NAWI-S-24-00165 Rev1

Discovery of a novel ejaculation mechanism in danceflies (Diptera: Empididae), with implications for genital elongation

Tomoki Hirose • Kazunori Yoshizawa*

Systematic Entomology, School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

*correspondence: psocid@agr.hokudai.ac.jp

Abstract

Genitalia are known to evolve rapidly and are among the most variable structures in insect morphology, making them a target of active research. However, function and evolutionary significance of internal genital structures remain less well understood. Here, we report the morphology and mechanism of a novel ejaculatory system that has evolved in the dancefly genus *Rhamphomyia* (Insecta: Diptera: Empididae). Using synchrotron μCT technology, we examined male genitalia of five dancefly species and identified an ejaculatory system resembling a leverage hydraulic jack, which is thought to have derived from a plunger-like pumping system. This jacking system amplifies the applied muscle power by up to 4.2 times, allowing the system to produce the same pumping power with much smaller muscles. However, the volume of the pumping muscle in the jacking system is comparable to that of the plunger system, indicating a significant increase in ejaculation power in this genus. We hypothesize that the greater pumping power evolved through sexual selection favoring strong ejaculation to rapidly pass semen through a thin and elongated phallus and spermathecal duct.

Keywords sperm pump • evolutionary novelty • lever system • genitalia • sexual selection

Introduction

The lever system is highly effective, especially when the effort arm (EA) is longer than the load arm (LA), as it amplifies the applied force according to the ratio of EA to LA. This power efficiency has led to the widespread use of lever systems in human tools, such as scissors, can openers, and hydraulic jacks. Lever systems are also commonly observed in insects, as in mechanisms like wing flapping hinges, jaw chewing apparatuses, and leg articulations (Chapman 1998). These systems are thought to have evolved simultaneously with the origin of these structures. There are instances of existing lever systems being strengthened and achieved new function, as seen in the wasp's abdomen and the beetle's horn (Hashimoto 1996; Weber et al. 2023). However, if not entirely absent, it is a very rare phenomenon that a pre-existing mechanism is completely replaced by a lever mechanism in insects while retaining the original function.

Male flies (Diptera) possess a pumping structure at the base of the phallus, known as a sperm pump, which acts as an ejaculator of semen. The sperm pump comprises a cylindrical sperm sac and an apodeme, or the ejaculatory apodeme. The ejaculatory apodeme, an endoskeleton projecting anteriorly from the sperm sac, provides an attachment site for muscles that generate the pumping power necessary for ejaculation. In its ancestral form, the ejaculatory apodeme moves like a piston that directly compresses the sperm sac like plunger (Figs. 1A, 2A) (Downes 1965; Ovchinnikova 1989). Two or three pairs of muscles attached to the ejaculatory apodeme are aligned nearly parallel to its pushing axis, acting as direct compressors of the ejaculatory apodeme. Because there is no muscle functioning as a decompressor, the compressed sac presumably returns to its original condition by its own resilience (Downes 1965; Ovchinnikova 1989).

Here, we report the morphology and function of a novel lever-jacking system identified in the sperm pump of a genus of danceflies (Empididae), a family representing a basal lineage of the higher Diptera (suborder Brachycera). Using μCT analyses, we confirmed that, except for species of a particular group, two pairs of muscles attached to the ejaculatory apodeme are aligned nearly parallel to its pushing axis (Trehen 1961, 1962). These muscles likely act as direct compressors as has been reported in both lower and higher Diptera (Downes 1965; Ovchinnikova 1989). This suggests that these empidid species retain a pumping system similar to the dipteran ground plan condition and have no decompressor muscles.

In contrast, the novel pumping system we identified involves a lever mechanism, where one muscle pair functions as a compressor and the other as a decompressor. Further modifications similar to a hydraulic jack were also detected. We examined the sperm pump structures and associated muscles of five empidid species to elucidate the evolutionary novelties related to the

origin of this lever-jacking system. Additionally, we discuss the evolutionary background of the novel ejaculatory system in relation to genital elongation in danceflies.

Material and methods

The following five dancefly species (Insecta: Diptera: Empididae) were subjected for the present examinations. Tribe Empidini: *Rhamphomyia* (*Calorhamphomyia*) *longistigma*, *Rhamphomyia* (*Rhamphomyia*) sp. (*sulcata* group), *Empis* (*Euempis*) sp. (*tessellata* group), and *E.* (*Polyblepharis*) *multinodosa*; tribe Hilarini: *Hilara* sp. The subgeneric name, with an abbreviated generic name, is used to refer to each species in the following lines. Samples were fixed with hot water then preserved in 80% ethanol. Samples were dehydrated with 80–100% ethanol in ascending order before critical point drying (EM CPD300, Leica, Wetzlar, Germany). Samples (one specimen for each species) were scanned using synchrotron μCT at the BL20XU or BL20B beamelines (Uesugi et al. 2012) of the Super Photon ring-8 GeV (SPring-8: Hyogo, Japan). We used ITK-SNAP 3.6 (Yushkevich et al. 2006) to obtain 3D representations. Muscle volume was calculated based on the pixel numbers in the reconstructed 3D models. The length of the hind femur was used as an index of body size. The pushing axis was defined as a line connecting the anterior meeting point of the M1/M2 muscles and the middle of the base of the apodeme (for the direct compressing system) or the tip of the load arm (for the lever systems). The power direction produced by each muscle was estimated by connecting the midpoint of its origination and insertion sites. A detailed account of the methods is given in the electronic supplementary material. Raw data and reconstructed 3D model of all species examined are available from FigShare at DOI: 10.6084/m9.figshare.26377348.

Results and discussion

The empidid sperm pump is composed of the following elements in all five species (Fig. 1): the enlarged sperm sac (ss) connected dorsally to the testis via a tube (tu) and ventrally to the inner tube of aedeagus (ad); the ejaculatory apodeme (ea) extending from the anterior surface of the sperm sac and consisting of a vertical and a horizontal plate, forming a cross-section a "+" shape; and two sets of muscles (dorsal M1 and ventral M2) originating from the ejaculatory apodeme (both vertical and horizontal plates) and attaching to the 9th sternum extending and continuing to the sperm sac. These structural and muscular characters (Fig. 1) are consistent with previous observations of the other

empidid species (Trehen 1961, 1962; Ulrich 1972).

In all species except for those in the genus *Rhamphomyia* (subgenera *Rhamphomyia* and *Calorhamphomyia*), the ejaculatory apodeme extends anteriorly, with its base tightly united with the sperm sac (Fig. 1A). Both ejaculatory muscles are large and comparable in volume (ca. 1:1 to 2:1 in M1:M2 volume ratio: Table 1) and are arranged nearly parallel to the pushing axis of the ejaculatory apodeme (Fig. 1A). The similarity in volume and nearly symmetrical arrangement of the two ejaculatory muscles suggests they work together to provide direct compressing power to the sperm sac, functioning like a plunger (a direct compressing system, or DC system: Fig. 2A). No decompressor muscle was detected in the DC system (Fig. 1A), suggesting that the sperm sac returns to its original state by the resilience of the sac sclerite (Downes 1965; Ovchinnikova 1989). This empidid ejaculation system agrees with the ancestral plunger-like mechanism of the dipteran sperm pump (Downes 1965; Ovchinnikova 1989). Lever-like modifications of the ejaculatory apodeme have been previously mentioned as an autapomorphy of the superfamily Empidoidea, without examining muscle conditions (Cumming et al. 1995; Sinclair 2000; Sinclair and Cumming 2006). However, our present examinations revealed that at least some dancefly species (representing two tribes, Empidini and Hilarini) retain the dipteran ancestral pumping system.

In contrast, the M2 muscle in *R.* (*Rhamphomyia*) is significantly smaller than M1 (approximately 1/9 in volume; Table 1) and is arranged nearly perpendicular to the M1 muscle (Fig. 1B). The M1 muscle is nearly parallel to the pushing axis of the apodeme, but the M2 muscle is arranged at a right angle to this axis (Fig. 1B), indicating that it cannot function as a direct compressor. In the examined specimen, the sperm sac was almost completely compressed, but the base of the aedeagal tube (the articulation point) remained nearly undeformed (Fig. 1B). The reconstructed 3D model showed that the apodeme is united to the sperm sac, and there is an articulation between the posteroventral corner of the apodeme and the base of the inner tube of aedeagus (asterisk in Fig. 1B). These muscular and structural features suggest that the apodeme functions as a lever (the lever-compressing system: LC), with the articulation serving as a fulcrum, and that M1 acts as a compressor while M2 functions as a decompressor (Fig. 2B). The ejaculatory apodeme (effort arm: EA) and its base (load arm: LA) are arranged in an L-shape, with the articulation point at the corner (a type 1 lever: Figs. 1B, 2B). The formation of the LC system required not only the structural innovations mentioned above but also changes in the muscle control system (e.g., impulse pattern), transitioning from synchronous M1-M2 compressions to alternating compressions (Fig. 2).

The EA to LA length ratio in *R.* (*Rhamphomyia*) is approximately 1:1.25 (Fig. 1B). A lever system can amplify pumping power according to EA/LA. However, in *R.* (*Rhamphomyia*), EA/LA

< 1, so this system does not amplify muscle power. Nevertheless, the LC system in *R.* (*Rhamphomyia*) is probably more efficient mechanically than the DC system of other danceflies or dipterans. The absence of a decompressor muscle in the DC system implies that the sperm sac must have strong resilience to recover from compression. In contrast, the LC system includes a decompressor muscle, eliminating the need for strong resilience in the sperm sac. Compressing a highly resilient sperm sac requires much greater muscle power than compressing a sac with low resilience, likely making the LC system in *R.* (*Rhamphomyia*) more efficient mechanically compared to the DC system in other dipterans (Downes 1965; Ovchinnikova 1989).

Further modification of the ejaculatory system, likely evolved via the LC system, was detected in *R.* (*Calorhamphomyia*) (Fig. 1C). The ejaculatory apodeme extends anteroventrally (Fig. 1C) rather than anteriorly (Fig. 1AB), with its base separated from the sperm sac by a membranous region (Supplementary Movie), except for the ventral tip firmly articulated with the base of the aedeagal tube (asterisk in Fig. 1C). The apodeme also lacks the lower half of the vertical plate (attachment site of the M2 muscle), while the horizontal plates extend ventrolaterally, forming a cross-section resembling an inverted "Y" shape. Two sets of muscles were confirmed, but the M2 muscle is extremely reduced in volume (approximately 1/370 of M1: Fig. 1C, Table 1) and attaches to the lower crotch of the inverted Y-shaped apodeme (Fig. 1C). As in *R.* (*Rhamphomyia*), M2 does not function as a compressor but is assumed to act as a decompressor (Fig. 1C, Supplementary Movie). Compression by M1 causes a dorsal shift of the apodeme, but due to its separation by a membrane, it does not modify the sperm sac itself (Supplementary Movie). Instead, the base of the apodeme surrounded by a membrane pushes the semen, like a hydraulic jack (a lever-jacking system, or LJ system). In this species, the effort arm is much longer than the load arm, and the pumping power produced by this LJ system can be estimated as approximately 4.2 times the applied muscle force (Fig. 1C). Additionally, the membrane surrounding the base of the apodeme allows the jacking system to operate with lesser power than the compressing systems. Our experiments also showed that the apodeme of *R.* (*Calorhamphomyia*) can be moved very easily and has little resiliency (Supplementary Movie). This suggests that the LJ systems can generate more effective pumping power with much smaller muscles compared to the DC or LC systems.

However, despite the power efficiency of LC and LJ systems compared to the DC system, the total muscle volume of the sperm pump system in *R.* (*Rhamphomyia*) (LC system) and *R.* (*Calorhamphomyia*) (LJ system) is approximately comparable to (or even larger than) that of other danceflies with the DC system (Fig. 3). This implies that *R.* (*Rhamphomyia*) and *R.* (*Calorhamphomyia*) ejaculate semen with much greater force than other empidids. We assumed two possibilities which likely interact to drive the evolution of leverage pumping systems: (1) The

aedeagus of *R.* (*Rhamphomyia*) and *R.* (*Calorhamphomyia*) is thinner, longer, and more sinuous compared to that of other danceflies examined (Fig. 2CD). Semen is a viscous liquid that requires significant force to be pumped through a thin and long tube, necessitating greater muscle power (Matsumura et al. 2019). Elaborate genital structures are generally thought to be formed as a result of sexual selection (Eberhard 1985). In danceflies and other dipterans, the length of the female's spermathecal duct typically corresponds to that of the male aedeagus (Downes 1965; Akbar et al. 2022), suggesting a coevolutionary relationship between male and female genitalia. This further implies that the elongated and sinuous aedeagus of *R.* (*Rhamphomyia*) and *R.* (*Calorhamphomyia*) likely evolved due to sexual selection. If seminal viscosity acts as a physical constraint on genital evolution, the formation of lever-type ejaculatory mechanisms may have enabled males to respond to sexual selection favoring elongated and sinuate aedeagus; (2) Strong pumping power itself might be favored and evolved through sexual selection. Many dancefly females only allow males to copulate while they are eating a nuptial gift (Svensson et al. 1990). In the hangfly *Hylobittacus apicalis* (Mecoptera), females also accept mating only during the consumption of a nuptial gift. It has been reported that the narrow and elongated spermathecal duct in this hangfly hinders rapid sperm transfer from males, and only males offering a large prey item can transfer enough sperm to ensure their paternity (Thornhill 1976). If this is also the case for danceflies, strong sperm pumping may be also advantageous for males to transfer sperm quickly and certainly.

The lever-like ejaculatory apodeme was once considered an autapomorphy of Empidoidea (Cumming et al. 1995; Sinclair 2000; Sinclair and Cumming 2006). Therefore, although the lever system was only detected in the genus *Rhamphomyia* in this study, it might be more widely distributed in the superfamily. For example, genital photographs of *R.* (*Pararhamphomyia*) showed a pumping system similar to LJ system (Barták et al. 2015), which has been confirmed here only in *R.* (*Calorhamphomyia*). Observing the sperm pump system in a wide variety of danceflies is necessary to uncover the diversity, transitional patterns, and evolutionary background of the leverage pumping system. The mating system of danceflies are also highly diverse and complex, including swarming, nuptial gifting, active multiple couplings by female, and sex-role reversals (e.g., Svensson et al. 1990; Funk and Tallamy 2000). These factors are crucial for understanding genital evolution and sexual selection in danceflies. Establishment of a robust phylogeny for Empididae is also a key to elucidating morphological evolution and its relation to the behavioral traits within this family. It also remains unclear why the evolution of a highly efficient lever system did not lead to a reduction in the volume of pumping muscles, necessitating detailed functional and behavioral analyses. While the evolution of elaborate genitalia as a consequence of sexual selection is a very actively studied area, the relationship between the structural evolution and the ejaculating muscles and/or seminal hydrodynamics has less attracted (Matsumura et al. 2019). It should also be

noted that female dipterans also have a sperm uptake system at their spermathecal duct (Cumming and Wood 2017). The evolution of empidid genital structures could provide intriguing insights into the physical constraints on genital evolution.

Acknowledgements.

We thank Toyohei Saigusa for continuous guidance on dipterology, Yoko Matsumura for discussion, and Kentaro Uesugi, Akihisa Takeuchi and Masayuki Uesugi for technical support at SPring-8. Research at SPring-8 was approved through project numbers 2022A1201 and 2023A1173. This study was partly supported by JSPS Grant 19H03278 and 24K02080 to KY.

Competing interests. The authors declare no competing of interests.

References

Akbar SA, Kanturski M, Barták M, Wachkoo AA, Maqbool A (2022) SEM studies and discovery of an intriguing new *Rhamphomyia* (*Pararhamphomyia*) (Diptera, Empididae, Empidinae) species from the Kashmir Himalayas. Eur Zool J 89:1325–1350.

Barták M, Kubík S (2015) Palaearctic species of *Rhamphomyia* (*Pararhamphomyia*) *anfractuosa* group (Diptera, Empididae). ZooKeys 514:111–127.

Chapman RF (1998) The Insects, Structure and Function. Cambridge Univ Pr, Cambridge

Cumming JM, Sinclair BJ, Wood DM (1995) Homology and phylogenetic implications of male genitalia in Diptera–Eromoneura. Entomol Scand 26:121–151.

Cumming JM, Wood DM (2017) Adult Morphology and Terminology. In: Kirk-Spriggs AH, Sinclair BJ (eds) Manual of Afrotropical Diptera. Vol 1. South African National Biodiversity Institute, Pretoria, pp 89–134.

Downes JA. 1965 Note on the organs and processes of sperm-transfer in the lower Diptera. Can Entomol 100:608–617.

Eberhard WG (1985) Sexual selection and animal genitalia. Harvard University Press, Cambridge

Funk DH, Tallamy DW (2000) Courtship role reversal and deceptive signals in the long-tailed

dance fly, *Rhamphomyia lingicauda*. Anim Behav 59:411–421.

Hashimoto Y (1996) Skeletomuscular modifications associated with the formation of an additional petiole on the anterior abdominal segment in aculeate Hymenoptera. Jpn J Entomol 64:340–356.

Matsumura Y, Michels J, Rajabi H, Shimozawa T, Gorb SN (2019) Sperm transfer through hyper-elongated beetle penises – morphology and theoretical approaches. Sci Rep 9:10238.

Ovchinnikova OG (1989) Muskulatura genitaliy samtsov dvurylykh Brachycera-Orthorrhapha (Diptera). Trudy Zool Inst Akad Nauk SSSR 190:1–160.

Sinclair BJ (2000) Morphology and terminology of Diptera male terminalia. In Contributions to a Manual of Palaearctic Diptera, volume 1. Papp L, Darvas B (eds) Science Herald, Budapest, pp 53–74

Sinclair BJ, Cumming JM (2006) The morphology, higher-level phylogeny and classification of the Empidoidea. Zootaxa 1180:1–172.

Svensson BG, Petersson E, Frisk M (1990) Nuptial gift size prolongs copulation duration in the dance fly *Empis borealis*. Ecol Entomol 15:225–229.

Thornhill R (1976) Sexual selection and nuptial feeding behavior in *Bittacus apicalis* (Insecta: Mecoptera). Am Naturalist 110:529–548.

Trehen P (1961) Contribution a l'étude de morphologique des segments génitaux du mâle d'*Hilara maura* Fab. (Dipteères Empidides). Bull Soc Zool France 86:285–295.

Trehen P (1962) Contribution a l'étude de l'anatomie de l'hypopygium dans la famille des Empidinae (Diptères-Empidides). Bull Soc Zool France 87:498–508.

Uesugi K, Hoshino M, Takeuchi A, Suzuki Y, Yagi N (2012) Development of fast and high throuput tomography using CMOS image detector at SPring-8. Developments in X-Ray Tomography VIII, Proc SPIE 8506:850601.

Ulrich H (1972) Zur Anatomie des Empididen-Hypopygiums (Diptera). Veröff Zool Staatssamml München 16:1–28.

Yushkevich PA, Piven J, Hazelett HC, Smith RG, Ho S, Gee JC, Gerig G (2006) User-guided 3D active contour segmentation of anatomical structures: Significantly improved efficiency and reliability. NeuroImage 31:1116–1128.

Weber JN, Kojima W, Boisseau RP, Niimi T, Morita S et al. (2023) Evolution of horn length and

lifting strength in the Japanese rhinoceros beetle *Trypoxylus dichotomus*. Curr Biol 33:4285–4297.

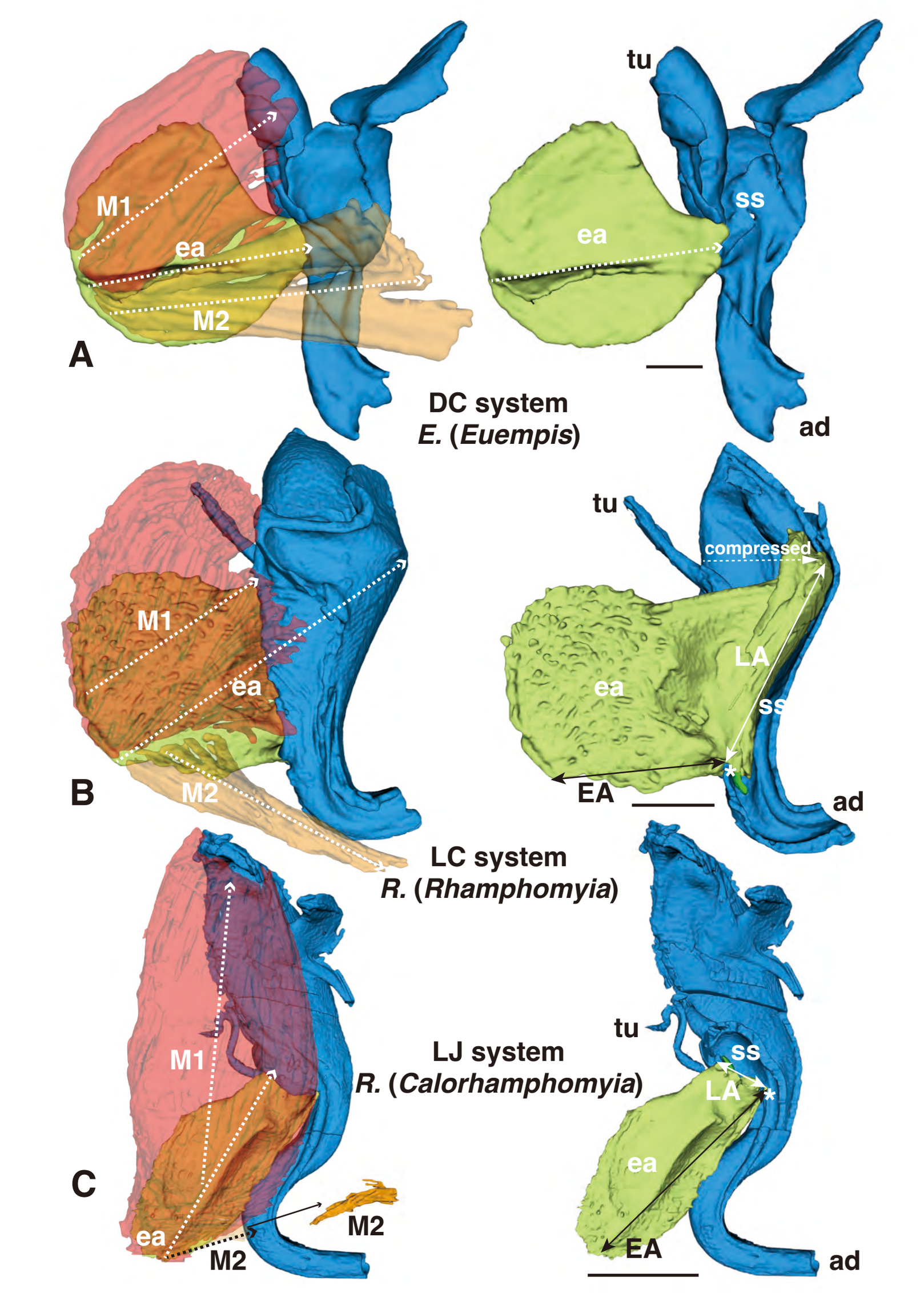
Fig. 1. The sperm pump of (A) *E*. (*Euempis*), (B) *R.* (*Rhamphomyia*), (C) *R.* (*Calorhamphomyia*). Left ones show full structure (muscles in half-translated), and right ones omit the muscles and the foreground sac sclerite. Dotted arrow indicates the direction of the compression of each muscle or pushing direction of the ejaculatory apodeme. Abbreviations: * – fulcurum; ad – aedeagal tube; ea – ejaculatory apodeme; EA/LA – effort/load arm; M1/2 – muscle #1/#2; ss – sperm sac; tu – tube to the testis. Scale = 0.1 mm.

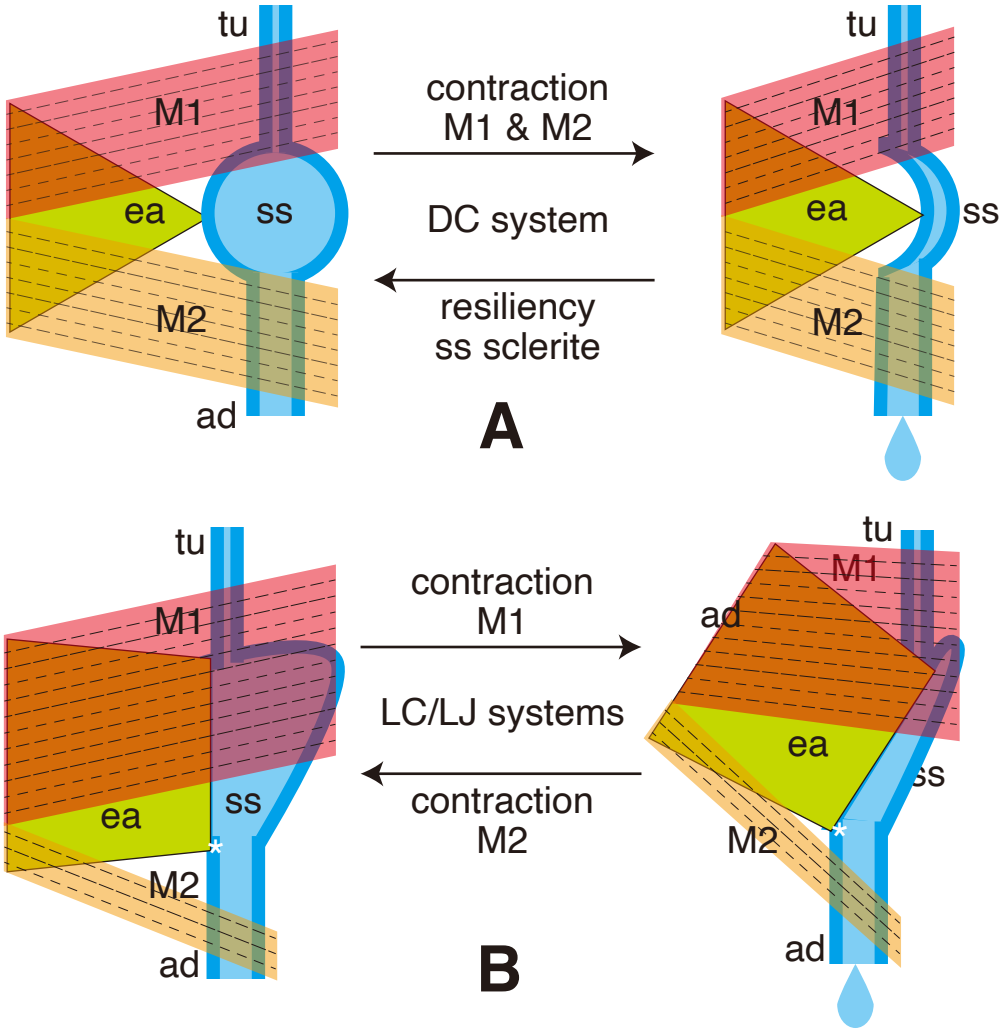
Fig. 2. (AB) Schematic illustrations showing the mechanism of (A) the direct compressing (DC) and (B) leverage compressing/ leverage jacking systems (LC/LJ). Left ones show relaxed condition and right ones show compressed condition. Note that LC and LJ are mainly differed in the ejaculatory apodeme connected with (LC) or separated from (LJ) the sac sclerite. See Fig. 1 for abbreviations. (CD) The male terminalia of (C) *E.* (*Euempis*) (with simple aedeagus and DC system) and (D) *R.* (*Calorhamphomyia*) (with elongated and sinuate aedeagus and LJ system). Blue arrow heads indicate the tip of aedeagus.

Fig. 3. Relationships between the hind femur length and the sperm pumping muscle volume of each species examined.

Table 1. Measurements of the sperm pump muscles and hind femur.

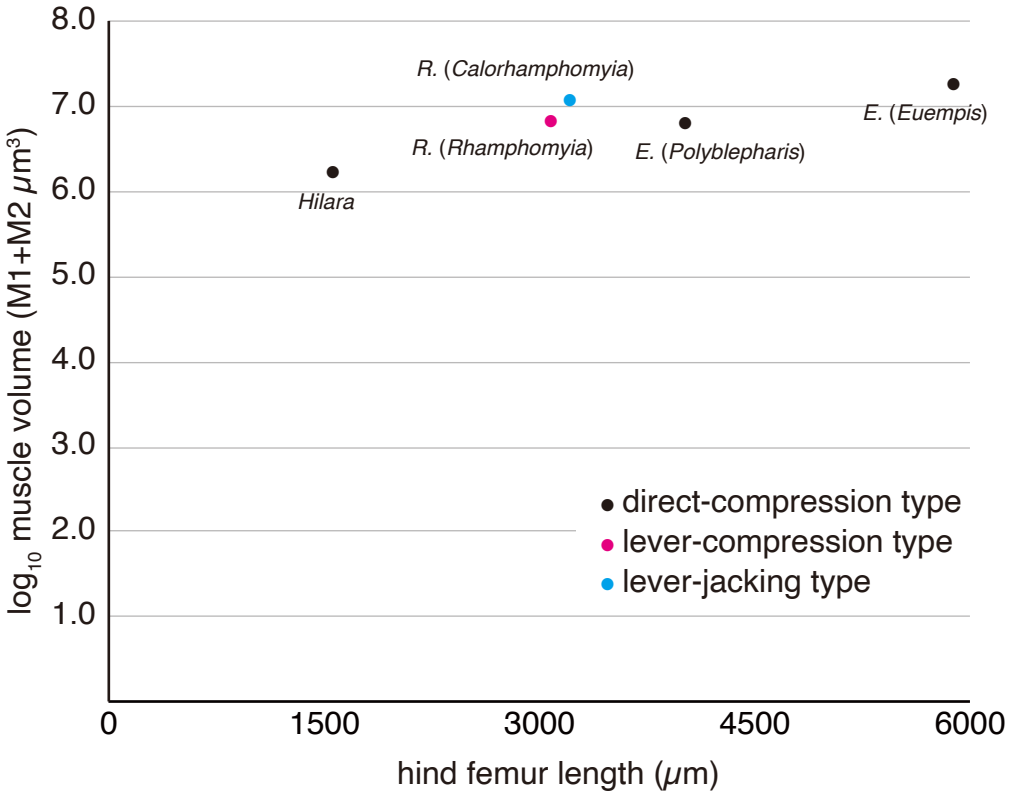
Supplementary Movie. Manipulation of the lever-jacking sperm pumping system of *Rhamphomyia* (*Calorhamphomyia*) *longistigma.*


A
M1
ea
M2
tu
ss
ea
ad
DC system
E. (Euempis)
B
M1
ea
M2
tu
compressed
LA
ea
ss
*
EA
ad
LC system
R. (Rhamphomyia)
C
M1
ea
M2
M2
tu
ss
LA
*
ea
EA
ad
LJ system
R. (Calorhamphomyia)


tu
M1
ea
ss
M2
ad
contraction
M1 & M2
DC system
resiliency
ss sclerite
A
tu
M1
ea
ss
M2
tu
M1
ad
ea
ss
M2
ad
contraction
M1
LC/LJ systems
contraction
M2
B


ea
ad
C


ea
ad
D


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
log10 muscle volume (M1+M2 μm3)
R. (Calorhamphomyia)
R. (Rhamphomyia)
Hilara
E. (Polyblepharis)
E. (Euempis)
direct-compression type
lever-compression type
lever-jacking type
0
1500
3000
4500
6000
hind femur length (μm)

	M1 volume (µm3)	M2 volume (µm3)	M1/M2 ratio	M1+M2 volume (µm3)	Hind femur length (µm)
Hilara	1,286,223	600,145	2.14	1,886,369	1528
E. (*Euempis*)	12,367,411	7,984,865	1.55	20,352,275	5851
E. (*Polyblepharis*)	3,430,247	3,465,645	0.99	6,895,892	3979
R. (*Rhamphomyia*)	6,466,227	719,303	8.99	7,185,529	3044
R. (*Calorhamphomyia*)	13,331,637	35,963	370.7	13,367,600	3179