

# Ethological uniqueness of a damselfly with no near relatives: the relevance of behaviour as part of biodiversity

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## Abstract

*Ethological uniqueness of a damselfly with no near relatives: the relevance of behaviour as part of biodiversity.* Taxonomically isolated species may contribute unique characters to biological diversity, particularly at the level of ethodiversity. To test this idea, we analysed the territorial and reproductive behaviour of *Pseudolestes mirabilis* (Zygoptera, Pseudolestidae), an endemic damselfly from Hainan island, China, and the only representative of its family. Our hypothesis was that the uniqueness of this taxon would be evident in its behaviour. We found that the agonistic encounters between males were usually very short (less than 2 min) and consisted of a face-to-face display with both males maintaining a close distance while flying using only the forewings. No other odonate flies with only two wings in territorial contests. Furthermore, a small proportion of fights were escalated and lasted about one hour, with clear exhibition of the coloured hindwings. Males also confronted wasps (*Eustenogaster nigra*) that used the same microhabitat in a similar way, albeit for short time. Females were found in low numbers. This limited copulatory frequency and most males did not mate in the whole day. Unexpectedly for a damselfly with coloured wings, precopulatory courtship was almost absent, suggesting that intrasexual selection is behind the evolution of coloured wings in this species. Copulation lasted an average of seven minutes, with a first stage of rivals' sperm removal (64% of sperm removed) and a second stage of insemination. In agreement with our initial hypothesis, copulatory behaviour was unique: males did not translocate sperm to their vesicle before each mating but translocated sperm after copulation, a behaviour that cannot be easily explained. These exclusive characteristics point to the relevance of this species as an exceptional taxon that merits high conservation priority.

Key words: Ethodiversity, Odonata, China, *Pseudolestes mirabilis*, Sexual selection, Sperm competition

## Resumen

*Singularidad etológica de una damisela sin parientes cercanos: la importancia del comportamiento como parte de la biodiversidad.* Las especies taxonómicamente aisladas pueden aportar caracteres únicos a la diversidad biológica, particularmente en el ámbito de la diversidad etológica. Para comprobar esta idea, analizamos el comportamiento territorial y reproductor de *Pseudolestes mirabilis* (Zygoptera, Pseudolestidae), una damisela endémica de la isla de Hainan, en China, y el único representante de su familia. Nuestra hipótesis era que la singularidad de este taxón sería evidente en su comportamiento. Encontramos que los combates agonísticos entre machos fueron generalmente muy cortos (menos de 2 min) y consistieron en un vuelo de presentación cara a cara a una distancia cercana usando únicamente las alas anteriores. Ningún otro odonato vuela solo con dos alas en luchas territoriales. Además, una pequeña proporción de las peleas se intensificó y duró alrededor de una hora; en estas peleas los machos mostraron de forma evidente sus alas posteriores coloreadas. Los machos también se enfrentaron de manera similar, aunque durante poco tiempo, a las avispa (*Eustenogaster nigra*) que utilizaron el mismo microhábitat. El hecho de que se encontraran pocas hembras limitó la frecuencia de cópula, lo que determinó que la mayoría de los machos no se apareara durante todo el día. A diferencia de lo que cabría esperar para una libélula con alas coloreadas, el cortejo anterior a la cópula fue prácticamente inexistente, lo que sugiere que la selección intrasexual está detrás de la evolución de las alas coloreadas en esta especie. La cópula duró un promedio de siete minutos y estuvo compuesta por una primera etapa de eliminación del esperma de los rivales (64% de esperma retirado) y una segunda

etapa de inseminación. De acuerdo con nuestra hipótesis inicial, el comportamiento de cópula fue único: los machos no transfirieron el esperma a su vesícula antes de cada apareamiento, sino que lo hicieron después de la cópula, un comportamiento que no se puede explicar fácilmente. Estas características exclusivas apuntan a la importancia de esta especie como taxón excepcional que merece una alta prioridad de conservación.

Palabras clave: Diversidad etológica, Odonata, China, *Pseudolestes mirabilis*, Selección sexual, Competencia espermática

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## Introduction

There is an emerging consensus in the scientific community that characterises our time as an era of fast human-driven extinctions (Levinton, 2001). Many species are becoming rare and others have already disappeared, and this is a general process, affecting vertebrates (e.g. Galán Regalado, 2015) and invertebrates (e.g. Gibbs, 1998), and terrestrial (Ceballos and Ehrlich, 2002) and marine ecosystems (Roberts, 2003), generating a truly irreversible change (Dirzo and Raven, 2003). The word 'biodiversity' has been instrumental in promoting species conservation efforts (Takacs, 1996). However, much biodiversity is currently not evaluated in conservation programs, because it is related to levels of organic complexity which usually escape monitoring. Among these neglected levels of biodiversity, ethodiversity is prominent (Cordero-Rivera, 2017). Some behaviours vanish before being scientifically studied (Caro and Sherman, 2012), although species may persist. Furthermore, when species with unique behaviours are lost, these elements of biodiversity disappear forever. Therefore, documenting species-specific, population-specific and individual-specific behaviours is a priority to fully embrace biodiversity conservation (Cordero-Rivera, 2017).

Freshwater systems are among the most threatened ecosystems in the world due to overexploitation (water extraction), pollution, and, in the case of forest streams, intensification of forestry, with substitution of forests by plantations of exotic trees (Cordero-Rivera et al., 2017) or their transformation to agricultural systems (Revenga, et al., 2005). Mitigation of these negative effects is of primary importance, particularly because freshwater systems are home to 6% of world species, even though they represent only 0.8% of the world surface (Dudgeon et al., 2006). Odonates are a key taxon in the ecological networks of small forested streams, where they may dominate the food webs (e.g. Yule, 1996). They are sensitive to changes in land use, and to the loss of forests (Cordero-Rivera, 2006).

The behaviour and ecology of odonates is well studied, particularly in temperate regions, and for this reason this order has been the focus of much research (Corbet, 1999; Córdoba-Aguilar, 2008). Odonates have become text-book examples of sexual selection and sperm competition since the discovery of the dual function of male genital ligula as a device for rivals' sperm removal and transfer (Waage, 1979). Nevertheless, the diversity of reproductive strategies is understudied, and we lack information on the basic ethology of many taxa, especially those confined to tropical forests (Córdoba-Aguilar and Cordero-Rivera, 2008). Among these understudied taxa, the Hainan endemic *Pseudolestes mirabilis* Kirby, a species so unique that its taxonomic position has been the centre of much controversy (Yu and Bu, 2011), is now proposed as a monotypic family (Dijkstra et al., 2014). It shows several morphological and ethological particularities (Reels, 2008). This species is restricted to small forested streams and has its habitat threatened by human activities (Zhu et al., 2015; Xue et al., 2017). The larva, with unique characters such as abdominal gills, is found in moderate numbers

in these streams (Yu and Bu, 2011). This species has expanded since the last glacial period, but it shows signatures of slight decline in the last centuries, which might be exacerbated by current land use changes (Xue et al., 2017). Our goal was therefore to test the hypothesis that taxonomically isolated species show peculiar and unique behaviours, and are therefore of special interest at the level of ethodiversity (Cordero-Rivera, 2017). We concentrated on the study of territorial and reproductive behaviour, using marked animals in forested streams.

## Methods

### Field sites

Field work was done at two localities in Hainan island (China) in May–June 2014 (for further details see Garrison et al., 2015). First, we completed exploratory surveys to become familiar with the species and locate suitable streams for detailed behavioural observations. Between 27 and 30 May we sampled several streams at Diaoluoshan Forest Reserve, in Lingshui county (coordinates 18.727933°N, 109.880182°E, 900 m altitude). A second period of preliminary sampling was completed between 31 May and 1 June at Shuinan village, Wuzhishan (18.894678°N, 109.672557°E, 700 m). This second locality was found to have high density of *P. mirabilis* (in agreement with Xue et al., 2017) and therefore we selected a stream of about 1 m in width and 0.1–0.5 m in depth, which was visited daily between 13 and 23 June.

### Field methods

We studied a sector of 275 m of the stream (of which 70 m were dry) running thru secondary forest and tea plantations, and visited the site to mark and observe the animals, for a mean of  $6.23 \pm 0.58$  hours ( $\pm$  SE), and a total of 68.5 hours. Observations started at around 9:00 h and ended at 17:00 h, except when rainy conditions (common after 13:00 h) shortened field-work. From June 13 to June 15, we concentrated on behavioural observations. From June 16 to June 23, we marked all males found in the stream to estimate movements and survival.

On 13 June we installed a datalogger to record air and water temperature every 5 minutes (Gemini Dataloggers, UK). The logger was situated in a small tree at the shore of the stream, about 1 m from the water surface, and a probe was placed in the stream. The logger was covered with leaves to avoid direct sun exposure. Water temperature showed very small changes, with a mean of  $22.3 \pm 0.005^\circ\text{C}$  ( $N = 2,939$  readings; range 21.68–23.54). Air temperature showed repetitive daily changes over the study period, starting at about  $25^\circ\text{C}$  at 9:00 h, with a maximum of  $28\text{--}29^\circ\text{C}$  at 15:00 h, and a mean of  $23.8 \pm 0.037^\circ\text{C}$  (range 20.45–29.65).

From 16 to 23 June, we captured and marked all the males found on the stream, writing a number on their wing using a permanent marker (Faber–Castell Multimark, 1523) (see fig. 3A). They were then released immediately in the same spot. Marked males returned

to the same place after marking (albeit with around 10 min of delay) and behaved normally. Marked animals were not recaptured and their identification number was read without disturbing them.

It was not possible to record data blind because our study involved focal animals in the field. We conducted a focal observation period of 10 min of marked males. If a male was not marked, we observed it and marked it at the end of the observations. For each male we recorded the time observation began, the number of flights, the number of agonistic interactions with other males (or male wasps, see Results), the number of grooming behaviours (cleaning of head, eyes, legs or abdomen) and the number of wing displays. Wing displays are defined as spontaneous movements of the wings, opening and closing them in a fast sequence (video 1 in supplementary material); this makes the males highly conspicuous, at least to human observers. If a focal male was observed in a fight, we measured fight duration and whether the male returned to his previous perch/territory. Some fights were also recorded with a video camera, including high-speed video (300 frames/second, Casio EX-F1). Given the limited number of males in the stream (see Results), we observed the same marked males at different times of the day, on different days, trying to get one focal observation per male within each hour interval between 9 and 16 h, the period of maximum activity at the stream.

#### Reproductive behaviour

When a female was detected near the water, we followed her closely to describe her reproductive behaviour. No females were marked and they were collected at the end of the focal observation. Whenever possible, copulations were video-recorded for further analyses. To study sperm competition mechanisms we collected females after mating ( $N = 5$ ; presumed to have full sperm load), and females at the end of stage I ( $N = 5$ ; expecting to have a smaller sperm volume if sperm removal occurs). We also collected four females found alone at different times of the day, to estimate the sperm volume of females arriving at the stream. Sample size was limited by the number of females found and the numbers of copulations observed. The sperm volume of the storage organs was estimated by measuring the area of the sperm mass and multiplying by a uniform thickness (Cordero and Miller, 1992).

#### Analysis of mark–recapture data

To analyse the recapture histories of marked males, we used the full time-dependent Cormack–Jolly–Seber model (CSJ), as implemented in Mark 8.1 (White and Burnham, 1999). First, we estimated the degree of fit of the CJS model to the data, by using TEST 2 and TEST 3 of program Release, from within Mark. Although data were limited, there were no significant deviations, indicating a reasonable fit (Goodness of Fit Results TEST 2 + TEST 3;  $\chi^2_{11} = 5.08$ ,  $p = 0.927$ ). We then estimated the value of the extra-multinomial variance ( $c$ -hat) parameter using two strategies, and

selected the most conservative. We computed the estimated  $c$ -hat from the CJS full model and divided this value by the mean  $c$ -hat from the bootstrap procedure in Mark. This  $c$ -hat indicated underdispersion (0.6780). The second estimation was obtained by dividing the observed deviance of the CJS model by the mean deviance of the bootstrap procedure. The value obtained (1.2489) was used to correct estimates of parameters and confidence intervals. We estimated the number of males found in the transect following the methods of Jolly (1965) and Manly and Parr (1968), using the software Popan 5 (Arnason et al., 1998).

Throughout the text, mean values are presented with their standard errors and sample size (in parenthesis). Statistical analyses were performed using xIStat 2017 ([www.xlstat.com](http://www.xlstat.com)).

## **Results**

### Demography and general activity

Over the study period, we marked 36 males and resighted all but four on subsequent days. On average, males were resighted on  $4.6 \pm 0.4$  different days, and one male, marked on the first day of study, was observed every day until the end of the fieldwork, always at the same spot of the stream. This high site-fidelity resulted in high recapture rates. The best model to explain variability in recapture histories is the simplest model  $\{\Phi(\cdot) p(\cdot)\}$ , with constant survival and recapture rates (table 1). The remaining models had low statistical support ( $\Delta \text{QAIC} > 6.8$ ; table 1). The estimated survival rate was 0.8989 (SE = 0.0272; confidence interval: 0.8318–0.9412), and the recapture rate was 0.9252 (SE = 0.272; CI: 0.8514–0.9639). This survival rate translates into an expected longevity of 9.4 days, using the formula of Cook et al. (1967) [ $\text{lifespan} = -1/\log_e(\text{survival})$ ].

The methods of Jolly (1965) and Manly and Parr (1968) yielded almost identical estimates of population size for the period between 16 and 22 June (no valid estimates were obtained before 16 June). The estimated number of males was 17–21 individuals (average  $20.4 \pm 1.0$  (11) males), suggesting stable and low population density. This number is concordant with field observations because we found between 16 and 24 males each day.

Males were observed arriving at the stream between 8:50 and 9:30 h and leaving about 16:30–17:00 h, although some were still at the stream after 17:00 h. They spent most of their time perched. The most frequent behaviour was 'wing display', which peaked between 13 and 15 h, with 5–7 displays per 10 min. This behaviour was rarely observed after 15 h (1 display/10 min; fig. 1; video 1 in supplementary material). The second most common activity was spontaneous flight, also peaking at 13:00–15:00 h, but with less variation over the day (1.4–3.1 flights/10 min). Grooming and agonistic interactions (fighting) were infrequent, with an average of fewer than 0.5/10 min (fig. 1).

Table 1. Results of model selection for *P. mirabilis*, using Mark software. The most supported model (in bold) is also the simplest, with only two parameters. Survival ( $\Phi$ ) and recapture rates ( $p$ ), and model notation follow Lebreton et al. (1992), where  $t$  indicates variation of the parameter over time, and a dot indicates constant value.

Tabla 1. Resultados de la selección de modelos para *P. mirabilis*, utilizando el programa informático MARK. El modelo con mayor respaldo estadístico (en negrita) también es el más sencillo, con solo dos parámetros. Índices de supervivencia ( $\Phi$ ) y recaptura ( $p$ ) y la notación del modelo siguen lo establecido en Lebreton et al. (1992), donde  $t$  es la variación del parámetro en el tiempo y el punto indica valor constante.

Model	QAICc	Delta QAICc	QAICc weights	Model likelihood	Num.Par	QDeviance
<b>{Phi(.) p(.)}</b>	<b>138.0020</b>	<b>0.0000</b>	<b>0.9618</b>	<b>1.0000</b>	<b>2</b>	<b>63.5148</b>
{Phi(t) p(.)}	144.8118	6.8098	0.0319	0.0332	11	50.4384
{Phi(.) p(t)}	148.0921	10.0901	0.0062	0.0064	11	53.7187
{Phi(t) p(t)}	158.2188	20.2168	0.0000	0.0000	20	41.0956

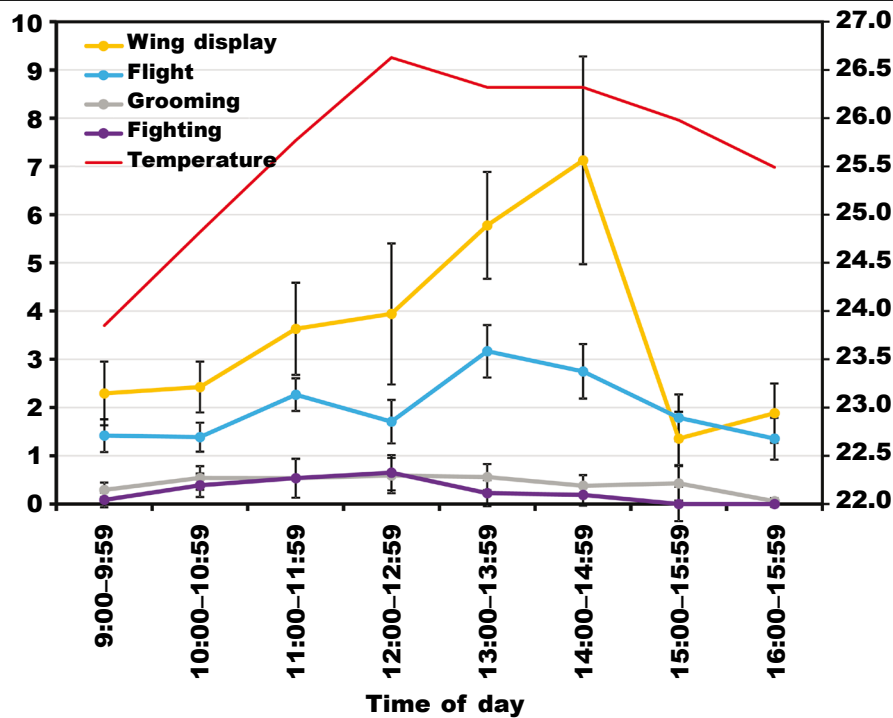


Fig. 1. Variation in activity of male *P. mirabilis* and air temperature over the day (average from 13–23 June 2014). Data represent the frequency of each behaviour per 10 min of observation ( $\pm$  SE). This plot is based on 162 focal observations of 10 min each, involving 28 marked males and eight unmarked males. No male was observed more than once on each day.

Fig. 1. Variación en la actividad de los machos de *P. mirabilis* y en la temperatura del aire a lo largo del día (promedio del 13 al 23 de junio de 2014). Los datos representan la frecuencia de cada comportamiento en 10 minutos de observación ( $\pm$  EE). La figura está basada en 162 observaciones focales de 10 minutos cada una, de 28 machos marcados y ocho no marcados. Ningún macho fue observado más de una vez al día.

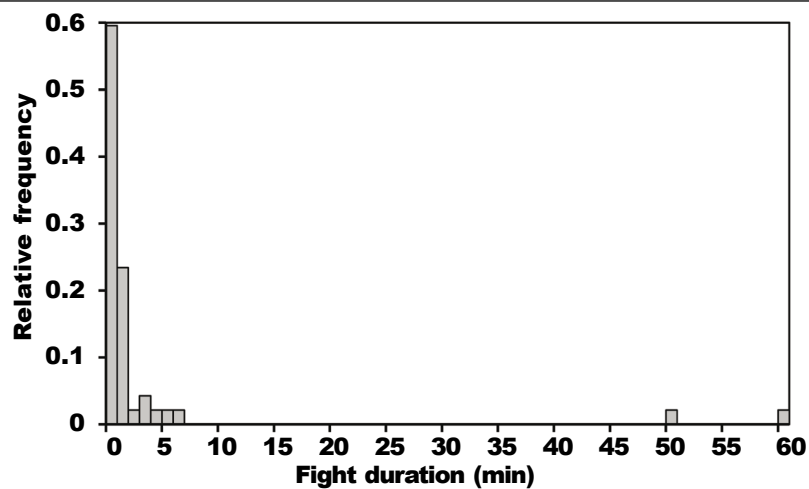


Fig. 2. Histogram of duration of 47 agonistic encounters between males of *P. mirabilis*, in classes of one minute. Note that the distribution is bimodal, with very short or very long fights.

Fig. 2. Histograma de la duración de 47 encuentros agonísticos entre machos de *P. mirabilis*, en clases de un minuto. Obsérvese que la distribución es bimodal, con peleas muy cortas o muy largas.

#### Territorial behaviour

Males defended a territory of about 0.5–1 m in radius, which included one or more damp logs, used by females to lay eggs. The same area was defended on consecutive days, showing high site fidelity. A few non-territorial males were observed perching on the shore vegetation; they were not attacked by residents unless they flew into a territory.

Males were highly aggressive against conspecific males. We timed 47 interactions involving 15 marked males and 16 unmarked males. These data indicate that fight duration is bimodal (fig. 2). Most fights (83%) lasted less than 2 minutes and were face-to-face encounters where males exhibited their blue face while mimicking behaviour of the other (fig. 3B). Neighbour territorial males were frequently observed engaging in these interactions. A few cases (13%) lasted up to 7 min, and two (4%) lasted 50 and 60 min. These were escalated fights that started with very close face-to-face displays (at about one body length of distance) and with the abdomen of both males pointing upwards (fig. 3C, 3D and video 2 in supplementary material), intercalated with hovering periods (fig. 3E) of both males flying only with their forewings (video 3 in supplementary material). From high-speed video, forewing beat frequency can be estimated as 11.2–12.2 hz. Both males approached and retreated alternatively, maintaining a distance of 20–40 cm, while moving to treetops. The two escalated fights observed may have lasted more than one hour because it was difficult to follow the pair into the forest canopy. Escalated fights were sometimes interrupted by short periods (2–3 minutes) of perching in the shoreline vegetation.

Males also interacted aggressively with wasps (*Eustenogaster nigra* Saito and Nguyen, 2006), with short face-to-face confrontations similar to those observed with conspecific males (fig. 3F, 3G). These interactions were common (N = 29 cases) but never lasted more than a few seconds. Sometimes males were attacked by the wasps while they were perched. It was unclear whether the wasps acted as territorial or as predators, but male *P. mirabilis* were apparently acting as territorial when they confronted the wasps.

#### Reproductive behaviour

When a male detected a female perched near his territory, there was a brief courtship flight, of a few seconds, in which the male exhibited his coloured hindwings. Females were observed approaching the stream and started to lay eggs on dead logs if no males were around. Males readily tried to grasp ovipositing females in tandem, with no or very brief courtship. Immediately, the male grasped the female prothorax, a precopulatory tandem was formed (5–6 sec), and the pair copulated. Two females rejected copulation after having been grasped in tandem. There was no intra-male sperm translocation before copulation.

Copulation (fig. 3H) occurred at low frequency, at any time between 10:50 and 15:14 h (fig. 4), with a maximum of three matings observed per day and a total of 14 matings over the study period. Copulation lasted on average  $7.10 \pm 0.98$  (7) min, with a range from 2.6 to 10.0 min (fig. 4). A generalized linear model with copulation duration as the response variate and time of day and air temperature as predictors suggested that copulation duration was not

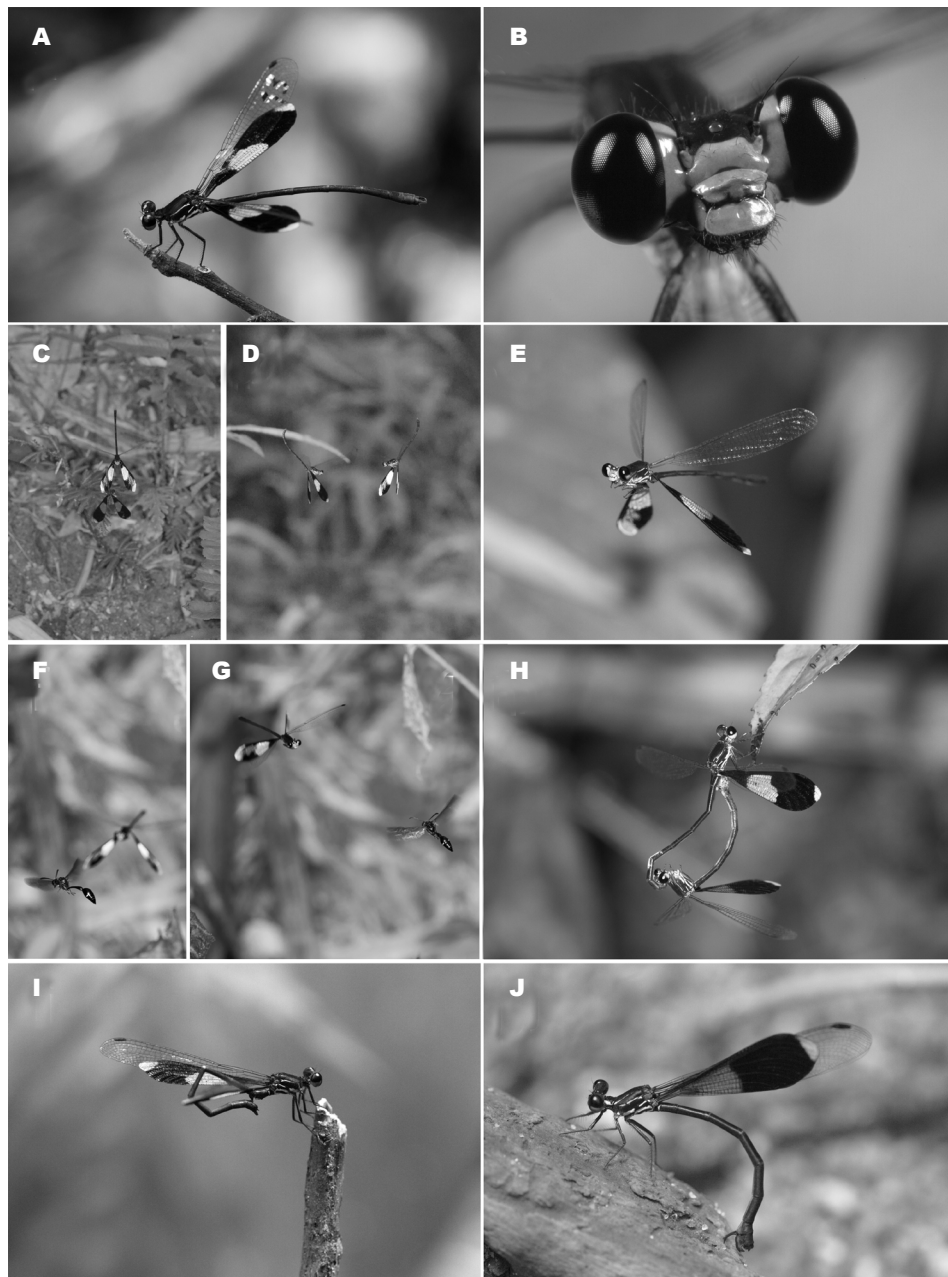


Fig. 3. Agonistic and reproductive behaviour of *P. mirabilis*: A, a territorial male (marked on the right forewing); B, detail of the blue face of the male; C, D, agonistic behaviour of high intensity, where the males show coloured hind wings, and upward-oriented abdomen; E, a male in the static agonistic flight, with the abdomen in horizontal position, and situated about 20–40 cm from its opponent; males fly only with their forewings; F, G, agonistic encounter with the wasp *Eutenogaster nigra*, which patrolled the same area of the stream; H, copulation; I, intra-male sperm translocation, which takes place after copulation; J, one ovipositing female, inserting eggs in a damp log on the stream. (Photos: ACR.)

Fig. 3. Comportamiento agonístico y reproductor de *P. mirabilis*: A, un macho territorial (marcado en el ala delantera derecha); B, detalle de la cara azul del macho; C, D, comportamiento agonístico de alta intensidad, donde los machos exhiben las alas posteriores coloreadas, y el abdomen orientado hacia arriba; E, un macho en vuelo agonístico estático, con el abdomen en posición horizontal, y situado a unos 20–40 cm de su oponente; los machos solo vuelan con sus alas anteriores; F, G, encuentro agonístico con la avispa *Eutenogaster nigra*, que patrullaba la misma zona del arroyo; H, cópula; I, la transferencia de esperma hacia la vesícula seminal del propio macho, que tiene lugar después de la cópula; J, una hembra en puesta, insertando huevos en un tronco húmedo en el arroyo. (Fotos: ACR.)

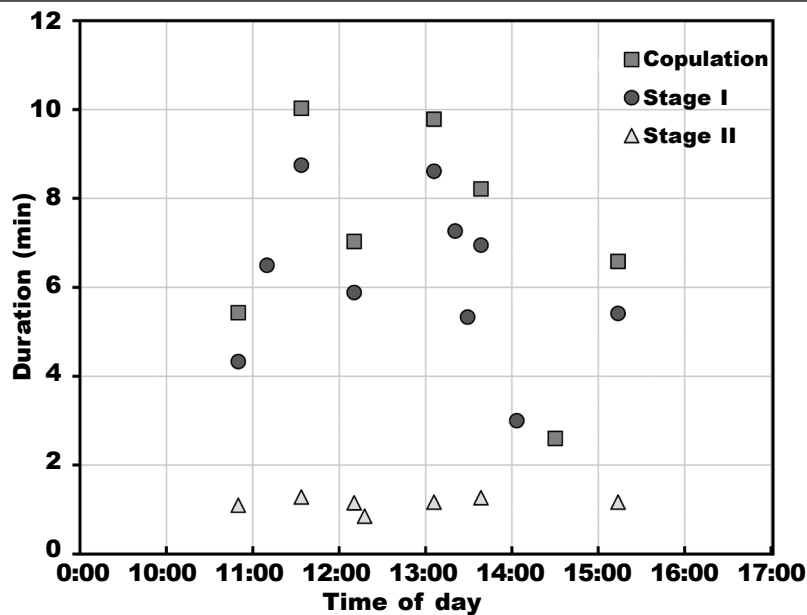


Fig. 4. The relationship between time of day and copulation duration (and its stages) in *P. mirabilis*. Note that stage II shows very little variation in duration.

Fig. 4. Relación entre la hora del día y la duración de la cópula (y sus etapas) en *P. mirabilis*. Obsérvase que la etapa II muestra muy poca variación en su duración.

affected by these variables ( $F_{3,3} = 2.134$ ,  $p = 0.275$ ). Copulation consisted of two phases (video 4 in supplementary material), similar to those described for other Zygoptera (Miller and Miller, 1981). Stage I was the most variable phase (average:  $6.21 \pm 0.57$  (10); range: 3.0–8.8 min) and occupied 87% of copulation (fig. 4). Stage II was short, and almost identical in duration among different pairs (average:  $1.14 \pm 0.05$  (7); range: 0.9–1.3 min; fig. 4).

Females had a large spherical *bursa copulatrix* and no spermatheca (fig. 5A–5C). Male genital ligula had a flexible head culminated by two processes, covered by backward oriented spines (fig. 5D–5E). Movements of the male abdomen during stage I of copulation were suggestive of sperm removal by male genitalia (video 4 in supplementary material). To test this idea, we preserved females found alone, mated females after copulation, and interrupted females at the end of stage I, presumably before insemination. Upon dissection, we found that females when copulation was interrupted (mean volume:  $0.0233 \pm 0.0076$  (5)  $\text{mm}^3$ ), compared to pre-copula females ( $0.0644 \pm 0.0133$  (4)), which in turn had a sperm volume very similar to females captured after copulation ( $0.0678 \pm 0.0125$  (5)). There were significant differences between groups (ANOVA,  $F_{2,11} = 5.095$ ,  $p = 0.027$ ). One of the females whose copulation was interrupted had the *bursa* empty, which could indicate that she had never mated previously. If this female were excluded, the sperm volume in interrupted fe-

males would be  $0.0292 \pm 0.0063$  (4). A comparison between this value and the mean sperm volume of precopula females indicated marginal differences ( $t = -2.388$ ,  $p = 0.054$ ).

After copulation, most females expelled a drop of sperm (six out of eight females closely observed) by active movements of their genital valves (video 3 in supplementary material). One such drop was examined under microscope and its volume was similar to the volume of sperm stored in postcopula females. After copulation, the male remained close to the female, and then flew to a perch in the territory. We concentrated on female postcopulatory behaviour, until we discovered that the intra-male sperm translocation took place within 2 min of the end of copulation (fig. 3I). The last three matings observed, when we specifically followed the male to check for this behaviour, all completed intra-male sperm translocation (lasting about 20 sec) after copulation (video 3 in supplementary material). Oviposition was observed on dead wood inside the stream (fig. 3J; video 5 in supplementary material), with males remaining not far from their mate, although close guarding could not be confirmed.

## Discussion

In agreement with our initial hypothesis, we found that *P. mirabilis* possess several unique behaviours when compared to other damselflies. Given their



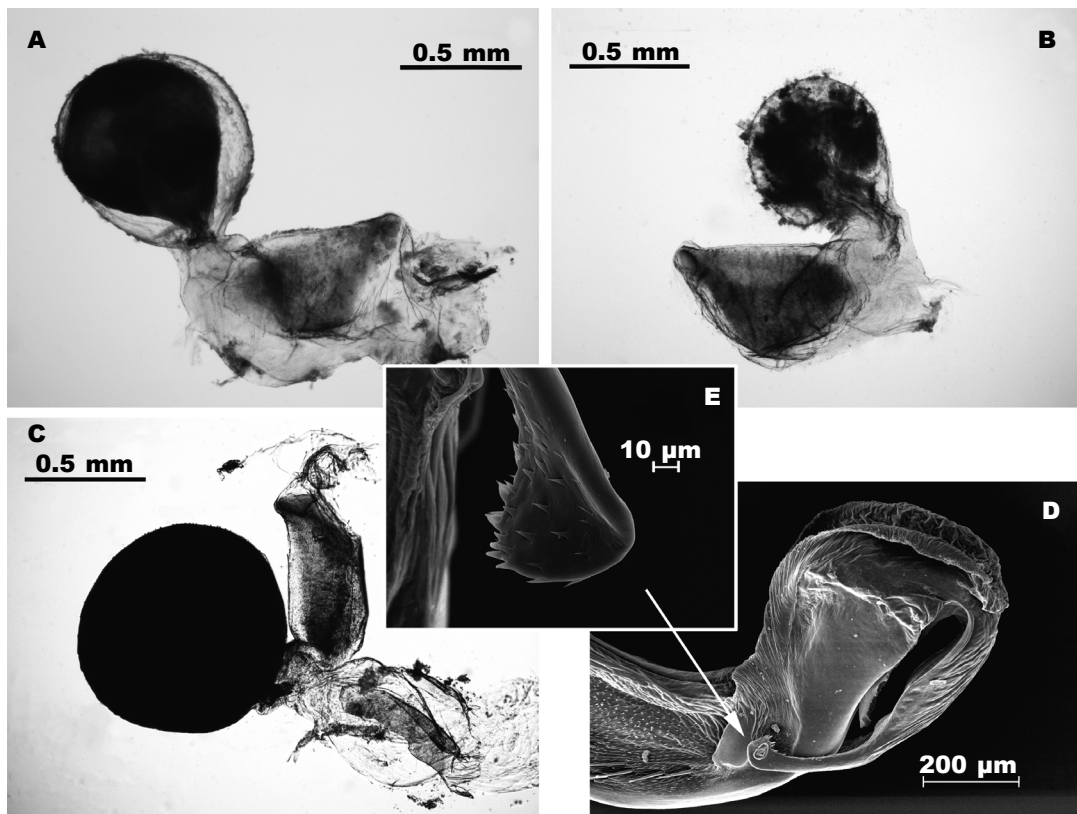


Fig. 5. Female and male genitalia of *P. mirabilis*: Bursa copulatrix and genital plates of a female collected before copulation (A), compared to a female whose copulation was interrupted at the end of stage I, before insemination (B), and a female collected immediately after copulation (C). The sperm volume of interrupted females was clearly reduced. Male genital ligula at SEM (D), with a detail (E) of the spines in the genital process. This anatomical evidence points to sperm removal by male genital processes. Images edited to remove background dust.

Fig. 5. Genitales masculinos y femeninos de *P. mirabilis*. Bursa copulatrix y placas genitales de una hembra capturada antes de la cópula (A), en comparación con una hembra cuya cópula fue interrumpida al final de la fase I, antes de la inseminación (B), y una hembra capturada inmediatamente después de la cópula (C). El volumen de esperma encontrado en hembras al interrumpir la cópula se redujo claramente. Ligula genital masculina observada al SEM (D), con un detalle (E) de las espinas en el proceso genital. Este rasgo anatómico apunta a la eliminación del esperma por los procesos genitales masculinos. Imágenes editadas para eliminar el polvo de fondo.

distinctiveness, limited distribution and threats to their specialised habitat (Zhu et al., 2015), it is a priority taxon for conservation (Xue et al., 2017). Furthermore it is phylogenetically isolated, to the extent that it has been considered a new (sub)family since its original description (Kirby, 1900), and there currently seems to be a consensus to consider it a monotypic family (Dijkstra et al., 2014; Reels and Zhang, 2015; Yu and Bu, 2011).

Our results confirm that *P. mirabilis* is highly territorial (Reels, 2008). Male density during study period was about one male per meter in favourable zones, with a mean of 20 individuals in a section of 200 m. Our population had low density, but the species was

dominant in the studied stream. Our fieldwork was probably completed at the end of the flight season so the density might be higher earlier in the season. Females were rarely seen on the stream.

Males are long-lived, showing very little activity for hours, but sometimes engaging in conspicuous and elaborate aggressive behaviour. The estimated survival rate of 0.8989 per day is similar to that of Calopterygidae (Cordero-Rivera and Stoks, 2008), a family that also shows high site tenacity, territoriality, and in some species, escalated agonistic encounters (Córdoba-Aguilar and Cordero-Rivera, 2005). However, in many respects, the morphology and behaviour of *P. mirabilis* recalls that of the genus *Chalcopteryx*, an endemism of

the Amazon forest streams (Guillermo–Ferreira et al., 2014; Resende and De Marco, 2010), which belongs to a distant family (Polythoridae). Both *Pseudolestes* and *Chalcopteryx* have long hyaline forewings, short hindwings with conspicuous coloration. Both also show high territoriality and engage in aerial contests between neighbouring males, sometimes involving three or more males. There are therefore surprising ethological and morphological convergences between these two distantly related taxa. Similar ecological pressures might be behind their convergent behaviour and morphology, which is also found at the larval stages, with both genera having abdominal gills, albeit completely different in structure (dos Santos and Costa, 1987; Yu and Bu, 2011).

The most conspicuous behaviour of *P. mirabilis* is the agonistic male–male encounters (Reels, 2008). For most of the time, activity of territorial males was reduced, and some even remained motionless for more than one hour. Wing displays broke this immobility, again recalling behaviour of males of *Chalcopteryx*, which remain motionless for long periods and exhibit their iridescent hindwings in dark spots of the forests (Resende and De Marco, 2010). Fights in *P. mirabilis* were highly ritualised, consisting of short chases of a few seconds or minutes, with males confronting each other and displaying their blue face and coloured hind–wings (fig. 3). It is noteworthy that some fights escalated and lasted for almost one hour (fig. 2). Other damselflies apparently use their face ornamentation during territorial displays as honest signals (Vilela et al., 2017), but it is not known whether this applies to *P. mirabilis*.

During these displays, we observed that the hindwings remained motionless (video 2 and video 3 in supplementary material), and the males beat their forewings at only 11–12 Hz, which is considerably slower than other territorial damselflies (e.g. 29.5 Hz in *Chlorocypha cancellata*; Günther, 2015). This behaviour is likely to be very costly in terms of energy. In one of the long fights observed, the resident male lost the territory after this interaction, as sometimes occurs with the escalated fights in *Calopteryx* (Marden and Waage, 1990). Future studies of the phenotypic variables associated with flight endurance and the tendency to escalate agonistic encounters are needed to fully understand the territorial behaviour of *P. mirabilis*.

An unexpected result from our fieldwork was the interspecific agonism between damselflies and wasps (fig. 3F; see also Garrison et al., 2015). Wilson and Reels (2001) described *P. mirabilis* as giving 'a strong bee–like impression'. It seems that its hymenopteran mimicry is enough to produce mistaken identity between the two species. Interspecific territoriality is not uncommon among similar species, particularly congeneric ones that might be unable to distinguish between conspecific and heterospecific females (Anderson and Grether, 2010). This is not the case for *P. mirabilis* and *E. nigra*. Both species might be paying the costs of sharing the habitat and having similar aggressive behaviour. It would be interesting to compare sympatric and allopatric populations of the two species, to detect possible character displacement.

Reproductive behaviour of *P. mirabilis* also shows several unique characters. First, copulation was not preceded by the intra–male sperm translocation, which typically occurs in Zygoptera before each mating (Cordero–Rivera and Córdoba–Aguilar, 2010). This behaviour is needed because odonates do not have internal connection between the testis and the intromittent organ (Cordero–Rivera and Córdoba–Aguilar, 2010). Sperm translocation after mating has previously been recorded only in the small coenagrionid *Mortona-grion hirosei* (Naraoka, 2014), and in the genus *Cora* (Polythoridae; Fraser and Herman, 1993), and we observed the same behaviour in Chinese members of the family Euphaeidae (Cordero–Rivera, unpublished). Given that males were never observed translocating sperm to their seminal vesicle before mating, we assume that they were inseminating the sperm already stored in that organ, translocated after the last mating. This is counterintuitive, because mating frequency was found to be very low: most males would not find a female to mate with for over several days. Therefore, we hypothesize that the seminal vesicle of *P. mirabilis* has physiological mechanisms to maintain sperm alive for longer periods than most odonates. Alternatively, males could translocate sperm each morning, discarding previous unused sperm, although we did not observe this behaviour. In any case, this unusual behaviour needs further study. We are currently reviewing the diversity of intra–male sperm translocation in the entire order Odonata, as an example of ethological diversity (Cordero–Rivera, 2017).

A second unusual fact about *P. mirabilis* reproductive behaviour is the almost complete absence of precopulatory courtship typically found in odonates with coloured wings (Outomuro et al., 2013; Svensson and Waller, 2013). Another taxonomically isolated damselfly, *Hemiphysalia mirabilis*, considered by some authors as the oldest living zygopteran (e.g. Fraser, 1955), was found to have a highly specialised courtship (Cordero–Rivera, 2016), suggesting that courtship behaviour might have evolved early in the damselflies. In agreement with this, recent fossil evidence also points to the development of specialised structures likely used for courtship in Cretacic damselflies (Zheng et al., 2017). Nevertheless, in *P. mirabilis*, the coloured wings have apparently evolved in the context of intra–sexual selection. Therefore, wing pigmentation might be an honest signal of male quality, and a study of inter–individual and inter–population variation in wing pigmentation would be of interest.

Copulatory behaviour of *P. mirabilis* was typical, however, in the sense that it follows the copulatory stages already described for Coenagrionidae (Miller and Miller, 1981) and found to be widespread in Zygoptera (Córdoba–Aguilar and Cordero–Rivera, 2008). This points to a very old origin of sperm removal in the Odonata (Cordero–Rivera, 2016). The genital ligula of this species has two distal appendages 'each of which terminates in a white pad covered with microscopic recurved prickles' (Needham, 1931) (see fig. 5D–5E). This morphology is in agreement with the ability to remove sperm from the *bursa copulatrix*. We provide evidence for sperm removal in this spe-

cies, and this information is of relevance to track the evolution of sperm displacement in the Odonata (Cordero–Rivera and Córdoba–Aguilar, 2010). It is noteworthy that females do not have spermathecae, which may indicate that males are controlling fertilization, because females usually retain control over the spermathecal sperm (Córdoba–Aguilar and Cordero–Rivera, 2008). Yet we found that after mating, most females expelled a drop of sperm in such a large quantity that it was clearly visible with the naked eye (video 4 in supplementary material). There are two possible explanations for this behaviour. Females might simply be expelling the sperm that the male removed during copulation (Lindeboom, 1998), or they might be cryptically choosing between ejaculates and expelling part of the sperm from the last male (Córdoba–Aguilar, 2006). These two possibilities are not mutually exclusive. A genetic analysis is needed to understand this behaviour.

In conclusion, we found that a taxonomically isolated species of damselfly shows remarkable ethological features (see also Cordero–Rivera, 2016) and a striking resemblance to an ecologically similar group of damselflies from a different family (Polythoridae). The agonistic behaviour of *P. mirabilis* is unique and has no equivalents in odonates. This diversity is therefore not explained by the usual three levels of biodiversity assessments, which include genes, species and ecosystems but neglect behaviour (Cordero–Rivera, 2017).

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**Supplementary material**

Video 1. Feeding and wing displays of male *P. mirabilis*.

*Video 1. Alimentación y exhibición de las alas del macho de P. mirabilis.*

<https://youtu.be/UFAUZdSsuWE>

Video 2. Agonistic interactions between territorial males of *P. mirabilis*. Note the different position of the abdomens at the start of interactions, and the increasing distance between males as the interaction continues. Males use only the forewings to confront each other, and maintain the abdomen in a horizontal position.

*Vídeo 2. Interacciones agonísticas entre machos territoriales de P. mirabilis. Obsérvese la diferente posición del abdomen al inicio de las interacciones y la creciente distancia entre los machos a medida que la interacción continúa. Los machos solo utilizan las alas posteriores en los enfrentamientos y mantienen el abdomen en posición horizontal.*

<https://youtu.be/oBgFNACxPng>

Video 3. High speed video (300 frames/second) of a male *P. mirabilis* in static confrontation with another male. Note that hindwings are not used in these agonistic displays.

*Vídeo 3. Vídeo a alta velocidad (300 fotogramas por segundo) de un macho de P. mirabilis en confrontación estática con otro macho. Obsérvese que las alas posteriores no se utilizan en estas exhibiciones agonísticas.*

<https://youtu.be/UY9Z4AVVwx8>

Video 4. Copulatory behaviour of *P. mirabilis*. Mating lasts about seven minutes, and has a first phase (stage I), which is used by males to remove sperm from the spermatheca, the only organ for sperm storing of females of this species. Stage II lasts about one minute and constitutes the phase of insemination. After copulation, most females expel a drop of sperm. Males do not translocate sperm before mating, but they do perform this behaviour a few minutes after copulation.

*Vídeo 4. Comportamiento de cópula de P. mirabilis. La cópula dura unos siete minutos y tiene una primera fase (etapa I), que los machos utilizan para extraer el esperma de la espermateca, que es el único órgano de almacenamiento de esperma que tienen las hembras de esta especie. La etapa II dura aproximadamente un minuto y constituye la fase de inseminación. Tras la cópula, la mayor parte de las hembras expulsa una gota de esperma. Los machos no transfieren el esperma antes de la cópula, pero sí lo hacen pocos minutos después.*

<https://youtu.be/HEh203Pt57k>

Video 5. Oviposition behaviour by female *P. mirabilis* on damp logs in forested streams. Females were sometimes guarded by males.

*Vídeo 5. Comportamiento de ovoposición de la hembra de P. mirabilis en troncos húmedos de arroyos de zonas boscosas. En ocasiones, los machos vigilaban a las hembras.*

<https://youtu.be/PphV-HGz-vg>