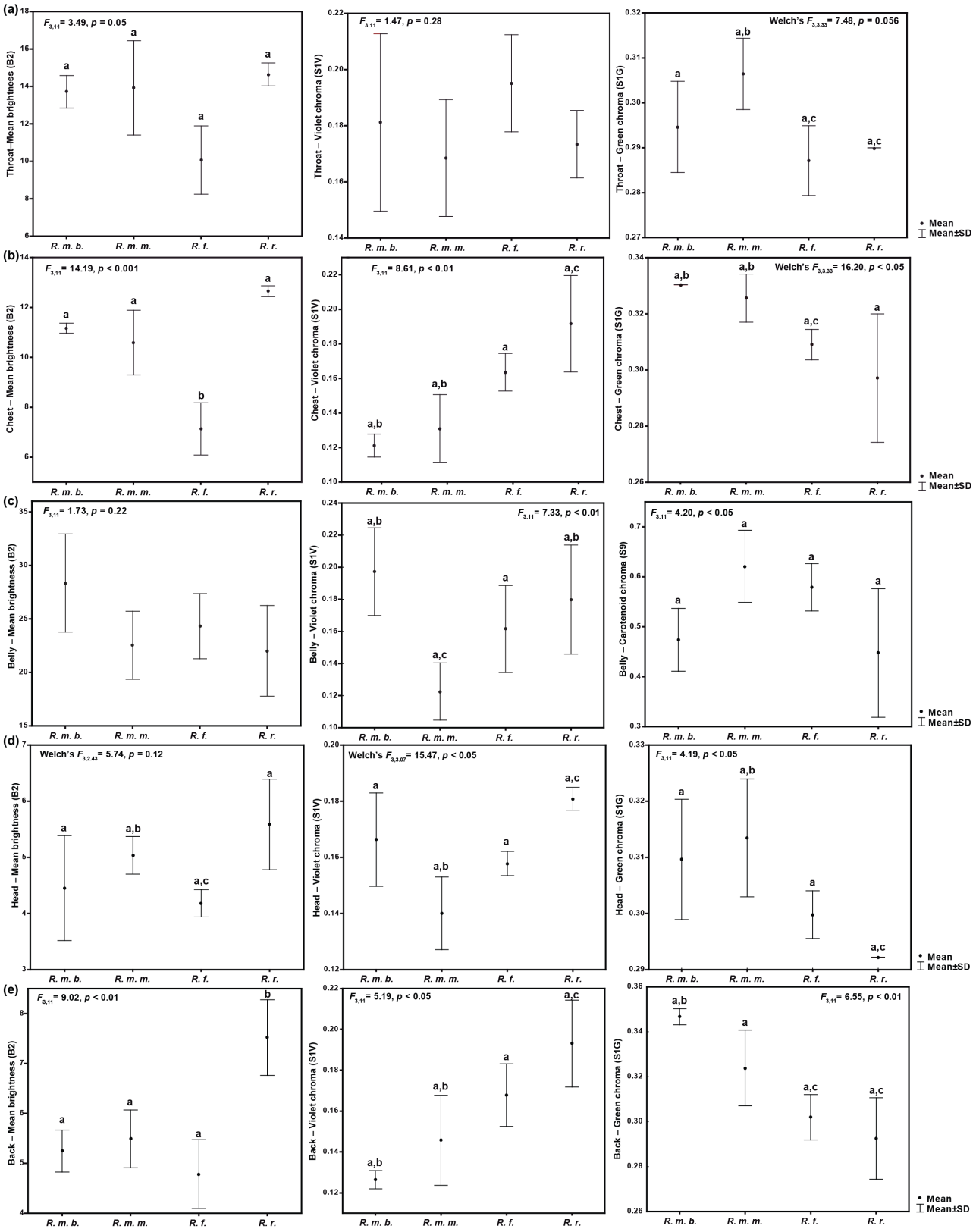

Figure A1. Results from the analysis of three spectral variables measured on each of the five plumage patches measured on museum skins of *Ramphotrigon* flycatchers. Mean (dot) ± SD (whiskers) depicted in all cases. (a, b, d, e) Mean brightness, violet chroma and green chroma for the throat, chest, head and back. (c) Mean

brightness, violet chroma and carotenoid chroma for the belly. The ANOVA's or Welch's *F* statistic and the significance of the tests (*p*) are shown within each panel. Pairs of taxa that differed significantly after Tukey's post hoc comparisons (or Dunnett's T3 for the Welch's *F*-test) are identified with different letters. *R. m. b.*, *R. megacephalum bolivianum*; *R. m. m.*, *R. m. megacephalum*; *R. f.*, *R. fuscicauda*; *R. r.*, *R. ruficauda*.

Supporting Tables

Table A1. Museum skins used in morphological and coloration analyses. All individuals correspond to adult males deposited at the Coleccion Boliviana de Fauna (CBF), the Colección Nacional de Aves, Instituto de Biología de la Universidad Nacional Autónoma de México (CNAV), and the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN).

Taxa	Catalogue number	Year	Locality
Deltarhynchus flammulatus[*]	CNAV 3603	1974	Jalisco, Mexico
D. flammulatus[*]	CNAV 26743	1989	Guerrero, Mexico
Ramphotrigon fuscicauda	CBF 2268	1991	Pando, Bolivia
R. fuscicauda	CBF 4576	2009	La Paz, Bolivia
R. fuscicauda	CBF 4640	2009	La Paz, Bolivia
R. fuscicauda	MACN-Or 71934	2009	La Paz, Bolivia
R. megacephalum bolivianum	CBF 1726	1990	La Paz, Bolivia
R. m. bolivianum	CBF 4628	2009	La Paz, Bolivia
R. m. megacephalum	MACN-Or 34409	1953	Misiones, Argentina
R. m. megacephalum	MACN-Or 36988	1954	Misiones, Argentina
R. m. megacephalum	MACN-Or 36989	1954	Misiones, Argentina
R. m. megacephalum	MACN-Or 36990	1954	Misiones, Argentina
R. m. megacephalum	MACN-Or 36995	1954	Misiones, Argentina
R. m. megacephalum	MACN-Or 71126	2007	Misiones, Argentina
R. m. megacephalum	MACN-Or 72897	2012	Misiones, Argentina

Ramphotrigon ruficauda	CBF 2236	1991	Pando, Bolivia
R. ruficauda	CBF 512	1986	Pando, Bolivia

*not included in the coloration analyses

Table A2. Factor loadings of the three beak size linear variables (length, width and depth) measured on museum skins of males of *D. flammulatus* and the three species of *Ramphotrigon*. One principal component (PC) with eigenvalue > 1 was extracted from the principal component analysis (PCA). All original variables correlated significantly (factor loadings values| ≥ 0.7) with PC1 (which is unrotated).

Original variables	PC1
Beak length	0.97
Beak width	0.91
Beak depth	0.76
Percentage of explained variance	79%

Table A3. Individual body mass information obtained from some of the museum skins measured for the morphological and coloration analyses (Table A1) and complemented from data downloaded from VertNet (www.vertnet.org).

Taxa	**Catalogue number**	**Locality**	**Weight (g)**
Ramphotrigon fuscicauda	CBF 2268	Pando, Bolivia	17.5
R. fuscicauda	MACN-Or 71934	La Paz, Bolivia	18.4
R. fuscicauda	CBF 4640	La Paz, Bolivia	19.3
R. fuscicauda	CBF 4576	La Paz, Bolivia	20.9
R. megacephalum bolivianum	CBF 4628	La Paz, Bolivia	14.2
R. m. bolivianum	CBF 1726	La Paz, Bolivia	15
*R. m. bolivianum**	KU 131676	Cusco, Peru	16.5

R. m. megacephalum	MACN-Or 71126	Misiones, Argentina	11.9
R. m. megacephalum	MACN-Or 72897	Misiones, Argentina	12.7
Ramphotrigon ruficauda	CBF 512	Pando, Bolivia	20.5
R. ruficauda	CBF 2236	Pando, Bolivia	21
*R. ruficauda**	ROM 138414	Suriname	16
*R. ruficauda**	KU 88835	Guyana	17.3
*R. ruficauda**	YPM 101773	Suriname	17.7
*R. ruficauda**	KU 88340	Guyana	18
*R. ruficauda**	UMMZ 210512	Suriname	18.5
*R. ruficauda**	ROM 125884	French Guiana	19
*R. ruficauda**	KU 90391	Guyana	19.2
*R. ruficauda**	KU 94867	Guyana	20

*mined from VertNet

Table A4. Mean ± SD for the morphological variables measured on museum skins of *Deltarhynchus* and *Ramphotrigon* flycatchers. Numbers between parentheses indicate sample sizes. All measurements are in milimetres (mm).

Taxa	Beak length (mm)	Beak width (mm)	Beak depth (mm)	Wing length (mm)	Tail length (mm)	Tarsus length (mm)
Deltarhynchus flammulatus (n = 2)	15.41 ± 0.92	7.09 ± 0.27	4.56 ± 0.33	75.99 ± 1.78	74.55 ± 2.25	19.56 ± 0.08
Ramphotrigon fuscicauda (n = 4)	14.12 ± 0.45	6.46 ± 0.23	4.53 ± 0.19	72.51 ± 1.58	76.08 ± 4.69	17.48 ± 0.60
R. megacephalum bolivianum (n = 2)	11.91 ± 0.83	5.64 ± 0.07	4.18 ± 0.21	60.02 ± 1.14	56.83 ± 2.59	15.90 ± 0.35
R. m. megacephalum (n = 7)*	11.33 ± 0.36	5.72 ± 0.31	4.11 ± 0.10	62.51 ± 1.45	58.07 ± 2.72	15.45 ± 0.61
R. ruficauda (n = 2)	13.63 ± 0.61	5.97 ± 0.34	4.79 ± 0.23	77.91 ± 0.52	75.00 ± 2.83	15.52 ± 1.14

*n = 6 for beak measurements

Table A5. Mean noise-corrected perceptual distances in the avian color space (*ΔS*, *jnd*) between species of *Ramphotrigon* flycatchers for the three plumage patches measured. For each mean *ΔS* we also report a 95% confidence interval estimated from 1,000 pseudoreplicates of bootstrap per comparison.

Patch	Pairwise comparison	*ΔS* (*jnd*)	95% confidence interval (*jnd*)
Throat	*R. megacephalum bolivianum vs R. m. megacephalum*	0.23	0.21–0.66
	R. megacephalum bolivianum vs R. fuscicauda	1.02	0.32–1.73
	R. megacephalum bolivianum vs R. ruficauda	0.32	0.13–0.84
	R. megacephalum megacephalum vs R. fuscicauda	0.99	0.55–1.49
	R. megacephalum megacephalum vs R. ruficauda	0.44	0.33–0.63
	R fuscicauda vs R. ruficauda	0.79	0.37–1.30
Chest	*R. megacephalum bolivianum vs R. m. megacephalum*	0.95	0.27–1.63
	R. megacephalum bolivianum vs R. fuscicauda	2.24	1.60–2.89
	R. megacephalum bolivianum vs R. ruficauda	2.16	0.97–3.37
	R. megacephalum megacephalum vs R. fuscicauda	1.30	0.66–1.96
	R. megacephalum megacephalum vs R. ruficauda	1.23	0.20–2.35
	R fuscicauda vs R. ruficauda	0.21	0.19–1.27
Belly	*R. megacephalum bolivianum vs R. m. megacephalum*	1.78	1.01–2.69
	R. megacephalum bolivianum vs R. fuscicauda	1.04	0.27–1.77
	R. megacephalum bolivianum vs R. ruficauda	0.26	0.20–1.58
	R. megacephalum megacephalum vs R. fuscicauda	0.74	0.13–1.67
	R. megacephalum megacephalum vs R. ruficauda	2.03	0.73–3.42
	R fuscicauda vs R. ruficauda	1.29	0.20–2.57
Head	*R. megacephalum bolivianum vs R. m. megacephalum*	0.98	0.15–2.07

	R. megacephalum bolivianum vs R. fuscicauda	0.38	0.20–1.16
	R. megacephalum bolivianum vs R. ruficauda	0.39	0.35–1.17
	R. megacephalum megacephalum vs R. fuscicauda	0.78	0.28–1.26
	R. megacephalum megacephalum vs R. ruficauda	0.92	0.40–1.43
	R fuscicauda vs R. ruficauda	0.14	0.07–0.39
Back	*R. megacephalum bolivianum vs R. m. megacephalum*	1.22	0.71–1.80
	R. megacephalum bolivianum vs R. fuscicauda	2.11	1.82–2.41
	R. megacephalum bolivianum vs R. ruficauda	1.96	1.33–2.61
	R. megacephalum megacephalum vs R. fuscicauda	0.89	0.36–1.37
	R. megacephalum megacephalum vs R. ruficauda	0.78	0.25–1.52
	R fuscicauda vs R. ruficauda	0.28	0.27–0.74

Table A6. Mean ± SD for the three spectral variables measured on each plumage patch of *Ramphotrigon* flycatchers analyzed. Numbers between parentheses indicate sample sizes. B2: mean brigthness; S1V: violet chroma; S1G: green chroma; S9: carotenoid chroma.

Patch / Taxa	Throat			Chest			Belly			Head			Back		
	B2	S1V	S1G	B2	S1V	S1G	B2	S1V	S9	B2	S1V	S1G	B2	S1V	S1G
R. fuscicauda (n = 4)	10.06 ± 1.82	0.20 ± 0.02	0.29 ± 0.01	07.13 ± 1.05	0.16 ± 0.01	0.31 ± 0.01	24.32 ± 3.05	0.16 ± 0.03	0.58 ± 0.05	4.18 ± 0.24	0.16 ± 0.00	0.30 ± 0.00	4.79 ± 0.69	0.17 ± 0.02	0.3.0 ± 0.01
R. m. bolivianum (n = 2)	13.71 ± 0.87	0.18 ± 0.03	0.29 ± 0.01	11.17 ± 0.20	0.12 ± 0.01	0.33 ± 0.00	28.35 ± 4.59	0.20 ± 0.03	0.47 ± 0.06	4.45 ± 0.93	0.17 ± 0.02	0.31 ± 0.01	5.25 ± 0.42	0.13 ± 0.00	0.35 ± 0.00
R. m. megacephalum (n = 7)	13.92 ± 2.52	0.17 ± 0.02	0.31 ± 0.01	10.59 ± 1.30	0.13 ± 0.02	0.33 ± 0.01	22.53 ± 3.18	0.12 ± 0.02	0.62 ± 0.07	5.04 ± 0.33	0.14 ± 0.01	0.31 ± 0.01	5.49 ± 0.58	0.15 ± 0.02	0.32 ± 0.02
R. ruficauda (n = 2)	14.64 ± 0.61	0.14 ± 0.01	0.29 ± 0.00	12.65 ± 0.22	0.19 ± 0.03	0.30 ± 0.02	22.01 ± 4.24	0.18 ± 0.03	0.45 ± 0.13	5.59 ± 0.81	0.18 ± 0.00	0.29 ± 0.00	7.52 ± 0.76	0.19 ± 0.02	0.29 ± 0.02