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Geographical variation of prementum size in Iberian *Cordulegaster boltonii* (Odonata: Cordulegastridae) populations

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All relevant data are within the paper.

Abstract. Within wide geographical areas, Odonata populations can show biometric differences as a consequence of both biotic (e.g., predation, competition) and abiotic factors (mainly temperature). These differences can occur in the larval stage, although reliable characters are needed to detect differences. We analyzed whether *Cordulegaster boltonii* larvae from 18 Iberian populations differ regarding head width and prementum size (maximum width, minimum width, and maximum length), using measurements taken on final stage exuviae. Prementum length was greater in southern populations than in northern ones. Geographic latitude and temperature were the variables that best explained this variation in females, whereas latitude and altitude above sea level offered the best explanation among males.

Key words. Dragonfly, exuvia, population variation, prementum size, Portugal, Spain

Introduction

In Anisoptera, the sexes differ in size in the final stadia, females being larger than males (e.g., Ferreras-Romero, 1997; Ferreras-Romero & Corbet, 1999). Those size differentials recorded in the larval stage do not always correspond with those observed in adult stage (Serrano-Meneses et al., 2007).

Cordulegaster boltonii (Donovan, 1807) is a large odonate (Anisoptera) widely distributed throughout the Western Palearctic (Boudot & Holusa, 2015); in the Iberian Peninsula, it mainly occupies small mountain streams. In *C. boltonii*, exuvia head width can be used in biometric comparisons of different populations (El Haissoufi et al., 2018). Likewise, prementum maximum and minimum width, as well as maximum length of the prementum, are proportional to total exuvial length (El Haissoufi et al., 2018) and, therefore, are good indicators of the larva body size. *Cordulegaster boltonii* is a partivoltine species, slow-growing during its larval stage which lasts 2–3 years in the southernmost part of its range (Ferreras-Romero & Corbet, 1999), and several years at higher latitudes (Donath, 1987; Schütte, 1997).

In exuviae of *C. boltonii* from the Iberian Peninsula and Northern Morocco, three traits (head width, length of the gonapophysis and the length of the lateral spine on the 9th sternite) have been used in population differentiation (Casanueva et al., 2020). The present work analyzed whether the exuvial prementum is also a valid trait for analysis of the differentiation of Iberian populations. Specifically, our objectives were: a) if prementum size varies in the area studied, b) if this variation is similar in males and females; and c) how prementum size is related to environmental temperature, altitude and latitude.

Material and methods

Exuviae belonging to final instar of *C. boltonii* collected at 18 Iberian streams (three in Portugal and 15 in Spain) were analyzed (Table 1, Fig. 1). Streams were sampled from May to July during the years 2016 to 2020 (with the exception of the Bejarano Stream, which was sampled from 1989 to 1992). In the laboratory, the following measurements were taken from each exuvia: (1) head width (He) (i.e., the maximum distance between the compound eyes), (2) maximum width (W+) and minimum width (W-) of the prementum and prementum maximum length (ML) (Fig. 2), determined as previously described by Verschuren (1989) and Casanueva et al. (2017). These variables were measured to the nearest 0.1 mm using a binocular stereo microscope with an eyepiece micrometer. Head capsule on exuviae is open dorsally along the exuvial line. To get repeatable accurate measurements of its width, the two halves of the head are approximate, using a fine point entomological tweezers, until the split between them seems to disap-

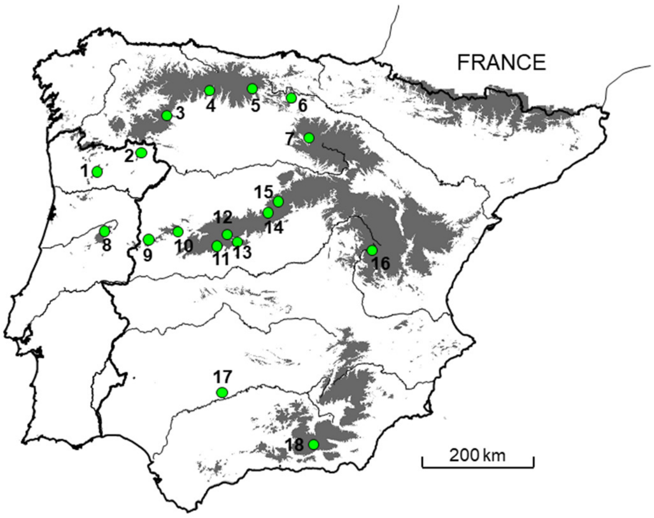

Figure 1. Map showing the distribution of the 18 streams sampled (green dots) on the Iberian Peninsula. 1 – Cova, 2 – Sabor, 3 – Eria, 4 – Curueño, 5 – Lazán, 6 – Sedanillo, 7 – Arlanzón, 8 – Zêzere, 9 – Frío, 10 – Francia, 11 – Barbellido, 12 – Gama, 13 – Alberche, 14 – Eresma, 15 – Cega, 16 – Júcar, 17 – Bejarano, 18 – Monachil. Shaded area: altitude ≥ 1000 m a.s.l.

Table 1. Country and geographic coordinates (decimal grades) from 18 Iberian sites where exuviae of *Cordulegaster boltonii* were collected. Altitude as m a.s.l. Tm: mean annual temperature (°C). Tm was obtained from WorldClim (www.wordclim.org) historical climate data (Hijmans et al., 2005).

Stream	Country	North	West	Altitude	Tm
1. Cova	Portugal	41.5821	-7.7847	329	12.3
2. Sabor	Portugal	41.9025	-6.7364	665	11.8
3. Eria	Spain	42.2619	-6.4344	1117	9.1
4. Curueño	Spain	42.8466	-5.4027	1001	9.9
5. Lazán	Spain	43.0222	-4.4783	1210	8.5
6. Sedanillo	Spain	42.7181	-3.7500	740	10.3
7. Arlanzón	Spain	42.2272	-3.3049	1170	8.9
8. Zêzere	Portugal	40.2419	-7.3144	685	11.3
9. Frío	Spain	40.3154	-6.6347	830	12.4
10. Francia	Spain	40.5055	-6.0472	595	13.3
11. Barbellido	Spain	40.3167	-5.2144	1450	8.6
12. Gama	Spain	40.4186	-5.2158	1642	8.1
13. Alberche	Spain	40.4383	-5.0611	1425	9.2
14. Eresma	Spain	40.8488	-4.0179	1200	10.1
15. Cega	Spain	41.0380	-3.4287	1255	9.3
16. Júcar	Spain	40.3244	-1.8141	1245	9.7
17. Bejarano	Spain	37.9366	-4.8705	400	16.5
18. Monachil	Spain	37.1222	-3.5072	1016	13.1

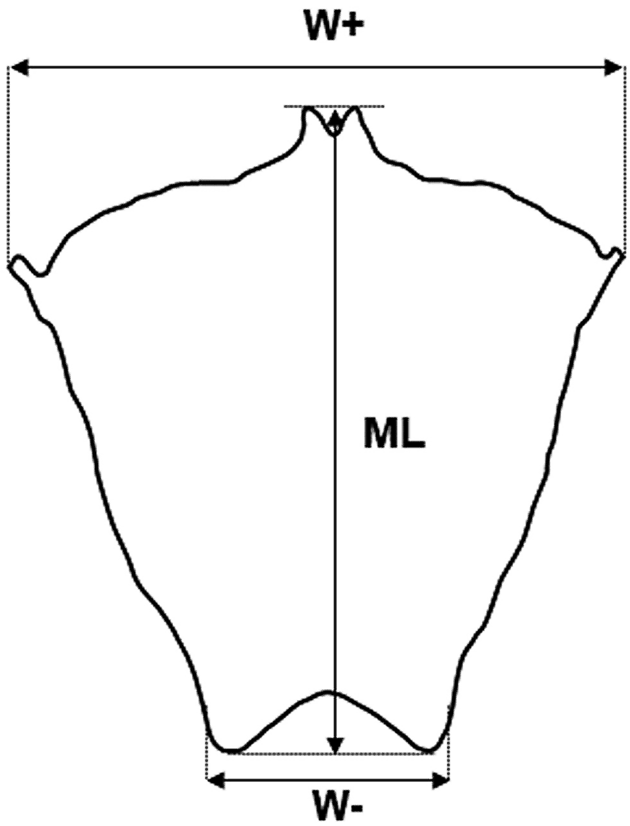

Figure 2. Measurements made in prementum of *Cordulegaster boltonii* exuviae: prementum length (ML), maximum width (W+) and minimum width (W-).

Table 2. Sample sizes (N) and mean values ± SD (mm) of four measurements in male and female exuviae of *Cordulegaster boltonii* from 18 Iberian populations analysed. He: head width. ML, W+, W-: maximum length, maximum width, and minimum width of the prementum. N: sample size.

	Males					Females				
River	**N**	**He**	**ML**	**W-**	**W+**	**N**	**He**	**ML**	**W-**	**W+**
1. Cova	14	8.06±0.19	6.78±0.24	2.54±0.08	6.68±0.19	13	8.63±0.24	7.27±0.18	2.70±0.07	7.18±0.26
2. Sabor	35	7.86±0.17	6.39±0.20	2.40±0.09	6.34±0.19	37	8.45±0.19	6.93±0.20	2.60±0.06	6.88±0.21
3. Eria	20	7.78±0.28	6.59±0.20	2.35±0.06	6.24±0.20	21	8.45±0.17	7.10±0.19	2.59±0.09	6.79±0.14
4. Curueño	27	7.93±0.15	6.32±0.21	2.37±0.09	6.21±0.16	33	8.49±0.18	6.85±0.27	2.59±0.08	6.80±0.20
5. Lazán	42	7.75±0.19	6.17±0.17	2.32±0.09	6.06±0.16	40	8.36±0.20	6.70±0.19	2.55±0.08	6.58±0.19
6. Sedanillo	31	7.97±0.21	6.60±0.18	2.46±0.06	6.30±0.16	40	8.48±0.26	7.09±0.26	2.67±0.10	6.84±0.20
7. Arlanzón	50	7.96±0.35	6.46±0.27	2.39±0.12	6.33±0.30	26	8.27±0.20	6.72±0.16	2.51±0.09	6.60±0.18
8. Zêzere	14	7.93±0.20	6.71±0.18	2.42±0.07	6.36±0.14	21	8.66±0.23	7.38±0.24	2.68±0.09	7.06±0.24
9. Frío	34	7.99±0.28	6.67±0.29	2.43±0.10	6.47±0.26	53	8.59±0.24	7.24±0.26	2.71±0.18	7.10±0.26
10. Francia	55	7.88±0.22	6.48±0.27	2.41±0.08	6.40±0.21	55	8.53±0.24	7.12±0.27	2.63±0.11	7.01±0.27
11. Barbellido	58	7.97±0.16	6.42±0.15	2.37±0.06	6.42±0.15	50	8.63±0.18	6.99±0.21	2.61±0.09	7.00±0.20
12. Gama	29	7.91±0.28	6.59±0.18	2.34±0.06	6.22±0.17	34	8.54±0.21	7.11±0.21	2.56±0.07	6.73±0.19
13. Alberche	83	7.88±0.18	6.38±0.19	2.33±0.07	6.22±0.16	75	8.49±0.22	6.99±0.24	2.56±0.09	6.78±0.22
14. Eresma	231	7.96±0.20	6.59±0.20	2.39±0.07	6.27±0.16	245	8.54±0.20	7.15±0.22	2.60±0.08	6.83±0.18
15. Cega	136	7.85±0.18	6.46±0.20	2.38±0.07	6.21±0.15	118	8.43±0.19	7.00±0.22	2.59±0.08	6.73±0.19
16. Júcar	36	8.28±0.19	7.09±0.17	2.63±0.09	6.63±0.13	24	8.80±0.21	7.59±0.22	2.81±0.08	7.08±0.16
17. Bejarano	51	8.09±0.20	7.13±0.21	2.54±0.07	6.65±0.19	52	8.82±0.19	7.82±0.19	2.81±0.08	7.34±0.18
18. Monachil	52	8.03±0.14	6.82±0.14	2.48±0.07	6.46±0.14	50	8.54±0.16	7.39±0.17	2.66±0.09	6.94±0.18

pear. These four variables are highly reliable and easily repeated in exuviae belonging to *C. boltonii*. (Casanueva et al. 2017). These measurements do not change significantly as the period of emergence progresses, neither in central Spain (Casanueva et al., 2017), nor in Morocco (El Haissoufi et al., 2018).

For each biometric variable, a normality test (Shapiro-Wilk) and homogeneity (Barlett's) test were performed. When these conditions were fulfilled, variables were compared among streams through ANOVA and when they were not fulfilled, they were compared using the Kruskal-Wallis test.

To assess what measurement best indicates the differences between the streams compared, principal component analysis (PCA) was used. When a variable was identified, linear regressions between that variable and the factors of temperature, latitude and altitude were carried out to determine if there was significant correlation.

Finally, to discover which model best explains the size variation of the exuviae according to latitude, altitude and environmental (air) temperature, a generalized linear model (GLM), as well as a gaussian distribution and an identity link, were carried out. The relative fit of the possible models was compared with the Akaike Information Criterion AIC (Akaike, 1974). AIC values were used to calculate the Δ AIC (difference in AIC with respect to the AIC of the best candidate model) and the "Akaike weight" w_i, which expresses the conditional probability for each of the models.

Models in which Δ AIC < 2 are considered to have substantial support; models in which 3 < Δ AIC < 7 are considered to have little support, and models in which Δ AIC > 10 indicate no support (Burnham & Anderson, 2002). A model with wi ≈ 1 presents unambiguous support for the data, while models with approximately the same weight have similar support for the data (Johnson & Omland, 2004).

In all cases, the minimum significance value was $p < 0.05$. These calculations were made by STATA 12.1 software. These calculations were carried out using the Past 3.15 pro software (Hammer et al., 2001).

Results

A total of 1985 exuviae were collected (998 male and 987 female). The highest values of He, ML, W+ and W- were recorded in exuviae from Júcar, Bejarano and Monachil streams (Table 2), watercourses located in the S-SE half of the Iberian Peninsula. Likewise, exuviae collected at Cova and Zêzere streams, both in Portugal, had values close to those found in the aforementioned streams, while the lowest values were found in exuviae collected at Curueño, Lazán, and Arlanzón streams, located in the more septentrional mountains. The sizes of these four variables differs significantly between streams, both in males and females (Table 3).

Principal Component Analysis was carried out in order to determine which variable best demonstrates

Table 3. Statistical values comparing measurements of head width (He), prementum lenght (ML), maximum (W+) and minimum (W-) width of prementum. H: Kruskal-Wallis test. F: Anova. *p*: probability.

	Females		Males	
He	$F_{(17, 913)} = 15.34$	$p < 0.0001$	H = 184.91	$p = 0.0001$
ML	H = 453.60	$p = 0.0001$	H = 469.16	$p = 0.0001$
W-	H = 308.90	$p = 0.0001$	H = 347.11	$p = 0.0001$
W+	H = 369.56	$p = 0.0001$	H = 365.73	$p = 0.0001$

the differences between the streams studied. PC1 explains 77.7% of the variance for females and 77.9% for males. ML was the variable with larger loading in both females (0.70) and males (0.69) (Fig. 3). Henceforth, ML was used for population analysis and environmental variables.

In females, linear regression between ML and mean air temperature was positive (r = 0.54, $p < 0.0001$), while negative with latitude and altitude (r = -0.55, $p < 0.0001$ and r = -0.34, $p < 0.0001$, respectively). Similarly, in males, linear regression between ML and mean air temperature was positive (r = 0.47, $p < 0.0001$) while negative with altitude and latitude (r = -0.49, $p < 0.0001$; r = -0.16, $p < 0.0001$, respectively).

According to GLM, the model that best expressed ML differences in females was latitude × temperature, and the next best model was the one that included the variables latitude, temperature and altitude (Table 4). However, the best model in males was latitude × altitude, and the second-best model was the one that included the variables latitude, temperature and altitude (Table 5).

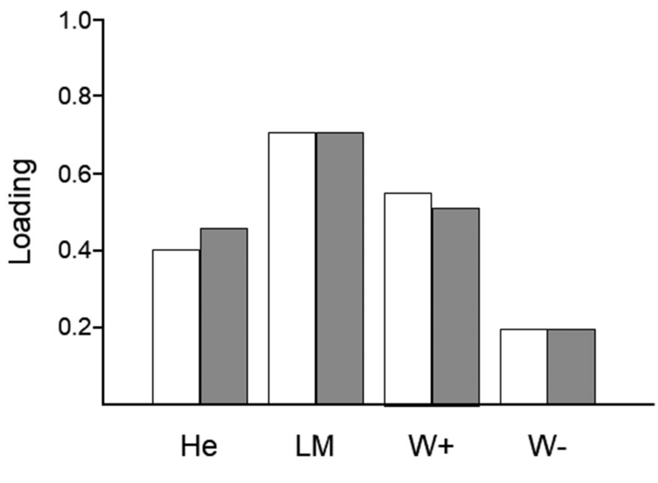

Figure 3. Loading values of the four variables analysed (head width (He), prementum lenght (ML), maximum (W+) and minimum (W-) width of prementum) obtained from male (shaded bars) and female (empty bars) exuviae of *Cordulegaster boltonii* collected at the Iberian Peninsula.

Table 4. GLM values in female exuviae of *Cordulegaster boltonii* collected from 18 Iberian streams. Lat: latitude. Alt: altitude as m a.s.l. Tm: mean annual temperature.

	AIC x N	Δ AIC	w_i
Lat	221.1867	97.8117	0.0000
Tm	233.4255	110.0505	0.0000
Alt	462.5082	339.1332	0.0000
Lat x Tm	123.3750	0.0000	0.4231
Lat x Alt	124.7568	1.3818	0.2120
Tm x Alt	167.9874	44.6124	0.0000
Lat x Alt x Tm	123.6711	0.2961	0.3649

Table 5. GLM values in male exuviae of *Cordulegaster boltonii* collected from 18 Iberian streams Lat: latitude. Alt: altitude as m above sea level. Tm: mean annual temperature.

	AIC	Δ AIC	w_i
Lat	118.8618	69.8600	0.0000
Tm	138.5224	89.5206	0.0000
Alt	296.6056	247.6038	0.0000
Lat x Tm	57.9838	8.9820	0.0072
Lat x Alt	49.0018	0.0000	0.6407
Tm x Alt	99.4008	50.3990	0.0000
Lat x Alt x Tm	50.1994	1.1976	0.3521

Discussion

Prementum length is a trait used in Odonata larvae and exuviae taxonomy (e.g., Conesa, 2021; Doucet, 2010; Tennessen, 2019), as well as populational analyses (Nunes et al., 2021). Present work confirms that it is also a valid trait to use in the analysis of variations between Iberian populations of *C. boltonii* using exuviae.

Our data agree with previous studies which found that female and male larvae of *C. boltonii* have different body sizes in final instar (Ferreras-Romero & Corbet, 1999), and that size varies with latitude (Casanueva et al., 2020; Johansson, 2003). In Iberian populations of *C. boltonii*, size variation in males relates more to latitude and altitude, while in females it relates more strongly to latitude and temperature. Altitude and temperature are two factors directly related to each other (Montgomery, 2006). Consequently, in both sexes, latitude seems to be the determining factor in the biometric differences found between the analyzed populations.

Several reasons have been proposed to explain the relationship of size variation with latitude. Bergmann's rule (Bergmann, 1847) states that endothermic animals inhabiting cooler climates will be larger than related species from warmer climates. The temperature-size rule proposes that with increasing temperature the development rate increases and, therefore, the body size of adult specimens may be smaller (Atkinson, 1994). Converse to Bergmann's rule, however, it has also been proposed that the size is smaller in cold climates than in warm climates (Mousseau, 1997).

The exuviae from Iberian southern populations of *C. boltonii* are larger than the northern populations. This fact suggests that *C. boltonii* conforms to Bergmann's inverse rule. The availability of resources has been invoked as a factor largely responsible for the size of animals (Atkinson & Sibly, 1997); the more resources there are, the larger their size will be. Since productivity and latitude are usually negatively correlated (Zeuss et al., 2017), it is possible that *C. boltonii* has adjusted its size to greater availability of resources in southern Iberia, where the temperature is higher than in central and northern Iberia (IGN, 2019).

In several species of odonates, Johansson (2003) found a U-shaped relationship between body size and temperature throughout their geographic distribution ranges. Apparently, the Iberian populations of *C. boltonii* do not fit this model, perhaps because a larger study area is needed, also covering central and northern Europe. Johansson (2003) suggested that in low latitude the length of the growth period is longer than in high latitude, and thus the larvae have more time for development and can reach a larger size. On the other hand, Hassal (2013) suggested that the relationship between latitude and body size can be explained by the photoperiod and temperature, in that the specimens that occupy southern latitudes grow faster and are smaller, while those occupying northern latitudes are larger. In cold areas, the odonates can increase body size by extending the duration of life cycle for several years, i.e., by partivoltinism (Zeuss et al., 2017).

In southern Spain, *C. boltonii* is a partivoltine species, although a small percentage of the larval population completes the life cycle in two years (semivoltinism) (Ferreras-Romero & Corbet, 1999). Life cycle duration of *C. boltonii* in central and northern Iberia is not known. It is probably longer than in the south, as could indirectly be deduced from two facts: a) in colder areas of Europe the larval period lasts up to 4 and 5 years (Donath, 1987; Schütte, 1997); and b) in *Boyeria irene*, a reophylous, large anisopteran that occupies the same habitat as *C. boltonii* (Santamaría et al., 2019), populations from central Iberia have a longer larval cycle than those from southern (Velasco et al., 2018). Because it is unknown whether *C. boltonii* follows this same strategy, the deductions of Hassall (2013) and Zeuss et al. (2017) should not be applied to it.

In the populations analyzed in this work, the second model that best explains the variation in size of each sex contains the following variables: temperature, latitude, and altitude (Tables 4 and 5). This suggests that none of these three variables exclusively explains the recorded geographic variation, and that other variables probably need to be considered as well.

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