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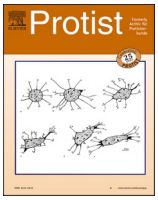

Tracing the Glow: Rethinking Luciferin Biosynthesis in Dinoflagellates☆

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ABSTRACT

Bioluminescence is a striking feature of many dinoflagellates, yet the origin of the luciferin substrate that underlies light emission remains unresolved. Previous studies have noted that luciferin is structurally similar to chlorophyll and related catabolites, but its presence in heterotrophs is puzzling, as such organisms have no need to produce chlorophyll. Hypothetical and sometimes conflicting views on several luciferin biosynthesis reactions have been proposed, but a conclusive template for the pathway is missing. Here, we integrate existing evidence into a model for luciferin biosynthesis based on three testable hypotheses. First, we posit that phototrophic and heterotrophic dinoflagellates are capable of *de novo* luciferin synthesis through a plastid-derived pathway. Secondly, we surmise that luciferin is derived from a specific pathway for chlorophyll degradation that includes the structurally similar pyropheophorbide *a*. Finally, we revisit the role of P630 as a likely biosynthetic precursor rather than a mere oxidation artefact. We then outline experimental strategies to test these hypotheses within the broader framework of tetrapyrrole metabolism. Resolving luciferin biosynthesis will elucidate not only the biochemical, spatial, and regulatory underpinning of bioluminescence but also illuminate its evolutionary origins and how ancestral metabolic pathways can be retooled for novel cellular functions.

1. Dinoflagellate bioluminescence

Over 70 recorded dinoflagellate species are capable of bioluminescence (Marcinko et al., 2013) which they likely use as a defensive mechanism (Abrahams and Townsend, 1993; Buskey et al., 1983; Guilford and Cuthill, 1989; Hanley and Widder, 2017; Huang et al., 2024; Valiadi et al., 2014; Valiadi and Iglesias-Rodriguez, 2013; White, 1979), although it may have arisen for other purposes such as oxygen detoxification (Timmins et al., 2001; Wilson and Hastings, 2013). Light emission can deter predators such as copepods (Prevett et al., 2019) and its resulting impact on marine nutrient cycles highlights the need to understand its molecular mechanism and evolutionary origin. Studies on dinoflagellate bioluminescence to date have focused on the luciferase enzyme and luciferin-binding protein (LBP) which regulate the bioluminescent reaction, on the triggering pathway (Fajardo et al., 2019; Fogel and Hastings, 1971; Hastings and Sweeney, 1957), and on the circadian regulation of bioluminescence (Morse et al., 1989; Sweeney and Hastings, 1957). However, how dinoflagellates synthesise the luciferin substrate, whose enzymatic oxidation leads to light emission, is not yet known.

The bioluminescent reaction takes place in an organelle unique to dinoflagellates called the scintillon (Fogel et al., 1972). The scintillon contains the molecular machinery required to emit light: luciferase, LBP, and luciferin (Johnson et al., 1985). Structurally, active scintillons are vacuolar invaginations connected to the cytoplasm by a narrow neck (Nicolas et al., 1985). The close association between the scintillon and the vacuole underpins the bioluminescent reaction. LBP binds luciferin at pH ~8 and protects it from auto-oxidation, whilst luciferase binds luciferin at pH ~6, and catalyses luciferin oxidation, to produce a photon of blue light (peak 474 nm) (Hastings and Sweeney, 1957). The reaction is triggered by a rapid shift in pH, which makes luciferin available to enzymatic oxidation. This is mediated by the flooding of protons into the scintillon through a recently discovered proton channel in the vacuolar membrane (Rodriguez et al., 2017).

The proton channels are activated by an action potential that runs along the vacuolar membrane and is propagated by their opening (Eckert, 1965a, 1965b; Rodriguez et al., 2017). This action potential is in turn triggered by a signalling pathway which likely involves G-proteins, TRP channels and intracellular calcium stores. The signal is known to be initiated by mechanical stress on the plasma membrane (Chen

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et al., 2007; Hamman and Seliger, 1982; Lindström et al., 2017a; McFarland et al., 2025; Von Dassow and Latz, 2002).

Another special characteristic of dinoflagellate bioluminescence is that it exhibits circadian regulation, at least in phototrophic species, where light emission is mechanically inducible only at night (Sweeney and Hastings, 1957). In *Lingulodinium polyedra*, bioluminescent phase seems to be regulated by the disappearance and re-construction of the scintillon organelle and contents (Fritz et al., 1990), whereas in *Pyrocystis lunula*, the scintillon is always present, but the intracellular location changes from the centre of the cell, during the day, to the cell periphery at night (Knaust et al., 1998; Sweeney, 1981, 1982; Widder and Case, 1982). All bioluminescent heterotrophs tested thus far seem to lack this circadian regulation (Buskey et al., 1992; Esaias et al., 1973).

2. Luciferin biosynthesis

Dinoflagellate luciferin is an open-chain tetrapyrrole. It is prone to autooxidation, making it unstable and difficult to work with. Without the presence of reducing agents, it has a half-life of 25 min at 0 °C (Bode and Hastings, 1963). The first clues into the structure of luciferin came in 1980, when Dunlap et al. (1980) found that the light-emitting systems of dinoflagellates and euphausiid shrimp (*Meganyctiphanes norvegica*) are cross-reactive. Studies of the two substrates revealed that they are open chain polypyrroles of a molecular weight of around 550 Da. Oxidation experiments and analysis of the products further revealed that dinoflagellate luciferin is related in structure to chlorophyll. The researchers also noted that long-term storage of luciferin in the absence of a reducing agent results in the formation of a blue product with an absorption maximum of ~620 nm (Fresneau et al., 1988; Nakamura et al., 1989).

The exact structure of dinoflagellate luciferin was elucidated by spectroscopy, mass spectrometry, and nuclear magnetic resonance in comparison to the shrimp luciferin (Nakamura et al., 1989). The structures of four different compounds isolated from *P. lunula* were elucidated: dinoflagellate luciferin, oxyluciferin (the product of the enzymatic reaction with a carbonyl group at the position $C13^2$), an air-oxidation product, and a blue air-oxidation product (Fig. 1A). *P. lunula* was chosen as it has the highest luciferin concentration of any known bioluminescent dinoflagellate, and the luciferin is always present across the circadian cycle (Knaust et al., 1998; Wang and Liu, 2017). The first air oxidation product has a hydroxyl group at the C15 position. The blue air-oxidation product has a double-bond on the beta bridge and corresponds to the compound with an absorption maximum of ~620 nm observed earlier (Dunlap et al., 1980). Interestingly, studies on luciferin in the mid-80s by Fresneau and colleagues also found the blue air-oxidation product but called it P630 due to its absorption maximum at 630 nm (Fresneau et al., 1986) (Fig. 1B). The absorption spectra of Fresneau's P630 and Nakamura's blue air-oxidation product almost perfectly align and both compounds thus likely represent the same molecule (Fajardo et al., 2020). The slight differences in absorption maximum may be due to different solvents used during spectroscopy, or simply down to interpretation. Fresneau et al. (1986) also describes the isolation of an NAD(P)H-reductase of ~47 kDa which can reduce P630 into a product with the spectral characteristics and light-emitting properties of luciferin. They conclude that P630 appears to be the precursor of luciferin rather than its product, however this idea gained little traction in subsequent literature (Fig. 1B) (Fajardo et al., 2020). The reduction of P630 into luciferin was considered to be a metabolic process, possibly acting as an egress of excess reducing power generated by photosynthesis and respiration (Fresneau and Arrio, 1988). This proposal is in keeping with the discovery of a specific chlororespiration pathway in chloroplasts (Bennoun, 1982) and similar reactions in cyanobacteria (Omata and Murata, 1985; Peschek, 1982), but it would imply further modifications of the pathway in bioluminescent heterotrophs.

The structure of luciferin bears much similarity to chlorophyll derivatives, most markedly in the presence of the ring E and identical functional groups (Dunlap et al., 1981; Nakamura et al., 1989). The key difference is that luciferin is cleaved at the delta linkage of its tetrapyrrole ring, which is highly unusual in chlorophyll derivatives, and further reduced. Photooxidation experiments by Topalov and Kishi (2001) demonstrate that pyropheophorbide *a* (pyropheide *a*) modified at the C20 position is cleaved identically to luciferin (Fig. 1C). They speculate that dinoflagellate luciferin may be derived by a new pathway from a chlorophyll catabolite hydroxylated at C20. Similar cleavage of the delta link has been observed in pyropheide *a* conjugates with Zn (Iturraspe and Gossauer, 1991) (Fig. 1D). Interestingly, chemical reduction of the cleaved molecule produces a blue compound that lacks the metal group and loses one double bond (Iturraspe and Gossauer, 1992) in a way structurally similar to P630 and luciferin (Fig. 1D). Wu et al. (2003) investigated luciferin relationships further by adding isotopically labelled chlorophyll precursors, glycine and glutamate, into the growth medium of *P. lunula*. Both chlorophyll *a* and luciferin incorporated the label thus suggesting they may be derived from the same precursor pathway. However, direct evidence is needed given difficulties with interpreting isotope labelling, especially given that δ-aminolaevulinic acid synthase (part of the C4 pathway using glycine) appears to be absent in dinoflagellates (Cihlář et al., 2016; Janouškovec et al., 2017).

The evidence of luciferin being chlorophyll derived, however, is puzzling when we consider that many bioluminescent dinoflagellates are also heterotrophic. This poses the question of how heterotrophs acquire luciferin. One hypothesis is that they uptake chlorophyll from their prey and have a luciferin biosynthesis pathway that degrades prey-derived chlorophyll. However, one study suggests otherwise. Yamaguchi and Horiguchi (2008) showed that the heterotroph *Protoperidinium crassipes* fed on rice flour retained its bioluminescence over nearly two years, which indicates that it synthesises luciferin *de novo*. Equally intriguing is that all bioluminescent heterotrophs examined to date appear to retain the ancestral tetrapyrrole biosynthesis pathway, which is required for haem production (Cooney et al., 2024; Janouškovec et al., 2017). It is therefore possible that the non-photosynthetic plastids of heterotrophic dinoflagellates might also contain a luciferin biosynthesis pathway (Janouškovec et al., 2017).

3. Our perspective

We integrate current evidence about dinoflagellate luciferin biosynthesis into a new model (Fig. 1E) that relies on three independent, testable proposals. Firstly, we hypothesise that most dinoflagellates, including at least some heterotrophs, synthesise their luciferin *de novo*. This proposal builds on evidence that heterotrophic dinoflagellates were ancestrally photosynthetic and kept tetrapyrrole biosynthesis in non-photosynthetic plastids similarly to the malaria parasite *Plasmodium* (Janouškovec et al., 2017; Waller and McFadden, 2005). Accordingly, molecular phylogenies have shown that the heterotrophic *Noctiluca scintillans* and *Polykrikos kofoidii* have surprisingly close photosynthetic relatives in *Spatulodinium pseudonoctiluca* (Gómez et al., 2010) and *Polykrikos hartmannii* (Hoppenrath et al., 2010; Hoppenrath and Leander, 2007), respectively. Whilst it remains possible that these heterotrophs acquire luciferin from prey-derived chlorophyll, they may well have adapted their chlorophyll biosynthesis pathway for luciferin biosynthesis. Interestingly, such adaptation need not involve chlorophyll *a* itself: its precursor chlorophyllide can be directly converted into pheophorbide. The enzymatic conversions leading to luciferin in any dinoflagellate might indeed involve reordering, rerouting (e.g., via pyropheophytin) and even bypassing (is chelation required?) of some reactions compared to chlorophyll metabolism. However, we base our model on the parsimonious assumption that both pathways share common intermediates (Fig. 1E).

Secondly, we propose that luciferin is derived from a specific chlorophyll degradation pathway, which includes pyropheide *a* as a key

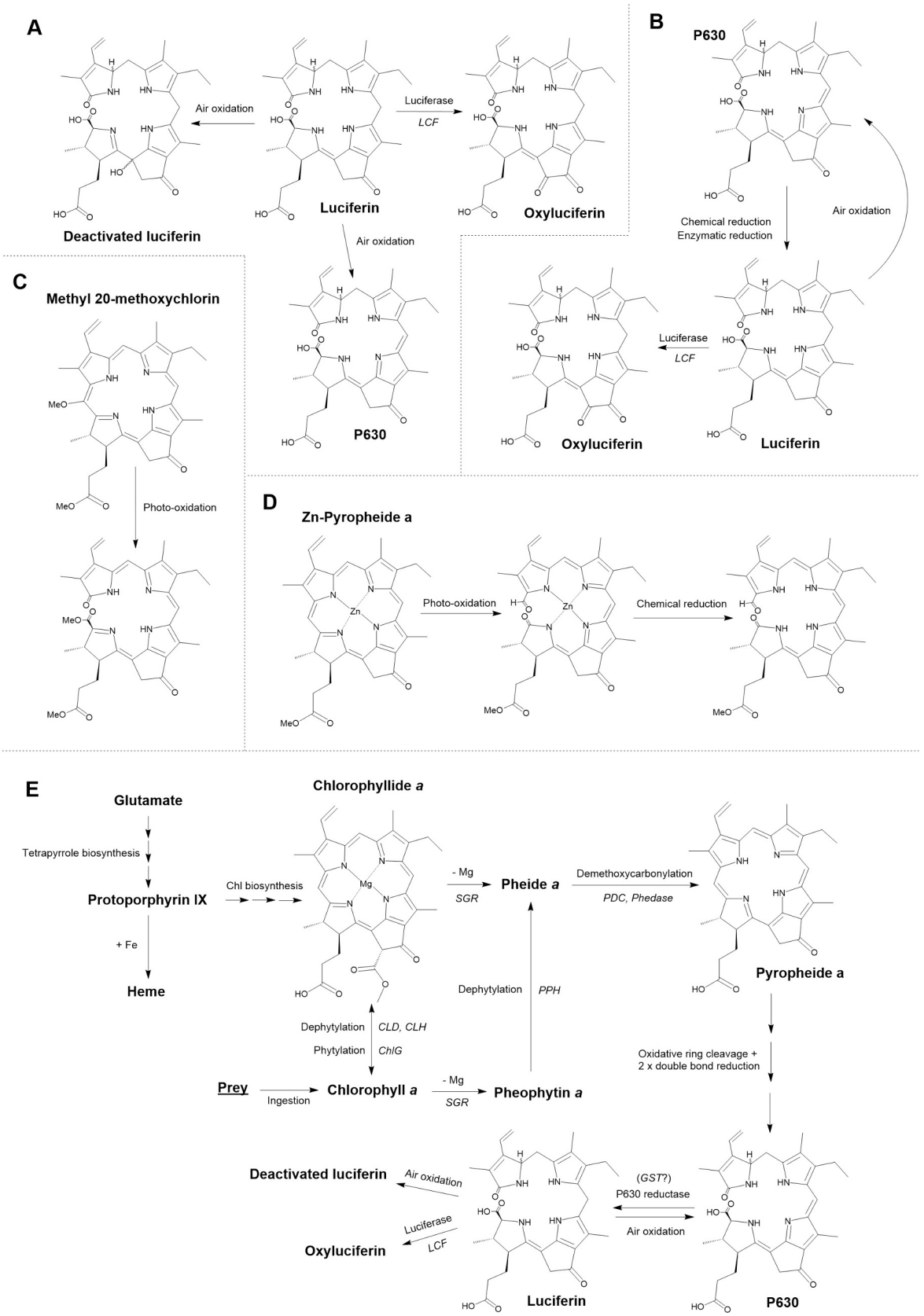

Fig. 1. Chemical reactions predicted to be involved in luciferin biosynthesis. (A) Paradigm of luciferin oxidation to different products, adapted from Nakamura et al., 1989. (B) Hypothesis of P630 as a precursor of luciferin, adapted from Fresneau et al., 1986 (C) Photo-oxidative ring opening of a modified pyropheide *a* molecule, adapted from Topalov and Kishi, 2001. (D) Photo-oxidative ring opening and subsequent reduction of a Zn conjugate of pyropheide *a*, adapted from Iturraspe and Gossauer, 1992. (E) An integrated scheme of luciferin biosynthesis proposed in this study.

intermediate (Fig. 1E). Pyropheide *a* and dinoflagellate luciferin share a striking structural similarity and three differences from chlorophyll: the lack of the phytol tail, conjugated metal and carboxymethyl group on ring E. As such, pyropheide *a* is likely derived by the action of a dephytylase such as pheophytinase or chlorophyllase (Schelbert et al., 2009), dechelatase (Shimoda et al., 2016) and yet unidentified demethoxycarbonylase (Suzuki et al., 2002). Although the role of pyropheide *a* in chlorophyll degradation in algae is poorly understood, it is present in green and brown microalgae (Doi et al., 2001; Spooner et al., 1994) and is commonly found in marine sediments (Louda et al., 1998). Only a few transformations - a ring cleavage by oxygenation and as little as two reductions - are required to convert pyropheide *a* into P630.

Finally, we propose that P630 has a dual relationship to dinoflagellate luciferin. It is both the immediate biosynthetic precursor of luciferin (Fajardo et al., 2020; Fresneau et al., 1986), and, under certain conditions, its air-oxidation product *in vitro* (Nakamura et al., 1989) (Fig. 1E). The traditional interpretation that P630 arises solely from air oxidation of luciferin during storage is supported by the observation that luciferin slowly converts to P630 in the absence of reducing agents (Bode and Hastings, 1963; Dunlap and Hastings, 1981; Nakamura et al., 1989). However, this view does not account for the fact that P630 is detectable in fresh extracts of *P. lunula* when reducing agents are removed from the extraction buffer. Fresneau's extraction protocols in the 1980s, which did not include reducing agents, consistently recovered P630 from extracts, whereas methods using reducing buffers by other groups only detected P630 after long-term storage. This suggests that the strong reducing conditions used in standard extraction protocols may mask the true abundance of P630 present *in vivo*. In addition, such conditions could lead to the detachment of bound factors, such as a previously described 4.8 kDa peptide in *P. lunula* (Fresneau et al., 1988), which may be guiding reductive power toward the beta bridge of the tetrapyrrole ring. The differential presence of such factors could explain why experimental reduction of differently prepared P630 in the Fresneau and Nakamura studies yielded different amounts of active luciferin substrate for the bioluminescence assay (Fresneau et al., 1986; Nakamura et al., 1989).

Crucially, biochemical assays demonstrated that P630 can be converted into luciferin either chemically (via reducing agents) or enzymatically. Fresneau et al. (1986) reported an NAD(P)H reductase that reduces P630 to luciferin, which then emits light in the presence of luciferase. Although the amino acid sequence of this enzyme, which we refer to as P630 reductase (Fig. 1E), has not yet been identified, the activity itself is reproducible, present in multiple species, and strongly indicates that P630 is an authentic intermediate in luciferin biosynthesis. It should be noted that the reduction of the P630 molecule by the P630 reductase was hypothesised to be catalysed by a glutathione S-transferase (GST) (Fajardo et al., 2019, 2020) which was found to have the same N-terminal domain as luciferase and LBP (Okamoto and Hastings, 2003). This N-terminal domain could link the protein to bioluminescence activity, although this remains to be proven experimentally. *In vivo*, the stability of luciferin is likely maintained through binding to LBP or the 4.8 kDa peptide, while the balance between oxidised (P630) and reduced (luciferin) states is controlled by the P630 reductase. We therefore argue that P630 represents the final intermediate in luciferin metabolism: a molecule that accumulates both as a natural biosynthetic intermediate in luciferin metabolism, and as a by-product of air-oxidation *in vitro*.

4. Future Directions

Despite decades of research on dinoflagellate bioluminescence, the biosynthetic origin of luciferin remains unresolved. Elucidating this pathway will require an integrated approach, combining modern biochemical, omics and physiological techniques.

Experimental perturbation of the chlorophyll biosynthesis pathway represents a promising avenue to examine its relationship to luciferin. Building upon the amino acid feeding experiments of Wu et al. (2003), this could be achieved by using isotopically labelled specific precursors of the pathway, such as δ-aminolaevulinic acid, and analytical techniques to quantify luciferin and its biosynthetic intermediates, such as HPLC, spectroscopy, and mass spectrometry. A complementary approach may involve specific inhibitors of tetrapyrrole metabolism (gabaculin, 4-amino 5-fluoropentanoic acid; Gardner et al., 1988; Hoult et al., 1986), or its putative enhancers such as copepodamides (Gonzalo-Valmala et al., 2025; Lindström et al., 2017b; Prevett et al., 2019).

These experiments would be particularly interesting with bioluminescent heterotrophs, which lack photosynthesis but retain tetrapyrrole biosynthesis that includes chlorophyll precursor protoporphyrin IX (Fig. 1E). As such, heterotrophs provide a natural test case for whether luciferin can be synthesised either *de novo* or from prey-derived chlorophyll, especially if they are fed on non-photosynthetic prey or by osmosis. The culturing and experimental manipulation of heterotrophic dinoflagellates may therefore have a unique role in tackling the current questions.

On the molecular side, transcriptomics and proteomics both hold potential for uncovering luciferin biosynthesis enzymes downstream of chlorophyll. Differential gene expression analyses under precursor supplementation or inhibition, or other conditions, could reveal both candidate enzymes and pathway regulators. Similarly, proteomic characterisation of purified scintillons may help uncover novel proteins associated with bioluminescence and luciferin metabolism. Although chlorophyllide and chlorophyll are produced in the plastid, terminal luciferin biosynthesis steps have yet unknown intracellular location and might well be spatially and functionally associated with the scintillon. Improved methods for scintillon purification, possibly including flow-cytometry based sorting, could reduce contamination by chloroplasts and other organelles observed previously (Rodriguez et al., 2017).

Finally, targeted biochemical studies could help to isolate enzymes responsible for terminal biosynthetic steps including the reduction of P630 to luciferin. Molecular identification of the P630 reductase, as well as the 4.8 kDa peptide reported by Fresneau and colleagues (Fresneau et al., 1986, 1988), would clarify whether these factors mediate luciferin stability, metabolism and oxidoreductive balance *in vivo*. Demonstrating their function would not only provide insight into luciferin biosynthesis, but also into the mechanisms underpinning the regulation of flash emission, biogenesis and degradation of scintillons, and circadian regulation of bioluminescence.

Together, the approaches listed above offer paths toward disentangling the elusive biosynthesis of dinoflagellate luciferin and the nature and distribution of the associated genes. Beyond resolving a complex biochemical puzzle, such findings could address long-standing mysteries about bioluminescence itself. Some of the most interesting of questions include how light emission is regulated and integrated with other cellular processes, what is its environmental function, and why it emerged and spread across evolutionarily diverse dinoflagellates in the first place.

CRediT authorship contribution statement

James F.E. Vanstone: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Jan Janouškovec:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Jan Janouskovec reports financial support was provided by Ministry of Education Youth and Sports of the Czech Republic (CZ.02.01.01/00/22_008/0004624). If there are other authors, they declare that they have no known competing financial interests or personal relationships that

could have appeared to influence the work reported in this paper.].

Data availability

No data was used for the research described in the article.

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