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Sexually-dimorphic post-eclosion behaviour in the European stag beetle *Lucanus cervus* (L.) (Coleoptera: Lucanidae)

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The recent discovery of a unique post-eclosion, mycangium-related behaviour in several Japanese stag beetles seems to have solved the enigma of the transportation of their symbionts from larva to adult. Here, to confirm this behaviour in the European stag beetle *Lucanus cervus* (L.), and to compare the post-eclosion behaviour between females and males, we observed five females and three males during the period of pupation and adult eclosion under a controlled environment. The mycangium-related behaviour was repeatedly observed in all five females, which is almost the same as has been reported in the Japanese species; they everted the mycangium and swept the cocoon walls. In *L. cervus*, the mycangium everted two and a half hours after eclosion and the onset of the sweeping behaviour occurred two hours after that; it continued intermittently for about four hours. Although males exhibited no sweeping behaviour, they eclosed with everted genitalia and retracted them within four hours. In both cases, the teneral imago exuded a huge amount of transparent liquid droplets from the dorsal intersegmental gap of the abdominal tip during the post-eclosion behaviour; we observed this loss of fluid in two females and one male. The origin of the liquid and the adaptive significance of these droplets are also discussed.

Keywords: genitalia, Lucanidae, mycangium, post-eclosion behaviour

INTRODUCTION

Pupation and eclosion are the most dynamic processes in insects with complete metamorphosis, and there are many crucial events and behaviours associated with those stages. Exoskeletal tissues of adults are newly formed below the pupal skin, and they are exposed to the outer environment soon after eclosion. Since those external tissues are not completely hardened at eclosion in most cases, the post-eclosion processes are very critical to finish the adult morphogenesis. For example, in stag beetles the adult wings are folded ventrally in the pupa and during eclosion they need to be extended over the back before they are hardened; later the softwings need to be intricately folded after they have dried up (Lai & Hsin-ping 2008; Hendriks & Fremlin 2012). Possibly, this is also the case with their male and female copulatory organs, which are part of the exoskeleton and have partially sclerotised segments. The genital morphology of the carabid beetles (Coleoptera: Carabidae), which has a complex three-dimensional structure and thus performs as a lock-and-key system in mating (Takami 2003), may need to be rearranged during this stage.

However, the post-eclosion behaviour associated with the morphogenesis of genitalia has not been fully investigated in stag beetles.

Moreover, some wood-feeding insects possess external organs that convey symbiotic microorganisms, usually refered to as mycangia, in various parts of their exoskeleton (Batra 1969; Beaver 1989; Happ *et al.* 1971; Tanahashi *et al.* 2010; Toki *et al.* 2012). Historically, mycangium (pl. mycangia) is a word coined by Batra (1963) - myco 'fungus' + angeion 'vessel', from Greek - for structures that carry viable fungal inoculum at all times. These structures were first discovered in fungus-farming beetles (Francke-Grosmann 1956). Since then, mycangia have been found on various parts of the body of wood-feeding beetles, from the oral cavity to procoxae, prothorax, mesothorax, and elytral bases, among others (Beaver 1989; Francke-Grosmann 1967). Basically, there are three types of mycangium: pit (with no to few setae), sac (pocket/tube) or setal brush; sometimes they are associated with complex glands (Beaver 1989; Happ *et al.* 1971). Nevertheless, the mycangia could not possess any microorganisms initially, because of their exoskeletal origin, therefore, the adults need to transfer the essential microorganisms from somewhere to the mycangium after the eclosion.

In the Lucanidae, the mycangium is a female-specific saccate organ hidden in a dorsal fold of the integument between the last two tergal plates (Tanahashi *et al.* 2010). This organ, which is rather apparent in a dissected female body, is a few millimetres across; its colour varies from yellow to black, depending on the species; it is even present in well preserved specimens. Therefore, it is perhaps surprising that it was overlooked for so long; there is no mention of it in major morphological studies (for example, Holloway 2007; Imura 2010). But there are two exceptions. Franciscolo represented it clearly, albeit without a description, in drawings of the dorsal view of the female reproductive system for a couple of species: *Lucanus cervus* (Linnaeus, 1758) and *L. tetraodon* (Thunberg, 1806) (Franciscolo 1997); he opted for ventral views of all the other species, in which case the mycangium is obscured by the reproductive organs. Hawes (2013) also observed it independently in dissected females of *L. cervus* and managed to culture mycangial yeasts in xylose. But the study of the mycangium was carried further by the Japanese researchers and has become a work in progress because it has raised many questions.

The way in which these yeasts are transmitted during their metamorphosis and from generation to generation has been revealed gradually. It is understood that when the females oviposit, they inoculate the vicinity of each egg with mycangial secretions (Tanahashi *et al.* 2010). The larva acquires the inoculum as soon as it starts feeding. The larva will moult twice in the pabulum, each time shedding the foregut and hindgut, which unlike the midgut are of ectodermal origin. Thus, it keeps its symbionts in the midgut as do stinkbugs, for example (Kikuchi *et al.* 2007). However, when a third instar larva of *L. cervus* reaches maturity it stops eating, leaves the pabulum and goes deep into the soil to make a cocoon. This time, the larva will line the cocoon wall with the contents of its gut (Hendriks & Fremlin 2012), thus spreading the symbiotic yeasts onto the cocoon wall. After that, it will moult twice inside the cocoon. This raised the important question of how the female re-acquires the mycangium symbionts.

The discovery of a mycangium-related post-eclosion behaviour, which has been first reported in the 8th Symposium on the Conservation of Saproxylic Beetles (Tanahashi & Fukatsu 2015), seems to provide the answer. Soon after eclosion, the

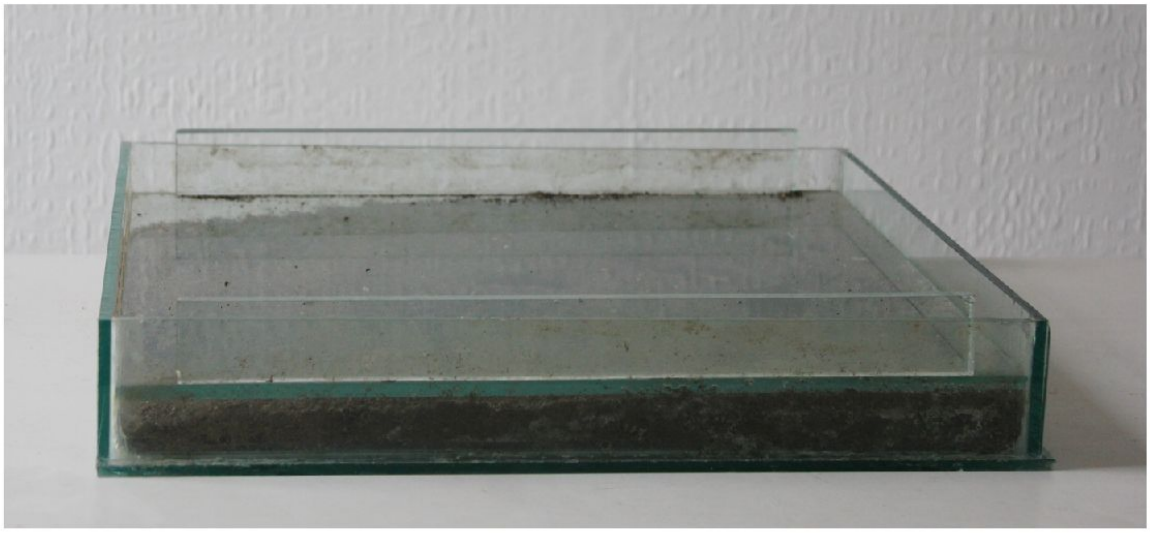

Fig. 1. Terrarium filled with soil and with the lid on. Base dimensions: 360 mm x 325 mm x 60 mm, glass thickness 4.6 mm. Lid dimensions: base 346 mm x 311 mm, glass thickness 6.2 mm; handles: 301 mm x 29 mm, glass thickness 8.4 mm.

female everts the mycangium and rubs this organ against the wall, accompanied by the exudation of droplets; thus suggesting that during this evertion of the mycangium the teneral imago is re-acquiring the symbionts left by the larva (Tanahashi & Fukatsu 2014). This unique mycangium-related behaviour has been observed in the Japanese species of *Dorcus*, *Prosopocoilus* and *Platycerus* (Tanahashi *et al.* unpublished). On the other hand, the male behaviour at their eclosion has not been well studied yet. Therefore, the aim of this study was to observe the post-eclosion behaviour of male and female *L. cervus*, and to reveal the sexual dimorphism of their behaviour.

MATERIAL AND METHODS

Terrarium. Third instar (L3) mature *L. cervus* larvae were placed in a specially prepared terrarium, designed and built by Paul Hendriks (unpublished) (Fig. 1). It was filled with a ~22 mm layer of moist, sandy soil. This thickness of soil was intended to force the larvae to keep a 'window' in their cocoon (Hendriks, unpublished). With smaller larvae some 'windows' had to be enlarged when the pre-pupa had emptied its gut and was quiescent. The soil was pressed down firmly and levelled to avoid collapse of the cocoons. In case some larvae were not yet ready to pupate, one fourth of the terrarium was filled with the pabulum from which they had been collected. Prior to adding the larvae to the box, some depressions were made in the levelled soil in order to accommodate them. After adding the larvae the lid was put in place in order to prevent the soil from drying out; the terrarium was kept indoors away from direct sunlight, at room temperature.

Larvae. Observations were carried out in the terrarium from June 2013 and May 2014 onwards. Initially, in each season, four mature L3 larvae of the same sex were selected. They were collected, before they had left the pabulum to pupate in the soil, in the first author's back garden (51° 54' N; 0° 54' E). Mature L3 larvae can be recognized because they have accumulated a lot of fat and have a yellowish hue.

Sexing was done by examining the medio-ventral region of the larva's 9th abdominal segment with a hand lens for the presence of the terminal ampulla (or, Herold organ) (Herold 1815; Martinez & Lumaret 2005). It is absent in the females; their ovaries are visible through gaps in the fat body between the 7th and 8th segments (Fremlin & Hendriks 2014). At a later stage, more individuals were moved into vacated cocoons: one pre-pupa larva and one pupa, July 2013, and one pre-pupa, August 2014; they were sourced from another terrarium. In 2013 a female pupa was later moved out and placed on a rudimentary cocoon in order to observe rear views during post-eclosion.

In total, eleven individuals were selected for observations during this study. Wherever possible, some biometric data was collected from these individuals either at the beginning or during their development: head capsule width, body length excluding the mandibles, and body weight. The instruments used were: digital callipers, to 0.01 mm accuracy, and a Scalix CB-310 electronic scale, to 0.01 g accuracy.

Photographic equipment. The equipment used was an EOS 550D SLR Canon camera fitted with a Sigma 105 mm objective; a Canon Speedlight 430EXII was sometimes used. The camera was either set on a Jessops copystand or a Velbon tripod (PH-248 Head). Photography in the terrarium was with the lid removed.

RESULTS

Pupation and eclosion behaviour

Of the eleven individuals selected for this study, eight reached the final moult: four females in the first year and three males and one female in the following one. In both years, one of the four larvae, which were initially introduced in the terrarium, took very long to settle down and did not survive for unknown reasons; one died in the pre-pupal stage and the other soon after it pupated. Another individual was killed by a centipede soon after pupation.

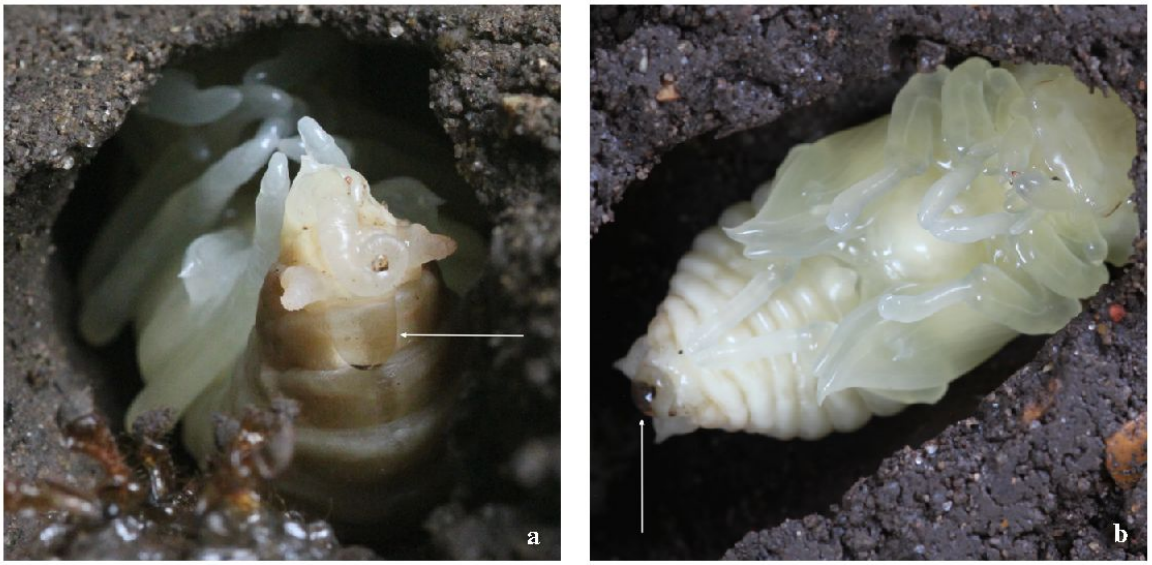

Fig. 2. *Lucanus cervus* pupae displaying a droplet, one hour after pupation, arrow: (a) male; (b) female.

The larvae, which settled well, took 36 days to pupate (n=4); the final moult took an additional 34 days (n=4), at room temperature. Placing the larvae in a shallow terrarium allowed for close-up observations of what was going on inside the truncated cocoons, but after the hindwings expanded, they obscured the view of the last abdominal segment and this hampered observations. Therefore, good rear views were only possible with a female, which moulted in a rudimentary cocoon, 2013, and a couple of imagos which eclosed in a boomerang shaped cocoon, 2014.

The metamorphosis of male and female larvae was identical. During each moult both lost a fair amount of fluid; this was apparent because of the way in which soil clung to the tip of their body and the wetness on the base of the cocoon.

The pre-pupa moulted on its back; the exuvia was left at the end of the cocoon away from the pupa, which also reclined on its back. Soon after ecdysis, a droplet was observed in the pupa's abdomen; in the male it hung from the dorsal side of the pupa's last abdominal segment (Fig. 2a). This was only observed in one male because the pupation of the other two occurred while the observer was absent from the country. In the female the droplet was at the very tip of the pupa's abdomen (n=4) (Fig. 2b), it was visible for a few hours.

The pre-imago moulted on its front, but this time the exuvia was tucked underneath the body. During ecdysis the elytra extended and moved over the back of the abdomen, which was very distended. The tips of the hindwings appeared soon after. The elytra were very pale but from then on melanised, gradually. This moult was videoed with two individuals, which eclosed in the boomerang shaped cocoon, right from the beginning for several hours (Fremlin 2014a, 2014b).

Post-eclosion dimorphic genitalia-everting behaviour

As melanisation was identical in both sexes, we are going to relate the genitalia-everting behaviour to the wings development.

1) Hindwings unfurled. As soon as the old cuticle had been tucked under the body, the last abdominal segment could be properly viewed for the first time. In the male, the genital capsule was already everted and the whip-like fagellum (or, permanently everted sac) soon assumed a curled upright posture. In contrast, the female's ovipositor never everted; only the last tergite (t9) was visible at this stage. Soon after that, both started exuding droplets, intermittently (Fig. 3). The male seemed do it much more often than the female, huge droplets. In the male the fluid emerged from under the penultimate tergite (t8) and ran over the genital capsule. In the female, it flowed from the penultimate inter-segmental region and ran over the last tergite (t9). During this stage the imagos had their legs bent at the tarsi; they moved, very slowly, while pushing against the anterior end of the cocoon (Fig. 3).

2) Hindwings expanded. About one hour after eclosion, the hindwings were fully expanded. At this stage, the male was still exuding droplets (Fig. 4a). The female's mycangium everted for the first time, about two and half hours after eclosion. The mycangium was an elliptical organ with a distinct texture from the surrounding integument. Its colour was a pristine pale cream (Fig. 4b). Three and a quarter hours after the hindwings had expanded, the male retracted the genitalia and stopped exuding fluid. This behaviour was recorded step-by-step only with one male, it lasted a bit over four hours (Fremlin 2014a). The teneral imago (38 mm in body length)

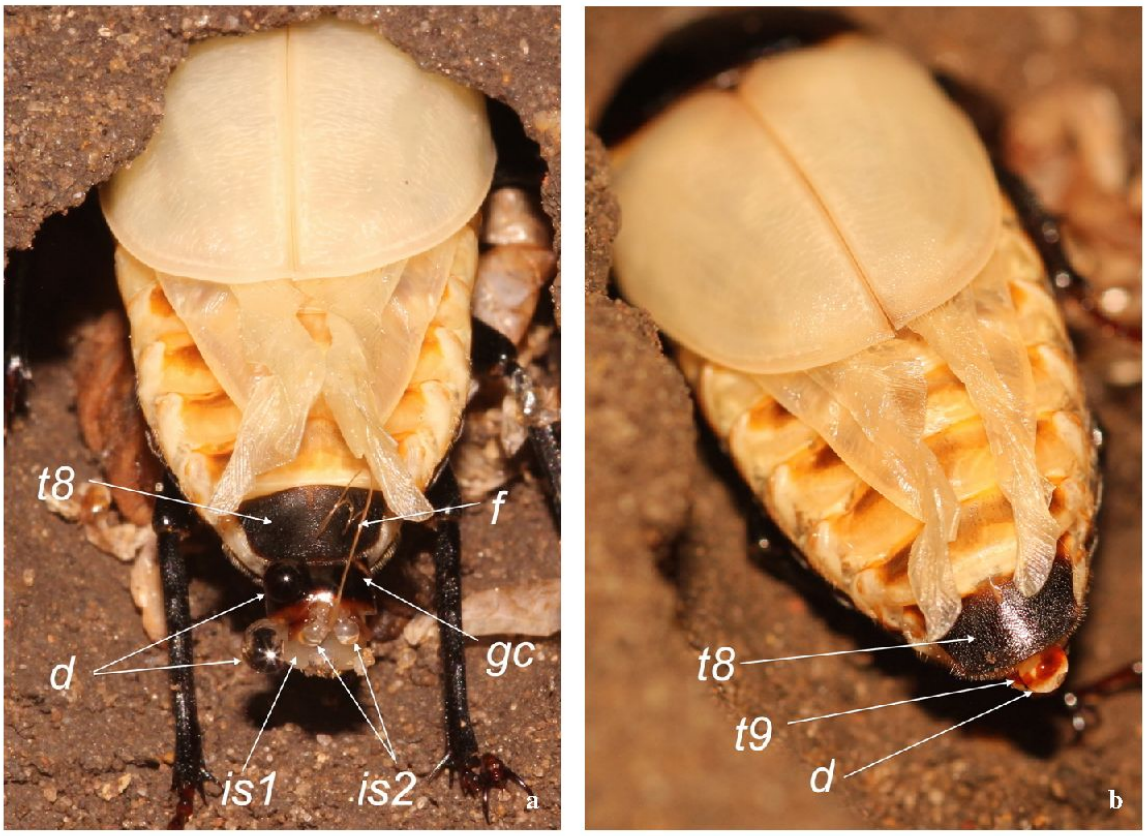

Fig. 3. Post-eclosion *L. cervus* imagos, with fully distended abdomen and unfurled hindwings, exuding droplets: — (a) male with everted genitalia, thirty minutes after eclosion; note the hindlegs bent at the tarsi; — (b) female, fifty minutes after eclosion. — Abbreviations: d, droplet; f, flagellum (or, permanently everted sac); gc, genital capsule; is1, large inner sac; is2, small inner sac/s (or, endophallus); t8, 8th tergite; t9, 9th tergite (divided into two hemitergites).

weighed 2.30 g. In normal cocoons, the evertion of the genitalia was reproducible with two more males.

Nearly two hours after the mycangium eversion, the female exhibited a distinctive behaviour, for the first time. She curled down the fully expanded abdomen tip in such a way that the mycangium had good contact with the surface of the cocoon and she started sweeping it in a rhythmic sideways motion, like a metronome. She did it with the legs bent at the tarsi; that way she was much closer to the ground. At the same time she exuded droplets of fluid, intermittently. This behaviour may last up to twenty minutes and was repeated several times, periodically. The female retracted the mycangium and rested in between. The mycangium got darker and dirtier in the process. In one individual observed step-by-step there were altogether three episodes during a period of four hours. The last one ended six hours after the eversion of the mycangium (eight and a half hours after eclosion) (Fremlin 2014b). This teneral female (26 mm in body length and 1.32 g in weight) lost 41 per cent of her weight from the pupal stage to the end of the observations. Roughly the same behaviour was observed with three more females. They seemed to loose a lot of fluid, sometimes this fluid collected at the bottom of the cocoon, due to their shallowness (Fig. 5a). This female (31 mm in body length and 2.15 g in weight) lost 60 per cent of her weight from larva to teneral imago.

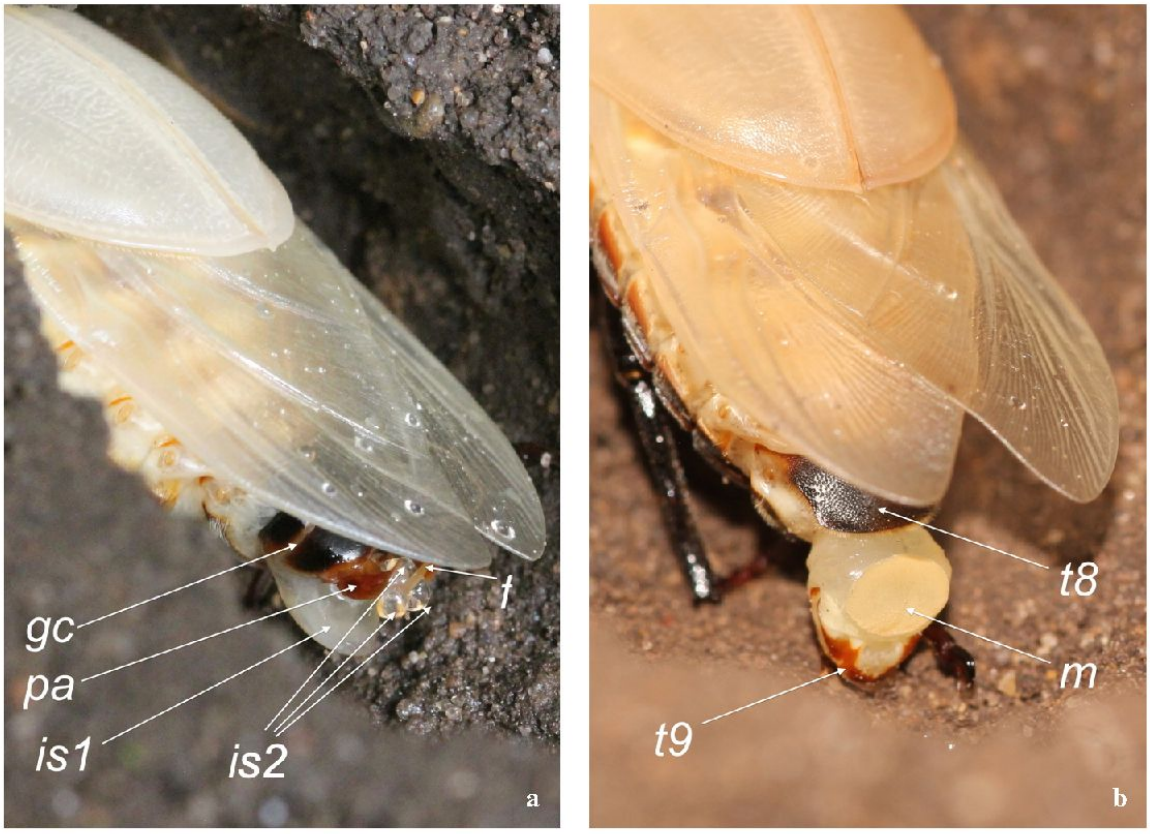

Fig. 4. Lateral view of *L. cervus* imagos with expanded hindwings, still very wet. — (a) Male's everted genitalia, one hour and forty minutes after eclosion. — (b) Female's freshly everted mycangium, three hours after eclosion. — Abbreviations: f, flagellum (or, permanently everted sac); gc, genital capsule; is1, large inner sac; is2, small inner sac/s (or, endophallus); m, mycangium; pa, parameres; t8, 8th tergite; t9, 9th tergite (divided into two hemitergites).

Serendipitously, an interesting behaviour was observed with the female whose pupa was placed on the rudimentary cocoon. This female was found on her back and, judging by the dark colour of her elytra, must have eclosed in the previous hours during the night; she could not turn round. Consequently, her hindwings did not develop properly, and were sticking out in an unnatural manner. However, shortly after she was placed on her feet, she started exuding droplets and within a bit over two hours she exhibited the normal 'sweeping' behaviour. At one time she flipped on her back and started retracting the mycangium (Fig. 5b); then she resumed that behaviour once back on her feet. She carried on for about three and a half hours, intermittently, exuding droplets and sweeping, exactly like a normal female would have. This female (27 mm in body length and 1.54 g in weight) lost 42 % of her weight from the pupal stage till the end of these observations.

3) Hindwings folded, elytra chestnut brown. After the hindwings were folded, the females were observed only sporadically and no more sweeping episodes were recorded.

Both sexes remained with a distended abdomen until their cuticle hardened up, but their last abdominal segment was no longer telescoped. This lasted until the next day. After that, they no longer presented a distended abdomen, hence the 9th tergite and the 8th and 9th sternites were no longer everted. From then on the adults

Fig. 5. — (a) *L. cervus* female after exhibiting the mycangium-related behaviour, twenty minutes before the hindwings were folded; note the cocoon wetness (arrow) and the light brown elytra. — (b) Abnormal eclosion female retracting the mycangium soon after flipping on her back; note the malformed hindwing. — Abbreviations: m, mycangium; s7, 7th sternite; s8, 8th sternite; s9, 9th sternite (divided in two hemisternites, or gonocoxites); t8, 8th tergite; t9, 9th tergite (divided into two hemitergites).

remained quiescent *in situ* for eight to nine months until their emergence the following year.

DISCUSSION

Overall, their metamorphosis was reproducible and predictable. Some researchers have reported on this (Harvey & Gange 2003; Lai & Hsin-ping 2008; Hendriks & Fremlin 2012). Regardless of their size, there was no difference between their moulting times; speed of melanisation; hindwings development or straightening of the head; but their post-eclosion behaviour seems to have been unreported in the past. As expected, *L. cervus* females behaved virtually the same way as the previously tested Japanese species: *Dorcus*, *Prosopocoilus* and *Platycerus* (Tanahashi *et al.*, unpublished); but the latter exhibited their mycangium-related behaviour after the hindwings were folded and we have probably missed that. A female, which had an abnormal eclosion, resumed the mycangium-related behaviour as soon as she was turned on her feet, and it lasted for a good length of time, indicating that this behaviour is switched on by some other external stimulus or antecedent behaviour.

The male's genitalia-everting behaviour came as a total surprise. The unique male's post-eclosion everted genitalia has been captured by Lai & Hsin-ping (2008) in a post-eclosion series with *Dorcus curvidens binodulosus* (Waterhouse, 1874) and is evident in photos of other stag beetle species posted in the Internet by hobbyists; but, to our knowledge, it has has not been described. What we observed during post-eclosion is rather different from what occurs during copulation. Then the genitalia rotate sideways as they evert from the genital capsule (Fremlin pers. obs.); but this rotation mechanism has also not been described.

The abdomen of a hardened imago also telescopes when gentle pressure is applied (Franciscolo 1997), or when one is trodden on (Fremlin pers. obs.); in both cases the male's genital capsule everts but usually only a bit of the parameres and the flagellum are visible. The description of *L. cervus* genitalia by Franciscolo (1997) fits with our observations regarding the flagellum and the parameres; the endophallus (is2) is cognate with the penis, which is a semi-transparent membranous organ. The complex structure of the endophallus when manually inflated has

been extensively studied for *Platycerus* species (Imura 2007, 2010), but not yet for this species. The bigger sac (is1) is probably related to the integument, which connects the genitalia with the genital capsule, but its eversion needs further investigation.

There also seem to be no reports about the exudation of liquid either by the pupa or the imago. Even though both male and female exuded droplets soon after the eclosion, it is interesting to note that the male seemed to do it more liberally than the female; but this could be linked to the fact that the observed females were smaller than the male. The duration of the male genitalia-related behaviour was much shorter than in the female and it started right after eclosion. The female took a good two hours to evert the mycangium, and then a couple of hours to start sweeping; then she exuded droplets with greater frequency. We have scant data of their weight loss from pupa to imago, about forty percent, but this needs to be investigated and compared with that of the male.

However, the male genitalia-everting behaviour raises many questions. Perhaps this behaviour is necessary for the male to arrange the inner sacs of his genitalia? Why both exuded droplets right after the eclosion? It is clear that in the female this loss of fluid is associated with the mycangium-related behaviour. The female would have a vested interest in maintaining a high level of humidity in her cocoon; it would have a direct influence in the survival of the purged larval gut symbionts and the efficiency of symbiont uptake from the cocoon. However, its importance to the male is not so clear. The fluid is probably from the haemolymph, but it needs to be analysed. Also its source needs to be located accurately; the fact that there was loss of fluid soon after pupation suggests that the morphology of this exocrine system, like the genitalia, could already be fairly developed at that stage; this needs to be investigated further. From our observations in the teneral imago the fluid seems to originate from the same integument in both sexes: the dorsal region of the penultimate inter-segmental membrane. In the male, this membrane was not everted the same way, but the liquid dripped from above the genital capsule; in the female it dripped below the mycangium. In either case, it is not being discarded via the gut. The anal opening in the female is in the ventral area (Franciscolo 1997; Holloway 2007); in the male the rectum goes through the genital capsule and opens above the flagellum (Wanat 2007; Fremlin pers. obsv.).

CONCLUSION

The post-eclosion behaviour of the stag beetle, *Lucanus cervus*, was recorded in both male and female. The female exhibited the mycangium-related behaviour, which is almost the same as has been known in several Japanese stag beetle species of different genera, suggesting that this kind of behaviour is common in the Lucanidae, as well as the universality of the female-specific mycangium. The genitalia-everting behaviour in the male was observed for the first time. We also discovered that both male and female exuded a huge amount of liquid from the abdominal tip, not via the gut, along with these sexually dimorphic post-eclosion behaviours. The mechanism and the adaptive significance of such exudation of liquid should be investigated in future studies.

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