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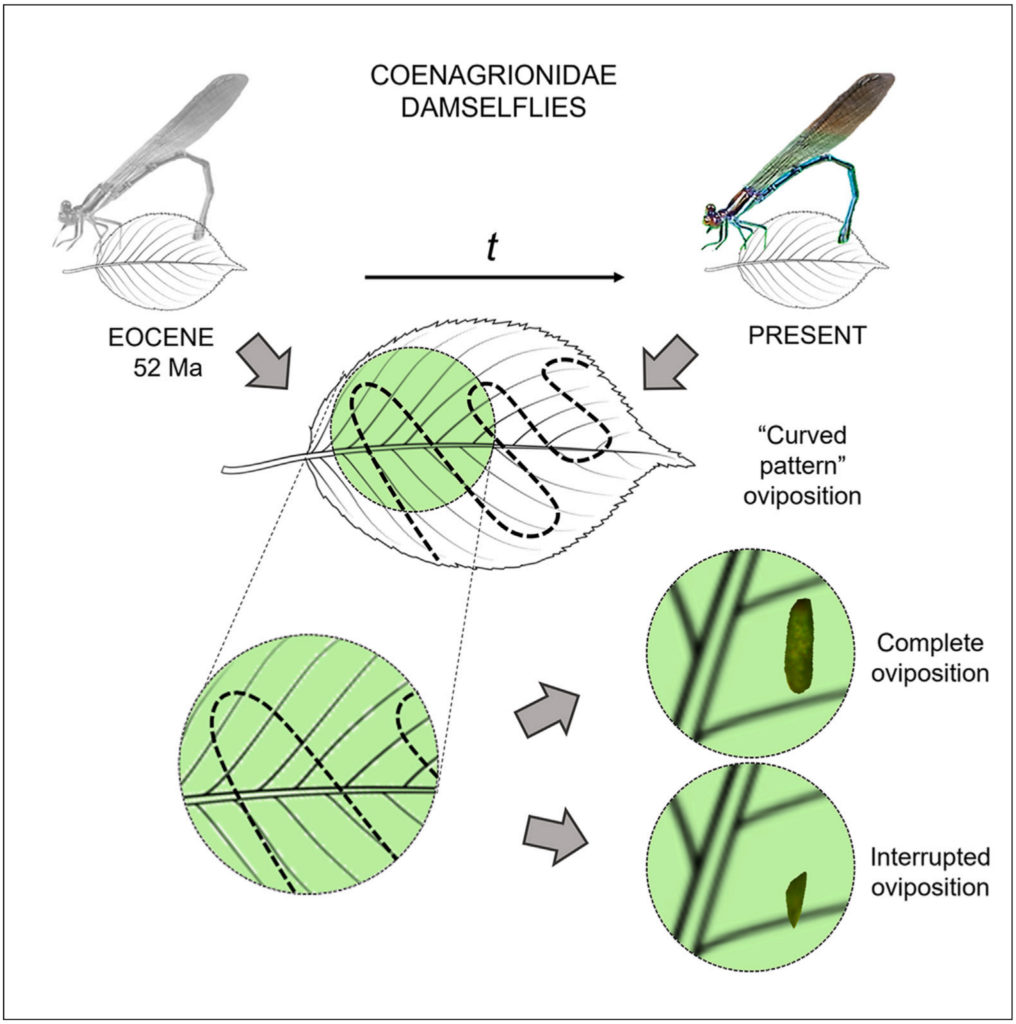

Eugenia Romero-Lebrón, Marcos Fernández-Monescillo, Natalia Matushkina, Xavier Delclòs, Raquel M. Gleiser

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Highlights

"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

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

Eugenia Romero-Lebrón,[1,2,8,*] Marcos Fernández-Monescillo,[2,3] Natalia Matushkina,[4] Xavier Delclòs,[5,6] and Raquel M. Gleiser[1,2,7]

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 1,800 endophytic eggs from recent coenagrionids indicates that these scars were caused by ovipositor incisions, but without egg insertion. The scar correlates ($\chi 2$-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 that Coenagrionidae damselflies (narrow-winged damselflies or pond damselflies) have been avoiding leaf veins for at least 52 million years.

INTRODUCTION

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

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

Odonatoptera is one of the oldest groups of Pterygota (winged insects), and their earliest records date to the later Namurian (Bashkirian/Serpukhovian; Lower Carboniferous), about 320 to 313 Ma[7,8] thereafter, they have a relatively rich fossil record.[9] Evidence for endophytic oviposition of Odonatoptera is known from the Paleozoic (examples in[2,10–13]). Odonata is the only order of Odonatoptera that persists today, whose earliest known records are in the Permian.[14–16] This group is currently very diverse and abundant (about 6,000 living species and 600 extinct species[17]) and represents one of the most charismatic groups of insects in the world. Fossil damselflies egg traces are very similar in morphology and oviposition patterns to those of extant representatives.[10,11,18,19] Endophytic eggs of Odonata are laid in a regular pattern, which is a common feature recorded both in fossils[6,20–25] and in extant species.[26,27] Within damselflies, the family Lestidae is characterized by ovipositing in a straight pattern (Lestidae-Typ *sensu* Hellmund and Hellmund[20]), whereas the family Coenagrionidae is characterized by ovipositing in a curved (or zigzag) pattern (Coenagrionidae-Typ *sensu* Hellmund and Hellmund[20]). Krassilov et al.[23] suggest that oviposition patterns of modern damselflies evolved under the influence of leaf venation pattern evolution, cuticle anisotropy, epidermis, and mesophyll of plant leaves.

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

Endophytic oviposition has been described as avoiding the leaf veins (e.g., Moisan et al.[24]; Donovan et al.[28]; Xu et al.[29]) or being located parallel to the leaf veins (primary and secondary) (e.g., Laaβ and Hoff[11]; Bomfleur et al.[30]; Gnaedinger et al.[31]). In fact, the earliest records of this behavior are mentioned in the Carboniferous (Early Pennsylvanian) by Dernov.[12] All these authors rely on descriptive observations and, so far, there have been no studies on whether this behavior is casual or deliberate.

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

RESULTS

Oviposition behavior

The oviposition behavior of damselflies under natural conditions was observed in females of the Coenagrionidae family. These females were identified using the taxonomic keys illustrated by Garrison et al.[32] and von Ellenrieder and Garrison[33] as *Argia joergenseni* Ris, 1913 and *Acanthagrion lancea* Selys, 1876. Methodology specifications are detailed in the STAR Methods. We describe the oviposition behavior of both species

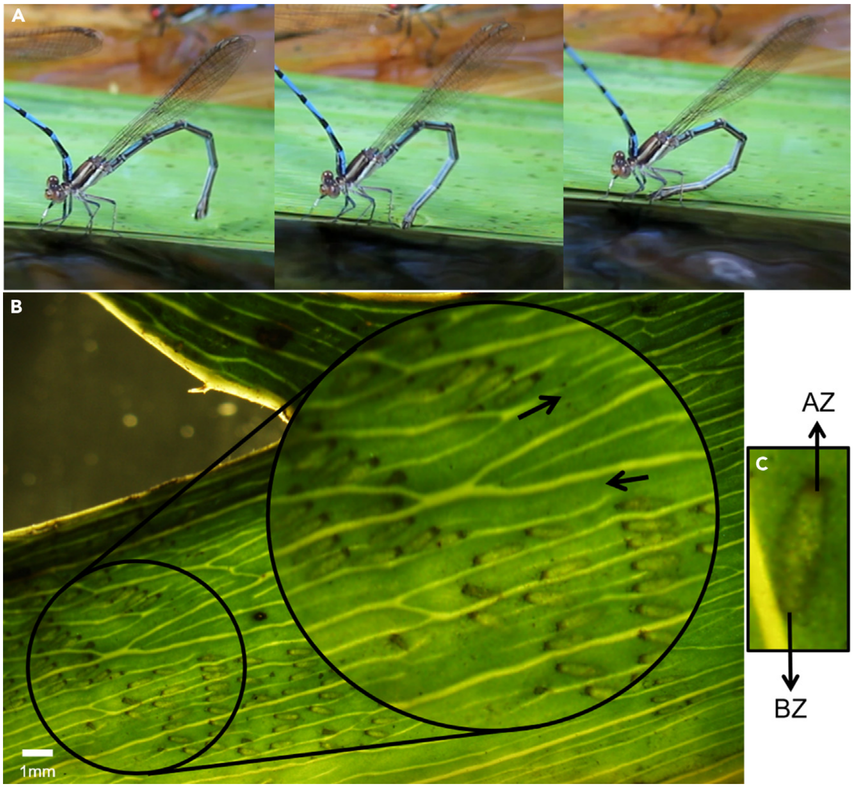

Figure 2. Endophytic oviposition and ovipositor position variation in *Acanthagrion lancea* (Zygoptera, Coenagrionidae)

(A) *Acanthagrion lancea* laying eggs endophytically, folding the abdomen, and varying the ovipositor position.

(B) Extant leaf of *Eryngium agavifolium* (Apeaceae) showing directional change of the egg (black arrows), because of changes in the position of the abdomen and ovipositor of *Acanthagrion lancea*. Scale: 1mm.

(C) Detail of the egg zones. Abbreviation: *AZ*, apical zone (black); *BZ*, basal zone.

as: The female perches on the leaf surface and then makes repetitive movements of the abdomen to the right, left, up, and down, resulting in the deposition of eggs over a wide area of the leaf, shifting her position to the apex or base of the leaf. The female folded her abdomen, contacting the ovipositor with the leaf surface, and touched the substrate numerous times before inserting it. Upon laceration of the plant tissue, the cut valves of the ovipositor began to move, gradually penetrating the tissue and generating a small pocket under the leaf epidermis. When the cutting valves were fully inserted into the plant, the female remained motionless for an instant, moving the tip of her abdomen slowly up and down. Once the pocket was made, she inserted an egg. Then, the female removed the ovipositor and maintaining its position, moved its abdomen laterally, repeating the process, generating a curved row of successive eggs. The average distance between consecutive eggs was 0.5 mm.

The number of eggs per row, and thus the total length of the curved row of eggs, was limited by the length of the damselfly abdomen, because of space restrictions and/or, apparently, by the presence of areas interpreted as undesirable for oviposition, e.g., the proximity of leaf veins, mainly those of large-caliber veins such as the midrib (principal leaf vein). At the beginning of a new row of eggs, the female could remain in the same position and just bend her abdomen, varying the position of the ovipositor in relation to her body (Figure 2A), or she could simply walk a few steps forward. The distance between successive curved rows varied between 1.1 mm and 2.1 mm. This behavior resulted in a curved (or zigzag) pattern.

Initially, the female oviposits with her abdomen extended backwards and the ovipositor behind her hind legs, and once she has oviposited a set of eggs, she folds her abdomen anteriorly under her body, and

Table 1. Extant material analyzed: identification, localities, egg measurements (length average ±standard error) expressed in mm, number of eggs per leaf, number of incisions per leaf, χ^2 (Chi Square) value, and p value

Extant leaf specimen number	Species	Localities	Egg length	Number of eggs per leaf	Number of associated scars per leaf	χ^2 value	p
1	*Eryngium agavifolium* Griseb [a]	Arroyo Los Hornillos	0.77 ± 0.003	385	45	325.44	<0.0001
2	*Eryngium agavifolium* Griseb [a]	Arroyo Los Hornillos	0.81 ± 0.005	108	44	111.17	<0.0001
3	*Eryngium agavifolium* Griseb [a]	Arroyo Los Hornillos	0.84 ± 0.01	39	24	50.89	<0.0001
4	*Polygonum punctatum* Elliott [a]	Arroyo Los Hornillos	0.77 ± 0.01	40	5	6.27	0.0123
5	*Cyperus* sp.[a]	Arroyo Los Hornillos	0.75 ± 0.02	127	3	130	<0.0001
6	*Iris pseudacorus* L. [b]	Arroyo Los Molles	1.65 ± 0.05	82	14	96	<0.0001
7	*Iris pseudacorus* L.[b]	Arroyo Los Molles	1.86 ± 0.03	107	11	106.28	<0.0001
8	*Iris pseudacorus* L. [b]	Arroyo Los Molles	1.96 ± 0.03	51	7	39.87	<0.0001
9	*Ludwigia grandiflora* (Michx.) Greuter & Burdet [a]	Río Suquía	0.69 ± 0.01	150	44	48.48	<0.0001
10	*Ludwigia grandiflora* (Michx.) Greuter & Burdet [a]	Río Suquía	0.73 ± 0.01	120	36	35.04	<0.0001
11	*Ludwigia grandiflora* (Michx.) Greuter & Burdet [a]	Río Suquía	0.69 ± 0.01	84	14	5.33	0.0209
12	*Alternanthera philoxeroides* (Mart.) Griseb [a]	Río Suquía	0.76 ± 0.01	224	39	58.39	<0.0001
13	*Alternanthera philoxeroides* (Mart.) Griseb [a]	Río Suquía	0.74 ± 0.01	79	12	26.56	<0.0001
14	*Alternanthera philoxeroides* (Mart.) Griseb [a]	Río Suquía	0.73 ± 0.01	191	21	76.85	<0.0001

[a] *Acanthagrion lancea*.
[b] *Argia joergenseni*.

sometimes the ovipositor is in front of her front legs (see Figure 2A, and Romero-Lebrón et al.[34]). At this position, she repeats the process of lacerating the tissue and placing an egg in each incision, with the particularity that the eggs are arranged in reverse (Figure 2B) compared to eggs deposited when the ovipositor does not exceed its forelegs.

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

Ovipositor insertion frequency

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

We also identified these associated scars in fossil leaves from the Eocene of Laguna del Hunco and Río Pichileufú, similar to those produced in extant leaves by the "Interrupted oviposition" (Table 2, Figure 5). It is important not to confuse the traces of the ovipositor insertion with traces of bites generated by the mouthparts of sucking insects (piercing and sucking) used to perforate the leaves superficially and thus

Table 2. Fossil material analyzed: identification, localities, trace measurements (length average ±standard error) expressed in mm, number of traces per leaf, number of incisions per leaf, $\chi 2$ (Chi Square) value, and p 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	*Myrcia deltoidea* Berry	Laguna del Hunco	0.84 ± 0.02	46	4	11.26	0.0008
MPEF-IC-1370	*Celtis ameghenoi* 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	*Eucalyptus chubutensis* Berry	Laguna del Hunco	0.84 ± 0.04	28	8	13.71	0.0002
MPEF-IC-1377	*Cassia argentinensis* Berry	Laguna del Hunco	0.98 ± 0.03	30	12	22.4	<0.0001
MPEF-IC-1378	*Lomatia occidentalis* 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

feed on internal fluids.[35] These types of traces are circular to oval in shape, with an average diameter of 0.3 mm, whose extreme values reach 1.2 mm in diameter[1] and frequently appear above the leaf veins.[36]

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

DISCUSSION

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

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

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

Several substrate characteristics play important roles in the choice of oviposition sites. In natural observations, some factors driving oviposition site selection are foliage stiffness, tissue thickness and trichome density, which provide clues about desiccation risk, accessibility to trophic resources, or predation risk

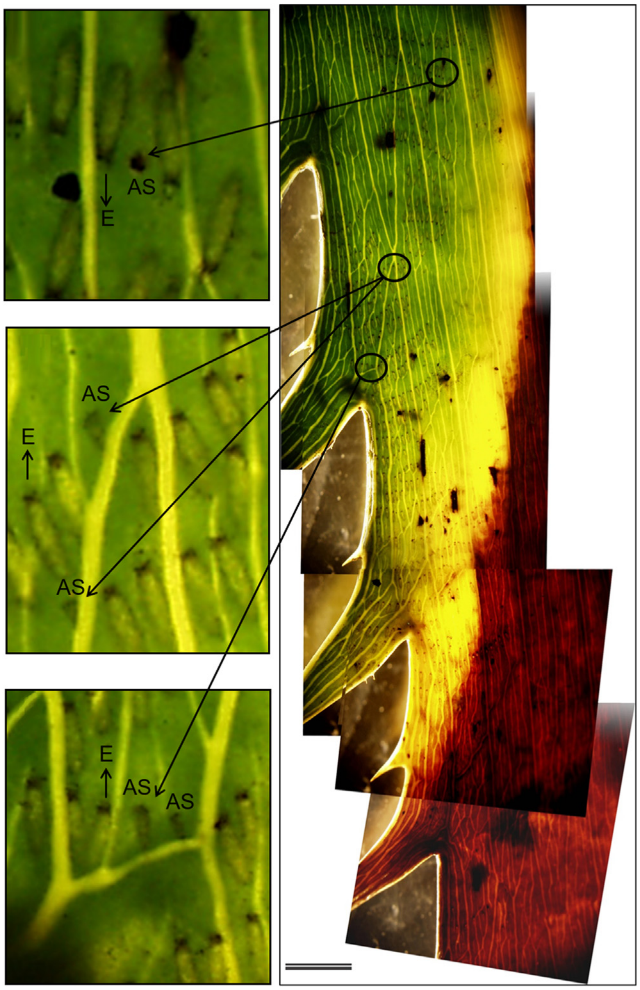

Figure 3. Extant leaf of *Eryngium agavifolium* (Apeaceae) showing the associated scar generated by the insertion of the ovipositor of *Acanthagrion lancea* into the leaf tissue without laying eggs
AS, associated scar; *E*, egg. Scale: 5 mm.

for their progeny, among others (e.g., Pasquier-Barre et al.[37,38]; Griffin and Yeargan[39]; Lundgren et al.[40]). Tissue stiffness may be a key factor in the selection of oviposition substrates, as has been demonstrated in some insects with endophytic oviposition, such as odonatans or hemipterans (e.g., Matushkina and Gorb[26]; Grunert[41]; Constant et al.[42]). In Zygoptera, exists a direct correlation between the stiffness of the ovipositor and the stiffness of their preferred plant substrates.[26,27] Occasionally, in Coenagrionidae it has been observed (pers. obs.) that when the substrate is soft enough, or when oviposition occurs at an early stage of leaf development, the veins are not necessarily an impediment, and may even represent the only place on the leaf surface stiff enough to hold the eggs and, therefore, substrate stiffness is a vitally important phenomenon to be considered.

Other factors besides stiffness, such as the distance to the point of convergence of the leaf veins, play a role in the choice of oviposition site.[42] In Zygoptera, the female's decision to oviposit is not only based on the successful penetration of plant tissues but also on other factors, such as plant anatomy.[26,27] Different structures of the ovipositor are endowed with sensory organs with which females would be able to recognize the stiffness that occurs in the ovipositor during penetration into the plant tissue, and thus determine the suitability of the oviposition site.[43–45] Thus, during oviposition, Zygoptera females can detect the proximity of leaf veins at the time of insertion and adjust the position of the egg in relation to the position of the leaf vein (or fibers) during tissue penetration.[44] Avoidance of

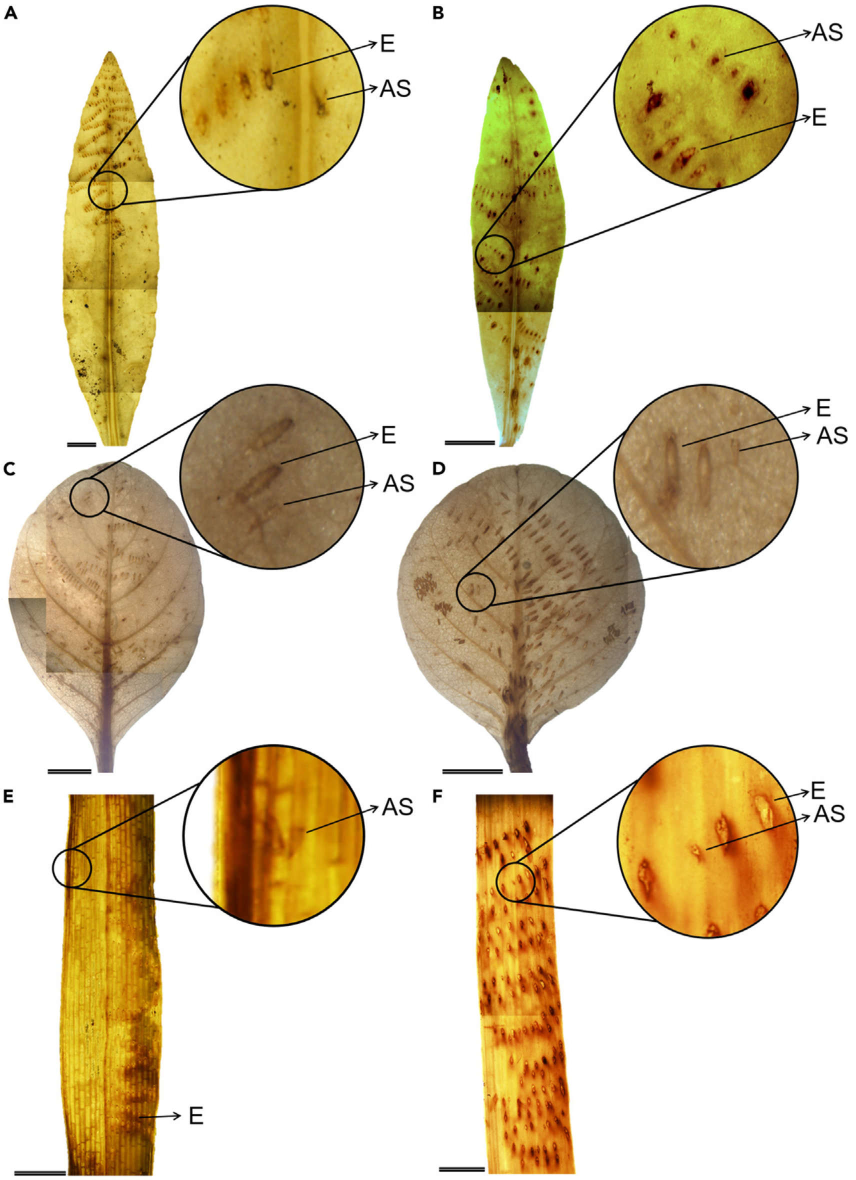

Figure 4. Extant leaves showing eggs and associated scars generated by the insertion of the ovipositor into the leaf tissue without laying eggs

(A and B) *Alternanthera philoxeroides*.

(C and D) *Ludwigia grandiflora*.

(E) *Cyperus* sp.

(F) *Iris pseudacorus*. Abbreviation: *AS*, associated scar; *E*, egg. *Acanthagrion lancea* eggs in A, B, C, D, and E; *Argia joergenseni* eggs in F. Scale: 5 mm.

undesirable areas is necessary for normal embryo development.[46] Therefore, orienting the egg with respect to the substrate fibers (without crossing a leaf vein) would decrease the damage to the eggs.[44]

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

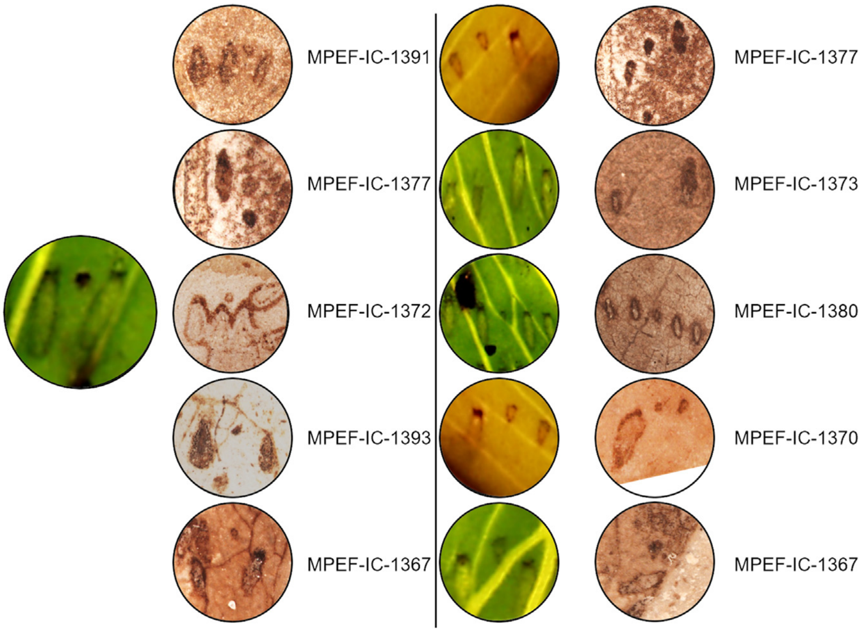

Figure 5. Comparison between extant (first and third columns) and fossil (second and fourth columns) leaves of the eggs/traces and the associated scar generated by the insertion of the ovipositor into the leaf tissue without laying eggs

pattern of oviposition in Coenagrionidae damselflies would be a fixed and specific behavior on a large scale, the leaf veins and the stiffness of the substrate used would generate a smaller-scale influence, specific to the location of each egg.

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

Conclusions

Five conclusions can be drawn from this study.

1) We define ''Complete oviposition'' and ''Interrupted oviposition''. Scars produced by the ovipositor without eggs are reported for the first time, revealing that such scars can fossilize and were previously unrecognized as such.
2) Each insertion of the ovipositor generates a scar in the leaf (plant substrate) with (''Complete oviposition'') or without (''Interrupted oviposition'') egg insertion.
3) Female damselflies of the Coenagrionidae family may apparently detect the proximity of a leaf vein when inserting their ovipositor into plant tissue, and in such cases, they avoid laying eggs. However, this behavior is highly dependent on the stiffness of the substrate.
4) Leaf veins do not generally influence the curved oviposition pattern of Coenagrionidae damselflies, but they may influence the individual location of some eggs.
5) Scars generated by the damselfly ovipositor to avoid leaf veins have also been observed in Eocene materials from Patagonia, Argentina, suggesting that females would have been performing this behavior for at least 52 million years ago.

Limitations of the study

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

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - ○ Lead contact
  - ○ Data and code availability
- MATERIALS AVAILABILITY
  - ○ Fossil material
  - ○ Extant material
- METHOD DETAILS
  - ○ Laboratory methodology
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - ○ Data analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106865.

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

The authors declare that they have no conflicts of interest.

INCLUSION AND DIVERSITY

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

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
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
Myrcia deltoidea Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1368
Celtis ameghenoi Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1370
Dicotyledonous	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1372
Eucalyptus chubutensis Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1373
Cassia argentinensis Berry	Museo Egidio Feruglio collection, Trelew, Chubut, Argentina	MPEF-IC-1377
Lomatia occidentalis 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

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact Eugenia Romero-Lebrón, eugeniaromerolebron@gmail.com.

Data and code availability

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

MATERIALS AVAILABILITY

Fossil material

The available material belongs to the Eocene of the Argentinian Patagonian localities of Laguna del Hunco in Chubut (Ypresian, 52 Ma[47]) and Río Pichileufú in Río Negro (Lutetian, 48 Ma[47]) (Figure 1). We studied in detail twenty-four fossil angiosperm leaf specimens (identified by P. Wilf) containing traces of endophytic oviposition with curved pattern (*Paleoovoidus arcuatum sensu* Sarzetti et al.[6]). Fossil remains of several damselfly families have been found in the Eocene of Argentinian Patagonia: Frenguelliidae,[48–50] Austroperilestidae,[51] and Synlestidae.[52,53] Most of them are discarded as possible candidate producers for these traces because they would oviposit in a straight pattern, but Frenguelliidae would oviposit in a curved pattern. This last attribution is discarded because Sarzetti et al.[6] and Romero-Lebrón et al.[19,34] have studied these fossil materials in particular, and concluded on the basis of detailed morphological studies as well as geometric morphometrics and classical morphometrics that these traces belong to the family Coenagrionidae. The fossil collection is housed in the Museo Egidio Feruglio (MEF collection), Trelew, Chubut Province, Argentina.

Extant material

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

METHOD DETAILS

Laboratory methodology

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

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

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

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis

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

To assess whether "Interrupted oviposition" could be explained by the presence of leaf veins, the proportion of "Complete oviposition" versus "Interrupted oviposition" crossing or not crossing a leaf vein was compared using χ^2 test (frequency under independence) using InfoStat (2020 version[54]).