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Regeneration interferes with fluctuating asymmetry analysis in odonate larvae

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ABSTRACT

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When an odonate larva loses a leg, it has the ability to regenerate it in the next moult. In this study, our goal was to test whether this ability affects fluctuating asymmetry analyses (FA) using *Calopteryx virgo* (Linnaeus, 1758) larvae. We observed that asymmetries in the femur and tibia caused by regeneration in field samples are not always detected as outliers, and therefore they are not automatically eliminated by statistical tests. However, they increased the value of the composite index of asymmetry by approximately 30 % with respect to the sample where all of the cases of regeneration were eliminated. Next, we wanted to test whether costs associated with leg regeneration had an effect on the development of other structures by increasing the level of fluctuating asymmetry in this species. The results confirmed that the value of the composite index of asymmetry calculated using antennal and mask measures was significantly higher for individuals that had a regenerating leg. Thus, prior knowledge of the biology and the physiology of the traits of the species under study should be an essential topic in fluctuating asymmetry studies to guarantee reliable results, as regeneration ability clearly interferes in fluctuating asymmetry analysis in odonate larvae.

Key words: Regeneration, outliers, Odonata, *Calopteryx virgo*, larva, leg segments.

RESUMEN

Los procesos de regeneración interfieren en los análisis de asimetría fluctuante en larvas de odonatos

Cuando una larva de odonato pierde una pata, ésta tiene la capacidad de regenerarla en la siguiente muda. En este trabajo queremos comprobar si esta capacidad interfiere en el análisis de la asimetría fluctuante (FA) en larvas de Calopteryx virgo *(Linnaeus, 1758). Se comprueba que las asimetrías de los fémures y tibias causadas por la regeneración en las muestras de campo no siempre son detectadas como "outliers", y por tanto no son desechadas en los análisis estadísticos automáticamente pero, incrementan el valor del índice compuesto de asimetría en casi un 30 % con respecto a la muestra en donde todos los casos de regeneración fueron eliminados. En segundo lugar, comprobamos si los costes debidos al crecimiento de la pata tras la amputación de la misma, pueden tener algún efecto en el desarrollo de otras estructuras incrementando el nivel de asimetría fluctuante en esta especie. Los resultados confirmaron que el valor del índice compuesto de asimetría, calculado usando las medidas de las antenas y de la máscara, fue significativamente mayor en aquellos individuos con patas regenerándose. Por ello, el buen conocimiento de la biología y fisiología de las estructuras de la especie bajo estudio debería ser un tema esencial en los trabajos de asimetría fluctuante para garantizar unos resultados fiables, puesto que la capacidad de regeneración claramente interfiere en los análisis de asimetría fluctuante en larvas de odonatos.*

Palabras clave: *Regeneración, "outliers", Odonata,* Calopteryx virgo*, larva, patas.*

INTRODUCTION

The development of the structures on the two sides of an organism with bilateral symmetry is presumably under the influence of identical genetic control, and because they are products of the same genome, they are expected to be identical. However, random accidents occurring during development can lead to deviations from perfect symmetry. Fluctuating asymmetry (FA) refers to population-level patterns of small random differences between left and right sides in bilateral structures of symmetrical organisms (Van Valen, 1962; Palmer, 1994). FA has been used as a measure of "general health" (e.g., Zakharov, 1992; Møller, 1997) because it is asymmetry that results from the inability of individuals to resist or buffer such developmental accidents (Van Valen, 1962).

FA may be caused by different factors, such as different types of genetic and environmental stress that affect developmental mechanisms (see Møller & Swaddle, 1997). Thus, individuals of low quality or that inhabit poor environments are expected to show larger differences between sides. Consequently, FA has become a subject of interest in fields as diverse as behavioural ecology, evolutionary biology, conservation biology and ecotoxicology (reviewed by Van Dongen, 2006). In fact, the simplicity in measuring FA has enabled a large number of studies to examine this variable. However, the subject remains controversial, mainly due to the inconsistency of results and weak and unclear stress-FA relationships. For example, some studies proposed the use of FA as an indicator of stress (Leary & Allendorf, 1989; Parsons, 1990; Clarke, 1993; De Block *et al.*, 2008). Others, however, failed to detect any relationship between FA and stress (Woods *et al.*, 1999; Hardersen, 2000; Hogg *et al.*, 2001).

Calopteryx virgo (Linnaeus, 1758) is an abundant zygopteran (Odonata) in Galicia (NW Spain) (Outomuro *et al.*, 2010) with larvae
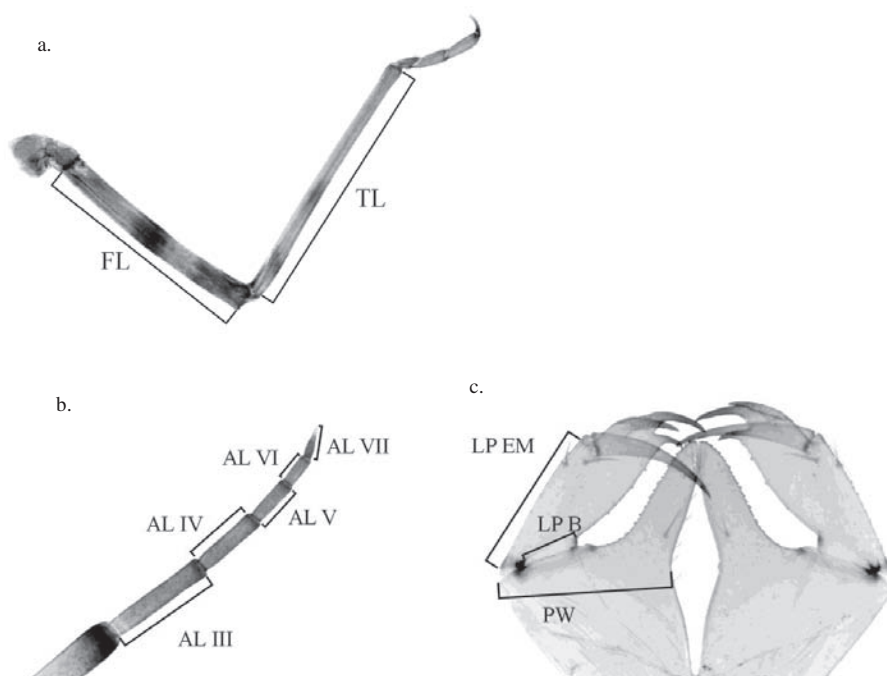
Figure 1.   (a) Landmarks used for measuring the length of the femur (FL) and tibia (TL). (b) Landmarks used for measuring the length of antennal traits. AL III, IV, V, VI, VII: antennal segments. (c) Landmarks used for measuring the length of mask traits. LP EM: external margin of the labial palps; LP B: base of the labial palps; PW: prementum width. *(a) Puntos de referencia usados para medir la longitud del Fémur (FL) y de la Tibia (TL). (b) Puntos de referencia usados para medir la longitud de los caracteres de las antenas. AL III, IV, V, VI, VII: segmentos de las antenas. (c) Puntos de referencia usados para medir la longitud de los caracteres de la máscara. LP EM; margen externa de los palpos labiales; LP B: base de los palpos labiales; PW: anchura del prementum.*

present in streams and rivers with clean, relatively rapid flowing water (Askew, 1988). Larval odonates are aggressive towards conspecifics and individuals of other species and frequently wound each other during these aggressive interactions. As a consequence they lose lamellae, legs, antennae or labia; the loss of legs and lamellae are the most common type of injury (Baker & Dixon, 1986). In addition, it is well known that odonate larvae show a remarkable ability to autotomise legs (Child & Young, 1903), with separation occurring at the trochanter-femur articulation. This process usually occurs when the larva has been grasped by a predator (Legrand, 1974). Interestingly, as in other Arthropods (Maruzzo *et al.*, 2005), when an odonate loses an appendage, it has the ability to regenerate it during successive moults (see Child & Young, 1903; Baker & Dixon, 1986), although the new leg is usually shorter than the original one. There are few studies addressing the regeneration of legs in odonate larvae (Child & Young, 1903; Parvin & Cook, 1968; Baker & Dixon, 1986), and no studies have used *C. virgo* as a study species. Furthermore, none of the studies of FA in odonate larvae that we have reviewed mention leg regeneration (Piscart *et al.*, 2006; Campero *et al.*, 2007; Chang *et al.*, 2007a,b); all of the reviewed studies attempt to relate FA and stress originated by chemicals. However, because differences between sides of symmetrical traits are often very small and sometimes of the same magnitude as measurement error, great care must be taken during measurement and analysis to maximise the likelihood of detecting FA differences among samples (Palmer, 1994). Thus, for example, extreme values must be eliminated, as they may be due to errors of measurement or they may also arise as a consequence of injury or trauma, and problems not related to those that might occur during the development of paired structures. As a consequence, the only method to eliminate cases of regeneration and avoid interference in FA analyses is statistical detection and the elimination of outliers.

Despite the obvious benefits of autotomy, as it allows the individual to avoid fatal situations, the regeneration of an appendage has several costs. These include costs related to performance and to the reallocation of resources (Maginnis, 2006), as resources needed for somatic growth or reproduction would be deviated to regrowth of the lost part (Uetz *et al.*, 1996; Stoks, 2001; Vilisics *et al.*, 2005).

Thus, in view of previous literature and assuming that the inspected individuals were regenerating cases, the aim of this study is twofold. First, we aim to understand the extent of leg asymmetries caused by regeneration and to test whether these asymmetries are detected as outliers by currently used statistical tests, as all these cases should be eliminated in FA studies because they are not related to developmental instability. Second, if regeneration of appendages implies a reallocation of resources for regrowth, we aim to test whether this cost might have an effect on the development of other structures by increasing the level of FA.

MATERIALS AND METHODS

Sampling and mounting

Larvae of intermediate instars of *C. virgo* (approximately instar F4-F6, estimated by the degree of development of their wing pads) were collected with a Surber net from the Con River (42°36′53″N; 08°43′14″W) in Galicia (NW Spain), in February and October 2006. This river is almost unaltered in its upper section, which was the sampling location, and drains into a granitic catchment, resulting in oligotrophic water (pH: 6.13, medium water temperature: 12.9 °C, conductivity: 53 μS/cm). All samples were fixed with 4 % formaldehyde and labelled before transport to the laboratory. Prementa were removed and placed in a warm 10 % potassium hydroxide solution for two hours to clear the muscle (Clarke *et al.*, 1995). Cleared prementa were then placed in 80 % alcohol. Antennae, legs and caudal gills needed no clearing. All appendages were mounted on microscope slides using a glycerine gel mounting medium (gelatine powder 10 g, glycerol 54 ml, distilled water 60 ml, crystal phenol 0.5 g).

Trait selection and measurement protocol

To study whether regenerated legs can be detected as outliers, we measured the length of the femur (FL 1, FL 2, FL 3) and the tibia (TL 1, TL 2, TL 3) of the three leg pairs (Fig. 1a) in 50 individuals. The femur and tibia are normally the most reduced structures when a leg is regenerating, but they are also the most commonly used in FA studies (Piscart *et al.*, 2006; Campero *et al.*, 2007; Chang *et al.*, 2007a, b). Legs where regeneration was obvious (strikingly small legs already detected during the mounting procedure that will clearly be eliminated in any FA study) were not used in the analysis (8 cases).

As previously mentioned, we are not aware of any study addressing leg regeneration in *C. virgo*. However, during visual inspection for landmark selection, regenerated legs always showed fewer segments than normal legs (usually one or two, in contrast with the three segments of normal legs), as described by Child & Young (1903) for *Zygoptera*. Thus, when sizes of regenerating legs are almost normal, the only way of distinguishing them is by observing the tarsus. To assess the possible effect of regeneration of the femur and/or tibia on developmental stability of the organisms and thus compare the levels of FA between individuals with regenerating legs (tarsus with one or two segments) and organisms with normal legs, we initially selected 19 metric traits located on different body parts (antennae, prementum, wing pads and cerci). However, after a preliminary visual analysis, we rejected several traits due to their fragility (antennal scape and pedicel) and the lack of well-defined landmarks (wing pads, cerci and tarsi). No meristic traits were considered to be suitable for FA analysis in *C. virgo* because most of the setae and protuberances were inconspicuous or very difficult to count.

The traits chosen were the length of the antennal segments (AL) III, IV, V, VI, VII, the width of the base of the labial palps (LP B), the external margin of the labial palps (LP EM) and the prementum width (PW). All had clear landmarks and measurements were straightforward (Fig. 1b, c).

Microscope slides were viewed and photographed using a Nikon Eclipse 80i microscope equipped with a digital camera (resolution: 12 total megapixels). The length of the traits was quantified using an image analysis system (Nikon EclipseNet 1.20 for Windows) (Barca-Bravo *et al.*, 2008). All measurements were taken twice from the same trait (Measure 1: M1, Measure 2: M2), each time in a random order, and all data were automatically sent to a database, thus the researcher had no knowledge of the first measurement when obtaining the second. All measurements were conducted by the same person to ensure the exclusion of effects caused by measurements taken by multiple researchers. All values are given in micrometres.

Statistical analysis

Statistical analysis was performed as recommended by Palmer (1994) and Palmer & Strobeck (1986, 2003). Thus, for the first part of this study, the detection of regenerating legs as outliers, we made a visual inspection of scatter plots of replicate measures (M1-M2) of the

Table 1. Data on femur (FL) and tibia (TL) measurements, including the number of individuals with regenerating legs and the number and percentage of pairs where one leg was regenerating that were detected as outliers. *Datos de las medidas del fémur (FL) y de la tibia (TL), incluyendo el número de individuos con patas regenerándose y el porcentaje de patas que se estaban regenerando y fueron detectadas como "outliers".*

Structure	N	N without aberrant measures	Regenerating legs	Regenerating legs detected as outliers	% detected
FL 1	34	32	8	1	12.500
FL 2	33	32	5	2	40.000
FL 3	40	40	7	3	42.860
TL 1	37	36	9	7	77.770
TL 2	33	33	5	1	20.000
TL 3	42	40	7	3	42.860

femurs and tibias to detect deviant values due to problems during measurement. If one or more values were suspected to be extreme we applied Grubb's test as recommended by Palmer & Strobeck (2003) to reduce subjectivity. The deviation of a potential outlier is expressed as Grubb's test statistic (t_G), which indicates deviation of the observed value from the sample mean, expressed as a proportion of the sample standard deviation. The values of Grubb's test sta-
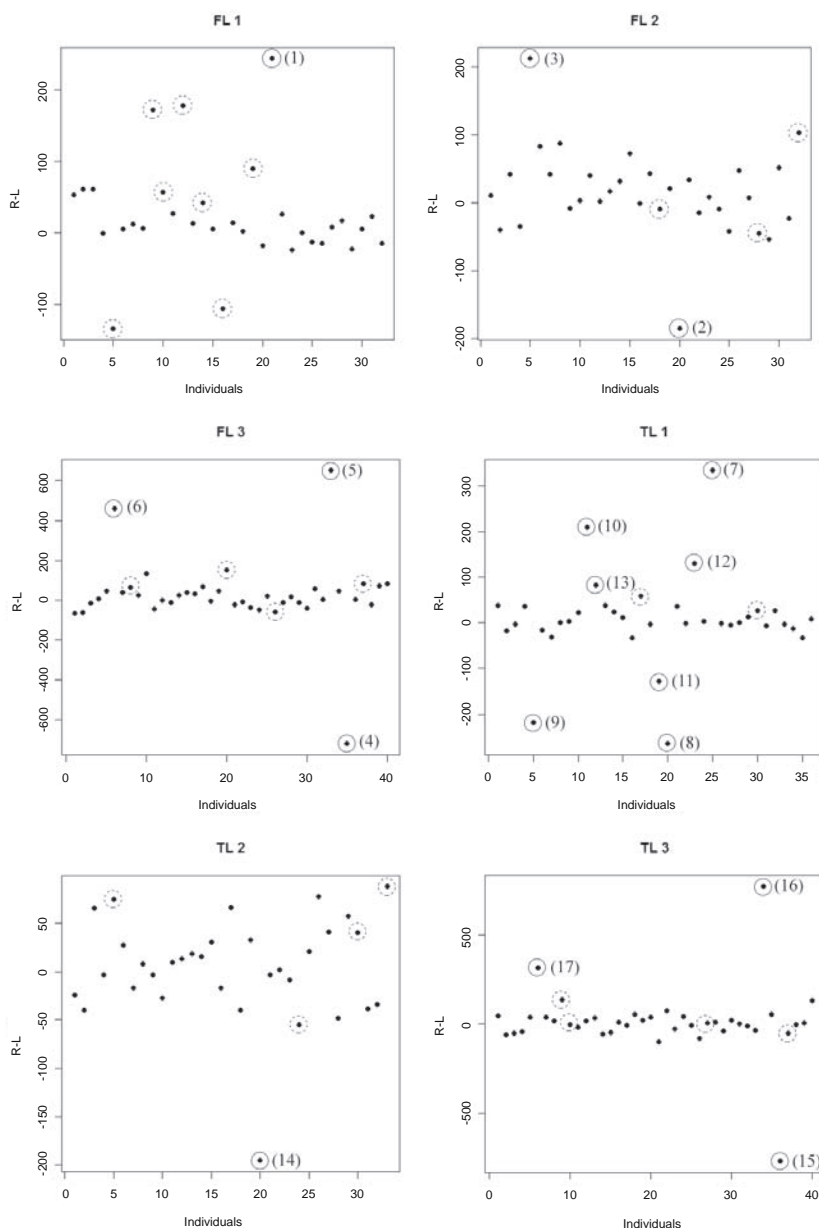
Figure 2. Scatter plots of the differences between sides (R-L = Right-Left) of the femur and tibia of the three pairs of legs. Points into full circles are detected outliers using Grubb's test. Points into dashed circles are cases of regeneration that were not detected as outliers. Labels of full circles correspond to those of Table 2. *Diagramas de dispersión de las diferencias entre lados (R-L = Derecho-Izquierdo) del fémur y de la tibia de los tres pares de patas. Los puntos rodeados por círculos completos son detectados como "outliers" usando el test de Grubb. Los puntos dentro de círculos incompletos son casos de regeneración que no fueron detectados como "outliers". Las etiquetas de los círculos completos corresponden a los presentados en la Tabla 2.*

tistic were then compared against tabled critical values (Sokal & Rohlf, 1995). Once aberrant measures were corrected or deleted, we attempted to identify outliers of the differences between the two sides of the body (R-L), which may be the result of an injury or trauma, by visual inspection of scatter plots and, as in the previous step, we applied Grubb's test. Statistics and graphics were computed with the Outliers package (Komsta, 2006) for R software, version 2.9.2 (R Development Core Team 2009).

As tests failed to detect several regenerating legs as outliers (see the Results section), exploratory analyses recommended to check whether a certain trait shows ideal FA (Palmer, 1994; Palmer & Strobeck, 1986, 2003) might also reveal distortions due to their presence. We performed these tests using two different data sets: one set retaining regenerating legs not detected as outliers (RegLegs in Table 3) and the same set but without the outlier values (eliminated after visual inspection of tarsi, NotRegLegs in Table 3). Thus, we first assessed measurement error (ME), which can inflate the between-sides variance and mask the variance due to FA (two-way ANOVA, sides x individuals). Moreover, it was also necessary to ensure that FA, and not other types of asymmetry, was present in the selected traits, as well as discard any dependence of FA on the trait size (Palmer & Strobeck, 1986). We also checked for correlations between FA values of the leg segments; if there is a developmental connection, then the effects of perturbations can be transmitted directly between traits, producing a statistical relationship between the directions of the trait asymmetries (Klingenberg, 2003). Detailed explanations of the procedures followed in the present study can be found in Servia *et al.* (2004a, b). Values were considered statistically significant at the 5 % level. When using multiple analyses for testing the same hypothesis sequential Bonferroni correction was used to adjust the observed probability values (Rice, 1989). The comparison of FA levels between the two groups of larvae was conducted using a composite index of asymmetry for all of the traits (CFA2, Leung *et al.*, 2000). Statistical analyses were performed using SPSS for Windows version 16.0.

Table 2. Results of Grubb's test applied to 17 measurements suspected to be outliers in Figure 3. All were confirmed as outliers. *Resultados del test de Grubb para "outliers" aplicado a 17 medidas dudosas en la Figura 3. Todas fueron confirmadas como outliers.*

Possible outlier		Test vs. mean	
Label	(R-L) (μm)	t_G (mean)	$P_{2\text{-tail}}$
(1)	243.42	3.060	0.014
(2)	−185.45	3.131	0.010
(3)	212.57	3.554	0.000
(4)	−720.35	4.188	0.000
(5)	650.97	4.590	0.000
(6)	459.83	4.909	0.000
(7)	334.75	3.365	0.004
(8)	−264.86	3.313	0.005
(9)	−218.36	3.411	0.003
(10)	209.67	3.168	0.001
(11)	−127.46	3.281	0.005
(12)	130.14	3.451	0.002
(13)	82.89	2.779	0.044
(14)	−195.56	3.772	0.000
(15)	−769.72	4.165	0.000
(16)	769.72	5.256	0.000
(17)	316.7	4.239	0.000

For the second part of the study, i.e., to test whether the costs of leg regeneration are linked to impaired development of other body structures, the analysis of FA was performed following the steps mentioned above using antennal and mask traits.

RESULTS

Concerning the detection of regenerating legs as outliers, scatter plots of the replicate measurements (M1-M2) revealed six aberrant measures that were further confirmed as outliers with Grubb's test (Table 1). These values were deleted because the legs were not well mounted and lacked well defined landmarks. Scatter plots of the differences between sides (R-L) also showed several extreme values (Fig. 2, points into full circles) that were confirmed as outliers with Grubb's test (Table 2). All outliers corresponded to pairs of legs with one regenerating leg. However, after checking the tarsi of all of the individuals, we confirmed that not all pairs with regenerating legs were detected as outliers (see Table 1 and Fig. 2, points into dashed circles);

Table 3. Descriptive statistics for the data sets, one including cases of regeneration not detected as outliers (RegLegs) and the other excluding these cases after visual inspection of tarsi (NotRegLegs). See Materials and Methods section for further details. (R + L)/2: trait size (μm). Slope: slope of the regression of |R-L| on (R + L)/2. (R-L): right-left. MSm: mean square of the measurement error (*p < 0.05). *Estadística descriptiva para los grupos de datos, uno incluyendo los casos de regeneración no detectados como "outliers" (RegLegs) y otro excluyendo eses datos después de la inspección visual de los tarsos (NotRegLegs). Ver Material y métodos para más detalles. (R + L)/2: tamaño del carácter (μm). Slope: pendiente de la regresión de |R-L| versus (R + L)/2. (R-L): derecho-izquierdo. MSm: media cuadrática del error de medida. (*p < 0.05).*

Group	Trait	n	(R + L)/2		(R − L)			MSm
			Mean ± SE	Slope (SE; Prob)	Mean ± SE	Skew ± SE	Kurtosis ± SE	
	FL 1	31	2959.471 ± 124.304	−0.005 (0.013; 0.699)	16.833 ± 10.865	0.478 ± 0.421	2.909 ± 0.821	174.941
RegLegs	FL 2	30	3631.964 ± 188.630	−0.002 (0.005; 0.647)	15.758 ± 7.479	0.272 ± 0.427	−0.495 ± 0.833	216.864
	FL 3	37	4253.126 ± 180.803	−0.003 (0.005; 0.519)	16.134 ± 8.441	0.597 ± 0.388	0.266 ± 0.759	190.157
	TL 1	29	3664.446 ± 177.248	0.004 (0.003; 0.267)	6.894 ± 4.254	0.231 ± 0.434	−0.284 ± 0.845	162.306
	TL 2	32	4170.330 ± 196.607	0.004 (0.004; 0.255)	10.270 ± 6.993	0.269 ± 0.414	−0.804 ± 0.809	251.792
	TL 3	37	4594.648 ± 183.959	−0.002 (0.005; 0.700)	4.746 ± 8.455	0.506 ± 0.388	0.780 ± 0.759	232.847
	FL 1	24	2997.732 ± 153.304	0.008 (0.005; 0.089)	9.362 ± 4.863	0.813 ± 0.472	0.412 ± 0.918	110.134
NotRegLegs	FL 2	27	3659.009 ± 198.055	0.001 (0.005; 0.880)	17.535 ± 7.234	0.061 ± 0.456	−0.546 ± 0.887	173.141
	FL 3	33	4345.004 ± 190.746	0.000 (0.005; 0.933)	10.738 ± 7.756	0.496 ± 0.409	0.365 ± 0.798	174.863
	TL 1	27	3644.135 ± 189.583	0.002 (0.003; 0.416)	6.433 ± 3.861	0.046 ± 0.448	−0.530 ± 0.872	133.755
	TL 2	28	4191.157 ± 211.329	0.008 (0.004; 0.041)	7.442 ± 6.657	0.305 ± 0.448	−0.576 ± 0.872	238.687
	TL 3	33	4671.704 ± 195.124	0.004 (0.005; 0.448)	0.094 ± 8.076	0.261 ± 0.409	0.949 ± 0.798	227.127

several showed a more advanced regeneration stage that made them similar in size to the normal leg.

Exploratory analyses recommended for checking whether a certain trait shows ideal FA showed no distortions caused by regenerating legs. Thus, both data sets used, one that retained regenerating legs not detected as outliers (RegLegs in tables and figures) and the same set but without the regenerating legs values (NotRegLegs), showed that the ME was always smaller than non-directional asymmetry in all of the traits (Table 3, MSm), thus, we considered the measurements to be reliable. The two-way ANOVA used to test for ME provided no evidence of directional asymmetry, and the assumption of normality was satisfied in all of the traits (no presence of significant platykurtosis (Table 3, R-L), thus there was no evidence of antisymmetry in any trait. Therefore, we can assume that all traits in the two data sets present ideal FA (Table 3).

We did not find any correlation between trait size and FA (see Table 3, (R + L)/2), thus it was unnecessary to correct trait FA for trait size in subsequent analyses (Palmer, 1994). Concerning between-trait correlation for FA values of the different traits, no significant values were found

Table 4. Correlation between FA values of the leg segments. Data sets as in Table 3. FL 1, FL 2, FL 3: femurs of the three pairs of legs; TL 1, TL 2, TL 3: tibias of the three pairs of legs (no significant values were detected). *Correlación entre los valores de FA de los segmentos de las patas. Grupos de datos igual que en la Tabla 3. FL 1, FL 2, FL 3: fémur de los tres pares de patas; TL 1, TL 2, TL 3: tibia de los tres pares de patas. (No se detectaron valores significativos).*

| Trait	Correlat. coeff.	Correlat. coeff.
	RegLegs	NotRegLegs
FL 1-FL 2	−0.183	0.018
FL 1-FL 3	−0.100	0.418
FL 1-TP 1	0.221	0.125
FL 1-TP 2	0.468	0.496
FL 1-TP 3	−0.122	0.222
FL 2-FL 3	0.192	−0.043
FL 2-TP 1	−0.033	0.113
FL 2-TP 2	0.336	0.165
FL 2-TP 3	0.221	−0.082
FL 3-TP 1	0.201	0.189
FL 3-TP 2	−0.034	0.232
FL 3-TP 3	0.368	0.319
TP 1-TP 2	−0.037	−0.268
TP 1-TP 3	−0.059	0.000
TP 2-TP 3	−0.066	−0.290

(Table 4). This implies that the selected traits develop independently during ontogeny and can be used for FA analyses (Palmer, 1994).
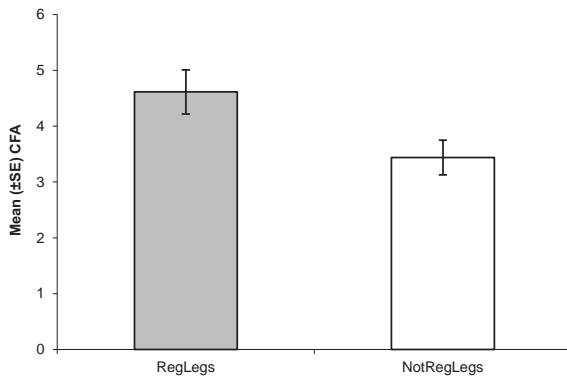
Figure 3. Mean (± SE) composite index of fluctuating asymmetry (CFA) for legs in the sample that include cases of regeneration not detected as outliers by Grubb's test (RegLegs) and the sample where all of the cases of regeneration were eliminated (NotRegLegs). *Media del índice compuesto (± SE) de asimetría fluctuante (CFA) para las patas en las muestras que se incluyen casos de regeneración no detectados como "outliers" por el test de Grubb (RegLeg) y la muestra en donde todos los casos de regeneración fueron eliminados (NotRegLegs).*

As all of the exploratory tests indicated the existence of ideal FA in both data sets, we calculated the value of the CFA index of asymmetry for both. Remarkably, even if some between-sides differences of pairs where one leg was regenerating were close to zero, those cases increased the value of the index by approximately 30 % compared with the sample where all of the cases of regeneration were eliminated (Fig. 3).

Concerning the possible relationship between leg regeneration and FA levels of antennal and mask traits, exploratory tests allowed us to assume that all traits presented ideal FA (Table 5). Thus, we observed that the ME was always smaller than non-directional asymmetry in all of the traits (Table 5, MSm), indicating that measurements were reliable. The two-way ANOVA used to test for ME provided no evidence of directional asymmetry, and the assumption of normality was satisfied in all of the traits. We did not find any correlation between trait size and FA (see Table 5, $(R + L)/2$). Concerning between-trait correlation for FA values of the different traits, significant values were found for three cases (Table 6), but the inconsistency of this result within traits suggests a type I error. Thus, we consider that the selected traits develop
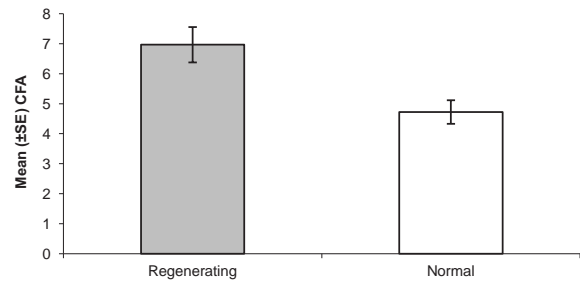
Figure 4. Mean (± SE) composite index of fluctuating asymmetry (CFA) using antennal and mask traits for a sample of individuals with regenerating legs and a sample of individuals with normal legs. *Media del índice compuesto (± SE) de asimetría fluctuante (CFA) usando los caracteres de las antenas y la máscara para una muestra de individuos con patas regenerándose y una muestra de individuos con patas normales*

independently during ontogeny and can be used for FA analyses (Palmer, 1994).

The CFA index showed significant differences between the two groups (Fig. 4), with the group of individuals with one or more regenerating legs showing higher levels of FA than the group with normal legs (t-test, $t = 3.164$, df = 39, $p = 0.002$).

DISCUSSION

Studies of FA in odonates are focused on adults and in most cases they measure the relationship between wing asymmetry and male mating success, as well as the effect of larval exposures to pollutants in the final wing morphology in adults (Carchini *et al.*, 2000; Hardersen, 2000). Contrastingly, studies in larvae are scarce, and none of the studies, to the best of our knowledge, make any mention of problems of leg regeneration when measuring FA using leg segments (Piscart *et al.*, 2006; Campero *et al.*, 2007; Chang *et al.*, 2007a, b). Indeed, only Stoks *et al.* (1999) and Stoks (2001) have addressed the consequences of lamella autotomy, but studies on odonate leg regeneration are strikingly scarce (note that we have only found four papers for the 20th century: Child & Young (1903), Parvin & Cook (1968), Baker & Dixon (1986), and McPeek (1990)).

Outliers may be caused by physical harm or severe deformities and are not related to FA (Palmer, 1994; Uetz *et al.*, 1996; Vilisics *et al.*,

2005). Moreover, they disrupt the distributions of the data and, for example, tests of normality can be influenced by a single outlier (Hardersen & Wratten, 1998; David *et al.*, 1998; Hardersen & Framptom, 1999; Pélabon *et al.*, 2006). However, despite the problems outliers pose, they are often not assessed in FA studies (e.g., Forbes *et al.*, 1997; Carchini *et al.*, 2000; Ribeiro *et al.*, 2007). In this study, data on tibia and femur length were screened for outliers using statistical tests. However, due to the ability of odonates to replace lost appendages in succeeding moults, pairs of legs that showed advanced regeneration could not be detected as outliers. Outstandingly, these values were indistinguishable within the data set, and all of the exploratory tests indicated that both samples, the one including regeneration cases and the one where all of these cases were eliminated after visual inspection, showed ideal FA. Therefore, no cue on the presence of these abnormal data was obtained using all recommended tests for FA analysis. However, these R-L differences are

not caused by the developmental instability but by injury, resulting in unreliable FA levels, and they clearly increased overall FA values (see also a preliminary study on the terrestrial isopod *Trachelipus rathkii* by Vilisics *et al.*, 2005). Thus, when using odonate legs in FA studies, previous visual inspection of tarsi is compulsory to avoid including asymmetries not related to developmental instability but to a single damage event. Researchers studying FA in organisms with regeneration abilities should be aware of this problem, as inclusion of regenerating traits when comparing FA among samples may lead to spurious results.

Additionally, many invertebrate taxa show the ability to autotomise appendages (e.g., legs, tails, caudal lamellae, etc.) in the escape from predators (e.g., Punzo, 1997; Stoks & De Block, 2000; Brueseke *et al.*, 2001) or during intraspecific interactions (Baker & Dixon, 1986; Robinson *et al.*, 1991; Stoks, 1998). During the regeneration of damaged or lost structures, the energy and materials required cannot be used for other proces-

Table 5. Descriptive statistics for the data sets, one set for individuals with one or more regenerating legs (Regenerating) and the other for normal individuals (Normal). (R + L)/2: trait size (µm). Slope: slope of the regression of |R-L| on (R + L)/2. (R-L): right-left. MSm: mean square of the measurement error (no significant values were detected). *Estadística descriptiva de los grupos de datos, un grupo serían los individuos con una o más patas regenerándose (Regenerating) y otro grupo serían los individuos normales (Normal). (R + L)/2: tamaño del carácter (µm). Slope: pendiente de la regresión de |R-L| versus (R + L)/2. (R-L): derecho-izquierdo. MSm: media cuadrática del error de medida. (No se detectaron valores significativos).*

Group	Trait	n	(R + L)/2		(R − L)			MSm
			Mean ± SE	Slope (SE; Prob)	Mean ± SE	Skew ± SE	Kurtosis ± SE	
	AL III	16	409.040 ± 33.619	0.021 (0.011;0.069)	2.141 ± 2.815	0.367 ± 0.524	−0.767 ± 1.014	6.781
Regenerating	AL IV	22	255.537 ± 11.822	0.025 (0.028; 0.387)	−3.587 ± 2.627	−0.021 ± 0.491	−0.337 ± 0.953	5.002
	AL V	22	165.365 ± 5.124	0.063 (0.057; 0.278)	0.075 ± 2.064	0.564 ± 0.491	0.586 ± 0.953	3.814
	AL VI	20	120.652 ± 4.052	0.075 (0.051; 0.155)	−1.513 ± 1.564	−0.067 ± 0.512	−0.248 ± 0.992	3.790
	AL VII	21	146.461 ± 3.041	−0.033 (0.039; 0.406)	0.13 ± 1.028	0.598 ± 0.501	−0.891 ± 0.972	3.799
	LP EM	17	939.648 ± 54.081	0.002 (0.012; 0.883)	−5.01 ± 3.361	−1.136 ± 0.550	1.247 ± 1.063	35.984
	LP B	22	335.841 ± 18.400	0.022 (0.020; 0.286)	−1.039 ± 2.522	−0.118 ± 0.491	0.712 ± 0.953	52.060
	PW	22	1005.625 ± 49.271	−0.010 (0.006; 0.108)	2.662 ± 1.900	0.394 ± 0.491	0.002 ± 0.953	41.770
	AL III	24	316.823 ± 33.494	0.014 (0.007; 0.072)	−0.398 ± 2.215	−0.084 ± 0.472	−0.556 ± 0.918	8.060
Normal	AL IV	23	228.272 ± 14.613	0.022 (0.017; 0.227)	−1.5 ± 1.939	−0.593 ± 0.481	−0.413 ± 0.935	5.562
	AL V	25	158.702 ± 6.340	−0.002 (0.026; 0.955)	1.760 ± 1.572	−0.351 ± 0.464	−0.972 ± 0.902	5.701
	AL VI	25	115.031 ± 3.695	0.004 (0.047; 0.931)	−2.331 ± 1.245	−0.178 ± 0.464	−0.405 ± 0.902	4.294
	AL VII	23	144.699 ± 3.810	0.006 (0.044; 0.893)	1.821 ± 1.174	0.395 ± 0.481	−0.675 ± 0.935	4.035
	LP EM	17	853.764 ± 69.702	−0.002 (0.008; 0.846)	2.346 ± 3.798	−1.02 ± 0.550	1.383 ± 1.063	33.701
	LP B	21	310.218 ± 20.315	0.030 (0.019; 0.128)	−8.095 ± 2.403	0.343 ± 0.501	−0.057 ± 0.972	52.926
	PW	20	930.662 ± 58.459	0.010 (0.006; 0.118)	4.50 ± 2.883	−0.310 ± 0.512	−0.752 ± 0.992	23.870

Table 6. Correlation between FA values of the selected traits. Data sets as in Table 5. AL III, IV, V, VI, VII: antennal segments; LP B: base of the labial palps; LP EM: external margin of the labial palps; PW: prementum width (*$p < 0.05$). *Correlación entre los valores de FA de los caracteres seleccionados. Grupo de datos igual que en la Tabla 5. AL III, IV, V, VI, VII: segmentos de las antenas; LP B: base de los palpos labiales; LP EM: margen externa de los palpos labiales; PW: anchura del prementum. (*p < 0.05).*

Trait	Correlat. coeff. Regenerating	Correlat. coeff. Normal
AL III-AL IV	0.367	0.284
AL III-AL V	0.002	−0.368
AL III-AL VI	0.066	0.033
AL III-AL VII	−0.282	0.125
AL III-LP EM	0.585	0.205
AL III-LP B	0.073	0.266
AL III-PW	0.085	0.047
AL IV-AL V	0.112	−0.005
AL IV-AL VI	0.384	0.266
AL IV-AL VII	−0.329	0.585*
AL IV-LP EM	0.064	0.197
AL IV-LP B	0.313	0.064
AL IV-PW	−0.100	0.231
AL V-AL VI	0.165	−0.137
AL V-AL VII	−0.227	0.076
AL V-LP EM	−0.026	−0.285
AL V-LP B	0.030	−0.326
AL V-PW	0.102	−0.214
AL VI-AL VII	−0.131	0.040
AL VI-LP EM	−0.142	0.386
AL VI-LP B	0.522*	0.255
AL VI-PW	−0.119	−0.245
AL VII-LP EM	0.110	−0.263
AL VII-LP B	−0.039	−0.107
AL VII-PW	0.118	−0.276
LP EM-LP B	0.362	−0.110
LP EM-PW	−0.115	−0.028
LP B-PW	−0.539*	0.036

ses, such as growth and/or reproduction (Juanes & Smith, 1995; Ramsay *et al.*, 2001). For example, lamella autotomy reduces the mass of *Lestes sponsa* (Odonata: Zygoptera) adults by approximately 10 % in field (Stoks *et al.*, 1999) and laboratory conditions (Stoks, 2001). Moreover, lamellae autotomy contributes to reduced growth rate, which results in increased hind wing asymmetry in adults (Stoks, 2001). Similarly, autotomy of either one or two hind legs in the last instar of *Schistocerca americana* (Orthoptera) nymphs resulted in abnormal wing formation (Ortego & Bowers, 1996).

Thereby, although the benefits of autotomy and regeneration are obvious and could be considered adaptive in nature, they apparently show an important trade-off due to different costs, such as the increase of FA levels as found in our study. As a correlative but not causal relationship was established in this work, we cannot eliminate the possibility that individuals with greater FA, and therefore poor quality, were more susceptible to limb loss (and hence regeneration). However, despite the numerous studies on the fitness consequences of FA (see meta-analyses by Leung & Forbes, 1996 or Beasley *et al.*, 2013), we were unable to find evidence to support this hypothesis in previous references. In contrast, Maginnis (2006) and Bely & Nyberg (2010) reviewed the costs of autotomy and present evidence of regeneration costs related to the allocation of resources. Experiments designed to establish causality between regeneration and the increase in FA would help to elucidate this question.

Thus, strong prior knowledge of the biology and physiology of the traits of the study species should be an essential topic in FA studies to guarantee reliable results, as regeneration ability clearly interferes in FA analysis in odonate larvae. Indeed, evidence exists that trait regeneration during the larval stage can affect adult morphology (Ortego & Bowers, 1996; Stoks, 2001), and further research is needed to understand the extent of these effects to assure adequate trait selection for FA analysis.

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