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Damselflies (Coenagrionidae) have been avoiding leaf veins during oviposition for at least 52 million years

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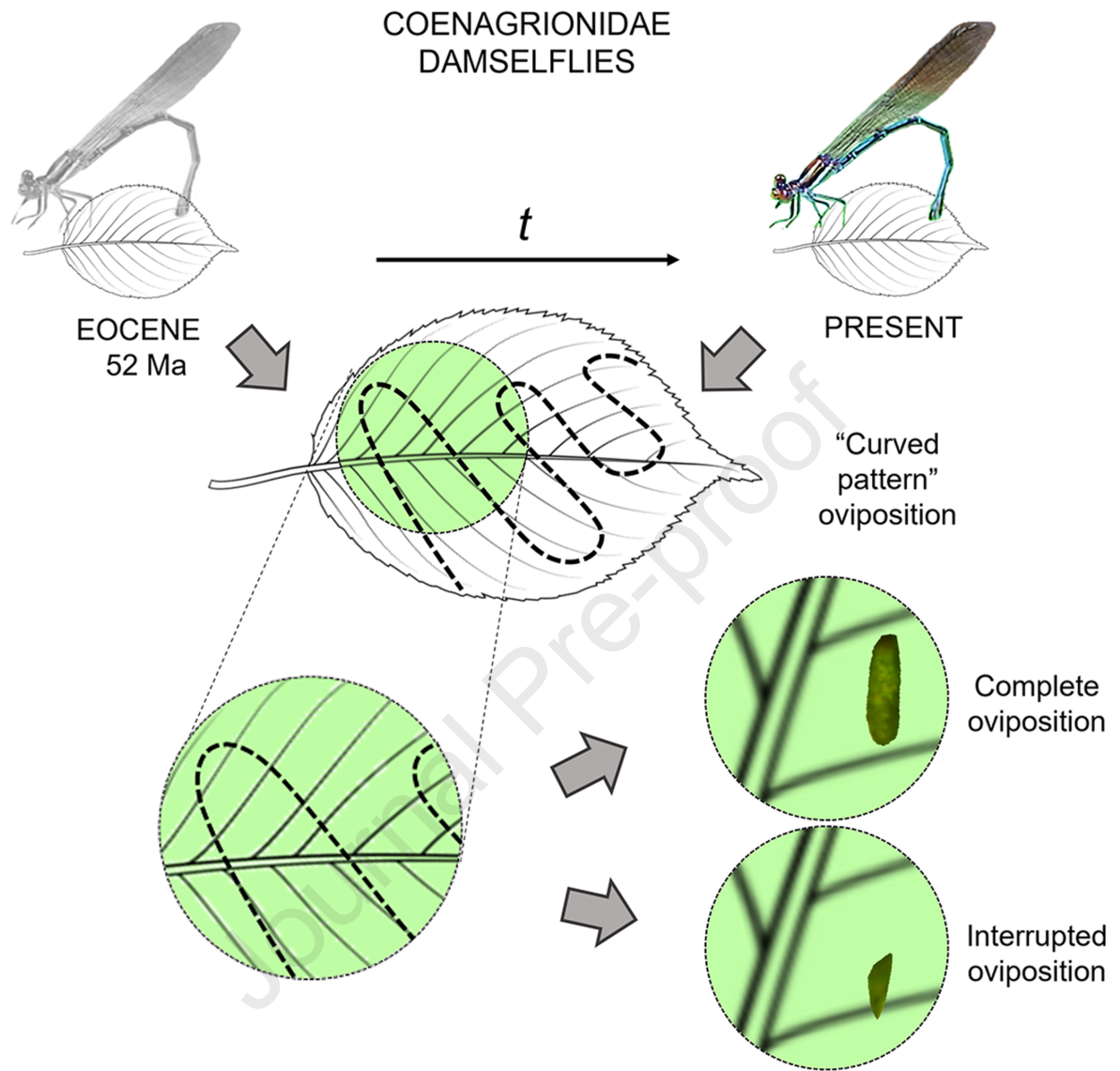
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Damselflies (Coenagrionidae) have been avoiding leaf veins during oviposition for at least 52 million years

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SUMMARY

Plant-insect interactions can provide extremely valuable information for reconstructing the oviposition behavior. We have studied about 1350 endophytic egg traces of coenagrionid damselflies (Odonata: Zygoptera) from the Eocene, identifying triangular or drop-shaped scars associated with them. This study aims to determine the origin of these scars. Our behavioral study of about 1800 endophytic eggs from recent coenagrionids indicates that these scars were caused by ovipositor incisions, but without egg insertion. The scar correlates (χ^2 -test) with leaf veins in both fossil and extant species. We infer that a female would detect the proximity of a leaf vein and avoid egg-laying, generating a scar that also fossilizes. For the first time, a scar produced by the ovipositor has been identified, indicating the existence of undesirable areas for oviposition. Accordingly, we recognize

35 that Coenagrionidae damselflies (narrow-winged damselflies or pond damselflies) have been avoiding
36 leaf veins for at least 52 million years.

37

38 INTRODUCTION

39 In fossil records, different types of evidence may indicate the presence of organisms that are no
40 longer present ^{1,2}. Plant-insect interactions can provide exceptional information on ancient
41 ecological and behavioral relationships between the two main groups of organisms that have lived
42 on Earth ³. Oviposition of fossil insects on plants is one of the most informative interactions found in
43 the fossil record ⁴ as related damage can provide fundamental information for understanding plant-
44 insect relationships and reconstructing insect mating behavior ⁵.

45 Oviposition inside leaves (endophytic) generates complex plant damage ¹. Reliable identification of
46 producers is often possible, especially when the oviposition biology of fossil organisms and that of
47 their modern counterparts are well studied ⁶. However, numerous plant-insect interactions have
48 been ignored, incorrectly assessed, or misinterpreted (see ²).

49 Odonoptera is one of the oldest groups of Pterygota (winged insects), and their earliest records
50 date to the later Namurian (Bashkirian/Serpukhovian; Lower Carboniferous), about 320 to 313 Ma ⁷,
51 ⁸ thereafter, they have a relatively rich fossil record ⁹. Evidence for endophytic oviposition of
52 Odonoptera is known from the Paleozoic (examples in ^{2, 10-13}). Odonata is the only order of
53 Odonoptera that persists today, whose earliest known records are in the Permian ¹⁴⁻¹⁶. This group
54 is currently very diverse and abundant (about 6000 living species and 600 extinct species ¹⁷) and
55 represents one of the most charismatic groups of insects in the world. Fossil damselflies egg traces
56 are very similar in morphology and oviposition patterns to those of extant representatives ^{10, 11, 18, 19}.
57 Endophytic eggs of Odonata are laid in a regular pattern, which is a common feature recorded both
58 in fossils ^{6, 20-25} and in extant species ^{26, 27}. Within damselflies, the family Lestidae is characterized by
59 ovipositing in a straight pattern (Lestidae-Typ *sensu* Hellmund and Hellmund ²⁰), whereas the family
60 Coenagrionidae is characterized by ovipositing in a curved (or zigzag) pattern (Coenagrionidae-Typ
61 *sensu* Hellmund and Hellmund ²⁰). Krassilov et al. ²³ suggest that oviposition patterns of modern
62 damselflies evolved under the influence of leaf venation pattern evolution, cuticle anisotropy,
63 epidermis, and mesophyll of plant leaves.

64 Endophytic oviposition has been described as avoiding the leaf veins (e.g., Moisan et al. ²⁴; Donovan
65 et al. ²⁸; Xu et al. ²⁹) or being located parallel to the leaf veins (primary and secondary) (e.g., Laaß

66 and Hoff ¹¹; Bomfleur et al. ³⁰; Gnaedinger et al. ³¹). In fact, the earliest records of this behavior are
67 mentioned in the Carboniferous (Early Pennsylvanian) by Dernov ¹². All these authors rely on
68 descriptive observations and, so far, there have been no studies on whether this behavior is casual
69 or deliberate.

70 Under this scenario and in order to study oviposition behavior in fossil and extant Coenagrionidae
71 damselflies and its influence of leaf veins, we (1) analyzed oviposition behavior in extant
72 Coenagrionidae from the Province of Córdoba, Argentina; (2) statistically analyzed Coenagrionidae
73 eggs (and traces) and other scars associated with extant and Eocene leaf veins from Patagonia,
74 Argentina (Fig. 1); (3) statistically compared extant and fossil data; and (4) conclude the similarities
75 in oviposition behavior of Eocene and extant Coenagrionidae damselflies.

76

77 RESULTS

78 Oviposition behavior

79 The oviposition behavior of damselflies under natural conditions was observed in females of the
80 Coenagrionidae family. These females were identified using the taxonomic keys illustrated by
81 Garrison et al. ⁴¹ and von Ellenrieder and Garrison ⁴² as *Argia joergenseni* Ris, 1913 and
82 *Acanthagrion lancea* Selys, 1876. Methodology specifications are detailed in the STAR Methods.
83 We describe the oviposition behavior of both species as: The female perches on the leaf surface and
84 then makes repetitive movements of the abdomen to the right, left, up, and down, resulting in the
85 deposition of eggs over a wide area of the leaf, shifting her position to the apex or base of the leaf.
86 The female folded her abdomen, contacting the ovipositor with the leaf surface, and touched the
87 substrate numerous times before inserting it. Upon laceration of the plant tissue, the cut valves of
88 the ovipositor began to move, gradually penetrating the tissue and generating a small pocket under
89 the leaf epidermis. When the cutting valves were fully inserted into the plant, the female remained
90 motionless for an instant, moving the tip of her abdomen slowly up and down. Once the pocket was
91 made, she inserted an egg. Then, the female removed the ovipositor and maintaining its position,
92 moved its abdomen laterally, repeating the process, generating a curved row of successive eggs.
93 The average distance between consecutive eggs was 0.5 mm.

94 The number of eggs per row, and thus the total length of the curved row of eggs, was limited by the
95 length of the damselfly abdomen, due to space restrictions and/or, apparently, by the presence of
96 areas interpreted as undesirable for oviposition, e.g., the proximity of leaf veins, mainly those of

97 large-caliber veins such as the midrib (principal leaf vein). At the beginning of a new row of eggs, the
98 female could remain in the same position and just bend her abdomen, varying the position of the
99 ovipositor in relation to her body (Fig. 2A), or she could simply walk a few steps forward. The
100 distance between successive curved rows varied between 1.1 mm and 2.1 mm. This behavior
101 resulted in a curved (or zigzag) pattern.

102 Initially, the female oviposits with her abdomen extended backwards and the ovipositor behind her
103 hind legs, and once she has oviposited a set of eggs, she folds her abdomen anteriorly under her
104 body, and sometimes the ovipositor is in front of her front legs (see Fig. 2A, and Romero-Lebrón et
105 al. ³⁹). At this position, she repeats the process of lacerating the tissue and placing an egg in each
106 incision, with the particularity that the eggs are arranged in reverse (Fig. 2B) compared to eggs
107 deposited when the ovipositor does not exceed its forelegs.

108 Egg morphology shows two differentiated zones: a rounded basal zone (the zone that first enters
109 the plant tissue) and a sharper dark-colored apical zone that sometimes rests partially outside the
110 plant tissue (Fig. 2C). The mean length of the extant eggs analyzed ($n = 1787$) was 0.85 ± 0.01 mm
111 (*Argia joergenseni* 1.84 ± 0.03 mm, and *Acanthagrion lancea* $0.76 \pm 2.3 \times 10^{-3}$ mm; see Table 1).

112

113 **Ovipositor insertion frequency**

114 A total of 168 leaves were analyzed, but only 14 extant and 10 fossil leaves met all criteria for
115 analysis (see Materials and Methods). The 14 extant leaves had 1787 eggs and 319 associated scars
116 (Table 1), and the 10 fossil leaves had 701 egg traces and 76 associated scars (Table 2). Our analysis
117 during oviposition allows us to infer that when the cut ovipositor valves detect a leaf vein, the
118 female stopped the oviposition process, removed the valves, and shifted their position. This
119 behavior, which does not deposit an egg, creates triangular or drop-shaped associated scars in the
120 leaf tissue (Figs. 3 and 4) and represents 0.18 % of the total number of incisions. This associated scar
121 measures 0.27 ± 0.19 mm (min – max: 0.07 – 0.57 mm) in length by $0.10 \pm 4.3 \times 10^{-3}$ mm (min – max:
122 0.04 – 0.17 mm) in width.

123 We also identified these associated scars in fossil leaves from the Eocene of Laguna del Hunco and
124 Río Pichileufú, similar to those produced in extant leaves by the "Interrupted oviposition" (Table 2,
125 Fig. 5). It is important not to confuse the traces of the ovipositor insertion with traces of bites
126 generated by the mouthparts of sucking insects (piercing and sucking) used to perforate the leaves
127 superficially and thus feed on internal fluids ⁴³. These types of traces are circular to oval in shape,

128 with an average diameter of 0.3 mm, whose extreme values reach 1.2 mm in diameter ¹ and
129 frequently appear above the leaf veins ⁴⁴.

130 Statistical analysis using χ^2 test indicated that, for each of the leaves of the extant and fossil plants
131 analyzed, the proportion of eggs (or their traces) crossing a leaf vein is lower than the proportion
132 expected by chance. Alternatively, the proportion of incision scars without eggs (or trace) near the
133 leaf veins is higher than the proportion expected by chance ($p < 0.05$; Tables 1 and 2).

134

135 **DISCUSSION**

136 Oviposition behavior and associated scars suggest that Coenagrionidae damselfly females would
137 avoid laying eggs if they had to or could pass through a leaf vein and, that this behavior creates a
138 distinctive and potentially fossilizing scar. For the first time, a scar produced by the female
139 ovipositor at the time of oviposition is identified, which indicates the existence of possible
140 undesirable areas for oviposition. This study adequately documents the morphological
141 characteristics and size of these scars, avoiding future misinterpretations or identifications, present
142 in both extant and fossil samples. In fossils, it is imperative to be able to distinguish the insertions of
143 the ovipositor without leaving an egg ("Interrupted oviposition"), as these scars fossilize and could
144 be confused with damage caused by other organisms such as hemipteran insects, mites, viruses, or
145 fungi.

146 Previous studies have indicated that endophytic Odonatoptera egg traces are positioned parallel to
147 the leaf veins (principal or secondary leaf veins) because this direction makes it easier for females to
148 lay eggs ¹¹ or avoid vascular tissue ²⁹. In fact, that the traces are placed parallel to the leaf veins,
149 without crossing it, is so relevant that it has an ichnospecies (e.g., *Paleoovoidus marginatus*
150 Gnaedinger et al. ³¹). In endophytic insects, the location of traces and their avoidance of leaf veins
151 has been widely reported in the literature, with at least 39 publications describing this behavior
152 from the Middle Carboniferous to the Upper Miocene (see Supplemental information). Therefore, it
153 could be inferred that leaf vein avoidance behavior would have been present for at least 318.5
154 million years, long before the present research. In any case, these authors reported observations,
155 until now, it was unknown whether this was a casual or deliberate behavior of the female during
156 egg laying.

157 We have not been able to find any mention in the literature that quantitatively describes the
158 endophytic behavior of females in case of detecting the possibility of the egg passing through a leaf
159 vein, nor the resulting associated scar.

160 Several substrate characteristics play important roles in the choice of oviposition sites. In natural
161 observations, some factors driving oviposition site selection are foliage stiffness, tissue thickness
162 and trichome density, which provide clues about desiccation risk, accessibility to trophic resources,
163 or predation risk for their progeny, among others (e.g., Pasquier-Barre et al. ^{45, 46}; Griffin and
164 Yeorgan ⁴⁷; Lundgren et al. ⁴⁸). Tissue stiffness may be a key factor in the selection of oviposition
165 substrates, as has been demonstrated in some insects with endophytic oviposition, such as
166 odonatans or hemipterans (e.g., Matushkina and Gorb ²⁶; Grunert ⁴⁹; Constant et al. ⁵⁰). In
167 Zygoptera, exists a direct correlation between the stiffness of the ovipositor and the stiffness of
168 their preferred plant substrates ^{26, 27}. Occasionally, in Coenagrionidae it has been observed (pers.
169 obs.) that when the substrate is soft enough, or when oviposition occurs at an early stage of leaf
170 development, the veins are not necessarily an impediment, and may even represent the only place
171 on the leaf surface stiff enough to hold the eggs and, therefore, substrate stiffness is a vitally
172 important phenomenon to be considered.

173 Other factors besides stiffness, such as the distance to the point of convergence of the leaf veins,
174 play a role in the choice of oviposition site ⁵⁰. In Zygoptera, the female's decision to oviposit is not
175 only based on the successful penetration of plant tissues but also on other factors, such as plant
176 anatomy ^{26, 27}. Different structures of the ovipositor are endowed with sensory organs with which
177 females would be able to recognize the stiffness that occurs in the ovipositor during penetration
178 into the plant tissue, and thus determine the suitability of the oviposition site ⁵¹⁻⁵³. Thus, during
179 oviposition, Zygoptera females can detect the proximity of leaf veins at the time of insertion and
180 adjust the position of the egg in relation to the position of the leaf vein (or fibers) during tissue
181 penetration ⁵². Avoidance of undesirable areas is necessary for normal embryo development ⁵⁴.
182 Therefore, orienting the egg with respect to the substrate fibers (without crossing a leaf vein) would
183 decrease the damage to the eggs ⁵².

184 Vasilenko ³ and Romero-Lebrón et al. ² infer that evolutionarily, the spatial pattern of the
185 endophytic eggs is insect specific and would not depend on the plant species used as substrate.
186 Other authors (Krassilov et al. ²³; Gnaedinger et al. ³¹) proposed that the spatial arrangement of
187 Odonatoptera eggs has been developed in relation to the reticular venation of host plants. Our
188 study proposes that, although the spatial pattern of oviposition in Coenagrionidae damselflies

189 would be a fixed and specific behavior on a large scale, the leaf veins and the stiffness of the
190 substrate used would generate a smaller-scale influence, specific to the location of each egg.

191 The behavior of inserting the ovipositor without laying eggs to avoid leaf veins generates scars that
192 can be observed in the Eocene of Argentinian Patagonia and in modern-day specimens, indicating
193 that the oviposition behavior of Coenagrionidae damselflies has been preserved for at least 52
194 million years.

195

196 **CONCLUSIONS**

197 Five conclusions can be drawn from this study:

198 1) We define "Complete oviposition" and "Interrupted oviposition". Scars produced by the
199 ovipositor without eggs are reported for the first time, revealing that such scars can fossilize and
200 were previously unrecognized as such.

201 2) Each insertion of the ovipositor generates a scar in the leaf (plant substrate) with ("Complete
202 oviposition") or without ("Interrupted oviposition") egg insertion.

203 3) Female damselflies of the Coenagrionidae family may apparently detect the proximity of a leaf
204 vein when inserting their ovipositor into plant tissue, and in such cases, they avoid laying eggs.
205 However, this behavior is highly dependent on the stiffness of the substrate.

206 4) Leaf veins do not generally influence the curved oviposition pattern of Coenagrionidae
207 damselflies, but they may influence the individual location of some eggs.

208 5) Scars generated by the damselfly ovipositor to avoid leaf veins have also been observed in
209 Eocene materials from Patagonia, Argentina, suggesting that females would have been performing
210 this behavior for at least 52 million years ago.

211

212 **Limitations of study**

213 This study has been conducted exclusively on the Coenagrionidae family; therefore, extrapolating
214 these results to all damselflies could lead to errors. In addition, it should also be noted that the
215 stiffness of the leaves/veins may influence the choice of oviposition, resulting in variations in the
216 results

217

218 **STAR METHODS**

219 Detailed methods are provided in the online version of the manuscript and include the following:

220 • **RESOURCE AVAILABILITY**

- 221 ○ Lead contact
- 222 ○ Materials availability
- 223 ○ Data and code availability

224 • **EXPERIMENTAL MODEL**

- 225 ○ Fossil material
- 226 ○ Extant material

227 • **METHODS DETAILS**

- 228 ○ Laboratory methodology

229 • **QUANTIFICATION AND STATISTICAL ANALYSIS**

- 230 ○ Data analysis

231

232 **SUPPLEMENTAL INFORMATION**

233 Supplemental information can be found online at <https://>

234

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239

240 **AUTHOR CONTRIBUTIONS**

241 E.R-L. and R.M.G. conceived the study. E.R-L. photographed the fossil and extant specimens,
242 conducted the statistical analyses, writing, and original draft preparation; E.R-L. and M.F-M.
243 conducted fieldwork on the extant specimens and made the figures; E.R-L., M.F-M., N.M., X.D., and
244 R.M.G. interpreted the results, wrote, and approved the manuscript. R.M.G. supervised the project.

245

246 **DECLARATION OF INTERESTS**

247 The authors declare that they have no conflicts of interest.

248

249 INCLUSION AND DIVERSITY

250 We support inclusive, diverse, and equitable conduct of research.

251

252 Figure legends

253 **Figure 1.** Location map of the five areas studied in Argentina, three current localities: Arroyo Los Molles (Río
254 Los Reartes, Intiyaco, Córdoba), Arroyo Los Hornillos (Los Pozos Verdes, Río Ceballos, Córdoba) and Río
255 Suquía -Río Primero- (Córdoba), and two fossil localities: Río Pichileufú (Middle Eocene, Lutetian, 48 Ma, Río
256 Negro) and Laguna del Hunco (Lower Eocene, Ypresian, 52 Ma, Chubut).

257

258 **Figure 2.** Endophytic oviposition and ovipositor position variation in *Acanthagrion lancea* (Zygoptera,
259 Coenagrionidae). (A) *Acanthagrion lancea* laying eggs endophytically, folding the abdomen, and varying the
260 ovipositor position. (B) Extant leaf of *Eryngium agavifolium* (Apeaceae) showing directional change of the egg
261 (black arrows), due to changes in the position of the abdomen and ovipositor of *Acanthagrion lancea*. Scale:
262 1mm. (C) Detail of the egg zones. Abbreviation: AZ, apical zone (black); BZ, basal zone.

263

264 **Figure 3.** Extant leaf of *Eryngium agavifolium* (Apeaceae) showing the associated scar generated by the
265 insertion of the ovipositor of *Acanthagrion lancea* into the leaf tissue without laying eggs. Abbreviation: AS,
266 associated scar; E, egg. Scale: 5 mm.

267

268 **Figure 4.** Extant leaves showing eggs and associated scars generated by the insertion of the ovipositor into
269 the leaf tissue without laying eggs. (A and B) *Alternanthera philoxeroides*. (C and D) *Ludwigia grandiflora*. (E)
270 *Cyperus* sp. (F) *Iris pseudacorus*. Abbreviation: AS, associated scar; E, egg. *Acanthagrion lancea* eggs in A, B, C,
271 D, and E; *Argia joergenseni* eggs in F. Scale: 5 mm.

272

273 **Figure 5.** Comparison between extant (first and third columns) and fossil (second and fourth columns) leaves
274 of the eggs/traces and the associated scar generated by the insertion of the ovipositor into the leaf tissue
275 without laying eggs.

276

277 Table legends

278 **Table 1.** Extant material analyzed: identification, localities, egg measurements (length average \pm standard
279 error) expressed in mm, number of eggs per leaf, number of incisions per leaf, χ^2 (Chi Square) value, and p
280 value. ⁽¹⁾*Acanthagrion lancea*; ⁽²⁾*Argia joergenseni*.

Extant leaf specimen number	Species	Localities	Egg length	Number of eggs per leaf	Number of associated scars per leaf	χ^2 value	p
1	<i>Eryngium agavifolium</i> Griseb ⁽¹⁾	Arroyo Los Hornillos	0.77 \pm 0.003	385	45	325.44	<0.0001

2	<i>Eryngium agavifolium</i> . Griseb ⁽¹⁾	Arroyo Los Hornillos	0.81 ± 0.005	108	44	111.17	<0.0001
3	<i>Eryngium agavifolium</i> Griseb ⁽¹⁾	Arroyo Los Hornillos	0.84 ± 0.01	39	24	50.89	<0.0001
4	<i>Polygonum punctatum</i> Elliott ⁽¹⁾	Arroyo Los Hornillos	0.77 ± 0.01	40	5	6.27	0.0123
5	<i>Cyperus</i> sp. ⁽¹⁾	Arroyo Los Hornillos	0.75 ± 0.02	127	3	130	<0.0001
6	<i>Iris pseudacorus</i> L. ⁽²⁾	Arroyo Los Molles	1.65 ± 0.05	82	14	96	<0.0001
7	<i>Iris pseudacorus</i> L. ⁽²⁾	Arroyo Los Molles	1.86 ± 0.03	107	11	106.28	<0.0001
8	<i>Iris pseudacorus</i> L. ⁽²⁾	Arroyo Los Molles	1.96 ± 0.03	51	7	39.87	<0.0001
9	<i>Ludwigia grandiflora</i> (Michx.) Greuter & Burdet ⁽¹⁾	Río Suquía	0.69 ± 0.01	150	44	48.48	<0.0001
10	<i>Ludwigia grandiflora</i> (Michx.) Greuter & Burdet ⁽¹⁾	Río Suquía	0.73 ± 0.01	120	36	35.04	<0.0001
11	<i>Ludwigia grandiflora</i> (Michx.) Greuter & Burdet ⁽¹⁾	Río Suquía	0.69 ± 0.01	84	14	5.33	0.0209
12	<i>Alternanthera philoxeroides</i> (Mart.) Griseb ⁽¹⁾	Río Suquía	0.76 ± 0.01	224	39	58.39	<0.0001
13	<i>Alternanthera philoxeroides</i> (Mart.) Griseb ⁽¹⁾	Río Suquía	0.74 ± 0.01	79	12	26.56	<0.0001
14	<i>Alternanthera philoxeroides</i> (Mart.) Griseb ⁽¹⁾	Río Suquía	0.73 ± 0.01	191	21	76.85	<0.0001

281

282 **Table 2.** Fossil material analyzed: identification, localities, trace measurements (length average ± standard
 283 error) expressed in mm, number of traces per leaf, number of incisions per leaf, χ^2 (Chi Square) value, and p
 284 value.

Fossil material	Species	Localities	Trace length	Number of traces per leaf	Number of associated scars per leaf	χ^2 value	p
MPEF-IC-1367	Dicotyledonous	Laguna del Hunco	0.95 ± 0.01	294	7	41.86	<0.0001
MPEF-IC-1368	<i>Myrcia deltoidea</i> Berry	Laguna del Hunco	0.84 ± 0.02	46	4	11.26	0.0008
MPEF-IC-1370	<i>Celtis ameghenoi</i> Berry	Laguna del Hunco	1.35 ± 0.02	65	9	28.49	<0.0001
MPEF-IC-1372	Dicotyledonous	Laguna del Hunco	1.18 ± 0.02	21	1	18.18	<0.0001
MPEF-IC-1373	<i>Eucalyptus chubutensis</i> Berry	Laguna del Hunco	0.84 ± 0.04	28	8	13.71	0.0002
MPEF-IC-1377	<i>Cassia argentinensis</i> Berry	Laguna del Hunco	0.98 ± 0.03	30	12	22.4	<0.0001
MPEF-IC-1378	<i>Lomatia occidentalis</i> Berry	Laguna del Hunco	1.10 ± 0.01	84	8	15.17	<0.0001
MPEF-IC-1380	Dicotyledonous	Laguna del Hunco	0.91 ± 0.03	31	5	17.42	<0.0001
MPEF-IC-1391	Dicotyledonous	Río Pichileufú	0.81 ± 0.02	58	12	56.39	<0.0001
MPEF-IC-1393	Dicotyledonous	Río Pichileufú	1.34 ± 0.02	44	10	17.73	<0.0001

285

286

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417

418 **STAR METHODS**

419 Detailed methods are provided in the online version of this manuscript and include the following

420 **RESOURCE AVAILABILITY**421 **Lead contact**

422 Further information and requests for resources and reagents should be directed to the lead contact

423 Eugenia Romero-Lebrón, eugeniaromerolebron@gmail.com

424

425 **Materials availability**

426

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Extant plant leaves (n=144) with Coenagrionidae endophytic eggs	This paper, IMBIV-UNC	N/A
Dicotyledonous	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1367
<i>Myrcia deltoidea</i> Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1368
<i>Celtis ameghenoi</i> Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1370
Dicotyledonous	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1372
<i>Eucalyptus chubutensis</i> Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1373
<i>Cassia argentinensis</i> Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1377
<i>Lomatia occidentalis</i> Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1378
Dicotyledonous	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1380
Dicotyledonous	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1391
Dicotyledonous	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1393

427

428 Data and code availability

429 This published article includes all datasets generated or analyzed during this study.

430

431 EXPERIMENTAL MODEL**432 Fossil material**

433 The available material belongs to the Eocene of the Argentinian Patagonian localities of Laguna del
434 Hunco in Chubut (Ypresian, 52 Ma³²) and Río Pichileufú in Río Negro (Lutetian, 48 Ma³²) (Fig. 1).
435 We studied in detail twenty-four fossil angiosperm leaf specimens (identified by P. Wilf) containing
436 traces of endophytic oviposition with curved pattern (*Paleoovoidus arcuatum sensu Sarzetti et al.*⁶).
437 Fossil remains of several damselfly families have been found in the Eocene of Argentinian
438 Patagonia: Frenguelliidae³³⁻³⁵, Austroperilestidae³⁶, and Synlestidae^{37, 38}. Most of them are
439 discarded as possible candidate producers for these traces because they would oviposit in a straight
440 pattern, but Frenguelliidae would oviposit in a curved pattern. This last attribution is discarded
441 because Sarzetti et al.⁶ and Romero-Lebrón et al.^{19, 39} have studied these fossil materials in
442 particular, and concluded on the basis of detailed morphological studies as well as geometric
443 morphometrics and classical morphometrics that these traces belong to the family Coenagrionidae.
444 The fossil collection is housed in the Museo Egidio Feruglio (MEF collection), Trelew, Chubut
445 Province, Argentina.

446 Extant material

447 One hundred forty-four angiosperm leaves (identified by ER-L) with endophytic oviposition of
448 recent damselflies (Odonata: Zygoptera, Coenagrionidae) were collected from three localities in
449 Córdoba Province, Argentina: Arroyo Los Molles (Río Los Reartes, Intiyaco, 31°56'41.7"S;
450 64°39'05.7"W), Arroyo Los Hornillos (Los Pozos Verdes, Río Ceballos, 31°09'05.7"S; 64°21'11.2"W)
451 and Río Suquía -Río Primero- (Córdoba, 31°24'20.2"S; 64°11'45.6"W) (Fig. 1). Copulation and
452 oviposition behaviors of damselflies were observed and recorded (Cannon 550D, 55–250 mm).
453 Some of the specimens we observed ovipositing in leaves were captured for subsequent taxonomic
454 identification.

455

456 METHODS DETAILS**457 Laboratory methodology**

458 Once oviposition was complete, the leaves were cut, moved to the IMBIV-UNC microscopy
459 laboratory (Córdoba, Argentina), and photographed using a digital camera Olympus DP71 adapted
460 to an Olympus SZX16 stereoscopic microscope.

461 We only considered angiosperm leaves showing ovipositions with a "curved pattern" (*sensu*
462 Romero-Lebrón et al. ²), one egg per incision, eggs *in situ*, and developed plant leaves with
463 identifiable leaf veins (primary and secondary). Stems have not been considered since we cannot
464 compare them with the fossil counterpart.

465 Egg lengths were measured inside the leaf, in their original position, for transparency, instead of
466 removing them from the plant material in order to make direct comparisons with the fossil material.
467 Once photographed, eggs and traces were measured digitally using ImageJ 1.51n.

468

469 **QUANTIFICATION AND STATISTICAL ANALYSIS**

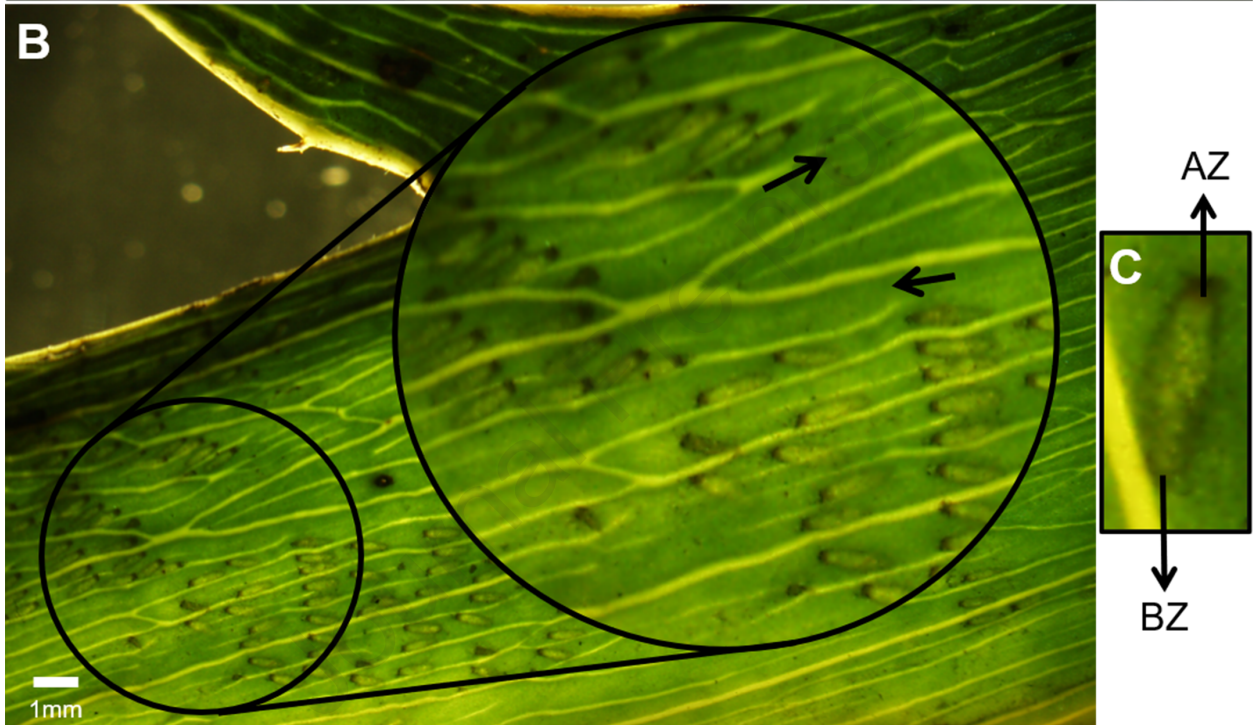
470 **Data analysis**

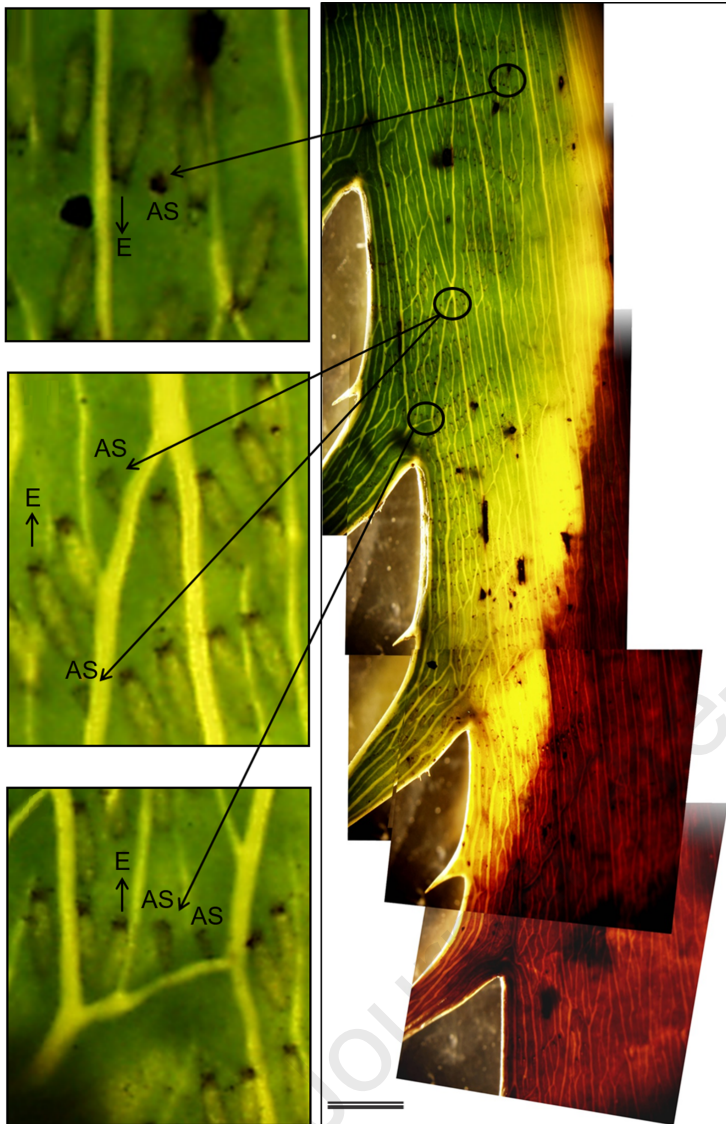
471 We defined "Complete oviposition" as an ovipositor incision that endophytically left an egg and,
472 "Interrupted oviposition" as an ovipositor incision that left a scar without an egg. In order to explore
473 whether this scar was associated with the presence of leaf veins, on each leaf, both fossil and
474 extant, the lengths of the eggs (or traces) were measured (maximum length) using ImageJ 1.51n.
475 For the case of "Interrupted oviposition", the distance from the beginning of the ovipositor's
476 incision scar to the nearest leaf vein was calculated in two distance categories: a) Not crossing: A
477 distance equal to or greater than the mean length of the egg (or trace) was considered as "Egg (or
478 trace) not crossing the leaf veins" and, b) Crossing: A distance less than the mean length of the egg
479 (or trace) was considered as "Egg (or trace) crossing the leaf veins". For "Complete oviposition" it
480 was counted whether the leaf veins were crossed or not. In addition, we incorporate a list of
481 publications mentioning the endophytic insect trace and its position relative to leaf vein
482 (Supplementary information).

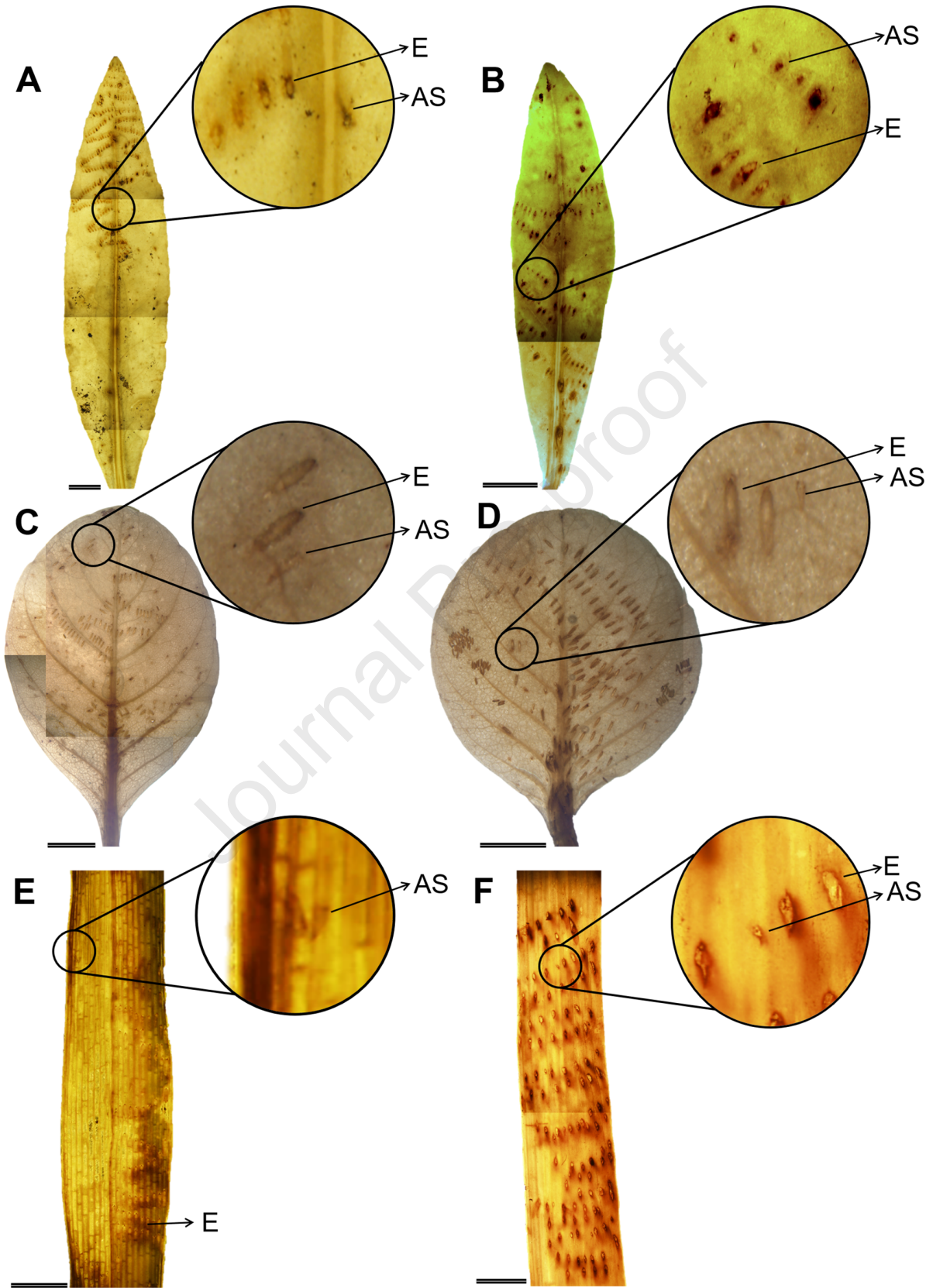
483

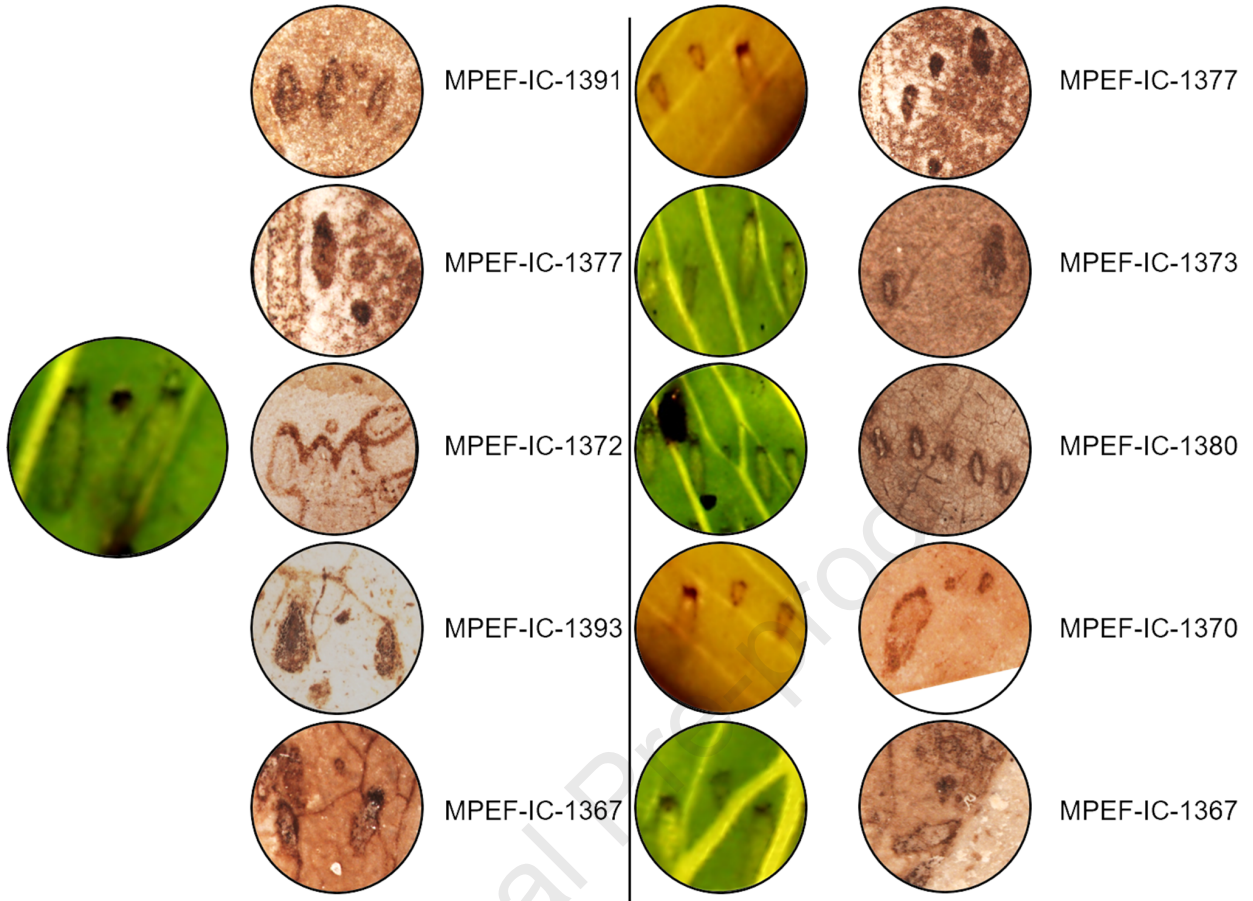
484 To assess whether "Interrupted oviposition" could be explained by the presence of leaf veins, the
485 proportion of "Complete oviposition" versus "Interrupted oviposition" crossing or not crossing a leaf
486 vein was compared using χ^2 test (frequency under independence) using InfoStat (2020 version ⁴⁰).











"Complete oviposition" and "interrupted oviposition" are defined for the first time

Coenagrionidae can detect leaf veins with ovipositor and avoid laying eggs in those cases

Leaf veins may influence egg location, but do not affect the curved oviposition pattern

Coenagrionidae have been performing this behavior for at least 52 million years

Journal Pre-proof