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[SHORT COMMUNICATION]

Is there a relationship between egg luminescence and the formation of a spherical germ rudiment in fireflies?

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ABSTRACT

In two firefly families, Lampyridae and Rhagophthalmidae, a spherical germ rudiment (SGR) forms through the deep invagination of the germ disk into the yolk, moving toward the egg's center. The SGR then elongates to form the embryo and amnion, with embryogenesis continuing while the embryo remains submerged in yolk until just before embryonic revolution. These developmental patterns are unique to these families and not observed in other insects. Notably, all Lampyridae eggs are luminescent throughout the egg period, whereas no egg luminescence has been reported in Rhagophthalmidae. We observed a change in the luminescence of *Aquatica lateralis* (formerly *Luciola lateralis*) during its egg period. Specifically, the newly laid egg emits persistent dim green light across the entire egg, but as days pass, the luminescence diminishes. Instead, two or three small yellow-green spots of light begin to appear during the second half of the egg period. Based on the discovery of two luciferases (Luc1 and 2) with distinct amino acid sequences in *A. lateralis*, we hypothesize that the overall egg luminescence results from Luc2. We assume that Luc2 and messenger RNA transcribed from *Luc2* are incorporated into the newly laid eggs, and these substances are evenly diffused in the egg, resulting in the luminescence of the entire egg. On the contrary, the spot-like luminescence is assumed to be attributed to Luc1, most of which is newly formed in mid-egg stage and localized in the photogenic organ of the mature embryo's eighth abdominal segment. From this, we infer that the early movement of the non-luminescent SGR to the center of the egg, by which the entire egg surface becomes filled with luminescent yolk, is adaptive by maintaining the egg's luminescence through Luc2 until the mid-egg stage. The SGR's early formation likely supports egg luminescence and helps maintain an aposematic signal to deter egg predators. However, in Rhagophthalmidae, where non-luminescent eggs receive maternal care throughout development, the adaptive significance observed in Lampyridae would not apply. We then propose that the problem regarding the relationship between the SGR and egg luminescence should be verified by examining the embryogenesis of luminescent and non-luminescent groups of Elateridae, the sister group of fireflies.

KEYWORDS Lampyridae, Rhagophthalmidae, Phengodidae, embryology, luciferin, luciferase

Formation of the spherical germ rudiment in firefly embryogenesis

In this study, we define fireflies as those belonging to three coleopteran families, i.e., Lampyridae, Rhagophthalmidae, and Phengodidae, all of which are luminescent, at least in their larval stages. The formation of the spherical germ rudiment (SGR) in Lampyridae was first described over a century ago in two American species: *Photuris pennsylvanica* and *Photinus consanguineus* (Williams 1916). Later, its formation was also reported in four Japanese species: *Nipponoluciola cruciata* (formerly *Luciola cruciata*), *Aquatica lateralis* (formerly *Luciola lateraris*), *Luciola parvula* (formerly *Hotaria parvula*), and *Pyrocoelia rufa* (Ando and Kobayashi 1975; Kobayashi and Ando 1985; Kobayashi 1987; Kobayashi et al. 2006; Kobayashi 2008). In these species, the SGR forms when the circular germ disk (Fig. 1A), located near the anterior pole of the egg, becomes sac-shaped and sinks into the yolk (Fig. 1B, C). The SGR then migrates toward the egg's center while maintaining its spherical shape (Fig. 1D). During further development, the SGR elongates and differentiates into a thin amnion on one side and an embryo (germ band) on the opposite side. Both the

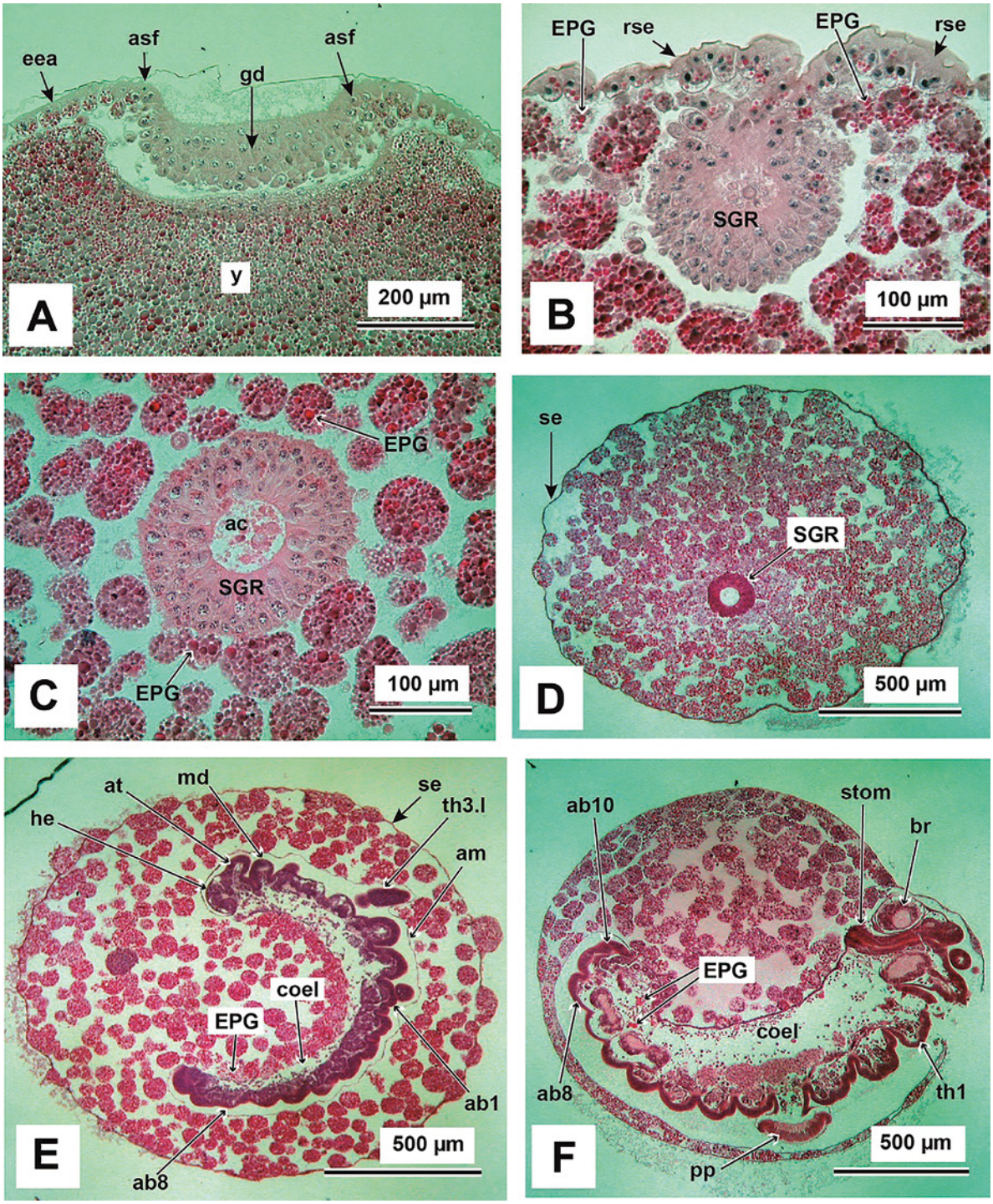

Fig. 1 Embryonic development of *Pyrocoelia rufa*. Eggs embedded in paraffin were sectioned at 5- or 7-μm thickness and stained with Delafield's hematoxylin and eosin. The histological procedures were previously described by Kobayashi et al. (2006). A. Approximately 2-day-old egg showing the germ disk (gd) and amnioserosal fold (asf). B. Approximately 3-day-old egg showing the spherical germ rudiment (SGR), rudimentary serosa (rse), and eosin-positive granule (EPG) in the yolk. C. Approximately 5-day-old egg showing the SGR, developing amniotic cavity (ac), and EPG. D. Approximately 31-day-old egg during winter diapause, showing the SGR near the egg's center. Winter diapause lasts around 160 days, from 11 to 170 days after oviposition, with the SGR retaining its spherical shape and a diameter of about 220 μm. E. Sagittal section of a 195-day-old egg showing a developing embryo. F. Sagittal section of a 205-day-old egg shortly before embryonic revolution. ab1, 8, 10: first, eighth and 10th abdominal segments, am: amnion, at: antenna, br: brain, coel: coelom, he: head lobe, md: mandible, pp: pleuropodium, se: serosa, stom: stomodaeum, th1: prothoracic segment, th3.l: metathoracic leg, y: yolk.

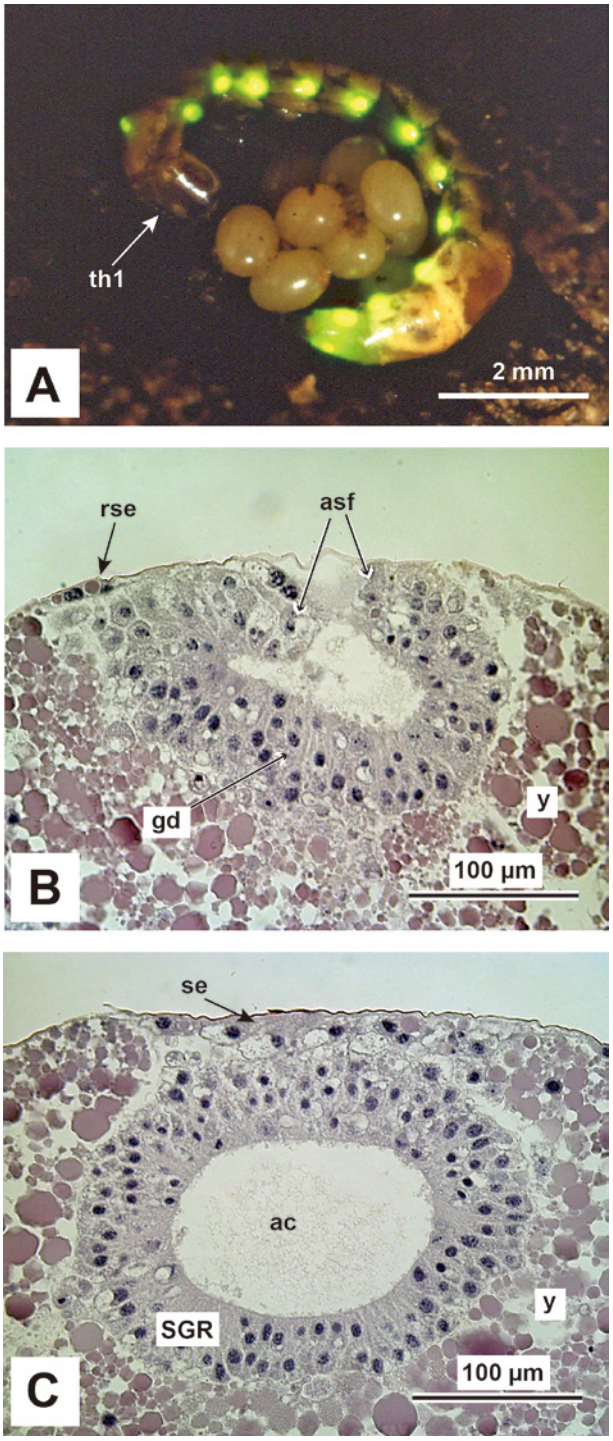

Fig. 2 A. Female adult *Rhagophthalmus ohbai* with her egg mass (photo courtesy of the late Dr. Nobuyoshi Ohba). Luminescent organs are segmentally located on the mesothorax, metathorax, and the first nine abdominal segments. Female body length is about 11 mm. Egg luminescence has not been confirmed (Kawashima, personal communication). B, C. Sections of *R. ohbai* eggs in spherical germ rudiment (SGR) formation. Eggs embedded in paraffin were sectioned at 5-µm thickness and stained with Delafield's hematoxylin and eosin. Histological procedures were previously described by Kobayashi et al. (2002). B. Approximately 3.5-day-old egg showing the invaginating germ disk (gd) and amnioserosal fold (asf). C. Approximately 4-day-old egg, showing the completed SGR. ac: amnionic cavity, rse: rudimentary serosa, se: serosa, th1: prothoracic segment, y: yolk.

amnion and embryo elongate while immersed in the yolk (Fig. 1E). As embryonic revolution approaches, the entire embryo becomes visible on the egg surface (Fig. 1F). The formation of the almost perfectly spherical SGR and embryonic development occurring while the SGR is deeply immersed in the yolk are unique developmental features not observed in other insects. Interestingly, a similar developmental process has been described for *Rhagophthalmus ohbai* (Kobayashi et al 2002) (Fig. 2B, C) in Rhagophthalmidae, which is closely related to Lampyridae. Therefore, SGR formation in the yolk may be considered an apomorphy shared by Lampyridae and Rhagophthalmidae, although recent phylogenetic studies revealed the sister group relationship between Rhagophthalmidae and Phengodidae, of which embryogenesis is entirely unknown (Amaral et al. 2019; Kusy et al. 2021).

The change of egg luminescence states

For over a century, it has been reported that lampyrid species exhibit weak luminescence not only from their eggs but also from their ovaries (e.g., Fabre 1913). In firefly larvae and adults, luminescence is produced by light released when firefly luciferase catalyzes the conversion of luciferin to oxyluciferin in the photocytes of the photogenic organs. In recent years, two types of luciferases were identified in *Nipponoluciola cruciata* and *Aquatica lateralis*: luciferase 1 (Luc1), found primarily in the photogenic organs of larvae and adults, and luciferase 2 (Luc2), found in eggs, pupae, and female adult ovaries (Oba et al. 2010, 2013). The two luciferases differ by approximately 30% in their amino acid sequences and are derived from distinct genes (*Luc1* and *Luc2*).

The entire egg of *A. lateralis* emits a persistent, faint green light (Fig. 3A); this luminescence diminishes over time. However, around the mid-egg stage, two or three egg areas begin emitting a strong yellow-green light (Fig. 3B, C), and this spot-like luminescence persists until hatching. The luminescence from the whole egg is due to Luc2, as Oba et al. (2013) detected only Luc2 in eggs on the first day after oviposition, although these authors did not address the cause of the spot luminescence that appears in the latter half of the egg period. Since the ovary contains Luc2 (Oba et al. 2013), it is assumed that the luminescence of the whole egg is due to Luc2 incorporated into the yolk during oogenesis. The SGR of *A. lateralis* completes by about 30 h after oviposition (Kobayashi and Ando, 1985). Then the possibility that Luc1 (but not Luc2) participate in the luminescence of the newly formed SGR may be considered. That possibility, however, is extremely low, because the newly laid egg (1 day after oviposition) of this species emits light with maximum wavelength of 543 nm, which is almost identical with

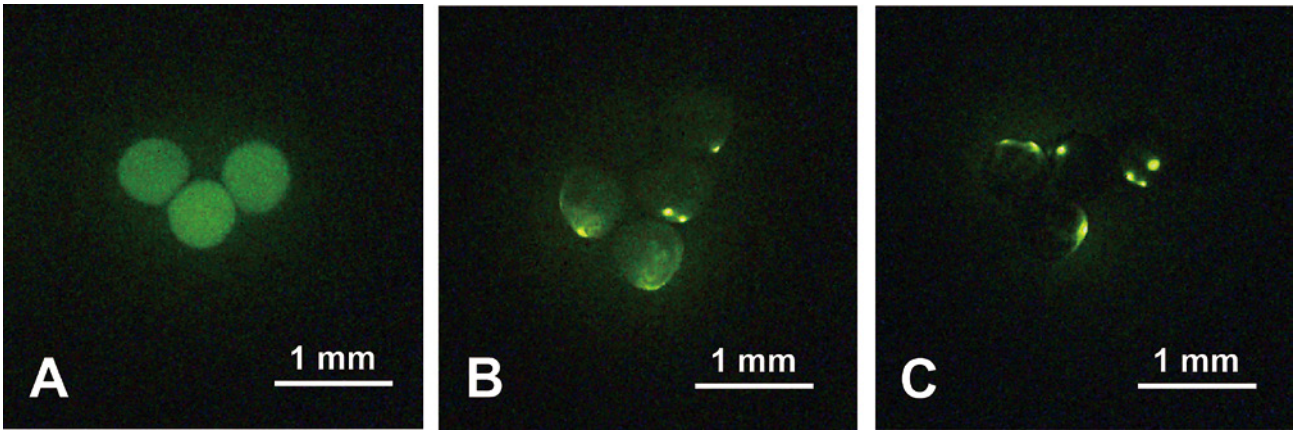

Fig. 3 Change in luminescence during *Aquatica lateralis* egg development. Eggs require 20–30 days to hatch. A. 1-day-old egg. B. 7-day-old egg. C. 16-day-old egg. Luminescence images were captured using a DP70 color CCD camera (Olympus) mounted on a BX51 microscope (Olympus) with a 4× objective lens. BX51 imaging lens was replaced with a bioluminescence imaging lens (Ogoh et al. 2014), reducing magnification to 0.8×. The exposure time was 5 min.

that of recombinant Luc2 (539–540 nm) but largely differs from that of recombinant Luc1 (550–597 nm, variable in different pH) (Oba et al. 2013). Based on comparisons with *N. cruciata*, which undergoes development nearly identical to that of *A. lateraris*, it is hypothesized that the latter's spot-like luminescence occurs when the embryo is still completely immersed in the yolk (7–8 days after oviposition). By this stage, the segmentation of the embryonic abdomen is complete; thus, this luminescence is believed to emanate from the vicinity of the photogenic organ in the eighth abdominal segment of the future larva. Specifically, the shift in luminescence state midway through the egg stage is thought to be due to a switch from Luc2 to Luc1 luminescence. This suggests an adaptive significance in the early movement of the non-luminescent SGR to the egg's center early in embryogenesis, surrounding the non-luminescent young embryo with luminescent yolk and maintaining whole-egg luminescence through Luc2 until the mid-egg stage. Although no experimental evidence exists for the ecological importance of egg luminescence, previous studies have indicated that, akin to larval and adult luminescence, egg luminescence may serve as an aposematic signal to predators (Oba et al. 2013; Bessho-Uehara et al. 2017; Powell et al. 2022).

Even if the adaptive significance of whole-egg luminescence is acknowledged, SGR formation in *Rhagophthalmus ohbai*, where no egg luminescence is observed, cannot be explained (Fig. 2A). The absence of egg luminescence in this species may be attributed to the maternal care provided by the apterous and larviform female adult, which emits continuous, likely aposematic, light throughout the care period (Fig. 2A). Nevertheless, as this species' first instar larvae already emit light (three luminescent spots on each body segment from the thorax to the abdomen; Ohba et al. 1996), it is assumed that the larval photogenic organs are fully developed in the egg by the time of hatching. However, the amino acid sequence of luciferase in *R. ohbai* differs markedly from that of Luc1 in Lampyridae (around 50% homology), with no equivalent to Luc2 having been identified (Oba et al. 2010, 2013). Additionally, there is a marked difference in the stainability of yolk granules between the yolk systems of *Pyrocoelia rufa* in Lampyridae and *R. ohbai* in Rhagophthalmidae, despite the same fixation method using hot alcoholic Bouin's solution (Kobayashi et al. 2002, 2006): the yolk of the former contains many eosin-positive granules (EPGs; Fig. 1B, C, E, F), which are stained red by eosin, whereas the yolk of the latter does not (Fig. 2B, C). Most EPGs appear to be protein yolk granules, diminishing due to consumption by the embryo during development. However, some EPGs are found in the embryonic coelom and fat bodies, near the eighth abdominal segment (Fig. 1E, F: EPG) where the larval photogenic organs form, and may be precursors of photocyte granules. Moreover, yolk-like granules in the embryo's fat bodies have also been observed in *N. cruciata* (Kobayashi 1987) and are potentially yolk-derived. This suggests that some EPGs may not be yolk but rather granules containing peroxisomes to which Luc1 is bound, and thus the EPGs may be involved in the luminescence from larval photogenic organs. Notably, the larval photogenic organ is known to originate from fat bodies (Williams 1916; Okada 1935).

Supposed distribution of egg luciferases and luciferin

As mentioned before, since the ovary of *Aquatica lateralis* contains Luc2 (Oba et al. 2013), it is implicitly assumed that Luc2 and messenger RNA transcribed from *Luc2* are incorporated into newly laid eggs from the mother insect. On the other hand, the origin of luciferin, or whether it is derived from the ovaries or it

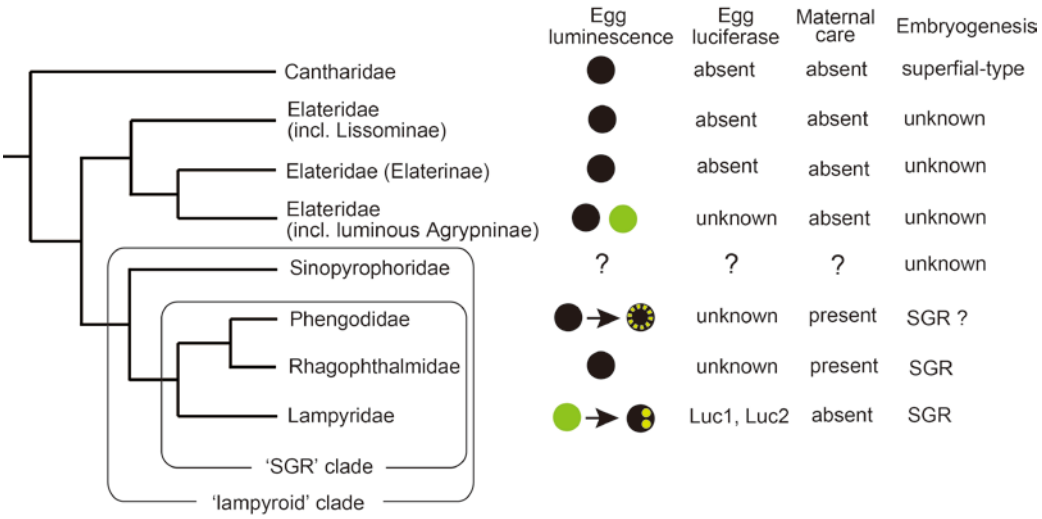

Fig. 4 Characters concerning egg luminescence and embryogenesis, mapping on the phylogenetic tree of elateroid families proposed by Kusy et al. (2021). Black circle: non-luminescence of egg, black circle with two yellow-green dots: spot-like egg luminescence in Lampyridae, black circle with yellow-green dotted circle: belt-like luminescence in Phengodidae, yellow-green circle: dim-green luminescence of whole egg. SGR: spherical germ rudiment.

is newly formed in the egg, has not yet been clarified. However, because firefly luciferin is a low molecular weight compound of 280 dalton, it is assumed to be easily diffused throughout the egg. Then, judging from the fact that the newly laid egg emits light from the entire egg surface, it is natural to consider that both Luc2 and luciferin are evenly diffused in the whole egg. These Luc2 and messenger RNA of maternal origin decompose as the development proceeds, resulting in stopping the emission of the dim green light from the entire egg. On the other hand, since the newly laid eggs do not contain Luc1 and messenger RNA transcribed from *Luc1* (Oba et al. 2013), transcription of *Luc1* probably starts when the light organ is differentiated in the embryonic eighth abdominal segment, and then Luc1 is accumulated in or around this segment. As mentioned before, it should be noted that some EPGs are found in the embryonic coelom and fat bodies, near the eighth abdominal segment of *Pyrocoelia rufa* (Fig. 1E, F; EPG). The photocytes of the adult firefly *Pteroptyx tener* contain eosin-positive granules (or EPG-like granules), and IHC staining with polyclonal anti-luciferase and DAB chromogen suggested that these granules correspond to luciferases bound to peroxisomes (Nur Khairunnisa et al. 2016). If the EPGs of *P. rufa* contain numerous peroxisomes, the EPGs might be incorporated into fat bodies. The fat bodies containing the EPGs then differentiate into photocytes in which the expression of *Luc1* starts, resulting in the photocytes to arise spot-like luminescence in the eighth abdominal segment.

Unlike the eggs of Lampyridae, those of Rhagophthalmidae probably lack the luciferase orthologous with Luc2, then the eggs are non-luminescent from the beginning. However, as mentioned before, luciferase is detected from the luminescent adult females of *Rhagophthalmus ohbai* and its amino acid sequence is largely different from that of the lampyrid species (about 50% difference) (Ohmiya et al. 2000). Although there is no evidence that this adult luciferase is the equivalent of the larval luciferase, since the newly hatched larvae are luminescent (Ohba et al. 1996), there is the possibility that the transcription of adult *Luc* begins in the photogenic tissues of mature embryos. In that case, the luciferase will be distributed in the photocyte or around the peroxisome of the photocyte in the tissue, but not throughout the egg.

Perspective

A recent phylogenomic study suggests that Sinopyrophoridae, or luminescent click beetles discovered first in Asia and elected as a distinct family, forms the sister group relationship with Lampyridae + (Phengodidae + Rhagophthalmidae) (Kusy et al. 2021). These four families are luminescent and thus designated as the 'lampyroid clade' (Fig. 4). This clade is the sister group of Elateridae, which also include many other luminescent click beetles largely belonging to the subfamily Agrypninae, although some other studies suggest that Elateridae is paraphyletic to this clade (e.g., Douglas et al. 2021).

The newly laid eggs of Phengodidae are non-luminescent, but they become luminous in mid- or later-egg stages (Wiliams 1917; Tiemann 1969). This egg luminescence is due to the 'luminous bands' of the developing embryo, but not to the glow of yolk

(Williams 1917). The larvae and adult females of this family also emit light from their heads (orange) and bodies (mesothorax to 9th abdominal segments with yellow green) (Branham 2005). Amino acid sequences of two luciferases responsible for the emission of these colors are determined (Viviani et al. 1999; Arnoldi et al. 2010), but the luciferase involved in egg luminescence are not identified. Non-luminescence of newly laid eggs is probably attributed to the absence of an enzyme corresponding to Luc2 found in Lampyridae. Interestingly, adult females (larviform) with continuous spot-like luminescence from their bodies, care her eggs until when the eggs start luminescence in mid-egg stages (Tiemann 1967) or until hatching (Viviani and Bechara 1997). Judging from the sister group relationship between Phengodidae and Rhagophthalmidae and maternal care habit in both families, it is strongly suggested that the SGR is formed also in the egg of Phengodidae. In that case, Lampyridae, Rhagophthalmidae, and Phengodidae probably form a 'SGR' clade (Fig. 4).

Luminescent click beetles in Agrypninae exhibit luminescent eggs, with even observed in unfertilized eggs in ovaries (Dubois 1886). Several luciferases are identified in several species mostly belonging to *Pyrophorus* (Agrypninae) (Stolz et al. 2003; Amaral et al. 2012). However, the luciferase involved in egg luminescence has not been specified. The identified luciferases have been suggested to arise from non-luminescent click beetles (Fallon et al. 2018); hence, they have an independent origin from those of the 'SGR' clade. In the recently discovered Sinopyrophoridae, there is no information about preimaginal stages.

In summary, as shown in Fig. 4, irrespective of relatively much information about egg luminescence and luciferases in Elateridae, embryogenesis has been entirely unknown in both non-luminescent and luminescent groups of this family. On the other hand, in Cantharidae, the presumed sister group of Elateroidea, the germ band forms on the surface of the yolk mass, thus the germ band belongs to the superficial type, as observed during embryogenesis in many other coleopterans (Fujiwara and Kobayashi 1987). Therefore, the presence or absence of the SGR in Elateridae is of great interest. If the SGR forms in luminescent groups but not in non-luminescent groups, the close relationship between SGR formation and egg luminescence must be considered. On the contrary, if the superficial typed germ band forms in both luminescent and non-luminescent groups, SGR formation must be an autapomorphic or phylogenetically fixed character in the 'SGR' clade, and it is difficult to assume the direct relationship between the SGR and egg luminescence.

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