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Transient leg deformations during eclosion out of a tight confinement: A comparative study on seven species of flies, moths, ants and bees

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ABSTRACT

Legs in dipteran pupae are tightly packed in a zigzag configuration. Changes in the shape or configuration of long podomeres during eclosion have been overlooked because they occur rapidly (in a few minutes) and the legs are hidden inside a tight opaque confinement: the puparium in the Cyclorrhapha, the obtect pupa in mosquitoes. We fixed insects at different times during eclosion and obtained a temporal description of changes in leg shape. At the start of eclosion in *Calliphora vicina* and *Drosophila melanogaster*, femora are buckled in between the joints. Later, the chain of podomeres straightened, pointing posterad. Initial deformation and further stretching were passive, exerted by forces external to the legs. The prerequisites for this are pliability of the tubular podomeres and anchoring of the tarsi to the confinement. Each femur was strongly crooked instead of buckled in the mosquito *Aedes cantans*. The site of bending shifted distad in the course of eclosion: a sort of peeling. In contrast, other insects (the moth *Bombyx mori*, the ants *Formica polyctena* and *Formica rufa*, the honey bee *Apis mellifera*) left their tight confinements without any change in the initial zigzag leg configuration and without transient deformations of initially straight femora and tibiae.

1. Introduction

Reversible leg deformations have been found by Frantsevich (2016) in the blow fly *Calliphora vicina* extricating from the puparium: (i) before eclosion, the legs of the pharate imago inside the puparium are packed in a zigzag (Z-configuration): coxae retracted, trochanters elevated, femora pointing dorsad and anterad, front and middle tibiae and tarsi pointing posterad, hind femora and tibiae crooked about 90°; (ii) the fly gets out of the puparium with legs stretched straight and directed posterad; (iii) hence, the femora must turn by 90° or even more, but there is no space for such a turn inside the puparium. Leg straightening is concealed *in vivo* inside the opaque puparium which obstructs direct observation. Therefore flies were fixed at different moments of extrication in order to obtain a temporal series of leg configurations. Transformation of femoral position was achieved by steep bucklings in one or two sites within the pliant femur, as it is illustrated in the Graphical abstract (the left specimen). The fold among the femur behaved like a sort of a hinge: parts of the femur could rotate about the fold. Instead of turning the straight femur, the fly extended the chain of short subpodomeres. Buckling was repaired *in vivo* by stretching of all legs during the further extrication. Crooked hind tibiae were also stretched, at least partly.

Evidently, the blow fly could not get out of the puparium without leg buckling. Most probably, this species which is able to deform own podomeres and to repair the straight shape is not unique among insects. If extrication without this ability is impossible, then the peculiar deformation mechanism must be preformed before appearance of *Calliphora*, or the Cyclorrhapha, or other flies, or even earlier. Zigzag leg packing is inherent in dipteran pupae: all pupae, depicted in the monograph by Brauns (1954), demonstrate this leg configuration (60 species of 35 families). The

Abbreviations: A. c., Aedes cantans; *A. m., Apis mellifera*; *B. m., Bombyx mori*; *C. v., Calliphora vicina*; *D. m., Drosophila melanogaster*; *F. p., Formica polyctena*; *F. r., Formica rufa*; R1, R2, R3, right front, middle and hind leg.

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Definitions of terms in the text

Buckling	deformation of a bent pipe due to the loss of the elastic stability, when the convex face of the pipe expands, the concave face constricts, the induced strains at both faces include force components directed to the middle line of the pipe; the pipe flattens and abruptly forms a fold
Crooking	smooth bending of the pipe or the segment of the pipe, without alteration in the sign of curvature
Eclosion	the emergence of an insect from a pupa
Extrication	the release from entanglements, such as a hard confinement or the soil, after or during eclosion
Exuvium	the empty pupal integument after release of the imago
Pharate imago	the almost ripe adult insect, formed and hidden inside the pupal shell
Puparium	the unshed and hardened larval integument protecting the pupa inside it
Ranking	arrangement of specimens of a given sample in the order of monotonous change of some trait (traits); ordinal numbers of specimens are their ranks. If, due to approximation, two or more specimens are compared equal, they receive the same rank

same was depicted for pupae of *Drosophila melanogaster* (Bainbridge and Bownes, 1981, Figs. 19 and 20). Champions in tight packing are mosquito pupae. They accommodate the long legs of the future imago under the thorax, because the abdomen is used for swimming and must be free (Nachtigall, 1962; Brackenbury, 1999). Pupal legs in the Culicidae were depicted by Darsie (1951, Fig. 1) and Brauns (1954, Fig. 19): in addition to the zigzag arrangement of coxae, femora, and tibiae, the hind tarsus enscribes additional loops under the wing pad.

On the other hand, mosquito eclosion was demonstrated in the film "Microcosmos" (Nuridsany et al., 1996, 1:09:00–1:09:30). With permission of the cited authors and the studio, we illustrate eclosion postures as a collage of still frames (Fig. 1). All legs seen above the water are straight and point posterad. The pupal shell is a tight confinement with scarce space for leg maneuvers. How the mosquito manages straightening of its legs, is concealed underwater.

The same style of leg packing is characteristic not only for Diptera. Pupae with legs, tucked in Z-configuration, were illustrated for the noctuid moth *Barathra brassicae* by Obenberger (1964, Fig. 177), for the honey bee *Apis mellifera* by Lavrekhin and Pankova (1969, Fig. 34), for the red wood ant *Formica rufa* by Dlusskiy (1967, Figs. 1,4).

We inspected several species, close or far relatives to *C. vicina,* which encountered similar obstacles during eclosion. Taking into account the short duration of eclosion – few minutes only – we selected insects which could be cultivated or collected in the field and provide abundant samples with synchronized eclosion.

Selected candidates are listed in Table 1. Legs of pupae in these species are packed in zigzag. All candidates have been treated uniformly; their fixed and dissected legs were arranged in temporal series and compared. We show below that flies use various modes of leg deformation and further straightening during eclosion, in contrast to representatives of the Lepidoptera and the Hymenoptera, which do not straighten their legs at all, their leg podomeres are not malformed in pharate imagines.

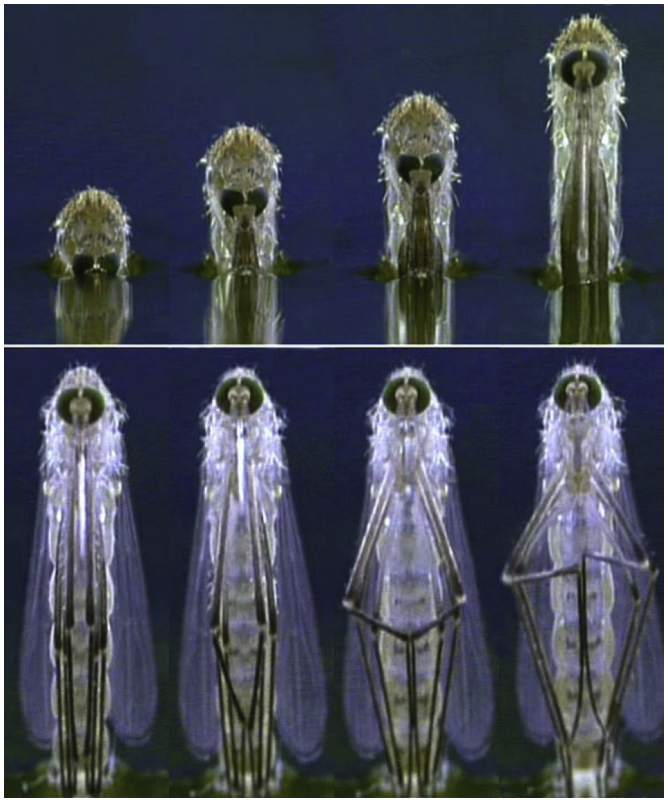

Fig. 1. Eclosion of a mosquito (collage of still frames from the film "Microcosmos"). Top panel – beginning of eclosion; bottom panel – leg extraction from the exuvium. Front leg long podomeres in the bottom right frame are clearly recognized as the femur, tibia, tarsus. In the bottom left frame, all podomeres are stretched posterad in a line.

2. Materials and methods

2.1. Rearing and pre-fixation

C.v.: postfeeding larvae were placed into a glass jar with sand for pupation. Puparia were transferred to plastic Petri dishes and kept under a wet cloth at 24–26 °C. After emergence of the first adults, puparia were under watch in order to intercept flies during extrication. Extricants were captured and placed at once in 70% ethyl alcohol: their movements stopped in 2–3 s.

D. m.: larvae were reared on the standard nutritional medium (Roberts, 1998) at 20 °C in glass tubes with transparent plastic plates, inserted vertically for pupation. Plates were transferred to Petri dishes (with few water droplets inside) for observation of extrication under a dissection microscope. Flies ready to extricate have been recognized by a swollen ptilinium. When the fly advanced out of the puparium, the plate with all attached puparia was flooded with hot water (5 ml, over 50 °C), thus the extricating specimen was momentarily immobilized together with all its neighbors on this plate. Then the plate was stored in 70% ethyl alcohol. The selected specimen was detached from the plate with the aid of a sliver of a hard steel razor blade. Alternative immobilization with ethyl alcohol caused ejection of a specimen out of its puparium.

A. c.: larvae and pupae, caught in a deep pit in the forest, were reared in the laboratory at 15–20 °C in 3 l jars filled with the water from their native water body. Pupae were transferred to 300 ml cups for observations of eclosion. Exuvia with eclosing mosquitoes were captured with a small forceps and transferred into vials with 96% ethyl alcohol.

Table 1
List of inspected species (Insecta, Holometabola).

Species	Taxonomy	Pupa and its shell	Type of tight confinement	Source of the sample
Calliphora vicina Robineau-Desvoidy, 1830	Calliphoridae, Diptera-Cyclorrhapha (Calyptrata)	Libera, soft	Hard puparium (opaque)	Commercial culture of larvae used as fish bait (Poland)
Drosophila melanogaster (Meigen, 1830)	Drosophilidae, Diptera-Cyclorrhapha (Acalyptrata)	Libera, soft	Hard puparium (semitranspa-rent)	Wild type laboratory strain *Canton S*
Aedes cantans Meigen, 1818	Culicidae, Culicoidea, Diptera-Nematocera	Obtecta, hard	Cephalothorax of the pupa	Field sample in the wildlife preserve "Holosiivsky", Kiev
Bombyx mori Linnaeus, 1758	Bombycidae, Bombycoidea, Lepidoptera	Obtecta, hard	Dense silk cocoon	Race "Whitecocoon-2 improved", Laboratory of Sericulture, Kharkov
Apis mellifera sossimai (Engel, 1999)	Apidae, Apoidea, Hymenoptera-Aculeata	Libera, soft	Loose silk cocoon glued to the cell, dense wax cell with a porous wax cap	Ukarinian honey bee (private apiary)
Formica rufa Linnaeus, 1761; *Formica polyctena* Förster, 1850	Formicidae, Vespoidea, Hymenoptera-Aculeata,	Libera, soft	Thin silk cocoon	Field samples in the landscape park "Theophania", Kiev

B. m.: circa 60% of cocoons, obtained from a producer, were circumcised down the equator with a stainless razor and halved, free pupae were stored in cardboard cases for observation of eclosion. Specimens were immobilized at different stages of eclosion by injection of 0.1–0.2 ml of 96% ethyl alcohol into the abdomen; movements stopped in 3–5 s. Specimens within their exuvia were stored in 70% ethyl alcohol. Specimens eclosing in cocoons were recognized by the advent of a transparent drop at the anterior pole of the cocoon, which later on dissolved the silk and the moth advanced through this aperture. The moth was immobilized with the same injection through the tiny hole drilled in the cocoon. Such specimens were stored in a freezer at − 18° C until further processing.

By cooling flies and moths during the night at 5 °C (Fraenkel and Hsiao, 1965) we obtained partial synchronization of eclosion when pupae were returned to the room temperature.

A. m.: combs with the sealed brood were inspected at 20 °C. A bee inside the wax cell is inobservable. Wax caps were forcedly opened, pupae with pharate adults inside were extracted with a forceps and placed into 70% ethyl alcohol. Normally, eclosed adults make their way out by gnawing the porous caps. They were captured at halfway and prefixed in ethanol as well as free young bees of varying age.

F. p., *F. r.*: collected cocoons and workers were transferred to the laboratory. 200 cocoons of *F. p.* with live ants inside were isolated from the colony and left for long-term observation. 40 cocoons were cut artificially and juvenile ants got out by themselves. Duration of their release was recorded. Workers cutting the cocoon shells were photographed in a Petri dish. Cocoons, opened by workers and containing emerging juvenile ants (ten workers per 50 cocoons of each species), were fixed in 70% ethyl alcohol.

2.2. Dissection and photography

Specimens were ranked by advance out of the confinement (Section 3.3). General views of all specimens were photographed against the contrast background; specimens were submerged in 96% ethyl alcohol in order to neutralise nitidulous light reflection from the body surface. Most specimens were photographed with the camera Canon EOS 550D (Canon Inc., Tokyo, Japan) attached to the ocular of the dissection microscope MBS-9 (former USSR). *B. m.* were macro photographed with the standard lens of the same camera, cocoons were photographed dry. Each specimen was photographed several times at different focal depth.

Specimens were postfixed in Bouin's solution for several days in order to preserve the leg configuration. Later on, specimens were rinsed in distilled water and ethyl alcohol and placed on a white rubber scaffold in a drop of ethyl alcohol or glycerol. Residues of the confinement, the head and the abdomen were discarded, the body halved and legs were isolated by chopping the coxae from the thorax. Legs were stored in tagged droplets of glycerol.

Legs were photographed submerged in ethyl alcohol, against a blue background in order to accentuate the contrast between the background and the leg stained in yellow by pycric acid from the fixative. Tiny legs of *D. m.* were embedded in droplets of the glycerol-chloral hydrate-gum medium (Pantin, 1948) under cover glasses and photographed in the microscope MBI-3 (former USSR). In most cases, we used photographs of a right leg viewed from behind (images of left legs were flipped horizontally).

Serial photographs were compiled with the software Adobe Photoshop 5.5 (Adobe Systems, Inc., San Jose, CA, USA). Combined plates were edited also in Adobe Photoshop. We recommend to view images at magnification 200%.

2.3. Statistical treatment

We measured deformation of long podomeres on photographs where the limb was positioned in the plane of bending. This approach provided only a flat projection of a 3D curve. Reference points on the leg were clicked on the image in the software Sigma Scan Pro (SPSS Inc., Chicago, IL, USA). Electronic tables of coordinates were processed in Microsoft Excel 97 (Microsoft Corporation, Redwood, WA, USA).

Orientation of each straight subpodomere was defined as a vector along this subpodomere, directed distad. Crooking or buckling were defined in the kinematic sense: as the angle between the distal and proximal vectors. Relative position of buckling or steepest bending was defined as the ratio of the length of a broken line from the base of the podomere till the extreme of buckling (or crooking) to the total podomere length, also down the broken line. Serial measurements were processed uniformly with a simple custom program written in Turbo Basic 1.3 (Borland International, Inc., Austin, TX, USA). Flat photographs of 3D objects, clicking by eye, and approximation of a curve by a broken line provided only a semiquantitative description.

Plots were constructed in the software Microsoft Excel 97, their artwork edition – in Corel Draw 8 (Corel Corp., Ottawa, Ontario, Canada).

3. Results

Amount of materials is shown in Table 2. Only circa 11% of the initially live insects were ranked and postfixed for morphological study. The causes of loss were variable: from devastating method of

Table 2
Amount of studied materials.

Species	Reared or collected	Ranked & postfixed	Primary photographs
C. v.	>160	59	>300[a]
D. m.	~900	43	169
A. c.	~500	75	191
B. m.	100	39	76
A. m.	~240	19	42
F. p., F. r.	~450	24	104
Totals	~2350	259	>880

[a] 30 juvenile adults fixed and measured without photographs.

fixation in *D. m.* (single specimen of a plate containing 20–30 puparia) till loss of insects emerged out of control (in *C. v.* or *B. m.*); some specimens were used in other tests or stored.

3.1. Leg configurations in pharate imagines

Confinements of inspected species are shown in Fig. S1 in Supplementary materials. They illustrate peculiar properties, listed in Table 1. We deal with two types of pupae: *pupa libera* with free legs or *pupa obtecta* with legs closely packed under the body and covered with the common dense cuticle. In the latter case appendages are divided with suturae, their hidden parts are covered with the soft sheath, e. g., in the mosquito pupa (Fig. 2C). Imaginal structures develop under the pupal shell and finally form their own integument inside the pupal one. Even in general photographs, one notices that all femoro-tibial joints point anterad, the femora and tibiae are oriented oppositely. Dissected legs in all inspected species were evidently packed in Z-configurations. After dissection, some legs retained their pupal sheath around the tarsi (Fig. 2B, also in A and C, view at high magnification!). Femora and tibiae of flies were more or less crooked or even buckled in *C. v.*, tarsi of *A. c.* were curved or recurved. Long podomeres in *B. m.* and in hymenopterans were straight. Shapes in *F. p.* were identical to those in *F. r.*

3.2. Opening of the confinement

Methods of opening are well known; we illustrate them with original photographs. Puparia of *C. v.* and *D. m.* are sealed with cups (*operculi*), which are discarded upon pressure from inside provided by the ptilinium (Laing, 1935; Atkins, 1949). Operculi open along the preformed cleavage lines (Brauns, 1954). The operculum in *C. v.* is conical and disintegrates into two valves, one of them may rest at the place and hinder the normal extrication (Fig. S1A in Supplementary materials). The operculum in *D. m.* is flat, situated at the ramp antero-dorsal face of the puparium. It does not disintegrate into halves, but in some cases one side remains attached to the puparium, therefore the emerging fly must move sidewards (Fig. S2A,B in Supplementary materials).

The pupal cephalothorax of *A. c.* cracks down the H-shape cleavage lines: the straight longitudinal fissure along the mesonotal sheath and two transverse fissures – between the head and the notum and between the meso- and metanotum (Darsie, 1951). The trunk of the juvenile mosquito moves forward out of the exuvium (Fig. 3), which retains its initial shape throughout the whole eclosion, because it must support buoyancy and equilibrium of the adult protruding above the water.

B. m. is protected by two hard confinements, one inside the other. The pupal shell cracks down the H-shape cleavage lines: the longitudinal fissure runs along the thoracic terga, transverse fissures divide the head from the thorax and the thorax from the abdomen (Fig. 4, ranks 1–4). Due to peristaltic movements of the abdomen, segments of the exuvium draw backwards telescopically: one over the other (Fig. 4, rank 5). In natural conditions, the moth ecloses inside the cocoon (Mikhaylov, 1950), the compact exuvium occupies a small place inside the cocoon. Then the moth gets out through the anterior pole of the cocoon, dissolving the coating of the silk threads with a drop of the labial gland secret (Chapman et al., 2013). The network of threads becomes loose, the moth draws the threads apart with the aid of the head and front legs. A moth at the initial stage of exit out of a cocoon is illustrated in Fig. S2C in Supplementary materials.

The pupal sheath in *A. m.* is soft. It is shed before attempts to get out of the wax cell (Lavrekhin and Pankova, 1969). This sequence is confirmed indirectly by comparing the short wing pads of the pupa (Figs. 2E, 5A) to the elongated but soft wings of active bees just before or at the very beginning of exit out of the cell (Fig. 5B–D). The bee makes its way out on its own, gnawing a hole in the wax cup with the mandibles.

Red wood ants *F. p.* and *F. r.* get out of their silk cocoons only with the aid of worker ants which tear the antero-ventral part of the cocoon with their mandibles (Dlusskiy, 1967); this behaviour

Fig. 2. Z-configuration of legs in pupae. Legs are depicted as right ones seen from behind and arranged in the order: R1 (front), R2 (middle), R3 (hind), as indicated in (F). (A) *Calliphora vicina*, femora flexed, tibiae bent. (B) *Drosophila melanogaster*, femora slightly bent, hind tibia bent. (C) *Aedes cantans*, head and wing vestige ablated, tibiae slightly bent, tarsi recurved, hind tarsi – recurved twice. (D) *Bombyx mori*, long podomeres straight. (E) *Apis mellifera*, long podomeres almost straight. (F) *Formica rufa*, long podomeres straight. Vertical scale bars for the body length in (A, D–F) 5 mm, (B, C) 1 mm; horizontal bars for the leg size in (A, D–F) 2.5 mm, (B, C) 0.5 mm.

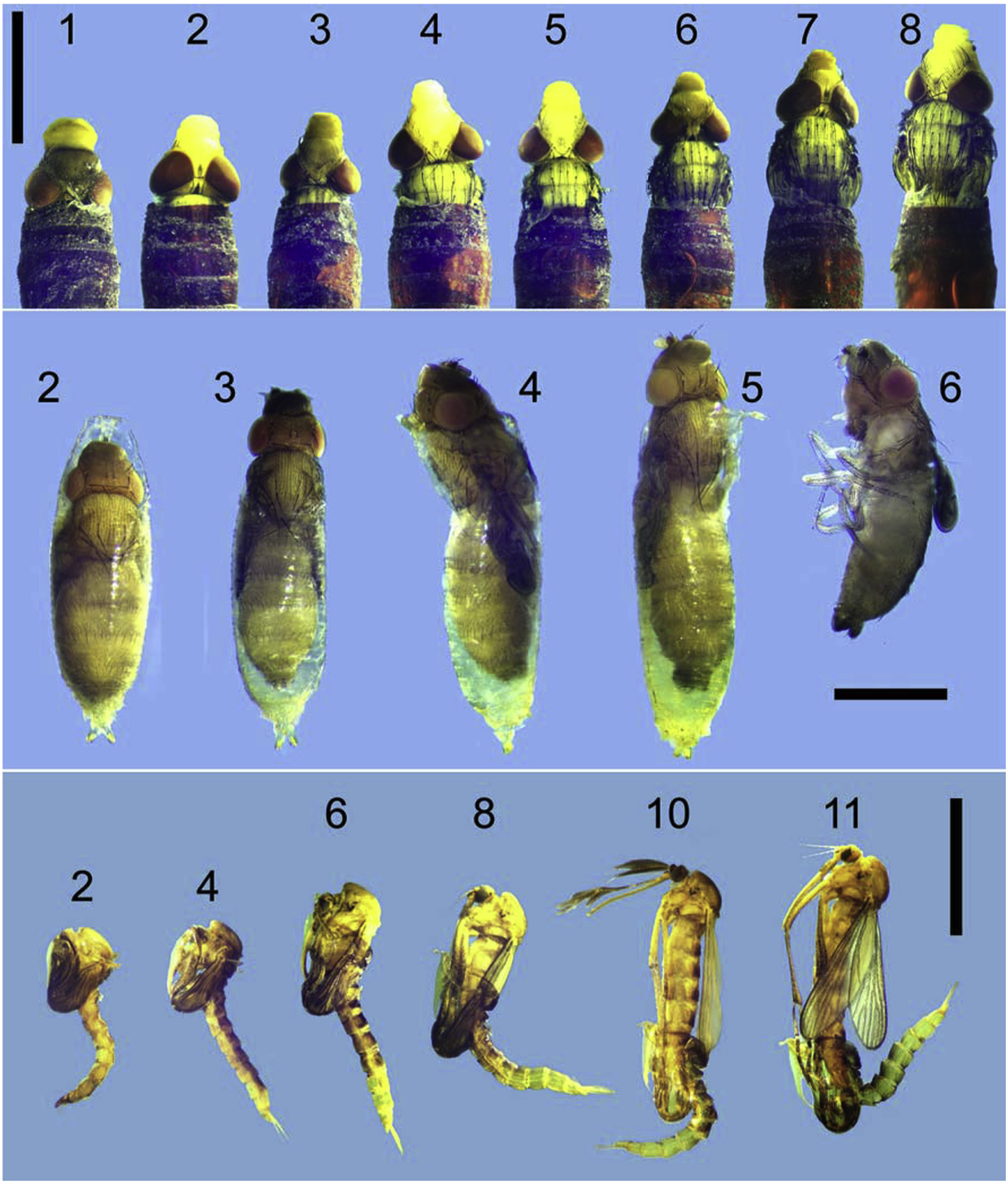

Fig. 3. Ranking of fixed specimens according to their advance out of the confinement. (Top row) *Calliphora vicina* extricants, scale bar 5 mm. (Middle row) *Drosophila melanogaster* extricants and the juvenile imago, scale bar 1 mm. (Bottom row) *Aedes cantans* eclosing from the pupal exuvia, only even ranks 2–10 are shown. Scale bar 5 mm.

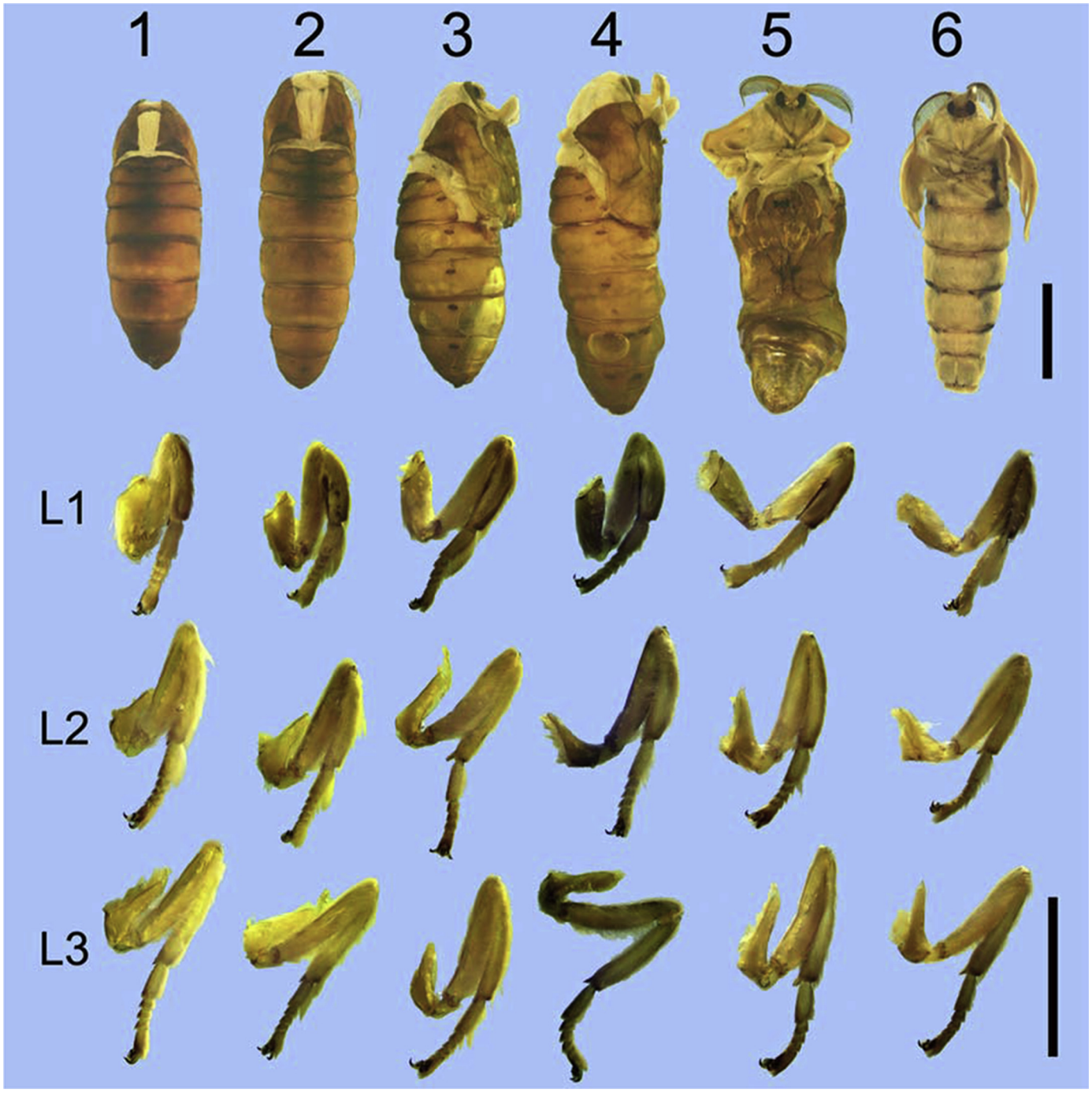

Fig. 4. Partial leg stretching without deformation during eclosion in *Bombyx mori*. Top row – moths eclosing from free pupae, ranked by their advance. Specimens (1, 2) are viewed from above, (3, 4) from the side, (5, 6) from below. Lower three rows – leg configurations in the same specimens (left front (L1), middle (L2) and hind (L3) legs, anterior view). Scale bars for moths and for legs – 5 mm.
The antero–ventral pupal shield in the specimen 5 is shifted far behind the thorax of the advancing imago (view at magnification 200%).

is typical for all ants, which spin cocoons as larvae (Wheeler, 1910). We illustrate this act with original photographs (Fig. S3C,D in Supplementary materials). All 200 cocoons of *F. p.*, deprived of workers' aid, perished. However, 40 ants safely escaped from cocoons cut artificially. The pharate imago is wrapped in a soft thin pupal sheath, which covers the body and appendages (Fig. S3A): in due time, this sheath is shed and shifted posterad, either by the juvenile ant itself or with the aid of workers (Fig. S3B).

3.3. Ranking

Ranking of eclosing and promptly fixed specimens was based on morphological traits which did not take into account the shape of the legs. Ranking for *C. v.* was based on body structures, which became visible in front of the anterior edge of the open puparium (Fig. 3, top row). Ranking of specimens which had left the puparium was based on the state of the ptilinium, wings, and tanning of the cuticle (Table S1 in Supplementary materials). Ranking for *D. m.*

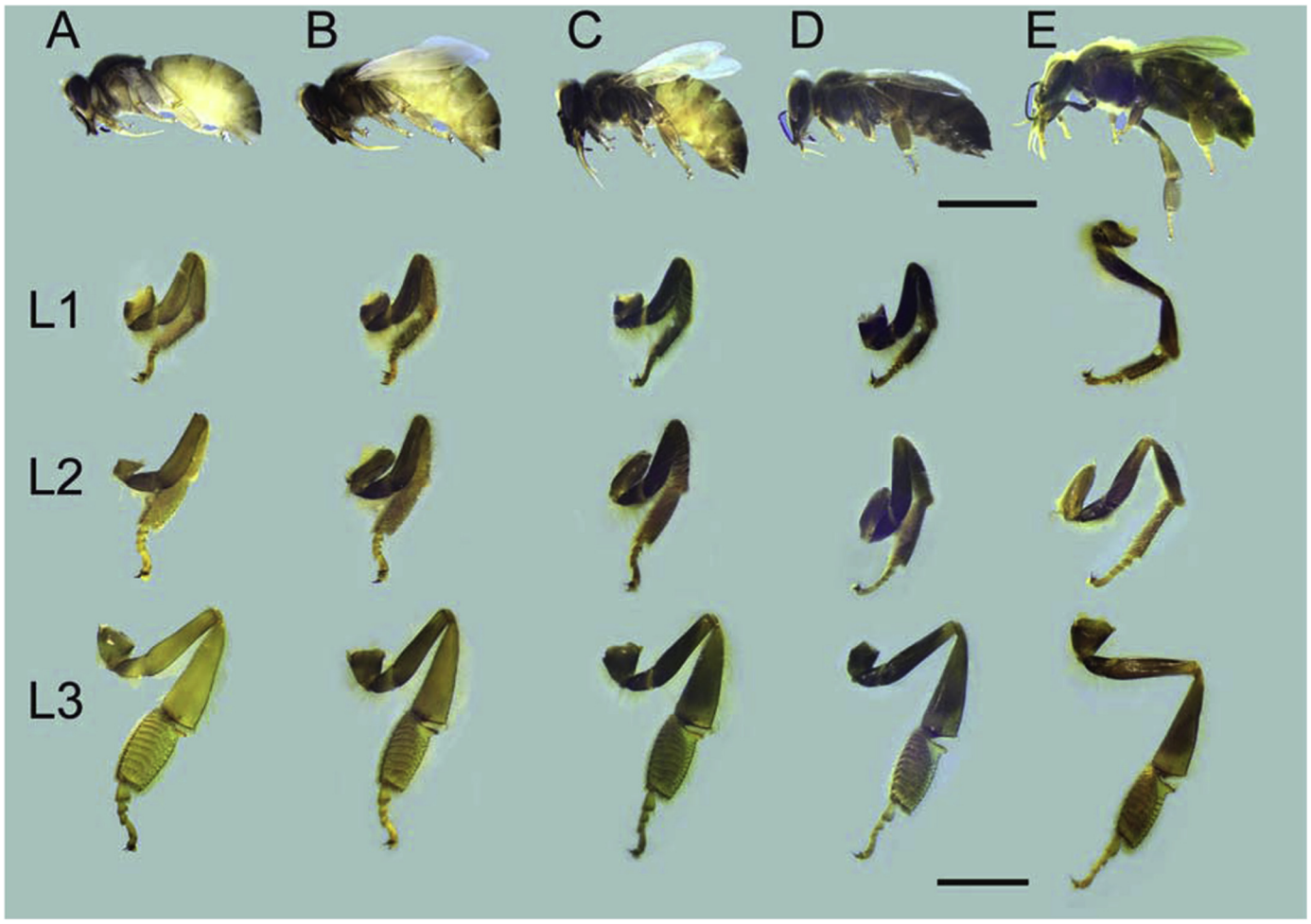

Fig. 5. Leg configurations in young honey bees *Apis mellifera*. Top row – general appearance of bees, lower three rows – legs of same specimens: left fore (L1), middle (L2), hind (L3) legs. (A) pharate imago with short vestigial wings, (B) pharate imago with long and soft wing vestiges, able to move appendages, (C) pharate imago with distinct wing venation, able to move appendages (specimens A-C were cut out of sealed cells), (D) a bee gnawing through the wax seal, (E) juvenile bee emerged from its cell. Scale bars: bees 5 mm, legs 2.5 mm.

was based on the state of the ptilinium and the operculum, but mainly on the level of advance of the body, seen inside the transparent puparium; free specimens were ranked using the same traits as for *C. v.* (Fig. 3, middle row, Table S2 in Supplementary materials). Ranking for *A. c.* was done in greater detail, using clefts in the pupal integument and advance of the abdomen, visible through the semitransparent exuvium (Fig. 3, bottom row, Table S3 in Supplementary materials). Ranking for *B. m.*, eclosing from the free pupa, used the pattern of fissures in the hard pupal shell and size of the moth's body exposed outside of the exuvium (Fig. 4, Table S4 in Supplementary materials).

Ranking for the bee and for red forest ants, using their advance during eclosion from the soft pupal sheath, was impossible inside the sealed wax cell and the opaque cocoon, respectively. We judged the maturation state of imagines, obtained by natural or artificial liberation, by the grade of tanning of the cuticle and the state of wing vestiges or wings. Such qualitative ranking was used in Fig. 5 (*A. m.*) and Fig. 10 (*F. p.*), showing imagines and shapes of their legs. Results, obtained in *F. r.*, were identical to those from *F. p.*

3.4. Leg deformation and stretching during eclosion in flies

Deformations of long podomeres in *D. m.* and *A. c.* share two common traits with extricating *C. v.* (Frantsevich, 2016, Fig. 7): (i) legs were straightened almost completely till the end of extrication, each leg pointing posterad; (ii) at the very beginning of extrication, femora were bent or even buckled between the trochantero-femoral and the femoro-tibial joints, this deformation was repaired during straightening. Typical deformations in three species are depicted in Fig. 6.

Bucklings in *C. v.* were situated near the base (Fig. 7A,B) and in the middle of the femur (Fig. 7A,C). They formed a tuck or a notch from the inner (antero-dorsal) side of the buckling. Bucklings occurred only in non-sclerotized and presumably pliant parts of the femur. Basal bucklings preexisted in pharate legs and increased drastically in front and middle femora at the start of extrication (rank 1). In hind femora, they appeared with some delay and had a lesser span. During extrication, angles of basal bucklings diminished significantly in all three leg pairs (see statistical evaluations in Table S5 in Supplementary materials), but the complete repair of this deformation lasted till maturation. Medial bucklings appeared in middle femora and to a lesser extent in hind ones, but their scatter was so big that no regular trend was noticed during extrication. This deformation was repaired only in mature adults. Crooking of the hind tibia diminished abruptly at the start of extrication and smoothly fell to almost zero at complete maturation (Frantsevich, 2016, Fig. 9F).

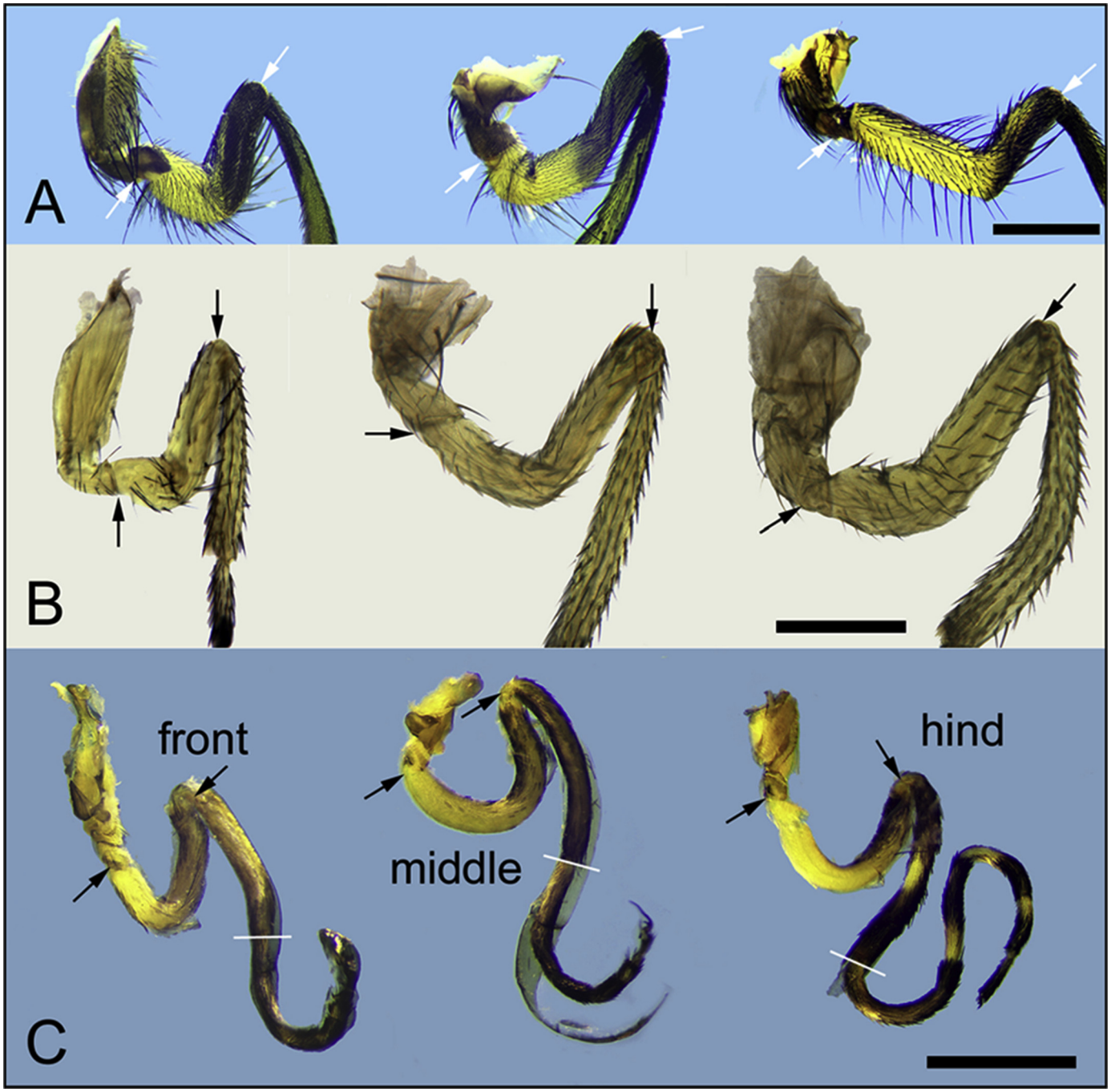

Fig. 6. Deformation of the femur in extricating/eclosing flies. (A) Buckling in *Calliphora vicina*, rank 3; (B) buckling in *Drosophila melanogaster*, rank 2; (C) crooking in *Aedes cantans*, ranks 3, 4. Legs are oriented as right ones, viewed from behind. Left column – front legs, middle column – middle legs, right column – hind legs, as indicated in (C). Proximal and distal joints of each femur are indicated with arrows, tibio-tarsal joints in (C) are crossed by white dashes. Scale bars in (A, C.) 1 mm, in (B) 0.25 mm.

Distinct sclerotized areas were not seen in the tiny legs of *D. m.*, in most observations the femur buckled only once in the middle. Bucklings in front and middle femora drastically increased since opening of the operculum: from 5°–14° to 63°–90° and from 14°–27° to 69°–96°, respectively. Buckling in the hind femora increased steeply till the end of extrication (Fig. 7E). It is rather easy to arrange leg images for *D. m.* from strongly buckled till straightened ones (Fig. 8), by the template for *C. v.* However, correlation with the rank, established by the grade of advance out of the puparium, was non-significant. Correlation was significant only for all ranks 2–8 and confirmed straightening of femora from beginning of extrication till the end of maturation. Hind tibiae, crooked in the pharate *D. m.*, straightened rather smoothly and significantly throughout extrication and maturation (Fig. 7F).

Deformation in *A. c.* was of another type than in the two flies described above: no buckling, but smooth and yet sharp crooking (Fig. 6C). The process of leg straightening is evident in ranked specimens of the mosquito (Fig. 9). The leg shapes at the very beginning of eclosion (rank 1) were identical to those in pupae, but at the next step (rank 2), femora strongly bent in the middle occurred. Later on, the straightened proximal part of the femur became longer, the bending site was situated closer to the femoro-

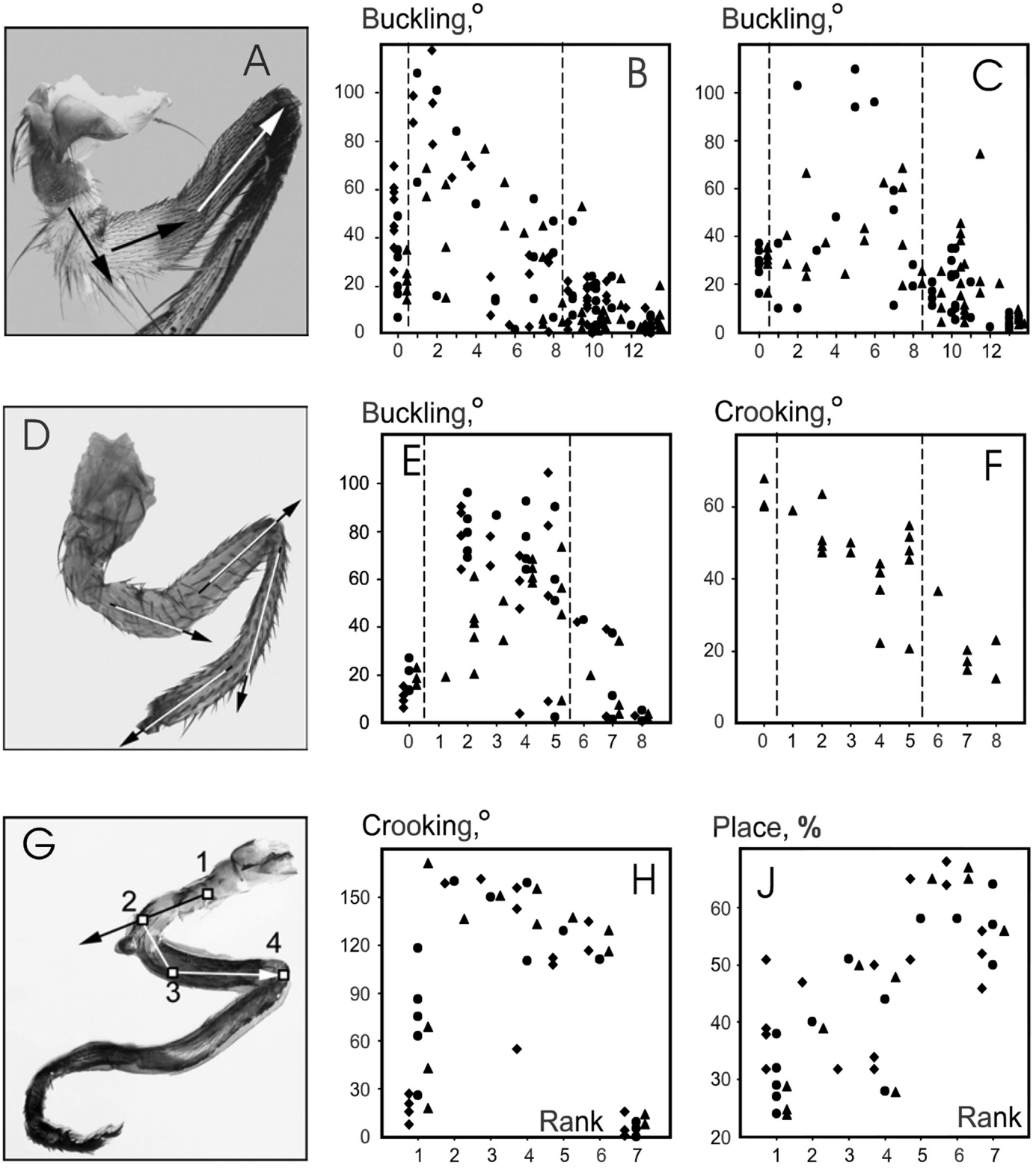

Fig. 7. Measurement of leg deformation versus rank in eclosing flies: (A–C) *Calliphora vicina*, (D–F) *Drosophila melanogaster*, (G–J) *Aedes cantans*. (A, D, G) leg photographs with vectors of subpodomeres, indicated by arrows. (B) buckling in the basal femoral hinges, (C, E) bucklings in the medial femoral hinges, (F) crooking of the hind tibia, (H) crooking of the femur, (J) relative position of the site of crooking. Markers for front legs – diamonds shifted a bit to the left, for middle legs – circles, for hind legs – triangles shifted a bit to the right. Dashed lines in (B, C, E, F) indicate start and end of eclosion; in (H, J) only ranks during eclosion are plotted. Further comments in the text.

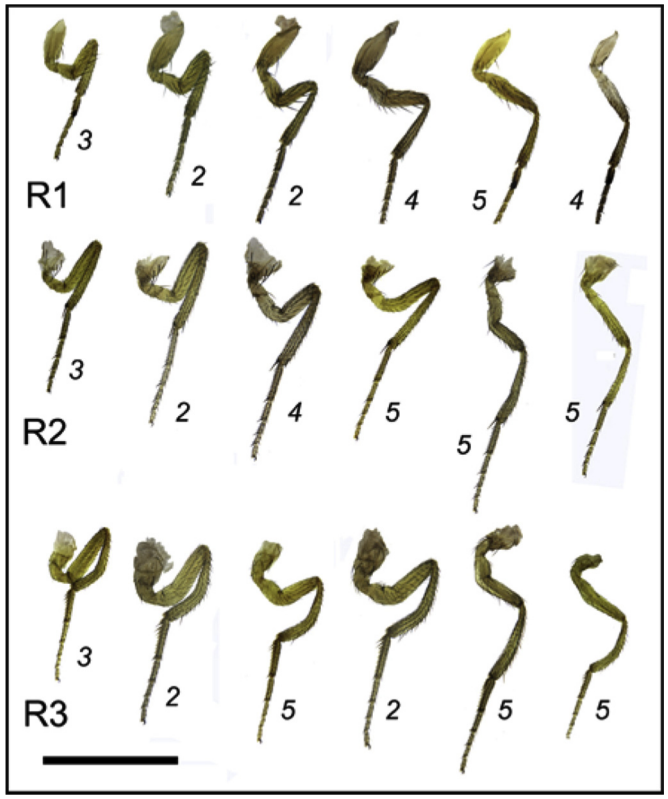

Fig. 8. Representative leg configurations in *D. melanogaster*, arranged by the grade of leg stretching. (Top row) front legs, (middle row) middle legs, (bottom row) hind legs. Ranks by the advance of the body are indicated at the tarsi of specimens. Buckling of the hind femur occurs later than in the front and middle legs. Legs of ten specimens of different size are depicted. Scale bar 1 mm.

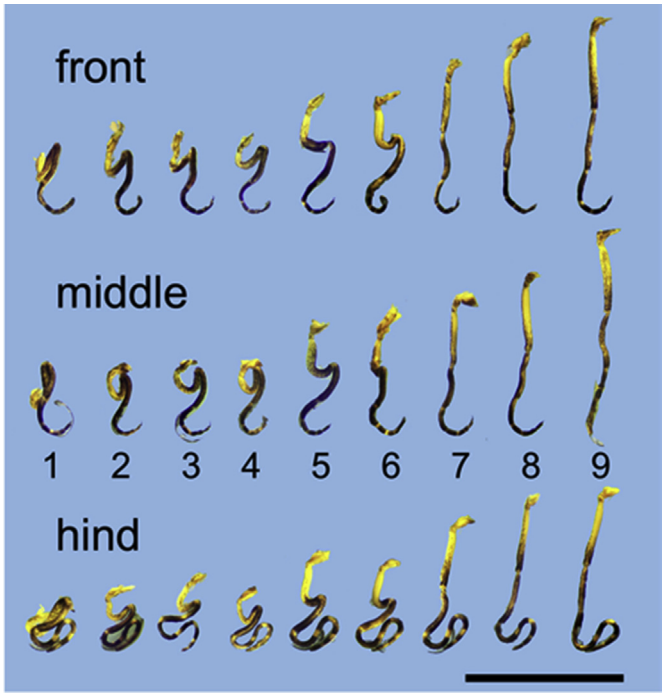

Fig. 9. Leg configurations in eclosing imagines of *Aedes cantans*. (Top row) front legs, (middle row) middle legs, (bottom row) hind legs. Ranks are indicated in the middle row. Legs are levelled by their tarsi as parts that are anchored to the tarsal segments of exuvia. Scale bar 5 mm.

tibial joint. Lightly crooked tibiae straightened in the late ranks of eclosion, the femoro-tibial joint extended to the limit. Tarsi retained their curved or recurved shapes till the latest ranks, because they remained inside the hard tarsal shells of the exuvium. The hind tarsal chain left the exuvium as last, repeating all recurved flexures of the shell.

Quantitatively, all three leg pairs behaved rather similarly (Fig. 7H), straightening of the femora was significant. More interesting is the distal shift of the steepest place of crooking (Fig. 7G), evaluated down the broken line which roughly outlines two straight parts of the femur (points 1–2 and 3–4) and the crooked segment in between (points 2–3). Correlation between position and the rank of eclosion was positive and significant for each leg pair as well as for their pooled samples. Tibiae in pharate adults and in eclosing mosquitoes were also initially crooked about 20°–40° and became almost straight at the latest ranks of eclosion. All joints extended during eclosion, and the chain of podomeres became straight.

3.5. Leg configurations in the silk moth, the honey bee, and ants

We illustrate leg configurations in *B. m.* only for free pupae (Fig. 4). Irrespective of ranks, legs in this moth were tucked in Z-configuration, femora pointed dorsad and anterad, tibiae pointed ventrad. Long podomeres were always straight or a bit curved, neither buckling nor steep crooking were observed. Legs were never stretched backwards, a moth got out of the exuvium with its "knees" (femoro-tibial joints) pointing forward.

The same results were obtained in *A. m.* (Fig. 5), *F. p.* (Fig. 10), and in *F. r.* (identical to *F. p.* and not illustrated here).

4. Discussion

4.1. Observations of leg positions during eclosion-extrication

Data on duration of metamorphosis and duration of eclosion/extrication by previous publications and by original observations are collected in Table 3. Normal eclosion/extrication lasts only 0.01–0.07% of the pupal stage duration. Due to such ephemeral display, details of leg movements escaped observation, especially in higher flies, hidden inside puparia, or in mosquitoes, hidden inside the exuvium and under water.

Pupae of higher flies, excised from puparia, were observed *in vivo* several times. Žďárek and Friedman (1986) photographed swaying movements, performed by the early pupa of *Sarcophaga bullata* one hour after head evagination; they recorded also freshly everted legs packed in Z-configuration (Fig. 3E in the cited article). Similar early events have been recorded in *D. m.*: head eversion, driven by peristaltic movements of the body, was accompanied by eversion of leg imaginal disks; half an hour later, leg podomeres reached their normal length due to cell differentiation. Z-configuration of legs in freshly everted disks was attained before podomere elongation (Fortier et al., 2006, Figs. 3C and 4A) and even in disks everting *in vitro* without inflation (Milner, 1977, Fig. 1H).

Extrication out of the puparium in *D. m.* was video recorded by Park et al. (2003) at the standard frame rate (25 s^{-1}): the dorsal side of the puparium was oriented to the camera, the operculum was artificially opened in order to observe peristalsis of the body, inflation of the ptilinium, and filling of tracheae with air. Undercranking filming (1 s^{-1}) of extricating *D. m.* was performed on puparia, glued by their ventral side, the dorsal side was excised above the head and the thorax (Baker et al., 1999). Legs were not observable in both cases. Observations on pupae, extracted from their puparia, would be artificial, because conditions of confinement would have been lost.

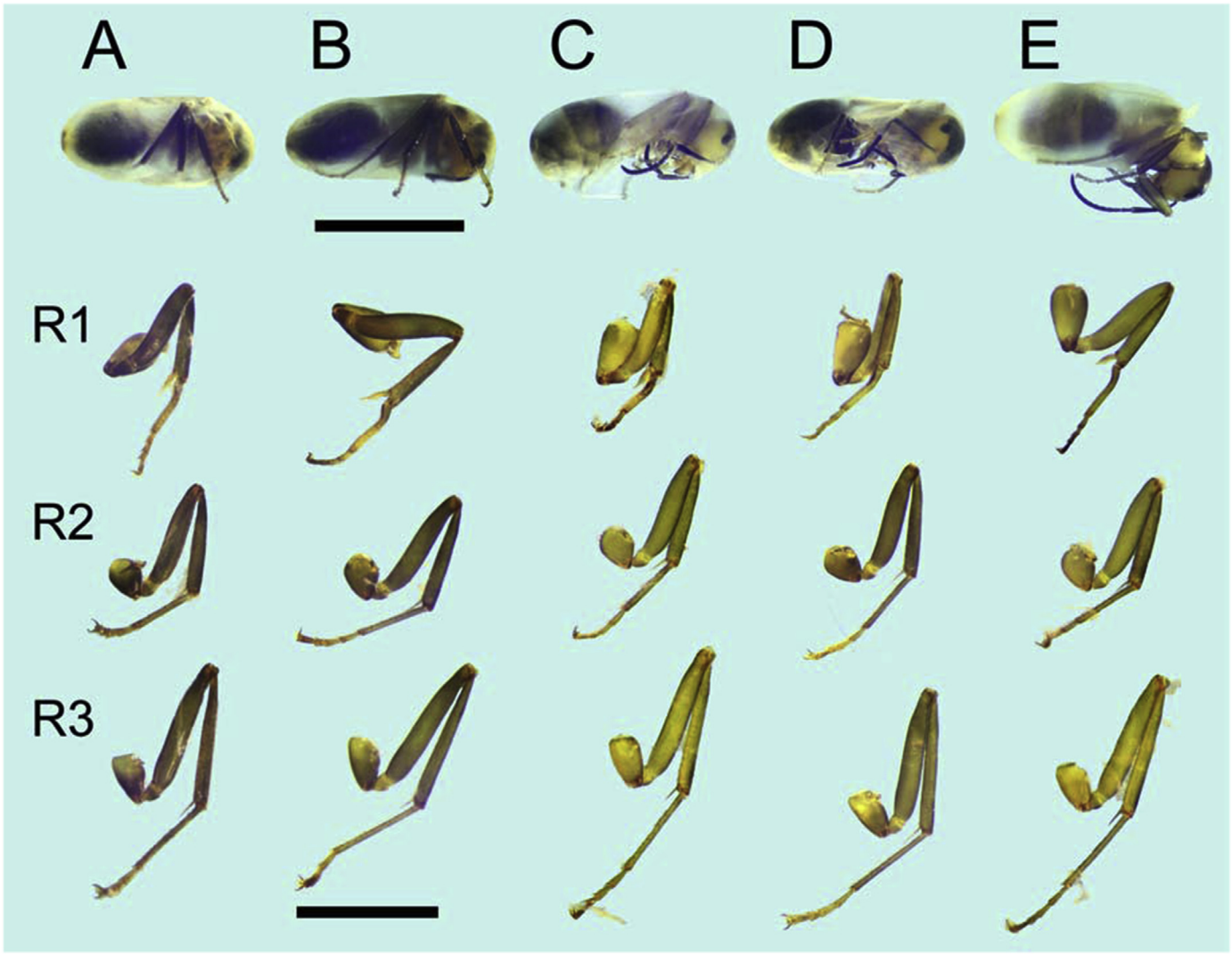

Fig. 10. Leg configurations in workers of *Formica polyctena*, released from cocoons by adult workers. Specimens were ranked by the grade of their liberation out of the cocoon. R1, R2, R3 right front, middle, hind legs. Scale bars for whole specimens 5 mm, for legs 2.5 mm.

Table 3
Duration of pupal metamorphosis and of eclosion/extrication.

Species	Pupal phase		Eclosion/extrication		Reference
	T, °C	Time	Conditions	Duration	
C. v.	24–26	≥10 d	In a smooth Petri dish	1–3 min	Original
D. m.	25		Intact puparium	<0.1 till several min	Original
	25	~100 h	–	–	Bainbridge and Bownes, 1981
	_	–	Intact puparium	~20 s	Bochicchio et al., 2013
	_	–	Intact puparium	~3 min	Park et al., 1999
	_	–	Operculum excised	54 ± 18 s	Park et al., 2003
	_	–	Dorsal side of puparium excised	1 s	Baker et al., 1999
A. c.	15–20	9–11 d	Intact pupa	10.4 ± 0.65 min	Original (mean ± m. error, $n = 10$)
B. m.	25	≥10 d	Free pupa	<3 min	Original
	24–28	9 d	–	–	Tikhomyrov, 1914
A. m.	35	9 d	–	–	Lavrekhin and Pankova, 1969
	35		Gnawing out the wax cell (video)	~4 min	Tatarenkov, 2014
F. p.	26–28	10–14 d	–	–	Zakharov, 1972, Fig. 6
					Zakharov, 2015
	_	–	Exit out of artificially cut cocoons	Median 3 min, range 1–23 min	Original ($n = 40$)

Residual leg deformations were noticed in newly emerged flies or in pharate imagines: legs in *C. v.* "were folded and bent as they were in the pupa" (Cottrell, 1962, p. 321); curved tibiae were photographed in *D. m.* and described as "abnormal" (Klein and Campos-Ortega, 1977, Figs. 5B and 7H; Monge et al., 2001, Fig. 7H) or "malformed" (D'Avino and Thummel, 1998, Fig. 3A–C; Fortier et al., 2006, Fig. 3A,B).

Out of several films on extrication in *D. m.*, posted in the Web, the most instructive is the rapid film (~60 s^{-1}) of Castanier (2013): the insect is seen at a skew from the side and below. Due to a partly discarded operculum, the fly's body is twisted sidewards, extrication is retarded and lasts about five minutes. Only one front leg is seen distinctly, and at the first distinct frame (time 2:51) this leg is already stretched backwards, but half of the tibia and the tarsus are still captured inside the puparium. Thus the possible moment of femur deformation is missing. The interesting feature of this film is the active movement of the half-free front leg: each time when the inflated head of the fly turns ventrad, the leg performs a series of jerks with a real frequency of about 7 s^{-1}. The leg stands still when the head is elevated. Before liberation out of the puparium, only this front leg is seen distinctly. How the legs turn backward from their initial Z-configuration, remains unclear. One of the present authors (L. F.) also failed to film these intrinsic processes in several specimens of *C. v.* which extricated out of their dark-brown puparia by vigorous peristaltic bouts.

Instantaneous fixation at different moments of eclosion with the following hard fixation of leg configurations appears to overcome this challenge, providing temporal snap-shots of eclosion.

4.2. Forces, exerting leg deformations, in flies

Three flies, inspected by us, demonstrated transient leg deformations during eclosion, namely, buckling or crooking amidst the femur, not concerning joints. One may assume three ways of deformation: (i) cell differentiation, (ii) action of intrinsic muscular forces, and (iii) passive deformation upon forces, external with respect to the given leg. Deformations evolve within the initial ranks of eclosion, during the first minute or even quicker. Cell differentiation, active during leg formation after eversion of imaginal disks, lasts at least 1–2 orders of magnitude longer (references in Section 4.1).

In both cases of muscular activity, forces are applied to a pliant long podomere. Legs in just emerged flies are able to walk, to groom, to right immediately after the exit out of the puparium (Reid et al., 1987; Ždárek and Denlinger, 1992; Frantsevich, 2016) or out of the pupa (Nuridsany et al., 1996). Moreover, if the legs in *Glossina brevipalpis* were surgically liberated just before extrication, they were able to move, and the whole puparium walked without attempts to start peristaltic body movements and attempts to leave the puparium (Ždárek and Denlinger, 1992). Even during extrication, the partially free leg was able to move in its own rhythm (Castanier, 2013).

The femur contains two long pennate muscles, inserting onto the internal head of the tibia, namely the flexor (depressor) and the extensor (elevator), described in *D. m.* by Soler et al. (2004, Fig. 6A–C). The span of extension-flexion in the femoro-tibial joint is close to 180°, therefore, the tendon excursion in the extensor is close to 150% of the tibial head size. If the tibia is fixed, contraction of the extensor may pull the tendon inward by about 5% of the length of the femur in our three fly species (evaluations by leg photographs). May this contraction, applied to the dorsal side of the pliant femur, cause crooking or buckling? *E. g.*, a mosquito pupa has proportions of the abdomen, similar to proportions of femora in our flies, and this pupa is able to bend the abdomen by ±180° dorsad or ventrad (Brackenbury, 1999). Bending is caused by one-side contraction of intersegmental muscles. Application of this mechanism to our case seems wrong, because functions of tibial muscles in adults are quite different, the femoro-tibial joint is not fixed during leg stretching, and, moreover, the site of bending shifts distad versus the rank in *A. c.*

The idea of a passive deformation of the legs, pulled forward by the advancing body and anchored by their distal parts to the confinement, was proposed for *C. v.* by Frantsevich (2016). The first forward thrust of the body pulls the coxa, the trochanter, and the proximal part of the femur forward, while the distal part remains behind. Thus the femur tucks at the base and/or in the middle by about the right angle in *C. v.* or *D. m.* Buckling evolves from bending if the deformation of the tubular structure drives to the loss of elastic stability (Alexander, 1968, Chapter IV; Brazier, 1927). This process was demonstrated on models: plastic pipes for cold drinks (Frantsevich, 2016, Supplementary materials). Upon stretching, legs or plastic pipes restore their cylindrical shape. Stretching is applied by the still advancing body.

Evidently, elastic properties of mosquito legs differ from those in higher flies, because, at the very beginning of eclosion, a crooking about 150° appears in the basal part of the femur instead of buckling. Later on, the place of bending migrates distad (Fig. 7H,J). One may imagine peeling of the femur which is attached to the exuvial shell, but is pulled anterad by the coxa and trochanter and thus is torn away from the shell step by step, while the distal part of the podomere is still fixed to the shell. The larger the bending (in the kinematic sense), the lesser is the force necessary to tear the pipe (or the sticky band) off the substrate (Kendall, 1975). Sharp crooking *per se* is easy to demonstrate using lavsan pipes for infusion.

During eclosion/extrication, legs are passively stretched into chains of straight podomeres. Few minutes spent in this dynamic straight state are too short for biochemical hardening and tanning of the cuticle. The question is why femora and tibiae do not return to their crooked or buckled configurations after release from external forces. Presumably, strains in the tubular shell after stretching are close to zero (that means that some areas were prestressed or plastic in distorted legs of the pharate imago).

4.3. Divergence of eclosion modes among Diptera, Lepidoptera, and Hymenoptera

Seven species are not plenipotentiaries for three insect orders numbering 450 thousand species, but additional information on other species is lacking, in particular for flies, which demonstrated transient deformation in the femur. Two methods: buckling or peeling – are based on leg pliability. They ensure, among other adaptations, safe liberation of an imago out of the pupa or puparium. Both methods need anchoring of the leg tips to the confinement. Buckling in *D. m.* looks almost the same as in *C. v.* These species represent main subdivisions of the Cyclorrhapha-Schizophora: the Acalyptrata and the Calyptrata, respectively. The molecular phylogenetic tree of the Diptera, analyzed by Wiegmann et al. (2011), places the clades Ephydroidea (including the Drosophilidae) and Oestroidea (including the Calliphoridae) as sister groups, although the Calyptrata as the whole were designated as a paraphyletic group. Rich branching of the Schizophora occurred in the Paleocene, 65 MA ago, due to emergence of the ptilinium. Leg buckling presumably accompanied evolution of the ptilinium or even was prior to the latter. Leg deformation, based on pliability of long podomeres, was found in *A. c.* (Culicidae). Clades ascending to the Culicomorpha and to the Cyclorrhapha diverged at the very base of the phylogenetic tree of the Diptera: about 230–240 MA ago (Wiegmann et al., 2011, Fig. S3). Historical studies on eclosion with leg deformation in the Diptera need inspection of several key

groups which may provide species fitting to the method of anatomical "snapshots" of the quick eclosion process.

Eclosion without change of Z-configuration and without any podomere deformation was found in the moth *B. m.*, in the honey bee *A. m.* and in the ants *F. p.* and *F. r.* The Hymenoptera are considered as the sister group of the Mecopterida (Ronquist, 1999), fossil hymenopterans were discovered since the second half of Triassic (Rasnitsyn, 1996). The fossil Mecoptera and Trichoptera were discovered yet in the Permian, isolation of clades to the recent Diptera and Lepidoptera is dated at the Triassic (Rasnitsyn, 1998; Grimaldi and Engel, 2006). From the viewpoint of the present article, the most interesting, but difficult objects to record the eclosion process are scorpion flies (Mecoptera, Panorpidae).

Contribution

L. F. reared synchronized parties of *C. v.*, selected, fixed and ranked specimens. I. K. did the same with *D. m.*, Y. D. – with *A. c.*, T. M. – with *B. m.*, I. S. – with *A. m.*, S. S. – with *F. p.* and *F. r.* L. F. photographed selected specimens, dissected and photographed their legs, composed figures, and wrote the text.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.asd.2017.05.002.

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