Multi-level convergence of complex traits and the evolution of bioluminescence

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ABSTRACT

Evolutionary convergence provides natural opportunities to investigate how, when, and why novel traits evolve. Many convergent traits are complex, highlighting the importance of explicitly considering convergence at different levels of biological organization, or 'multi-level convergent evolution'. To investigate multi-level convergent evolution, we propose a holistic and hierarchical framework that emphasizes breaking down traits into several functional modules. We begin by identifying long-standing questions on the origins of complexity and the diverse evolutionary processes underlying phenotypic convergence to discuss how they can be addressed by examining convergent systems. We argue that bioluminescence, a complex trait that evolved dozens of times through either novel mechanisms or conserved toolkits, is particularly well suited for these studies. We present an updated estimate of at least 94 independent origins of bioluminescence across the tree of life, which we calculated by reviewing and summarizing all estimates of independent origins. Then, we use our framework to review the biology, chemistry, and evolution of bioluminescence, and for each biological level identify questions that arise from our systematic review. We focus on luminous organisms that use the shared luciferin substrates coelenterazine or vargulin to produce light because these organisms convergently evolved bioluminescent proteins that use the same luciferins to produce bioluminescence. Evolutionary convergence does not necessarily extend across biological levels, as exemplified by cases of conservation and disparity in biological functions, organs, cells, and molecules associated with bioluminescence systems. Investigating differences across bioluminescent organisms will address fundamental questions on predictability and contingency in convergent evolution. Lastly, we highlight unexplored areas of bioluminescence research and advances in sequencing and chemical techniques useful for developing bioluminescence as a model system for studying multi-level convergent evolution.

I. INTRODUCTION

Evolutionary convergence, the repeated evolution of a similar phenotype, produces biological replicates useful for investigating fundamental questions about the interplay of conservation and divergence during the evolution of complex traits. Convergence is interpreted using either process-based or pattern-based definitions, which can lead to inconsistencies (Stayton, 2015). Process-based definitions attribute convergence to shared evolutionary processes (e.g. natural selection in response to comparable ecological problems). Process-based definitions are restrictive because: (*i*) it is difficult to infer processes for multiple convergent events, and (*ii*) process-based definitions do not consider non-adaptive or alternative hypotheses for producing evolutionary convergence (e.g. exaptation, stochastic processes, or different selective pressures). Alternatively, pattern-based definitions only interpret convergence as an observable pattern and accommodate hypotheses attributing evolutionary convergence to shared, different, and/or stochastic evolutionary processes.

Herein, we follow the pattern-based definition of evolutionary convergence as defined in the first sentence of this Introduction.

The classic approach to identifying cases of phenotypic convergence, called ancestral state reconstruction, uses a well-represented, robust phylogeny to map and trace the origin of a particular trait of interest (Joy *et al*., 2016). In the genomics era, we can extend this approach to examine phenotypic convergence at multiple biological levels (i.e. phenotype, organ, cellular, subcellular, molecular), or 'multi-level convergent evolution', especially as research moves towards characterizing complex trait systems at the cellular, subcellular, and molecular levels. Investigating convergent evolution with a hierarchical perspective is necessary because convergent phenotypes may or may not have conserved morphological or molecular mechanisms (Losos, 2011; Stern, 2013; Rosenblum, Parent & Brandt, 2014). Divergence or conservation in these mechanisms can be influenced by developmental, genetic, or functional/structural constraints (e.g. variations in developmental systems, gene pleiotropy, or the need to retain certain biochemical properties) (Gompel & Prud'homme, 2009; Stern & Orgogozo, 2009; Christin, Weinreich & Besnard, 2010; Frankel, Wang & Stern, 2012; Yeaman *et al*., 2018). Recognizing which biological levels of convergence are conserved and what affects their conservation can identify multi-level drivers of predictability in phenotypically convergent traits. Conversely, we can study multiple levels of convergent traits to understand how constraints led to different evolutionary pathways, and how these pathways ultimately converged on similar solutions.

Phenotypic convergence results from underlying genetic mechanisms that are either disparate or conserved. If conserved genetic mechanisms underlie phenotypic convergence, this is considered parallel evolution. It is important to recognize that there are many historical uses of the term 'parallel evolution', the two main ones being phylogenetic and molecular-based definitions. The former describes the independent evolution of a similar phenotype in closely related organisms, while the latter is used to describe the independent origin of a similar phenotype resulting from homologous molecular mechanisms. Although the meaning and use of the term 'parallel evolution' is often debated, herein we follow the definition presented in Rosenblum *et al*. (2014) and define parallel evolution as a special case of convergent evolution produced by homologous genetic mechanisms (Scotland, 2011; Rosenblum *et al*., 2014). Using this definition to explain convergent and parallel evolution will identify cases of phenotypic convergence that rely on the same genetic mechanisms and studying these will provide insight into the drivers and mechanisms of genetic conservation in convergent evolution. Therefore, we consider parallel evolution to be a subset of convergent evolution, and like convergence, it must be understood in a hierarchical fashion (i.e. regulatory mechanism, gene network, single gene). This hierarchical way of thinking is especially important when studying the convergent evolution of complex traits.

Unlike simple traits (e.g. melanism in pocket mice *Chaetodipus intermedius*) (Nachman, Hoekstra & D'Agostino, 2003), which are controlled by a simple genetic architecture, complex traits (e.g. eyes, venom, and bioluminescence) are multifaceted and functionally integrate many genes and their products (Ogura, Ikeo & Gojobori, 2004; Fernald, 2006; Haddock, Moline & Case, 2010; Pankey *et al*., 2014; Schendel *et al*., 2019; Arbuckle, 2020). Using a holistic approach to unravel, identify, and evolutionarily trace individual functionally important modules contributing to a complex trait is essential to understanding the evolution of the complex trait as a whole, especially since each module may have a different evolutionary history (Nilsson, 2009; Oakley &

Speiser, 2015; Suzuki, 2017). Knowledge of each module's evolutionary history generates hypotheses for when and how separate modules convened to produce extant complexity. However, complex phenotypes may consist of variable modules, and because of their inherent genetic complexity, the phenotypic convergence of complex traits may result from a combination of convergent and parallel genetic mechanisms. Therefore, in order to study the convergent evolution of complex traits systematically, we need to break down complex traits to identify their modules, then examine the multi-level convergent evolution of each module. This strategy merges the holistic approach used to understand complex trait evolution with the hierarchical lens used to study convergent evolution.

In this review, we introduce bioluminescence as an excellent trait for studying the multi-level convergent evolution of complex traits. Then, we use our proposed framework to summarize available information broadly at each biological level for luminous organisms that use convergent molecular mechanisms to produce light. Lastly, we identify gaps in knowledge that should be investigated to develop bioluminescence as a model system for studying convergence. We propose that bioluminescence systems provide exceptionally good opportunities for studying predictability in evolutionary trajectories and the roles of evolutionary and ecological constraints in complex trait convergence.

II. BIOLUMINESCENCE: AN EXCELLENT SYSTEM FOR STUDYING CONVERGENCE IN COMPLEX TRAITS

(1) Harnessing biochemical reactions to produce light

Bioluminescence, the biochemical production of light by a living organism, is generated by proteins called luciferases that facilitate the oxidation of substrates called luciferins. Photoproteins, a distinctive class of luciferase exhibiting different enzyme kinetics, are protein complexes that bind to luciferin and oxygen prior to initializing the biochemical reaction and require the addition of a cofactor to produce light (McCapra, 1976). Upon biochemical oxidation, oxidized luciferins enter an excited state and emit light as they return to their ground state (Hastings & Wilson, 1976). These biochemical reactions can be produced by the organism itself, called autogenic bioluminescence, or organisms can house and control luminous bacterial colonies in specialized light organs, called bacteriogenic bioluminescence. These biochemical reactions, harnessed by many organisms *via* autogenic or bacteriogenic bioluminescence, are used for diverse biological functions such as luring prey or mating, predator defence through the production of a startling display or an autotomized distraction, or counterillumination, a form of marine camouflage that uses ventrally produced light to match the intensity of downwelling light (Case *et al*., 1977; Morin, 1983; Young, 1983; Harper & Case, 1999; Marek *et al*., 2011; Gerrish & Morin, 2016; Hellinger *et al*., 2017). To harness this biochemical reaction in a bioluminescence system, luminous organisms use modules to perform several physiological functions: (*i*) production/dietary acquisition of a photoprotein/luciferase (Bessho-Uehara *et al*., 2020*b*); (*ii*) production/dietary acquisition of a luciferin (Thomson, Herring & Campbell, 1997); (*iii*) maintenance of an organ used to produce bioluminescence or house luminous bacterial symbionts; (*iv*) behavioural control of light production (Tong *et al*., 2009); (*v*) catabolism and/or recycling of bioluminescent molecules (Gomi, Hirokawa & Kajiyama, 2002), and in some cases, (*vi*) storage of bioluminescent

substrates (Cormier, Hori & Karkhanis, 1970; Fallon *et al*., 2016) and (*vii*) modification of light emission using biochemical or physical mechanisms (e.g. green fluorescent proteins, filters, chromatophores) (Denton *et al*., 1985).

However, not all bioluminescence systems have all these functions, and the modules used to achieve these functions can vary (Fig. 1). For example, organisms produce autogenic bioluminescence by using proteins that evolved from the parallel evolution of conserved genes (e.g. the independent evolution of calcium binding photoproteins from a homologous gene found in ctenophores, cnidarians, and non-luminous sponges) (Prasher, McCann & Cormier, 1985; Tsuji *et al*., 1995; Schnitzler *et al*., 2012), or non-homologous genes (e.g. luciferases in copepods and sea pansies) (Lorenz *et al*., 1991; Markova *et al*., 2004, 2015). Bioluminescence is a truly exceptional convergent system that is well suited for investigating factors responsible for determining whether disparate or parallel genetic mechanisms underlie convergence in its modules because it is a complex trait that was re-invented dozens of times using a mix of conserved, similar, and radically diverse ancestral genes.

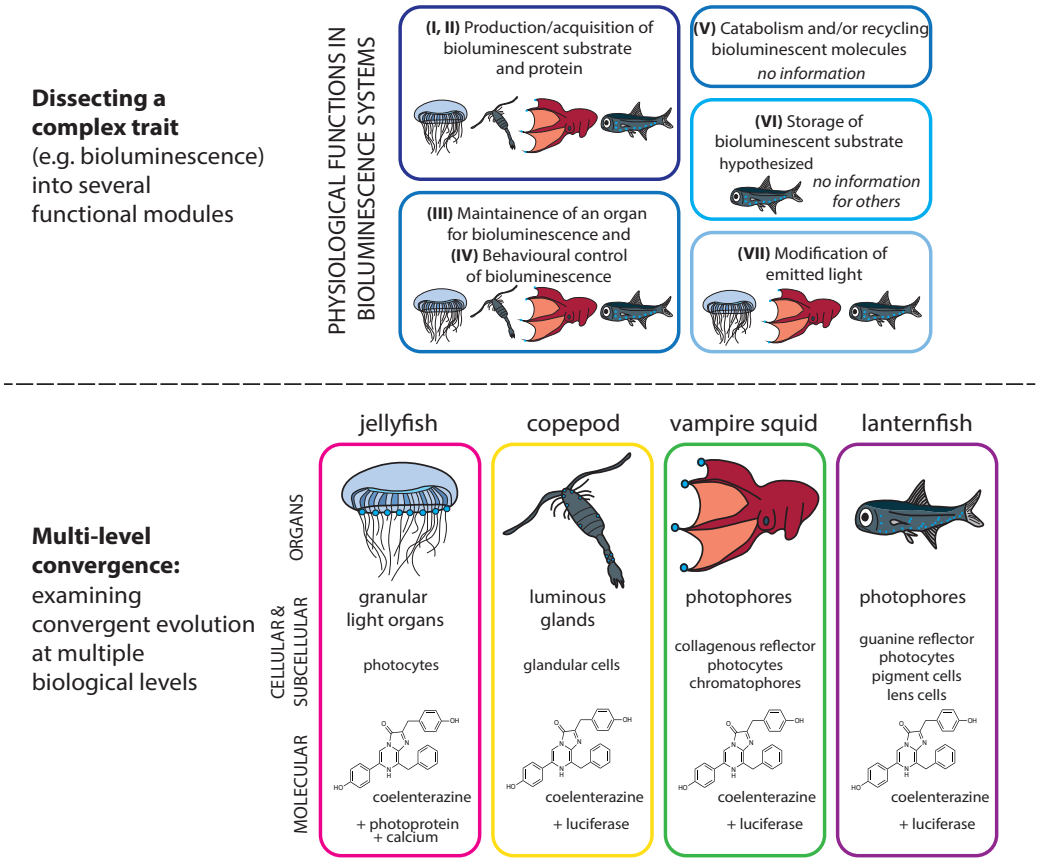

Fig 1. A systematic approach to investigating complex trait convergence involves dissecting a complex trait into several functional modules (top) and examining convergence at all levels of life (bottom). Luminous organisms may or may not share several functional modules to harness the ability to produce light. Although jellyfish, copepods, vampire squids, and lanternfishes evolved molecularly convergent proteins that produce light using the same luciferin substrate, this convergence is not necessarily found at other biological levels.

(2) Widespread convergent evolution of bioluminescence

Bioluminescence is found in freshwater, terrestrial, and marine environments, and evolved independently dozens of times across the tree of life. A previous review on convergence in bioluminescence estimated that bioluminescence evolved at least 40 times, and likely more than 50 times across lifeforms (Haddock *et al*., 2010). In their review, Haddock *et al*. (2010) also suggested that the number of independent origins could increase upon increasing the resolution of bioluminescence character mapping in ray-finned fishes and cephalopods; the former was done by Davis, Sparks & Smith (2016). Parsimony-based ancestral state reconstruction using a genus-level cephalopod phylogeny revealed that bioluminescence originated at least 10 times in cephalopods (Sanchez *et al*., 2018). Verdes & Gruber (2017) found at least eight independent origins of bioluminescence in Annelida. Recently, Bessho-Uehara, Francis & Haddock (2020*a*) examined the bioluminescence systems of deep sea anthozoans and estimated at least six origins of bioluminescence in Cnidaria: three in Anthozoa, two in Scyphozoa, and one in Hydrozoa. Gastropods independently evolved bioluminescence at least five times (in Polyceridae, Phylliroe, Planaxidae, *Quantula*, and *Latia*), but this conservative estimate may increase upon ancestral state reconstruction using a well-sampled, robust gastropod phylogeny (J.A. Goodheart, personal communication).

Calculating independent origins can be difficult because ancestral state reconstruction can overestimate or underestimate independent origins, depending on the assumptions used to describe the probability of character gain and loss. Assuming equal probabilities of gain and loss, ray-finned fishes evolved bioluminescence 29 times. However, if the cost to evolve bioluminescence is weighted twofold (i.e. a trait gain to trait loss ratio of 2:1), the number of origins is estimated to be 27. A bare minimum for the number of origins in fishes is 2 (bacteriogenic and autogenic), but that would imply an unreasonable number of losses (over 120). Aside from difficulties in calculation, defining an independent origin may not be straightforward because many luminous organisms do not produce their own luciferin and/or bioluminescent protein (i.e. organisms that rely on bioluminescent bacterial symbionts or organisms that acquire luciferins and/or bioluminescent proteins through their diet). Despite these caveats, Haddock *et al*. (2010) and Davis *et al*. (2016) included these cases in their estimates because these organisms evolved modules used for regulating bioluminescence (i.e. light organ, behavioural control of bioluminescence, modification of light emission). Following this line of reasoning and summarizing published estimates of bioluminescence in annelids, cnidarians, cephalopods, gastropods, and ray-finned fishes, we estimate at least 94 independent origins of bioluminescence across the tree of life (Fig. 2). Future improvements on the phylogenetic resolution and ancestral state reconstruction of other luminous phyla (e.g. Annelida, Arthropoda, Echinodermata, Mollusca) will likely increase this conservative estimate to at least 100 independent origins of bioluminescence. It is also important to note that, in addition to finding new luminous taxa or independent origins, subsequent investigations may disprove records of bioluminescence in enigmatic taxa such as pycnogonids or bryozoans.

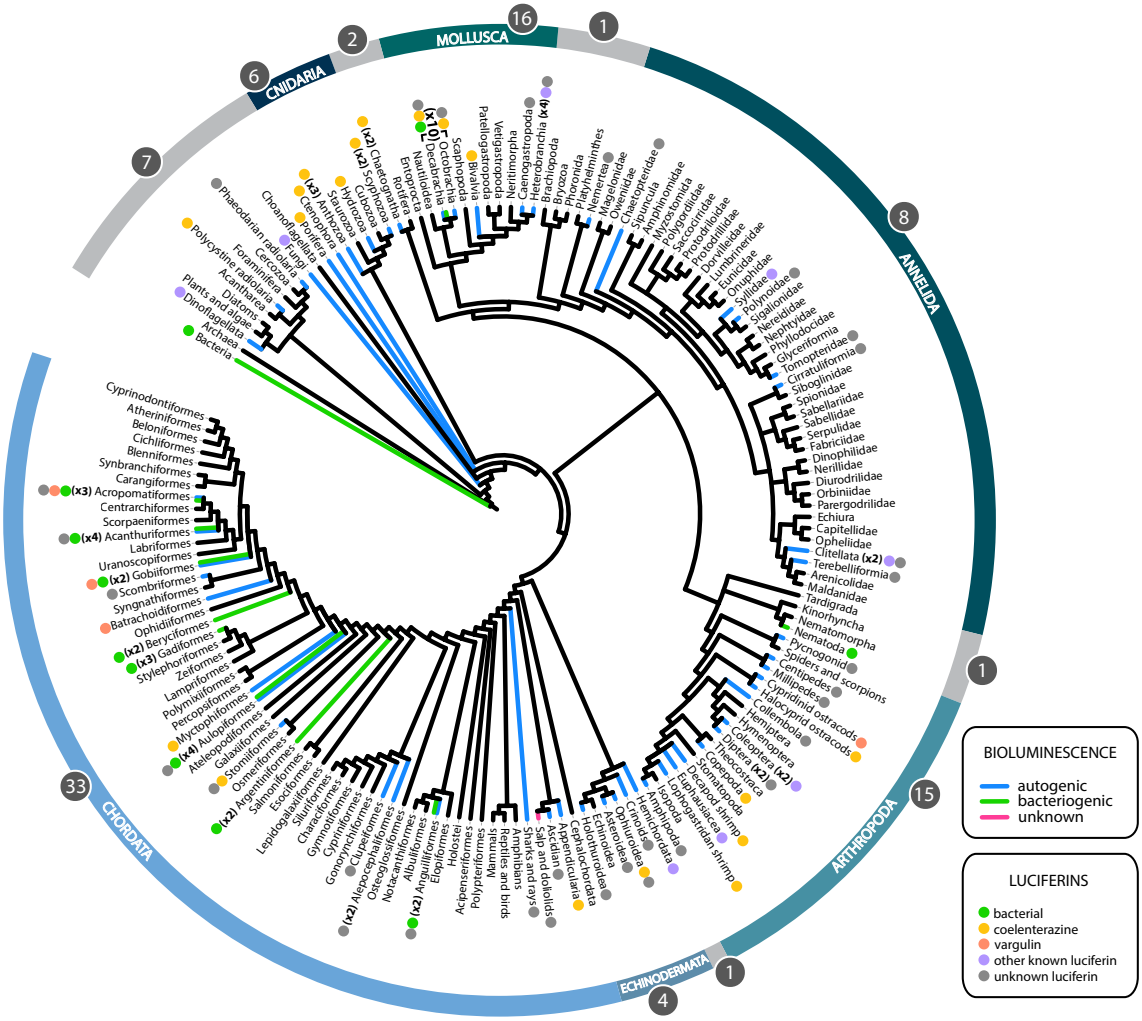

Fig 2. Phylogenetic tree showing at least 94 independent origins of bioluminescence. The blue external arcs group the tips falling under different phyla. Numbers in circles represent the number of independent origins within each arc. Black branches represent non-luminous taxa and coloured branches indicate luminous taxa, with blue, green, or pink branch colours corresponding to autogenic, bacteriogenic, or unknown-source bioluminescence, respectively. Autogenic and bacteriogenic bioluminescence can be found in the same taxon, as shown by bicoloured branches. Numbers next to taxon names indicate the number of origins within that taxonomic group. Coloured dots next to taxon names indicate the types of luciferins used in the taxon's bioluminescence systems (see key on bottom right). The supertree topology is based on previously published phylogenies (Dunn *et al*., 2008; Haddock *et al*., 2010; Regier *et al*., 2010; Oakley *et al*., 2013; Cannon *et al*., 2014; Misof *et al*., 2014; Davis *et al*., 2016; Weigert & Bleidorn, 2016; Sanchez *et al*., 2018; Cunha & Giribet, 2019; Marlétaz *et al*., 2019).

Bioluminescence systems use many different types and combinations of organs, cells, and biochemistries (Sweeney, 1980; Herring, 1987, 2000; Shimomura, 2006). The diversity of biological levels found within this complex trait is precisely what makes bioluminescence an excellent system for examining how conservation and divergence shape the evolution of complex

traits. While bioluminescence can be convergent at one biological level, this convergence may not be found at other levels (Fig. 3). For example, bioluminescence is phenotypically convergent, but its use as a behavioural mechanism (e.g. mating, defence, prey attraction) may not be convergent. Secreted bioluminescence, which is used for mating and defence, is discharged from light organs such as modified mouths, photophores, and appendages (Angel, 1968; Nicol, 1969; Barnes & Case, 1972; Abe *et al*., 2000; Robison *et al*., 2003; Wong *et al*., 2015). Alternatively, internal extracellular and intracellular bioluminescence, which is used for counterillumination, prey capture, mating, and communication, is produced in light spots associated with the epithelium, light organs connected to the digestive tract, luminous lanterns, or structurally complex photophores (Peterson & Buck, 1968; Arnold & Young, 1974; Baguet, 1975; Haddock & Case, 1999; Thacker & Roje, 2009; Galeazzo *et al*., 2019).

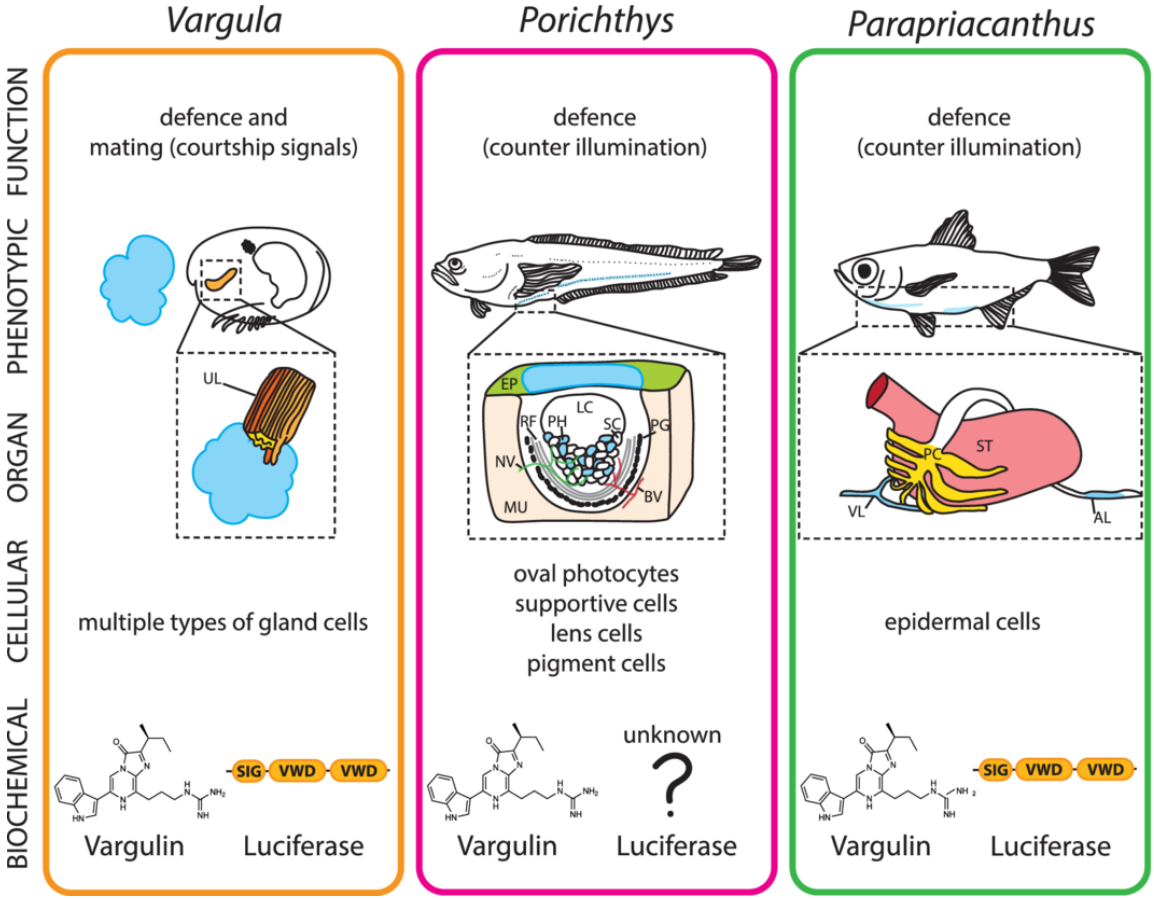

Fig 3. Cypridinid ostracods (*Vargula*), midshipman fishes (*Porichthys*), and golden sweepers (*Parapriacanthus ransonneti*) all use vargulin to produce light, but these organisms rely on different biological components (upper lip, photophore, and visceral light organs, respectively) to produce bioluminescence (exemplified in blue). The methods used to produce bioluminescence (secreted or intracellular) and its function (defence and/or mating) varies between ostracods and the fishes. *Vargula*: UL, upper lip. *Porichthys*: BV, blood vessel; EP, epidermis; LC, lens cells; MU, muscle; NV, nerves; PG, pigment cell; PH, photocyte; RF, reflector; SC, supportive cell. *Parapriacanthus ransonneti*: AL, anal light organ; PC, pyloric caeca; ST, stomach; VL, ventral light organ; SIG, signal peptide; VWD, Von Willebrand Type D domain.

We see divergence and conservation at an organ level, because the biological function of bioluminescence may be constrained by the types of organs associated with a particular behaviour. At a cellular level, we see divergence and conservation of the cell types found in light organs. Furthermore, convergent organ types do not necessarily contain morphologically similar cell types. For example, while photophores in the viperfish *Chauliodus sloani* contain secretory photocytes that are disparate from photocytes found in the midshipman fish *Porichthys*, the photocytes found in the squids *Watasenia scintillans* and *Bathothauma lyromma* contain crystal rods that are structurally similar to those found in krill photophores (Nicol, 1969; Sweeney, 1980). It is important to recognize that past bioluminescence research historically grouped these 'cell types' based on morphological features or functions (i.e. subcellular content, production of light). With advances in genetic sequencing at a cellular level, which we highlight in Section V.1, we can genetically define a cell type and produce cell-type phylogenies to understand how cells used in bioluminescence systems evolved.

At the molecular level (i.e. genes, proteins, substrates), we may see divergence, convergence, and in some cases, parallelism of the biochemical components used to produce light (e.g. luciferin, luciferase/photoprotein). Organisms that use different luciferins to produce light have non-convergent bioluminescent proteins. On the other hand, organisms that use identical luciferins for bioluminescence have molecularly convergent bioluminescent proteins. The molecular convergence in bioluminescent proteins can result from parallel evolution or convergent evolution (the evolution of homologous proteins in the former case, or non-homologous proteins in the latter). Despite many cases of molecular convergence, most known bioluminescent proteins exhibit wide molecular diversity and are not homologous across distantly related taxa, which suggests that most origins of bioluminescent proteins are the result of convergent, but not parallel, evolution (Hastings, 1983; Rees *et al*., 1998; Oba, Branham & Fukatsu, 2011).

Unlike the diversity of luciferases and photoproteins, there are only 11 known luciferins plus 2 additional candidates for the New Zealand glowworm *Arachnocampa luminosa* (insect) and *Ptychodera flava* (hemichordate) luciferins (Fig. 4) (Shimomura & Johnson, 1968; Kanakubo *et al*., 2005; Shimomura, 2006; Petushkov *et al*., 2014; Purtov *et al*., 2015; Oba *et al*., 2017*a*; Watkins *et al*., 2018; Kotlobay *et al*., 2019). The genetic bases responsible for luciferin biosynthetic pathways are only known for bacterial and fungal luciferins. In luminous bacteria, the lux operon encodes genes used to produce both subunits of bacterial luciferase in addition to bacterial luciferin (Meighen, 1991). Similarly, the genes involved in the biosynthesis of fungal luciferin are found in a genomic cluster, along with fungal luciferase (Kotlobay *et al*., 2018). Although the biosynthetic pathways responsible for coelenterazine and vargulin are unknown, the amino acids responsible for their formation are phenylalanine/tyrosine/tyrosine and arginine/isoleucine/tryptophan, respectively (Kato *et al*., 2004; Oba *et al*., 2009).

The chemical structures of most luciferins are distinct, apart from two paired examples (Fig. 4). Dinoflagellate and krill luciferins, molecules that are classified as tetrapyrroles, are structurally almost identical. The structural similarities observed in krill and dinoflagellate luciferins suggest that krill obtain their luciferin through their diet. Additionally, krill and dinoflagellate luciferins are structurally similar to chlorophyll, a molecule that is hypothesized to be the precursor for dinoflagellate luciferin (Dunlap, Hastings & Shimomura, 1981; Nakamura *et al*., 1988, 1989;

Shimomura, 1995). The only other luciferins that share a part of their chemical structure are coelenterazine and vargulin (Campbell & Herring, 1990; Shimomura, 2006). Coelenterazine and vargulin share an imidazolopyrazinone core, which is composed of a fused bicyclic ring containing three nitrogens and a ketone; however, these luciferins cannot be used interchangeably. Except for these two examples, luciferins have diverse chemical structures, thus showing that the independent evolution of luciferins was not constrained to one type of small molecule (Hastings, 1983).

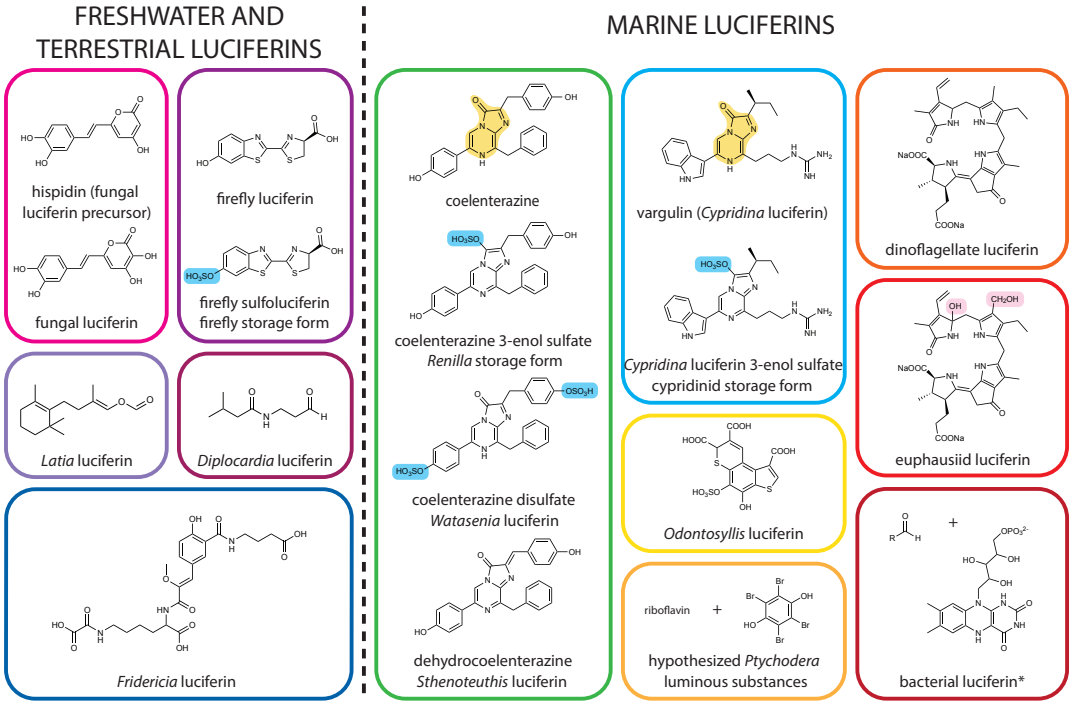

Fig 4. Chemical structures of known luciferins, their derivatives, and their storage forms. Vargulin and coelenterazine share imidazolopyrazinone cores (highlighted in yellow). Firefly sulfoluciferin and the 3-enol sulfated vargulin and coelenterazine are proposed to be luciferin-storage forms that can be enzymatically converted to active luciferins using a sulfotransferase (sulfates highlighted in blue). *Sthenoteuthis oualaniensis* and *Watasenia scintillans* use modified coelenterazine as their luciferins. * In addition to marine systems, luminous bacteria have a symbiotic relationship with terrestrial nematodes. Luminous bacteria reside in the nematode gut, and once expelled by the nematode, the bacterial bioluminescence might attract new larval hosts for the nematode (Patterson *et al*., 2015).

Although most luciferins are only used by the taxa that produce them, the luciferins coelenterazine and vargulin are also used as bioluminescent substrates obtained in the diet in phylogenetically distant organisms. Coelenterazine is the most common marine luciferin, shared across nine phyla (Porifera, Radiolaria, Cnidaria, Ctenophora, Mollusca, Arthropoda, Echinodermata, Chaetognatha, and Chordata), while vargulin is shared across two phyla (Arthropoda and Chordata) (Tsuji *et al*., 1971; Rees *et al*., 1990; Thomson *et al*., 1997; Haddock *et al*., 2010; Markova & Vysotski, 2015; Martini *et al*., 2020). Despite the prevalence of coelenterazine-based bioluminescence, most organisms that use it do not produce it themselves. Because the only organisms known to have coelenterazine biosynthesis capabilities are deep sea copepods,

ctenophores, and decapod shrimps (the former determined through tracing stable isotopes and latter two determined by observing bioluminescence in multiple generations despite being reared on a coelenterazine-free diet, or an increase in luciferin found in embryos, respectively), many other organisms that use coelenterazine for bioluminescence probably obtain it through their diet (Thomson, Herring & Campbell, 1995; Oba *et al*., 2009; Francis *et al*., 2015; Bessho-Uehara *et al*., in press). This is supported by experimental evidence showing that many bioluminescent organisms reared in the laboratory on a coelenterazine-free diet gradually lose the ability to luminesce, but can regain bioluminescence activity upon consuming food items containing coelenterazine (Frank, Widder & Case, 1984; Haddock, Rivers & Robison, 2001; Mallefet, Duchatelet & Coubris, 2020). Furthermore, coelenterazine can be detected in the digestive tracts, stomachs, and livers of both luminous and non-luminous organisms, suggesting that it is readily available in the marine food web (Shimomura, 1987; Rees *et al*., 1990; Mallefet & Shimomura, 1995). Similarly, only ostracod crustaceans are known to produce vargulin. Luminous fishes rely on the dietary acquisition of this substrate, and some fishes supply it to their young through maternal provisioning (Haneda & Johnson, 1958; Tsuji *et al*., 1971; Tsuji, Barnes & Case, 1972; Duchatelet *et al*., 2019). In addition to dietary acquisition of luciferin, a recent discovery found that the luminous fish *Parapriacanthus ransonneti* acquires both vargulin and luciferase from bioluminescent ostracods. This phenomenon, known as 'kleptoprotein bioluminescence', is a novel mechanism used in autogenic bioluminescence, which produces light by using a bioluminescent protein sequestered from the fish's diet (Bessho-Uehara *et al*., 2020*b*).

The rampant dietary acquisition of the luciferins coelenterazine and vargulin suggests that many luminous organisms do not produce their own luciferins. Of the Metazoa hypothesized to produce their own luciferins, all marine groups (i.e. cypridinid ostracods, ctenophores, decapod shrimps, deep sea copepods, syllid polychaetes) and two terrestrial groups (*Diplocardia longa* earthworms, *Latia neritoides* freshwater snails) contain taxa (some if not all) that are capable of secreting bioluminescence into the environment (Herring, 1985; Widder, Greene & Youngbluth, 1992; Verdes & Gruber, 2017). Is the evolution of these two characters, luciferin production and bioluminescence secretion, correlated? This hypothesis could be tested using a phylogenetic correlation test for these two binary characters (luciferin production and secretory bioluminescence) (Pagel & Meade, 2006). As exemplified here, we can use a holistic approach to examine bioluminescence systems, hypothesize how they evolved, and reach a better understanding of how luminous systems' functional modules evolved and are integrated.

(3) Evolutionary origins of bioluminescence

The ubiquitous nature of bioluminescence, coupled with the biochemical diversity found across bioluminescence systems, suggests that the evolutionary origins of bioluminescence are disparate. Like other complex traits, understanding the origin and evolution of bioluminescence requires dissecting and investigating each functional module used in this trait. In the case of the module responsible for the biochemical production of light, studying the evolutionary histories of luciferins and photoproteins/luciferases will provide testable hypotheses for how these substrates and proteins evolved across bioluminescence systems. For example, in luminous fungi and bacteria, genes for luciferin synthesis and luciferase production are found in a genomic cluster (Meighen, 1991; Kotlobay *et al*., 2018). Does this pattern of genetic organization extend to luminous metazoans? How did these genes and their corresponding products originate and evolve?

Although the functional modules in bioluminescence systems include more than just those used to produce light biochemically (luciferin, luciferases/photoproteins), an approach that emphasizes studying a complex trait's functional modules is historically constrained by earlier bioluminescence research that focused mainly on characterizing the biochemistry of light production in luminous systems. Now, given advances in specimen collection, imaging, and sequencing, we can begin to understand the diversity of modules used to perform the physiological functions comprising this complex trait [i.e. maintaining light organ(s), controlling light production, light modification, substrate storage].

E. Newton Harvey, a pioneer in bioluminescence research, observed that bioluminescence is widespread but he questioned why it is seemingly scattered randomly across lifeforms:

> *"Apparently there is no rhyme or reason in the distribution of luminescence throughout the plant or animal kingdom. It is as if the various groups had been written on a blackboard and a handful of sand cast over the names. Where each grain of sand strikes, a luminous species appears" (Harvey, 1920, p. 11).*

One hundred years later, we can investigate whether bioluminescence is truly distributed haphazardly across the tree of life, why some groups have more luminous species than others, and we can propose hypotheses by identifying and comparing selective pressures and possible evolutionary routes leading to bioluminescence. By exploring these routes in luminous and non-luminous organisms, we can test whether certain organisms are predisposed to bioluminescence and hypothesize why we see this scattered phylogenetic distribution of bioluminescence.

(a) *Origins of photoproteins and luciferases*

Most photoproteins and luciferases are molecularly disparate but produce bioluminescence by playing analogous roles as substrate binders and oxidation facilitators. How did luciferases/photoproteins evolve and, in most cases, how can non-homologous proteins produce light using the same substrate? Is the oxygenase function of a photoprotein or luciferase ancestral or derived? One hypothesis proposes that luciferins were non-specifically oxidized by pre-existing proteins. This 'promiscuous protein hypothesis' postulates that proto-luciferases/photoproteins may either function as an oxygenase and/or provide a hydrophobic environment for the oxidation of luciferin (Seliger, 1975; Hastings, 1983; Rees *et al*., 1998; Vassel *et al*., 2012; Adams & Miller, 2020). The latter can be accomplished by intrinsic protein pockets, or a protein-cage effect, which is hypothesized to provide an isolating environment to prevent quenching of luciferin's excited state (Rees *et al*., 1998). This hydrophobic environment hypothesis is supported by firefly luciferase, which evolved from an ancestral fatty acyl-CoA synthetase, thus showing that its oxygenase abilities are not ancestral but derived (Oba, Ojika & Inouye, 2003; Oba *et al*., 2006). Ancestral proteins that facilitated luminescence did not necessarily have to produce the full bioluminescence that we see today, but if they offered an evolutionary advantage, selection may have acted to improve substrate binding efficiency and light production. These hypotheses are supported by the diversity of extant, non-homologous photoproteins and luciferases, demonstrating that their convergent evolution is not constrained to one protein family (Fig. 5) (Gould, Keller & Subramani, 1987; Abe, Nagata & Hashizume, 1996; Oba *et al*., 2003, 2006; Oakley, 2005; Loening, Fenn & Gambhir, 2007; Markova *et al*., 2012; Schnitzler *et al*., 2012; Delroisse *et al*., 2017; Brugler *et al*., 2018; Fallon *et al*., 2018; Kotlobay *et al*., 2018; Schultz *et al*., 2018). The origins of luciferases/photoproteins and their ancestral functions are mostly

unknown. Understanding the origin and evolution of these proteins involves tracing the evolutionary histories of bioluminescent proteins, performing ancestral protein sequence reconstruction, then expressing and characterizing the function of these ancestral proteins (Takenaka *et al*., 2013). Ultimately, this workflow will reveal how diverse luciferases and photoproteins functionally converged to produce light.

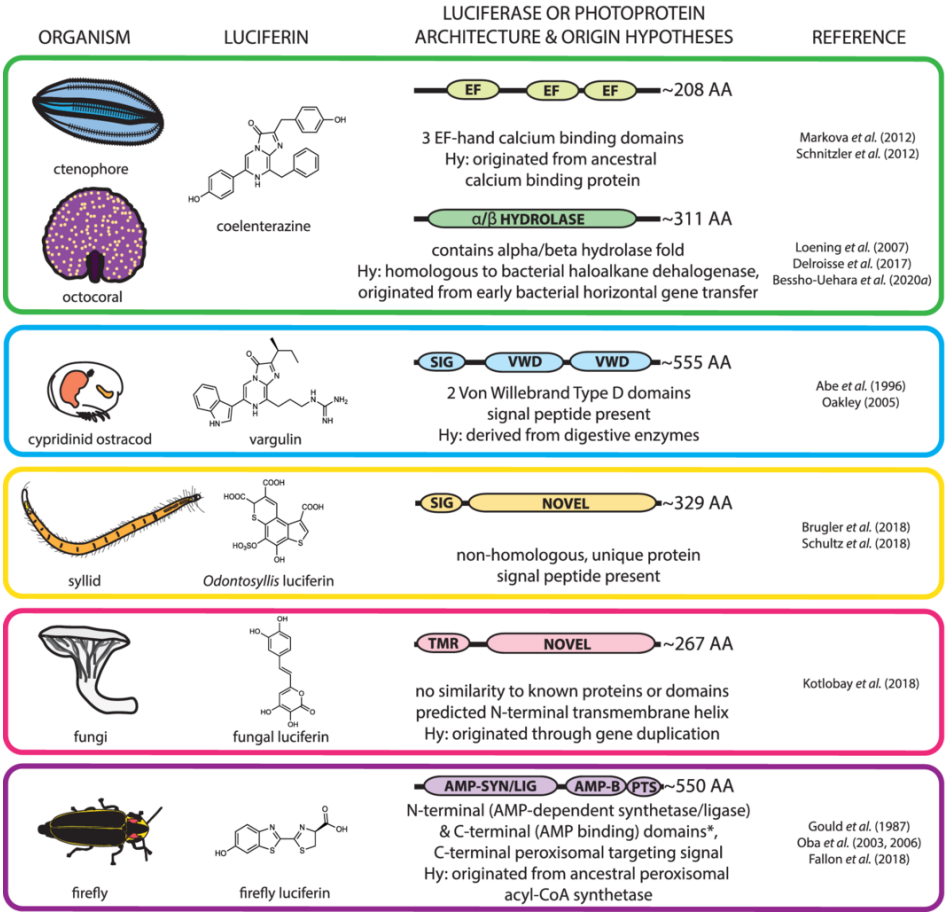

Fig 5. Subset of photoproteins and luciferases exemplifying the heterogeneity found in bioluminescent proteins. Photoproteins and luciferases vary in amino acid length, protein domain architecture, and evolutionary history. Most luciferases and photoproteins are non-homologous, even in organisms that use the same luciferin substrate (such as ctenophores and sea pansies). As a result of this disparity, bioluminescence research proposed different hypotheses (Hy) for the origins of these proteins. * The N-terminal AMP-dependent synthetase/ligase and C-terminal AMP binding domains were predicted using InterProScan.

(b) *Origins of luciferins*

Although luciferins are chemically distinct from each other, their roles in light-producing biochemical reactions are the same – luciferins are oxidized and produce chemiluminescence. Despite their disparate chemical structures, luciferins play convergent biochemical roles. Hypotheses for the origin and evolution of luciferins are strongly tied to the sensitivity of luciferins to oxygen and their intrinsic chemiluminescence. The oxidative stress hypothesis posits that

luciferins or their precursors, proto-luciferins, played a role in scavenging detrimental reactive oxygen species, which damage biological materials (Hastings, 1983; Rees *et al*., 1998). Combined with another hypothesis proposing that (proto-)luciferins had innate chemiluminescence capabilities, selection could act on (proto-)luciferins that produce light upon oxidation.

Although the oxidative stress hypothesis currently lacks widespread experimental support, future research on luciferin biosynthesis could reveal the roles of (proto-)luciferins, which may support this hypothesis. As an example, recent discoveries in the fungal bioluminescence system, specifically research on the biosynthetic pathway for fungal luciferin, provided some support for this hypothesis (Oba *et al*., 2017*b*). Fungi produce luciferin by modifying a luciferin precursor called hispidin (Oliveira *et al*., 2012; Purtov *et al*., 2015; Oba *et al*., 2017*b*; Kotlobay *et al*., 2018). Hispidin is also found in non-luminous fungi, where it is used as pigment precursor and has antioxidant biochemical properties (Bu'Lock, Leeming & Smith, 1962; Khushbaktova *et al*., 1996). Given this information, the chemiluminescence hypothesis could be tested by investigating whether the proto-luciferin hispidin has innate chemiluminescent properties. In the future, identifying the biosynthetic pathways of luciferins will ultimately reveal how different luciferins originated and became integrated into bioluminescence systems, and whether there are patterns in their evolutionary histories.

III. REVIEWING THE MULTI-LEVEL CONVERGENT EVOLUTION OF BIOLUMINESCENCE: AIMS AND SCOPE

We focus this part of our review on organisms that use the luciferins coelenterazine and vargulin because in addition to phenotypic convergence, they represent cases of interphylum convergence (at a molecular level) in luminous systems that use the same luciferins to produce light. Although krill and dinoflagellate luciferins are almost structurally identical, we do not include them here because there is no experimental evidence to support the hypothesis that krill obtain their luciferin from dinoflagellates. Additionally, although fireflies and click beetles evolved their bioluminescence systems and biochemistries independently through the parallel evolution of their luciferases, they are members of the same phylum, therefore we do not review their bioluminescence systems here (Fallon *et al*., 2018).

There is an uneven distribution of functional information for bioluminescence systems because much past bioluminescence research focused on identifying the molecular bases of bioluminescence biochemistries and the larger structural components of light organs. We anticipate that, with advances in research technologies, future bioluminescence research will characterize additional modules used to perform the various functions involved in luminous systems. Specifically, we hope our review will stimulate researchers to investigate: (*i*) the morphology and/or genetics of cell and subcellular components of luminous systems; (*ii*) the biochemistry of luciferin-storage mechanisms; (*iii*) genetic and physiological mechanisms involved in the behavioural control of light production; and (*iv*) mechanisms for catabolism and recycling of bioluminescent molecules.

There are many prior reviews that are focused on the biodiversity (Herring, 1987; Herring & Widder, 2001; Haddock *et al*., 2010; Widder, 2010; Oba *et al*., 2011, 2017*a*), biochemistry

(Viviani, 2002), biological function (Herring, 1990), sexual dimorphism (Herring, 2007), vision components (Locket, 1977; Warrant & Locket, 2004), and convergent evolution of bioluminescence systems (Harvey, 1956; Waldenmaier, Oliveira & Stevani, 2012; Oba & Schultz, 2014), but none that examined the diversity of biological levels found in molecularly convergent bioluminescence systems. Therefore, below we broadly highlight the diverse biological levels of organization used for light production in luminous systems that rely on the shared luciferins coelenterazine and vargulin and identify questions that arise from the use of a holistic and hierarchical approach to examine these systems.

(1) Coelenterazine systems

A number of cephalopods, chaetognaths, cnidarians, crustaceans, ctenophores, fishes, larvaceans, ophiuroids, and radiolarians independently evolved coelenterazine-based bioluminescence, and this taxonomic diversity is reflected in their diverse structures and mechanisms underlying light production. Coelenterazine-based bioluminescence can be secreted into the environment by using specialized glands and organs to eject the bioluminescence, and/or be produced intracellularly and/or housed within the organism in structured photophores or light organs. Interestingly, the cephalopod *Vampyroteuthis infernalis* can use its photophores to produce both secreted and intracellular bioluminescence (Robison *et al*., 2003). Decapod and lophogastridan shrimp secrete bioluminescence from their mouths and through a pore on the maxilla, respectively, while some copepods and halocyprid ostracods use glands to secrete bioluminescence through pores, chaetognaths secrete luminous particles from hexagonal-chambered light organs, and searsid fishes use a black-lined, post-cleithral organ to secrete bioluminescence (Angel, 1968; Barnes & Case, 1972; Herring, 1972, 1976; Thuesen, Goetz & Haddock, 2010; Wong *et al*., 2015). A number of decapod shrimps, many fishes, and most cephalopods produce internal and/or intracellular bioluminescence in photophores ranging in structural complexity (composed of structures such as lenses, reflectors, pigment, or photogenic tissue), while ctenophores, cnidarians, radiolarians, ophiuroids, and some copepods house bioluminescence in luminous areas of their bodies such as granular light organs along the rim of *Aequorea victoria*, the gelatinous outer layer of radiolarians, or the arms of *Amphiura filiformis* (Shimomura, Johnson & Saiga, 1962; Herring, 1976, 1979, 2000; Brehm & Morin, 1977). Thus, the organs used to produce coelenterazine-based bioluminescence are diverse despite having molecularly convergent biochemistries to produce bioluminescence.

Similar to the diversity of bioluminescent organs, the cells used to produce coelenterazine-based bioluminescence are morphologically variable and contain different subcellular contents both within and across taxa. Although photogenic organs are structurally variable, most contain photocytes. Similar to the generic use of the term ‘luciferases’ for non-homologous genes, photocytes are a type of cell defined solely by the common function of light production and may not be homologous. Within and across groups, did photocytes evolve from the same ancestral cell type? By distinguishing and morphologically characterizing the cell types associated with bioluminescence systems, especially photocytes, we can begin to form hypotheses on how these cells originated and evolved. In fishes, the hatchetfish *Argyropelecus olfersii*'s photophores have small photocytes that are densely packed and polyhedral, while the lanternfish family Myctophidae has photophores containing long, spindle-shaped photocytes (Nicol, 1969). Within photocytes, subcellular organization can vary even within a family, as seen in members of the squid family

Enoploteuthidae (Young & Bennett, 1988). Although these photocytes are morphologically dissimilar, did they evolve from similar ancestral cell types? If so, did parallel genetic mechanisms govern photocyte evolution, size, and organization? If not, how did photocytes convergently evolve from disparate cell types?

At a molecular level, both photoproteins and luciferases are capable of producing light with coelenterazine, but these proteins are not homologous across taxa and have no similarity beyond that expected by chance, suggesting that there are many routes that proteins can take to converge to produce bioluminescence (Markova & Vysotski, 2015). Although some closely related taxa use homologous photoproteins/luciferases, this is not always the case. In Cnidaria, the luminous scyphozoan *Periphylla periphylla* uses a luciferase, the hydrozoan jellyfish *Aequorea* uses a photoprotein, and the anthozoan sea pansy *Renilla* uses a luciferase (Ward & Cormier, 1978; Shimomura *et al*., 2001). Photoproteins using coelenterazine as a substrate can also vary in terms of the cofactors required to produce bioluminescence. For example, cnidarians, ctenophores, and radiolarians use the divalent cation Ca^{2+} as a photoprotein cofactor, but the squids *Dosidicus gigas* and *Sthenoteuthis oualaniensis* use a monovalent cation cofactor such as Na^{+} or K^{+} (Shimomura, 1985; Takahashi & Isobe, 1994; Tsuji *et al*., 1995; Galeazzo *et al*., 2019). This biochemical variability in bioluminescence reveals that there are numerous biological approaches to harnessing coelenterazine-based bioluminescence.

(2) Vargulin systems

Although not as widely used as coelenterazine, vargulin-based bioluminescence systems still exhibit some of the variability found in coelenterazine-based systems. Bioluminescence can be intracellular (e.g. in *Porichthys*) or secreted into the surrounding environment (e.g. in cypridinid ostracods), and organs housing internal and/or intracellular bioluminescence vary in morphology and origin (Tsuji, Lynch & Haneda, 1970; Anctil, 1977; Sweeney, 1980; Herring, 1985). The luminous fishes that produce autogenic bioluminescence in the Pempheridae, Apogonidae, and Batrachoididae families rely on the dietary acquisition of vargulin from their bioluminescent ostracod prey (Haneda & Johnson, 1958; Tsuji *et al*., 1971; Thompson, Nafpakntis & Tsuji, 1987; Thacker & Roje, 2009). Given that coelenterazine is so ubiquitous in marine systems, why do these fishes use vargulin? Do their diets lack coelenterazine, or was the evolution of their bioluminescence systems constrained by the ability of their digestive systems to absorb this substrate?

While vargulin-based bioluminescence independently originated fewer times than coelenterazine-based systems, there are still similar cases of morphological diversity in the light organs used for vargulin-based bioluminescence in ostracods and fishes. Despite being non-convergent at a biochemical level, how did luminous organisms convergently evolve structurally similar light organs? Similar to the oral light organs present in decapod/lophogastridan shrimps, cypridinid ostracods secrete bioluminescent mucus from a specialized light organ called the upper lip (see Fig. 3), which contains tusks ending in numerous nozzles (Huvard, 1993; Abe *et al*., 2000; Cohen & Morin, 2003). The photophores used for vargulin and coelenterazine-based bioluminescence also share similar structural components. The toadfishes *Porichthys* spp., commonly known as midshipman fishes, have hundreds of ventral dermal photophores composed of a pear-shaped lens, photogenic tissue, a reflector, and pigment (Nicol, 1957; Anctil, 1977) (Fig. 3). However, instead

of photophores, pempherid and apogonid fishes independently evolved visceral light organs that are morphologically unique to vargulin-based bioluminescence. The pempherid *Parapriacanthus ransonneti* produces bioluminescence in ventral thoracic and anal light organs, the former extending from the pyloric caeca (where luciferin is stored), and the latter connecting to the rectum and anus (Haneda & Johnson, 1958) (Fig. 3). Although pempherid and apogonid vargulin-based bioluminescence is produced in light organs that are connected to the gut, the light organs in pempherids are distinct structures containing numerous tubules, while those in apogonids are less structurally complex (Thacker & Roje, 2009). Why is this type of visceral light organ unique to vargulin-based bioluminescence? In addition to using vargulin stored in the pyloric caeca, *P. ransonneti* also sequesters and uses ostracod luciferase ('kleptoprotein bioluminescence') (Bessho-Uehara *et al*., 2020*b*). Did this dual functional requirement of sequestering both ostracod luciferin and luciferase constrain the evolution of pempherid light organs?

Like coelenterazine systems, cellular morphology and subcellular composition is variable across independent origins of vargulin based bioluminescence. In cypridinid ostracods including *Vargula hilgendorfii*, *V. tsujii*, and *Photeros graminicola*, the light organ, called the upper lip, houses different types of gland cells (estimated to be between two and six different cell types), with some types of gland cells localized to specific areas and others appearing in multiple regions of the upper lip. One particular cell type contains yellow vesicles hypothesized to hold vargulin, which is yellow/orange in colour when pure (Huvard, 1993; Abe *et al*., 2000). Midshipman fish photophores contain many types of cells: lens cells, photocytes, pigment cells, and supportive cells (Strum, 1969; Baguet & Zietz-Nicolas, 1979). Their photophore reflector is similar to those found in other luminous fishes (e.g. lanternfishes) and is comprised of cells containing guanine crystals, a reflective material also found in fish skin (Herring, 2000; Levy-Lior *et al*., 2008; Paitio *et al*., 2020). Given that other fishes with photophores use coelenterazine, do the cellular components of these photophores originate from similar progenitor cells or similar cell types? Comparing the cell types in pempherid and apogonid light organs, which evolved independently from the extension of the gut, will be especially interesting, because of organ-level differences in structural complexity. In addition to differences in structural complexity, do the cell types in pempherid and apogonid light organs differ?

Unlike any known coelenterazine-based luciferase or photoprotein, the luciferase in cypridinid ostracods contains two von Willebrand factor type D domains (Fig. 5), which are domains shared in mucins and glycoproteins (Oakley, 2005). Although the enzymatic function of the ancestral protein is unknown, the dual role of the ostracod upper lip (secreting bioluminescence and consuming food), coupled with the presence of von Willebrand factor type D domains, suggest that ostracod luciferase originated from an ancestral digestive enzyme (Abe *et al*., 1996; Oakley, 2005). As previously mentioned, *Parapriacanthus ransonneti* sequesters both vargulin and ostracod luciferase from its diet and uses them both to produce light. The recent discovery of kleptoprotein bioluminescence in *Parapriacanthus* may extend to *Pempheris*, the sister genus with similar light organs that also uses vargulin for bioluminescence (Haneda, Johnson & Shimomura, 1966). Because the apogonid *Jaydia* has a similar light organ, future work testing kleptoprotein bioluminescence in apogonids will reveal whether this recently discovered mechanism for producing autogenic bioluminescence evolved convergently in other taxa. Although the molecular basis of cypridinid ostracod bioluminescence is well known, it is unknown in midshipman fishes. Midshipman luciferase is difficult to extract; perhaps it is membrane bound,

or perhaps their photophores do not contain much luciferase (Cormier, Crane & Nakano, 1967; Tsuji *et al*., 1971). *In vivo* observations reveal that midshipman fish bioluminescence is much dimmer than that of ostracods. Differences in their bioluminescence biochemistry, coupled with differences in their bioluminescence emission spectra, suggest that these luciferases are unrelated (Cormier *et al*., 1967; Tsuji *et al*., 1975). It is unlikely that midshipman fishes exhibit kleptoprotein bioluminescence, because non-luminous specimens can luminesce after injecting vargulin into their stomachs (Barnes, Case & Tsuji, 1973). Thus, identifying the luciferase gene in midshipman fishes, the only other record of a molecularly convergent vargulin-based bioluminescence system, is crucial to understanding how this interphylum molecular convergence evolved.

IV. LUCIFERIN STORAGE AND REGULATION: AN UNEXPLORED AREA OF BIOLUMINESCENCE RESEARCH

In addition to investigating the traditional luciferin and luciferase/photoprotein bioluminescence system, we highlight that an untapped area in bioluminescence research is the presence of stored luciferins and the biochemical mechanisms used to store them. Filling that gap will not only reveal how various bioluminescence systems address the issue of unstable luciferins, which will improve our understanding of how functional convergence (preservation of a substrate) evolved through conserved or disparate genetic mechanisms, but will also improve our understanding of bioluminescence as a whole by characterizing the functional module used for substrate storage in this complex trait. Furthermore, by genetically characterizing the proteins used for storing luciferins, the results of this research can be applied to regulate the availability of active luciferins in bioluminescence-based biotechnological imaging assays, which currently rely on luciferins that are vulnerable to non-specific oxidation.

Luciferins are double-edged swords because they are typically unstable molecules that must be oxidized in order to perform their function and yet this very attribute makes them prone to non-specific oxidation by the surrounding environment. To prevent non-specific oxidation and/or to regulate usage, some animals such as fireflies, ostracods, and sea pansies biochemically modify their luciferins for storage (Cormier *et al*., 1970; Nakamura *et al*., 2014; Fallon *et al*., 2016). Additionally, lanternfish tissues contain low amounts of coelenterazine derivatives (relative to coelenterazine), but it is not known whether they have a mechanism to convert luciferin into a storage form (Duchatelet *et al*., 2019). Fungi and dinoflagellates have luciferin precursors that can be readily converted to active luciferins upon the addition of NADPH (Fresneau *et al*., 1986; Oba *et al*., 2017*b*). In addition to biochemical modification following luciferin biosynthesis or acquisition, organisms could possibly store luciferins by regulating the biochemical conversion of luciferin precursors to active luciferins. We hypothesize that evolution can find a way to regulate available luciferin by simply controlling luciferin biosynthesis.

Interestingly, fireflies, ostracods, and sea pansies use different luciferins but use or are proposed to use sulfotransferases to store the substrates in a sulfated form. However, the genetic basis for luciferin sulfotransferase is only known in fireflies (Fallon *et al*., 2016). The activity of this luciferin sulfotransferase is specific to firefly luciferin. Similar to the photoprotein/luciferase origin hypothesis, firefly luciferin sulfotransferase may have originated from an ancestral,

promiscuous sulfotransferase. Luciferins can also be stored by luciferin-binding proteins, as in the case of dinoflagellates, or 'substrate-binding fractions' in the luminous fly *Orfelia fultoni* (Lee *et al*., 1993; Viviani *et al*., 2020). Interestingly, sea pansies have both a storage form of luciferin and a luciferin-binding protein, the latter of which releases coelenterazine upon the addition of calcium (Anderson, Charbonneau & Cormier, 1974; Inouye, 2007). Currently, we only know of a few organisms that can actively store their luciferins (dinoflagellates, fireflies, sea pansies, and ostracods), and there are three hypothesized types of luciferin-storage mechanism: (*i*) biochemical modification into a sulfated form; (*ii*) luciferin-binding proteins; and (*iii*) biochemical regulation of luciferin production. Do organisms that share luciferins store luciferins in the same way? If so, how and why? Fireflies, ostracods, and dinoflagellates produce their own luciferins, yet they still store their luciferins. Can organisms with a dietary dependence on luciferin also store luciferin? Testing for luciferin-storage capabilities in bioluminescence systems and chemically identifying storage forms of shared luciferins such as coelenterazine and vargulin will be especially valuable, because it will reveal other cases of molecular convergence or parallelism within this complex trait.

V. PROMISING NEW TOOLS FOR BIOLUMINESCENCE RESEARCH

Characterizing bioluminescence systems incorporates many fields such as biology, ecology, chemistry, physics, and computational biology. The most comprehensive chemical and biochemical methods are outlined in a book by Osamu Shimomura, who shared the 2008 Nobel Prize in Chemistry with Martin Chalfie and Roger Y. Tsien for discovering the green fluorescent protein in *Aequorea victoria* and developing its use as a biotechnological tool (Shimomura, 2006). In the following sections, we highlight newly developed biological and chemical tools that will be useful for characterizing bioluminescence systems.

(1) Advances in genetic sequencing

Most past bioluminescence research examined bioluminescence at the organ or molecular levels. Sequencing at a cellular resolution will allow us to extend our knowledge of bioluminescence to a cellular level. The development of single-cell sequencing techniques such as single cell RNA sequencing (scRNA-seq) or single cell assay for transposase-accessible chromatin using sequencing (ATAC-seq), which generates RNA data and identifies accessible chromatin at a cellular level, respectively, will allow us to move towards an understanding of regulatory elements used to control bioluminescence, characterizing cell types associated with bioluminescence systems, and tracing the developmental history and evolutionary relationships of cells used in luminous systems (Buenrostro *et al*., 2015; Kolodziejczyk *et al*., 2015; Shafer, 2019). Using these sequencing methods at a cellular level rather than an organ level will offer a better genetic resolution of the organ as a whole. Furthermore, by using spatial transcriptomics technology, which sequences RNA from a section of tissue, we can spatially resolve transcriptomic data and connect morphological data with genetic data (Ståhl *et al*., 2016). For example, using scRNAseq, Sebé-Pedrós *et al*. (2018) identified ctenophore photocytes that expressed photoproteins and opsins. We can apply these technologies to identify genes unique to luminous photocytes or other cells involved in light production, followed by identifying the molecular basis of bioluminescence by functionally testing candidate genes, for example by using clustered regularly interspaced short

palindromic repeat (CRISPR) genome editing to test regulatory elements, or using protein expression systems to produce and test proteins functionally (Cregg *et al*., 2000; Schnitzler *et al*., 2012; Ran *et al*., 2013; Rosano & Ceccarelli, 2014).

(2) Mass spectrometry: improving resolution and advances in imaging

Mass spectrometry can be an extremely sensitive and useful tool in characterizing bioluminescence systems. For example, researchers used mass spectrometry to quantify coelenterazine using just a single halocyprid ostracod specimen, an animal that is about 1 mm long (Oba *et al*., 2004). Rather than mixing methanol extracts from luminous tissue with a known luciferase, a technique called 'cross reactivity' traditionally used to test for the presence of a specific luciferin, mass spectrometry can be used to analyse these extracts and chemically identify the luciferin. Given a luciferin standard, analysis tools (e.g. spectral matching tandem mass spectra, molecular networking) can identify components in the extract that are similar but not an exact match, which will be useful for identifying derivatives of luciferins such as the modified coelenterazine used in *Sthenoteuthis oualaniensis* and *Watasenia scintillans* bioluminescence (Guthals *et al*., 2012; Yang *et al*., 2013; Wang *et al*., 2016).

In addition to small molecule analyses, mass spectrometry is also used in proteomic workflows and top-down/bottom-up proteomics, which quantifies the mass and abundance of proteins, and identifies the amino acid sequence and its precursor gene given an extracted and purified protein, respectively (Aebersold & Mann, 2003; Bantscheff *et al*., 2007). Like single-cell genetic sequencing, mass spectrometry can also be used to identify proteins at a cellular level (Su, Shi & Wei, 2017; Specht & Slavov, 2018). Furthermore, a technique called mass spectrometry imaging can be used to determine the spatial distribution of molecules in a tissue sample (Stoeckli *et al*., 2001; McDonnell & Heeren, 2007; Stauber *et al*., 2010; Hamilton *et al*., 2020). This technique, used to track proteins and cell metabolism, has promising applications in bioluminescence research. We suggest that this approach could be applied to tracing and identifying molecular and genetic pathways involved in luciferin biosynthesis. Specifically, we envision that, as mass spectrometry imaging techniques improve for protein identification, this approach could be used to identify proteins in regions of the tissue that contain luciferins/luciferin-like molecules (Piehowski *et al*., 2020). After obtaining this information, we can propose and functionally test potential biosynthetic pathways using protein expression or genome-editing techniques. Using these chemical tools will bolster data generated from new sequencing techniques and will allow us to understand how bioluminescence systems function at cellular, biochemical, and genetic levels.

VI. CONCLUSIONS

1. Central goals in evolutionary biology are to understand how complexity originates, what shapes its evolution, and how complex traits with similar functions repeatedly evolve. We can systematically study the convergent evolution of a complex trait by using a hierarchical and holistic approach that investigates multi-level convergent evolution and functional modules.

2. Bioluminescence, an ecologically important trait, is an excellent system for studying the roles of divergence and conservation in convergent evolution because of its biological complexity, biodiversity, and ubiquity across lifeforms. We present an updated estimate of at least 94 independent origins of bioluminescence across the tree of life.
3. Developing bioluminescence as a system for studying convergence requires studying all levels of life and an interdisciplinary approach that incorporates computational biology, molecular biology, ecology, evolution, chemistry, biochemistry, and physics.
4. Organisms that use the same luciferins have bioluminescent proteins that are molecularly convergent. Despite this molecular convergence, we see both conservation and divergence in the biological functions, organs, and cells associated with the production of bioluminescence. We anticipate that, with advances in mass spectrometry and genetic sequencing, future bioluminescence research will further characterize the functional modules involved in bioluminescence systems (i.e. luciferin/luciferase production/acquisition, bioluminescent molecule recycling or catabolism, maintenance of light organs, behavioural control of light production, modification of light emission, and storage of luciferin).
5. Ultimately, using widespread convergent systems such as bioluminescence to study broad evolutionary questions will reveal how different evolutionary pathways can converge on similar traits and will further our understanding of the origins and evolution of biological complexity.

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