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Letters to the Editor

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Resolving Confusion in the Use of Concepts and Terminology in Intra-Puparial Development Studies of Cyclorrhaphous Diptera

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Developmental studies of necrophagous insects are strongly needed to support medico-legal investigations, because minimum post-mortem intervals ($_{min}$PMI) can be estimated from development data for species collected from a forensic scene together with accurate temperature information from that scene. The life cycle of cyclorrhaphous flies, which include some of the most used forensic indicators, shows an unusual feature as the pupal stage and the subsequent development of the pharate adult take place inside an opaque, barrel-like puparium, formed from the cuticle of the third-instar larva (Fraenkel and Bhaskaran 1973). Although unusual this feature is not unique among insects, or even among Diptera, despite the statement of Proença et al. (2014). The period from pupariation (i.e. puparium formation) until the emergence of the adult is of special importance for forensic studies as this period lasts for more than 50% of the total immature development. However, unlike the larval stage where a quantitative measure of age (e.g. body length) can be modelled in relation to time, the puparium shows virtually no external age-related changes (Amendt et al. 2011). Nevertheless, the puparium can be removed in order to determine morphological markers related to age on the insect inside, which can then be used for simple age estimation. Accordingly, a number of recently published studies (e.g. Pujol-Luz and Barros-Cordeiro 2012, Defilippo et al. 2013, Proença et al. 2014, Ma et al. 2015) have described age-related morphological landmarks in the intra-puparial development of several forensically important Diptera, chiefly blow flies (Calliphoridae). Our concern has to do with confusion regarding concepts and terminology frequently occurring in these kinds of intra-puparial development studies. It is very likely that much of the existing confusion is related to the wide use of the terms ‘pupa’, referring to any fly individual during its intra-puparial development (regardless of which developmental stage lies inside the puparium), and ‘pupal stage’ or ‘pupal period’, referring to the period from pupariation

to adult emergence in forensic entomology (e.g. Amendt et al. 2011). This terminology might be practical but it is incorrect, as it includes within the 'pupal period or stage' not only the actual pupal stage but also the prepupal stage and the final development of the pharate adult, even when the latter is significantly the longest intra-puparial stage in cyclorrhaphous flies (Hinton 1971).

Different authors have already highlighted the frequent confusion and misuse of terminology in studies on the metamorphosis of cyclorrhaphous flies (e.g. Hinton 1946, 1971, 1973; Jenkin and Hinton 1966; Fraenkel and Bhaskaran 1973). Although the readers can find more detailed descriptions in those publications, we believe that it is worthwhile compiling a short review here of the correct terms for the most important stages and events in the intra-puparial development to help reduce future confusion:

Pupariation refers to the formation of the puparium, it takes place when the contraction of the post-feeding larva is irreversible and it is different from 'pupation' or formation of the pupa, which takes place later. From pupariation to the first apolytic event the insect should be called a *prepupa*, as it is still attached to the puparium (i.e. the larval cuticle) (Fig. 1A–B). The term *pupa* should be used only when the *larval-pupal apolysis* (i.e. the separation of the epidermal cells of the pupa from the larval cuticle or puparium) is complete (Fig. 1C). At that time, the legs and wings have partially everted but not the head; the morphology of the pupa still resembles that of the prepupa and it should be called a *cryptocephalic pupa* (= "hidden head") (Fig. 1C). Then, within usually a relatively short period the head, legs and wings evert completely, and the cryptocephalic pupa becomes the *phanerocephalic pupa* ("visible head"), where head, thorax and abdomen are discernible (Fig. 1D). Shortly after the dramatic transformation of the cryptocephalic into the phanerocephalic pupa, the *pupal-adult apolysis* (i.e. the

separation of the adult epidermal cells from the pupal cuticle) starts (Fig. 1E), and at its completion the pupa has become the *pharate adult* (Fig. 1F), which will continue its development, usually for several days, until its emergence from the puparium.

Given that the term 'puparium' is a paronym of 'pupa', it is not surprising that there is frequent use of the latter as a malapropism. For example, Ma et al. (2015) give a "morphological description of pupae" of the blow fly *Chrysomya rufifacies* (Macquart), but what they actually describe is the morphology of the puparium. This malapropism also affects other words derived from 'pupa' and 'puparium'. For instance, Pujol-Luz and Barros-Cordeiro (2012) suggest that obligatory parasitic flies show "a much larger intra-pupal [sic] development", but we assume they refer to either the whole intra-puparial period, as the title of their paper suggests, or to the actual pupal stage, with no reference to the internal changes of the pupa. In the same way, Proença et al. (2014) discuss "the developmental time of intrapupae [sic]" of different *Chrysomya* Robineau-Desvoidy species, although their study focuses on the external morphology of the different intra-puparial stages. Also, in Proença et al. (2014) there is an account of the pupariation process with a description of the gradual "acquisition of pigmentation of the cuticle of the pupa" from white to black, which obviously refers to the darkening of the puparium, i.e. the hardened cuticle of the third-instar larva, not the cuticle of the pupa. Interestingly, Proença et al. (2014) write later that "after 66 h, the pupa showed gradual body pigmentation" although, according to their results (and their statement just a few lines above), at that time the insect is already the pharate adult, i.e. no longer a pupa. This misuse of the term 'pupa' for every intra-puparial developmental stage generates confusion and imprecise terms – like the 'pupal morphogenesis' of Ma et al. (2015), which is actually mostly focussed on the adult morphogenesis – and classifications –

like the division into ‘juvenile’ and ‘mature pupa’ (Defilippo et al. 2013, Ma et al. 2014) even when both divisions would include part of the pharate adult stage.

Determining and classifying the intra-puparial developmental stages is another frequent source of confusion and misinterpretation. Hinton (1971, 1973) convincingly argued for the appropriateness of defining the intra-puparial stages of cyclorrhaphous flies based on the apolyses rather than on ecdyses, as the larval-pupal apolysis is not followed by a larval-pupal ecdysis. Indeed, the adult sheds both larval and pupal cuticles simultaneously at emergence (Hinton 1973). A determination of the onset of pupal and pharate adult stages that is not based on completion of the apolysis (e.g. Defilippo et al. 2013, Proença et al. 2014, Ma et al. 2015) is therefore completely arbitrary. Moreover, it must be highlighted that determining when an apolysis is complete requires either histological (Fraenkel and Bhaskaran 1973) or virtual micro-computed tomographical sections (Fig. 1). Hence, the description of the larval-pupal apolysis process by simple macroscopic examination of *Chrysomya albiceps* (Wiedemann) prepupae by Pujol-Luz and Barros-Cordeiro (2012) is likely based on misinterpretations of concepts. Determining the timing of the different intra-puparial stages correctly and consistently is particularly crucial in forensic entomology, as a misinterpretation of concepts may lead to errors in $_{min}$PMI estimations. For example, Defilippo et al. (2013) consider that the cryptocephalic pupal stage starts at the same time as pupariation in *Calliphora vicina* Robineau-Desvoidy (compare Tables 1 and 2 in their paper). However, larval-pupal apolysis is not complete until several hours after pupariation in this species (approximately 18 hours at 24 ºC; see Fig. 1C), therefore the values determined by Defilippo et al. (2013) could lead to a significant error in $_{min}$PMI estimations.

It is not our aim to disregard the studies of Pujol-Luz and Barros-Cordeiro (2012), Defilippo et al. (2013), Proença et al. (2014), and Ma et al. (2015). Indeed, we are aware of the wide confusion regarding the terminology and concepts related to intra-puparial development and particularly in the forensic entomology literature (we have probably also sometimes misused the terms) and therefore the reigning confusion is understandable. We suggest replacing the widely and erroneously used ‘pupal stage’ referring to the whole period from pupariation to adult emergence by ‘intra-puparial period’ in forensic entomology literature. If ‘pupal stage’ is maintained because of its wide use, it would be advisable to briefly mention that this term is used in a broader sense, including the prepupal, pupal and pharate adult stages. Nevertheless, in the particular case of intra-puparial development studies, the correct terminology should be fastidiously applied as it is the only way of avoiding further confusion and misinterpretations.

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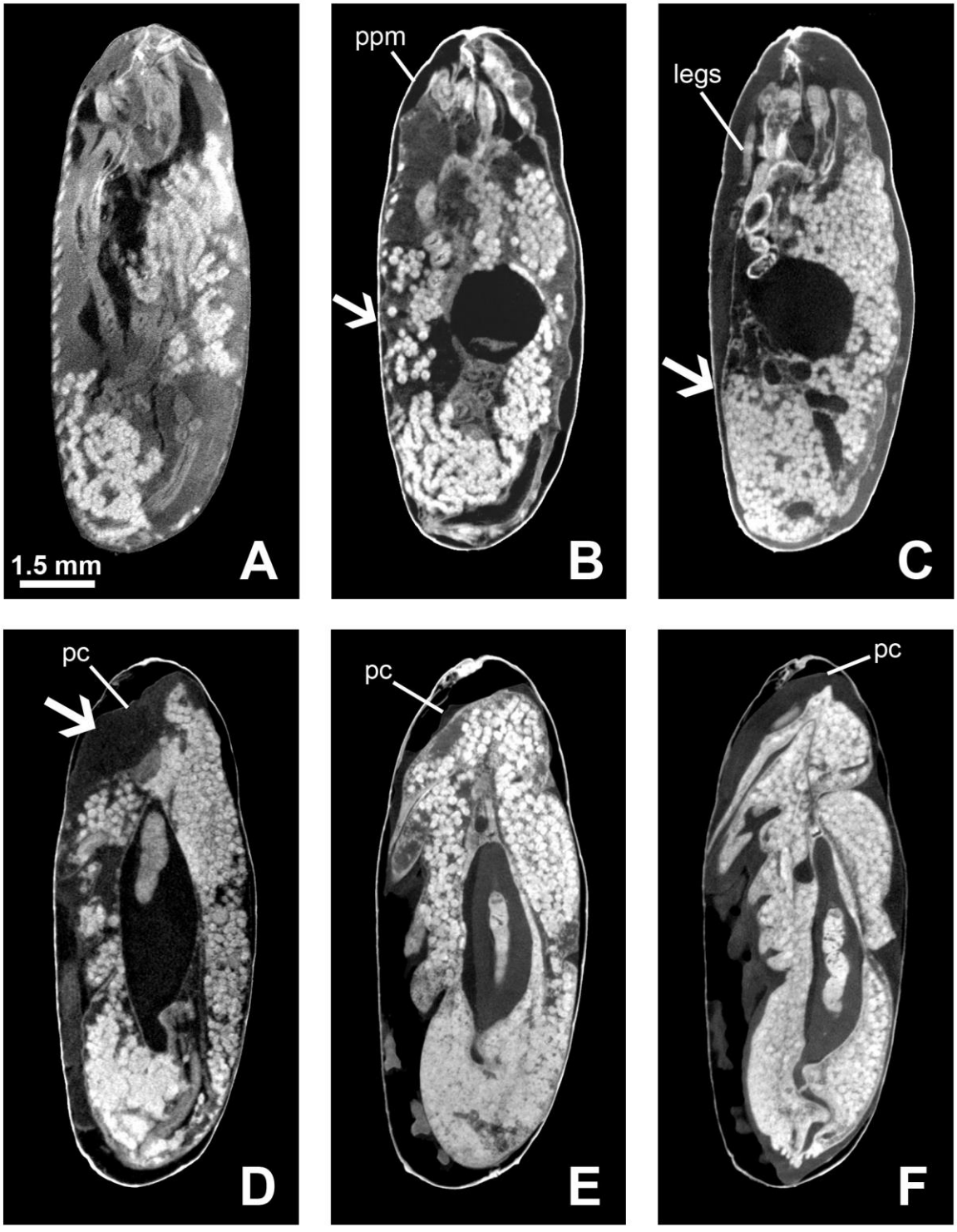
FIGURE CAPTIONS

Fig. 1. Micro-CT virtual sagittal sections of the blow fly *Calliphora vicina* Robineau-Desvoidy at different times after pupariation, stained in iodine 0.5M and scanned in a Nikkon Metrology HMX ST 225 system (exposure: 500 ms; voltage: 110–130 kV; current: 100 μA). (A) At pupariation, the prepupa is still attached to the puparium, i.e. to the third-instar larval cuticle. (B) 12 hours after pupariation, larval-pupal apolysis is taking place but it is still not complete, as the epidermis is still attached to the puparium in some areas of the abdominal region (arrow). (C) 18 hours after pupariation, larval-pupal apolysis is complete as the epidermis has detached from the puparium over the body (arrow); the legs and wings have partially everted and the prepupa has become the cryptocephalic pupa. (D) 30 hours after pupariation, the head has everted (arrow) although it will maintain a hyaline appearance until the migration of the fat bodies. The cryptocephalic pupa has transformed into the phanerocephalic pupa. (E) 48 hours after pupariation, the adult epidermis has detached from the pupal cuticle only in some areas; the pupal-adult apolysis is still not complete. (F) 72 hours after pupariation, the pupal-adult apolysis is complete as the pupal cuticle has detached over the body; the insect is now a pharate adult, i.e. no longer a pupa. Abbreviations: pc, pupal cuticle; ppm, puparium.



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