Glowing belowground: investigating the evolution and biological functions of *Photorhabdus* bioluminescence

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"Glowing belowground: investigating the evolution and biological functions of *Photorhabdus* bioluminescence"

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Abstract

Photorhabdus is a genus of bacteria living in symbiosis with soil-dwelling nematodes belonging to the genus *Heterorhabditis*. Together, they parasitize and kill small arthropods, including herbivorous insects belowground. *Photorhabdus* bacteria have the singular capacity to chemically produce and emit cyan light, a phenomenon known as bioluminescence. During the infection process, *Photorhabdus* bacteria produce bioluminescence resulting in the glow of the parasitized organism. Bioluminescence is a widespread trait present in more than 800 genera of organisms that has evolved independently at least 94 times through evolution. Nonetheless, *Photorhabdus* bacteria are unique as they are the only known terrestrial bacteria to possess this capability. While bioluminescence has captivated scientists for many decades, little is known about the biological functions of this trait in soil ecosystems. *Photorhabdus* bioluminescence is thought to originate from ancient horizontal gene transfer events. Various hypotheses have been proposed regarding its potential functions and some suggest that it may be a disappearing trait with no particular role. In this thesis, I took an interdisciplinary approach to investigate the evolution and biological functions of *Photorhabdus* bioluminescence. The main findings from this thesis include: (i) *Photorhabdus* bioluminescence displays a great inter- and intra-specific variability across the genus; (ii) *Photorhabdus* bioluminescence is an evolutionary dynamic trait that can rapidly evolve under laboratory conditions in a strain-specific manner; (iii) bioluminescence neither decreases nor disappears in the *Photorhabdus* genus through subsequent speciation events; (iv) bioluminescence is regulated by a multi-component network of genes in *Photorhabdus*; (v) bioluminescence is required for successful symbiosis between *Photorhabdus* bacteria and *Heterorhabditis* nematodes; (vi) *Photorhabdus* bioluminescence might prevent competitions in entomopathogenic nematodes; (vii) Plants produce defensive secondary metabolites in response to root exposure to *Photorhabdus* bioluminescence. This thesis contributes to a better understanding of the biological functions of belowground bioluminescence. It highlights the crucial roles played by *Photorhabdus* bioluminescence in the symbiotic relationship with *Heterorhabditis* nematodes, as well as its regulatory functions within soil ecosystems.

Keywords: Bioluminescence, *Photorhabdus*, evolution, *Heterorhabditis*, soil ecosystems

Résumé

Photorhabdus est un genre de bactéries vivant en symbiose avec des nématodes appartenant au genre *Heterorhabditis*. Ensemble, ils parasitent et tuent de petits arthropodes, y compris des insectes herbivores. Au cours du processus d'infection, les bactéries *Photorhabdus* produisent de la bioluminescence, rendant l'organisme parasité bioluminescent à son tour. La bioluminescence est une caractéristique répandue présente chez plus de 800 genres d'organismes et qui a évolué indépendamment au moins 94 fois au cours de l'évolution. Les bactéries *Photorhabdus* sont uniques car elles sont les seules bactéries terrestres connues à posséder cette capacité. Bien que la bioluminescence ait captivé les scientifiques pendant de nombreuses décennies, on sait peu de choses sur les fonctions biologiques de cette caractéristique dans les écosystèmes terrestres. Il a été postulé que la bioluminescence chez *Photorhabdus* résulte d'événements ancestraux de transfert horizontal de gènes. Diverses hypothèses ont été proposées concernant ses fonctions potentielles et certaines suggèrent qu'il pourrait s'agir d'un trait disparaissant sans rôle particulier. Dans cette thèse, j'ai adopté une approche interdisciplinaire pour étudier l'évolution et les fonctions biologiques de la bioluminescence chez *Photorhabdus*. Les principales conclusions de cette thèse incluent : (i) la bioluminescence de *Photorhabdus* présente une grande variabilité inter- et intra-spécifique ; (ii) la bioluminescence de *Photorhabdus* est un trait dynamique qui peut évoluer rapidement dans des conditions de laboratoire ; (iii) la bioluminescence ne diminue ni ne disparaît dans le genre *Photorhabdus* à travers des événements de spéciation successifs ; (iv) la bioluminescence est régulée par un réseau de gènes à composants multiples chez *Photorhabdus* ; (v) la bioluminescence est nécessaire à la symbiose entre les bactéries *Photorhabdus* et les nématodes *Heterorhabditis* ; (vi) la bioluminescence de *Photorhabdus* pourrait prévenir la concurrence chez les nématodes entomopathogènes ; (vii) les plantes produisent des métabolites secondaires de défense en réponse à l'exposition des racines à la bioluminescence de *Photorhabdus*. Cette thèse met en évidence les rôles cruciaux joués par la bioluminescence de *Photorhabdus* dans la relation symbiotique avec les nématodes *Heterorhabditis*, ainsi que ses fonctions régulatrices au sein des écosystèmes terrestres.

Mots-clefs : Bioluminescence, *Photorhabdus*, évolution, *Heterorhabditis*, écosystèmes terrestres.

List of abbreviations

ABA ..... abscisic acid
AHL ..... acylated homoserine lactone
AI-2 ..... autoinducer-2
ANOVA ..... analysis of variance
BHT ..... butylated hydroxytoluene
CDS ..... coding sequence
CRY ..... cryptochrome
DEG ..... differentially expressed gene
EPN ..... entomopathogenic nematode
FC ..... fold change
FDR ..... false discovery rate
FW ..... fresh weight
HIPV ..... herbivory-induced plant volatile
HSD ..... honestly significant difference
IAA ..... indole-3-acetic acid
IJ ..... infective juvenile
IPS 3,5-dihydroxy-4-isopropyl-trans-stilbene
JA ..... jasmonic acid
JA-Ile ..... jasmonic acid-isoleucine
LB ..... lysogenic broth
M-form ..... mutualistic form
NRP ..... non-ribosomal peptide synthetase
OD ..... optical density
P-form ..... pathogenic form
PCA ..... principal component analysis
PHOT ..... phototropin
PHY ..... pyhtochromes
PK ..... polyketide synthases
QS ..... quorum sensing
RLU ..... relative light unit
ROS ..... reactive oxygen species
RPM ..... revolutions per minute
RSA ..... root system architecture
SA ..... salicylic acid
SEM ..... standard error of the mean
WCR ..... western corn rootworm

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General introduction

Photorhabdus is a genus of bacteria that share a symbiotic relationship with microscopic soil-dwelling nematodes belonging to the genus *Heterorhabditis*. This symbiotic pair parasitizes and kills small arthropods belowground. *Photorhabdus* bacteria possess the singular ability to produce and emit cyan light, known as bioluminescence. Interestingly, *Photorhabdus* is the only known genus of terrestrial bioluminescent bacteria. The bioluminescence of *Photorhabdus* is believed to have originated from ancient horizontal gene transfer events. Despite its prevalence, its potential benefits remain poorly understood. During the infection process, *Heterorhabditis* nematodes enter into arthropods and release their *Photorhabdus* bacterial symbionts. Within arthropods hemocoel, *Photorhabdus* bacteria undergo exponential development and produce bioluminescence, resulting in a characteristic glow of the infected organisms in the soil. The subterranean environment is a rich habitat hosting an important biodiversity, including plants, nematodes, bacteria, and arthropods. The occurrence of bioluminescent infected organisms in this dark environment raises, among other questions, two fascinating ones: How does *Photorhabdus* bioluminescence impact the behavior and physiology of other soil-dwelling organisms? Does *Photorhabdus* bioluminescence play a role in the symbiosis with *Heterorhabditis* nematodes? In my thesis, I tackled these questions using an interdisciplinary approach. Ultimately, the aim of this work is to provide insights into the evolution and biological functions of *Photorhabdus* bioluminescence belowground.

The discovery and phylogeny of *Photorhabdus* bacteria

Photorhabdus is a genus of facultatively anaerobic, gram-negative *Gammaproteobacteria* belonging to the *Morganellaceae* family (**Adeolu et al., 2016; Boemare et al., 1993; Thomas and Poinar, 1979**). The name of this genus can be broken down into two parts: "photo" and "rhabdus", which can be literally translated from the Greek to "glowing-rod". This name is derived from the ability of bacteria from this genus to produce bioluminescence (**Boemare et al., 1993**). The earliest mentions of *Photorhabdus* bacteria might date back to the American Civil War, where reports indicate that wounds of some soldiers exhibited a faint blue glow. These accounts suggested that wounded soldiers with glowing wounds had a higher survival rate compared to others, giving rise to the term "Angel's glow". More recently, a scientific explanation for these folklore stories was proposed. It has been hypothesized that *Photorhabdus* bacteria could have developed on soldier wounds, causing the faint blue glow. This idea suggests that the antimicrobial activity of *Photorhabdus* bacteria might have prevented the growth of pathogenic bacteria on the wounds, leading to the observed higher survival rates in soldiers with the "Angel's glow". However, experimental evidence supporting this hypothesis is currently lacking. *Photorhabdus* bacteria are entomopathogens, and to date, only a few Australian strains from the species *P. asymbiotica* have been isolated from human hosts (**Mulley et al., 2015**).

Photorhabdus bacteria live in obligate symbiosis with entomopathogenic nematodes (EPNs) belonging to the *Heterorhabditis* genus (**Boemare et al., 1993; Thomas and Poinar, 1979**). Bacterial symbionts of EPNs were isolated for the first time more than sixty years ago (**Poinar and Thomas, 1966, 1965**). At this period, no distinction was made between *Photorhabdus* and *Xenorhabdus,* another genus of *Morganellaceae* bacteria that share symbiotic relationship with EPNs belonging to the *Steinernema* genus. The first clear mention of the isolation of *Photorhabdus* bacteria date from 1979, even though it was referred as *Xenorhabdus luminescens* (**Thomas and Poinar, 1979**). During the two following decades, the taxonomy of EPNs bacterial symbionts was examined using DNA-DNA hybridization techniques and led to the creation in 1993 of a new genus: *Photorhabdus*. This new genus was constituted of a single species, *P. luminescens* which comprised all bioluminescent strains previously classified as *Xenorhabdus luminescens* (**Boemare et al., 1993**). In 1999, Fisher-Le Saux *et al*. proposed to divide the *Photorhabdus* genus based on genetic and phenotypic characterizations into three

species: *P. asymbiotica*, *P. luminescens* and *P. temperata*, each of which included several subspecies (**Fischer-LeSaux et al., 1999**). Since the early 2000s, major improvements in sequencing technologies were made and quantitative phylogenetic methods were developed to replace the DNA–DNA hybridization approach. In 2018, a thorough revision of the *Photorhabdus* genus based on whole-genome sequencing was performed, leading to the elevation of most *Photorhabdus* subspecies to the species level (**Machado et al., 2018**). Currently, the *Photorhabdus* genus comprises 30 taxa, including 23 species, with 6 of them divided into different subspecies (**Castaneda-Alvarez et al., 2022; Machado et al., 2018, 2019, 2021a, 2021b, 2023**). Thanks to extensive research on this subject, the number of described species increases every year.

Photorhabdus symbiotic relationship with *Heterorhabditis* nematodes

Entomopathogenic nematodes: natural enemies of insects belowground

The pair formed by *Heterorhabditis* nematodes and their bacterial symbionts, *Photorhabdus,* are naturals enemies of small arthropods, including insects. *Heterorhabditis* nematodes form with *Steinernema* nematodes the insect-parasitic guild called EPNs. There is no formal convention for the term EPN, but a commonly accepted definition was proposed by Dillman *et al*. (**Dillman et al., 2012a**). It specifies two criteria:

- EPNs must rapidly and efficiently kill their hosts.
- EPNs have to display a stable and mutually beneficial association with a bacterial symbiont. This bacterial symbiont should be involved in insect pathogenicity.

For this association to be stable over generation, EPNs must transmit their bacterial symbionts to their progeny. The definition proposed by Dillman *et al.* is built from the historical context when the adjective "entomopathogenic" was initially used to describe *Photorhabdus* and *Xenorhabdus* bacteria and was applied by extension to the nematodes associated with these bacteria. In addition to *Heterorhabditis* and *Steinernema* nematodes, several species belonging to the *Oscheius* genus have been described as EPNs which is a matter of controversy (**Loulou et al., 2022; Pervez et al., 2013; Torrini et al., 2015; Ye et al., 2010; Zhang et al., 2008; K. Zhang et al., 2019)**. It was shown that *Oscheius tipulae* is able to rapidly kill *Ceratitis capitata* pupae, an insect considered a major pest in citrus production. However, the role of a putative bacterial partner in this pathogenicity has not been demonstrated (**Loulou et al., 2022**). *Oscheius* nematodes are commonly isolated from insect cadavers using baited traps, alongside EPNs (**Campos-Herrera et al., 2019, 2015; Jaffuel et al., 2018**). This co-occurrence and the confusion between entomopathogeny, phoresy, necromeny, and parasitism raise the potential for misclassification.

Since the second half of the 20th century, scientists have shown a growing interest in EPNs due to their potential utility as an alternative to chemical pesticides in sustainable crop management (**Koppenhöfer et al., 2020**). Entomopathogenic nematodes are safe to humans and while they have a wide host range (pathogenicity has been described with at least 17 orders of insects), they are generally considered as safe to the environment and non-target organisms (**Piedra-Buena et al., 2015**). Currently, five species of *Heterorhabdis* nematodes (*Heterorhabditis bacteriophora, H. indica, H. marelatus, H. megidis,* and *H. zealandica*) are

commercialized in Europe, North America, and Australia as biological control agents (**Kaya and Gaugler, 1993; Koppenhöfer et al., 2020**). Entomopathogenic nematodes are ubiquitous, being present in all continents with the exception of Antarctica, but our knowledge of their geographic distribution is strongly influenced by sampling efforts across the world (**Adams et al., 2006**). Historically, the majority of EPNs have been identified in North America, Asia, and Australia. Recent research has expanded our understanding, as evidenced by the identification of two new *Heterorhabditis* species in Rwanda and Mexico (**Bruno et al., 2020; Machado et al., 2021**). These investigations are crucial for selecting EPNs well-adapted to local conditions and target hosts, preventing potential ecological imbalances that may arise from introducing exotic EPN species (**Millar and Barbercheck, 2001**).

The search for a host

The parasitic lifestyle of EPNs, starts with the search of a suitable host. This critical step is performed by infective juveniles (IJs), a developmentally arrested stage that can live up to several weeks in the soil without feeding (**Gaugler, 2002**). The *Heterorhabditis* IJs harbor *Photorhabdus* bacteria in their intestines and act as vectors, releasing these bacterial symbionts into suitable arthropod hosts (**Ciche and Ensign, 2003**). Host-seeking EPNs adopt different foraging strategies that are commonly categorized into three groups. Cruisers, characterized by high mobility, intermediates with moderate mobility, and ambushers, employing a sit-and-wait approach. While cruisers have higher probability of finding sedentary and cryptic resources, ambushers are more effective at encountering resources with high mobility (**Campbell and Gaugler, 1993**). In the environment, host-seeking EPNs display great variety of behaviors. Cruisers and ambushers are endpoints of a continuum spectrum of behaviors displayed by host-seeking EPNs (**Campbell and Gaugler, 1993, 1997; Lewis et al., 1993, 1992**). In addition, different strategies are employed by individuals within a population and the foraging behavior of EPNs is plastic depending on various factors including the habitat type and the age of IJs (referred as the time after emergence from a depleted cadaver; **Ennis et al., 2010; Lee et al., 2016**). *Steinernema carpocapsae* nematodes illustrate well this complexity. While this species of EPNs is generally described as ambushers, it is common that a small proportion of its population, called sprinters, exhibit high mobility (**Bal et al., 2014**).

To localize suitable host, IJs sense and integrate multiple cues in the soil. These cues can be emitted directly by potential hosts or can derived from their activities. It has been shown that

carbon dioxide (CO_2) resulting from most biological activities, attract host-seeking EPNs (**Boff, 2002; Kaya and Gaugler, 1993**). Host-seeking EPNs also use volatiles emitted by plants fed by herbivorous insects. These herbivory-induced plant volatiles (HIPVs) are produced in large amounts upon feeding by herbivores and can diffuse over great distance in the soil matrix. While CO_2 can be seen as an unspecific signal, HIPVs attest indirectly of the presence of herbivorous organisms. In 2005, a team of researchers from the Universities of Neuchatel in Switzerland, and Jena in Germany showed that insect-damaged maize roots were highly attractive to EPNs (**Rasmann et al., 2005**). The results from this work revealed that European maize lines produce a sesquiterpene called (E)-β-caryophyllene when fed by the western corn rootworn (WCR) *Diabrotica virgifera virgifera.* This sesquiterpene has been shown to be responsible for the attractiveness of EPNs under laboratory conditions and in field (**Hiltpold et al., 2010; Köllner et al., 2008; Rasmann et al., 2005**). It was shown that EPNs infected significantly more WCR larvae in the vicinity of maize line naturally producing (E)-β-caryophyllene compared to control treatment with maize line not producing this compound, leading to a decrease in WCR adult emergence (**Rasmann et al., 2005**). Attraction of natural enemies of herbivores through the emission of HIPVs is part of plant indirect defense strategies. Such tritrophic interaction highlights the efficiency of the integration of indirect cues by EPNs to locate potential hosts. Since the publication of the work of Rasmann *et al.*, multiple studies focusing on the attractiveness of HIPVs for host-seeking EPNs were conducted. Notably, it was shown that HIPVs form several plants of agricultural interest, including cotton, citrus tree, potato, sugarcane, carrot, and wine modulate the behavior of host-seeking EPNs. Herbivory-induced plant volatiles can induce different responses in host-seeking EPNs that are more likely strain-specific than related to foraging strategies. For instance, among three tested strains of the ambusher *S. carpocapsae*, only one strain demonstrates chemotaxis towards linalool (**Ali et al., 2011; Laznik and Trdan, 2016a, 2016b, 2013**). Herbivory-induced plant volatiles typically comprise a complex blend of numerous compounds, potentially exceeding 30 terpenes. Consequently, accurate identification of each compound and understanding their respective significance in the modulation of the behavior of EPNs can be challenging (**Rasmann et al., 2012**).

Insects constantly release chemicals in their environment including pheromones, exudates, molting skins, and frass. In addition to HIPVs, it has been shown that cues directly emitted by

insects, such as nitrogen present in frasses, uric acids and urea, hypoxanthine, and xanthine are used by EPNs to locate hosts (**Boff et al., 2001; Campbell and Kaya, 2000; Schmidt and All, 1979**). The work conducted by Dillman *et al.* show that host-seeking EPNs exhibit a species-specific response to odors emitted by different insect species, likely contributing to an appropriate host selection. The strength of the EPNs response to these cues seems to be correlated to their foraging strategies. Specifically, EPN species categorized as ambushers are proposed to be less responsive to insect-derived signals compared to cruisers (**Dillman et al., 2012b**). It has been suggested that chemical cues play a more significant role for cruisers, whereas physical cues, such as air movement, are more relevant for ambushers (**Baiocchi et al., 2019; Gaugler, 2002**). To maximize the efficiency to encounter a host, EPNs favor location where multiple cues are detected. It was shown that EPNs exhibiting a cruiser strategy oriented preferentially toward combination of insect signals, HIPVs, and CO_2 rather than these cues alone (**Dillman et al., 2012b; Turlings et al., 2012; Zhang et al., 2021**). The results from some of these studies also suggest that host-seeking EPNs may prefer plant cues over herbivore odors (**Andaló et al., 2017; Tol et al., 2001**). In the vicinity of a potential host, EPNs have the ability to assess its quality and suitability, allowing them to decide whether or not to initiate infection. For example, EPNs avoid WCR larvae that have sequestered toxic plant secondary metabolites (**Robert et al., 2017**). The infection status of the host is also assessed and host-seeking EPNs can distinguish healthy from infected organisms and even discern between con- and hetero-specific infections (**Baiocchi et al., 2017; Grewal et al., 1997; Jagodič et al., 2017; Kunkel et al., 2006**). Organisms infected by EPNs emit specific odors which modulate multi-trophic interactions belowground. The response of host-seeking EPNs to these odors is likely strain- or species-specific. The results from experiments conducted with *Steinernema* nematodes indicate that while *S. riobrave* are attracted to insects that have recently been infected by conspecifics, *S. glaseri* and *S. feltia* are repelled by con- and hetero-specific infected insects (**Baiocchi et al., 2017; Lewis and Gaugler, 1994**). In addition, it was shown that uninfected hosts are attracted by EPN-infected cadaver, increasing the probability of emerging IJs to encounter a new suitable prey. Recent studies enable the identification of two volatiles involved in this process: 3-methyl-2-buten-1-ol (prenol) and butylated hydroxytoluene (BHT) (**Baiocchi et al., 2017; X. Zhang et al., 2019**). While prenol repel host-seeking EPNs and has been suggested to be a dispersal cue, BHT attracts them (**Baiocchi et al.,**

2017; X. Zhang et al., 2019). The production of prenol and BHT is dynamic over the course of the infection, and it is though that host-seeking EPNs respond to these volatiles in a dose-dependent manner (**Baiocchi et al., 2017**).

***Photorhabdus* bacteria: between virulence and symbiosis**

Once in the vicinity of a suitable host, *Heterorhabditis* IJs enter its body trough natural apertures such as the mouth, the anus, or the spiracles to reach the arthropod hemocoel. There, the nematodes regurgitate 50 to 200 *Photorhabdus* bacterial cells which are in the predominant pathogenic form (P-form) (**Ciche and Ensign, 2003**). The *Photorhabdus* P-form is characterized by its high virulence towards insects and its capacity to support *Heterorhabditis* growth (**Gerritsen et al., 1992; Somvanshi et al., 2012**). Inside the hemocoel, *Photorhabdus* bacteria multiply exponentially and secrete insecticidal toxins, leading to rapid insect death by septicemia and toxemia within 24 to 72 hours (**Bowen et al., 1998; Bowen and Ensign, 1998; Daborn et al., 2002**). While *Photorhabdus* bacteria are very effective at killing insects, it has been shown that axenic *Heterorhabditis* nematodes are capable of causing insect death without their symbiont. However, axenic IJs are much less effective at killing their host than IJs nematode harboring their bacterial symbiont (**Hallem et al., 2007**). Alongside toxins, *Photorhabdus* bacteria also excrete digestive enzymes, antibiotics preventing the proliferation of saprophytic bacteria, and growth factors essential for nematode development (**Richardson et al., 1988; Strauch and Ehlers, 1998**). These growth factors include crystalline inclusion proteins, that are rich in essential amino acids, though to be required as a nutritional source for normal *Heterorhabditis* development (**Bintrim and Ensign, 1998; Brachmann et al., 2007**). During this early-infection step, *Heterorhabditis* IJs also contribute to insect virulence by avoiding the insect immune system response and promoting favorable developing environment for *Photorhabdus* bacteria. It was shown that *Heterorhabditis* IJs produce excreted-secreted proteins that reduce the expression of diptericin, an insect antimicrobial peptide (**Kenney et al., 2019**). During the exponential growth phase, *Photorhabdus* bacteria start to emit the characteristic cyan bioluminescence. Next, *Photorhabdus* bacteria enter into the stationary growth phase, producing a maximum in bioluminescence intensity, and concomitantly, IJ nematodes undergo development into self-fertile hermaphrodite adults. This specific step is referred to as IJ recovery (**Waterfield et al., 2009**). *Photorhabdus* bacteria undergo a metabolic shift from insect pathogenicity towards a symbiosis-centered state,

thereby producing essential elements to support the growth and development of nematodes. *Heterorhabditis* nematodes feed on bacterial biomass, which originates from the digestion of insect tissues and organs by *Photorhabdus* bacteria. Subsequently, hermaphrodite nematodes lay eggs that hatch and develop into male, female, or hermaphrodite adults, and reproduce for two to four generations, depending on the amount of resource available. Once nutritive resources are depleted, *Heterorhabditis* gravid females re-associate with *Photorhabdus* bacteria. During this critical step, *Heterorhabditis* gravid females retain some eggs, allowing part of the progeny to develop within maternal bodies. In the meantime, a part of *Photorhabdus* bacterial cells switch to a phenotypically different form called the mutualistic form (M-form). This switch results of the stochastic inversion of the *mad fimbriae* promoter, resulting in the expression of the *mad*-operon. *Photorhabdus* bacteria expressing the *mad*-operon can adhere to the posterior region of the nematode intestine and produce a biofilm to progressively colonize the nematode progeny developing into the maternal body cavity (**Somvanshi et al., 2012, 2010**). Upon endotokia matricida, the newborn nematode generation engages a specific developmental pathway to develop into IJ nematodes. These IJ nematodes break out from the depleted carcass to start their parasitic lifecycle over again. A complete infection cycle typically occurs in two to three weeks and can lead to the emergence of several tens to hundreds of thousands of nematodes in the soil. During this complex lifecycle, *Photorhabdus* bacteria produce bioluminescence which results in the occurrence of bioluminescent infected organisms in the dark belowground environment. It has been shown under laboratory conditions that the two phenotypically different *Photorhabdus* forms do not produce bioluminescence at the same intensity. Investigation made with *in-vitro* *Photorhabdus* cultures indicate that M-form are much less bioluminescent than P-form cells (**Somvanshi et al., 2012**). In addition to the P- and M-forms, it has been observed that *Photorhabdus* cells also exist in two variants called primary and secondary cell types (**Akhurst, 1980**). While all the above-mentioned characteristics referred to primary cell type, it has been suggested that secondary cell type might be adapted to an *Heterorhabditis*-free life. This hypothesis relies on two distinctive features of the *Photorhabdus* lifecycle. Firstly, within an infected host, a fraction of *Photorhabdus* primary cells undergoes a random transition to a secondary cell type. Secondly, after the host carcass is depleted, only a limited number of *Photorhabdus* cells re-associates with *Heterorhabditis* nematodes. The presence in the

depleted carcass of *Photorhabdus* secondary cell types, proposed to be adapted to a nematode-free life, might facilitate the survival of *Photorhabdus* in the soil through an alternative lifestyle (**Eckstein et al., 2019; Langer et al., 2017**). This complex lifecycle is summarized in **Figure 1**.

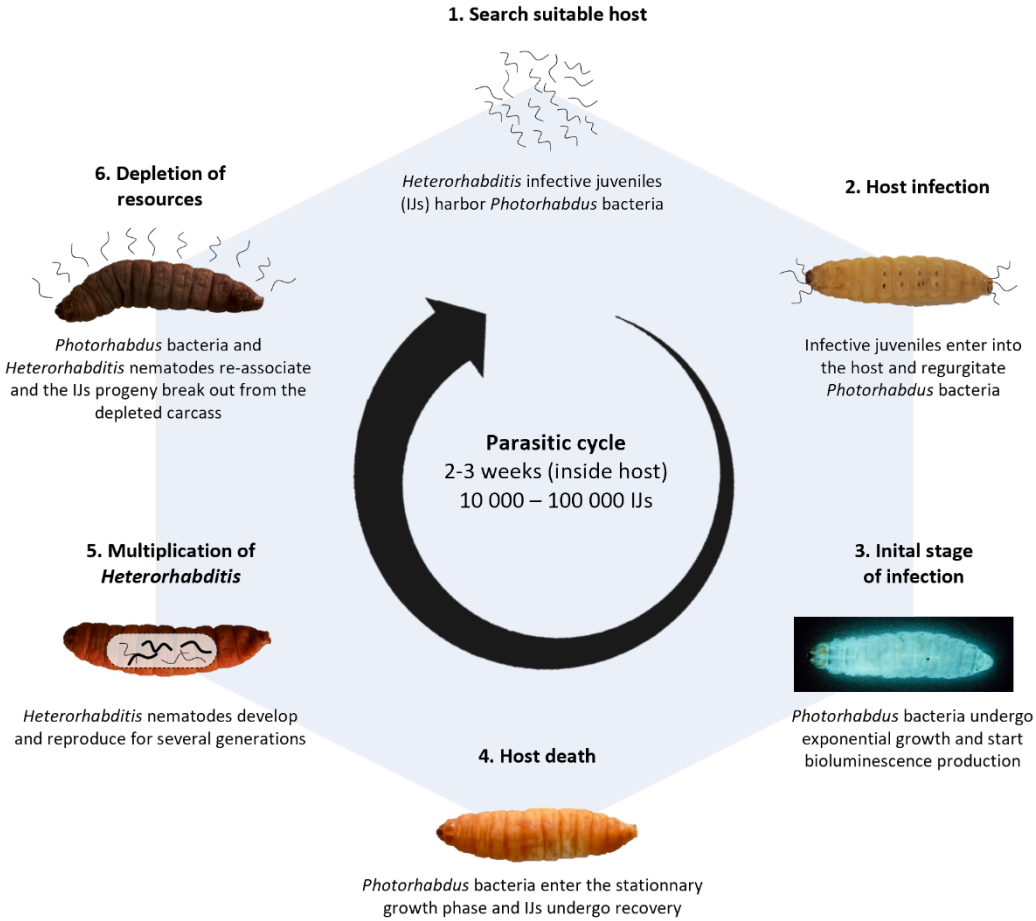

Figure 1. *Photorhabdus* bacteria and *Heterorhabditis* nematodes parasitize small arthropods in the soil. The cycle starts by the search of a suitable host by *Heterorhabditis* infective juveniles (IJs) in the soil. Infective juveniles enter the host and release their bacterial symbiont inside its hemocoel. *Photorhabdus* bacteria multiply and start to produce bioluminescence, resulting in the glow of the infected organism. The host rapidly dies due to septicemia and toxemia, while *Heterorhabditis* infective juveniles (IJs) undergo recovery. Then, *Heterorhabditis* nematodes reproduce for several generations and upon nutrition depletion they re-associate with *Photorhabdus* bacteria. Finally, the new generation of IJs break out for the depleted carcass and search for a new host to infect. Scale is not maintained for clarity.

Photorhabdus: the only genus of bioluminescent bacteria in terrestrial ecosystems

Bioluminescence is a widespread trait

All described species of *Photorhabdus* bacteria, except *P. Heterorhabditis* subsp. *aluminescens*, are bioluminescent (**Machado et al., 2021a**). Bioluminescence is defined as the chemical production of light by living organisms. It differs from fluorescence and phosphorescence as it does not rely on the activation or re-emission of another source of light (**Wilson and Hastings, 1998**). Bioluminescence is a widespread trait, present in more than 800 genera of organisms that has evolved independently at least 94 times through evolution (**Lau and Oakley, 2021**). At core, bioluminescent reactions are characterized by the oxygenation of substrates called luciferins by enzymes called luciferases. Although the chemical reactions share common features, luciferins and luciferases do not refer to specific classes of molecules and these molecules are not necessarily chemically related (**Wilson and Hastings, 1998**). In bacteria, the capacity to produced bioluminescence relies on the presence of the *lux*-operon (**Dunlap and Urbanczyk, 2013**). This operon is composed at minima of two genes, *luxA* and *luxB*, which encode the two subunits of the bacterial luciferase. Bacterial luciferase is an enzyme of approximatively 80 KDa which catalyze the oxidation of reduced flavin mononucleide and a long chain fatty aldehyde in presence of dioxygen (**Fisher and Raushel, 1995**). During this redox reaction, light is produced (**Baldwin et al., 1995**). The production of the two substrates necessary for the reaction is controlled by a fatty acid reductase complex and a flavin reductase. The genes coding for this fatty acid reductase complex are part of the *lux*-operon of most bioluminescent bacteria and are called *luxC, luxD,* and *luxE*, while the flavin reductase is encoded by the gene *fre*. In *Photorhabdus* bacteria, the presence of the *lux*-operon is thought to originate from ancient horizontal gene transfer events from their ancestral marine relatives (**Kasai et al., 2006; Urbanczyk et al., 2008**).

Bioluminescence displays significant variation in emission patterns across different organisms. For instance, in bacteria, it is emitted as a continuous glow lasting from several hours to several days. In contrast, dinoflagellates exhibit bioluminescence as a single flash emission, while in certain animals like the common glowworm (*Lampyris noctiluca*), it is produced as repetitive pulses (**Cock and Matthysen, 2001; Kahlke and Umbers, 2016; Wilson and**

Hastings, 1998). Bioluminescence also exhibits diversity in the wavelengths emitted. *Photorhabdus* bacteria, for example, emit a distinctive cyan light (with a peak emission at approximately 480 nm), falling within the range observed in bioluminescent marine bacteria (420-520 nm). In contrast, green-yellow bioluminescence (520-580 nm) is more commonly observed in terrestrial organisms (**Haddock et al., 2010; Kahlke and Umbers, 2016; Wilson and Hastings, 1998**). For example, the Jamaican firefly *Pyrophorus plagiophtalamus* emits bioluminescence with peak emission wavelengths between 550-562 nm, while an examination of Brazilian fireflies shows that adult specimens produce bioluminescence with maximum emission wavelengths ranging from 548 to 573 nm (**Biggley et al., 1967; Viviani and Bechara, 1995**).

Regulation of bioluminescence production in bacteria

The first insight into the regulation of bacterial bioluminescence led to the discovery of quorum sensing (QS), a key mechanism of bacterial communication, more than seventy years ago (**Farghaly, 1950; Kempner and Hanson, 1968**). This regulatory mechanism enables bacteria to initiate coordinated responses based on their population density. In two marine bioluminescent bacteria, *Aliivibrio fischeri* and *Vibrio campbellii*, bioluminescence production is under the control of the acylated homoserine lactone (AHL)-based QS regulatory circuit (**Farghaly, 1950; Kempner and Hanson, 1968**). This pathway involves two additional *lux* genes, *luxI* and *luxR*, which respectively code for the AHL autoinducer synthase and the LuxR transcriptional regulator (**Devine et al., 1988; Lupp et al., 2003**). As the population of *A. fischeri* or *V. campbellii* increases, the autoinducer AHL accumulates in their surrounding environment. Once a threshold concentration is reached, AHL binds to LuxR, activating the transcription of the *lux*-operon and leading to bioluminescence production.

However, it should not be assumed that bioluminescence production is always under QS control. Work conducted on *Photobacterium* bacteria showed that, bioluminescence production is not always dependent on cell densities, suggesting that other mechanisms regulate this process (**Katznelson and Ulitzur, 1977; Rosson and Nealson, 1981; Tanet et al., 2019**). Little is known about the regulation of bioluminescence production in *Photorhabdus*. *Photorhabdus* bacteria do not possess the gene *luxI* but the absence of this gene does not necessarily indicate that bioluminescence is not regulated by QS (**Duchaud et al., 2003**). This

is exemplified in one strain of *V. campbelli* called DS40M4, where bioluminescence production is under QS control despite the absence of *luxI* (**Simpson et al., 2021**). *Photorhabdus* bacteria possess another major QS signaling mechanism based on the autoinducer-2 (AI-2) synthesis protein coded by the gene *luxS* and the transcriptional regulator UvrY (**Duchaud et al., 2003**). Results from transcriptomic studies conducted with *luxS*- and *uvrY*-deficient *Photorhabdus* bacteria suggest that these genes might play roles in modulating *Photorhabdus* bioluminescence (**Krin et al., 2008, 2006**). The conclusion drawn from those studies suggest that UvrY might act upstream of LuxS to positively regulates bioluminescence production in an Al-2-dependent manner. The autoinducer-2 might indirectly enhances bioluminescence production by reducing the spermidine level, which in turn might affect the availability of aldehyde content, the substrate of luciferase. This hypothesis relies on the downregulation of polyamine metabolism genes in *luxS*-mutant. Yet, these results require nuanced interpretation, as bioluminescence production in two different strains of *Photorhabdus laumondii* subsp. *laumondii luxS*-mutant was demonstrated to either decrease or increase in a time-dependent manner (**Heinrich et al., 2017**). Given our present understanding, it is still unclear whether bioluminescence production in *Photorhabdus* is cell density-dependent.

Biological functions of bioluminescence

The first reports of bioluminescence date back to ancient Greece, and the ecological significance of bioluminescence has been holding the interest of scientists for many decades (**Delroisse et al., 2021; Kahlke and Umbers, 2016; Lau and Oakley, 2021; Nealson and Hastings, 1979**). Presently, bioluminescence is considered an important modulator of intra- and inter-specific biotic interactions and a regulator of abiotic stress tolerance (**Cock and Matthysen, 2001; Czyz and Wegrzyn, 2001; Marek et al., 2011; Sivinski, 1981; Underwood et al., 1997; Verdes and Gruber, 2017**). In terrestrial ecosystems, bioluminescence is involved in defense, reproduction, and foraging functions. Glowing millipedes from the genus *Motyxia* emit light as an aposematic warning signal, while other insects and millipedes release repulsive substances and more bioluminescence upon attack as defensive strategies (**Cock and Matthysen, 2001; Marek et al., 2011; Moosman, et al., 2009; Underwood et al., 1997; Viviani and Bechara, 1997**). The New Zealand glowworm, *Arachnocampa luminosa*, is thought to use bioluminescence to lure prey in its larval stage and later, as an adult, employs it as a mating signal (**Meyer-Rochow and Eguchi, 1984**).

In fungi, bioluminescence could favor spore dispersion, but this hypothesis is not consistently supported (**Herring, 1994; Sivinski, 1981, 1998; Weinstein et al., 2016**). In oceans, symbiotic relationships between bioluminescent bacteria and fish or squid are common. These relationships often take place in specific organs called photophores. While these photophores may present favorable conditions for bacterial growth, light production is a precious tool for the host. It can lure prey, serve as a defense mechanism to escape predation, or attract sexual mates (**Haddock et al., 2010; Hellinger et al., 2017; Johnsen et al., 2004; Kubodera et al., 2007**). Bioluminescence emitted by non-symbiotic marine bacteria has been suggested to attract marine zooplankton and fish, which feed on glowing particles. Bacteria that can survive digestion reach the guts of the animals, where they benefit from a rich nutritious environment and a fast-moving vector for wide propagation (**Zarubin et al., 2012**). Bioluminescence is also involved in DNA repair, UV-radiation protection, and oxidative stress resistance in marine bacteria (**Czyz et al., 2000; Szpilewska et al., 2003; Walker et al., 2006**). Bacterial luciferase, the central player in bioluminescence production, is thought to detoxify reactive oxygen species (ROS) under low oxygen conditions. This hypothesis is particularly relevant as both marine and terrestrial bioluminescent bacteria complete their life cycles in poor oxygen environments (**Dunlap, 2014; Dunlap and Urbanczyk, 2013**). Belowground, bioluminescence might contribute to defensive strategies, as evidenced by the release of bioluminescent substances by earthworms and potworms in response to stimulation (**Pes et al., 2016; Seesamut et al., 2021; Verdes and Gruber, 2017**).

Current knowledge about the biological functions of *Photorhabdus* bioluminescence

Photorhabdus bacteria initiate bioluminescence production during their exponential growth phase within the hemocoel of infected organisms. This production reaches a maximum concomitantly with the death of the host and decline during to the stationary growth phase of the bacteria. While bioluminescence is prevalent in the *Photorhabdus* genus, its biological function remains unclear. As in marine bacteria, bioluminescence might contribute to DNA repair and UV-radiation protection (**Czyz et al., 2000; Peat and Adams, 2008**). *Photorhabdus* bioluminescence has also been hypothesized to promote oxidative stress resistance by acting as a redox sink (**Peat and Adams, 2008; Stabb, 2005; Waterfield et al., 2009**). During the initiation of a host infection, *Photorhabdus* cells must face the production of ROS that are part of the innate defense mechanisms of insects. The timing of bioluminescence production aligns

with this hypothesis. In addition, bioluminescence emitted by *Photorhabdus*-infected cadavers was proposed to attract healthy prey in the vicinity of infected organisms, by positive phototaxis, to improve predation success (**Waterfield et al., 2009**). Infective juveniles break out from infected insect only when its carcass is depleted, usually several weeks after the beginning of the infection whereas bioluminescence production is a characteristic of the early-stage of infection. Taken together, these elements are not in favor of this hypothesis. Recently, Cimen examined a previously formulated hypothesis positing that bioluminescence might function as a protective mechanism against scavengers (**Cimen, 2023; Waterfield et al., 2009**). During the reproduction of *Heterorhabditis* nematodes inside an infected host, the insect cadaver might represent an easy target for scavengers in the soil. In that context, bioluminescence was proposed to be an aposematic signal deterring scavenger. Regarding the timing of bioluminescence production, this hypothesis is particularly pertinent to the early-stage of infection. In contrast, other cues, such as odors emitted by infected insects, may deter scavengers in the later stages of infection. Based on this investigation, it was proposed that bioluminescence does not deter scavengers. The choice of the control treatment for assessing the impact of bioluminescence on scavenger deterrence is a matter of discussion. Due to the unavailability of an aluminescent *Photorhabdus* mutant, a less bioluminescent strain, still capable of light production, was utilized as the control (**Cimen, 2023**). Finally, it has also been suggested that *Photorhabdus* bioluminescence is a remnant of a horizontal gene transfer with no particular biological function (**Peat et al., 2010**). At the time of the work conducted by Peat *et al.*, only three species of *Photorhabdus* had been described, which strongly limit the conclusion that can be drawn from this evolutionary investigation. While most of these hypotheses are biologically relevant, they should be approached cautiously, as access to modern molecular techniques was sometimes limited and there remains a need for further experimental validation (**Cimen, 2023; Ffrench-Constant et al., 2003; Peat et al., 2010; Peat and Adams, 2008; Waterfield et al., 2009**).

The aim of this thesis

The bioluminescence produced by *Photorhabdus* bacteria stands out as a remarkable trait among terrestrial bacteria, but our comprehension of its biological functions remains limited. In this thesis, I aim to fill this knowledge gap by employing an interdisciplinary approach that integrates molecular tools, metabolomic and volatile analyses, along with behavioral experiments. In the following paragraphs, I will concisely present the focus of the different chapters of this thesis

- In *Photorhabdus* bacteria, the molecular mechanisms responsible for bioluminescence production are known. However, the evolution and regulation of this process remains poorly understood. In the first chapter of this thesis, we used a panel of 59 strains representing all species and subspecies of the genus up to 2020, when this investigation was started, to deepen our understanding of the regulation and evolution of bioluminescence production in the *Photorhabdus* genus. Previous studies suggested that *Photorhabdus* bioluminescence might be a disappearing trait. To test this hypothesis, we assessed the evolution of *Photorhabdus* bioluminescence at both extended evolutionary timescales, utilizing phylogenetic analysis, and within shorter timescales, employing an experimental evolution approach. We utilized genomic and transcriptomic tools to examine the roles of both regulators described in existing literature and genes identified during our experimental evolution experiments on the variability in the levels of bioluminescence produced across the *Photorhabdus* genus.

- *Photorhabdus* bacteria share a symbiotic relationship with nematodes from the genus *Heterorhabditis.* Together, they parasitize and kill small arthropods belowground. Infection by this entomopathogenic pair lead to the occurrence of bioluminescence infected organisms in the soil. We hypothesize that *Photorhabdus* bioluminescence is a signal of infection by *Heterorhabditis* nematode which modulate the foraging behavior of host-seeking EPNs. In the second chapter of this thesis, we used genetically engineered aluminescent *Photorhabdus* strains, their bioluminescent wild type counterparts, and luminescent beads to test this hypothesis (**Fig. 2A**).

- During the initial infection stage, *Photorhabdus* bacteria undergo exponential growth and initiate bioluminescence production within the host, while *Heterorhabditis* nematodes remain developmentally arrested. The prevailing viewpoint suggests that *Heterorhabditis* nematodes transition to adulthood only upon detecting a signal from *Photorhabdus* bacteria, indicating favorable conditions for their development and reproduction. We hypothesize that *Photorhabdus* bioluminescence plays a role in this process, contributing to the success of symbiotic relationship with *Heterorhabditis* nematodes (**Fig. 2B**). We tested this hypothesis in the third chapter of this thesis by performing symbiotic assays and metabolomic analyses with aluminescent *Photorhabdus* mutant strains and their bioluminescent wild-type counterparts.

- The entomopathogenic pair formed by *Photorhabdus* bacteria and *Heterorhabditis* nematodes infect herbivorous insects, leading to the occurrence of bioluminescent infected organisms in the rhizosphere. This bioluminescent signal is likely sensed by plant roots. We hypothesize that *Photorhabdus* bioluminescence inform plants about the presence of herbivorous insects and their natural enemies, EPNs, in the rhizosphere (**Fig. 2C**). In the last chapter on this thesis, we exposed maize roots to insect larvae infected with *Photorhabdus* bacteria or to luminescent beads and assessed plant responses through transcriptomic, metabolomic and phytohormonal analyses.

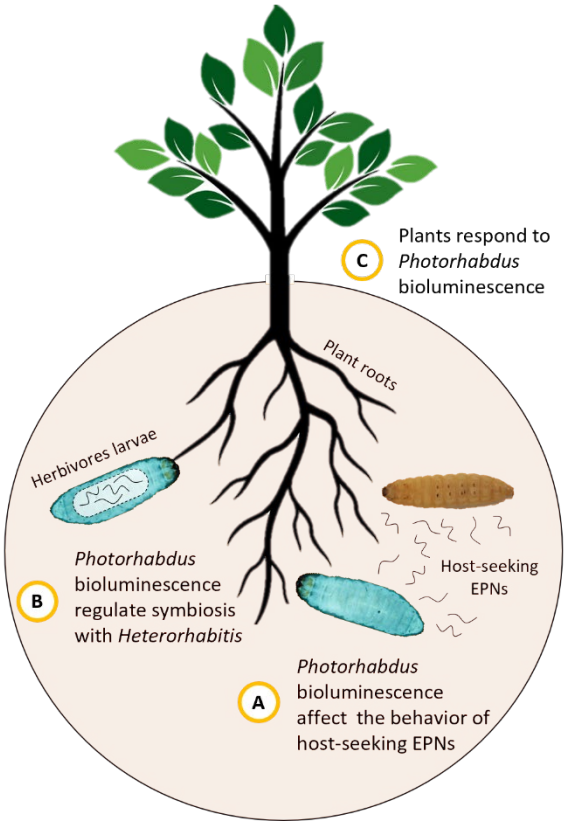

Figure 2. Contextual representation of the hypotheses that we investigate in this thesis about the putative biological functions of *Photorhabdus* bioluminescence. Infection by *Photorhabdus* bacteria result in the occurrence of bioluminescent infected organisms in the soil. We hypothesize that this bioluminescence might (**A**) affect the behavior of host-seeking EPNs, (**B**) play roles in the symbiosis with *Heterorhabditis* nematodes, (**C**) trigger responses in plants. These hypotheses are further discussed and tested in the different chapters of this thesis. Scale is not maintained for clarity.

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CHAPTER 1
Regulation and evolution of *Photorhabdus* Bioluminescence

Abstract

Bioluminescence, the chemical production of light by living organisms, is a widespread trait in aquatic bacteria but is present in only one genus of terrestrial bacteria: *Photorhabdus*. In all bacteria, this trait relies on the presence in the genome of the *lux*-operon. While the regulation of the *lux*-operon is under the control of quorum sensing in many marine bacteria, the mechanisms regulating the production of *Photorhabdus* bioluminescence remain unclear. *Photorhabdus* bacteria exhibit a complex lifecycle, sharing symbiotic relationship with soil-dwelling nematodes from the genus *Heterorhabditis*. During its lifecycle, *Photorhabdus* cells exist in two phenotypically different forms: a pathogenic form (P-form) which is virulent towards insects and a mutualistic form (M-form) which is thought to be involved in the colonization of *Heterorhabditis* nematodes. In addition, *Photorhabdus* cells also exist in two phenotypic variants called primary and secondary cells. While primary cells display all phenotypes involved in the symbiotic relationship with *Heterorhabditis* nematodes, secondary cells have been suggested to be adapted to a nematode-free life in the rhizosphere. Current knowledge on the regulation of *Photorhabdus* bioluminescence production is mainly built from studies focusing on cell types, or form variants using the model species *Photorhabdus laumondii* subsp. *laumondii.* In this work, a panel of 59 strains representing all species and subspecies of the genus up to 2020, when this investigation was started, was used to deepen our comprehension of the regulation and evolution of bioluminescence production in the *Photorhabdus* genus. Our characterization of bioluminescence production *in vitro* and *in vivo* reveals that this trait displays a high intra- and inter-specific variability. We investigated the evolution of *Photorhabdus* bioluminescence at both extended evolutionary timescales, utilizing phylogenetic analysis, and within shorter timescales, employing an experimental evolution approach. We provide evidence that *Photorhabdus* bioluminescence is an evolutionary dynamic trait that can rapidly evolve under laboratory conditions in a strain-specific manner but that it does not tend to disappear. Using molecular tools, we demonstrate that bioluminescence production is tightly controlled by a multicomponent network of genes, not limited to the regulators already described in the literature.

Introduction

Bioluminescence is the chemical production of light by living organisms (**Kahlke and Umbers, 2016**). It is a widespread trait in marine and freshwater bacteria, and only one genus of terrestrial bacteria is known to share this feature: *Photorhabdus* (**Herring, 1977; Meighen, 1991; Nealson and Hastings, 1979; Thomas and Poinar, 1979; Vannier et al., 2020; Waterfield et al., 2009**). In bacteria, bioluminescence production relies on a set of genes organized into the *lux*-operon. The composition of this operon differs depending on the species of bacteria, but all share at least the genes *luxA* and *luxB* that code the two subunits of bacterial luciferase and *luxC*, *luxD,* and *luxE* that code the three subunits of the fatty acid reductase synthesizing the aldehyde substrate needed for the bioluminescent reaction (**Dunlap et al., 2013**). It has been hypothesized that *Photorhabdus* bacteria acquired the *lux*-operon from ancient horizontal gene transfer events (**Kasai et al., 2006; Urbanczyk et al., 2008**). Despite the prevalence of bioluminescence in the *Photorhabdus* genus, it has been suggested that this trait is cryptic and tends to disappear (**Peat et al., 2010**). The study of bioluminescence production in the historical model species *Aliivibrio fischeri* (previously identified as *Vibrio fischeri* or *Photobacterium fischeri*) and *Vibrio campbellii* (previously identified as *Beneckea harveyi* or *Vibrio harveyi*) led to the discovery of quorum sensing (QS), a major means of bacterial communication, more than seventy years ago (**Farghaly, 1950; Kempner and Hanson, 1968**). While multiple examples of QS-controlled bioluminescence production have been described, the mechanisms regulating bioluminescence production in *Photorhabdus* are still unclear (**Anetzberger et al., 2009; Farghaly, 1950; Hayek et al., 2020; Kempner and Hanson, 1968; Swift et al., 1998**). In this work, we aim to enhance our understanding of the regulation and evolution of bioluminescence production in the *Photorhabdus* genus.

Photorhabdus is a genus of bacteria member of the family *Morganellaceae* (**Adeolu et al., 2016**). The name *Photorhabdus*, literally meaning “glowing-rod” is derived from the ability of bacteria from this genus to produce bioluminescence (**Boemare et al., 1993**). *Photorhabdus* bacteria live in obligate symbiosis with microscopic soil-dwelling nematodes belonging to the *Heterorhabditis* genus (**Boemare et al., 1993; Thomas and Poinar, 1979**). Together, they form an entomopathogenic pair that parasitizes and kills small arthropods belowground. *Heterorhabditis* nematodes share similarities in their lifecycles and ecological niches with another genus of entomopathogenic nematodes (EPNs): *Steinernema.* While *Xenorhabdus*,

the bacterial symbionts of *Steinernema* nematodes, were first isolated in 1966, *Photorhabdus* bacteria were isolated for the first time in 1979 (**Poinar and Thomas, 1966, 1965; Thomas and Poinar, 1979**). Until 1993, all bacterial symbionts isolated from *Steinernema* and *Heterorhabditis* nematodes were grouped in the genus of *Xenorhabdus*. During this time, *Photorhabdus* bacteria were classified as *Xenorhabdus luminescens* (**Boemare et al., 1993**). Since the early 2000s, major improvements in sequencing technologies and extensive research have enabled a thorough revision of the *Photorhabdus* genus. Currently, the *Photorhabdus* genus comprises 30 taxa, including 23 species, with 6 of them divided into different subspecies (**Castaneda-Alvarez et al., 2022; Machado et al., 2023, 2021a, 2021b, 2019, 2018**).

Bioluminescence is prevalent in the *Photorhabdus* genus, but its biological functions remain unclear. It has been suggested that *Photorhabdus* bioluminescence might contribute to DNA repair, UV-radiation protection, oxidative stress resistance, or prevent attack of *Photorhabdus*-infected cadavers by scavengers (**Baur et al., 1998; Cimen, 2023; Ffrench-Constant et al., 2003; Peat and Adams, 2008; Waterfield et al., 2009**). It has also been suggested that *Photorhabdus* bioluminescence is a disappearing trait, remnant of horizontal gene transfer events with no particular biological function (**Peat et al., 2010**). Access to modern molecular techniques was sometimes limited during these investigations and at the time of the work conducted by Peat *et al.*, only three species of *Photorhabdus* had been described. While these hypotheses are biologically relevant, they require further experimental validation and should be approached cautiously.

The first insight into the regulation of bacterial bioluminescence were derived from works carried out using the marine bioluminescent bacteria models *Aliivibrio fischeri* and *Vibrio campbellii*. In these two species, bioluminescence production is under the control of the acylated homoserine lactone (AHL)-based QS regulatory circuit (**Farghaly, 1950; Kempner and Hanson, 1968**). This pathway involves *luxI* and *luxR* genes coding, respectively, AHL autoinducer synthase and luxR transcriptional regulator (**Devine et al., 1988; Lupp et al., 2003**). It should not be assumed that bioluminescence production is always under QS control. Work conducted on *Photobacterium* bacteria indicated that for several strains of this genus, bioluminescence production is not dependent on cell density, suggesting that other

mechanisms are regulating this process (**Katznelson and Ulitzur, 1977; Rosson and Nealson, 1981; Tanet et al., 2019**). In *Photorhabdus, t*he regulation and characteristics of bioluminescence production are still unclear (**Dunlap et al., 2013**). *Photorhabdus* bacteria do not possess *luxI* (**Duchaud et al., 2003**). The absence of this gene in *Photorhabdus* does not necessarily indicate that bioluminescence is not regulated by QS. This is exemplified in *V. campbelli*, where light production is under QS control despite the absence of *luxI* (**Greenberg et al., 1979; Tanet et al., 2019**). *Photorhabdus* bacteria possess another major QS signaling mechanism based on the autoinducer-2 (AI-2) synthesis protein coded by the gene *luxS* (**Duchaud et al., 2003**). Results from transcriptomic studies conducted with *luxS*- and *uvrY*-deficient *Photorhabdus* bacteria suggest that both of those regulators play roles in modulating *Photorhabdus* bioluminescence (**Krin et al., 2008, 2006**). The conclusion drawn from those studies is that *uvrY* acts upstream of *luxS* to positively regulates bioluminescence production in an Al-2-dependent manner. The protein AI-2 would indirectly enhance bioluminescence by reducing the spermidine level, which in turn would affect the availability of aldehyde content, the substrate of luciferase. This hypothesis relies on the downregulation of polyamine metabolism genes in *luxS*-mutant. Yet, these results require nuanced interpretation, as bioluminescence production in two *luxS*-mutant strains of *Photorhabdus laumondii* subsp. *laumondii* (previously identified as *Photorhabdus luminescens* subsp. *laumondii*) was demonstrated to either decrease or increase in a time-dependent manner (**Heinrich et al., 2017**). Given our present knowledge, it is still unclear whether bioluminescence production is cell density-dependent or not in *Photorhabdus.*

Photorhabdus cells exist in two phenotypically different forms: the predominant pathogenic form (P-form) which is virulent towards insects and supports EPNs growth, and the mutualistic form (M-form) which produces less bioluminescence and is thought to allow the re-association of *Photorhabdus* with their symbiotic EPNs (**Gerritsen et al., 1992; Somvanshi et al., 2012**). Mutualistic-form appears from P-form spontaneously by stochastic inversion of the *mad fimbriae* promoter, resulting in the expression of the *mad*-operon. Besides form variation, *Photorhabdus* cells also exist in two phenotypic variants called primary and secondary cell types (**Akhurst, 1980**). Primary cells display all phenotypes involved in the symbiotic relationship with EPNs, whereas secondary cells do not support EPNs growth and produce less bioluminescence (**Joyce and Clarke, 2003**). While it has been suggested that secondary cells

might be adapted to an EPN-free life in the rhizosphere, free-living *Photorhabdus* bacteria have not yet been isolated from soil (**Eckstein et al., 2019; Langer et al., 2017**). Pathogenic-form and primary cells are more bioluminescent than their counterparts, and mechanisms involved in the transition process provides good hints on the regulation of bioluminescence. The protein MadJ has been identified as a potential regulator of the switch between P-form to M-form. In M-form, luciferase encoded by *luxA* and *luxB* is downregulated while the gene coding the LysR-type regulator HexA is strongly upregulated compared to P-form (**Somvanshi et al., 2012**). The protein HexA also plays an important role in the regulation of bioluminescence production in primary and secondary cells. Experimental evidence from genetically modified *Photorhabdus* strains led to the hypothesis that HexA indirectly represses bioluminescence production at the post-transcriptional level in secondary cells (**Langer et al., 2017**). It has also been suggested, based on experimental data, that ArcZ, a regulatory small RNA, might repress HexA by binding to its transcript thanks to the RNA chaperone Hfq (**Neubacher et al., 2020**). These studies suggest that HexA might indirectly repress bioluminescence production in secondary type and M-form cells. In contrast, in primary type and P-form cells, Hfq and the ArcZ/Hfq complex might repress HexA at the transcriptional and post-transcriptional levels, resulting in a de-repression of bioluminescence production. In summary, the production of bioluminescence in *Photorhabdus* is dependent on the form variant (P-form and M-form), and on the cell type (primary and secondary) in which the bacteria exist. The global regulators HexA and Hfq play important roles in this process.

Current knowledge on the regulation of *Photorhabdus* bioluminescence production are derived from studies focusing on cell types, or form variants in the model species *P. laumondii* subsp. *laumondii* (**Eckstein et al., 2019; Joyce and Clarke, 2003; Langer et al., 2017; Neubacher et al., 2020; Somvanshi et al., 2012; Waterfield et al., 2009**). The aim of our study is to widen our understanding of the regulation and evolution of bioluminescence production in the *Photorhabdus* genus. Therefore, we used a panel of 59 strains representing all species and subspecies of the genus up to 2020, when this investigation was started, to characterized *Photorhabdus* bioluminescence production both *in vitro* and *in vivo*. This approach provides a biologically relevant insight into the intra- and inter-specific variabilities of this trait. We investigated the evolution of *Photorhabdus* bioluminescence at both extended evolutionary timescales, utilizing phylogenetic analysis, and within shorter timescales, employing an

experimental evolution approach. Finally, to unravel the regulation of *Photorhabdus* bioluminescence production, we investigated the role of regulators described in the literature and genes identified in this work using genomic and transcriptomic technologies.

Material and methods

Photorhabdus culture

To maintain the panel of *Photorhabdus* strains used in this study, all strains were cultured onto lysogenic broth (LB) agar plates (Sigma-Aldrich, USA) and incubated at 28°C. The bacteria were re-plated on fresh LB agar plates every thirty days. After five re-plating cycles, the cultures were refreshed using glycerol stocks. Liquid *Photorhabdus* bacterial cultures were grown in liquid LB at 28°C with constant agitation (180 rpm) in darkness.

In vivo and *in vitro* bioluminescence measurements

To measure the bioluminescence emitted by *Photorhabdus* bacteria-infected insects, *Galleria mellonella* larvae were infected with the different bacterial strains, as described previously (**Castaneda-Alvarez et al., 2022**) Briefly, all *Photorhabdus* bacterial strains were individually grown for 16-20 hours in LB medium at 28 °C with constant agitation (180 rpm). The bacterial cultures were then collected, and their optical densities at 600 nm (OD_{600}) were measured. Subsequently, all cultures were diluted to obtain bacterial cultures with an OD_{600}=1, and 10 µL of the resulting bacterial cultures were injected into third-instar *G. mellonella* larvae. Three larvae per strain were injected and the experiments were conducted three independent times (n=9). Bioluminescence was measured at regular intervals for 48 h using 24-well microtiter plates (Greiner Bio-One, Au) and a Varioskan Flash multimode reader (Thermo Fisher Scientific). Infected larvae were photographed to document differences in bioluminescence production among the different bacterial strains. For this, a Canon EOS-1D camera equipped with a Canon 100 mm f/2.8 macro lens with image stabilization (Ultrasonic) was used, under the following settings: Aperture f/2.4, ISO 6400, Bulb mode (BLUB), and exposures ranging from 30 to 120 seconds. To measure the bioluminescence emitted by bacteria cultured *in vitro*, the different bacterial strains were cultured in 384-well microtiter plates (Greiner Bio-One, Au) as follows. All bacterial strains were grown individually for 16-20 h in LB medium at 28 °C with constant agitation (180 rpm). The bacterial cultures were then collected, and their OD_{600} were measured. All cultures were then diluted to obtain bacterial cultures with an OD_{600}=0.03-0.05. Ten microliters of these bacterial solutions were inoculated into 70 µL of half-strength LB medium (12,5 g/l; Carl Roth, DE). The plates were incubated at 28 °C. OD_{600} (as a proxy for bacterial growth) and bioluminescence measurements were carried out every

30-60 min for 48 h using a Varioskan Flash multimode reader (Thermo Fisher Scientific) or a SpectraMax 0480 microplate reader. The plates were gently shaken orbitally (4.5 mm amplitude and 5 s shaking cycles) before each measurement. Bioluminescence was expressed in relative light units (RLU). Photographs of bioluminescence on solid-medium cultured bacterium were made using an Amersham Imager 600 instrument (Cytiva, USA).

Bacterial genomic DNA extraction and sequencing

Genomic DNA was extracted from three- to five-day-old bacterial colonies of *Photorhabdus* using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) following the manufacturer's instructions. Briefly, bacterial cells were harvested from fresh colonies grown on LB agar plates and resuspended in a 2 mL microtube containing a mix of 180 µL of Lysis Solution T and 200 µL of Lysis Solution C (GenElute Bacterial Genomic DNA Kit), supplemented with proteinase K at 20 mg/mL. The mixture was vortexed thoroughly and heated at 55°C for 2-3 hours to ensure complete cell wall lysis. To obtain RNA-free genomic DNA, 20 µL of RNase A Solution (GenElute Bacterial Genomic DNA Kit) was added, and the microtube was incubated for 2 minutes at room temperature. Next, 200 µL of 100% ethanol was added to the cell lysate, which was then loaded onto a DNA binding column. The column was centrifuged at 6500 g for 1 minute. Subsequently, the column was washed twice with 500 µL of Wash Solution (GenElute Bacterial Genomic DNA Kit) and centrifuged for 3 minutes at 16000 g to dry. The genomic DNA was resuspended in 50 µL of Elution Solution (GenElute Bacterial Genomic DNA Kit). The purity and concentration of the extracted genomic DNA were assessed by electrophoresis on a 1% (v/v) TAE-agarose gel stained with 0.005% nucleic acid gel stain (SYBR Safe, Apex Bio) and using a NanoDrop spectrophotometer (IMPLEN, CA, USA). For Sanger sequencing, samples were sent to Microsynth AG (Balgach, Switzerland). The obtained sequences were manually curated using BioEdit 7.2.5 (**Hall, 1999**).

Phylogenetic trees

To reconstruct whole genome-based phylogenetic relationships, genomes were first aligned using Roary 3.13.0. Genes to be considered core had to be present in 85 % of the genomes with an 85 % protein identity (**Castaneda-Alvarez et al., 2022; Machado et al., 2021a, 2021b**). Obtained alignments were used to build phylogenetic trees using FastTree 2.1.10 based on

the generalized time reversible model. Graphical representation and edition of the phylogenetic trees were performed in R with the package "Phytools" (**Revell, 2012**).

Experimental evolution experiments

To infer the potential for rapid evolutionary changes in bioluminescence production in the *Photorhabdus* genus under laboratory conditions, we conducted experimental evolution experiments and measured and contrasted bioluminescence production in parental and in their corresponding laboratory evolved (lab-evolved) strains, using a panel of 59 strains representing all species and subspecies of the genus up to 2020, when this investigation was started. To generate the lab-evolved strains, each bacterial strain was individually cultured at 28 °C with constant shaking (180 rpm) in 14 mL round bottom polystyrene culture tubes (Corning, USA) filled with 3 mL of LB medium. After 24 h, subsamples of the resulting bacterial cultures were preserved as glycerol stocks, representing the parental strains. Then, 300 µL of the same bacterial cultures were transferred to 2.7 mL of fresh LB medium. Bacterial cultures were incubated and cultured in a similar manner for 24 h, and this procedure was repeated for a total of 30 cycles. At the end of the experiment, bacterial cultures were plated on LB agar plates to obtain cultures from single colonies. Three single-colony cultures were established for bacterial strain and preserved as glycerol stocks, representing the lab-evolved strains. To determine differences in bioluminescence production levels between the parental and lab-evolved strains, bioluminescence was measured two independent times with five biological replicates each time (n=10). Bacterial strains were cultured in 384-well plates in LB medium as described above (see section: "*In vivo* and *in vitro* bioluminescence measurements", page 53). To determine the genetic alterations associated with the observed changes in bioluminescence emissions following experimental evolution, genomic differences between the lab-evolved strains and their evolutionary ancestors were evaluated by variant calling analyses (VCA) using Snippy 4.6.0 (Victorian Bioinformatic Consortium, Au, https://github.com/tseemann/snippy). Detected variants were confirmed by sequencing polymerase chain reaction (PCR) products. Genomic DNA was isolated and sequenced as described above (see section: "Bacterial genomic DNA extraction and sequencing", page 54).

Bacterial RNA extraction, cDNA library preparation and RNA sequencing

Total RNA was extracted from three- to five-day-old bacterial colonies of *Photorhabdus* using the FastGene RNA Basic kit (NIPPON Genetics, JP) following manufacturer's instructions. 2-

Mercaptoethanol (Sigma-Aldrich, USA) was added at a final concentration of 1% (v/v) as a reductant in lysis buffer. Extraction was followed by a DNase I treatment step. Total RNA quality was controlled by electrophoresis on 1% (v/v) TAE-agarose gel stained with 0.005% nucleic acid gel stain (SYBR safe, Apex Bio) and DNA contamination was assessed by carrying out PCR. cDNA library was prepared from DNase I treated-RNA using the PrimeScript RT Reagent kit (Takara Bio, JP) following manufacturer's instructions. Total RNA was extracted after 10 h of growth in 5 mL of liquid LB displayed in 14 mL culture tubes from our panel of 59 *Photorhabdus* strains. The total gDNAse-treated RNA samples were sent to GENEWIZ (GENEWIZ Germany GmbH, DE) for differentially expressed genes profiling.

Phylogenetic comparative methods

To evaluate the link between bioluminescence production and evolutionary time or genes expression, we correlated bioluminescence levels and these factors. To account for the shared evolutionary history of the different strains used in this study, we statistically assessed the correlations between genes expression and bioluminescence by phylogenetic generalized least squares (PGLS) using the R packages "Ape", "nlme", and "Geiger" (**Harmon et al., 2008; Paradis et al., 2004; Pinheiro et al., 2013**). Non-phylogenetic-corrected correlations were assessed using Pearson's tests. Phylogenetic signals were calculated using Pagel's λ method with the R package "Phytools" (**Revell, 2012**). Ancestral state reconstructions were carried out using the R package "Phytools" (**Revell, 2012)**. Root-to-tip distances were calculated with the R package "adephylo" (**Jombart and Dray, 2010**). Phylogenetic trees used for the different phylogenetic comparative analyses were reconstructed based on core-genome sequences, as described above (see section: "Phylogenetic trees", page 54).

Statistical analyses

Statistic tests used to assess the different datasets are described in more detail in the figure legends. Normality and equality of variance were verified using Shapiro-Wilk and Levene's tests, respectively using Sigma Plot 14.5 (Systat Software Inc., San Jose, CA, USA). One-sample Student's t-test, Person's correlation tests, and two way-repeated measures ANOVA were conducted in R 4.3.2 (R Core Team 2023). Phylogenetically corrected correlations were statistically assessed by PGLS using the R packages "Ape", "Geiger", and "Nlme" (**Harmon et**

al., 2008; Paradis et al., 2004; Pinheiro et al., 2013). Phylogenetic signals were calculated by the Pagel's λ method using the R package "Phytools" (**Revell, 2012**).

Results

Cell density might not regulate bioluminescence production in *Photorhabdus*

To characterize bioluminescence production in *Photorhabdus* at the genus level, we used a panel of 59 strains representing all species and subspecies of the genus up to 2020, when this investigation was started (**Fig. 1 and Table S1**). We measured bioluminescence production *in vitro* using liquid cultures in LB medium. The profiles of bioluminescence production *in vitro* vary, ranging from bell-shape curves to double-peak curves or exponential phase followed by plateau (**Figs. 2 and S1**). Some strains display lag-phase bioluminescence production at low cell density, which is typical of QS control. However, it is not the case for all strains, and after reaching a maximum, usually between 18 h and 24 h, bioluminescence either enters a stationary phase or decreases. These latest profiles are inconsistent with cell density-dependent regulation of bioluminescence, suggesting that cell density might not regulate bioluminescence production in *Photorhabdus*.

Bioluminescence production displays an important intra- and inter-specific variability in *Photorhabdus*, but does not tend to disappear

In addition to *in vitro* measurements, we evaluated bioluminescence production *in vivo* by infecting *G. mellonella* larvae. Hence, we present a complete view of the levels of bioluminescence production at the *Photorhabdus* genus level (**Figs. 3 and 4**). To provide an evolutionary context, we present bioluminescence production measurements according to the phylogeny, highlighting that bioluminescence production is variable in an intra- and inter-specific manners. Strains belonging to the same species can produce highly different levels of bioluminescence, while strains that are genetically distant may display similar intensities with no discernible evolutionary pattern. It has been hypothesized that bioluminescence production is remnant of horizontal gene transfer events that did not have enough evolutionary time to disappear. To test this hypothesis, we built an ancestral state reconstruction of bioluminescence in *Photorhabdus* (**Fig. 5**). We did not detect a tendency of bioluminescence production towards a decline or an increase over evolutionary time. To strengthen our observation, we tested if there is a correlation between levels of bioluminescence production and evolutionary time (**Fig. 6**). The low coefficient of determination (R=0.09) and the high p-value (p-val=0.45) suggest that there is no correlation between these two variables. This result indicates that bioluminescence does not tend to decrease in the *Photorhabdus* genus.

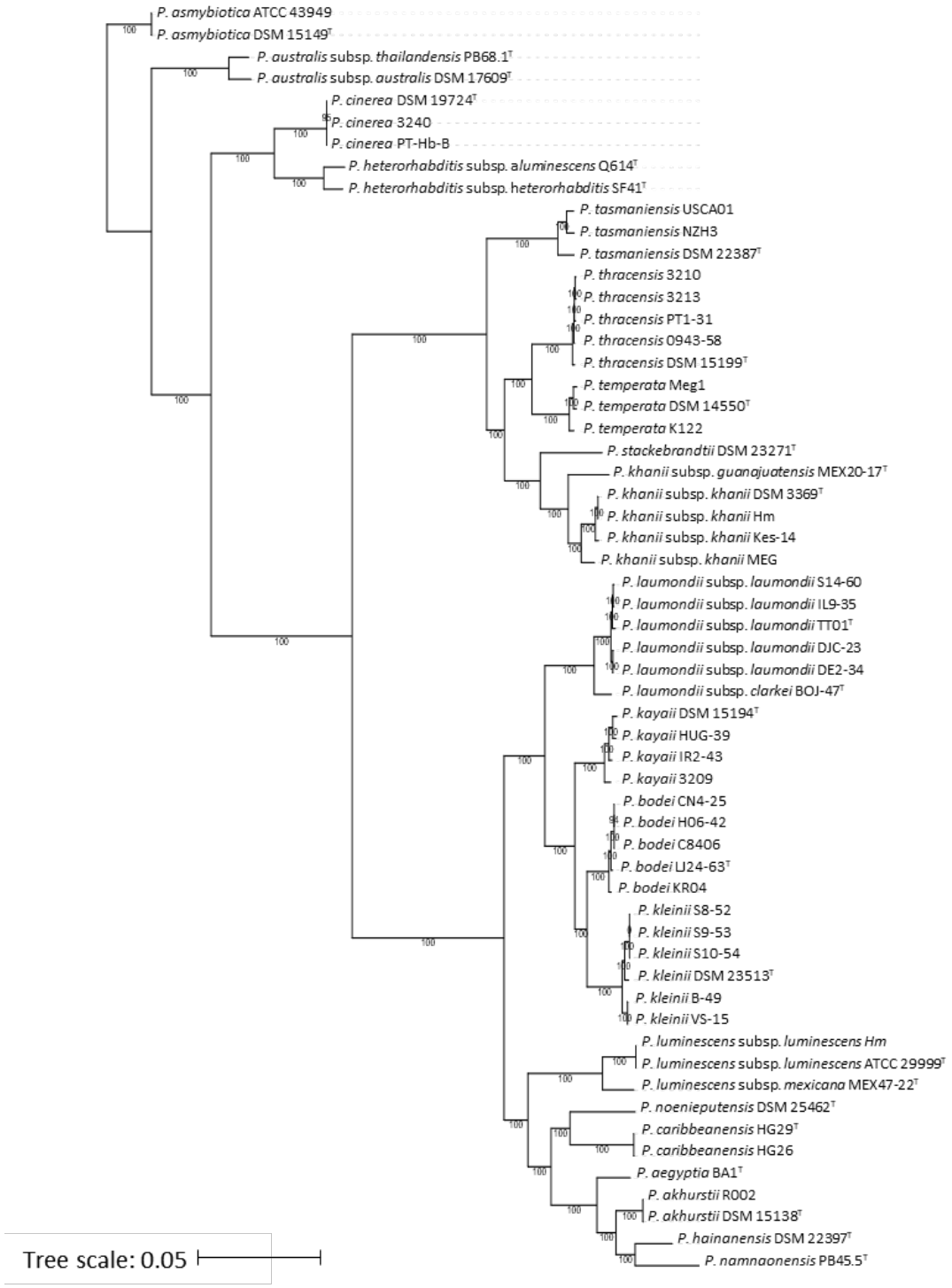

Figure 1. Bacterial strains used for this study cover all the diversity of the *Photorhabdus* genus. Maximum-likelihood phylogenomic tree based on core genome sequences of *Photorhabdus* bacterial strains used for this study. For the analysis, 2 238 990 nucleotide positions were considered. Numbers at the nodes represent bootstrap support. Bar length represents 0.05 nucleotide substitutions per sequence position. ^{T}: type strain.

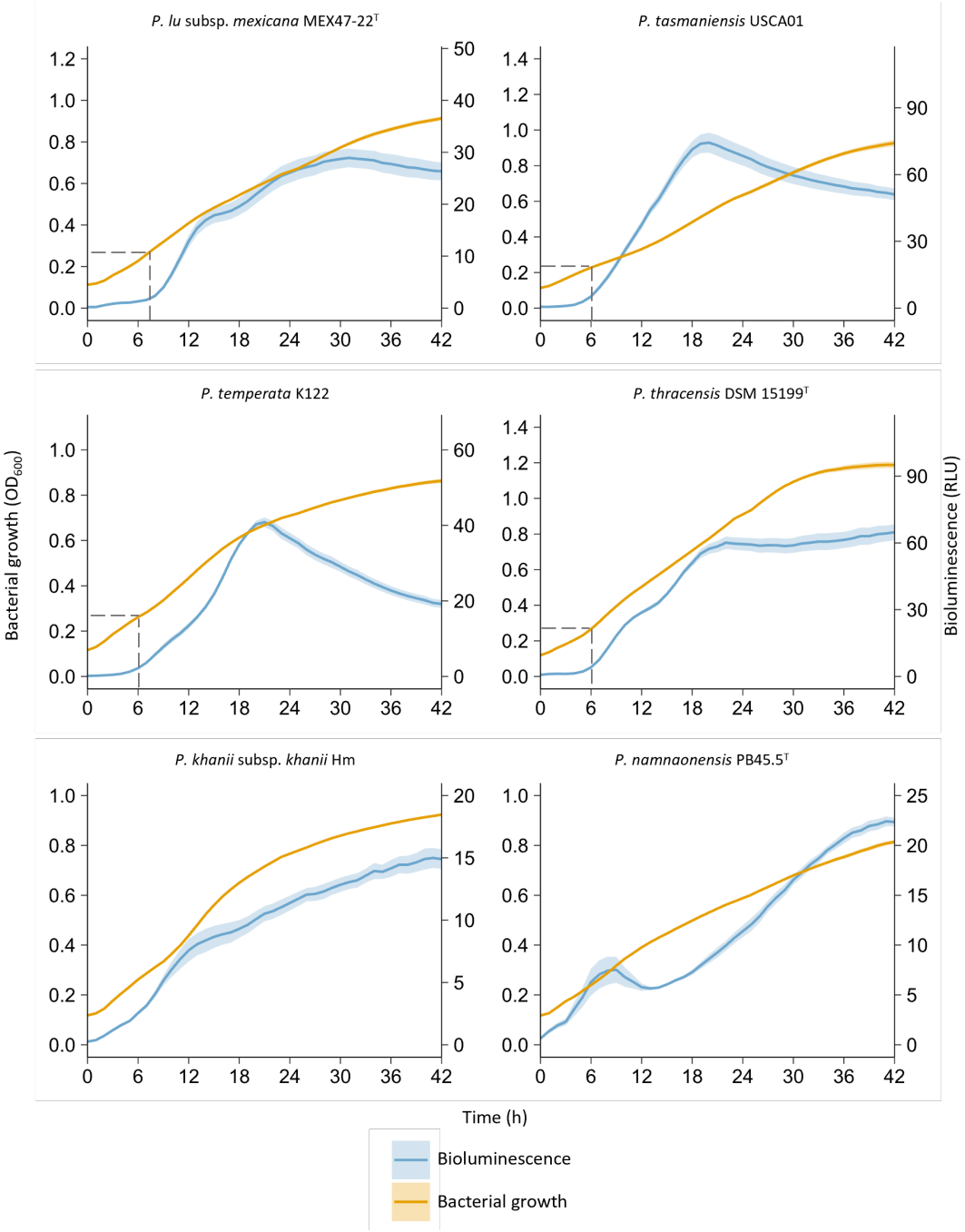

Figure 2. Bioluminescence production might not be cell density-dependent in the *Photorhabdus* genus. Kinetic curves of bioluminescence production and bacterial growth in six strains of our panel. These strains were chosen to highlight the diversity of shapes observed in bioluminescence production. A lag-phase in bioluminescence production (dashed lines) is present for the four upper strains but not for the two lower ones. Absence of lag-phase, bell-shape, double peaks and plateau do not align with the hypothese of a cell density-dependent regulation of bioluminescence production in *Photorhabdus.* Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). RLU: relative light units. ^{T}: type strain.

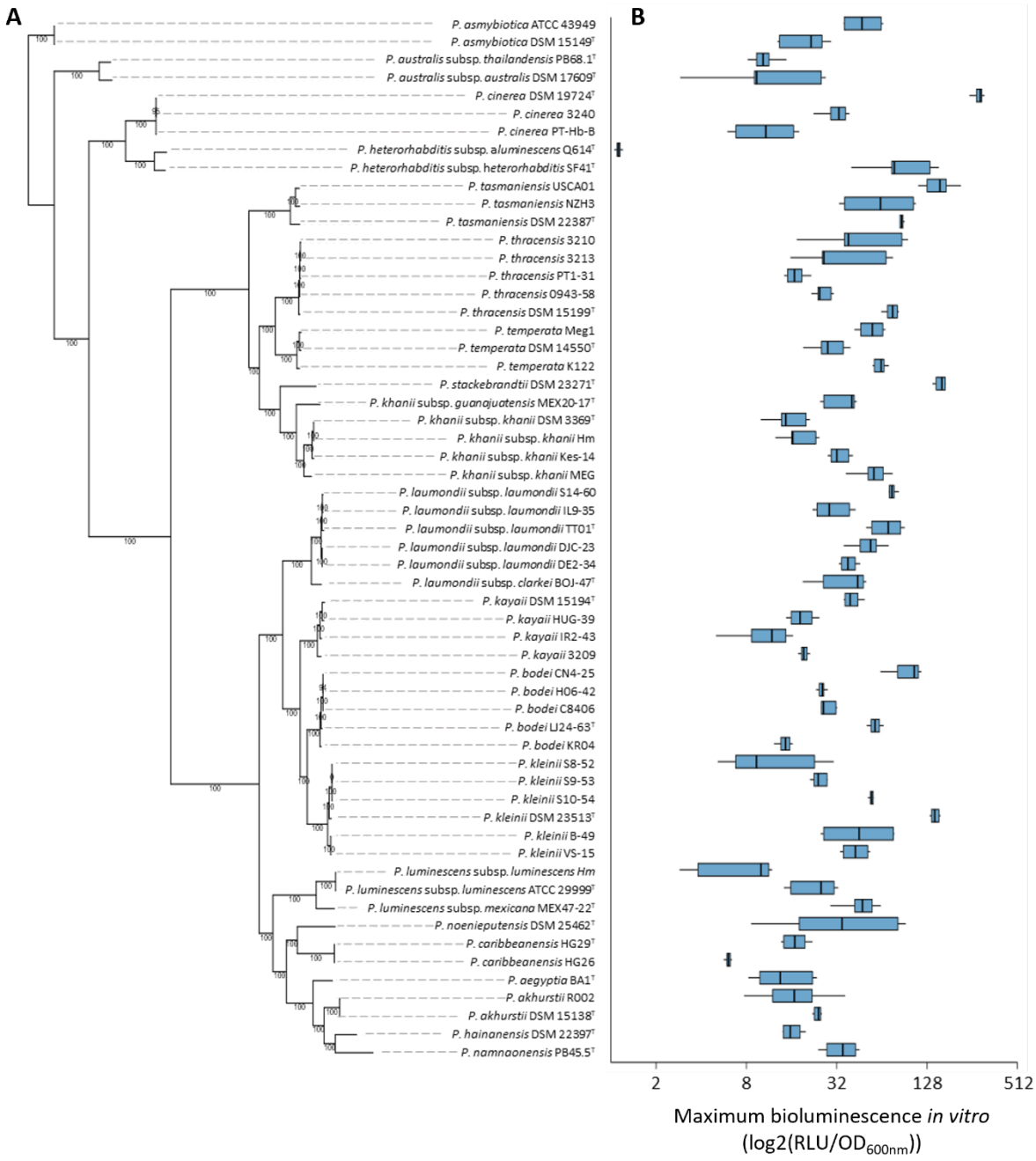

Figure 3. There is large inter- and intra-specific variability in the levels of bioluminescence produced *in vitro* in the *Photorhabdus* genus. (**A**) Core-genome phylogenomic tree of the *Photorhabdus* genus. (**B**) Maximum bioluminescence levels emitted by the different *Photorhabdus* strains *in vitro* within 44 h of bacterial growth at 28 °C in liquid LB medium. Box plots show minimum, first quartile, median, third quartile, and maximum values. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). The maximum bioluminescence is presented in log2 scale to better visualize the wide range of values. RLU: relative light units. ^{T}: type strain.

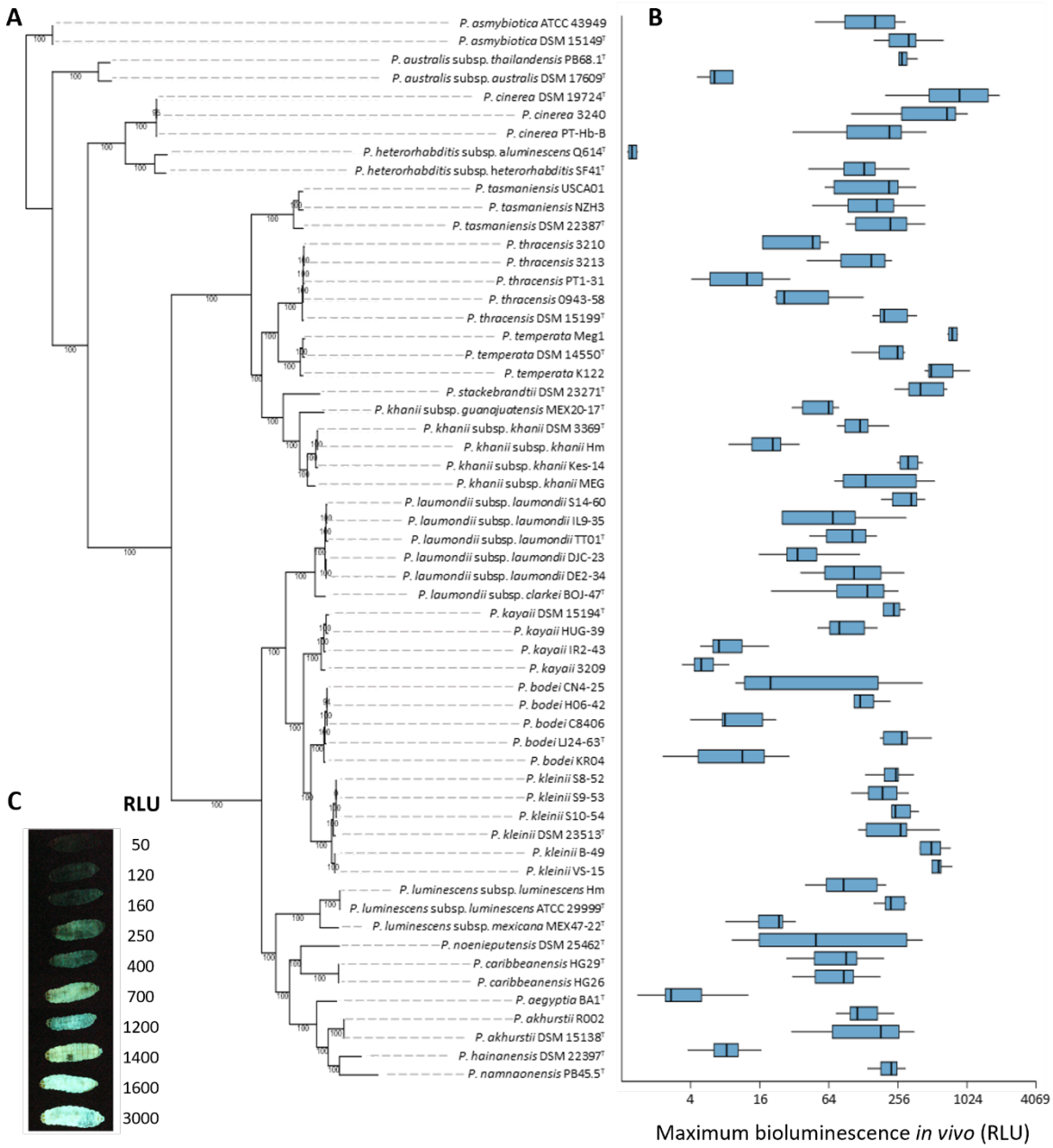

Figure 4. There is large inter- and intra-specific variability in levels of bioluminescence produced *in vivo* in the *Photorhadbus* genus. (**A**) Core-genome phylogenomic tree of the *Photorhabdus* genus. (**B**) Maximum bioluminescence levels emitted by the different *G. mellonella* larvae injected with the different *Photorhabdus strains*. Box plots show minimum, first quartile, median, third quartile, and maximum values. Bioluminescence levels were measured in three wells for each strain, and the experiments were conducted three independent times (n=9). The maximum bioluminescence is presented in log2 scale to better visualize the wide range of values. (**C**) Photographs of *G. mellonella* larvae infected with different *Photorhabdus* strains illustrating phenotypic variability in the bioluminescence emission intensities. RLU: relative light units .^{T}: type strain.

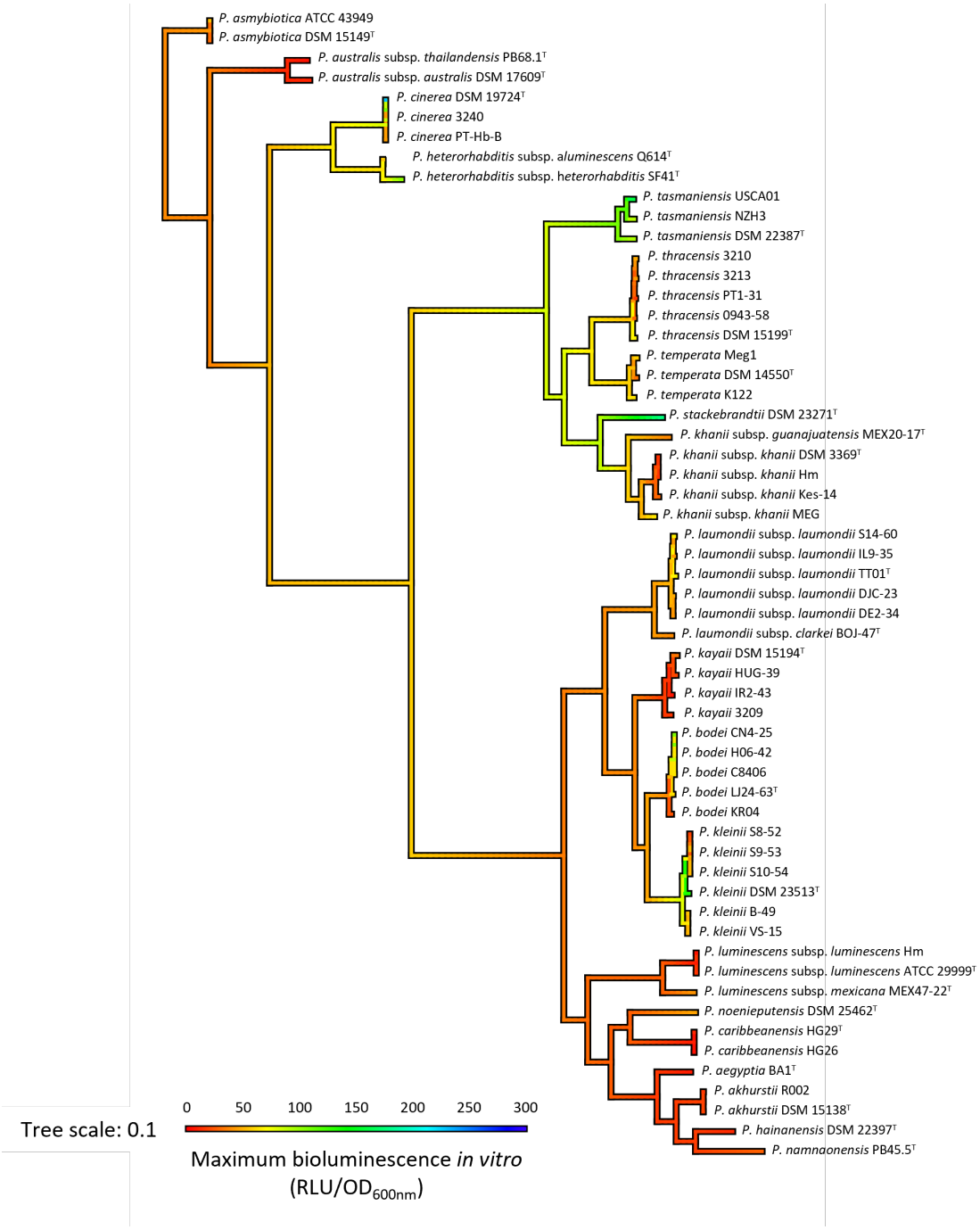

Figure 5. Ancestral state reconstruction of bioluminescence in the *Photorhabdus* genus. Ancestral state reconstruction of bioluminescence based on core genome sequences of *Photorhabdus* bacteria. For the analysis, 2 238 990 nucleotide positions were considered, and maximum bioluminescence levels emitted by the different *Photorhabdus* strains *in vitro* were used. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). Bar length represents 0.1 nucleotide substitutions per sequence position. RLU: relative light units .^{T}: type strain.

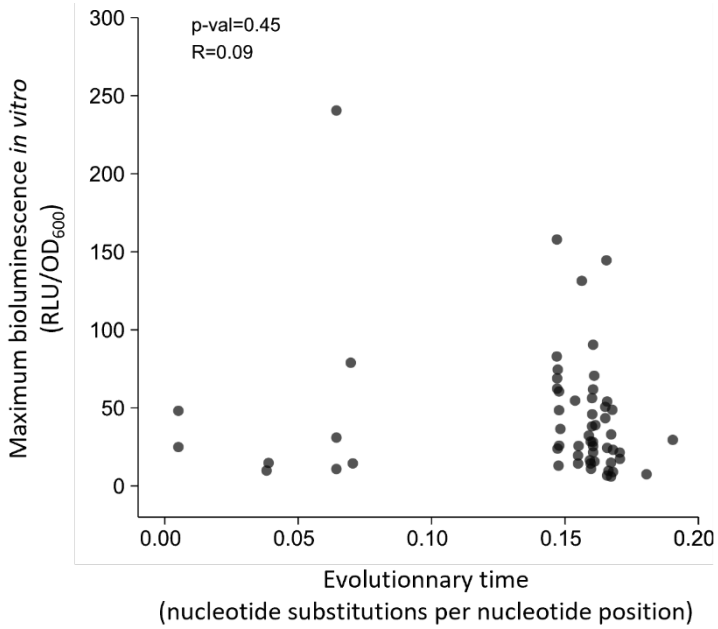

Figure 6. There is no evidence for a decline in bioluminescence production through evolution in the *Photorhabdus* genus. Correlation between maximum bioluminescence emitted by the different *Photorhabdus* strains *in vitro* and evolutionary time computed from the phylogenetic tip-to-root distances. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). Coefficient of determination (R) and Pearson p-value (p-val) are presented on the graphic. RLU: relative light units.

Photorhabdus bioluminescence is an evolutionary dynamic process

To investigate the evolutionary dynamic of bioluminescence production on a shorter timescale under laboratory conditions, we performed experimental evolution experiments (**Fig. 7**). Briefly, we grew all *Photorhabdus* strains from our panel (parental strains) in liquid LB medium, refreshing the medium every 24 h for 30 cycles to generate lab-evolved strains. We measured the changes in levels of bioluminescence produced by lab-evolved strains compared to parental strains (**Figs. 8, 9, S2, and S3**). Our results reveal that 62% of the lab-evolved strains exhibited significantly distinct levels of bioluminescence compared to their parental strains, demonstrating that this trait can rapidly evolve under laboratory conditions. These changes occurred in both directions in a strain-specific manner: bioluminescence production increased for some strains, while it decreased for others independently of the species they belong to. To exclude the possibility of form or cell type variations in lab-evolved strains, we performed a phenotypical examination of bacterial colonies (**Fig. 10**). The morphology of the colonies of lab-evolved strains exhibits typical phenotypic characteristics of the primary cell type and P-form, including the production of pigments, mucoid consistency, and opaque appearance. Taken together, these results highlight that *Photorhabdus* bioluminescence can rapidly evolve under laboratory conditions in strain-specific manner, indicating that it is an evolutionary dynamic trait, regardless of cell types and form variants.

Photorhabdus bioluminescence is modulated by a multicomponent network of genes

To identify the molecular mechanisms underpinning the changes in bioluminescence production, we selected a list of 17 lab-evolved strains that we sent for whole genome sequencing (**Table 1**). Using variant calling analysis, we identified genetic mutations in 8 of the lab-evolved strains (**Table 2**). Among the mutations observed, we could directly link only one to bioluminescence regulation. This mutation occurred in the *hexA* promoter region of the lab-evolved *P. tasmaniensis* strain USCA01 (**Fig. 11**). Other mutated genes include several two-component sensory systems signaling for environmental stress, RNA degradation regulators, vitamin biosynthesis, membrane lipopolysaccharide and peptidoglycan synthesis, and global transcriptome regulator (**Table 2**). To further explore the role of the genes mutated in lab-evolved strains, we assessed their expression levels in all the strains of our panel, and examined if we could draw correlations between their expression levels and the levels of bioluminescence production across the *Photorhabdus* genus (**Fig. 12**). Our results indicate that

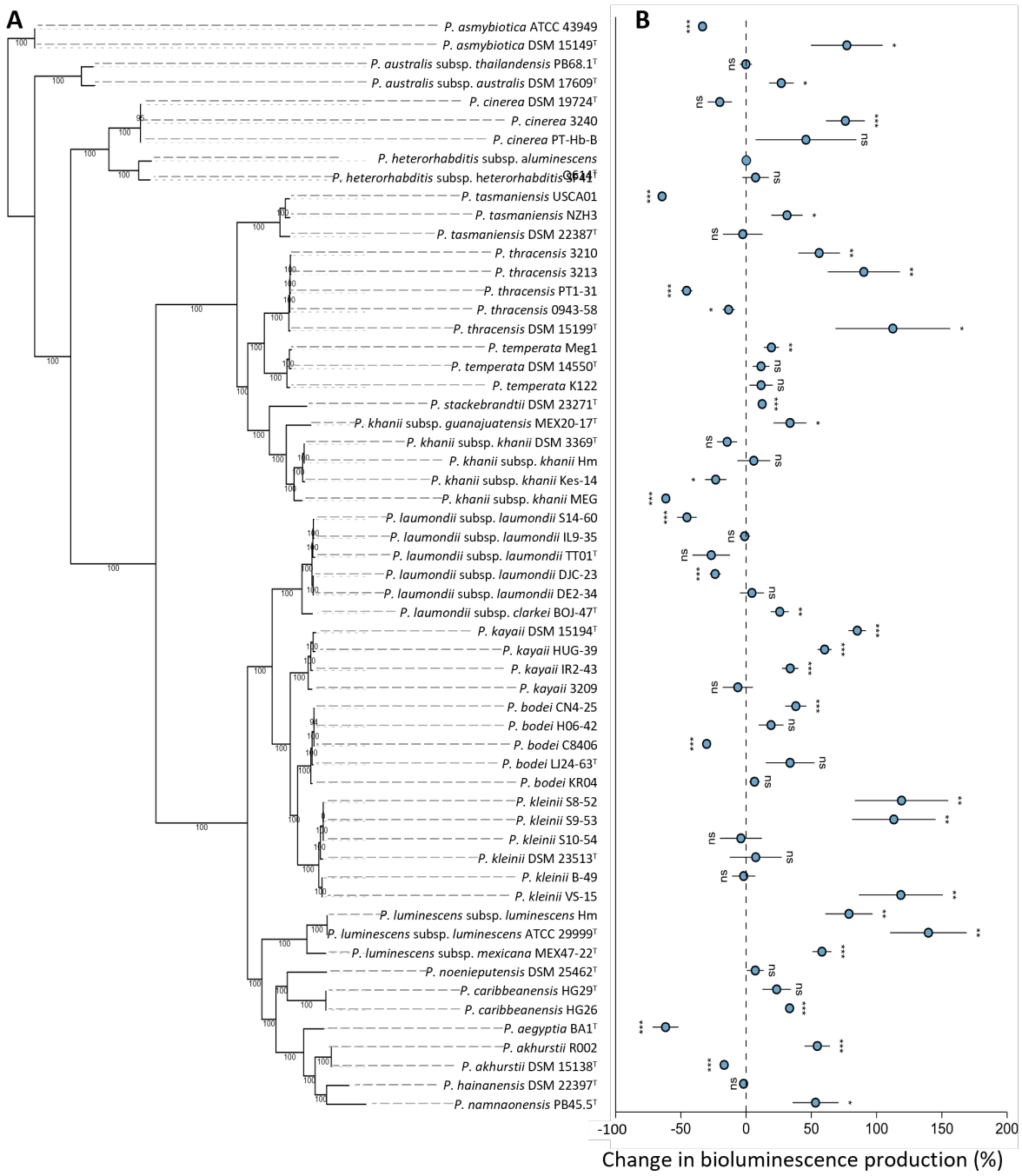

Figure 8. The intensity of bioluminescence emitted by *Photorhabdus* bacteria can rapidly evolve under laboratory conditions in a strain-specific manner. (**A**) Core-genome phylogenomic tree of the genus *Photorhabdus*. (**B**) Change in maximum bioluminescence levels emitted *in vitro* by lab-evolved *Photorhabdus* strain compared to parental strains. Positive values indicate an increase in levels of bioluminescence produced by lab-evolved strains compared to parental strains. Analogously, negative values indicate a decrease. Dots represent mean and bars represent SEM. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). Asterisks indicate that the mean value is statistically different from 0 (ns : not significant,*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ by one-sample t-test). ^{T}: type strain.

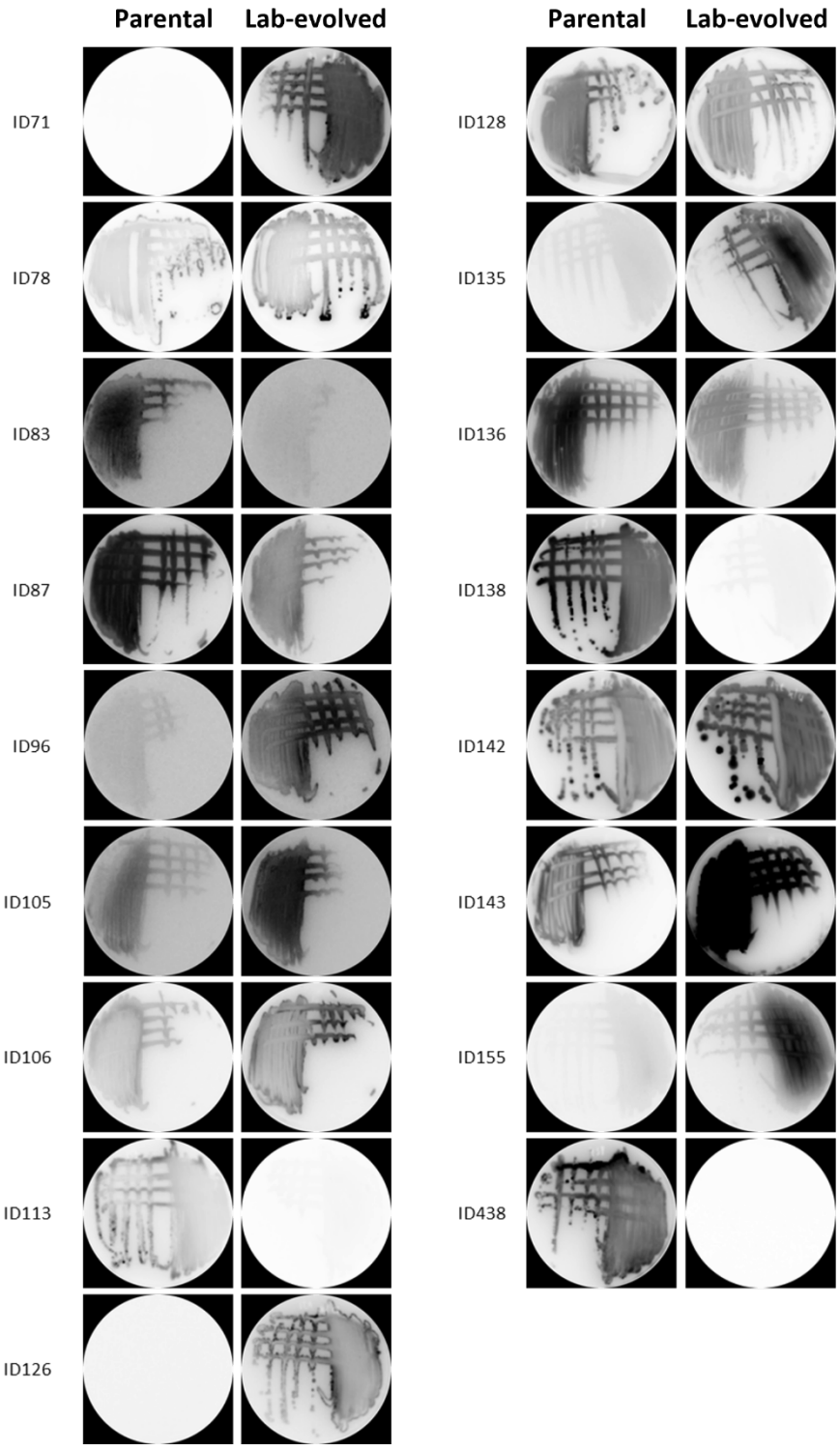

Figure 9. Bioluminescence production rapidly evolves under laboratory conditions in a strain-specific manner. Top-down greyscale photographs of 2- to 3-day-old *Photorhabdus* colonies grown on LB-agar medium. Darker colors indicate higher bioluminescence levels. To facilitate the reading, ID numbers are written instead of the full names of the strains. The correspondence between ID numbers and full names is presented in Table 1. Photographs were made using an Amersham Imager 600 (Cytiva, US). Picture colours were scaled to the minimum and maximum camera counts per pixel using ImageJ.

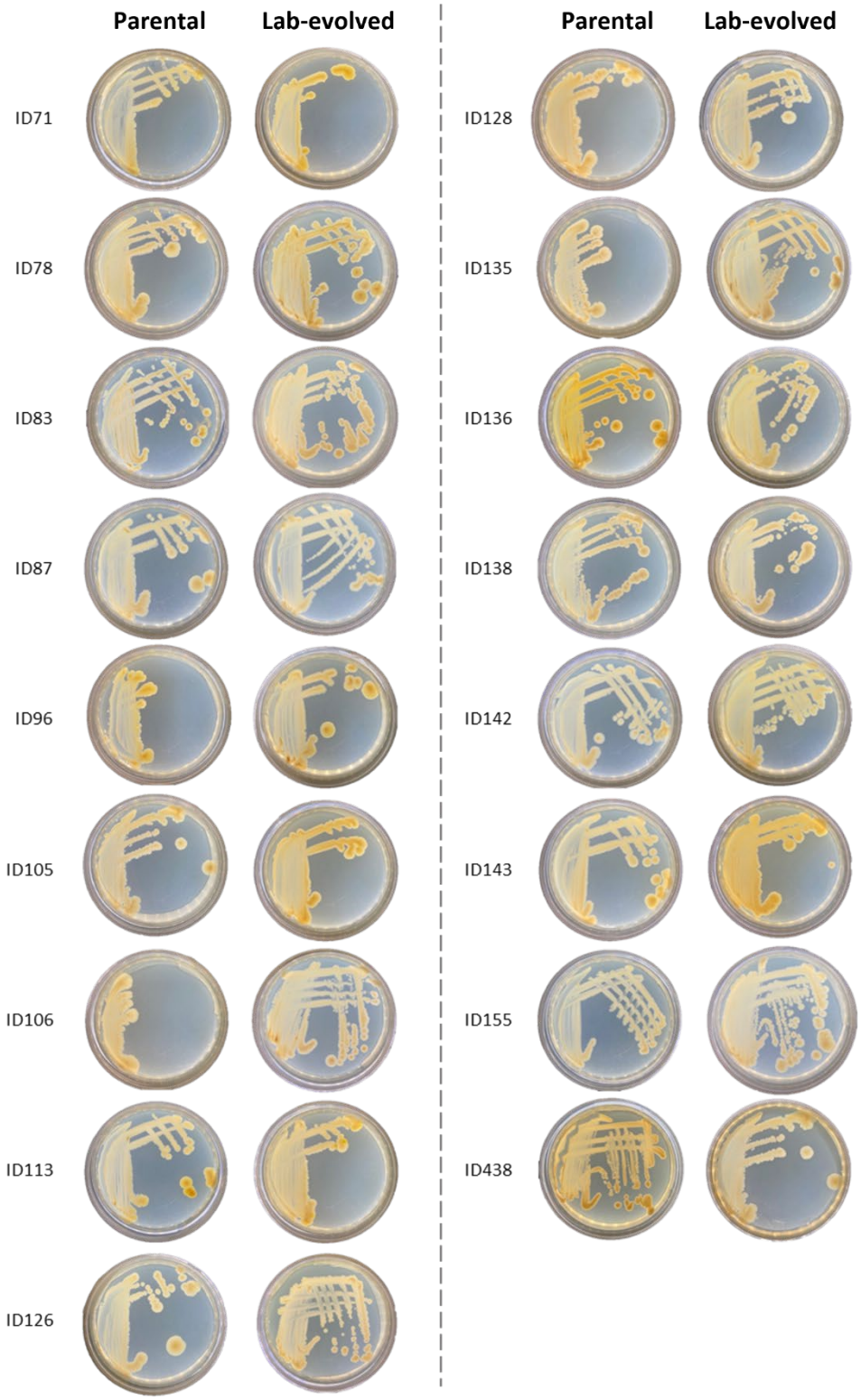

Figure 10. Lab-evolved strains and parental strains exhibit typical primary cell and P-form phenotypes. Pictures of 5- to 7-day-old *Photorhabdus* colonies grown on LB-agar medium were taken with a white light source placed under the Petri dishes. No phenotypical differences can be observed between lab-evolved strains compared to their relative parental strains. To facilitate the reading, ID numbers are written instead of the full names of the strains. The correspondence between ID numbers and full names is presented in Table 1.

Table 1. Description of the changes in the levels of bioluminescence produced by lab-evolved strains compared to parental strains. Only the strains that have been sent for genomic DNA sequencing are presented. ^{T}: type strain.

ID	Strain	Bioluminescence after selection
71	*P. kleinii* VS-15	Increase
78	*P. luminescens* subsp. *mexicana* MEX47-22^{T}	Increase
83	*P. bodei* KR04	Decrease
87	*P. thracensis* PT1-31	Decrease
96	*P. kayaii* IR2-43	Decrease
105	*P. kleinii* S8-52	Increase
106	*P. kleinii* S9-53	Increase
113	*P. laumondii* subsp. *laumondii* S14-60	Decrease
126	*P. luminescens* subsp. *luminescens* Hm	Increase
128	*P. namnaonensis* PB45.5^{T}	Decrease
135	*P. cinerea* 3240	Increase
136	*P. khanii* subsp. *khanii* MEG	Decrease
138	*P. tasmaniensis* USCA01	Decrease
142	*P. thracensis* 3210	Increase
143	*P. thracensis* 3213	Increase
155	*P. kayaii* DSM 15194^{T}	Increase
438	*P. laumondii* subsp. *laumondii* DJC-23	Decrease

Table 2. Description of the genetic mutations that occurred during the experimental evolution experiments. Mutations occurred in regulating and coding sequences of genes involved in diverse functions. Most of the genes mutated cannot be directly linked to bioluminescence production according to currently available literature. ^{T}: type strain.

ID	Strain	Type of mutation	Gene	Putative function	Change in bioluminescence	Reference
125	*P. asymbiotica* ATCC 43949	Stop gain(c.792C>A;p.Tyr264)	Sensor histidine kinase (rcsC)	Component of the Rcs signaling system	Increase	Stout and Gottesman, 1990
96	*P. kayaii* IR2-43	SNP (c.658C>T;p.220His>Tyr)	Cell shape-determining protein (mreB)	Cell shape maintenance	Increase	Doi et al., 1988
105	*P. kleinii* S8-52	Stop gain (c.1177G>T;p393Glu>*)	Poly(A) polymerase I (pcnB)	Global control of gene expression	Increase	Coa and Sarkar, 1992; Raynal and Carpousis, 1999
71	*P. kleinii* VS-15	SNP (c.323C>T;p108Ser>Phe)	Sensir histidine kinase (cpxA)	Histidine kinase member of the two-component regulatory system CpxA/CpxR	Increase	Danese et al., 1995; Yamamoto et al., 2006
136	*P. khanii* subsp. *khanii* MEG	Ins(c.-406insG of thiD gene) and/or	Hydroxymethyl pyrimidine kinase (thiD)	Thiamine (vitamin B1) biosynthesis	Increase	Mizote et al., 1999
		Ins (c.-640insG of pagP gene)	Lipid IV (A) palmitoyl transferase (pagP)	Lipopolysaccharides modification in the outer membrane		Bishop et al., 2000
100	*P. luminescens luminescens* Hm	SNP (c.680G>A;p227Ser>Asn)	Sensory histidine kinase/phosphatase (ntrB)	Regulatory systeme controlling the expression of nitrogenregulated genes	Increase	Weiss et al., 2002
157	*P. thracensis* DSM 15199^{T}	Stop gain (c.1114C>T;p372Gln>*)	Penicillin-binding protein activator (lpoA)	Regulator of peptidoglycan synthesis	Increase	Typas et al., 2010
138	*P. tasmaniensis* USCA01	SNP (c.45C>G of hexA gene)	Promoter region of hexA gene	Global transcriptional regulator repressing secondary metabolism and bioluminescence	Decrease	Joyce and Clarke, 2003; Langer et al., 2017

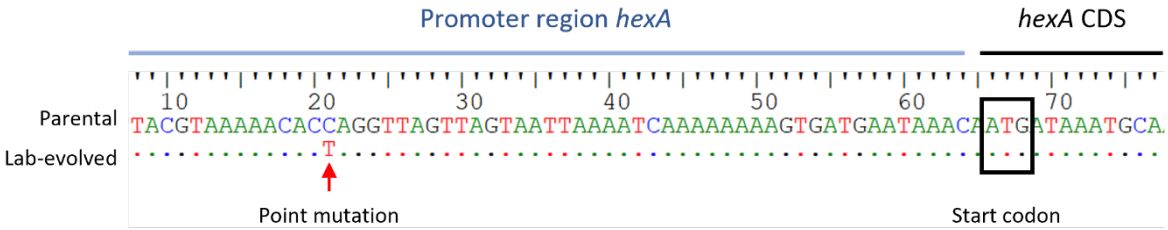

Figure 11. Point mutation in the promoter region of the gene *hexA* is responsible for the decrease in bioluminescence production in the lab-evolved strain *P. tasmaniensis* USCA01. A point mutation (cytosine replaced by adenosine) has been detected thanks to variant calling analysis in the promoter region of the gene *hexA* coding for a well-known global regulator involved in bioluminescence regulation in primary/secondary cell types and P-form/M-form.

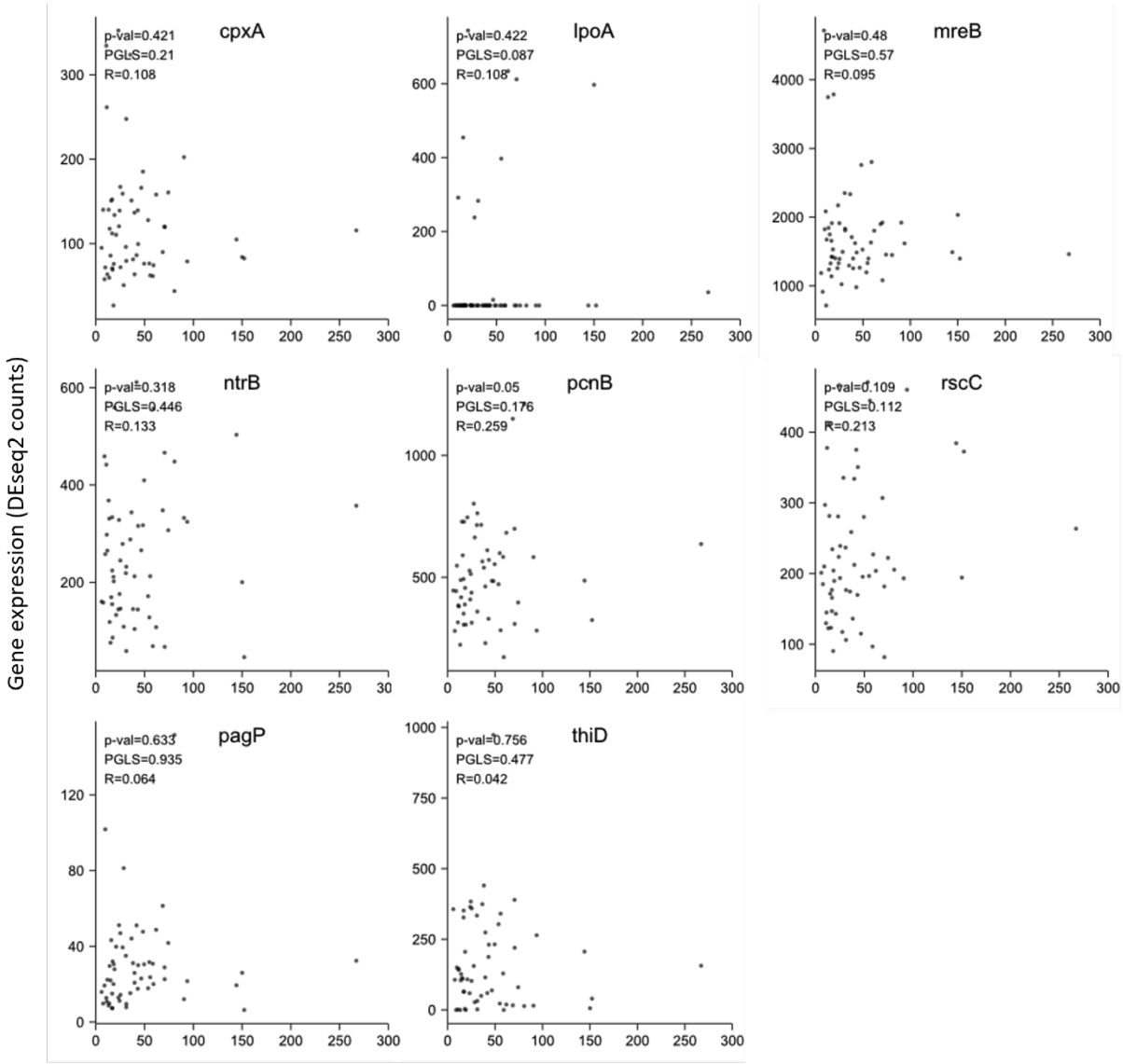

Figure 12. There are no correlations between the transcription levels of the genes mutated during the experimental evolution experiments and the levels of bioluminescence across the *Photorhabdus* genus. The scatter plots illustrate the relationship between transcription levels of genes mutated during experimental evolution and the levels of bioluminescence produced. Each data point corresponds to a *Photorhabdus* strain. Phylogenetically corrected correlations were statistically assessed by PGLS. Non-phylogenetically corrected correlations were statistically assessed by Pearson's test. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). RLU: relative light units.

there is no correlation between these two variables. Then, we focused on the expression levels of genes described in the literature as modulators of bioluminescence production (**Fig. 13**). We could establish significant correlations for *lux*-operon genes, but not for regulators such as *hexA* and *hfq*. We conclude that differences in expression levels of those regulators do not explain the intra- and inter-specific variabilities in the levels of bioluminescence production that we observe.

Lux-operon genes are the central elements of bioluminescence production. We speculated that the genetic structure of these genes might explain the variability in the levels of bioluminescence production across the *Photorhabdus* genus. To test this hypothesis, we computed the pairwise differences in nucleic acid sequences of the *lux*-operon genes. For each strain of our panel, we compared its *lux*-operon sequence with the sequences of the 58 other strains. Pairwise difference values were normalized to range between 0 and 1. Values close to 0 indicate that *lux*-operon sequences are almost identical, whereas values close to 1 indicate sequences with a lot of differences. We also calculated pairwise differences in maximum bioluminescence produced for all strains and built a scatter plot from these data (**Fig. 14**). We do not detect any tendency suggesting that differences in *lux*-operon sequences are correlated with differences in levels of bioluminescence production. Interestingly, our results highlight that strains with almost identical *lux*-operon sequences can produce very different levels of bioluminescence, whereas stains with different *lux*-operon can produce similar levels. This suggests that the variability in the levels of bioluminescence produced in the *Photorhabdus* genus is probably not caused by differences in the nucleic acid sequences of the *lux*-operon. These findings need to be taken carefully, as minor genetic alterations can result in significant phenotypical changes. A single point mutation in the coding sequence of a given gene can induce a structural modification in an essential domain of the encoded protein.

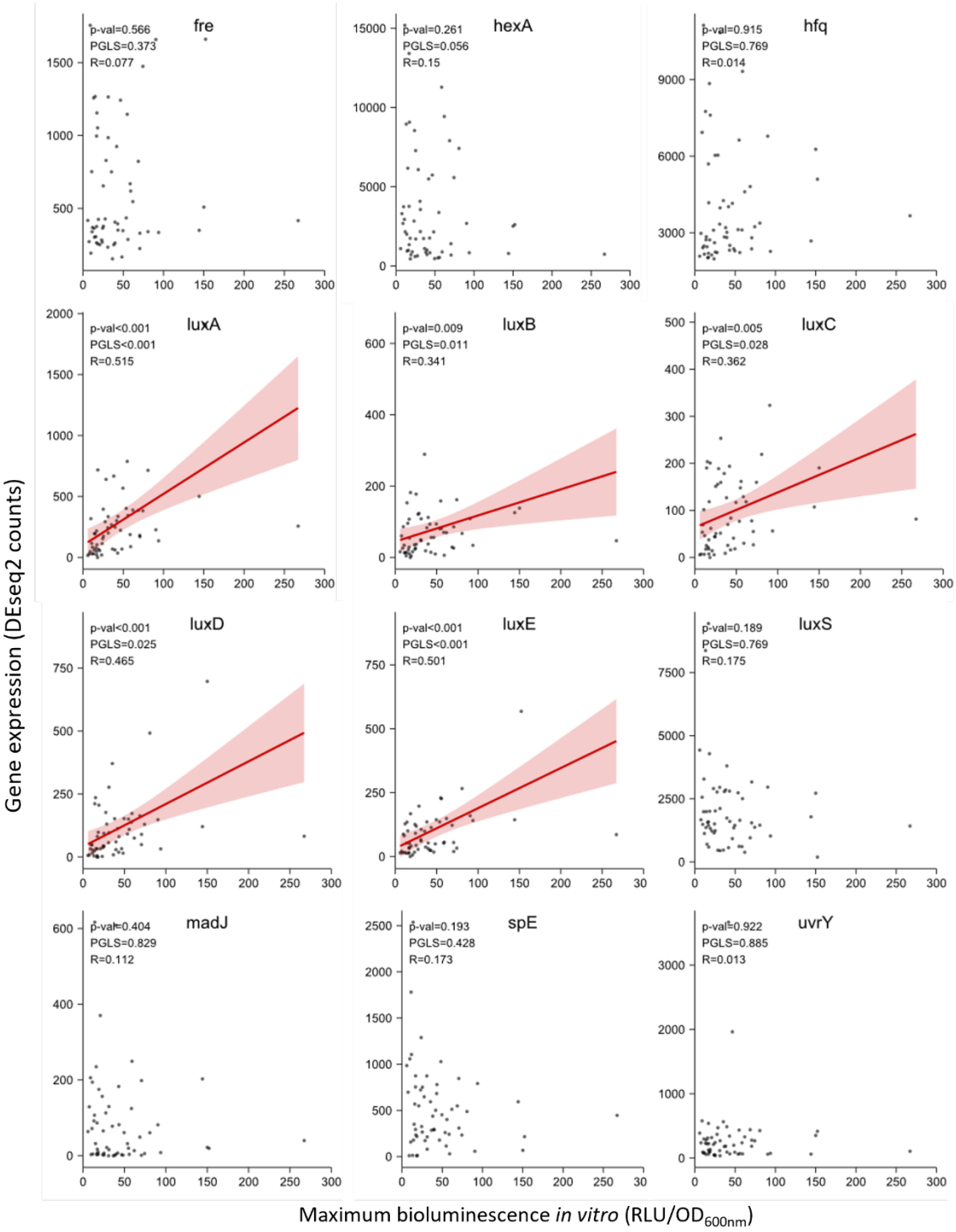

Figure 13. Expressions of *lux*-operon genes are correlated to the levels of bioluminescence produced in the *Photorhabdus* genus. The scatter plots illustrate the relationship between the levels of bioluminescence produced and the transcription levels of a set of genes that have been described as having a role in bioluminescence regulation. Each data point corresponds to a *Photorhabdus* strain. Phylogenetically corrected correlations were statistically assessed by PGLS. Non-phylogenetically corrected correlations were statistically assessed by Pearson's test. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). RLU: relative light units.

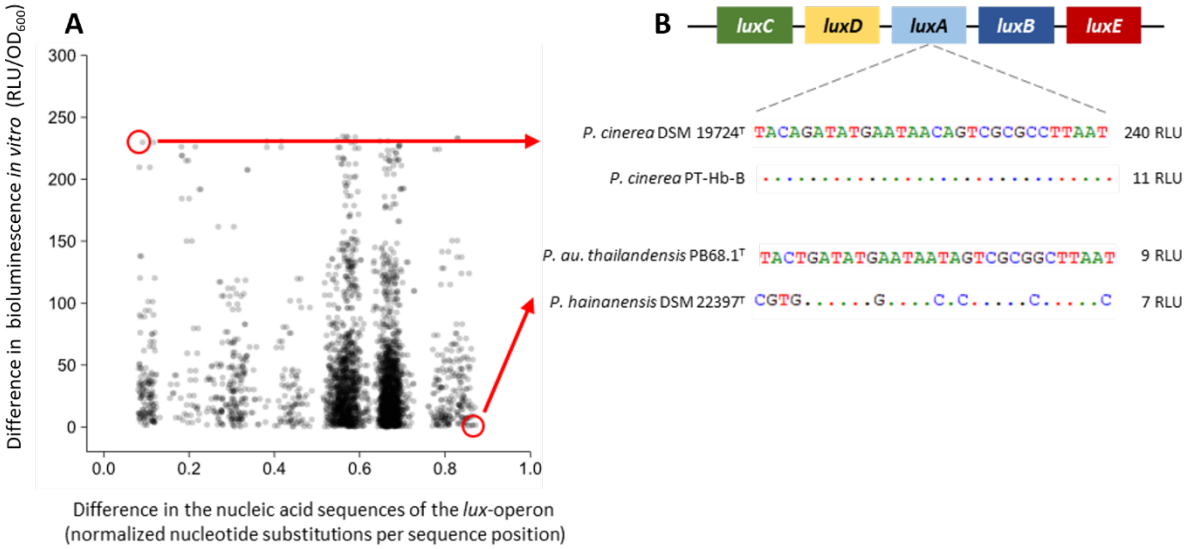

Figure 14. The variability in bioluminescence levels across the *Photorhabdus* genus appears to be unrelated to differences in the nucleic acid sequences of the lux-operon genes. (**A**) Scatter plot representing the correlation between the pairwise differences in the nucleic acid sequences of the *lux*-operon and the pairwise differences in the maximum levels of bioluminescence produced by each strain. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20) The pairwise differences in nucleic acid sequences represent the genetic divergence between two sequences in terms of substitutions per site. The values were normalized to range between 0 and 1. Values close to 0 indicate that the two sequences compared are highly similar, while values close to 1 indicate many differences. Red circles highlight two pairwise comparisons that are detailed in (**B**). Comparison of a portion of *luxA* sequence from *P. cinerea* DSM 19724^{T} and *P. cinerea* PT-Hb-B. Those two strains have highly similar *lux*-operon sequences but produce drastically different levels of bioluminescence. The same comparison made between *P. australis* subsp. *thailandensis* PB68.1^{T} and *P. hainanensis* DSM 22397^{T}, two strains that are genetically distant but produce similar levels of bioluminescence. RLU: relative light units .^{T}: type strain.

Discussion

Photorhabdus are the only known bioluminescent bacteria in terrestrial ecosystems. While the molecular mechanisms responsible for bioluminescence production in *Photorhabdus* are known, the evolution and regulation of this process remains poorly understood. It is often assumed that bacterial bioluminescence is under QS control, as it is in *V. campbellii* and *V. fischeri*, two historical model species (**Farghaly, 1950; Kempner and Hanson, 1968**). We asked the question whether it is also the case in *Photorhabdus.* Regulation of bioluminescence production by QS results in bioluminescence kinetic curves displaying a typical lag-phase. This lag-phase corresponds to the time it takes for the bacterial population to reach a threshold in cell density before producing bioluminescence. We characterized the growth and bioluminescence production in the *Photorhabdus* genus using a panel of 59 strains representing all species and subspecies of the genus up to 2020, when this investigation was started. We observed lag-phases for some strains of our panel, but overall, the profiles we obtained are inconsistent with cell density-dependent regulation of bioluminescence. While our observations suggest that in *Photorhabdus*, bioluminescence production might not be regulated by cell density, we acknowledge that more experimental evidence is required to confirm or refute this hypothesis. In addition, our results highlight that although strains from the same species or subspecies can exhibit similar levels of bioluminescence, this is not always the case. This phenomenon is exemplified in *P. cinerea* which is composed of the strain DSM 19724^{T}, one of the highest bioluminescence producers, and the strain PT-Hb-B a low bioluminescence producer and illustrate the important intra-specific variability in bioluminescence production across the *Photorhabdus* genus.

Previous studies have suggested that *Photorhabdus* bioluminescence is subject to disappearance over evolutionary time (**Peat et al., 2010**). However, considering the prevalence of this trait across the genus, we hypothesize that *Photorhabdus* bioluminescence is not disappearing. We investigated the evolutionary trajectory of *Photorhabdus* bioluminescence at both extended timescales using phylogenetic analysis and shorter timescales under laboratory conditions through experimental evolution. We built an ancestral state reconstruction of bioluminescence and investigated a putative correlation between the levels of bioluminescence production and evolutionary time in the genus. We did not detect a significant correlation between the intensities of bioluminescence and evolutionary time

across the *Photorhabdus* genus (p-val=0.45). Overall, we did not detect any tendency towards a decrease in bioluminescence production over extended timescales. This result, along with the observation that none of the more derived *Photorhabdus* strains are aluminescent, provides little support to the hypothesis of Peat et al., and shows that the intensity of bioluminescence has not decreased and this trait is not disappearing through speciation events in the *Photorhabdus* genus.

Applying an experimental evolution approach, we demonstrate that *Photorhabdus* bioluminescence production can rapidly evolve under laboratory settings in strain-specific manner, highlighting the dynamic nature of this trait under these conditions. The transition of form or cell type could not explain the observed changes in bioluminescence levels, as lab-evolved strains conserved typical phenotypic characteristics of the primary cell type and P-form. It is important to note that these experiments were performed in liquid cultures under conditions optimal for *Photorhabdus* bacterial growth *in vitro* (liquid LB at 28°C with constant agitation at 180 rpm in darkness). These controlled conditions allowed us to track the mutations acquired by *Photorhabdus* bacteria affecting their bioluminescence production. Through this approach, we obtained several mutants with altered bioluminescence production and were able to identify the genes involved. Whether mutations in the same genes will occur under natural conditions during the course of evolution is unknown, but possible.

Currently, our understanding on the regulation of *Photorhabdus* bioluminescence is limited. Most of the knowledge on this subject is derived from studies focusing on transcriptional changes in the different cell types and form variants in the model species *Photorhabdus laumondii* subsp. *laumondii* (**Eckstein et al., 2019; Langer et al., 2017; Neubacher et al., 2020; Somvanshi et al., 2012**). While the global regulators HexA and Hfq have been proposed as regulators of bioluminescence in *Photorhabdus*, we posit that these regulators alone may not fully account for the observed variability in bioluminescence levels across the *Photorhabdus* genus. We employed genomic tools to identify the mutated genes responsible for the changes in the levels of bioluminescence production through our experimental evolution experiments. Only one out of the eight mutated genes could be directly linked to bioluminescence regulation, while others display a wide range of functions, from environmental stress

responses to vitamin synthesis or cell shape maintenance (**Bishop et al., 2000; Cao and Sarkar, 1992; Danese et al., 1995; Doi et al., 1988; Mizote et al., 1990; Raynal and Carpousis, 1999; Stout and Gottesmanm 1990; Typas et al., 2010; Weiss et al., 2002; Yamamoto et al., 2006**). We used a global transcriptomic approach to investigate the role of the genes identified in our experimental evolution experiments and the role of regulators described in the literature across the *Photorhabdus* genus. We could not draw significant correlations between the expression levels of these genes or regulators and levels of bioluminescence production. These results suggest that the diversity in levels of bioluminescence produced across the genus is not solely caused by differences in the expression levels of these genes or regulators. Taken together, these results suggest that numerous regulatory pathways, not restricted to the regulators described in the literature, interplay to modulate bioluminescence production. Based on our results and on currently available literature, we propose a simplified network of *Photorhabdus* bioluminescence regulation in **Fig. 15**.

The expression levels of *lux*-operon genes are directly correlated to the levels of bioluminescence production across the *Photorhabdus* strain, which confirms that this operon is the central element of this process. We investigated if differences in the nucleic acid sequence of *lux*-operon genes could be responsible for the diversity in levels of bioluminescence production in *Photorhabdus*. Comparing the *lux*-operon gene sequences and levels of bioluminescence pairwise for each strain, we did not detect a correlation between these two factors. We hypothesize that the diversity of bioluminescence observed is not due to changes in the sequences of *lux*-operon genes. It is important to note that changes in the nucleic acid sequence of *lux*-operon genes can lead to change in bioluminescence production, such as a shift in the wavelength of light emitted (**Cline, 1974**). The three-dimensional resolution of the luciferase from *V. campbellii* has enabled the identification of important domains for the functioning of this protein (**Fisher, 1995**). It has been shown that a single point mutation in the luciferase of *V. campbellii* could lead to a wavelength shift in the bioluminescence produced (**Yen-Cheng 2004**). This mutation occurred in an amino acid critical for the molecular interaction between luciferase and flavin mononucleide. A second mutation in another important domain of luciferase of *V. campbellii* led to a severe reduction in the level of bioluminescence produced. These findings reveal that a little genetic change in the *lux*-operon gene can lead to important changes in bioluminescence production.

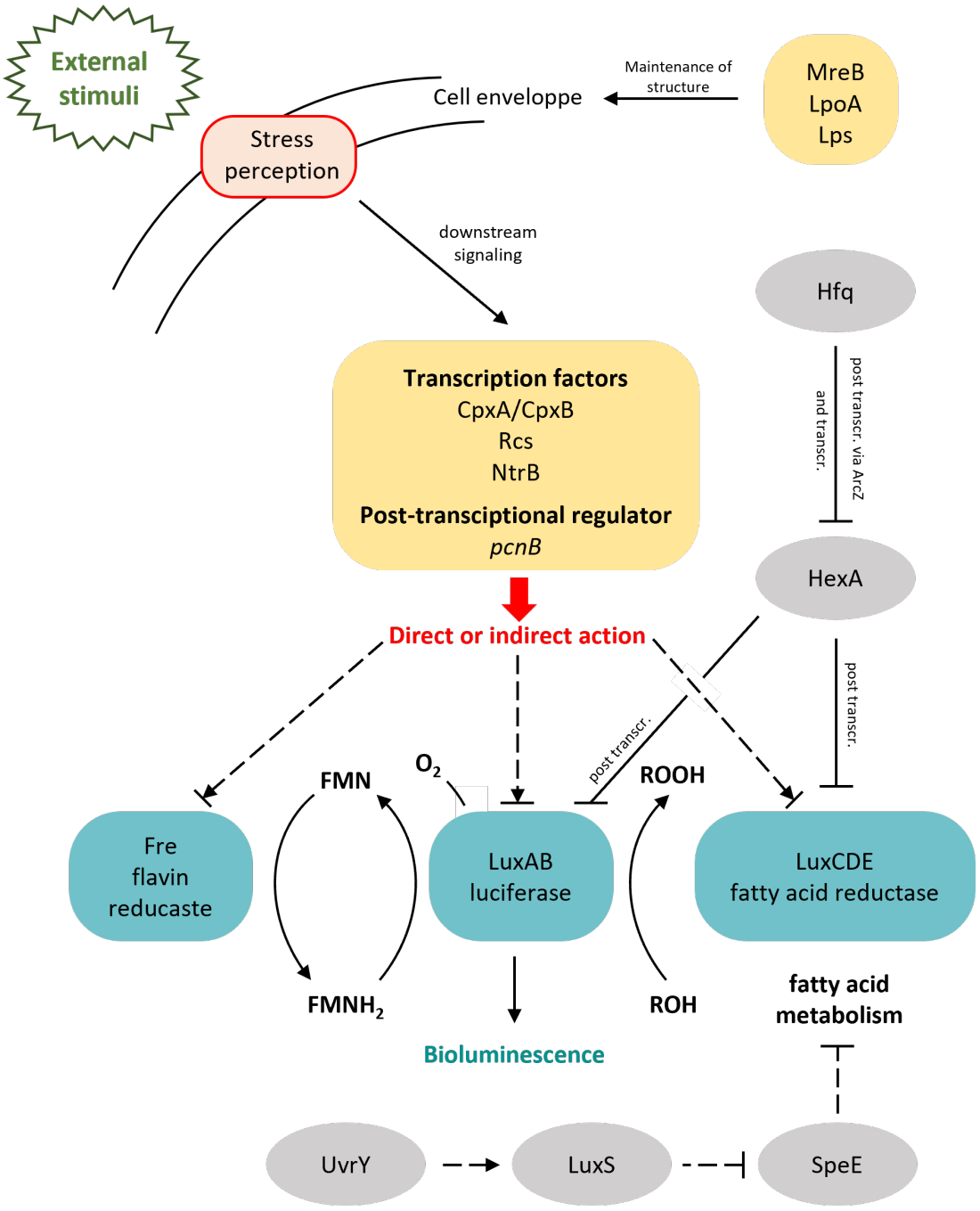

Figure 15. Proposed model for the regulation of *Photorhabdus* bioluminescence regulation. The model incorporates results from this work and information from the literature. The heterodimeric luciferase (LuxAB) catalyzed the redox reaction using long chain fatty aldehyde (ROH), reduced flavin mononuclease ($FMNH_2$) and dioxygene (O_2) as substrates. This redox reaction produces energy in the form of light. This reaction is tightly controlled by a multicomponent network of genes, not limited to the global regulators HexA and Hfq. The genes identified in this work are presented in yellow boxes. Arrows represent induction, horizontal lines represent repression, arrows with horizontal line represent regulation without specification, and dashed lines indicate hypothetical regulation.

Conclusions

The aim of our study is to investigate the regulation and evolution of bioluminescence production in the *Photorhabdus* genus. The assessment of bioluminescence production in the 59 strains of our panel reveals that this trait displays important intra- and inter-specific variabilities across the genus. Using both phylogenetic and an experimental evolution approaches we are able to show that bioluminescence is an evolutionary dynamic trait that can rapidly evolve under laboratory conditions in a strain-specific manner but that has not tendency to disappear over extended evolutionary time. Our genomic and transcriptomic analyses reveal that in *Photorhabdus*, bioluminescence production is tightly controlled by a multicomponent network of genes, not restricted to the regulators already described in the literature. Taken together, our work contributes to deepen our knowledge about the singular ability of *Photorhabdus* bacteria to produce bioluminescence belowground.

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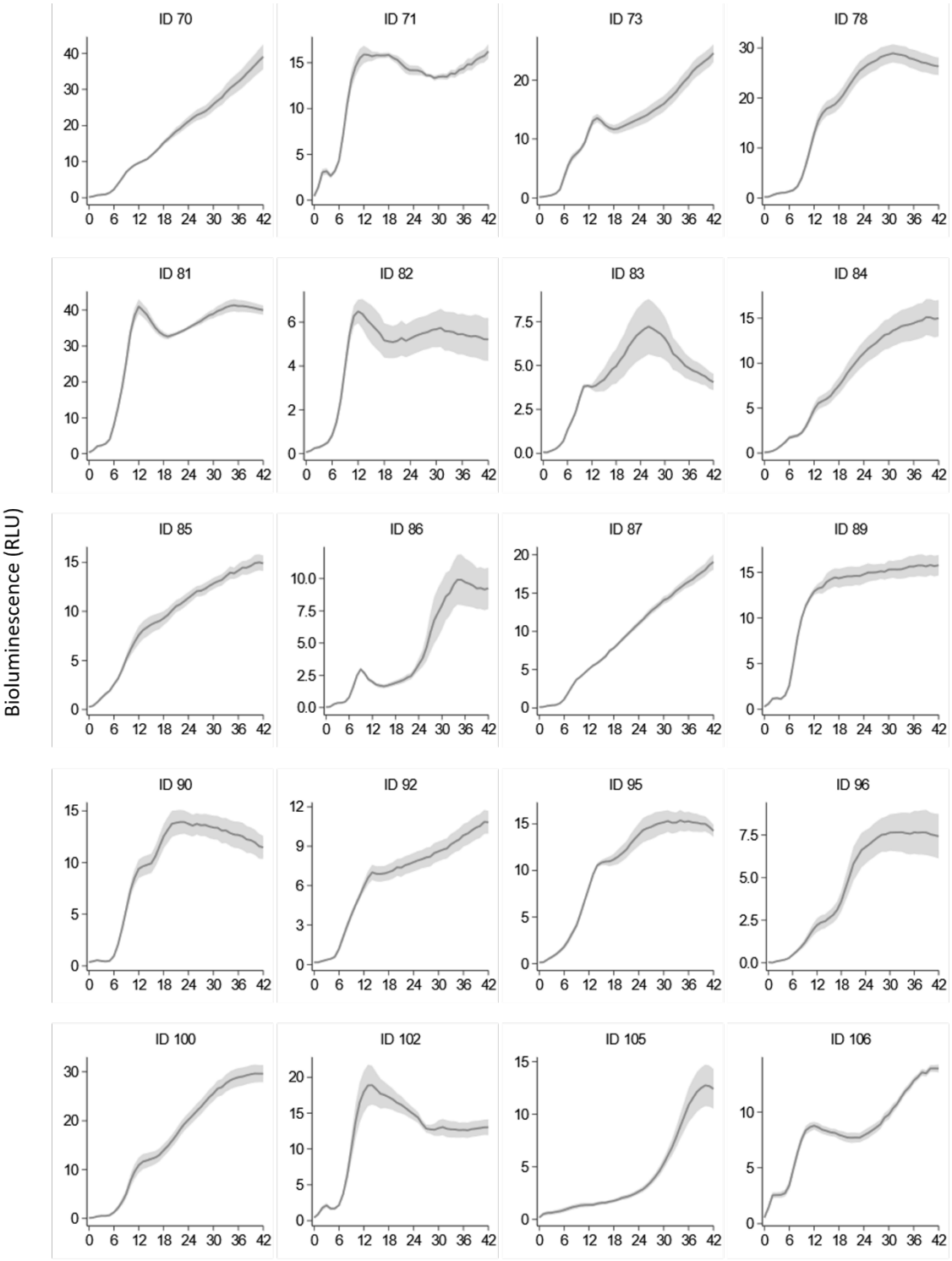
Supplementary materials



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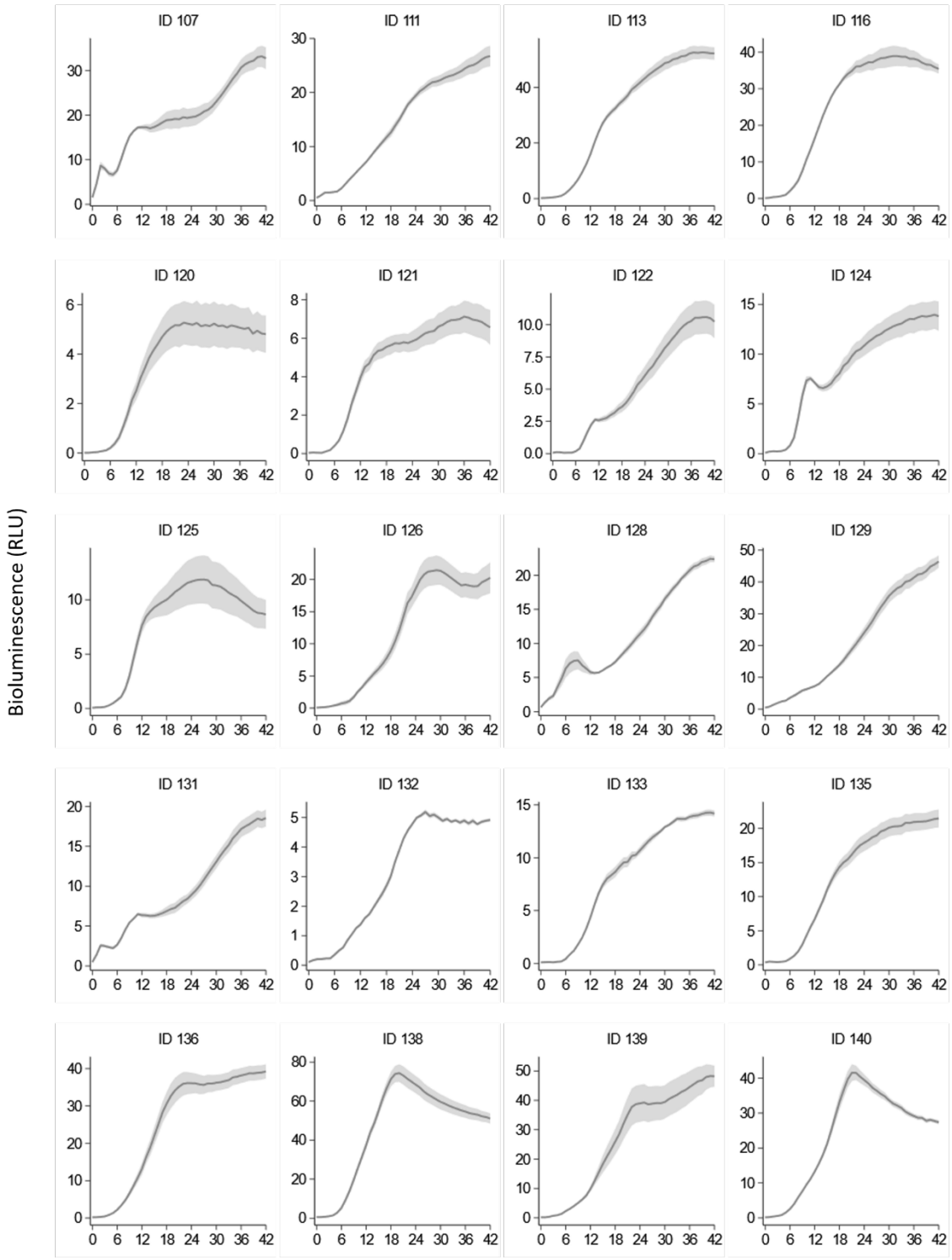

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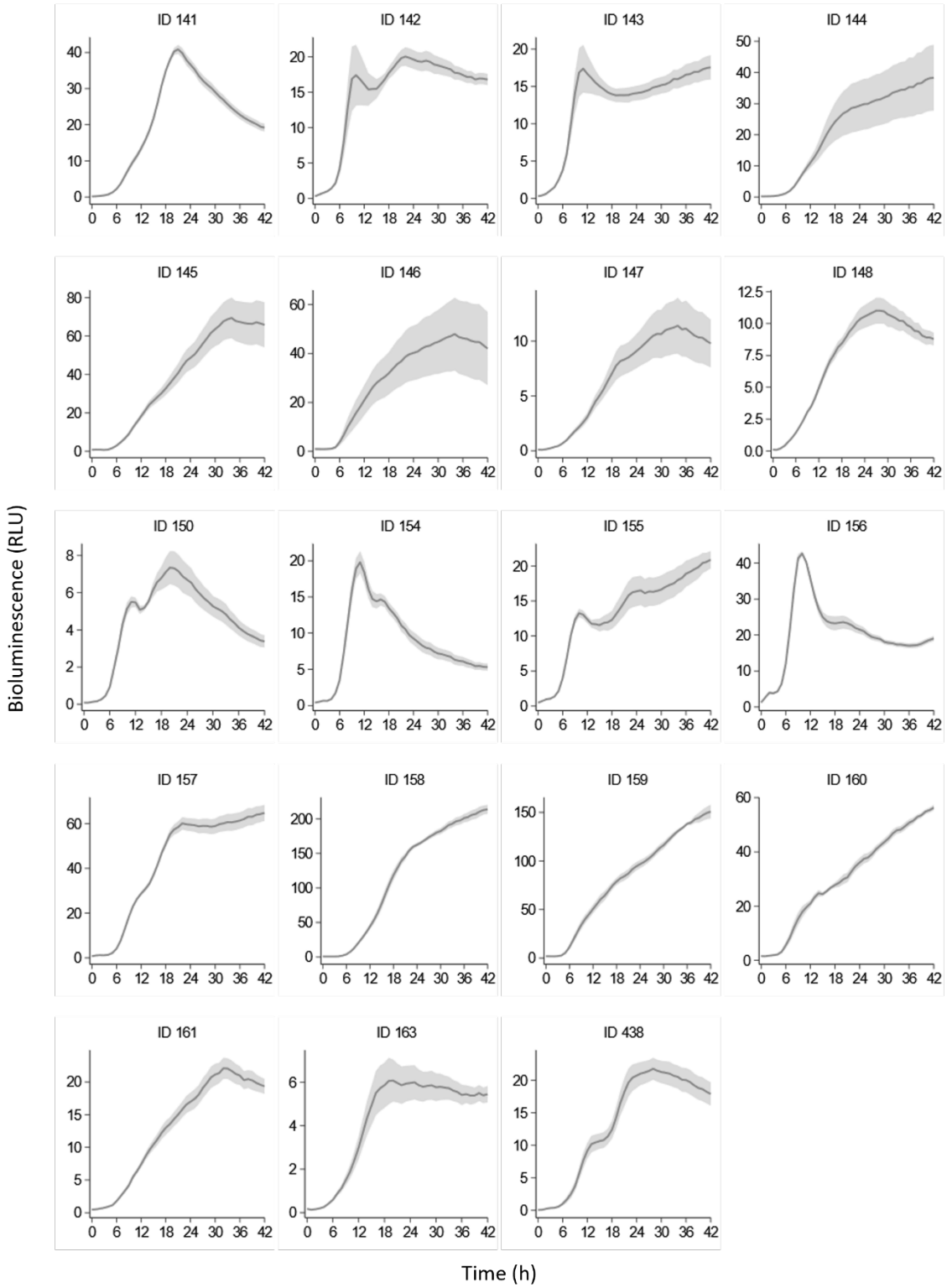
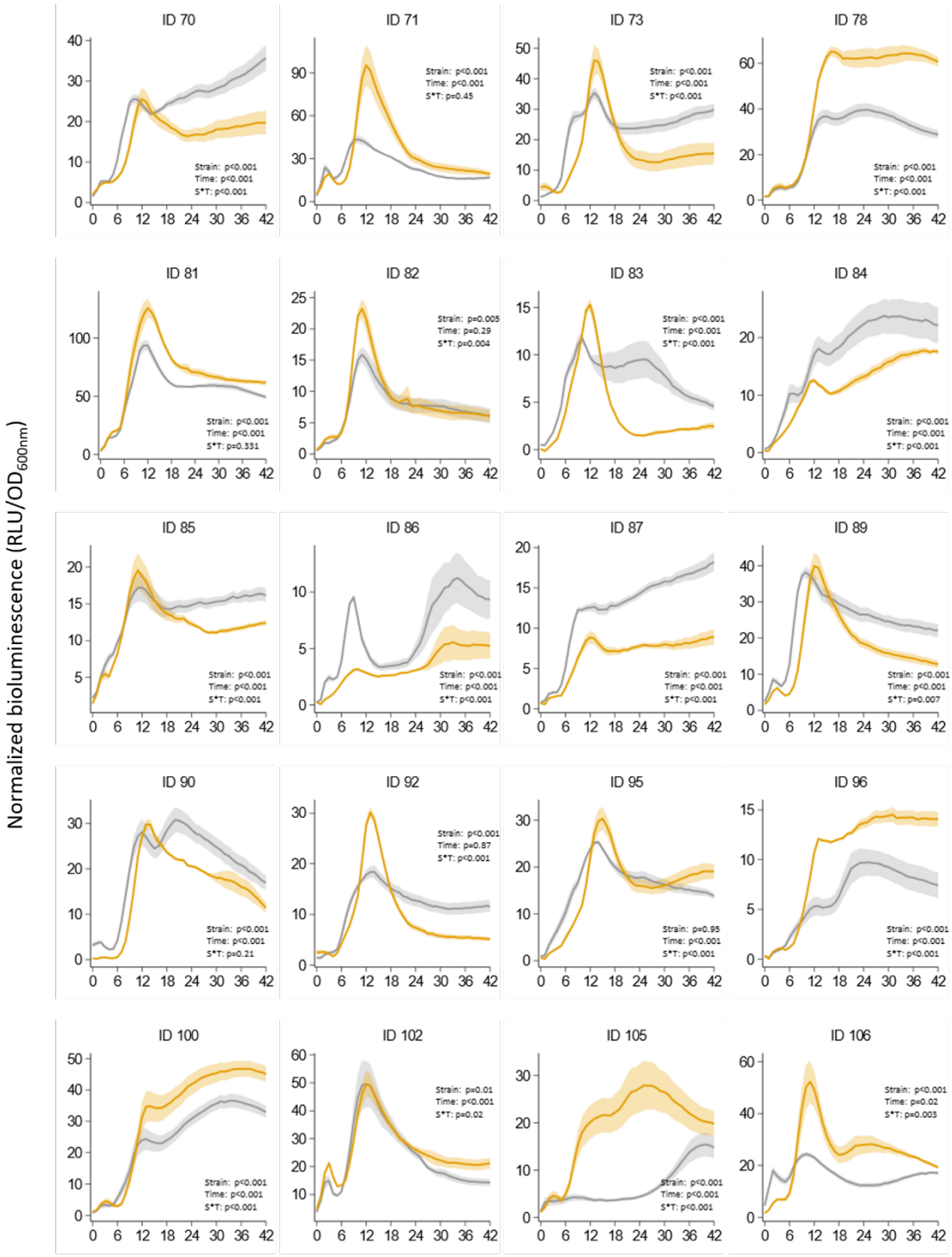


Figure S1. Kinetics of bioluminescence produced by parental *Photorhabdus* strains *in-vitro*. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). To facilitate the reading, ID numbers are written instead of the full names of the strains. The correspondence between ID numbers and full names is presented in Table S1. RLU: relative light units.



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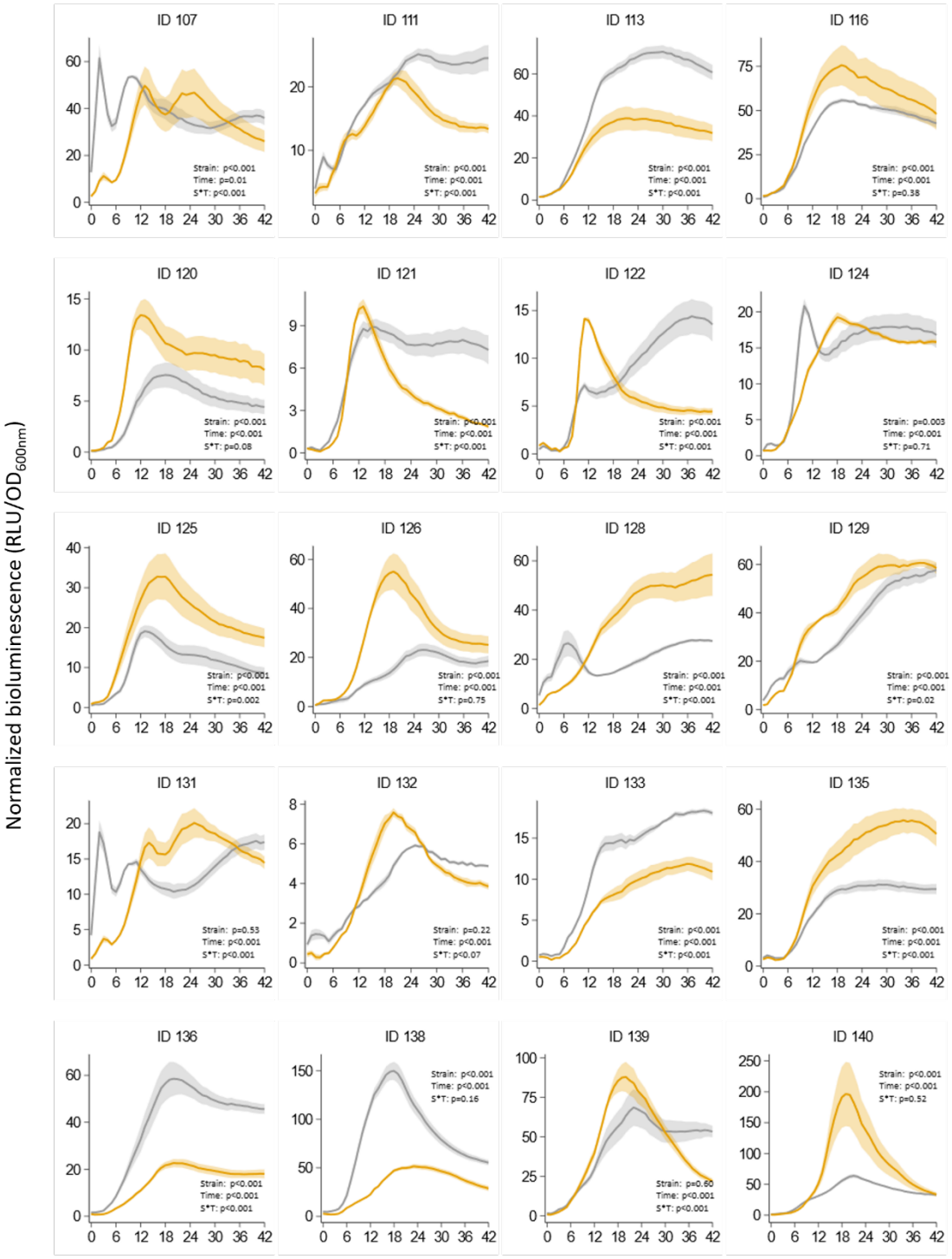

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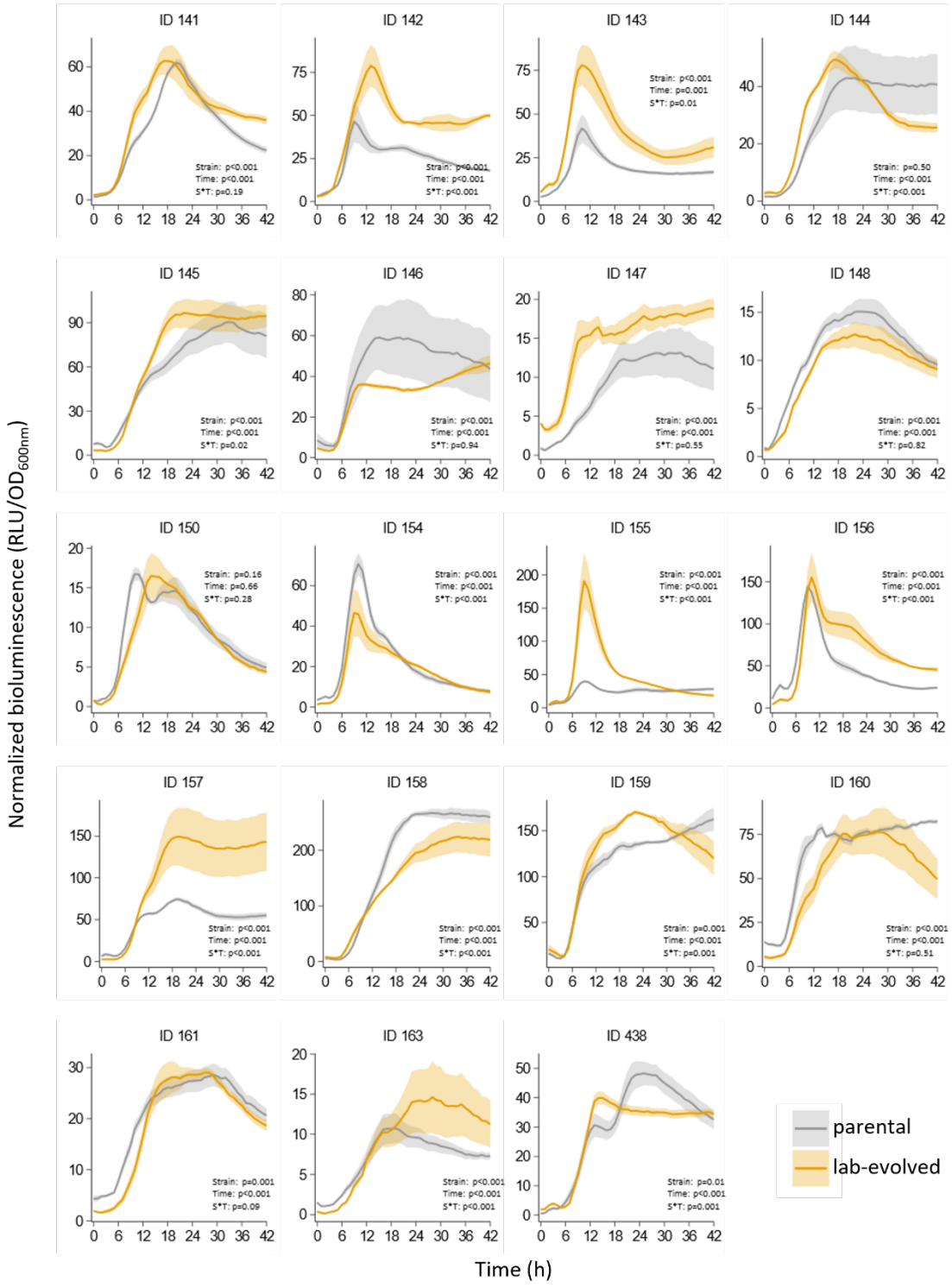
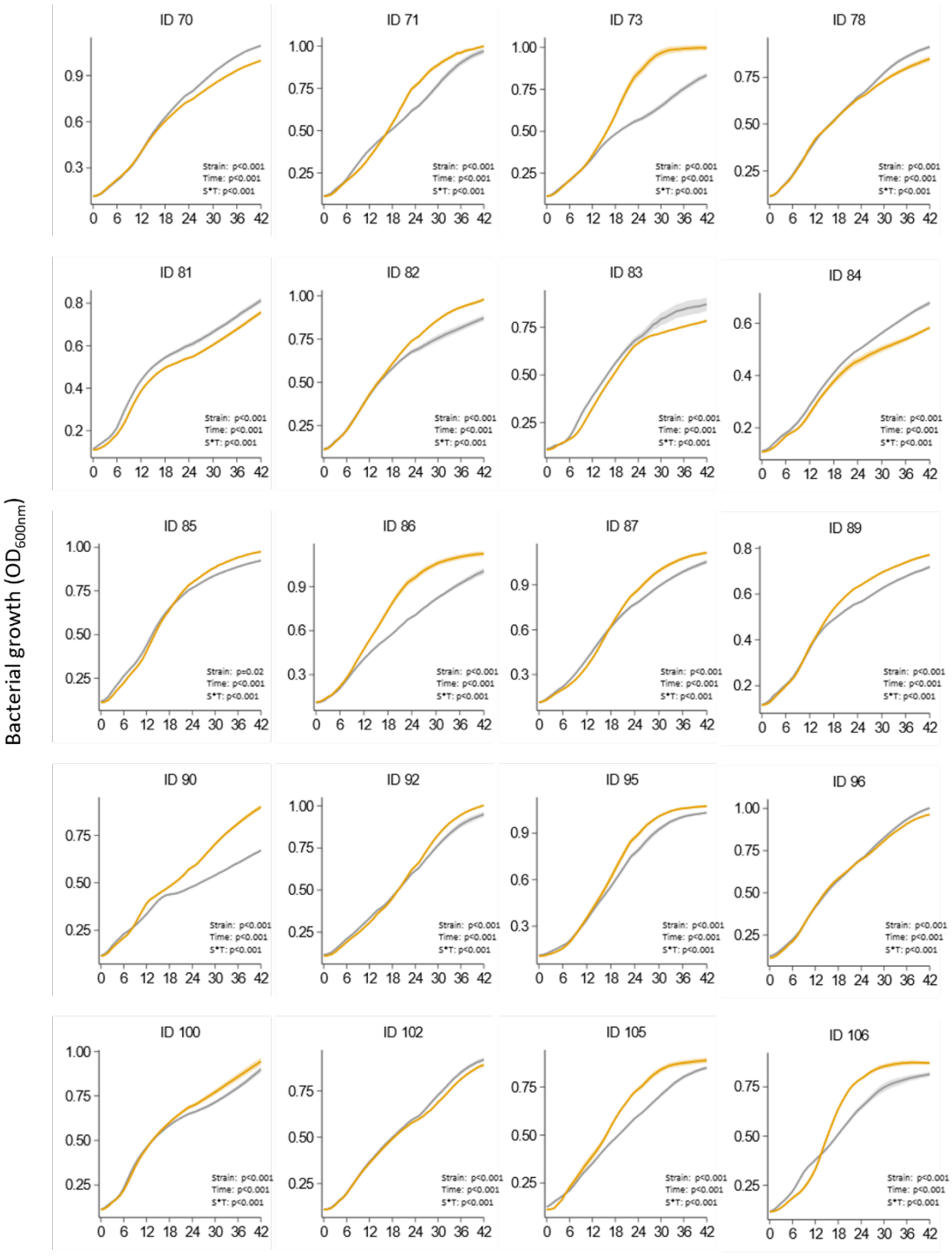


Figure S2. Kinetics of bioluminescence normalized by bacterial growth produced *in-vitro* by parental and lab-evolved *Photorhabdus* strains. Bioluminescence levels were measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). The normalized bioluminescence levels were statistically assessed by two-way repeated measures ANOVA. To facilitate the reading, ID numbers are written instead of the full names of the strains. The correspondence between ID numbers and full names is presented in Table S1. RLU: relative light units.



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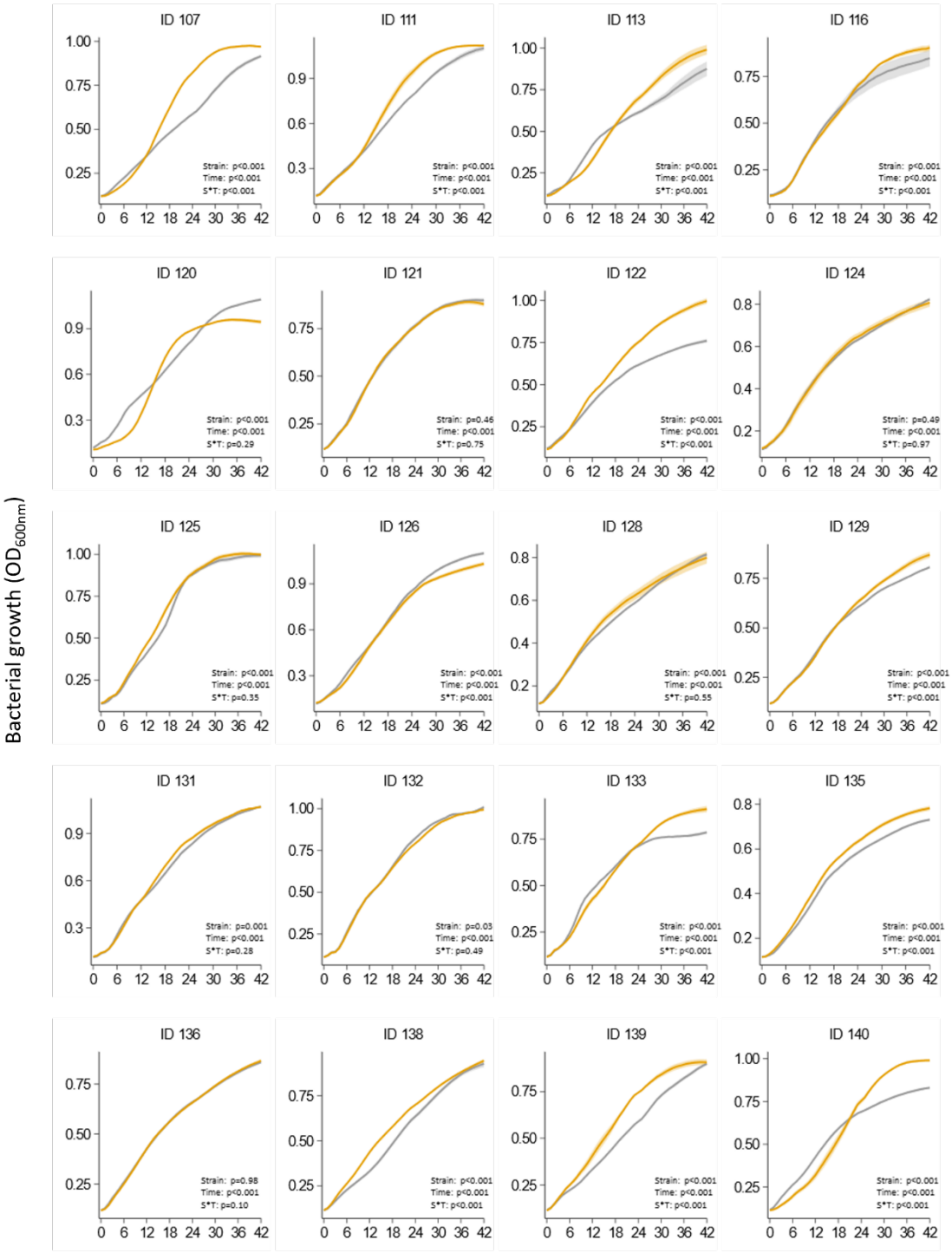

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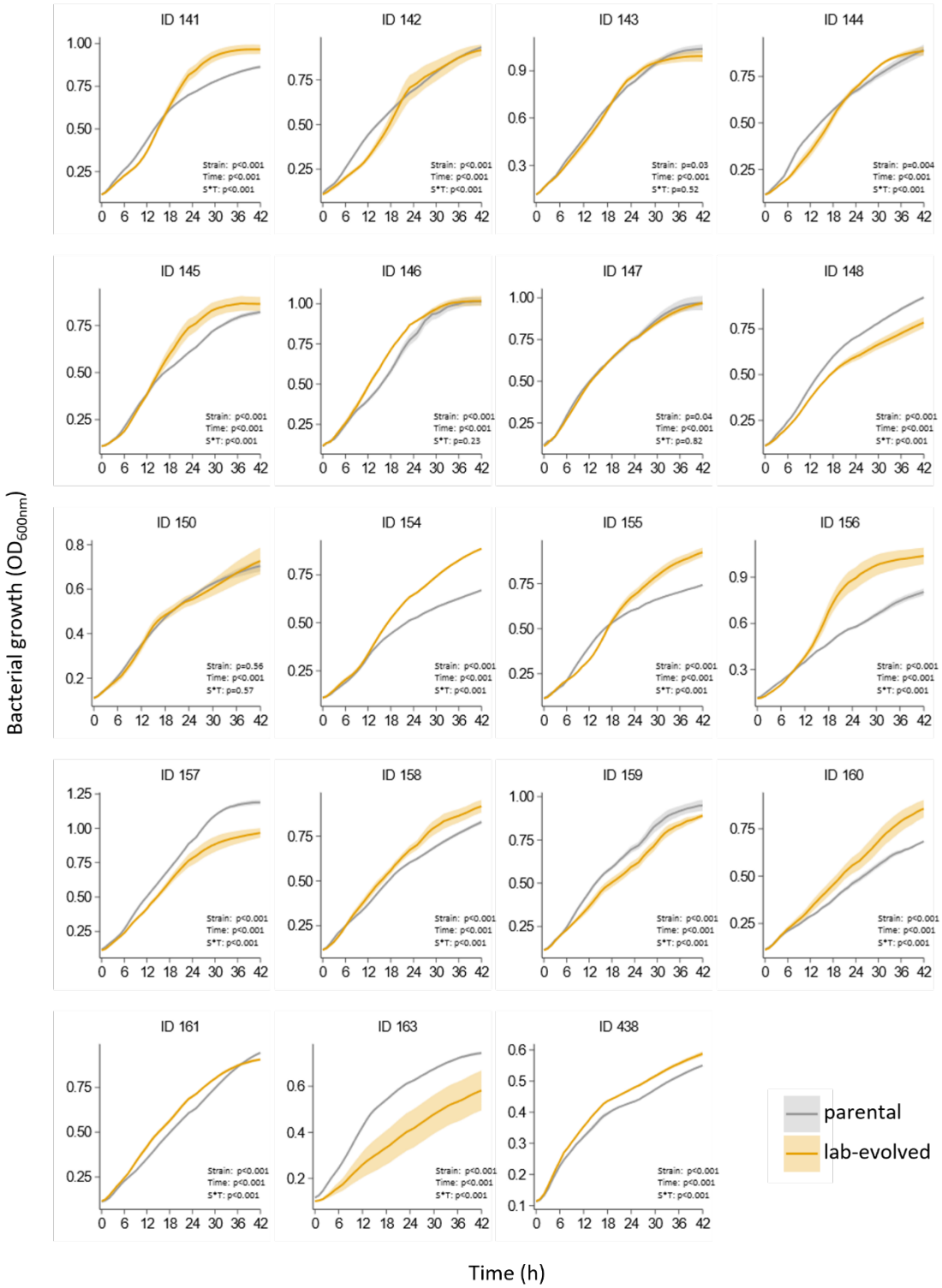

Figure S3. Kinetics of bacterial growth of parental and lab-evolved *Photorhabdus* strains *in-vitro*. Bacterial growth (OD_{600nm}) was measured in five cultures for each strain, and the experiments were conducted two to four independent times (n=10-20). The bacterial growth was statistically assessed by two-way repeated measures ANOVA. To facilitate the reading, ID numbers are written instead of the full names of the strains. The correspondence between ID numbers and full names is presented in Table S1.

Table S1. List of the bacterial strains used in this study and their respective ID numbers.

ID	Strain
70	*P. khanii* subp *khanii* Kes-14
71	*P. kleinii* VS -15
73	*P. khanii* subsp. *guanajuatensis* MEX20-17
78	*P. luminescens* subsp. *mexicana* MEX47-22
81	*P. bodei* CN4 25
82	*P. akhurstii* subsp. *akhurstii* R002-26
83	*P. bodei* KR04
84	*P. bodei* C8406
85	*P. khanii* subsp. *khanii* Hm
86	*P. aegyptia* BA1
87	*P. thracensis* PT1-31
89	*P. laumondii subsp. laumondii* DE2-34
90	*P. laumondii* subsp. *laumondii* IL9-35
92	*P. kayaii* HUG-39
95	*P. bodei* H06-42
96	*P. kayaii* IR2-43
100	*P. laumondii* subsp. *clarkei* BOJ-47
102	*P. kleinii* B-49
105	*P. kleinii* S8-52
106	*P. kleinii* S9-53
107	*P. kleinii* S10-54
111	*P. thracensis* 0943-58
113	*P. laumondii* subsp. *laumondii* S14-60
116	*P. bodei* LJ24-63
120	*P. luminescens* subsp. *luminescens* Hm
121	*P. australis* subsp. *thailandensis* PB68.1
122	*P. hainanensis* DSM 22397
124	*P. akhurstii* subsp. *akhurstii* DSM 15138
125	*P. asymbiotica* ATCC 43949
126	*P. luminescens* subsp. *luminescens* ATCC 29999
128	*P. namnaonensis* PB45.5
129	*P. temperata* Meg
131	*P. caribbeanensis* HG29
132	*P. caribbeanensis* HG26
133	*P. heterorhabditis* subsp. *aluminescens* Q614
135	*P. cinerea* 3240
136	*P. khanii* subsp. *khanii* MEG
138	*P. tasmaniensis* USCA01
139	*P. tasmaniensis* NZH3
141	*P. temperata* K122
142	*P. thracensis* 3210
143	*P. thracensis* 3213
144	*P. noeniputensis* DSM 25462
145	*P. heterorhabditis* subsp. *heterorhabditis* SF41
146	*P. asymbiotica* DSM 15149
147	*P. australis* subsp. *australis* DSM 17609
148	*P. khanii* subsp. *khanii* DSM 3369
150	*P. kayaii* 3209
154	*P. laumondii* subsp. *laumondii* TT01
155	*P. kayaii* DSM 15194
156	*P. kleinii* DSM 23513
157	*P. thracensis* DSM 15199
158	*P. cinerea* DSM 19724
159	*P. stackebrandtii* DSM 23271
160	*P. tasmaniensis* DSM 22387
161	*P. temperata* DSM 14550
163	*P. cinerea* PT-Hb-B
438	*P. laumondii* subsp. *laumondii* DJC 23

CHAPTER 2
Effect of *Photorhabdus* bioluminescence on the foraging behavior of entomopathogenic nematodes

Abstract

Entomopathogenic nematodes (EPNs) parasitize and kill insects belowground. They use multiple cues to locate their future hosts and are able to discriminate between uninfected, con-, and hetero-specific infected organisms. Nitrogen and 3-methyl-2-buten-1-ol (prenol) produced by EPN-infected organisms have been shown to repel host-seeking EPNs in a dose-dependent manner. These compounds are not thought to be specific to particular EPN species. It is likely that other cues, both chemical and non-chemical, also influence the behavior of host-seeking EPNs towards con- and hetero-specific infected organisms. At the beginning of the infection process by *Heterorhabditis* nematodes, their bacterial symbionts, *Photorhabdus*, produce bioluminescence, causing the glow of the infected organism. Based on the timing of its production, we hypothesize that bioluminescence is a signal of early-stage of infection by the *Heterorhabditis*/*Photorhabdus* pair that modulates the foraging behavior of host-seeking EPNs and contributes to the avoidance of intra- and inter-specific competition. Applying a homology-driven approach, we identified elements necessary for light perception in EPNs. To assess the effect of bioluminescence on the behavior of host-seeking EPNs we performed nematode choice assays with insects, genetically engineered aluminescent *Photorhabdus* strains, their bioluminescent wild type (WT) counterparts, and luminescent beads. None of the EPNs tested responded to the bioluminescence produced by insects infected by *Photorhabdus laumondii* subsp. *laumondii* strain DJC-23. Choice assays performed with luminescent beads, mimicking *Photorhabdus* bioluminescence, indicate that several strains of EPNs are responsive to higher intensities luminescence. Specifically, *H. georgiana* Hbb and *H. beicherriana* CN4 nematodes were repelled by both intermediate and high luminescence, while *H. zacatecana* MEX-41, *H. bacteriophora* TT01, *S. carpocapsae* Andermatt, and *S. feltiae* Jakub nematodes were repelled only by the highest luminescence level tested. These results suggest that *Photorhabdus* bioluminescence can modulate the behavior of host-seeking EPNs in an intensity- and species-dependent manner to avoid intra- and inter-specific competitions.

Introduction

Belowground, microscopic soil-dwelling nematodes parasitize and kill small arthropods, including insects. These entomopathogenic nematodes (EPNs) belong to the genera *Heterorhabditis* and *Steinernema* and exist in symbiosis with bacteria from the genera *Photorhabdus* and *Xenorhabdus*, respectively (**Boemare et al., 1993; Forst et al., 1997; Poinar and Thomas, 1966, 1965; Thomas and Poinar, 1979**). To locate host, the free-living stage of EPNs, called infective juveniles (IJs), sense and integrate diverse cues in the soil matrix (**Ali et al., 2010; Boff et al., 2001; Kaya and Gaugler, 1993; Rasmann et al., 2005; Tol et al., 2001)**. This host-seeking step is the initial phase of parasitism and is crucial for successful completion of the lifecycle of EPNs. Prior to infection, EPNs assess the suitability of their future host, discriminating between healthy, con- and hetero-specific infected insects (**Baiocchi et al., 2017; Grewal et al., 1997; Jagodič et al., 2017; Kunkel et al., 2006**). While host-seeking EPNs are repelled by late-stage infected insects, they exhibit different behavior towards early-stage infected insects by conspecific, some species being attracted to them (**Baiocchi et al., 2017; Grewal et al., 1997**). Different species of EPNs produce distinct blends of odors (**Grunseich et al., 2021**). Nitrogen, emitted as volatile ammonia, and 3-methyl-2-buten-1-ol (prenol) have been demonstrated to diffuse from EPNs-infected organisms and have been shown to repel host-seeking EPNs in a dose-dependent manner (**Baiocchi et al., 2017; Shapiro et al., 2000**). However, these two volatiles are not thought to be specific to particular EPN species. For instance, *Galleria mellonella* infected by two species of *Steinernema* nematodes produced similar quantities of prenol at similar rates of emission (**Baiocchi et al., 2017**). It is likely that in addition to nitrogen and prenol other cues, both chemical and non-chemical, influence the behavior of host-seeking EPNs towards infected organisms, especially regarding the discrimination between con- and hetero-specific infection (**Grunseich et al., 2021**). *Photorhabdus,* the bacterial symbionts associated with *Heterorhabditis* nematodes possess the singular ability to produce bioluminescence belowground (**Thomas and Poinar, 1979; Waterfield et al., 2009**). At the beginning of the infection process by *Heterorhabditis* nematodes, their bacterial symbionts produce bioluminescence, causing their host to glow belowground. Based on the timing of its production, we hypothesize that bioluminescence is a signal of early-stage of infection by the *Heterorhabditis*/*Photorhabdus* pair that modulates

the foraging behavior of host-seeking EPNs and contributes to the avoidance of intra- and inter-specific competition.

Heterorhabditis and *Steinernema* nematodes belong to the *Rhabditida* order, although they are not sister clades. Despite this distinction, these organisms exhibit similarities in their ecological niche, possess overlapping host ranges, and share numerous features in their parasitic lifecycle (**Poinar, 1979**). However, the symbiotic partners of *Steinernema* nematodes belong to the genus *Xenorhabdus*, which are aluminescent bacteria. Therefore, infection by the *Steinernema/Xenorhabdus* pair does not result in the glow of the infected organism. Both *Heterorhabditis* and *Steinernema* lifecycles start with the search of suitable hosts. This crucial step is executed by IJs, the only developmental stage capable of surviving for extended periods of time without food while searching for hosts in the soil (**Gaugler, 2002**).

Infective juveniles sense and integrate multiple cues from their environment to localize potential host. Yet, it has been shown that carbon dioxide (CO_2), emitted by most living organisms, is used by host-seeking EPNs to localize biological activities (**Boff, 2002; Kaya and Gaugler, 1993**). Carbone dioxide is an unspecific signal, and it has been suggested that relying solely on its exploitation could result in low foraging efficiency (**Boff, 2002; Tol et al., 2001**). Entomopathogenic nematodes can use CO_2 alone or in combination with insects or plants odors to locate hosts (**Hallem et al., 2011a, 2011b**). The rhizosphere represents an ecological hub influencing most groups of soil-dwelling organisms. Upon attack by root herbivores, plants emit large amounts of herbivore-induced plant volatiles (HIPVs) diffusing in the soil matrix. Multiple examples, including HIPVs emitted by maize, cotton, citrus tree, potato, sugarcane, carrot, and grape vine, highlight the importance of plant-derived volatiles in host-seeking behavior of EPNs (**Ali et al., 2010; Filgueiras et al., 2016; Kergunteuil et al., 2019; Laznik and Trdan, 2016, 2016, 2013; Rasmann et al., 2005; Rasmann and Turlings, 2008**). European maize lines produce (E)-β-caryophyllene when fed by the western corn rootworn (WCR) *Diabrotica virgifera virgifera.* It has been shown that this sesquiterpene was highly attractive to EPNs under laboratory conditions and in field (**Hiltpold et al., 2010; Köllner et al., 2008; Rasmann et al., 2005**). Tritrophic interaction involving plants, herbivores arthropods and their natural enemies, EPNs, are part of the indirect strategies employed by plants to defend themselves. Host-seeking EPNs also benefit from integrating HIPVs as these volatiles often lead to the localization of suitable hosts. In addition, EPNs are also able to use cues directly emitted by their preys, such as sex pheromones, molting skins, and frass (**Boff et al.,**

2001; Campbell and Kaya, 2000; Schmidt and All, 1979). The current view is that host-seeking EPNs integrate environmental cues in a hierarchical order and orient preferentially toward location where multiple cues are emitted (**Dillman et al., 2012; Turlings et al., 2012; Zhang et al., 2021**).

Once EPNs encounter a potential host, they assess its quality and suitability. Host-seeking EPNs are able to distinguish between uninfected and infected insects and even discern between con- and hetero-specific infections (**Baiocchi et al., 2017; Grewal et al., 1997; Jagodič et al., 2017; Kunkel et al., 2006**). While late-stage infected insects tend to repel host-seeking EPNs, it has been shown that IJs can infect early-stage infected insects (**Griffin, 2012**). It has been shown nitrogen, released as volatile ammonia from EPN-infected organisms, repels host-seeking EPNs in a dose-dependent manner. Low concentrations of this compound, associated with early-stage of infection, attract EPNs, while higher concentrations, associated with later stage of infection, repels EPNs (**Shapiro et al., 2000**). Two other volatiles, 3-methyl-2-buten-1-ol (prenol) and butylated hydroxytoluene (BHT), emitted by EPN-infected insects, are recognized as a late-stage infection cues (**Baiocchi et al., 2017; Zhang et al., 2019**). Both prenol and BHT attract uninfected hosts in the vicinity of EPN-infected cadaver, increasing the probability of emerging IJs to encounter a new suitable prey. Infectivity assays indicate that some EPNs (e.g. *S. riobrave*) are attracted to insects that have recently been infected by conspecifics while others are repelled by con- and hetero-specific infected insects (e.g. *S. glaseri* and *S. feltia*) suggesting that foraging behavior of host-seeking EPNs toward infected insects is strain- or species-specific (**Baiocchi et al., 2017; Lewis and Gaugler, 1994**). Organisms infected by different species of EPNs produce distinct blends of odors (**Grunseich et al., 2021**). While ammonia, prenol, and BHT are emitted by EPN-infected organisms, there are not known to be specific to particular EPN species but rather generally associated with EPN infection. In addition, the repellent action of prenol and BHT towards host-seeking EPNs is believed to be linked with the late-stage infection, while the differential behavior displayed by EPNs towards con- and hetero-specific infection is associated with the early-stage of infection (**Baiocchi et al., 2017; Grunseich et al., 2021; Zhang et al., 2019**).

The bacterial symbiont of *Heterorhabditis* nematodes, *Photorhabdus* possess the singular ability to produce bioluminescence belowground. During the beginning of the infection process, *Photorhabdus* bacteria multiply exponentially within the host and produce

bioluminescence. The production of bioluminescence reaches a maximum in intensity one to three days after the beginning of the infection, concomitantly with the insect death resulting in the bioluminescence of the cadaver in the soil. Next, the bacteria enter into the stationary phase of growth and the bioluminescence production gradually decrease. It has been suggested that soil inhabitants are strongly limited in their use of visual information but rather use chemical and tactile cues to communicate and behave (**Jones, 2002**). Nonetheless, studies focusing of earthworms and potworms suggest that bioluminescence might play ecological functions belowground (**Pes et al., 2016; Seesamut et al., 2021; Verdes and Gruber, 2017**). Moreover, the soil-dwelling nematode *Caenorhabditis elegans* is able to sense light and move away from its source of emission (**Gong et al., 2016; Ward et al., 2008**). While the protein LITE-1 has been shown to be the photoreceptor involved in this negative phototaxis behavior, the protein GUR-3 has been described as a photosensor and proved to confer light sensitivity to light-insensitive neurons and muscles in *C. elegans* (**Bhatla and Horvitz, 2015; Gong et al., 2016; Liu et al., 2010**). Based on the timing of its production, we hypothesize that bioluminescence is a signal of early-stage of infection by the *Heterorhabditis*/*Photorhabdus* pair that modulates the foraging behavior of host-seeking EPNs and contributes to the avoidance of intra- and inter-specific competition. To test our hypothesis, we first investigated EPNs genomes to identify putative photosensors (LITE-1 and GUR-3) and phototransduction pathway proteins. To assess the effect of bioluminescence on foraging EPNs behavior, we used insects, genetically engineered aluminescent *Photorhabdus* strains, their bioluminescent wild type (WT) counterparts, and luminescent beads to perform choice assays with several species of EPNs. To complete our analysis, we compared the volatiles emitted by insects infected by genetically engineered aluminescent and bioluminescent *Photorhabdus* strains. Taking advantage of modern molecular tools our work aims to reveal the potential role played by *Photorhabdus* bioluminescence in the modulation of the behavior of host-seeking EPNs.

Material and methods

Photorhabdus culture

The naturally bioluminescent *Photorhabdus laumondii* subsp. *laumondii* strain DJC-23 and the genetically-engineering aluminescent *Photorhabdus laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 were used for this work. To maintain these two strains, they were cultured onto lysogenic broth (LB) agar plates (Sigma-Aldrich, USA) and incubated at 28°C. Every thirty days the bacteria were refreshed from glycerol stocks.

Nematodes rearing

Nematodes were stored in IJ developmental stage in 50 mL of water in culture flasks at 10-13°C in darkness. Every two months the cultures were refreshed by infecting third-instar *Galleria mellonella* larvae with 100 IJs and collecting the emerging the progeny using the White trap method (**White, 1927**). The following nematode species and strains were used: *Heterorhbaditis bacteriophora* TT01, *H. beicherriana* CN4, *H. georgiana* Hbb, *H. ruandica* Rw14_N-C4a, *H. zacatecana* MEX-41, *Heterorhabditis* sp. S10, *Steinernema akhursti* AKH, *S. carpocapsae* Andermatt, *S. feltiae* Jakub, *S. litorale* Aichi, and *S. surkhetense* CS20.

Identification of nematodes photosensor and phototransduction proteins

The gene and protein sequences of the *C. elegans* photosensors LITE-1 (accession number: NP_509043.3) and GUR-3 (acc. num.: NP_509743.2) and of the phototransduction proteins DAF-1 (acc. num.: NP_001023159.1), GPA-3 (acc. num.: NP_001309498.1), GOA-1 (acc. num.: NP_492108.1), ODR-1 (acc. num.: NP_001362115.1), TAX-2 (acc. num.: NP_492427.3) and TAX-4 (acc. num.: NP_499033.1) were retrieved from the National Center for Biotechnology Information (NCBI) website (**Bethesda, 1988**). To identify homologs of these proteins in EPNs we used the basic local alignment search tool of Bioedit 7.2.5 using *C. elegans* protein sequences and *Heterorhabditis* and *Steinernema* annotated genomes (**Hall, 1999**). The following reference sequence genome assemblies (NCBI RefSeq assembly) were used: *C. elegans* GCA_000002985.3, S*. carpocapsae* GCA_000757645.3 and, *S. feltiae* GCA_007213375. For investigation on *Heterorhabditis* nematodes we used our unpublished genome assemblies and annotations. Information about the templates and queries used are summarized in **Table S1**. All the genomes and sequences used for this investigation are available within the electronic form of this thesis. Similarity scores were computed in Bioedit 7.2.5 using the substitution matrix BLOSUM62 (**Hall, 1999**). To build protein models we used the amino acid

sequences of the identified homologs and the protein structure homology-modelling server provided by the SWISS-MODEL website (**Waterhouse et al., 2018**).

Impact of the initial nematode inoculum size on reproductive output

To determine the impact of the number of nematodes initiating the infection on the reproductive output, we evaluated the number of IJs emerging from insect cadavers. To this end, we first infected *G. mellonella* larvae with different inoculum size, from 100 to 1000 IJs of the strain *H. bacteriophora* TT01. Then, we transferred infected insects to White traps, and emerging nematodes were regularly collected and counted until no further nematodes emerged.

Measure of (bio)luminescence emitted by *Photorhabdus*-infected insects and luminescent beads

To measure the bioluminescence emitted by *Photorhabdus*-infected insects, *G. mellonella* larvae were infected with the different bacterial strains, as described previously (**Castaneda-Alvarez et al., 2022**). Briefly, *Photorhabdus* bacterial strains were individually grown for 16-20 hours in LB medium at 28 °C with constant agitation (180 rpm). The bacterial cultures were then collected, and their optical densities at 600 nm (OD_{600}) were measured. Subsequently, all cultures were diluted to obtain bacterial cultures with an OD_{600}=1, and 10 µL of the resulting bacterial cultures were injected into third-instar *G. mellonella* larvae. Twenty-four larvae per strain were injected (n=24). Bioluminescence was measured after 24 h using a SpectraMax 0480 microplate reader. To measure the luminescence emitted by luminescent beads, we first exposed the beads to sunlight for 30 s (intermediate luminescence) or for 120 s (high luminescence). Three beads were placed in 12 wells of a 24-well microtiter plates (Greiner Bio-One, Au), and bioluminescence measurements were carried out using a SpectraMax 0480 microplate reader (n=12).

Nematode behavioral experiments

To evaluate whether EPNs integrate bioluminescence in their foraging decisions, we carried out two sets of choice experiments as follows. In the first set of experiments, nematodes from different species were released and allowed to choose between *G. mellonella* larvae infected with aluminescent *P. laumondii subsp. laumondii* Δ*luxC-E* strain DJC-23 bacteria or infected with their bioluminescent WT counterparts. *Galleria mellonella* larvae were infected with the

different bacterial strains as described above (see section: “Measure of (bio)luminescence emitted by *Photorhabdus*-infected insects and luminescent beads”, page 104). Twenty-four hours later, one aluminescent insect cadaver and one bioluminescent insect cadaver were transferred to 9 cm Petri plates filled with a 2 mm layer of phytagel medium (0.25% (m/v) phytagel (P8169; Sigma-Aldrich), 0.3% (m/v) sodium chloride, and 246 mg/L magnesium sulphate). The insects were placed 6 cm apart from each other. Besides bioluminescence production, no phenotypical differences were observed between *G. mellonella* larvae infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 bacteria or infected with their bioluminescent WT counterparts. In addition, *G. mellonella* larvae infected with either aluminescent mutant or bioluminescent WT *Photorhabdus* strains showed 100% mortality. To confirm that the observed mortality was not caused by our experimental design, we injected 10 µL of sterile LB liquid medium into third-instar *G. mellonella* larvae and observed no mortality in the injected larvae. Then, 60 nematodes were released in the center of the Petri plate, equidistantly from the insect cadavers. All Petri plates were incubated at 24 °C in darkness. Nematode choices were recorded 24 h later as this timing coincided with the maximum intensity of bioluminescence emitted by *Photorhabdus*-infected *G. mellonella* larvae (**Fig. S1**). A total of 15 choice arena types, each with 60 nematodes, were assayed (n=15). In the second set of experiments, we allowed nematodes from different species to choose between living *G. mellonella* larvae and living *G. mellonella* larvae lying next to luminescent beads. The insect larvae were placed into 1.5 mL glass vials (with pierced lids) to restrain their free movement while allowing diffusion of volatiles. These vials were then transferred to 14 cm Petri plates filled with a 2 mm layer of phytagel medium. The vials were placed 6 cm apart from each other. Following this, 100 nematodes were released in the center of the Petri plates, equidistantly from the vials. All Petri plates were incubated at 24 °C in darkness. Nematode choices were recorded 24h later. A total of 5 choice arena types, each with 100 nematodes were assayed, and the experiments were conducted two independent times (n=10). The following nematode species and strains were used: *H. bacteriophora* TT01, *H. beicherriana* CN4, *H. georgiana* Hbb, *H. ruandica* Rw14_N-C4a, *H. zacatecana* MEX-41, *Heterorhabditis* sp. S10, *S. akhursti* AKH, *S. carpocapsae* Andermatt, *S. feltiae* Jakub, *S. litorale* Aichi, and *S. surkhetense* CS20.

Untargeted volatile analyses of *Photorhabdus*-infected insects

To evaluate whether insect cadavers infected with bioluminescent and aluminescent bacterial strains differ in the quantity or quality of emitted volatiles, we measured the volatiles emitted by *G. mellonela* larvae injected with either the aluminescent *P. laumondii subsp. laumondii* Δ*luxC-E* strain DJC-23 bacteria or with their bioluminescent WT counterparts. Controls consisted of LB-injected insects and untreated insects. Eighteen hours after treatments, insects were transferred to glass bottles covered with aluminum foil (7 cm diameter, Verre & Quartz Technique SA, Neuchâtel, Switzerland) and clean humidified air was pushed through at a rate of 0.7 l/min and pulled through Haye-Sep filters (25 mg of Haye-Sepadsorbent, 80–100 mesh; Vassays systems, NY, USA) at a rate of 0.4 l/min. Volatiles were sampled over a period of 6 hrs. Then, volatile-trapping filters were collected, replaced by clean filters, and a new cycle of volatile collection was initiated. Sampling cycles were repeated at regular intervals for five consecutive days. Volatile-containing filters were eluted with 150 lµl of dichloromethane spiked with 100ng of N-octane and 100 ng of nonyl-acetate as internal standards (Sigma, Buchs, Switzerland). Volatiles were analyzed by gas chromatography and mass spectrometry as described previously (**Arce et al., 2021; Clancy et al., 2023**). For this an Agilent 7890B gas chromatograph (GC) instrument coupled to an Agilent 5977B gas chromatography/mass spectrometry detector (GC/MSD) instrument were used (Agilent Technologies, Santa Clara, CA, USA). Samples were injected into the injector port at 230 °C and pulsed in a spitless mode onto an apolar column (HP-5MS 5% Pheny Methyl Silox, 30 m x 250 µm internal diameter x 0.25 µm film thickness, J&W Scientific, Agilent Technologies SA, Basel, Switzerland). Helium, with a constant flow of 1 mL/min (constant pressure 8.2317 psi), was used as the carrier gas. Following injection, the column temperature was maintained at 40 °C for 3.5 min, then increased to 100 °C at a rate of 8 °C/min, and subsequently raised at 5 °C/min to 230 °C, followed by a post run of 3 min at 250 °C. Volatile identification was obtained by comparing mass spectra with those in the NIST17 Mass Spectra Library and commercial standards.

Statistical analyses

Statistic tests used to assess the different datasets are described in more detail in the figure legends. Normality and equality of variance were verified using Shapiro-Wilk and Levene's tests, respectively using Sigma Plot 14.5 (Systat Software Inc., San Jose, CA, USA). Nematode preferences were statistically assessed by generalized linear model under Poisson distribution

and corrected for overdispersion with quasi-binomial function, when necessary, followed by analysis of deviance and false discovery rate-corrected post hoc tests. These analyses were followed by residual analysis to verify the suitability of the error distribution and model fitting. The above analyses were conducted in R 4.3.2 (R Core Team 2023) using the packages “lme4”, “car”, “emmeans”, and “RVAideMemoire”(**Bates et al., 2014; Fox, 2019; Hervé, 2020; Lenth and Lenth, 2018**). The levels of (bio)luminescence emitted by infected insects and luminescent beads were statistically assessed by one-way ANOVA followed by Tukey HSD multiple comparison tests in R 4.3.2 (R Core Team 2023). Nematode reproductive output and volatile emitted by infected insects were statistically assessed by two-way repeated measures ANOVA followed by Holm’s test for multiple-comparisons using Sigma Plot 14.5 (Systat Software Inc., San Jose, CA, USA).

Results

Entomopathogenic nematodes possess homologs of *Caenorhabditis elegans* photosensor and phototransduction proteins

We hypothesize that foraging EPNs are able to sense *Photorhabdus* bioluminescence belowground. As a first step to assess this hypothesis, we conducted an analysis based on a literature review to identify photosensors and proteins associated with phototransduction in the genomes of EPNs. We focused on two photosensors, LITE-1 and GUR-3, and six proteins involved in phototransduction, DAF-1, GPA-3, GOA-1, ODR-4, TAX-2, and TAX-4, that have been described in the soil-dwelling nematode *C. elegans.* We identified a homolog of the photosensors GUR-3 and LITE-1 in the genomes of *Heterorhabditis* nematodes but not in those of *Steinernema* nematodes. The identified homolog exhibited a greater sequence similarity to GUR-3 than to LITE-1, leading us to conclude that this protein is a homolog of GUR-3 rather than a homolog of LITE-1. We identified homologs of all *C. elegans* proteins involved in phototransduction, expect DAF-1, in both *Heterorhabditis* and *Steinernema* nematodes. However, similarity scores were relatively low for ODR-1, TAX-2, and TAX-4 (**Table S2**). We built three-dimensional models of *C. elegans* proteins and of their homologs in *H. bacteriophora* to pinpoint structure similarities (**Fig. 1**). Based on the presence of a homolog of GUR-3 and homologs of proteins involved in phototransduction we postulate that *Heterorhabditis* nematodes might be able to sense *Photorhabdus* bioluminescence belowground. Drawing conclusions for *Steinernema* nematodes is more challenging, given that they harbor homologs of certain proteins involved in phototransduction but lack a homolog of a photosensor. This investigation represents a first step in the characterization of potential bioluminescence perception in EPNs. We acknowledge that this inference needs to be taken cautiously, as it relies on homology with *C. elegans* and has not yet been corroborated through functional experiments.

The initial nematode inoculum strongly impacts reproductive output

Infection by the *Heterorhabditis/Photorhabdus* pair results in the glowing of the host. We hypothesize that the perception of this bioluminescence by host-seeking EPNs can contribute to the avoidance of intra- and inter-specific competition. To strengthen the ecological relevance of our hypothesis, we investigated whether competition for a host can bring

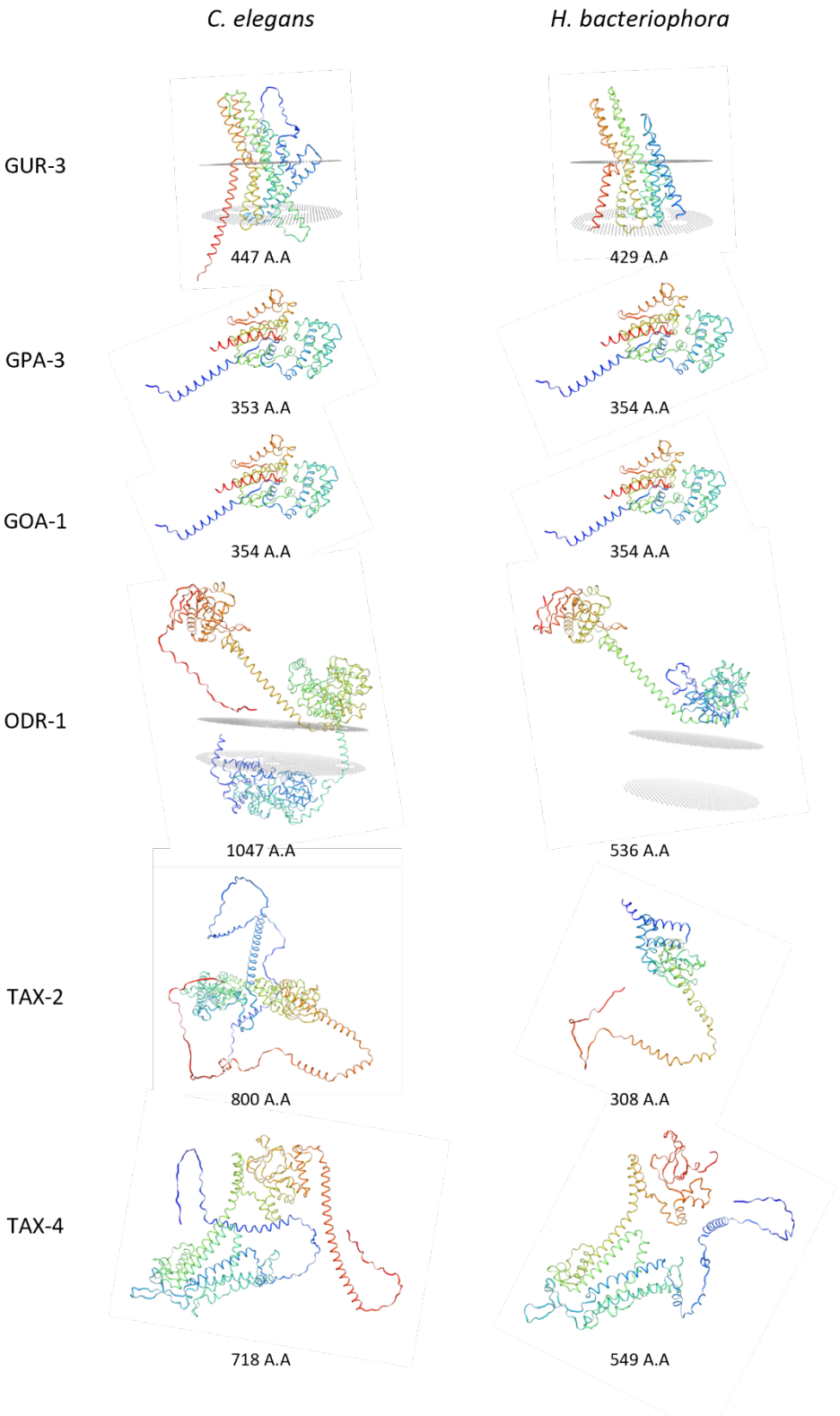

Figure 1. Homologs of the photosensor GUR-3 and of proteins associated with phototransduction are present in *Heterorhabditis* nematodes. Models of *C. elegans* photosensor GUR-3 and proteins involved in phototransduction. and their homologs in *H. bacteriophora.* Models were built from amino acid sequences using the protein structure homology-modelling server provided by the SWISS-MODEL website. Accession numbers of the sequences of *C. elegans* are listed in Table S1.

negative fitness consequences, by testing if the number of *Heterorhabditis* IJ individuals starting an infection affect the reproductive output. We infected insects with different numbers of IJs and evaluated the progeny resulting under each condition (**Fig. 2**). Our results suggest an inverse relationship between the initial inoculum size and the subsequent progeny yield. Lower initial inoculum sizes resulted in a higher number of progenies, whereas an increase in the initial inoculum size led to a reduction in reproductive output. Even a relatively small change in the initial inoculum size has an impact, exemplified by the two lowest initial inoculum sizes where we used: 100 and 200 IJs per larva. Indeed, after 14 days these two initial inoculum sizes resulted in the same reproductive output. However, if we assume that the 200 IJs treatment is equivalent to twice the 100 IJs treatment, we could expect that the reproductive output of the 200 IJs treatment would be twice as high as the one we observed. Our results indicate that excessively high number of IJs starting the infection can significantly decrease the reproductive output of EPNs and compromise the fitness of the species.

The foraging behavior of entomopathogenic nematodes is affected by (bio)luminescence in an intensity- and species-dependent manner

We hypothesize that bioluminescence is a signal of early-stage of infection by the *Heterorhabditis*/*Photorhabdus* pair that modulates the foraging behavior of host-seeking EPNs and contributes to the avoidance of intra- and inter-specific competition. To test this hypothesis, we performed a series of choice experiments. First, we used insect cadavers in early-stage of infection. We allowed IJs to choose between insect cadavers infected with either genetically engineered aluminescent *Photorhabdus* strains or their bioluminescent WT counterparts and recorded the proportion of each choice (**Fig. 3**). In the condition tested, none of the *Heterorhabditis* or *Steinernema* species were able to discriminate between aluminescent and bioluminescent insect cadavers. Based on the presence of homologs of both photosensor and phototransduction proteins in *Heterorhabditis* species, we expected to observe an effect of bioluminescence on the behavior of these nematodes. However, since *Steinernema* species lack a homolog of a photosensor, we did not have the same expectations for these nematodes. To pursue our investigation, we asked whether the absence of response toward bioluminescence could be attributed to the inability of nematodes to perceive this signal below a certain threshold of intensity. We conducted a second series of choice experiments where we offered the option to the IJs to choose between living *G. mellonella*

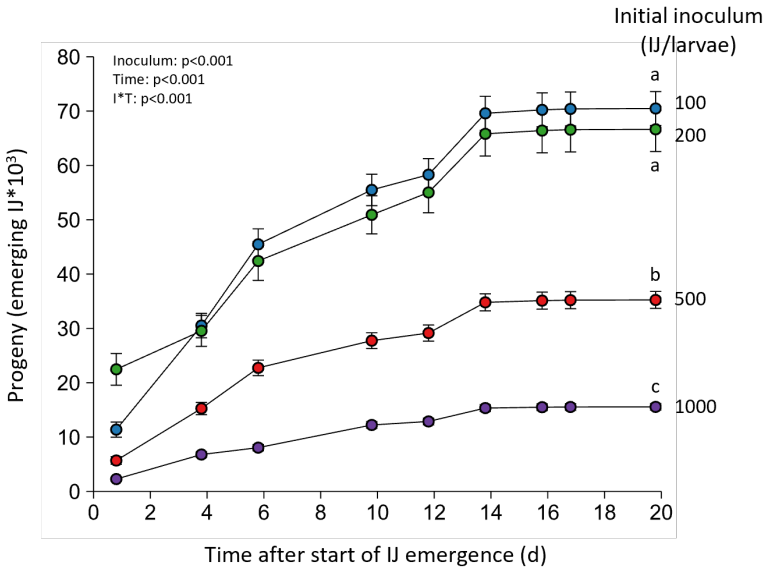

Figure 2. Excessively high inoculum size results in decrease of the reproductive output. Mean (± SEM) cumulative number of infective juvenile (IJ) nematodes emerging from *G. mellonella* larvae that were infected with 100. 200. 500. or 1000 IJ nematodes. Eight larvae per treatment were evaluated and the experiments were conducted two independent times (n=16). Different letters indicate statistically significant differences in total number of nematodes ($p<0.05$ by two-way repeated measures ANOVA with Holm's test for multiple comparisons).

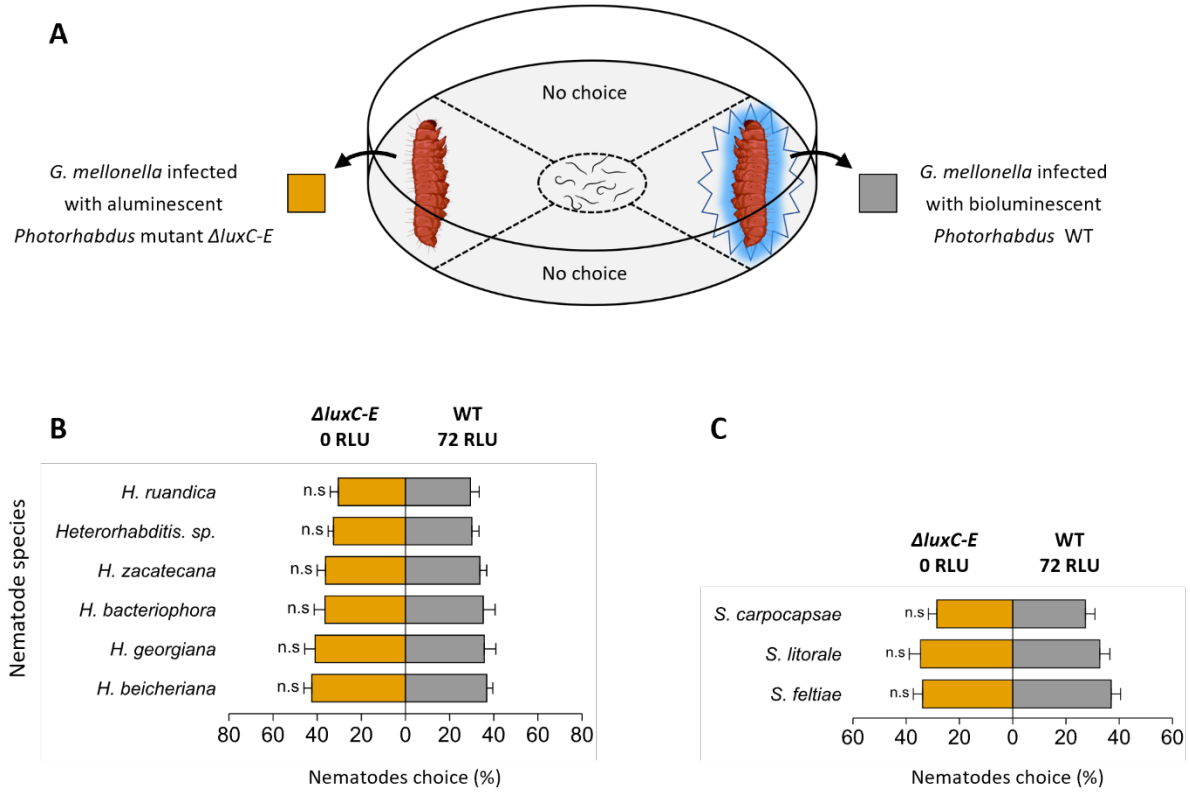

Figure 3. The foraging behavior of entomopathogenic nematodes is not affected by the bioluminescence produced by *Photorhabdus laumondii* subp. *laumondii* strain DJC-23. (**A**) Schematic representation of the experimental setup used. Proportions (Mean ± SEM) of (**B**) *Heterorhabditis* or (**C**) *Steinernema* nematodes orienting towards *G. mellonella* cadaver infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 bacteria (*ΔluxC-E)* or infected with their bioluminescent wild type counterparts (WT). Fifteen choice arenas. with each 60 nematodes. were assayed (n=15). Data were statistically assessed by generalized linear model followed by false discovery rate-corrected post hoc tests. n.s: not significant (p>0.05).

larvae or living *G. mellonella* larvae positioned next to luminescent beads. We tested two different intensities of luminescence that are within the range of the physiological levels of bioluminescence produced across the *Photorhabdus* genus (from 4 RLU for *P. aegyptia* strain BA1^T to 1033 RLU for *P. cinerea* strain DSM 19724^T, see chapter 1: "Regulation and evolution of *Photorhabdus* bioluminescence", page 47). The first intensity tested, designed as "intermediate", emitted 280 RLU, while the second, designed as "high", emitted 703 RLU. These intensities were respectively four times and ten times higher than the level of bioluminescence emitted by insects infected with *P. laumondii* subsp. *laumondii* strain DJC-23 bacteria (72 RLU, **Fig. S2**). To ensure precise determination of nematode choices, *G. mellonella* larvae were confined within glass vials with pierced lids, restricting their movement while allowing the diffusion of volatiles. Our observations revealed that certain *Heterorhabditis* and *Steinernema* species exhibited avoidance behavior towards insects positioned next to luminescent beads in an intensity-dependent manner (**Fig. 4**). Specifically, *H. georgiana* Hbb and *H. beicherriana* CN4 nematodes show higher responsiveness to luminescence than all other species, while *H. zacatecana* MEX-41, *H. bacteriophora* TT01, *S. carpocapsae* Andermatt, and *S. feltiae* Jakub nematodes responded exclusively to the highest luminescence level tested. Taken together, our results suggest that bioluminescence emitted by *Photorhabdus*-infected insects might serve as an occupancy signal used to prevent intra- and inter-specific competitions by certain EPN species and only at relatively high levels of bioluminescence. The luminescence levels emitted by luminescent beads fall within the spectrum of bioluminescence intensities produced by *Photorhabdus* strains, underscoring the biological relevance of this approach. Two of the tested species of *Steinernema* nematodes exhibit luminescence avoidance behavior under the highest intensity condition, despite our inability to identify a homolog of photosensors in these species. This indicates the potential presence of a light sensing system in *Steinernema* nematodes that does not rely on a homolog of GUR-3 or LITE-1.

Bioluminescence does not influence volatiles emitted by *Photorhabdus*-infected insects

Insects infected by EPNs emit specific volatiles derived from the bacterial metabolization of insect tissues. Foraging nematodes utilize these volatiles as cues to assess the suitability and quality of their future host. To validate our prior observations regarding the influence of *Photorhabdus* bioluminescence on foraging nematode behavior, we investigate whether

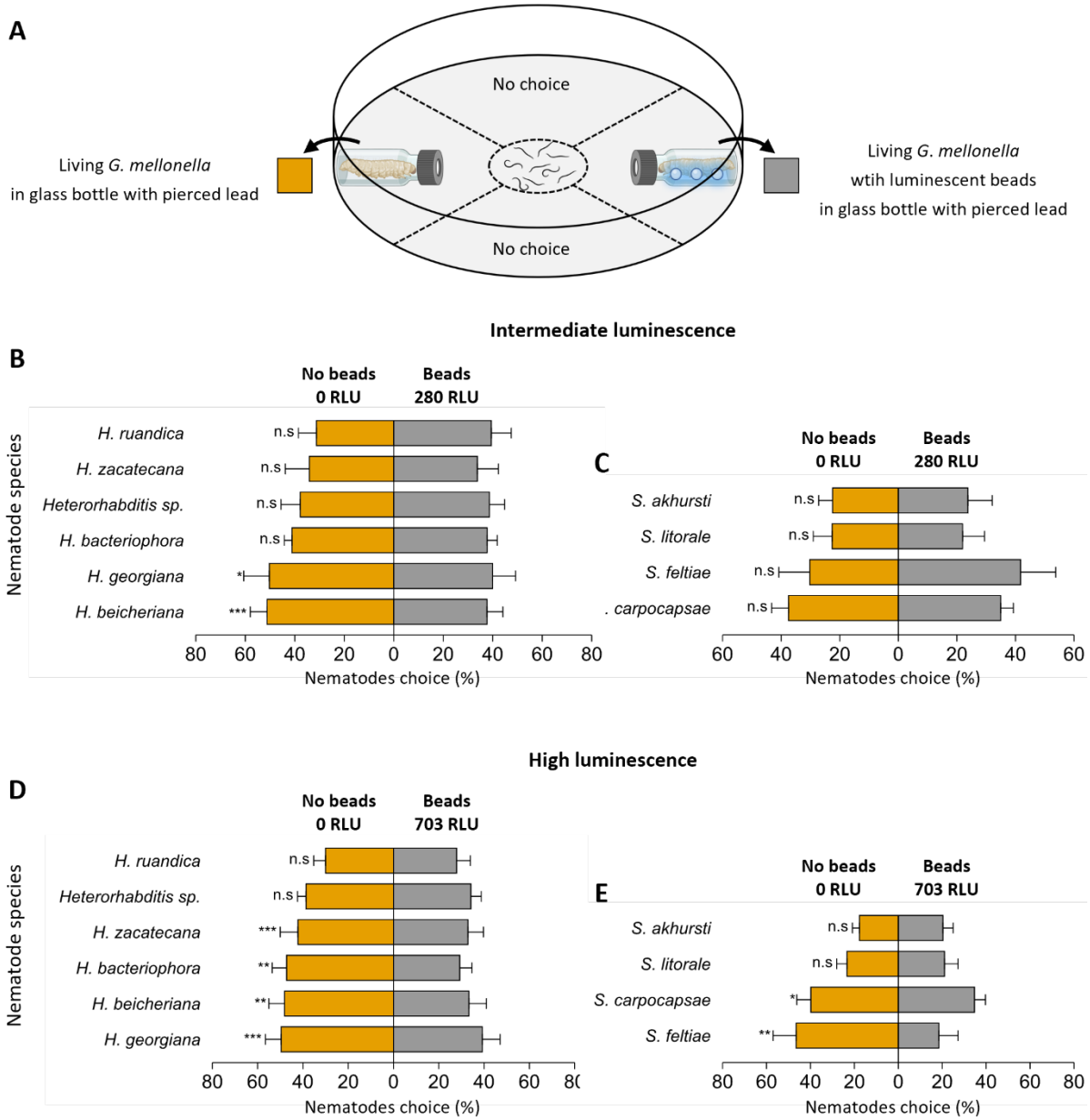

Figure 4. Luminescence repels foraging entomopathogenic nematodes in a species- and intensity-dependent manner. (**A**) Schematic representation of the experimental setup used. Living *G. mellonella* larvae were enclosed in glass bottles with pierced leads to restrict their movements. Proportions (Mean ± SEM) of (**B**) *Heterorhabditis* or (**C**) *Steinernema* nematodes orienting towards *G. mellonella* supplemented with beads producing intermediate luminescence or without beads. The same experiments were performed with beads producing high luminescence (**D** and **E**). Five choice arenas. with each 100 nematodes. were assayed and the experiments were conducted two independent times (n=10). Stars indicate statistically significant differences (n.s: p>0.05. *: p<0.05. **: p<0.01. ***: p<0.001 by generalized linear model followed by false discovery rate-corrected post hoc tests).

insect cadavers infected with bioluminescent and aluminescent bacterial strains differ in the quantity or quality of emitted volatiles. To address this question, we infected insects with either genetically engineered aluminescent *Photorhabdus* strains or their bioluminescent WT counterparts and collected the volatiles emitted by the infected cadavers over a five-day period. Our results reveal that both aluminescent and bioluminescent cadavers produce identical volatiles at similar emission rates (**Fig. 5**). This finding allows us to rule out the possibility of differential volatile emissions affecting the results of our choice experiments.

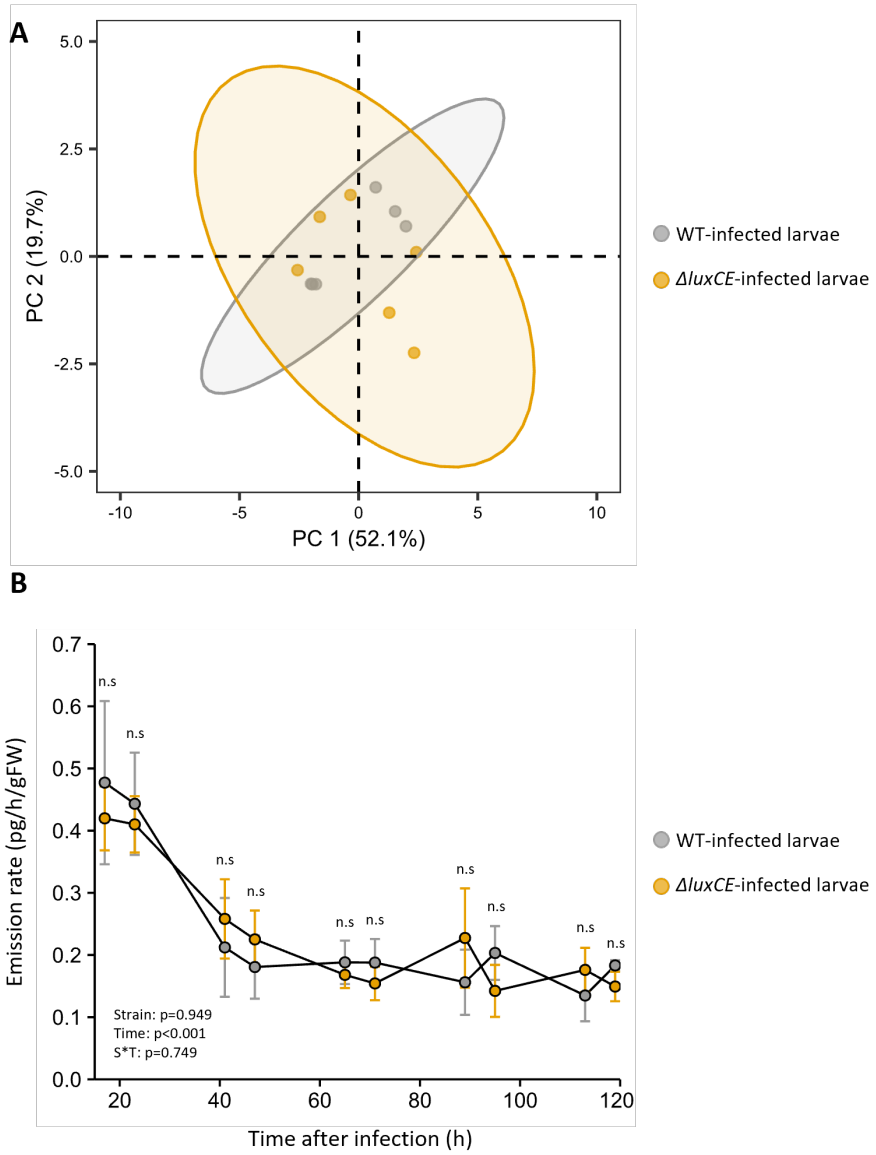

Figure 5. Bioluminescence does not influence volatile emissions of *Photorhadbus*-infected insects. (**A**) Principal component analysis of volatiles emitted by *G. mellonella* larvae infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 bacteria (*ΔluxC-E)* or infected with their bioluminescent WT counterparts (WT). (**B**) Mean (± SEM) emission rates of volatiles released by bacteria-infected *G. mellonella* larvae at different time points after infection. Volatiles were collected from six independent samples and each sample was composed of five larvae (n=6). Data were statistically assessed by two-way repeated measures ANOVA with Tukey HSD test for multiple comparisons. n.s: not significant (p>0.05).

Discussion

Infection of small arthropods by the *Heterorhabditis*/*Photorhabdus* pair results in the presence of bioluminescent infected organisms belowground (**Thomas and Poinar, 1979; Waterfield et al., 2009**). We hypothesize that host-seeking EPNs could use *Photorhabdus* bioluminescence as a signal of early-stage of infection by *Heterorhabditis* nematodes and adapt their foraging behavior accordingly. We investigated the genomes of EPNs to get a first hint whether they have the capability to detect *Photorhabdus* bioluminescence. We used a homology driven approach to identify EPNs proteins potentially involved in light perception and processing, based on current knowledge in the soil-dwelling relative *C. elegans*. Two photosensors sharing a high degree of similarity and belonging to the gustatory receptor family have been described in *C. elegans*: LITE-1 and GUR-3 (**Bhatla and Horvitz, 2015; Gong et al., 2016; Liu et al., 2010**). We identified a homolog of the photosensor GUR-3 in *Heterorhabditis* nematodes but not in *Steinernema* nematodes and speculated that this GUR-3 homolog could enable *Heterorhabditis* nematodes to perceive light. To process the signal produced by light perception, a functional phototransduction pathway is necessary. We identified several putative proteins involved in light signal processing in both *Heterorhabditis* and *Steinernema* species. *Steinernema* nematodes, symbiotically associated with aluminescent *Xenorhabdus* bacteria, lack a homolog of *C. elegans* photosensors while possessing putative light downstream signaling elements. The findings from this preliminary investigation suggest that while *Heterorhabditis* nematodes might possess all the elements necessary to sense and process *Photorhabdus* bioluminescence, it might not be the case for S*teinernema* nematodes. To further investigate this aspect, we utilized the sequence of the GUR-3 homolog from *H. bacteriophora* as a query to search for a homolog in *Steinernema* genomes. Given that *Steinernema* nematodes are more closely related to *Heterorhabditis* than to *C. elegans*, we anticipated a higher likelihood of obtaining relevant hits. However, we were unable to retrieve a homolog of GUR-3 in *Steinernema* genomes using this approach. It is possible that *Steinernema* nematodes possess a photosensor which is not homologous to the one of *C. elegans*, allowing them to perceive light signal.

We hypothesize that the bioluminescence produced by *Photorhabdus*-infected organisms might contribute to the avoidance of intra- and inter-specific competition by host-seeking EPNs. Subsequently to the investigation of EPNs capacity to sense light, we assessed whether

competition for a host can bring negative fitness consequences, by testing if the number of *Heterorhabditis* IJ individuals starting an infection affect the reproductive output. Our results confirmed previous findings indicating that for a number of approximately 100 individuals represented an optimal inoculum size to initiate the infection of *G. mellonella* larvae (**Selvan et al., 1993**). The presence of an optimum inoculum size supports the notion that conspecific infection can have either beneficial or detrimental effects, depending on the situation. Below the optimal inoculum size, conspecific infection may facilitate reaching the optimum size. Conversely, when the initial inoculum size is reached, conspecific infection could result in intraspecific competition, negatively impacting reproductive output (**Selvan et al., 1993; Zervos et al., 1991**). Experimental results from Selvan *et al*., suggest that excessively high inoculum size has a more adverse impact on reproductive output compared to inoculum sizes below the optimum (**Selvan et al., 1993**).

To test whether EPNs respond to bioluminescence emitted by *Photorhabdus*-infected insects, we performed choice assays with six strains of *Heterorhabditis* nematodes and three strains of *Steinernema* nematodes. In these assays, IJs were given the option to choose between aluminescent and bioluminescent insect cadavers. None of the tested EPNs preferentially moved toward or away from bioluminescent cadavers, indicating that in the tested conditions *Photorhabdus* bioluminescence does not influence nematode foraging behavior. Additionally, we demonstrated that aluminescent and bioluminescent insect cadavers produce qualitatively and quantitatively identical volatiles, eliminating the possibility of differential volatile production to affect our observations. Entomopathogenic nematodes exhibit different foraging strategies from highly motile cruisers to less motile ambushers (**Campbell and Gaugler, 1993**). *Heterorhabditis* IJs typically display high mobility and are classified as cruisers, whereas *S. feltiae* foraging behavior is classified as intermediate, and *S. carpocapsae* employs an ambushing strategy (**Campbell and Gaugler, 1997; Fallet et al., 2022; Gaugler et al., 1997; Selvan et al., 1993; Shapiro-Ilan et al., 2012**). According to the expected diversity of foraging behavior exhibited by the EPNs of our panel, it is unlikely that the generalized absence of response observed in our tests results from the immobility of IJs.

The bioluminescence produced by *Photorhabdus* bacteria is strain-specific and displays a broad spectrum of intensities (see chapter 1: “Regulation and evolution of *Photorhabdus*

bioluminescence", page 47). The *Photorhabdus* bacterial strain used in this study produces a mid-range intensity compared to other strains of this genus. To verify if higher level of bioluminescence intensity is necessary to induce a behavioral response in foraging nematodes, we performed a second series of choice assays. In this subsequent set of experiments, we allow IJs to choose between living *G. mellonella* larvae and living *G. mellonella* larvae positioned next to luminescent beads emitting two levels of luminescence: four times (intermediate) or ten times (high) higher than the one emitted by an insect infected by the bioluminescent *Photorhabdus laumondii* subsp. *laumondii* strain DJC-23 (WT). Only *H. georgiana* Hbb and *H. beicheriana* CN4 responded for both intensities while *H. zacatecana* MEX-41*, H. bacteriophora* TT01*, S. carpocapsae* Andermatt*,* and *S. feltiae* Jakub responded at the highest intensity. These results indicate that EPNs belonging to the genera *Heterorhabditis* and *Steinernema* respond to luminescence, revealing that EPNs are able to sense and respond to light signal. The fact that two species of *Steinernema* responded to luminescence suggests that a mechanism not relying on a photosensor homologous to the ones of *C. elegans* may be involved in light perception in this genus. The levels of luminescence emitted by luminescent beads are within the range of intensities produced by *Photorhabdus* strains suggesting that EPNs may be able to sense *Photorhabdus* bioluminescence.

The results from our two sets of experiments highlight a positive correlation between the intensity of (bio)luminescence and the number of EPNs species responding to this signal, which suggest that EPNs response to bioluminescence is intensity-dependent. All the strains which exhibited a response to (bio)luminescence exhibited avoidance of this signal. This behavior has multiple biological explanations. Host-seeking IJs may avoid insects infected by heterospecifics as they would need to compete for a resource that is partially depleted, risking the possibility of being outcompeted. Interspecific competition experiments reveal that some species tend to always outcompete other due to faster rate of development in the host (**Koppenhöfer et al., 1995**). While a compromised host might represent reduced risks to parasites, coexistence of different species of EPNs in the same host is unlikely because these organisms kill and consume their host, which would ultimately result in competition for resources (**Strand et al., 1990**). Entomopathogenic nematodes rely heavily on the development of their cognate bacterial symbionts inside their host for their own development and reproduction (**Adams et al., 2006; Mitani et al., 2004; Poinar and Thomas, 1966; Sicard**

et al., 2003). Parasitizing a host infected by heterospecifics in which non-cognate bacteria has already developed would unlikely resolved in successful development. On the other hand, infecting insects already infected by conspecifics can lead IJs to develop asynchronously with the nematodes already present in the host. This would likely result in the failure of IJs to undergo normal development. Moreover, competitive interactions impact both competitors negatively. In the case of conspecific competition, this would bring negative fitness consequences, which is evidenced by our investigation on the impact of the initial nematode inoculum size on reproductive output and previous study (**Grewal et al., 1997**). Nevertheless, we could postulate that the attraction of host-seeking nematodes to insect infected by conspecifics might be part of a cooperation strategy. It has been shown that the reproductive output is greatest when an optimum number of IJs initiate the infection (**Selvan et al., 1993; Zervos et al., 1991**). Attracting conspecifics during the early-stage of infection could serve to reach this optimum number of IJs within the host. Such mechanism could also lead to the attraction of conspecific individuals from other populations increasing gene flow during reproduction and reducing inbreeding. The results of our nematode behavioral experiments do not align with this latest hypothesis. On the contrary, our findings suggest that *Photorhabdus* bioluminescence acts as an early-infection signal, repelling host-seeking EPNs instead of attracting them. This mechanism might contribute to the avoidance of both intra- and inter-specific competition. We did not observe a response to (bio)luminescence in all the strains tested. Notably, *Heterorhabditis georgiana* Hbb and *H. beicheriana* CN4 were responsive at lower (bio)luminescence intensities compared to other EPNs. This result suggests that in addition to being intensity-dependent, EPNs response to bioluminescence is species-dependent.

In the environment, host-seeking EPNs sense multiple cues, including HIPVs that diffuse well in the soil matrix and can be very attractive for host-seeking EPNs (**Ali et al., 2010; Filgueiras et al., 2016; Kergunteuil et al., 2019; Laznik and Trdan, 2013, 2016a, 2016a; Rasmann et al., 2005; Rasmann and Turlings, 2008**). Previous experiments revealed that host-seeking EPNs favor location where multiple cues are originating and prefer HIPVs over herbivore odors alone (**Andaló et al., 2017; Tol et al., 2001**). The intrinsic characteristic of bioluminescence limits its dispersion though the soil matrix. Consequently, the ecological functions of bioluminescence belowground are likely to be confined to relatively short distances compared

to chemical cues such as HIPVs and volatiles emitted by organisms already infected by EPNs. Taken together, our results suggest that in the vicinity of an organism infected by *Heterorhabditis*, bioluminescence derived from *Photorhabdus* activity can be sensed by host-seeking EPNs in a species- and intensity-dependent manner. This bioluminescence repels host-seeking EPNs, contributing to the avoidance of intra- and inter-specific competition that bring negative fitness consequences.

Conclusions

Our work aims to test if bioluminescence emitted by *Heterorhabditis*-infected organisms deter host-seeking EPNs and contributes to the avoidance of intra- and inter-specific competition. As an initial step we confirmed that competition for a host reduce the reproductive output and, therefore, compromise the fitness of the species. Our investigation of EPN genomes suggest that *Heterorhabditis* nematodes possess all the elements necessary to sense and integrate light signal, while *Steinernema* nematodes might lack a photosensor. We pursue our work by conducting nematodes behavioral assays. None of the tested species responded to the bioluminescence emitted by insects infected by *P. laumondii* subsp. *laumondii* strain DJC-23 (72 RLU). Two out of ten tested species were repelled by the "intermediate" level of luminescence (280 RLU), while six out of ten tested species were repelled by the "high" level of luminescence (703 RLU). Our results suggest that EPNs, including *Steinernema* species, are able to sense and avoid bioluminescence in a species- and intensity-dependent manner, highlighting the adaptive nature of their response to (bio)luminescence in the context of foraging.

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Supplementary materials

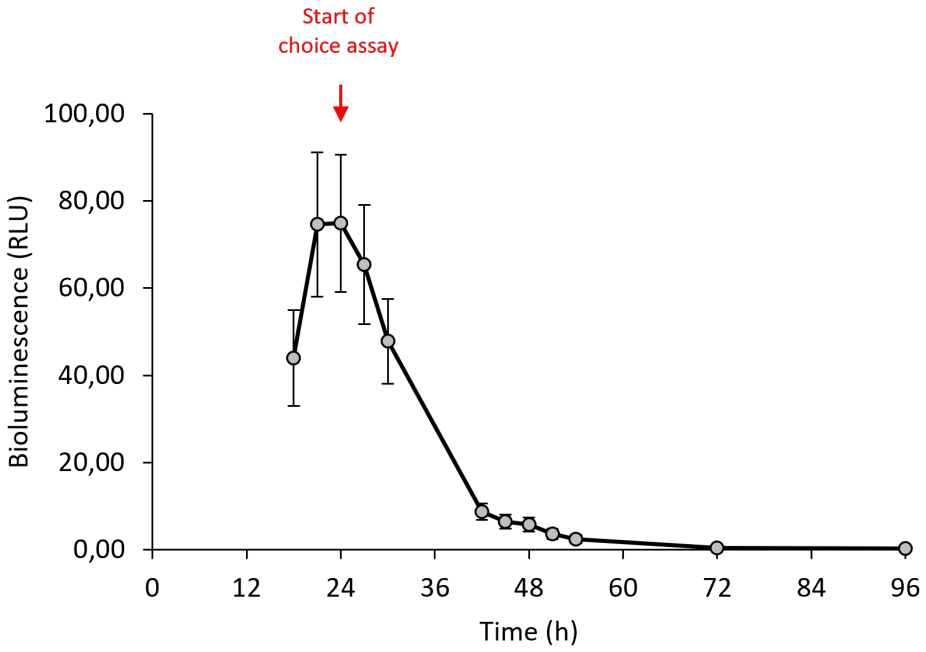

Figure S1. Kinetic curve of bioluminescence production *in vivo* in bioluminescent wild type *Photorhabdus* strain. Bioluminescence begins to be emitted within 18 h after the start of infection and reaches maximum intensity at approximately 24 h post-infection. After this peak, bioluminescence production gradually decreases, disappearing between 48-72 h post-infection. Nematode choice assays were conducted 24 h post-infection to maximize the potential impact of the bioluminescent signal on the behavior of host-seeking entomopathogenic nematodes. Bioluminescence levels were measured in six infected *Galleria mellonella larvae*, and the experiments were repeated independently twice (n=12). The bacteria used was *P. laumondii* subsp. *laumondii* WT strain DJC-23. RLU: relative light units.

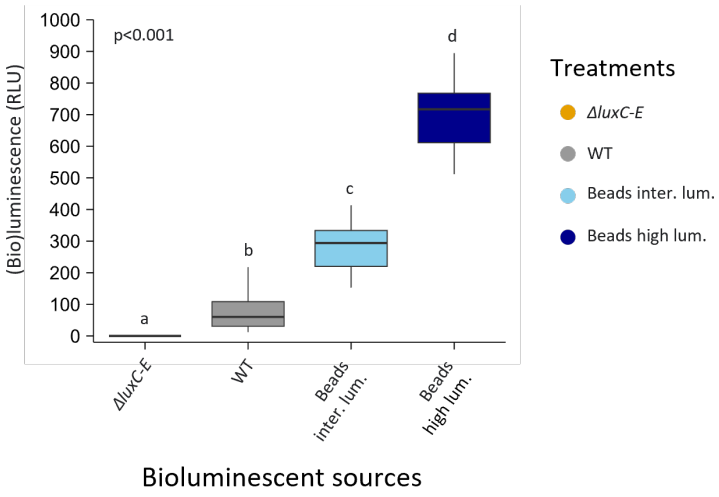

Figure S2. Levels of (bio)luminescence emitted by the different (bio)luminescent sources used for the nematodes choice assays. (Bio)luminescence levels emitted by luminescent beads exposed to sunlight for 30 s (beads intermediate luminescence). for 120 s (beads high luminescence). or by *G. mellonella* larvae infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 bacteria (*ΔluxC-E)* or infected with their bioluminescent WT counterparts (WT). Box plots show minimum. first quartile. median. third quartile. and maximum values. (Bio)luminescence levels were measured in twelve replicates for luminescent beads and in twenty-four replicates for infected *G. mellonella* (n=12-24). Different letters indicate statistically significant differences in (bio)luminescence emission levels ($p<0.05$ by one-way ANOVA with Tukey HSD test for multiple comparisons). RLU: relative light units.

Table S1. Description of the procedure used to retrieve the sequences of protein putatively involved in bioluminescence perception in entomopathogenic nematodes. Basic local alignment search tool was used to retrieve homologs of *C. elegans* proteins in entomopathogenic nematodes. 1 and 2: all the genomes and sequences used for this investigation are available within the electronic form of this thesis.

Protein name	Template[1]	Accession number template	Query[2]	Accession number query
H. bacteriophora GUR-3	Genome 1	Our data	*C. elegans* GUR-3	NP_509743.2
H. beicheriana GUR-3	Genome 2	Our data	*C. elegans* GUR-3	NP_509743.2
H. georgiana GUR-3	Genome 3	Our data	*C. elegans* GUR-3	NP_509743.2
H. ruandica GUR-3	Genome 4	Our data	*C. elegans* GUR-3	NP_509743.2
H. zacatecana GUR-3	Genome 5	Our data	*C. elegans* GUR-3	NP_509743.2
Heterorhabditis sp. GUR-3	Genome 6	Our data	*C. elegans* GUR-3	NP_509743.2
H. bacteriophora GPA-3	Genome 1	Our data	*C. elegans* GPA-3	NP_001309498.1
H. beicheriana GPA-3	Genome 2	Our data	*C. elegans* GPA-3	NP_001309498.1
H. georgiana GPA-3	Genome 3	Our data	*C. elegans* GPA-3	NP_001309498.1
H. ruandica GPA-3	Genome 4	Our data	*C. elegans* GPA-3	NP_001309498.1
H. zacatecana GPA-3	Genome 5	Our data	*C. elegans* GPA-3	NP_001309498.1
Heterorhabditis sp. GPA-3	Genome 6	Our data	*C. elegans* GPA-3	NP_001309498.1
S. carpocapsae GPA-3	Genome 7	GCA_000757645.3	*C. elegans* GPA-3	NP_001309498.1
S. feltiae GPA-3	Genome 8	GCA_007213375	*C. elegans* GPA-3	NP_001309498.1
H. bacteriophora GOA-1	Genome 1	Our data	*C. elegans* GOA-1	NP_492108.1
H. beicheriana GOA-1	Genome 2	Our data	*C. elegans* GOA-1	NP_492108.1
H. georgiana GOA-1	Genome 3	Our data	*C. elegans* GOA-1	NP_492108.1
H. ruandica GOA-1	Genome 4	Our data	*C. elegans* GOA-1	NP_492108.1
H. zacatecana GOA-1	Genome 5	Our data	*C. elegans* GOA-1	NP_492108.1
Heterorhabditis sp. GOA-1	Genome 6	Our data	*C. elegans* GOA-1	NP_492108.1
S. carpocapsae GOA-1	Genome 7	GCA_000757645.3	*C. elegans* GOA-1	NP_492108.1
S. feltiae GOA-1	Genome 8	GCA_007213375	*C. elegans* GOA-1	NP_492108.1
H. bacteriophora ODR-1	Genome 1	Our data	*C. elegans* ODR-1	NP_001362115.1
H. beicheriana ODR-1	Genome 2	Our data	*C. elegans* ODR-1	NP_001362115.1
H. georgiana ODR-1	Genome 3	Our data	*C. elegans* ODR-1	NP_001362115.1
H. ruandica ODR-1	Genome 4	Our data	*C. elegans* ODR-1	NP_001362115.1
H. zacatecana ODR-1	Genome 5	Our data	*C. elegans* ODR-1	NP_001362115.1
Heterorhabditis sp. ODR-1	Genome 6	Our data	*C. elegans* ODR-1	NP_001362115.1
S. carpocapsae ODR-1	Genome 7	GCA_000757645.3	*C. elegans* ODR-1	NP_001362115.1

S. feltiae ODR-1	Genome 8	GCA_007213375	*C. elegans* ODR-1	NP_001362115.1
H. bacteriophora TAX-2	Genome 1	Our data	*C. elegans* TAX-2	NP_492427.3
H. beicheriana TAX-2	Genome 2	Our data	*C. elegans* TAX-2	NP_492427.3
H. georgiana TAX-2	Genome 3	Our data	*C. elegans* TAX-2	NP_492427.3
H. ruandica TAX-2	Genome 4	Our data	*C. elegans* TAX-2	NP_492427.3
H. zacatecana TAX-2	Genome 5	Our data	*C. elegans* TAX-2	NP_492427.3
Heterorhabditis sp. TAX-2	Genome 6	Our data	*C. elegans* TAX-2	NP_492427.3
S. carpocapsae TAX-2	Genome 7	GCA_000757645.3	*C. elegans* TAX-2	NP_492427.3
S. feltiae TAX-2	Genome 8	GCA_007213375	*C. elegans* TAX-2	NP_492427.3
H. bacteriophora TAX-4	Genome 1	Our data	*C. elegans* TAX-4	NP_499033.1
H. beicheriana TAX-4	Genome 2	Our data	*C. elegans* TAX-4	NP_499033.1
H. georgiana TAX-4	Genome 3	Our data	*C. elegans* TAX-4	NP_499033.1
H. ruandica TAX-4	Genome 4	Our data	*C. elegans* TAX-4	NP_499033.1
H. zacatecana TAX-4	Genome 5	Our data	*C. elegans* TAX-4	NP_499033.1
Heterorhabditis sp. TAX-4	Genome 6	Our data	*C. elegans* TAX-4	NP_499033.1
S. carpocapsae TAX-4	Genome 7	GCA_000757645.3	*C. elegans* TAX-4	NP_499033.1
S. feltiae TAX-4	Genome 8	GCA_007213375	*C. elegans* TAX-4	NP_499033.1

Table S2. *Heterorhabditis* nematodes have homologs of the genes coding for *Caenorhabditis elegans* photosensor and phototransduction proteins in their genomes. Amino acid sequences of *C. elegans* genes were downloaded from the National Center for Biotechnology Information website. We used the basic local alignment search tool of Bioedit 7.2.5 to identify *Heterorhabditis* and *Steinernema* homologs using *C. elegans* proteins and *Heterorhabditis* and *Steinernema* annotated genomes. Similarity scores were computed in Bioedit 7.2.5 using the substitution matrix BLOSUM62. Accession numbers of the sequences of *C. elegans*. *S. carpocapsae* and. *S. feltiae* are listed in Table S1. N.A.: not applicable.

	Similarity scores of homologs with *C. elegans* proteins (%)							
Nematode strains	**LITE-1**	**GUR-3**	**DAF-1**	**GPA-3**	**GOA-1**	**ODR-1**	**TAX-2**	**TAX-4**
H. Bacteriophora TT01	N.A.	71	N.A.	88	98	37	32	60
H. beicheriana CN4	N.A.	62	N.A.	77	99	37	32	54
H. georgiana Hbb	N.A.	70	N.A.	88	99	37	32	54
H. ruandica Rw14_N-C4a	N.A.	70	N.A.	88	99	26	31	62
H. zacatecana MEX-41	N.A.	70	N.A.	88	99	42	58	59
Heterorhabditis sp. S10	N.A.	70	N.A.	88	99	37	21	39
S. carpocapsae Andermatt	N.A.	N.A.	N.A.	82	83	42	58	59
S. feltiae JAKUB	N.A.	N.A.	N.A.	84	74	45	58	56

CHAPTER 3
Roles of *Photorhabdus* bioluminescence in the symbiosis with *Heterorhabditis* nematodes

Abstract

Photorhabdus bacteria and *Heterorhabditis* nematodes form a symbiotic pair highly pathogenic to small arthropods, including insects. During the first step of the infection, *Photorhabdus* bacteria grow exponentially leading to the rapid insect death, while the nematodes remain in an arrested developmental stage called infective juvenile (IJ). Subsequently, *Photorhabdus* bacteria switch from pathogenic to symbiosis-centered metabolism and *Heterorhabditis* nematodes undergo development into adulthood. *Heterorhabditis* nematodes rely on specialized metabolites produced by *Photorhabdus* bacteria for their development and reproduction. Therefore, synchronization between IJs recovery and *Photorhabdus* bacteria metabolic transition is crucial for successful symbiosis. Current knowledge suggests significant roles for 3,5-dihydroxy-4-isopropyl-trans-stilbene (IPS) and alarmone, also called (p)ppGpp, in this process. *Photorhabdus* bacteria are unique in their ability to produce bioluminescence belowground. The timing of bioluminescence production, coupled with findings establishing connections between (p)ppGpp, IPS, and bioluminescence productions, suggest potential roles for bioluminescence in the symbiotic relationship between *Photorhabdus* bacteria and *Heterorhabditis* nematodes. To investigate these putative roles, we performed symbiotic assays and metabolomic analyses with aluminescent *Photorhabdus* mutant strains and their bioluminescent wild-type (WT) counterparts. Our results indicate that *Heterorhabditis* nematodes grown on lawns of aluminescent mutant strains do not recover into adulthood. These aluminescent mutant strains exhibit altered secondary metabolites production, including depletion in IPS production. Summarizing current knowledge and incorporating our findings, we propose a regulatory framework in which bioluminescence prime IJs to undergo recovery and initiate bacterial metabolic switch by promoting IPS production, ensuring the synchronization of these two processes necessary for successful symbiosis.

Introduction

Photorhabdus bacteria live in symbiosis with soil-dwelling nematodes from the genus *Heterorhabditis* (**Boemare et al., 1993; Thomas and Poinar, 1979**). Together, they form a pair that is highly pathogenic to small arthropods, including insects (**Thomas and Poinar, 1979**). *Heterorhabditis* nematodes in the developmental arrested stage, infective juvenile (IJ), harbor *Photorhabdus* bacteria in their intestine and release them into the hemocoel of their prey (**Ciche and Ensign, 2003**). *Photorhabdus* bacteria multiply exponentially and produce toxins causing rapid host death by septicemia and toxemia (**Bowen et al., 1998**). Subsequently, *Photorhabdus* bacteria enter into stationary growth phase and initiate a metabolic switch from insect pathogenicity toward symbiosis with *Heterorhabditis* nematodes (**Clarke, 2016, 2014; Joyce et al., 2011; Lango and Clarke, 2010**). Concurrently, *Heterorhabditis* nematodes that remained in the IJ stage undergo development to reach adulthood through a process known as recovery (**Strauch and Ehlers, 1998**). *Heterorhabditis* nematodes feed on the rich mixture of bacterial biomass and pre-digested insect tissue and relies on the production of essential metabolites by *Photorhabdus* for normal development and reproduction (**Bintrim and Ensign, 1998; Joyce et al., 2008**). The synchronization between *Photorhabdus* bacteria metabolic transition and *Heterorhabditis* nematodes recovery is a key element of the successful symbiosis between these two organisms. *Photorhabdus* bacteria are unique in their ability to produce bioluminescence belowground (**Thomas and Poinar, 1979; Waterfield et al., 2009**). *Photorhabdus* bacteria start to emit bioluminescence before the death of the prey, quickly reach a maximum in intensity, concomitant with a metabolic transition, and decrease during stationary growth phase. This intriguing timing suggest a potential function of bioluminescence in the symbiosis between *Photorhabdus* bacteria and *Heterorhabditis* nematodes.

Throughout its dualistic lifestyle, *Photorhabdus* bacteria must engage in both insect pathogenicity and symbiosis with *Heterorhabditis* nematodes in a specific timing. To fulfill these roles, they produce a wide variety of secondary metabolites in a time-dependent manner (**Shi and Bode, 2018**). *Photorhabdus* mutant deficient in the global post transcriptional regulator Hfq are impaired in secondary metabolites production and unable to establish normal symbiosis with *Heterorhabditis* nematodes. This finding highlight the

importance of *Photorhabdus* secondary metabolites for normal symbiosis with *Heterorhabditis* nematodes (**Langer et al., 2017; Tobias et al., 2017**). *Photorhabdus* secondary metabolites are mainly produced by non-ribosomal peptide synthetases (NRPSs) or polyketide synthases (PKSs ; **Bode, 2009**). *Photorhabdus* bacteria mutated in the *ngrA* gene that encodes phosphopantetheinyl transferase essential for NRPSs and PKSs activities are impaired in the production of secondary metabolites and unable to support nematode growth and development. This mutation hindered the production of 3,5-dihydroxy-4-isopropyl-trans-stilbene (IPS) and siderophore (**Aumann and Ehlers, 2001; Watson et al., 2005**). *Photorhabdus temperata* strain K122 bacteria with mutation in the *exbD* gene, responsible for siderophore production, display reduced iron uptake and fail to support nematode growth and development. However, a mutation of *exbD* in *Photorhabdus laumondii* subsp. *laumondii* strain TT01 has no discernible effect, complicating the interpretation of the role of siderophore in maintaining normal symbiosis with *Heterorhabditis* nematodes (**Watson et al., 2010**). Stilbenes, including IPS, are compounds typically associated with plants, and *Photorhabdus* bacteria, along with *Bacillus* bacteria, are the only known non-plant organisms capable of synthesizing this class of metabolites (**Clarke, 2014; Kumar et al., 2012; Paul et al., 1981**). Symbiotic assays conducted *in vitro* revealed a significant decrease in the recovery of IJs grown on lawns of *Photorhabdus* bacteria impaired in IPS production. Complementation with an ectopic addition of IPS partially restored IJ recovery. These findings suggest that, while IPS plays a crucial role, it is not sufficient alone for normal nematode development (**Joyce et al., 2008**). In *Photorhabdus,* the first step in the biosynthesis of IPS is the production of cinnamic acid by the phenylalanine-ammonium lyase encoded by the *stlA* gene. This step is negatively correlated with the availability of nutrients for the bacterial population, resulting in the production of IPS concomitant with the entry of *Photorhabdus* bacteria into the stationary growth phase. This event aligns with the initiation of IJ recovery process leading to the hypothesis that IPS may serves as a food signal (**Eleftherianos et al., 2007; Lango-Scholey et al., 2013**). Inside their prey, *Heterorhabditis* nematodes remain in arrested development stage until *Photorhabdus* bacteria enter stationary growth phase. *Photorhabdus* bacteria initially grow exponentially and prioritize pathogenicity towards the insect. Following the insect death, the bacteria undergo a metabolic shift towards a symbiosis-centered state, thereby producing essential elements to support the growth and development of nematodes. The current view is that *Photorhabdus* bacteria produce a food signal informing

Heterorhabditis nematodes that all requirements are met to support their development and growth (**Aumann and Ehlers, 2001**).

The synchronization of *Photorhabdus* bacteria metabolic transition and *Heterorhabditis* IJs recovery process is a key element of successful symbiosis. The alarmone (p)ppGpp, a signaling molecule accumulated upon nutrient limitation, might play an important role in this process. Transcriptional fusion experiments indicate that (p)ppGpp accumulation is required to promote *stlA* expression, linking nutrient limitation and IPS production (**Bager et al., 2016**). Similarly to (p)ppGpp accumulation, bioluminescence intensity produced by *Photorhabdus* bacteria rise during exponential growth phase and reach a maximum coincidentally with the metabolic transition towards symbiosis. The intriguing timing suggest potential roles of bioluminescence in the initiation of *Photorhabdus* bacteria metabolic switch or/and the recovery of *Heterorhabditis* nematodes. Here, we propose two mechanisms through which *Photorhabdus* bioluminescence could be involved in the symbiosis with *Heterorhabditis* nematodes. First, bioluminescence might be directly sensed by IJs, priming them to undergo the recovery process. *Heterorhabditis* nematodes possess homolog of the *Caenorhabditis elegans* photosensor and homologs of proteins involved in light signaling, suggesting that *Heterorhabditis* nematodes are able to sense light (**Bhatla and Horvitz, 2015; Gong et al., 2016; Liu et al., 2010; Ward et al., 2008**). Second, bioluminescence might be sensed by *Photorhabdus* bacteria themselves and trigger the metabolic switch driving to the subsequent production of the food signal, most likely IPS. Many nonphototrophic bacteria possess functional photosensors that help the bacteria to make important lifestyle decisions such as transition from single to multicellular biofilm or promotion of virulence (**Mussi et al., 2010; Purcell and Crosson, 2008; Tschowri et al., 2009; Van Der Horst et al., 2007**). While the presence of photosensors in *Photorhabdus* bacteria genomes has not been studied, it is plausible based on the widespread occurrence of bacterial photosensors predicted through bioinformatic analyses (**Gomelsky and Hoff, 2011**). The ability of *Photorhabdus* bacteria to sense bioluminescence is speculated, but findings presented in chapter 2 of this thesis suggest that *Heterorhabditis* nematodes sense bioluminescence in a species- and intensity-dependent manner (see chapter 2: “Effect of *Photorhabdus* bioluminescence on the foraging behavior of entomopathogenic nematodes”, page 97). Importantly, the two mechanisms we proposed do not conflict with previous findings. First, the level of (p)ppGpp might positively regulate the

emission of bioluminescence. Thus, (p)ppGpp might act upstream of bioluminescence to modulate symbiosis. This is supported by the timing of bioluminescence production, coinciding with (p)ppGpp accumulation, and the inability of mutant impaired in (p)ppGpp production to produce bioluminescence (**Bager et al., 2016**). Next, putative roles of bioluminescence do not exclude the requirement of IPS production to support nematode growth and development. We hypothesize that the two mechanisms we proposed exist concurrently. *Photorhabdus* bioluminescence might initiate the bacterial metabolic transition toward symbiosis and prime the IJ recovery, ensuring the synchronization of these two processes.

We tested our hypotheses by initially evaluating the capacity of aluminescent *Photorhabdus* bacteria to support nematode growth and development. To achieve this objective, we conducted *in vitro* symbiotic assays with *Heterorhabditis bacteriophora* nematodes, genetically modified aluminescent *Photorhabdus* strains, and their bioluminescent WT counterparts. Subsequently, we investigated potential alterations in the metabolism of aluminescent bacteria. This part involved both untargeted metabolomic analyses and IPS quantification, utilizing insects infected with either aluminescent or bioluminescent *Photorhabdus* strains. This work provides first insights into the potential function of *Photorhabdus* bioluminescence in the symbiotic relationship with *Heterorhabditis* nematodes and contributes to a better understanding of the fascinating lifestyle characteristic of these two organisms.

Material and methods

Photorhabdus culture

The naturally bioluminescent *Photorhabdus laumondii* subsp. *laumondii* strain DJC-23 and its relative genetically-engineering aluminescent Δ*luxA,* Δ*luxC,* Δ*luxE,* and Δ*luxC-E* mutant strains were used for this work. To maintain these five strains, they were cultured onto lysogenic broth (LB) agar plates (Sigma-Aldrich, USA) and incubated at 28°C. Every thirty days the bacteria were refreshed from glycerol stocks. The aluminescent *Photorhabdus* mutants were engineered in our laboratory, as described previously, by removing specific *lux*-operon genes (*luxA, luxC, luxE*), or the entire *lux*-operon, by allele exchange mutagenesis, from the model strain *Photorhabdus laumondii* subsp. *laumondii* WT strain DJC-23 (**Brachmann et al., 2007; Easom and Clarke, 2008**). To verify the fitness of these aluminescent mutant *Photorhabdus* strains, kinetic curves of their growth were performed *in vitro***.** The aluminescent mutant strains do not show altered growth compared to the bioluminescent WT strain (**Fig. S1**).

Nematodes rearing

The nematode strain *Heterorhabditis bacteriophora* TT01 was used for this work. Nematodes were stored in IJ developmental stage in 50 mL of water in culture flasks at 10-13°C in darkness. Every two months the cultures were refreshed by infecting third-instar *Galleria mellonella* larvae with 100 IJs and collecting the emerging the progeny using the White trap method (**White, 1927**).

Photorhabdus symbiotic capability evaluation

To evaluate whether bioluminescence production is required for the establishment and maintenance of symbiotic relationships between *Photorhabdus* and *Heterorhabditis* nematodes, we evaluated nematode development *in vitro* in the presence of bioluminescent or aluminescent strains, as described previously (**Addis et al., 2014**). Briefly, we cultured *H. bacteriophora* TT01 nematodes in semi-solid nematode growth gelrite (NGG) medium previously inoculated with either bioluminescent WT *P. laumondii* subsp. *laumondii* strain DJC-23 or with its relative genetically-engineering aluminescent Δ*luxA,* Δ*luxC,* Δ*luxE,* and Δ*luxC-E* mutant strains. The different bacterial strains were grown overnight in liquid LB medium. Thirty mL of the resulting bacterial cultures (OD_{600}=1) were then centrifuged, and the pellets were re-suspended in K-medium (3.1 g/L NaCl, 2.4 g/L KCl). After mixing, the cultures were

centrifuged again to remove all remaining K-medium. The resulting bacterial pellets were mixed with a similar volume of semi-solid phase nematode growth gelrite (NGG) medium (0.5 g casein peptone, 1.5 g NaCl, 0.75 g gelrite, 500 µL $CaCl_2 \cdot 2H_2O$ (147 g/L), 500 µL $MgSO_4 \cdot 7H_2O$ (246.6 g/L), 12.5 mL KH_2PO_4 (136 g/L) and 500 µL cholesterol (1 g/L in 99% ethanol) in 1 L distilled water). Then, 1.5 mL of the semi-solid NGG with bacteria was spread onto the surface of a 5 mm solid phase NGG layer (the same as semi-solid NGG but containing 1.5 g gelrite instead) in a sterilized Petri-dish (6 cm diameter, Greiner Bio-One GmbH, Frickenhausen, DE). After incubating at 28 °C for 48 h, one hundred IJs were released on the resulting semi-solid bacterial cultures. Nematode cultures were monitored daily and the numbers of developing adults and hermaphrodites were determined 3 days, 5 days, and 7 days after IJs were placed on the medium. Three nematode cultures per bacterial strain were established and the experiments were conducted two independent times (n=6).

***In vivo Photorhabdus* untargeted metabolomic analyses**

To assess whether aluminescent *Photorhabdus* mutant strains were impaired in secondary metabolites production, we carried out small molecular weight metabolomic analyses with insects infected with either aluminescent *Photorhabdus* strains or with their relative bioluminescent WT counterparts. Infected insects were flash frozen and then ground into a fine powder under liquid nitrogen. One hundred milligrams of the resulting powder were extracted using 0.5 mL of methanol:water:formic acid buffer (80:19.9:0.1 v/v/v). The sample extracts were then analyzed by ultra-high performance liquid chromatography - quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) using an Acquity UPLC™ I-Class coupled to a Synapt XS (Waters, Milford, USA). The system was controlled by MassLynx v.4.2. An Acquity UPLC™ HSS T3 column (100 × 2.1 mm, 1.8 µm) maintained at 25 °C was used at a flow rate of 0.5 ml/min. Mobile phases consisted of H_2O + 0.05% formic acid (solvent A) and acetonitrile + 0.05% formic acid (solvent B). A linear gradient from 0 to 100% B in 10.0 min was applied. The injection volume was 0.3 µl. MS detection was performed in positive electrospray ionization and using data-dependent acquisition (DDA). The resolution was set to 22'000 (at *m/z* 556). Data were acquired in continuum mode. The capillary voltage was set to 1.0 kV, the cone voltage to 25V, the source temperature to 140 °C, the desolvation temperature to 500 °C, the desolvation gas flow to 1000 L/h, the cone gas flow to 150 L/h, the nebulizer gas flow to 6.5 bars, and the collision gas (Ar) flow to 2.0 L/min. DDA settings were as follows: MS1

mass range and scan time 50–1200 Da and 0.1 s, respectively, top 7 MS/MS (0.05 s scan time each), intensity threshold 25'000 counts per s, MS/MS selection window 4 Da, peak deisotoping activated, dynamic exclusion of 1.5 s after acquisition. An exclusion list of the 200 most intense background ions was generated from a blank sample run just before the samples. For MS/MS acquisition, a ramped collision energy of 5-40 V (at *m/z* 50) and 20-70 V (at *m/z* 1200) was set. Quality control samples were prepared by pooling aliquots of all samples and run 4 times before the batch and about every 30 samples during the batch. The raw data were noise-reduced in MassLynx and imported into Progenesis QI (Waters) for peak picking. The following parameters in Progenesis QI were used: automatic determination of sensitivity (level 2), peak width 0.04 min, retention time limits 1.5-10 min, adducts $(M+H)^+$, $(M+NH_4)^+$, $(M+Na)^+$, $(2M+H)^+$, $(2M+Na)^+$. The generated feature list (.csv) and MS/MS fragment list (.msp) were exported to GNPS (https://gnps.ucsd.edu) to create a molecular network using the Feature-Based Molecular Networking (FBMN) workflow. The following parameters were used: mass tolerances of both precursor ion and MS/MS fragment ions 0.02 Da, minimum pairs cosine score 0.7, minimum matched fragment ions 6, network TopK 10, maximum connected component size 100, maximum shift between precursors 500 Da. The MS/MS spectra present in the network were then searched against GNPS spectral libraries. Information about the identification of metabolites that were differentially expressed across the treatments are presented in Table 1. Venn diagrams illustrating the number of differentially produced metabolites by the different treatments were created using the R package "ggvenn", and Principal component analyses (PCA) were carried out using the R package "factoextra" (**Kassambara and Mundt, 2020; Yan, 2021**). Metabolites produced *in vivo* by *Photorhabdus* bacteria were assessed in a total of three insects per treatment (n=3).

Stilbene quantification

To determine whether *in vivo* production of IPS was altered in aluminescent *Photorhabdus* mutant strains compared to their relative bioluminescent WT counterparts, we performed a targeted IPS quantification. The samples from the untargeted metabolomic analyses (see section: "*In vivo Photorhabdus* untargeted metabolomic analyses", page 140) were used but analyzed by ultrahigh performance liquid chromatrography-tandem mass spectrometry (UHPLC-MS/MS). Specifically, an Acquity UPLC I-Class (Waters) was coupled to a TQ-XS triple quadrupole (Waters) controlled by Masslynx 4.2 (Waters). The separation was performed on

an Acquity BEH C18 (50x2.1mm, 1.7 µm) column (Waters) in gradient mode. The mobile phases were H_2O + 0.05% formic acid (A) and acetonitrile + 0.05% formic acid (B) and the flow rate was 0.4 mL/min. The gradient started at 10% B and increased linearly to 70% B in 6 min, followed by a 1 min wash at 100% B and a 2 min reequilibration step at 10% B. The injection volume was 1 µL. The column temperature was 25°C. The mass spectrometer was used in positive electrospray ionization. The capillary voltage was +2kV, the cone voltage was +5V, the desolvation gas temperature and flow were 600°C and 1000 L/h respectively, and the cone gas flow was 350 L/h. Three MRM transitions were used: m/z 255/213 (CE 14 V), m/z 255/135 (CE 21 V), and m/z 255/107 (CE 26 V). TargetLynx XS (Waters) was used to process the data. A calibration curve with points at 1, 5, 20 and 50 ng/mL was built. Samples were diluted between 10 and 10'000-fold before injection depending on their concentrations. The production of IPS *in vivo* by *Photorhabdus* bacteria were assessed in a total of three insects per treatment (n=3).

Statistical analyses

Statistic tests used to assess the different datasets are described in more detail in the figure legends. Normality and equality of variance were verified using Shapiro-Wilk and Levene's tests, respectively using Sigma Plot 14.5 (Systat Software Inc., San Jose, CA, USA). Symbiotic capability of *Photorhabdus* strains and metabolite levels were statistically assessed by two-way repeated measures ANOVA followed by Holm's test for multiple comparisons using Sigma Plot 14.5 (Systat Software Inc., San Jose, CA, USA). More information about *Photorhabdus* metabolomic data are available in section: "*In vivo Photorhabdus* untargeted metabolomic analyses").

Results

Bioluminescence is required for normal development of *Heterorhabditis* nematodes *in vitro*

To test whether bioluminescence produced by *Photorhabdus* bacteria is required for normal development and growth of *Heterorhabditis* nematodes, we evaluate the symbiotic capability of aluminescent *Photorhabdus* bacterial strains. We cultured *Heterorhabditis* nematodes *in vitro* on lawns of genetically engineered aluminescent *Photorhabdus* strains, or on lawns of their bioluminescent WT counterparts and monitored the numbers of developing adult and hermaphrodite nematodes (**Fig. 1**). Our results indicate that after three days of culture IJs had started to develop into adults when placed on NGG medium inoculated with WT bioluminescent bacteria, while the first developing adults were observed after five days on NGG medium inoculated with the aluminescent Δ*luxA*, Δ*luxC,*and Δ*luxE* strains. In addition to the delayed timing of appearance, the number of developing adults was significantly reduced in plates inoculated with the aluminescent *ΔluxA*, *ΔluxC,*and *ΔluxE* strains, and no developing adults were observed on lawns of Δ*luxCE* mutant strain. Nematodes developed from adults into hermaphrodites in five days when grown together with bioluminescent WT *Photorhabdus* strain, and the number of individuals remained constant after seven days. On plates inoculated with aluminescent *Photorhabdus* mutants, no nematodes developed into hermaphrodites and most of developing adults died within seven days. Our results indicate that bioluminescence production, or at least functional *lux*-operon genes, are required for normal *Heterorhabditis* nematodes development.

Production of secondary metabolites is altered in aluminescent *Photorhabdus* mutant strains

Photorhabdus bacteria produce a variety of secondary metabolites playing important roles in symbiosis with their nematode partners or involved in pathogenicity towards insects. To get insight into the inability of aluminescent *Photorhabdus* strains to support nematode development and growth, we asked whether these mutants exhibit different metabolomic profiles compared to the bioluminescent WT. We infected insects with either aluminescent *Photorhabdus* mutant strains or with their relative bioluminescent WT counterparts and performed untargeted metabolomic analyses of the insect cadavers one, three, or five days after infection (**Fig. 2**). Our results suggest that the metabolism of aluminescent bacterial

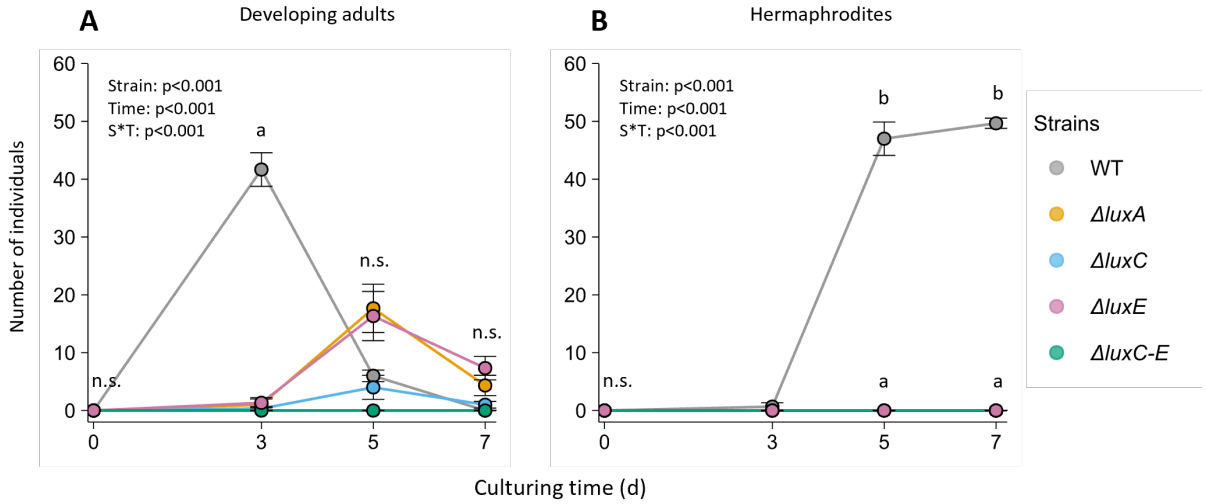

Figure 1. Aluminescent *Photorhabdus* mutants do no longer support nematodes growth *in vitro*. Numbers (Mean ± SEM) of *Heterorhabditis bacteriophora* strain TT01 nematodes developping on nematode growth gelrite medium inoculated with bioluminescent wild type (WT) *P. laumondii* subsp. *laumondii* strain DJC-23 or with its relative aluminescent *ΔluxA, ΔluxC, ΔluxE,* or *ΔluxC-E* mutant strains. Developing adults (**A**) and hermaphrodites (**B**) were counted 3 days, 5 days, and 7 days after infective juvenile nematodes were placed on the medium on day 0. Three nematode cultures per bacterial strain, each with one hundred nematodes, were established and the experiments were conducted two independent times (n=6). Different letters indicate significant differences ($p < 0.05$ by two-way repeated measures ANOVA with Holm's test for multiple comparisons). n.s.: not statistically significant.

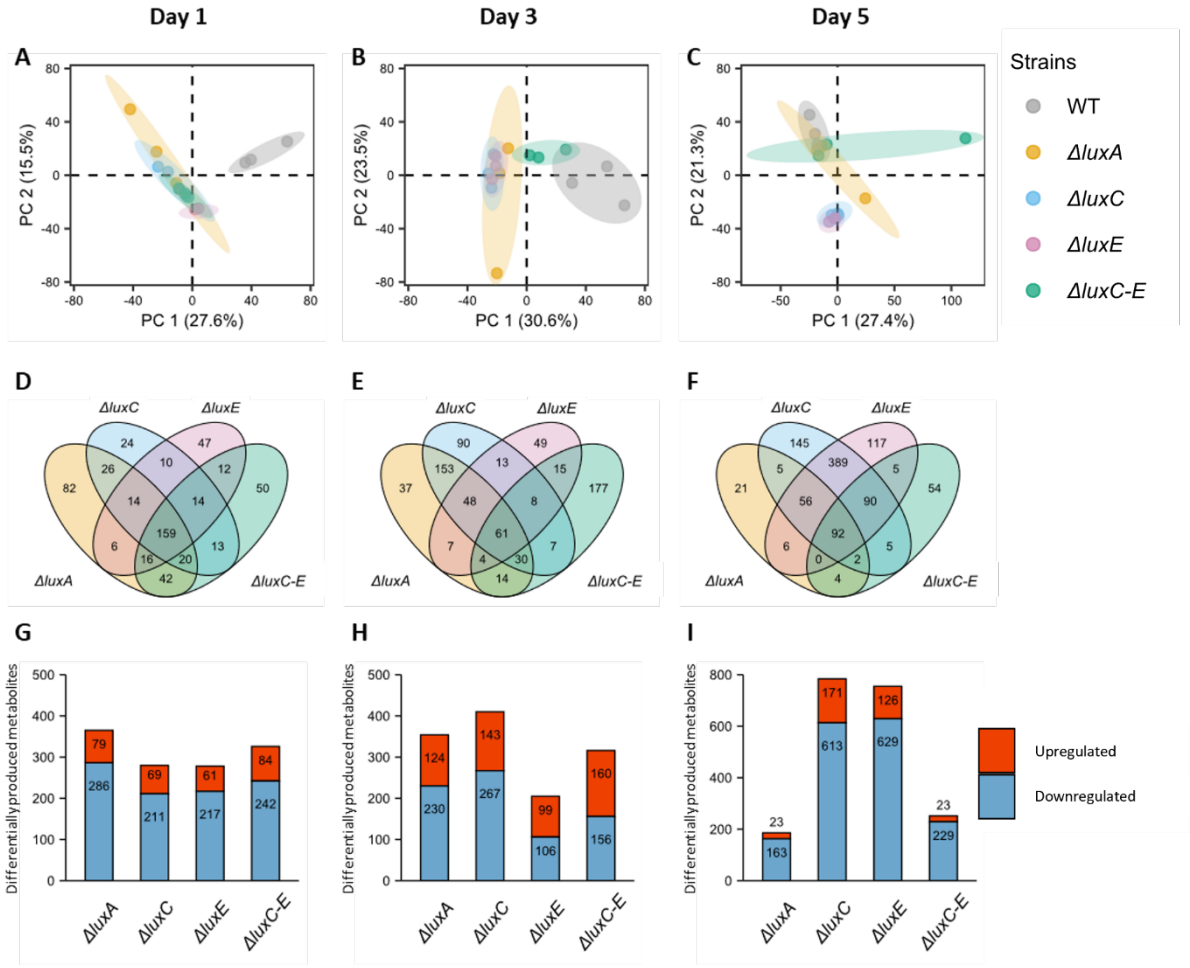

Figure 2. Aluminescent *Photorhabdus* mutant strains differ from their relative bioluminescent counterparts in secondary metabolites production in a time- and strain-dependant manner. (**A, B, and C**) Principal component analysis (PCA) plots of metabolites abundance in *G. mellonella* larvae infected with bioluminescent wild type (WT) *P. laumondii* subsp. *laumondii* strain DJC-23 or with its relative aluminescent *ΔluxA, ΔluxC, ΔluxE,* or *ΔluxC-E* mutant strains respectively 3 days, 5 days, or 7 days after infection. (D, E, and F) Venn diagrams illustrating the number of differentially regulated metabolites by the different treatments. (**G, H, and I**) Number of differentially regulated metabolites by the different treatments. Secondary metabolites were analyzed 3 days (panels A, D, and G), 5 days (panels B, E, and H), and 7 days (panels C, F, and I) after infection. Three replicates per treatment were analyzed (n=3).

strains is overall altered compared to the one of the bioluminescent WT one day after infection. Differences in metabolites production are evolving through time, and no clear group differentiation is present after five days between insect cadavers infected with bioluminescent WT strain and aluminescent ΔluxA and Δ*luxCE* strains. To follow up on our investigation, we focused on metabolites whose production were consistently altered in aluminescent bacterial strains through time and were able to detect fifteen of them (**Fig. 3 and Table 1**). All of these compounds were present in insect cadavers infected with WT bacteria and either absent or exhibit diminished levels in aluminescent mutant strains. Among these metabolites, we putatively identified phurealipid A, phrurealipid C, and lumizinone B, three compounds thought to be involved in insect pathogenicity by altering insect immune response (**Nollmann et al., 2015; Park and Crawford, 2016; Shi and Bode, 2018**). Our results suggest that metabolite production is altered in aluminescent *Photorhabdus* mutant strains compared to their relative WT counterparts in a time- and strain-dependent manner.

Aluminescent *Photorhabdus* mutant strains are impaired in IPS production

Previous work has highlighted the importance of IPS production in the symbiosis between *Photorhabdus* bacteria and *Heterorhabditis* nematodes. From these findings, the hypothesis that IPS serve as a food signal promoting IJ recovery process has emerged. We asked whether aluminescent *Photorhabdus* mutant strains are impaired in IPS production. We assessed the content of IPS in insect cadavers infected with aluminescent Photorhabdus strains or with their relative bioluminescent WT counterparts (**Fig. 4**). Our results indicate that one day after the beginning of the infection no IPS is detected in cadavers infected with the WT, or with the aluminescent *Photorhabdus* mutant strains. After three days, IPS accumulated to great amount (577 µg/gFW) only in cadavers infected with bioluminescent WT *Photorhabdus* strain. After five days, IPS content increased to reach 886 µg/gFW in cadavers infected with bioluminescent WT *Photorhabdus* strain, while it remained insignificantly different from 0 in other conditions. Our results indicate that aluminescent *Photorhabdus* mutant strains fail to produce IPS to normal level compared to bioluminescent WT. Taken together, our findings reveal that genetically engineered aluminescent *Photorhabdus* strains in which either individual *luxA, luxC,* or *luxE* genes or the whole *lux*-operon has been removed, exhibit altered secondary metabolism, including the inability to produce normal level of IPS, and do no longer support nematode growth and development *in vitro.*

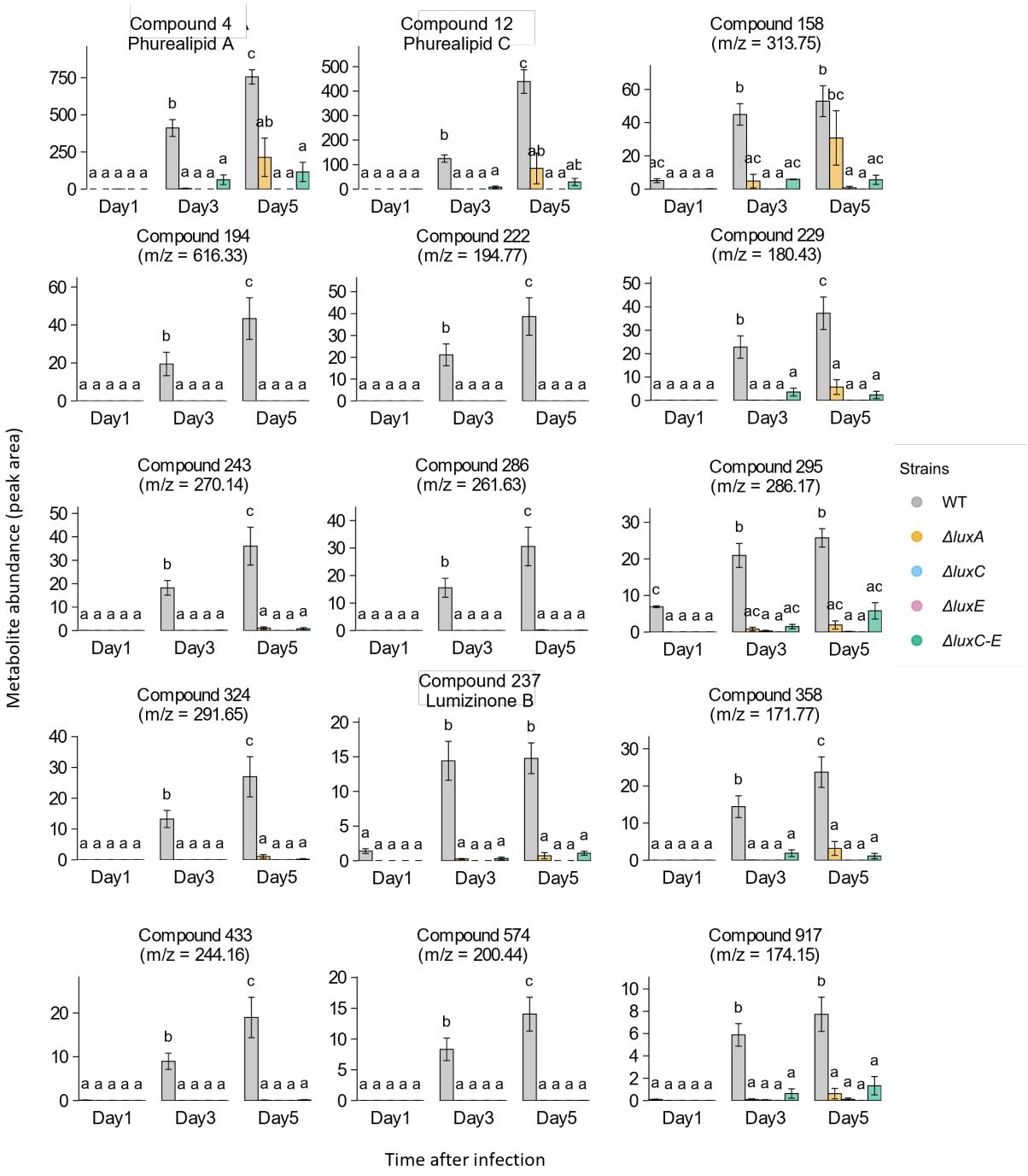

Figure 3. Secondary metabolites differentially produced *in-vivo* by aluminescent *Photorhabdus* mutant strains compared to their relative bioluminescent counterparts. Mean (±SEM) abundance (peak area) of low molecular weight metabolites in *G. mellonella* larvae infected with bioluminescent wild type (WT) *P. laumondii* subsp. *laumondii* strain DJC-23 or with its relative aluminescent *ΔluxA, ΔluxC, ΔluxE,* or *ΔluxC-E* mutant strains. Secondary metabolites were analyzed 3 days, 5 days, and 7 days after infection. Three replicates per treatment were analyzed (n=3). Different letters indicate statistically significant differences in metabolite abundance (P<0.05 by two-way repeated measures ANOVA with Holm's test for multiple comparisons). For information on the chemical identification of the different compounds, refer to Table S1.

Table 1. Characteristics of the secondary metabolites whose production were altered *in vivo* in aluminescent *Photorhabdus* mutant strains compared to their relative bioluminescent counterparts. For information on experimental procedure carried out, refer to Fig. 2.

Compound	m/z	RT (min)	Putative idenfication	Putative function	Reference
4	229.23	7.43	Phurealipid A	Inhibitor of insect immune response	Nollmann *et al.* 2010
12	257.26	8.59	Phurealipid C		Nollmann *et al.* 2015
158	313.75	2.64	Unidentified	-	-
194	616.33	1.23	Unidentified	-	-
222	194.77	1.23	Unidentified	-	-
229	180.43	1.23	Unidentified	-	-
243	270.14	1.21	Unidentified	-	-
286	261.63	1.22	Unidentified	-	-
295	286.17	2.76	Indol derivative	-	-
324	291.65	1.22	Unidentified	-	-
327	293.13	4.72	Lumizinone B	Modulate insect inflammatory signaling pathway	Park and Crawford 2016
358	171.77	1.23	Unidentified	-	-
433	244.16	1.22	Unidentified	-	-
574	200.44	1.24	Unidentified	-	-
917	174.15	1.69	Unidentified	-	-

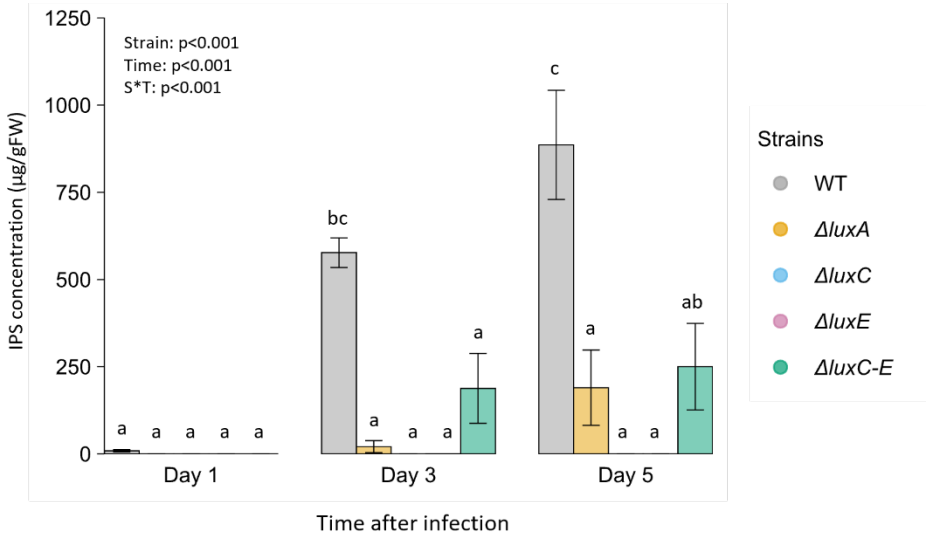

Figure 4. Production of IPS is impaired in aluminescent *Photorhabdus* mutant strains. Mean (±SEM) concentration (µg/gFW) of 3,5-dihydroxy-4-isopropyl-trans-stilbene (IPS) in *G. mellonella* larvae infected with bioluminescent wild type (WT) *P. laumondii* subsp. *laumondii* strain DJC-23 or with its relative aluminescent *ΔluxA, ΔluxC, ΔluxE,* or *ΔluxC-E* mutant strains. Quantifications of 3,5-dihydroxy-4-isopropyl-trans-stilbene (IPS) were performed 3 days, 5 days, and 7 days after infection.Three replicates per treatment were analyzed (n=3). Different letters indicate statistically significant differences in concentration (P<0.05 by two-way repeated measures ANOVA with Holm's test for multiple comparisons).

Discussion

Photorhabdus bacteria and *Heterorhabditis* nematodes are associated in an obligate symbiosis (**Boemare et al., 1993; Thomas and Poinar, 1979**). Within infected organisms, *Heterorhabditis* nematodes rely on their cognate bacterial symbionts to feed and develop (**Adams et al., 2006; Poinar and Thomas, 1966**). In addition, it is thought that *Heterorhabditis* IJs sense a food signal produced by *Photorhabdus* bacteria to recover into adulthood (**Aumann and Ehlers, 2001; Eleftherianos et al., 2007; Lango-Scholey et al., 2013**). We hypothesize that *Photorhabdus* bioluminescence plays a role in the synchronization between *Photorhabdus* bacteria metabolic switch towards symbiosis and *Heterorhabditis* nematodes recovery, contributing to the successful completion of their parasitic lifecycle.

We started our investigation by testing if bioluminescence was required for normal symbiosis between *Photorhabdus* bacteria and *Heterorhabditis* nematodes. To do so, we conducted symbiotic assays and observed the incapacity of aluminescent *Photorhabdus* mutant strains to support normal nematode growth and development. Our results suggest that this inability is caused by the hindrance of the recovery process. Nematodes cultured on lawns of aluminescent *Photorhabdus* mutant strains either failed to undergo development into adulthood, or the recovery was both delayed and severely diminished.

We hypothesize that the hindrance of *Heterorhabditis* nematodes to recover might be explained, at least partially, by an alteration of *Photorhabdus* secondary metabolism. We conducted untargeted metabolomic analyses to compare the production of secondary metabolites between insects infected with aluminescent *Photorhabdus* mutant strains and insects infected with their bioluminescent WT counterparts. Our results indicate that the production of secondary metabolites *in vivo* was altered in aluminescent *Photorhabdus* mutant strains. These mutant strains were impaired in the production of phurealipid A, phrurealipid C, and lumizinone B, three compounds thought to be involved in alteration of insect immune response (**Nollmann et al., 2015; Park and Crawford, 2016; Shi and Bode, 2018**). While our work focuses on putative roles of bioluminescence on symbiosis, this finding reveals that bioluminescence might play a role in *Photorhabdus* pathogenicity. During our investigations we did not notice alterations in pathogenicity of aluminescent *Photorhabdus* mutant strains, based on the mortality of infected larvae. However, our experiments were not

designed to assess this particular aspect, and precise timing of infected insect death, for example, were not monitored. In natural environments, infection by a single *Heterorhabditis* nematode individual can lead to insect death. For practical reasons, we directly injected bacterial culture into the insect hemocoel. Although the quantity of bacterial inoculum we administered is biologically relevant, it is important to note that it may be comparatively higher than the bacterial load resulting from infection by a single nematode individual. It is possible that putative alterations of pathogenicity in aluminescent mutant strains were overshadow by the relatively high bacterial load. Further investigation focusing on the pathogenicity of aluminescent *Photorhabdus* mutant strains would shed light on this interesting aspect of bioluminescence.

It has been suggested, based on experimental data, that IPS is, or is a part, or the food signal sensed by IJs to recover into adulthood (**Hapeshi et al., 2019; Joyce et al., 2008**). We were not able to identify IPS in our untargeted metabolomic analyses. Therefore, we specifically quantify the levels of IPS in insects infected either with aluminescent mutant or bioluminescent WT strains. Our results confirmed the previously described timing and quantity of IPS production in *Photorhabdus* WT strains (**Hu, 2000; Hu et al., 1997**). The production starts at least 24 h after the beginning of the insect infection, and IPS content can reach concentration from ~500 to ~1000 µg/gFW. Our analyses revealed that aluminescent *Photorhabdus* mutant strains are impaired in IPS production. This result suggests that the inability of aluminescent *Photorhabdus* mutant strains to support nematode growth and development is, at least partially, attributed to their deficient production of IPS. Furthermore, it underscores the requirement of bioluminescence, or at least a functional lux-operon, for IPS production.

Bioluminescence has often been considered a product of *Photorhabdus* secondary metabolism (**Clarke, 2016; Joyce et al., 2011**). Our results suggest that bioluminescence may play a role upstream, regulating secondary metabolism. In a previous study, it has been shown that *Photorhabdus* mutant strains deficient in (p)ppGpp biosynthesis are impaired in both bioluminescence and IPS production (**Bager et al., 2016**). Our results combined with previous findings, suggest that (p)ppGpp accumulation may directly or indirectly regulate bioluminescence and, consequently, promote IPS production through the activation of *stlA*

expression. We hypothesize that bioluminescence links the accumulation of (p)ppGpp to IPS production. Experiments carried out with exogenous applications of IPS on *Photorhabdus* bacteria cultures, reveal that IPS negatively regulates bioluminescence production (**Hapeshi et al., 2019**). Based on our results, we hypothesize the existence of a negative regulatory feedback loop, where bioluminescence promotes IPS production and IPS represses its own production by inhibiting bioluminescence. Transcriptomic analyses revealed that the expressions of *lux* genes were not altered upon IPS applications, suggesting that this inhibition likely occurs at the post-transcriptional level (**Hapeshi et al., 2019**).

In addition to the role played by bioluminescence on *Photorhabdus* secondary metabolism, we hypothesize that bioluminescence could be sensed by IJs and prime their recovery. While we did not experimentally test this last hypothesis, we propose in the next section, several experiments that would provide insights into the putative role played by *Photorhabdus* bioluminescence on the priming of *Heterorhabditis* IJs recovery (see section: “Conclusions”, page 153). Summarizing current knowledge and incorporating our findings, we propose a regulatory framework in which bioluminescence prime *Heterorhabditis* IJs to undergo recovery and initiate *Photorhabdus* metabolic switch by promoting IPS production, ensuring the synchronization of these processes necessary for successful symbiosis (**Fig. 5**).

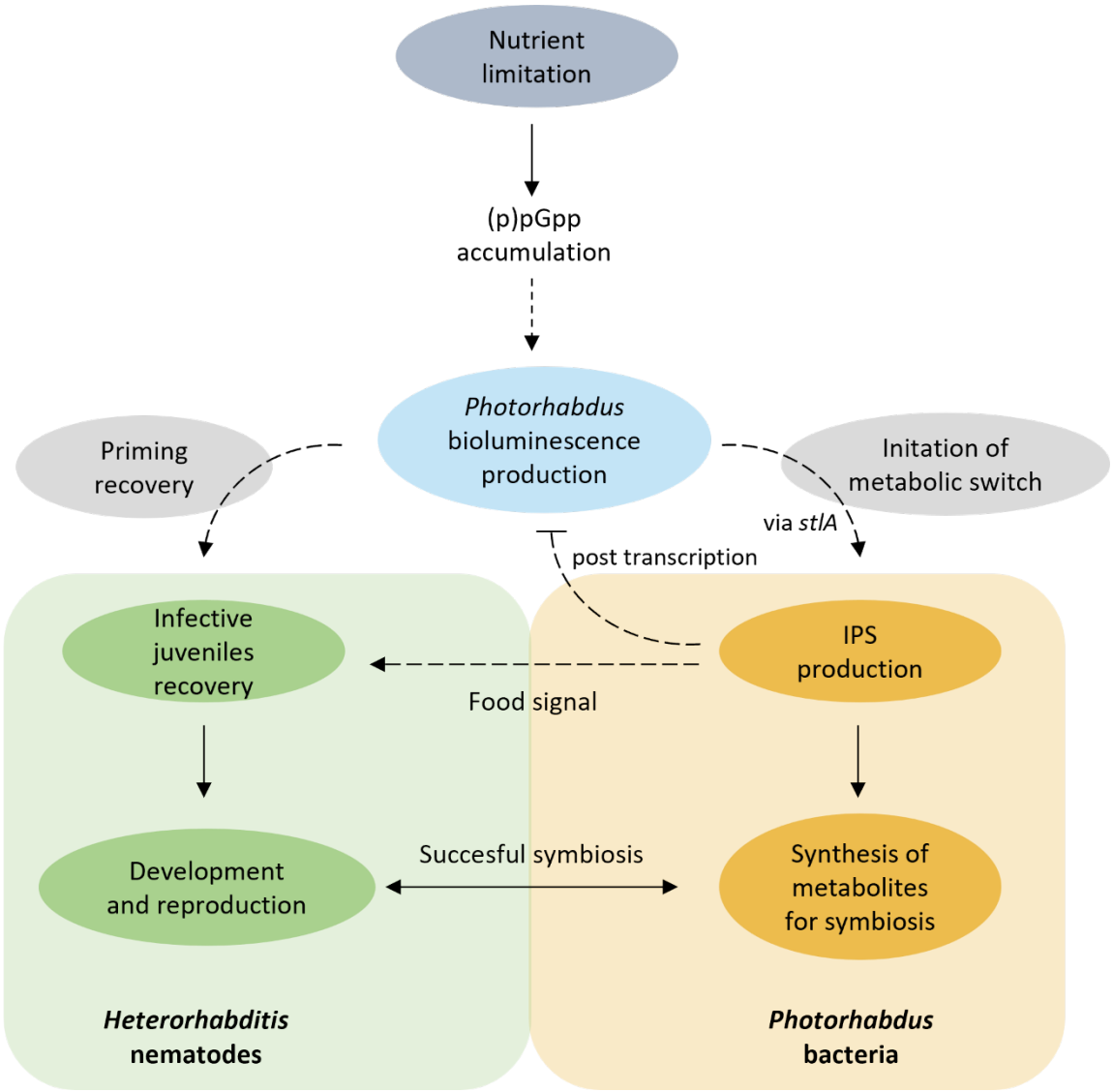

Figure 5. Proposed model of the regulatory framework involving *Photorhdbus* bioluminescecne and symbiosis between *Heterorhabditis* nematode of *Photorhabdus* bacteria. This model is based on currently available literature and findings from this study. Arrows represent induction or positive "action". Horizontal lines represent repression and dashed lines represent hypothetical regulation.

Conclusions

Our work aims to get insight into the putative roles of *Photorhabdus* bioluminescence in modulating symbiosis with *Heterorhabditis* nematodes. Our results reveal that *Heterorhabditis* IJs do not recover when cultured on laws of aluminescent *Photorhabdus* mutant strains, indicating that bioluminescence is required for normal *Heterorhabditis* nematodes growth and development. The inability of aluminescent *Photorhabdus* mutant strains to maintain normal symbiosis with *Heterorhabditis* nematodes is at least partly caused by the alteration of secondary metabolism, including depletion in IPS production. We propose a new model in which bioluminescence prime IJs to undergo recovery and initiate bacterial metabolic switch by promoting IPS production, ensuring the synchronization of these processes necessary for successful symbiosis. Experiments to guide future investigations aiming to confirm or refute this model are proposed in the general conclusions of this thesis, page 205. In conclusion, our research contributes to a better understanding of the symbiotic relationship between *Photorhabdus* bacteria and *Heterorhabditis* nematodes and highlights the potential roles played by bioluminescence in this fascinating process.

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Supplementary materials

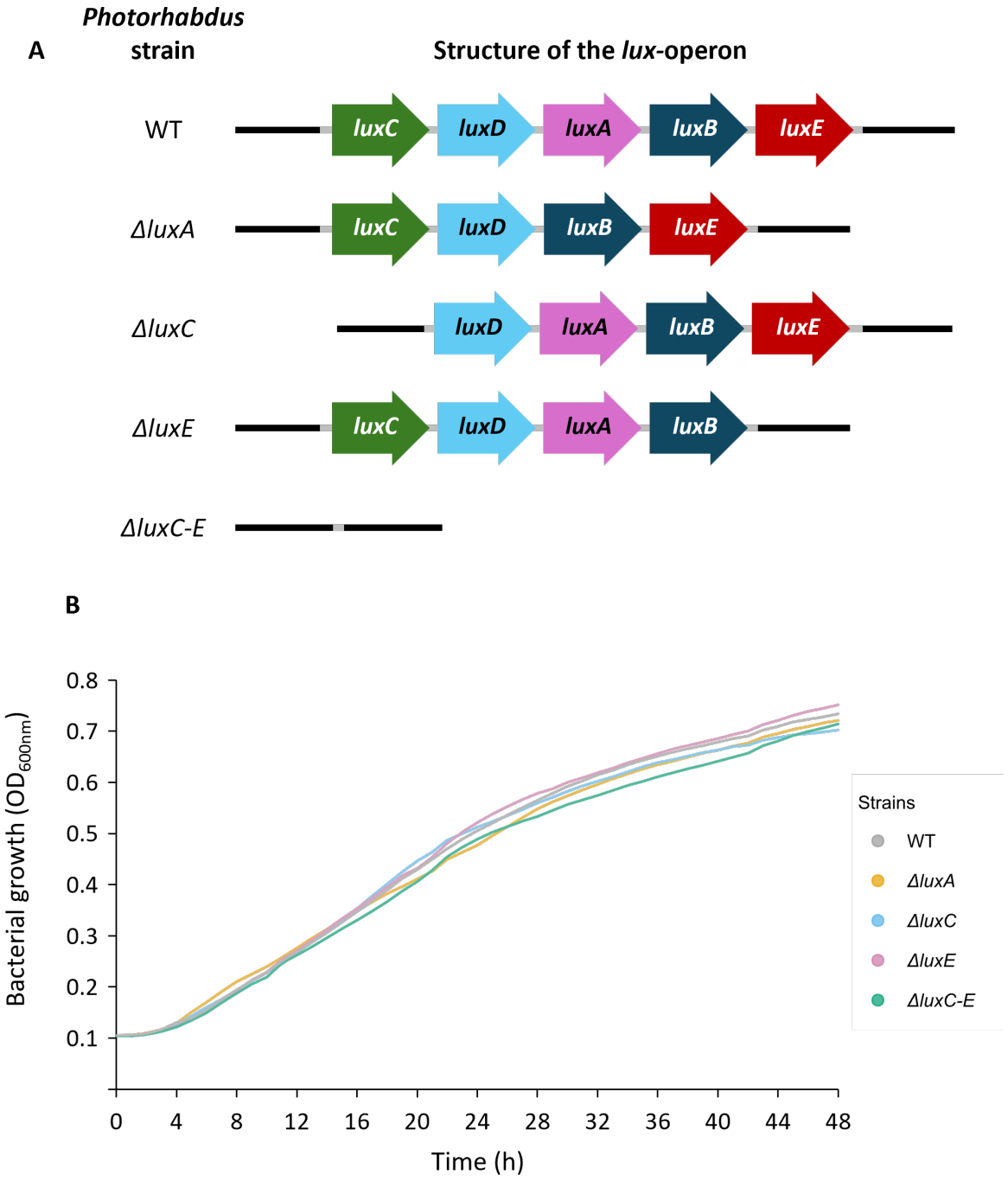


Figure S1. Molecular structure and fitness of the aluminescent mutant *Photorhabdus* strains. (**A**) Molecular structure of the *lux*-operon in bioluminescent wild-type (WT) and aluminescent mutant strains of *Photorhabdus*. The aluminescent bacterial strains were generated by removing specific *lux*-operon genes (*luxA, luxC, luxE*), or the entire *lux*-operon, by allele exchange mutagenesis, from the model strain *Photorhabdus laumondii* subsp. *laumondii* WT strain DJC-23. (**B**) Kinetic curves of the bacterial growth *in vitro* of WT and aluminescent mutant strains of *Photorhabdus*. The aluminescent mutant strains do not show altered growth compared to the bioluminescent WT strain. Bacterial growth was measured in four replicates for each strain, and the experiments were conducted 2 independent times (n=8).

CHAPTER 4
Plant responses to Photorhabdus bioluminescence exposure

Abstract

The symbiotic pair formed by *Heterorhabditis* nematodes and *Photorhabdus* bacteria parasitizes soil-dwelling herbivorous insects, leading to the occurrence of bioluminescent infected organisms in the rhizosphere. Plants sense light belowground, modulating root development and whole plant physiology. We hypothesize that plants are able to perceive and respond to the bioluminescence produced by *Photorhabdus* bacteria. This bioluminescence might inform plants about the presence of herbivorous insects and their natural enemies, entomopathogenic nematodes (EPNs), in the rhizosphere. To investigate this hypothesis, we exposed maize roots to insect larvae infected with *Photorhabdus* bacteria or to luminescent beads employing two experimental setups. In the first one, (bio)luminescent sources were inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant). In the second, they were placed in close proximity to the roots (Close). We assessed plant responses through transcriptomic, metabolomic and phytohormonal analyses. Control groups were composed of untreated maize plants or plants whose roots were exposed to insect larvae infected with genetically engineered aluminescent *Photorhabdus* strain. Our findings indicate that plants exhibit weak transcriptomic, metabolomic and phytohormonal responses upon bioluminescence exposure. Interestingly, we detected, using the distant experimental setup, an accumulation of two glycosided benzoxazinoids, 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose (DIMBOA-Glc) and 2-β-d-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA-Glc), in the leaves of plants whose roots were exposed to (bio)luminescent sources. The levels of DIMBOA-Glc and HMBOA-Glc were higher in plants whose roots were exposed to the two highest (bio)luminescence intensity treatments. Our results suggest that plants are capable of sensing *Photorhabdus* bioluminescence belowground and responding to it by activating defense responses aboveground in an intensity-dependent manner.

Introduction

Plants are sessile organisms that adapt to their environment by responding to external stimuli such as light, gravity, water, and mineral nutrients availability (**Dietrich et al., 2018; Jaffe et al., 1985; Ruppel et al., 2001**). While light, by driving photosynthesis, represents a major factor modulating plants growth aboveground, plant roots grow in rather dark subterranean environments. Plants are, however, able to sense the sunlight that can penetrate several centimeters under the soil surface thanks to photoreceptors and signaling component expressed in their roots (**Warnasooriya and Montgomery, 2011**). This light exposure influences both the root system architecture (RSA) and the overall metabolism and physiology of the seedling, with auxin playing a significant role in this process (**Gelderen et al., 2018; Silva-Navas et al., 2015; Yun et al., 2023**). Besides sunlight, there are other light sources, such as bioluminescence, present belowground (**Pes et al., 2016; Thomas and Poinar, 1979; Seesamut et al., 2021; Verdes and Gruber, 2017; Waterfield et al., 2009**). It remains unknown whether plants sense and respond to these alternative sources of light. Soil-dwelling nematodes from the genus *Heterorhabditis* parasitize and kill herbivorous insects (**Thomas and Poinar, 1979**). During the infection process, the nematode releases its bacterial symbiont, *Photorhabdus,* that produce bioluminescence resulting in the glow of infected organisms in the rhizosphere (**Thomas and Poinar, 1979; Waterfield et al., 2009**). We hypothesize that *Photorhabdus* bioluminescence can be sensed by roots, informing the plant about the presence of both herbivorous insects and *Heterorhabditis* nematodes in the rhizosphere, thereby triggering specific responses.

Light is a major environmental cue driving many aspects of plant life including, flowering, phototropism, and shade avoidance (**Gelderen et al., 2018; Kami et al., 2010**). Plants evolved very efficient light sensing and phototransduction systems, most of these elements being also present in plant roots (**Warnasooriya and Montgomery, 2011**). The sunlight, that can penetrate several centimeters under the soil surface, is integrated by roots and modulate the RSA (**Tester and Morris, 1987; Wolley and Stoller, 1978**). The RSA anchors the plant in the soil and optimizes its access to water and nutrients (**Silva-Navas et al., 2015**). Several classes of plant photoreceptors are involved in light perception belowground. These include, cryptochromes (CRYs), phototropins (PHOTs), and phytochromes (PHYs) that enable the perception of light of different spectra (**Briggs and Lin, 2012**). While blue light (320-500 nm)

is sensed by CRYs and PHOTs, red and far-red light sensing is performed by PHYs. Belowground, these photoreceptors are involved in a variety of developmental processes. Cryptochromes contribute to the regulation of primary root elongation. It was experimentally shown in *Arabidopsis thaliana* that CRY1 and CRY2 inhibit root growth by affecting free auxin levels (**Mo et al., 2015**). The blue light photoreceptor PHOT1, which is strongly expressed in roots close to the soil surface, is involved in root phototropism by modulating auxin transport (**Fankhauser and Christie, 2015; Galen et al., 2007; Wan et al., 2012; Zhang et al., 2013**). It has also been shown in *A. thaliana* that PHOT1 mediates root elongation (**Moni et al., 2015; Sakamoto and Briggs, 2002; Wan et al., 2008; Zhang et al., 2013**). The red and far-red photosensors PHYs have been shown to contribute to normal lateral root formation and root elongation (**Correll and Kiss, 2005; Salisbury et al., 2007; Silva-Navas et al., 2015**). Together, CRYs, PHOTs and PHYs play important regulatory roles, shaping the plant RSA.

During many decades, studies focusing on plant biology and physiology were performed with plant roots exposed to light. More recently, it has been proved that such exposure to light induces a reduction of both root growth and number of lateral roots. Experiments conducted with different light spectra highlighted that blue light has a predominant role on this effect. In addition, roots exposure to light affects ion accumulation and phytohormonal responses (**Silva-Navas et al., 2015**). It has been shown that plants whose roots were exposed to light displayed decreased auxin response, presumably due to the degradation of indole-3-acetic acid (IAA) which is known to be photolabile, while abscisic acid (ABA) and brassionolide sensitivity was increased (**Del Pozo and Manzano, 2014; Silva-Navas et al., 2015**). Root exposure to light induces strong burst of reactive oxygen species (ROS) signaling, which is typically associated with stress responses (**Manzano et al., 2014; Passaia et al., 2014; Tsukagoshi et al., 2010; Yokawa et al., 2011**). This light-induced stress has been hypothesized to add on other stresses such as high salinity, drought, or nutriment deprivation (**Silva-Navas et al., 2015**). Excessive light exposure can cause oxidative stress and DNA damage (**Ganguly et al., 2018; Hideg et al., 2013**). In response to this stress, the phenylpropanoid pathway is activated in an ABA-dependent manner (**Huang et al., 2019**).

Plants use small chemical messengers called phytohormones to orchestrate responses to environmental stresses. These phytohormones, including ABA, ethylene, jasmonic acid (JA),

and salicylic acid (SA), initiate cascades of reactions that are deeply interconnected in multiple signaling pathways to finely modulate the plant physiology (**Peleg and Blumwald, 2011; Wasternack, 2014**). Notably, phytohormones, together with ROS signaling, are involved in the dynamic production of plants secondary metabolites in response to external stimuli (**He et al., 2018**). In maize, a key crop worldwide, benzoxazinoids stand out as the most abundant defensive secondary metabolites. This class of compounds is involved in biotic defense against fungi, bacteria, herbivorous insects, and serves as allelochemical signals (**Sicker et al., 2000; Wouters et al., 2016**). Benzoxazinoids accumulation is stimulated by ABA, JA, JA–isoleucine (JA-Ile), and ethylene in a tissue-specific manner (**Sue et al., 2021**). In addition to phytohormones, other pathways are thought to modulate benzoxazinoids levels, and the regulation of benzoxazinoid biosynthesis by phytohormones remains unclear (**Malook et al., 2019**). Plants also activate indirect defensive strategies upon biotic stress, such as herbivore feeding. For example, European lines of maize emit important quantities of (E)-β-caryophyllene when fed by the western corn rootworm (**Rasmann et al., 2005**). This sesquiterpene attract entomopathogenic nematodes, that are natural enemies of this beetle, in the vicinity of the attacked plants (**Hiltpold et al., 2010; Köllner et al., 2008; Rasmann et al., 2005**). It was shown that ABA is locally and systematically accumulated following feeding by the western corn rootworm (**Erb et al., 2009**).

The sunlight is not the only light sources in belowground ecosystems. Soil-dwelling nematodes belonging to the *Heterorhabditis* genus live in obligate symbiosis with bioluminescent bacteria belonging to the *Photorhabdus* genus (**Boemare et al., 1993; Thomas and Poinar,1979**). Together, they parasitize and kill herbivorous insects. During the infection process, *Photorhabdus* produce cyan bioluminescence which lead to the glow of the infected host (**Thomas and Poinar, 1979; Waterfield et al., 2009**). Plant responses to belowground bioluminescence has, to our knowledge, not been investigated so far. We hypothesize that bioluminescence produced by *Photorhabdus*-infected insects is detected by root photosensors, informing the plant about the presence of both herbivorous insects and *Heterorhabditis* nematodes in the rhizosphere. Consequently, this luminous signal might activate defense mechanisms, mediated by phytohormones, in the plant. To investigate whether plants respond to bioluminescence belowground, we exposed maize roots to insect larvae infected with *Photorhabdus* bacteria or to luminescent beads employing two

experimental setups. In the first one, (bio)luminescent sources were inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant). In the second, they were placed in close proximity to the roots (Close). We assessed plant responses through transcriptomic, metabolomic and phytohormonal analyses. Control groups consisted of untreated maize plants or plants whose roots were exposed to insect larvae infected with genetically engineered aluminescent *Photorhabdus* strain. Our approach, leveraging modern molecular tools, provides insights into the potential effects of bacterial bioluminescence on plants and enhances our understanding of the ecological functions of bioluminescence belowground.

Material and methods

Photorhabdus culture

The following strains were used for this study: *Photorhabdus cinerea* strain DSM 19724^T (ID158), *Photorhabdus tasmaniensis* strain USCA01 (ID138), and the genetically-engineering aluminescent *Photorhabdus laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23. To maintain these strains, they were cultured onto lysogenic broth (LB) agar plates (Sigma-Aldrich, USA) and incubated at 28°C. Every thirty days the bacteria were refreshed from glycerol stocks.

Measure of (bio)luminescence emitted by *Photorhabdus*-infected insects and luminescent beads

To measure the bioluminescence emitted by *Photorhabdus*-infected insects, *G. mellonella* larvae were infected with the different bacterial strains, as described previously (**Castaneda-Alvarez et al., 2022**). Briefly, *Photorhabdus* bacterial strains were individually grown for 16-20 hours in LB medium at 28 °C with constant agitation (180 rpm). The bacterial cultures were then collected, and their optical densities at 600 nm (OD_{600}) were measured. Subsequently, all cultures were diluted to obtain bacterial cultures with an OD_{600}=1, and 10 µL of the resulting bacterial cultures were injected into third-instar *G. mellonella* larvae. Twenty-four larvae per strain were injected (n=24). After 24 h, both the measurement of bioluminescence levels and a scan across the wavelength range from 400 nm to 700 nm, at 10 nm intervals, were conducted using a SpectraMax 0480 microplate reader. To measure the luminescence emitted by luminescent beads (Beads), we first exposed the beads to sunlight for 30 s, subsequently three beads were placed in 12 wells of a 24-well microtiter plates (Greiner Bio-One, Au), and bioluminescence measurements were carried out using a SpectraMax 0480 microplate reader (n=12).

Exposure to (bio)luminescence in plant roots

To evaluate whether plants respond to *Photorhabdus*-induced bioluminescence exposure, plant roots were exposed to two types of (bio)luminescence sources: *Photorhabdus*-infected insects and luminescent beads. The *Photorhabdus*-infected insects consisted of third-instar *Galleria mellonella* larvae infected with either *P. cinerea* DSM 19724^T (ID158), a high bioluminescence producer, or with *P. tasmaniensis* USCA01 (ID138), an intermediate bioluminescence producer (**Fig. 1**). Larvae were infected as described above (see section:

"Measure of (bio)luminescence emitted by *Photorhabdus*-infected insects and luminescent beads", page 164). Luminescent beads emit luminescence with similar characteristics to real bioluminescence (**Fig. 1**). These (bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant) or placed in close proximity to the roots (Close) of 7-day-old maize plants (*Zea mays* var. Delprim). As controls, plant roots were exposed to insects infected with the genetically-engineering aluminescent *Photorhabdus laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 or were left untreated. Insects and luminescent beads were placed inside 2 mL polystyrene tubes (Kartell, Italy) to specifically test the effect of (bio)luminescence. Insects were replaced daily, and the plants were exposed to (bio)luminescence for six days. Luminescent beads were recharged daily by exposing them to sunlight for 30 seconds. The plants were then harvested, and roots and leaves were separated.

Plant transcriptomic analyses

To evaluate plant responses to bioluminescence at the transcript level, we carried out RNAseq experiments in plants whose roots were exposed or not to (bio)luminescence. (Bio)luminescence exposure treatments were carried out as described above (see section: "Exposure to (bio)luminescence in plant roots", page 163). The plant roots were flash frozen and subsequently ground into a fine powder under liquid nitrogen. One hundred milligrams of plant roots powder were used to extract the RNA. Total RNAwas isolated using the GeneJET Plant Purification Mini Kit (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to the manufacturer's instructions and complete DNA removal was performed using the Lucigen RNase-Free DNaseI kit (LubioScience, Zürich, Switzerland). RNA integrity, quality, and purity were initially assessed by gel electrophoresis and using a NanoDrop spectrophotometer (IMPLEN, CA, USA). Final RNA quality was assessed using the RNA Nano 6000 Assay Kit on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). For library construction, 1 µg of RNA per sample was used. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to their respective samples. PCR products were purified with the AMPure XP system (Beckman Coulter, Beverly, USA), and library quality was assessed using the Agilent 2100 (Agilent Technologies, Palo Alto, CA, USA). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster

generation, the library preparations were sequenced on an Illumina HiSeq 4000 platform, producing paired-end reads (2×150 bp). After data generation, the paired-end clean reads were mapped to the maize reference genome (Zm-B73-REFERENCE-NAM-5.0; NCBI accession number: GCF_902167145) using HISAT2 v2.0.5 (**Kim et al., 2015**). Gene expression levels were calculated using FeatureCounts (Subread v1.5.0 p3) with default parameters and are presented as fragments per kilobase of transcript per million fragments mapped (FPKM) (**Liao et al., 2014**). Differentially expressed genes (DEGs) were identified using the DESeq2 R package v1.20.0 with a false discovery rate (FDR) threshold lower than 0.2 and an absolute value of log2-transformed fold change (treatment/control) greater than 1.5 (**Love et al., 2014**). Venn diagrams illustrating the number of differentially expressed genes by the different treatments were created using the R package "ggvenn" (**Yan, 2021**). Principal component analyses (PCA) were carried out using the R package "factoextra" (**Kassambara and Mundt, 2020**). Plant root transcripts were assessed in a total of eight plants per treatment, and the experiment was conducted two independent times (n=8). Sequences of the transcripts used to build heatmaps are described in **Table S1**.

Plant untargeted metabolomic analyses

To evaluate plant responses to bioluminescence at the metabolic level, we carried out small molecular weight metabolomics experiments in plants whose roots were exposed or not to (bio)luminescence. The (bio)luminescence exposure treatments were carried out as described above (see section: "Exposure to (bio)luminescence in plant roots", page 163). Plant roots and leaves were flash frozen and then ground into a fine powder under liquid nitrogen. One hundred milligrams of the resulting plant roots and leaves powder were extracted using 1 mL of methanol:water:formic acid buffer (80:19.9:0.1 v/v/v). The sample extracts were then analyzed by ultra-high performance liquid chromatography - quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) using an Acquity UPLC™ coupled to a Synapt G2 instrument (Waters, Milford, USA). The system was controlled by MassLynx v.4.1. An Acquity UPLC™ HSS T3 column (100 × 2.1 mm, 1.8 µm) maintained at 25 °C was used at a flow rate of 0.4 mL/min. The mobile phases consisted of H_2O with 0.05% formic acid (solvent A) and acetonitrile with 0.05% formic acid (solvent B). A segmented gradient was applied from 0 to 50% in 7.5 min, followed by a transition to 100% B in 2.5 min. The injection volume was 2 µl. MS detection was performed in negative electrospray ionization and using data-dependent

acquisition (DDA). The resolution was set to 20'000 (at *m/z* 554). Data were acquired in centroid mode. The capillary voltage was set to -2.0 kV, the cone voltage to -25 V, the source temperature to 120 °C, the desolvation temperature to 400 °C, the desolvation gas flow to 900 L/h, the cone gas flow to 50 L/h, and the collision gas (Ar) flow to 2.0 L/min. DDA settings were as follows: MS1 mass range and scan time of 50–1200 Da and 0.15 s, respectively, top 3 MS/MS (0.15 s scan time each), intensity threshold 6500 counts per s, MS/MS selection window 4 Da, peak deisotoping activated, dynamic exclusion of 2.0 s after acquisition. An exclusion list of the 10 most intense background ions was generated from a blank sample run just before the samples. For MS/MS acquisition, a ramped collision energy of 5-40 V (at *m/z* 50) and 20-70 V (at *m/z* 1200) was set. Quality control samples were prepared by pooling aliquots of all samples and run four times before the batch and about every 30 samples during the batch. The raw data were imported into Progenesis QI (Waters) for peak picking using the following parameters: automatic determination of sensitivity (level 3), peak width 0.04 min, retention time limits 1.5-10 min, adducts $(M+H)^+$, $(M+NH_4)^+$, $(M+Na)^+$, $(2M+H)^+$, and $(2M+Na)^+$. The generated feature list (.csv) and MS/MS fragment list (.msp) were exported to GNPS (https://gnps.ucsd.edu) to create a molecular network using the Feature-Based Molecular Networking (FBMN) workflow. The following parameters were used for the FBMN: mass tolerances of both precursor ion and MS/MS fragment ions were set to 0.02 Da, minimum pairs cosine score was set at 0.7, the minimum matched fragment ions were 6, network TopK was 10, the maximum connected component size was 100, and the maximum shift between precursors was 500 Da. The MS/MS spectra present within the network were then searched against GNPS spectral libraries. Information about the identification of metabolites that were differentially expressed across the treatments are presented in Table S2. Venn diagrams illustrating the number of differentially produced metabolites by the different treatments were created using the R package "ggvenn", and PCA were carried out using the R package "factoextra" (**Kassambara and Mundt, 2020; Yan, 2021**). Plant root and leaf metabolites were assessed in a total of eight plants per treatment, and the experiment was conducted two independent times (n=8).

Benzoxazinoid quantification

To determine whether plant benzoxazinoid levels were altered upon (bio)luminescence exposure treatments, benzoxazinoid abundance data were extracted manually from the plant metabolomic datasets described above (see section: "Plant untargeted metabolomic analyses", page 166) using the exact masses of the following compounds: 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose (HDMBOA-Glc), 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose (DIMBOA-Glc), 2-β-d-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA-Glc), 6-methoxy-2-benzoxazolinone (MBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), 2-(2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIM_2BOA-Glc), 2-(2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one)- β -D-glucopyranose (HDM_2BOA-Glc), 2-(2-hydroxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HBOA-Glc), and 2-(2,4-dihydroxy-1,4-benzoxazin-3-one)-β-d-glucopyranose (DIBOA-Glc).

Phytohormone quantification

To evaluate plant responses to bioluminescence at the hormonal level, we measured phytohormones in plants whose roots were exposed or not to (bio)luminescence. The (bio)luminescence exposure treatments were carried out as described above (see section: "Exposure to (bio)luminescence in plant roots", page 163). Abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA), jasmonoyl-l-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (OPDA), and salicylic acid (SA) were quantified essentially as described previously (**Glauser et al., 2014; Machado et al., 2016, 2013**). To this end, plant material was ground into a fine powder under liquid nitrogen. Fifty mg of the resulting powder were extracted with using 1 mL of ethyl acetate and formic acid (99.5:0.5, v/v) containing 1 ng of isotopically labelled hormones as internal standards (d5-JA, d6-ABA, d6-SA, and 13C6-JA-Ile). Samples were subjected to extraction in a mixer mill for 3 min at 30 Hz, with 5-8 glass beads (1.25-1.65 mm diameter), and supernatants were recovered after centrifugation at 14,000 × g for 3 min. Pellets were reextracted with 500 µL of solvent. Samples were then evaporated to dryness and re-suspended in 200 uL of methanol and water (50:50, v/v). Phytohormones were analyzed using ultra-performance liquid chromatography coupled with tandem mass spectrometer (UHPLC-MS-MS). For this purpose, an Acquity UPLC instrument (Waters, USA) equipped with an Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 µm particle size, Waters,

USA) and connected to a QTRAP 6500+ (Sciex, USA) was used. Two µL of sample were injected. The mobile phase A consisted of H_2O with 0.05% formic acid, and mobile phase B was a mixture of acetonitrile and 0.05%formic acid. A gradient from 5-65% B in 6.5 min was applied, followed by column washing at 100% B and re-equilibration at 5% B for 2 min each. The flow rate was set at 0.4 mL/min, and the column temperature was maintained at 35 °C. The mass spectrometer was operated in electrospray negative ionization mode using the multiple reaction monitoring (MRM) technique. A six-point calibration curve (0.02, 0.1, 0.5, 5, 20, and 100 ng/mL, all containing labelled standards at 5 ng/mL) was used for quantification. Linear regressions weighted by 1/x were applied, and Analyst v.1.7.1 was used to control the instrument and process the data. Each phytohormone peak was normalized to that of its corresponding labelled form, except for JA and OPDA, which were normalized to that of 13C6-JA-Ile. Plant hormonal levels were assessed in a total of eight plants per treatment, and the experiment was conducted two independent times (n=8).

Statistical analyses

Statistic tests used to assess the different datasets are described in more detail in the figure legends. Normality and equality of variance were verified using Shapiro-Wilk and Levene's tests, respectively using Sigma Plot 14.5 (Systat Software Inc., San Jose, CA, USA). The levels of (bio)luminescence emitted by infected insects and luminescent beads, phytohormonal levels, benzoxazinoid levels and levels of metabolites differentially expressed across (bio)luminescent treatments were statistically assessed by one-way ANOVA followed by Tukey HSD multiple comparison tests in R 4.3.2 (R Core Team 2023). More information about maize transcriptomic and metabolomic data are available in sections: “Plant transcriptomic analyses” and “Plant untargeted metabolomic analyses”).

Results

Root exposure to bioluminescence induce weak transcriptomic responses in plant

To assess the plant responses to bioluminescence, we exposed maize roots to (bio)luminescent sources by inserting them in the rhizosphere 3-4 cm apart from the plant stem (Distant) or by placing them in close proximity to the roots (Close). We used *Photorhabdus*-infected insects or luminescent beads as (bio)luminescent sources, while untreated plants and insects infected with aluminescent *Photorhabdus* bacteria served as controls. To verify the biological relevance of this approach, we compared the light emitted by *Photorhabdus*-infected insects and luminescent beads (**Fig. 1**). Our characterization confirmed that the luminescence emitted by the beads is equivalent to natural *Photorhabdus* bioluminescence in terms of intensity and wavelength range. After the validation of our experimental approach, we investigated the global transcriptomic plant responses (**Fig. 2**). Despite the fact that there are DEGs in the different treatments for both experimental setups, we did not observe clear group differentiation on the PCA. This suggests that, overall, bioluminescence does not induce discernible transcriptomic responses in plants. We hypothesize that plants are capable of sensing *Photorhabdus* bioluminescence belowground and responding to it by activating defensive mechanisms. In maize, benzoxazinoids are the most abundant defensive secondary metabolites, while phytohormones, including ABA, ethylene, JA, and SA are involved in stress responses. Plants also use indirect defense strategies by emitting volatiles, such as terpenes. To pursue our investigation, we examined changes in the transcript levels of genes involved in light signaling, phytohormone signaling, benzoxazinoids biosynthesis, and terpene biosynthesis pathways (**Figs. 3, 4, 5, and 6**). Our results indicate that only a few genes exhibit differential expression, and these changes were not consistently observed across treatments and experimental setups. Plants are also able to reconfigure their primary metabolism in response to environmental changes. To evaluate this aspect, we examined differential expression of genes involved in the citric acid cycle and did not detect significant changes (**Fig. 7**). Taken together, our results suggest that (bio)luminescence exposure triggers minor transcriptomic responses in plants.

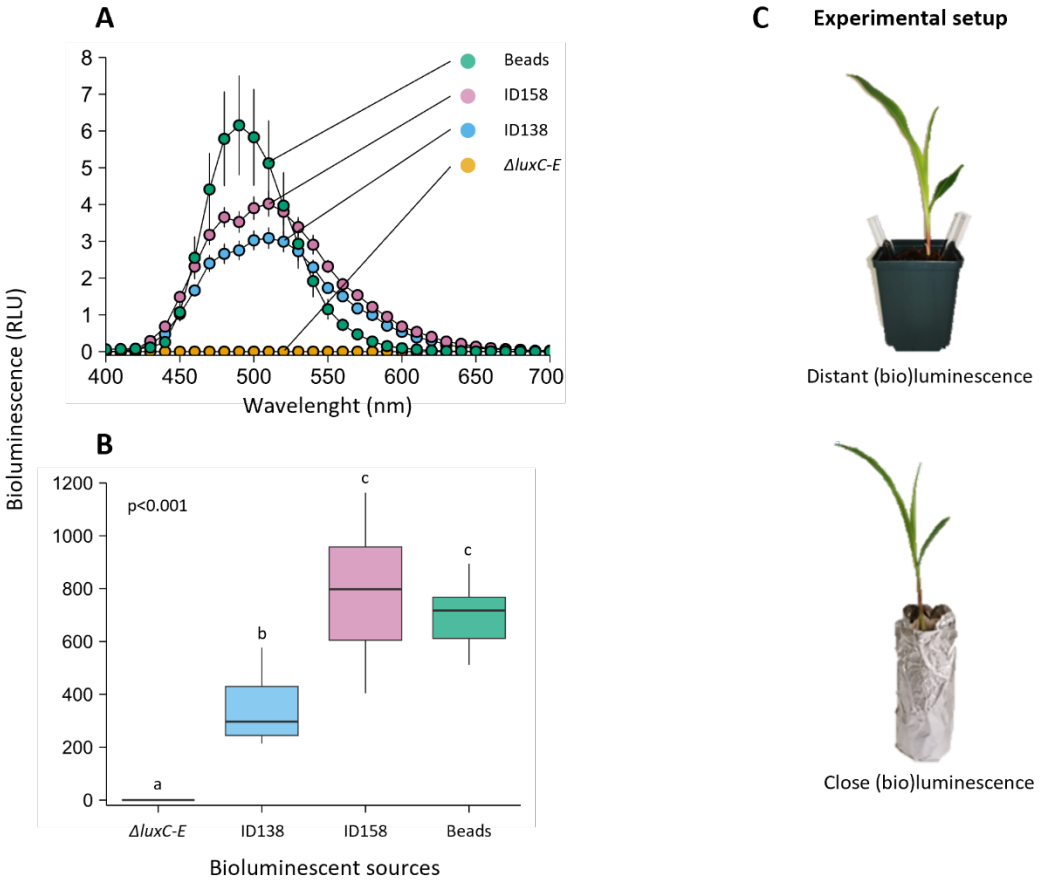


Figure 1. Characteristics of (bio)luminescent sources and experimental setups used for (bio)luminescence exposure treatments. (**A**) Scan of the mean (± SEM) intensities of (bio)luminescence for wavelength from 400 nm to 700 nm emitted by luminescent beads exposed for 30 s to sunlight (Beads), or by *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724^{T} (ID158), *P. tasmaniensis* strain USCA01 (ID138), or *P. laumondii* subps. *laumondii ΔluxC-E* strain DJC-23 (*ΔluxC-E*). (**B**) Total (bio)luminescence levels emitted by luminescent beads exposed to sunlight for 30 s (Beads), or by *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724^{T} (ID158), *P. tasmaniensis* strain USCA01 (ID138), or *P. laumondii* subps. *laumondii ΔluxC-E* strain DJC-23 (*ΔluxC-E*). Box plots show minimum, first quartile, median, third quartile, and maximum values. (**C**) Experimental setups used to induce plants. (Bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant (bio)luminescence) or placed in close proximity to the roots (Close (bio)luminescence). (Bio)luminescence levels were measured in twelve replicates for each treatments (n=12). Different letters indicate statistically significant differences in (bio)luminescence emission levels ($p<0.05$ by one-way ANOVA with Tukey HSD test for multiple comparisons). RLU: relative light units.

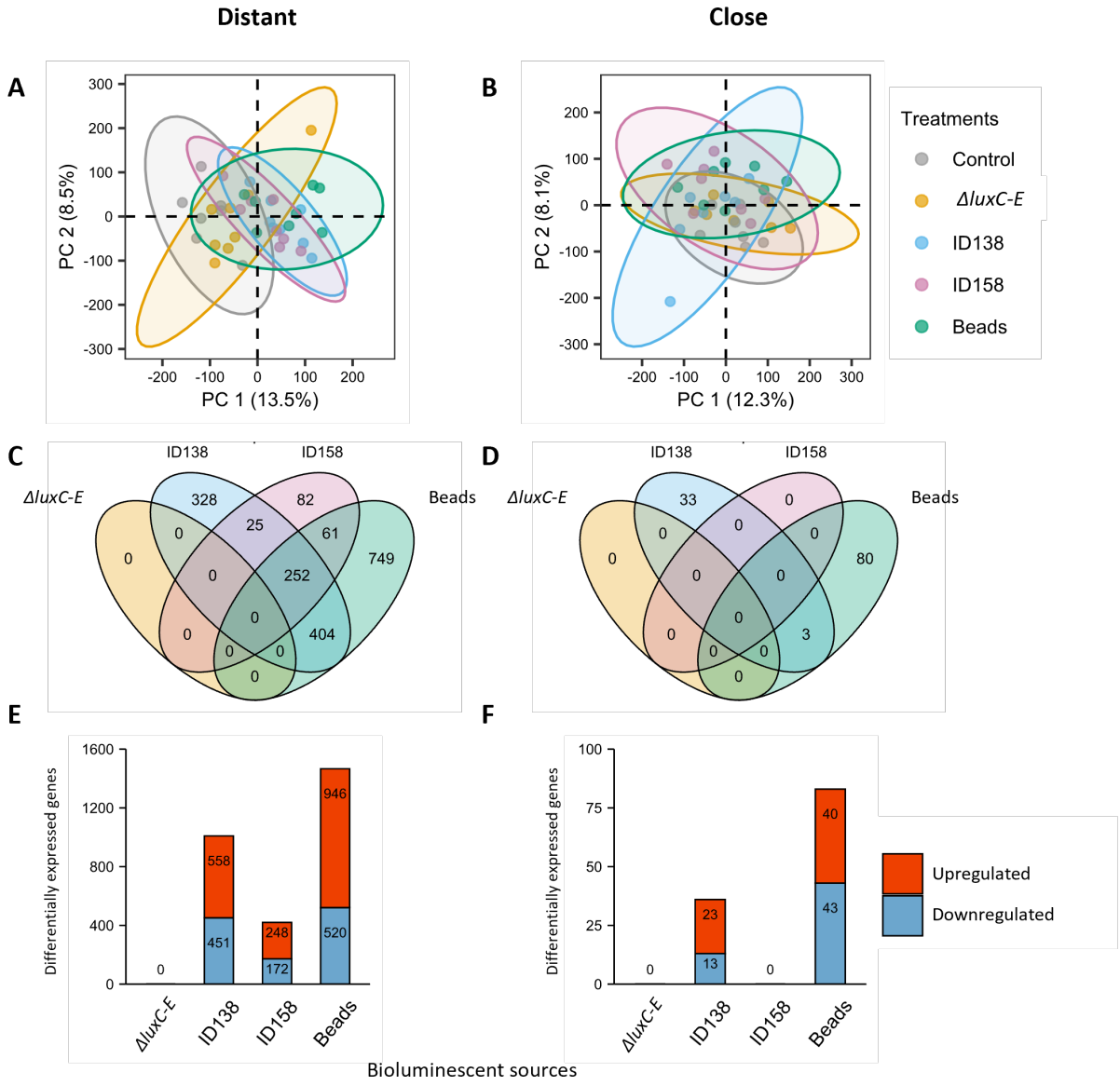
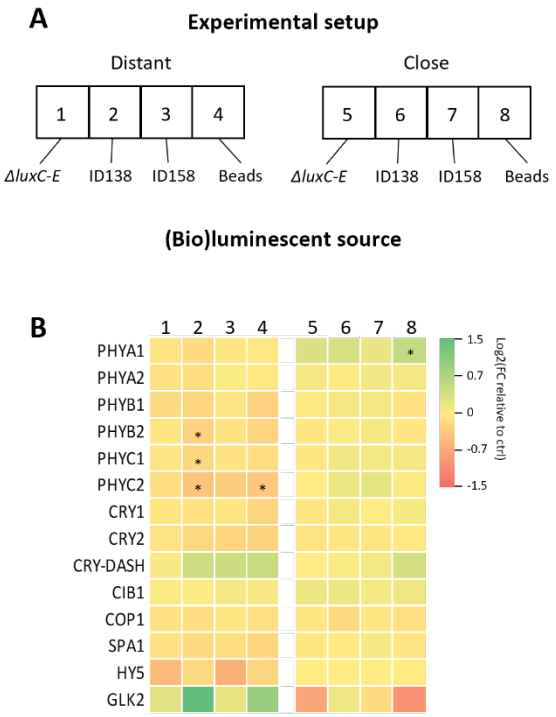


Figure 2. Roots exposure to (bio)luminescent sources induce weak root transcriptomic responses in maize. (**A-B**) Principal component analysis (PCA) plots of root transcript abundance upon (bio)luminescence exposure treatments. (**C-D**) Venn diagrams illustrating the number of differentially expressed genes (DEGs) by the different treatments. (**E-F**). Number of differentially expressed genes (DEGs) by the different treatments. (Bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant, panels A, C, and E) or placed in close proximity to the roots (Close, panels B, D, and F). Roots were exposed to luminescent beads (Beads) or to *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724T (ID158), a high bioluminescence producer, or with P. *tasmaniensis* strain USCA01 (ID138), an intermediate bioluminescence producer. As controls, plant roots were exposed to insects infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 *(ΔluxC-E*) or were left untreated. Eight replicates per treatment were analyzed (n=8).



C

Pathway	Function	Gene name
Photochrome	Red/far red photoreceptor	PHYA1
		PHYA2
		PHYB1
		PHYB2
		PHYC1
		PHYC2
Cryptophore	Blue photoreceptor	CRY1
		CRY2
	Light signaling	CRY-DASH
	Blue light signaling	CIB1
		COP1
		SPA1
		HY5
		GLK2

Figure 3. Roots exposure to (bio)luminescent sources induce weak transcriptomic responses of genes related to light signaling. (**A**) Description of treatments and their arrangement in the heatmaps. (**B**) Log2-fold changes (FC) in transcript abundance of genes related to light signaling upon the different treatments compared to controls. (**C**) Description of the genes analyzed. Fold changes values are represented in a red-yellow-green color scale. Red color indicates downregulation and green color indicates upregulation of gene expression. Asterisks indicate statistically significant differences between control and treatments ($P<0.05$ by t-test followed by Benjamini-Hochberg's False Discovery Rate (FDR) corrections).

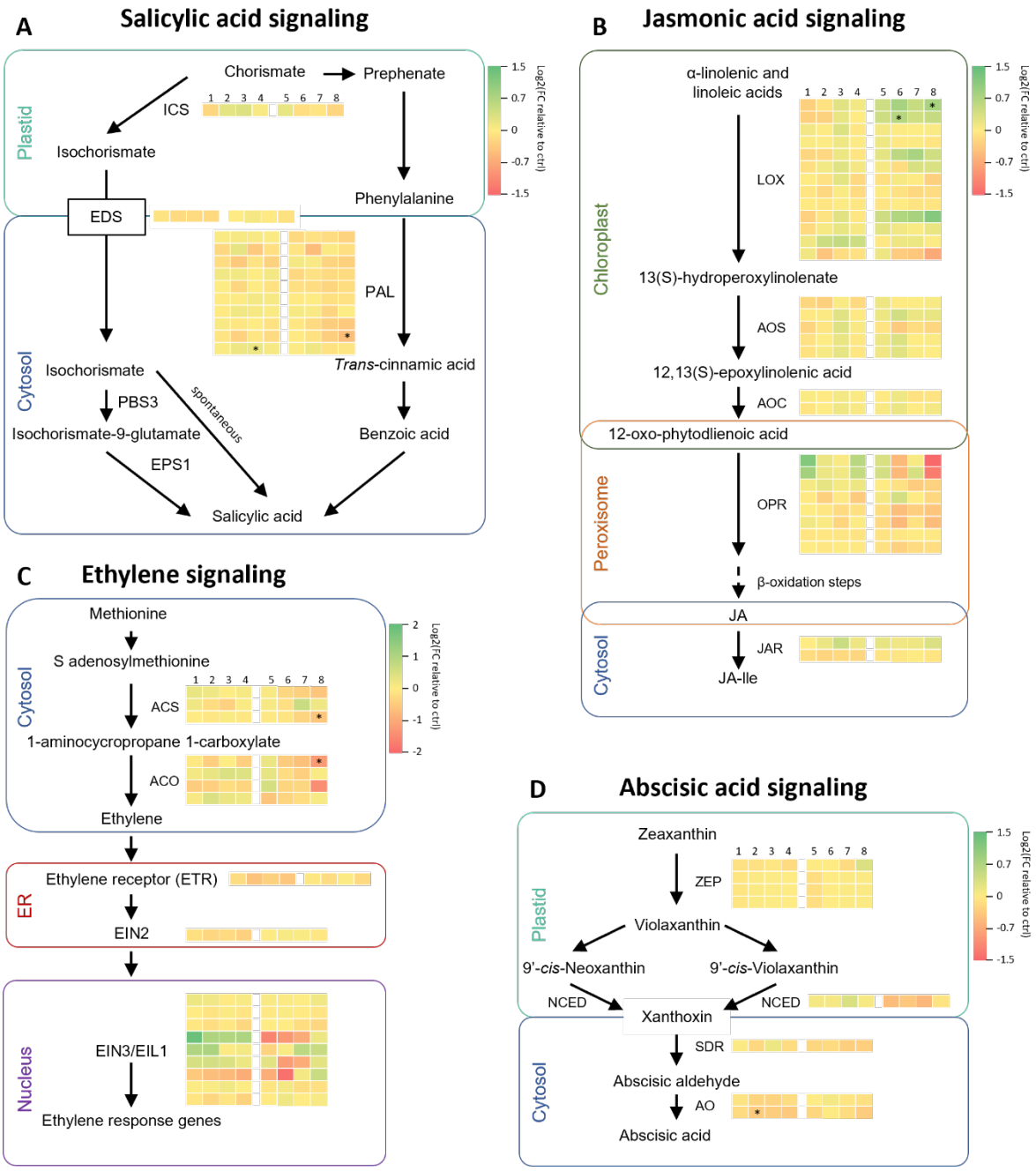


Figure 4. Roots exposure to (bio)luminescent sources induce weak transcriptomic responses of genes related to phytohormone signaling. (**A-D**) Log2 fold changes (FC) in transcript abundance of genes related to phytohormone signaling upon the different treatments compared to controls. (**A**) Genes related to salicylic acid signaling. (**B**) Genes related to jasmonic acid signaling. (**C**) Genes related to ethylene signaling. (**D**) Genes related to abscisic acid signaling. For the description of treatments and their arrangement in the heatmaps, refer to Fig. 3A. Fold changes values are represented in a red-yellow-green color scale. Red color indicates downregulation and green color indicates upregulation of gene expression. Asterisks indicate statistically significant differences between control and treatments (P<0.05 by t-test followed by Benjamini-Hochberg's False Discovery Rate (FDR) corrections).

A

Precursors	Enzymes	Products
GPP	PTS1/2/6/11	linalool
	TPS6/11	α-thujene sabinene
	TPS6/11/26	α-terpinolene β-myrcene limonene
	TPS26	α-terpineol γ-terpinene gerniol
GGPP	TPS2	geranyllinalool
FPP	TPS1/2	nerolidol
	TPS1	farnesol
	TPS1/4/5/10	β-farnesene
	TPS4/5	β-curcumene γ-curcumene sesquithujene
	TPS4/5/10	sesquisabinene a α-bergamotene
	TPS8/10/23	β-caryophyllene
	TPS4/5/6/10/11	β-bisabolene
	TPS6/11	β-macrocarpene
	TPS7	t-cadinol
	TPS10	α-muurolene δ-cadinene zingiberene sesquiphellandrene
	TPS8/10	α-copaene germacrene d
	TPS17	eudesmanediol
	TPS21	β-selinene
Geranyllinalool	CYP92C6	TMTT
Nerolidol	CYP92C5	DMNT

B

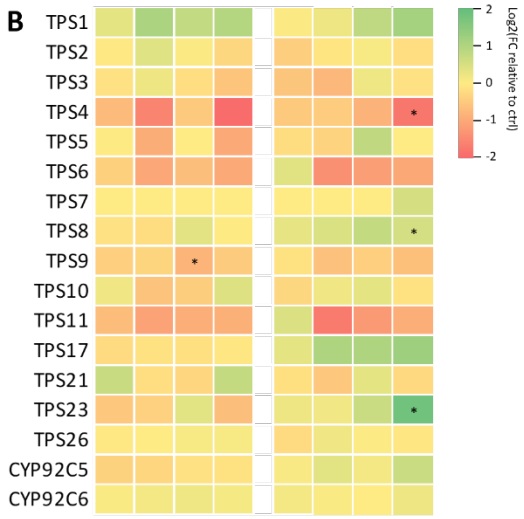


Figure 5. Roots exposure to (bio)luminescent sources induce weak transcriptomic responses of genes related to terpene biosynthesis. (**A**) Description of the enzymes involved in the production of volatile terpenes in maize. GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate; TPS, terpene synthase; CYP92C5 and CYP92C6, cytochrome P450 monooxygenases; TMTT, (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene; DMNT, (E)-3,8-dimethyl-1,4,7-nonatriene. (**B**) Log2 fold changes (FC) in transcript abundance of genes related to terpene biosynthesis upon the different treatments compared to controls. For the description of treatments and their arrangement in the heatmaps, refer to Fig. 3A. Fold changes values are represented in a red-yellow-green color scale. Red color indicates downregulation and green color indicates upregulation of gene expression. Asterisks indicate statistically significant differences between control and treatments ($P<0.05$ by t-test followed by Benjamini-Hochberg's False Discovery Rate (FDR) corrections).

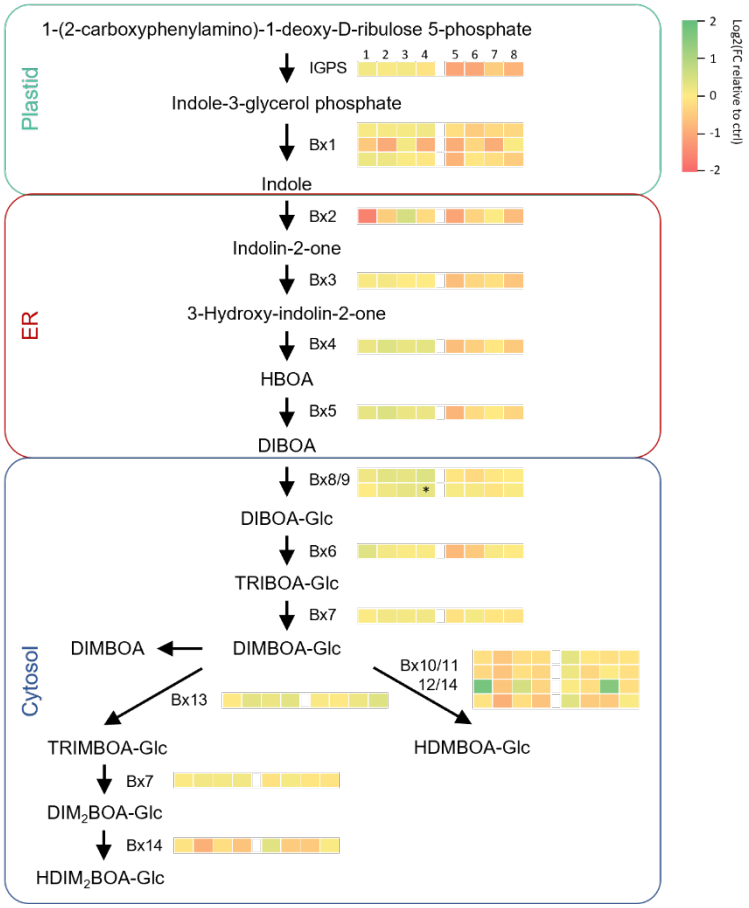


Figure 6. Roots exposure to (bio)luminescent sources induce weak transcriptomic responses of genes related to benzoxazinoid biosynthesis. Log2-fold changes (FC) in transcript abundance of genes related to benzoxazinoid biosynthesis upon the different treatments compared to controls. For the description of treatments and their arrangement in the heatmaps, refer to Fig. 3A. Fold changes values are represented in a red-yellow-green color scale. Red color indicates downregulation and green color indicates upregulation of gene expression. Asterisks indicate statistically significant differences between control and treatments ($P<0.05$ by t-test followed by Benjamini-Hochberg's False Discovery Rate (FDR) corrections).

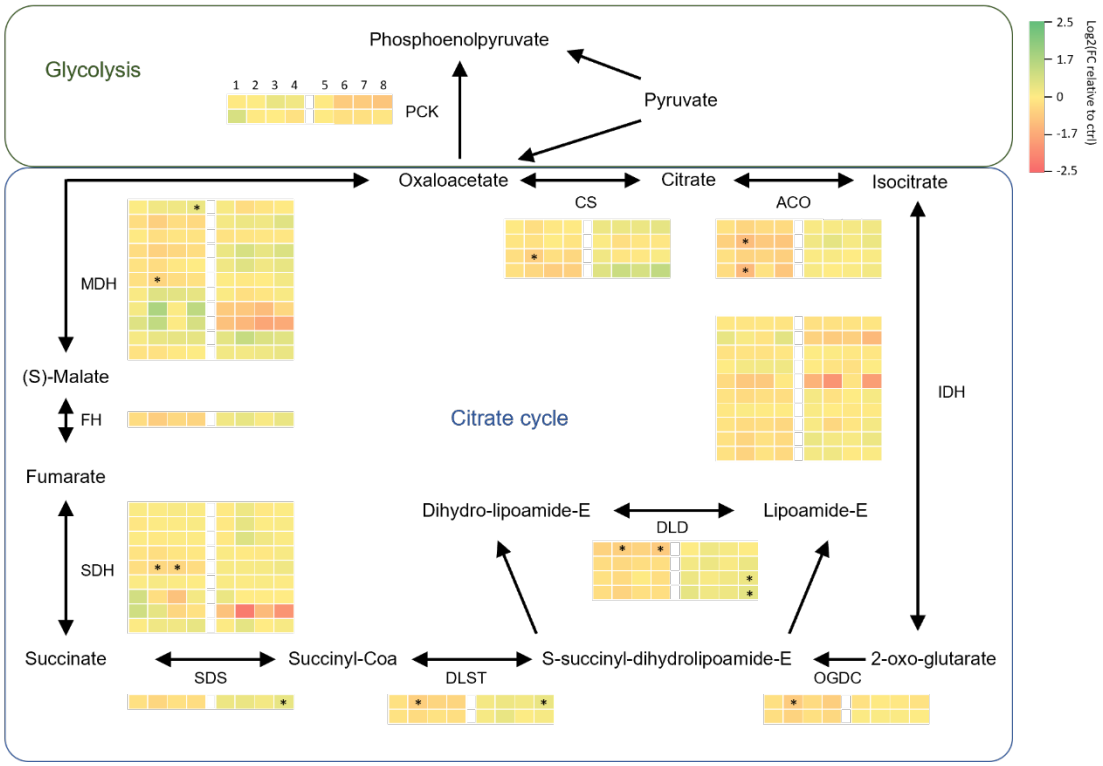


Figure 7. Roots exposure to (bio)luminescent sources induce weak transcriptomic responses of genes related to citric acid cycle. Log2-fold changes (FC) in transcript abundance of genes related to citric acid cycle upon the different treatments compared to controls. For the description of treatments and their arrangement in the heatmaps, refer to Fig. 3A. Fold changes values are represented in a red-yellow-green color scale. Red color indicates downregulation and green color indicates upregulation of gene expression. Asterisks indicate statistically significant differences between control and treatments ($P<0.05$ by t-test followed by Benjamini-Hochberg's False Discovery Rate (FDR) corrections).

Bioluminescence exposure does not lead to major alteration of phytohormonal levels

Phytohormones are key messengers orchestrating plant responses to environmental cues, such as light. Their levels are tightly controlled by multiple signaling pathways and feedback mechanisms. Small transcriptomic changes can lead to important phytohormonal level changes. To follow up on our investigations, we measured phytohormonal level changes in plants whose roots were exposed or not to (bio)luminescence (**Fig. 8**). We detected an increase in ABA levels in plants whose roots were closely exposed to insects infected by *P. cinerea* DSM 19724^{T} (ID158). This is the only change we observed, and it is not corroborated in the distant experimental setup. In summary, our results reveal that exposure to (bio)luminescence can lead to minor changes in phytohormone levels, as evidenced by a slight increase in ABA. However, such exposure does not significantly alter overall phytohormonal levels.

Root exposure to bioluminescence induce weak metabolomic responses in plant

Plants dynamically respond to their environment through the production of secondary metabolites. To deepen our study, we evaluated if bioluminescence emitted by *Photorhabdus*-infected insects in the rhizosphere induces a metabolomic response in plants. We performed an analysis of small molecular weight metabolites produced either in roots or in leaves of plants whose roots were exposed or not to (bio)luminescence. We observed weak responses at the global level, with few metabolites differentially produced in both roots and leaves (**Figs. 9 and 10**, respectively). To refine our approach, we focused on most abundant defensive secondary metabolites in maize, benzoxazinoids (**Fig. 11**). In the close experimental setup, control treatments (untreated plants and plants whose roots were exposed to aluminescent insects) exhibit a twofold increase in total benzoxazinoid levels compared to these control treatments in the distant experimental setup. Our results indicate an increase in total benzoxazinoid levels in maize leaves when the roots were exposed to (bio)luminescence in the distant experimental setup, contrasting with the absence of such an increase in the close experimental setup. This increase in total benzoxazinoid level is explained by the accumulation of DIMBOA-Glc and HMBOA-Glc in the leaves of (bio)luminescent exposed plants (**Fig. 12**). This accumulation is higher in plants whose roots were exposed to insect infected with *P. cinerea* strain DSM 19724^{T} (ID158), and in plants whose roots were exposed to luminescent beads (Beads), the two highest (bio)luminescence intensity treatments.

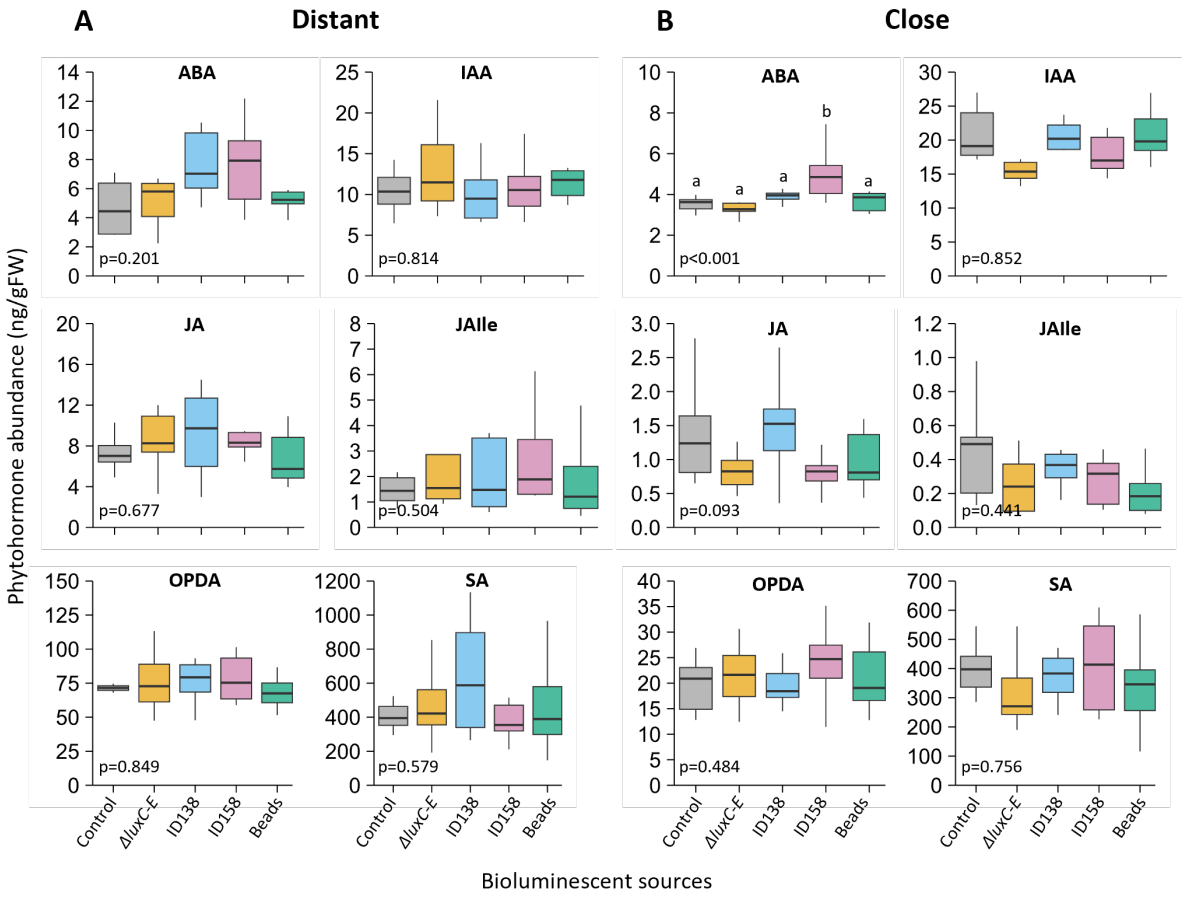


Figure 8. *Photorhabdus* bioluminescence does not lead to major alteration of phytohormonal levels. Phytohormone levels in the roots of plants whose roots were exposed or not to (bio)luminescence. The following phyotohormones were measured: abscisic acid (ABA), indole-3- acetic acid (IAA), jasmonic acid, jasmonoyl-l-isoleucine (JAIle), 12-oxo-phytodienoic acid (OPDA), and salicylic acid (SA). (Bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant, **panel A**) or placed in close proximity to the roots (Close, **panel B**). Roots were exposed to luminescent beads (Beads) or to *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724T (ID158), a high bioluminescence producer, or with P. *tasmaniensis* strain USCA01 (ID138), an intermediate bioluminescence producer. As controls, plant roots were exposed to insects infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 (*ΔluxC-E*) or were left untreated. Eight replicates per treatment were analyzed (n=8). Boxplots show minimum, first quartile, median, third quartile, and maximum values. Different letters indicate statistically significant differences in phytohormone levels (P<0.05 by one-way ANOVA with Tukey HSD test for multiple comparisons).

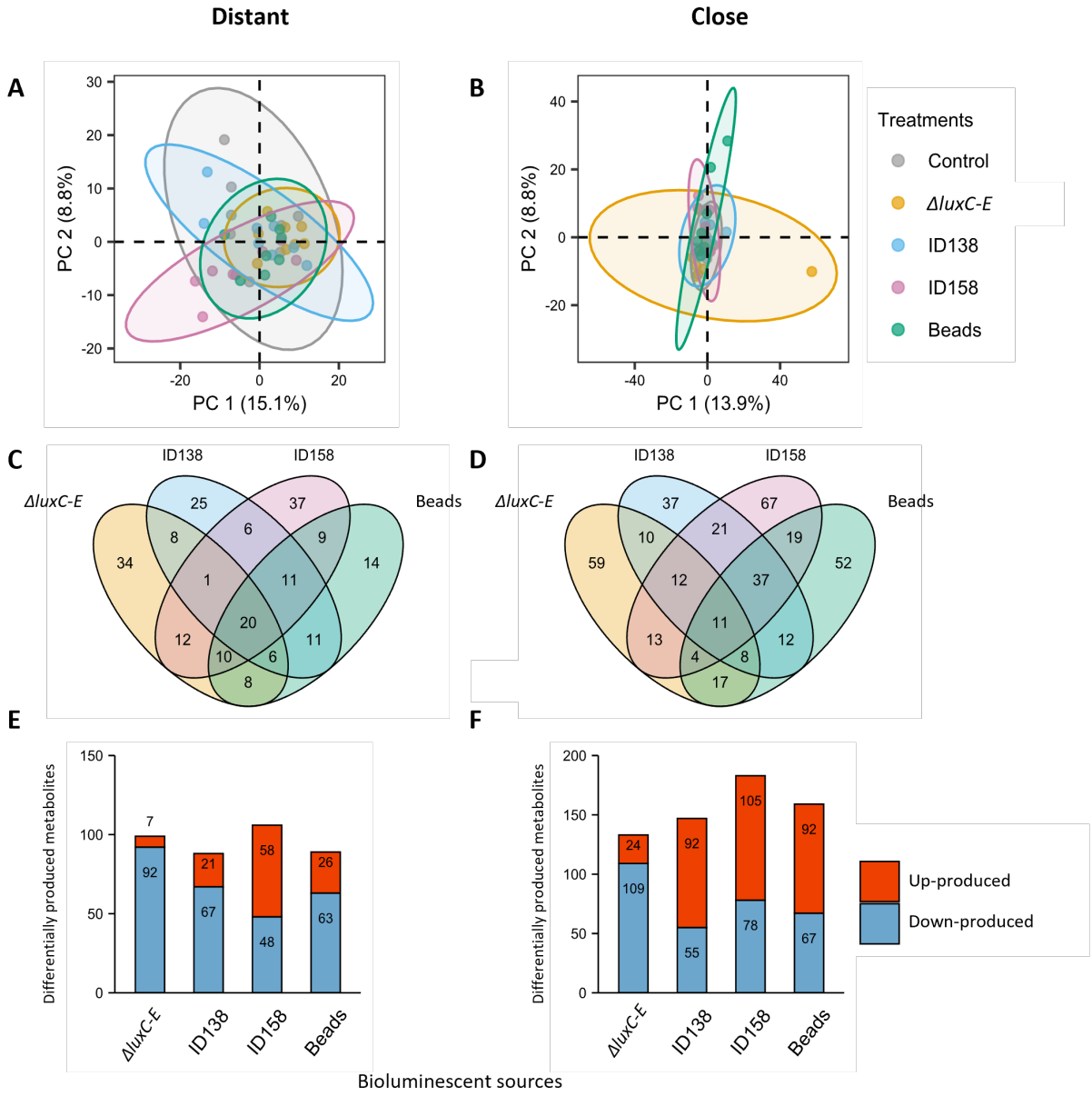


Figure 9. Roots exposure to (bio)luminescent sources induce weak root metabolomic responses. (**A-B**) Principal component analysis (PCA) plots of root metabolites abundance upon (bio)luminescence exposure treatments. (**C-D**) Venn diagrams illustrating the number of differentially produced metabolites by the different treatments. (**E-F**). Number of differentially produced metabolites by the different treatments. (Bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant, panels **A, C, and E**) or placed in close proximity to the roots (Close, panels **B, D, and F**). Roots were exposed to luminescent beads (Beads) or to *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724T (ID158), a high bioluminescence producer, or with P. *tasmaniensis* strain USCA01 (ID138), an intermediate bioluminescence producer. As controls, plant roots were exposed to insects infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 *(ΔluxC-E*) or were left untreated. Eight replicates per treatment were analyzed (n=8).

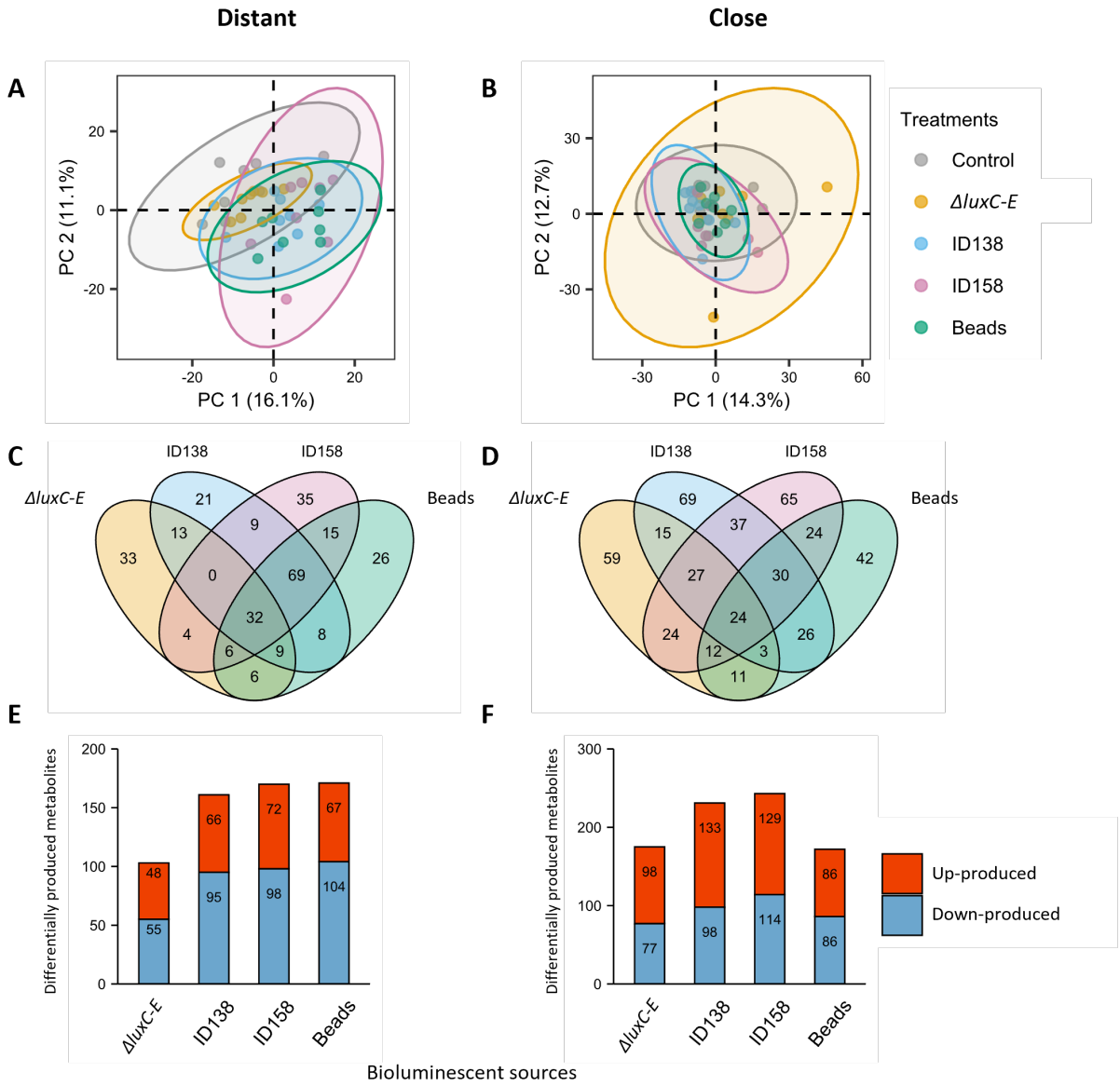


Figure 10. Roots exposure to (bio)luminescent sources induce weak leaf metabolomic responses. (**A-B**) Principal component analysis (PCA) plots of leaf metabolites abundance upon (bio)luminescence exposure treatments. (**C-D**) Venn diagrams illustrating the number of differentially produced metabolites by the different treatments. (**E-F**). Number of differentially produced metabolites by the different treatments. (Bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant, panels **A, C, and E**) or placed in close proximity to the roots (Close, panels **B, D, and F**). Roots were exposed to luminescent beads (Beads) or to *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724T (ID158), a high bioluminescence producer, or with P. *tasmaniensis* strain USCA01 (ID138), an intermediate bioluminescence producer. As controls, plant roots were exposed to insects infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 *(ΔluxC-E*) or were left untreated. Eight replicates per treatment were analyzed (n=8).

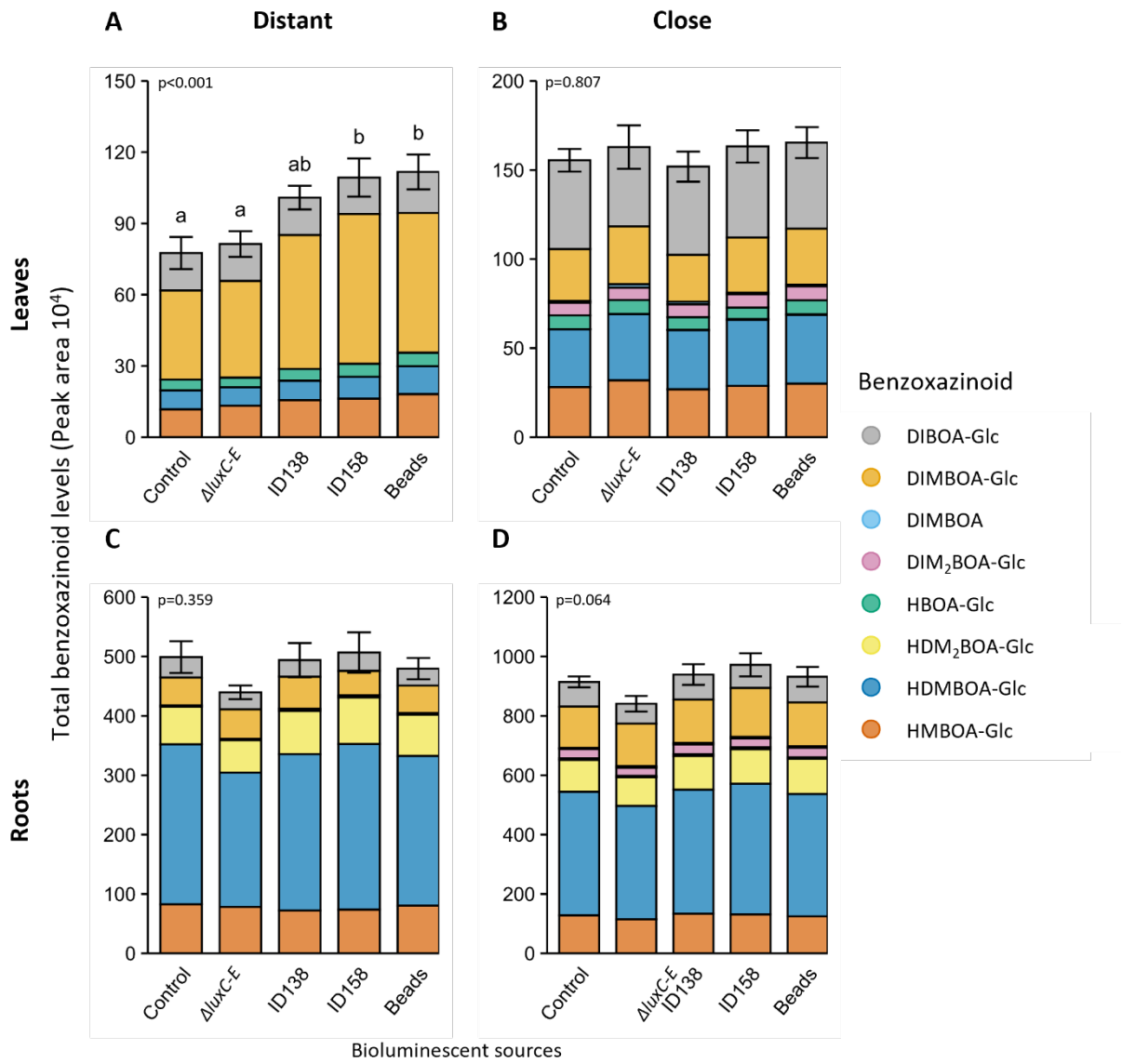


Figure 11. Roots exposure to (bio)luminescent sources induces the production of benzoxazinoid in a tissue-specific manner. Total benzoxazinoid levels in leaves (**A, B**) and roots (**C, D**) of plants whose roots were exposed or not to (bio)luminescence. (Bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant, panels **A and C**) or placed in close proximity to the roots (Close, panels **B and D**). Roots were exposed to luminescent beads (Beads) or to *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724T (ID158), a high bioluminescence producer, or with P. *tasmaniensis* strain USCA01 (ID138), an intermediate bioluminescence producer. As controls, plant roots were exposed to insects infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 *(ΔluxC-E*) or were left untreated. Eight replicates per treatment were analyzed (n=8). Different letters indicate significant differences in benzoxazinoid levels ($p < 0.05$ by one-way ANOVA with Tukey HSD test for multiple comparisons).

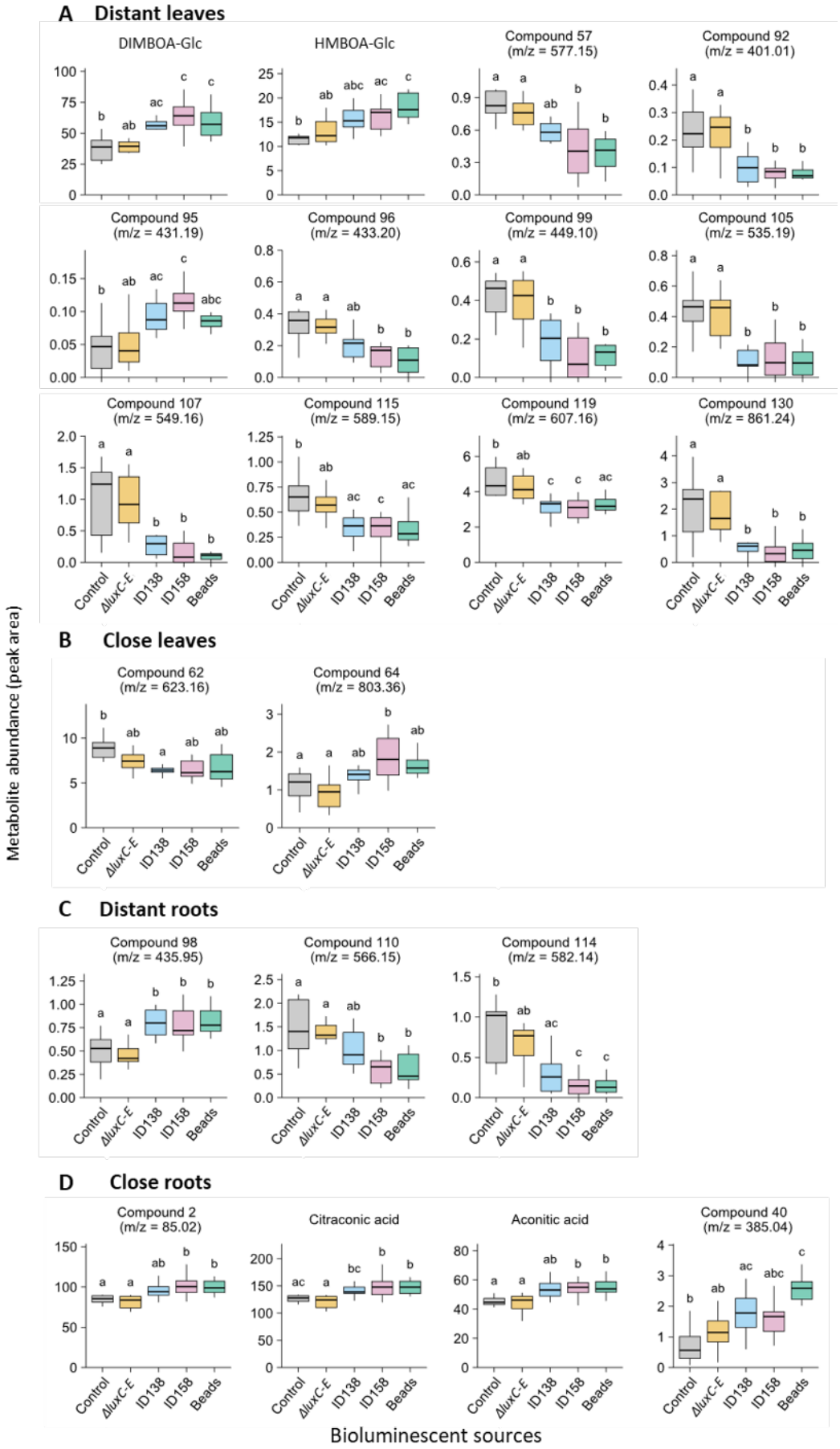

A Distant leaves
DIMBOA-Glc
HMBOA-Glc
Compound 57 (m/z = 577.15)
Compound 92 (m/z = 401.01)
Compound 95 (m/z = 431.19)
Compound 96 (m/z = 433.20)
Compound 99 (m/z = 449.10)
Compound 105 (m/z = 535.19)
Compound 107 (m/z = 549.16)
Compound 115 (m/z = 589.15)
Compound 119 (m/z = 607.16)
Compound 130 (m/z = 861.24)
B Close leaves
Compound 62 (m/z = 623.16)
Compound 64 (m/z = 803.36)
C Distant roots
Compound 98 (m/z = 435.95)
Compound 110 (m/z = 566.15)
Compound 114 (m/z = 582.14)
D Close roots
Compound 2 (m/z = 85.02)
Citraconic acid
Aconitic acid
Compound 40 (m/z = 385.04)
Control
ΔluxC-E
ID138
ID158
Beads
Metabolite abundance (peak area)
Bioluminescent sources

Figure 12. Plant metabolomic responses to root (bio)luminescence exposure. Abundance (peak area) of low molecular weight metabolites in leaves (A, B) and roots (C, D) of plants whose roots were exposed or not to (bio)luminescence. (Bio)luminescent sources were either inserted in the rhizosphere 3-4 cm apart from the plant stem (Distant, panel A and C) or placed in close proximity to the roots (Close, panel B and D). Roots were exposed to luminescent beads (Beads) or to *G. mellonella* larvae infected with either *P. cinerea* strain DSM 19724T (ID158), a high bioluminescence producer, or with P*. tasmaniensis* strain USCA01 (ID138), an intermediate bioluminescence producer. As controls, plant roots were exposed to insects infected with aluminescent *P. laumondii* subsp. *laumondii ΔluxC-E* strain DJC-23 (*ΔluxC-E*) or were left untreated. Eight replicates per treatment were analyzed (n=8). Boxplots show minimum, first quartile, median, third quartile, and maximum values. Different letters indicate statistically significant differences in metabolite abundance ($P<0.05$ by one-way ANOVA with Tukey HSD test for multiple comparisons). For information on the chemical identification of the different compounds, refer to Table S1.

Only a few metabolites exhibited consistent differential regulation across all (bio)luminescent treatments when compared to controls. Specifically, 12 metabolites in leaves and 3 in roots exhibited such regulation in the distant experimental setup, whereas 2 metabolites in leaves and 4 in roots did so in the close experimental setup. Additionally, there was no overlap in those metabolites between tissues and experimental setups (**Fig.12 and Table S2**). Our results suggest that plants exhibit tissue-specific metabolomic responses to bioluminescence exposure. These responses are dependent on the intensity of bioluminescence exposure.

Discussion

Plants sense the sunlight that diffuse through the first centimeters under the soil surface and respond to it by adapting their RSA (**Gelderen et al., 2018; Mo et al., 2015; Silva-Navas et al., 2015; Yun et al., 2023**). While subterranean environments are rather dark, light sources, such as bioluminescence are present (**Pes et al., 2016; Thomas and Poinar, 1979; Seesamut et al., 2021; Verdes and Gruber, 2017; Waterfield et al., 2009**). Soil-dwelling nematodes belonging to the *Heterorhabditis* genus live in obligate symbiosis with bioluminescent bacteria belonging to the *Photorhabdus* genus. Together, they parasitize small arthropods, including root herbivores, leading to the occurrence of bioluminescent infected organisms in the rhizosphere. We hypothesize that bioluminescence produced by *Photorhabdus*-infected insects is detected by root photosensors, informing the plant about the presence of both herbivorous insects and *Heterorhabditis* nematodes in the rhizosphere.

In nature, the sunlight diffusing in the soil modulate plant RSA (**Gelderen et al., 2018; Mo et al., 2015; Silva-Navas et al., 2015; Yun et al., 2023**). This biological process is enabled by the presence of photoreceptors and light signal transduction elements in the roots (**Warnasooriya and Montgomery, 2011**). *Photorhabdus* bacteria produce cyan bioluminescence with a peak emission at approximately 480 nm. Among plant photoreceptors present in their roots, CRYs and PHOTs sense light spectrum from 320 to 500 nm (**Briggs and Lin, 2012**; **Mo et al., 2015**). The intrinsic characteristic of light limits its dispersion though the soil matrix, with red and far-red light penetrating deeper that blue light (**Mo et al., 2015**). Herbivore insects can be parasitized by the *Heterorhabditis/Photorhabdus* pair while feeding and plant roots. This results in the glow of the infected insects inside the rhizosphere. These two aspects support the hypothesis that *Photorhabdus* bioluminescence could be detected by plants belowground.

It was shown under laboratory settings that roots exposure to light induces strong burst of ROS signaling, and therefore, can be considered a stress (**Manzano et al., 2014; Passaia et al., 2014; Tsukagoshi et al., 2010; Yokawa et al., 2011**). We hypothesize that *Photorhabdus* bioluminescence might represent a stress for plant. Our results reveal that roots exposure to bioluminescence induces minor changes in phytohormonal levels and weak transcriptomic responses of phytohormones biosynthetic pathways. We detected an increase in ABA levels in plants whose roots were closely exposed to insects infected by *P. cinerea* DSM 19724^{T}

(ID158). It has been shown that excessive light exposure can activate the phenylpropanoid pathway in an ABA-dependent manner (**Huang et al., 2019**). Abscisic acid might accumulate upon light exposure in an intensity-dependent manner. Therefore, *Photorhabdus* bioluminescence might trigger the accumulation of ABA in plants only when a high number of insects are infected in the rhizosphere. It has been experimentally shown that auxin response is impaired in plants whose roots were exposed to light, affecting root phototropism (**Del Pozo and Manzano, 2014**). The blue photoreceptors CRY1, CRY2 and PHOT1 are suggested to play role in this process by affecting auxin transport and free auxin levels (**Fankhauser and Christie, 2015; Galen et al., 2007; Mo et al., 2015; Wan et al., 2012; Zhang et al., 2013**). We did not detected change in IAA levels after root exposure to *Photorhabdus* bioluminescence. Nevertheless, it is possible that *Photorhabdus* bioluminescence affects auxin-dependent physiological processes by modulating auxin transport or cell compartmentalization. Such putative mechanisms could influence root phototropism and thereby modulate the plant RSA. Exploring this aspect further could provide valuable insights into the interplay between *Photorhabdus* bioluminescence, phytohormone signaling and plant development. In *A. thaliania*, root exposure to light induces the expression of *phyB* in the root cap and the expression of *phyD* in the whole roots (**Goosey et al., 1997; Somers and Quail, 1995a, 1995b**). We did not detect differential expression of these genes in our transcriptomic investigations. However, these photoreceptors are involved in red and far-red light perception and *Photorhabdus* bacteria produce cyan bioluminescence.

We hypothesize that *Photorhabdus* bioluminescence inform plants about the presence of both herbivore insects and their natural enemies, EPNs, in the rhizosphere. Plants respond to biotic stress, such as herbivory, by producing secondary metabolites. We pursue our investigation by assessing if *Photorhabdus* bioluminescence triggers metabolomic responses in plants. We detected, in the distant experimental setup, an increase in total benzoxazinoid content in the leaves of exposed plants, caused by the accumulation of both DIMBOA-Glc and HMBOA-Glc in this tissue. This metabolic plant response to bioluminescence is intensity-dependent. Benzoxazinoids are typically stored in their glucoside forms in plants and are activated upon plant injury by enzymatic conversion into agluconic forms (**Hopkins et al., 2009**). Our result suggests that exposure to bioluminescence belowground could activate defense aboveground, exemplified by the accumulation of DIMBOA-Glc, which is known for its toxicity

towards a wide range of herbivores (**Sicker et al., 2000**). It is common for herbivorous insects, such as the western corn rootworm, to dwell in the soil during their larval stage and then transition to living aboveground as adults, where they feed on the shoot parts of plants (**Chiang, 1973**). It was shown in maize that root herbivory by the western corn rootworm can induce plant defense aboveground defense, resulting in increased resistance against the aboveground herbivore *Spodoptera littoralis* (**Erb et al., 2009**). Similarly, we hypothesize that the accumulation of DIMBOA-Glc and HMBOA-Glc in leaves of plants whose root were exposed to *Photorhabdus* bioluminescence might contribute to systemic plant defense.

We observed benzoxazinoid accumulation in only one of the two experimental setups we used. This disparate result might be attributed to the stress inherent to the close experimental setup. In this specific setup, the (bio)luminescent sources, placed in 2 mL polystyrene tubes, were inserted as close as possible to the roots. While great care was taken during the insertion of the (bio)luminescent sources to avoid damaging the roots, the surrounding soil was disturbed. This manipulation might have affected soil microorganisms at the interface between roots and soil. Rhizosphere microorganisms play significant roles in plant development and are involved in multiple processes such as nitrogen fixation, pesticides detoxification, improvement of biotic and abiotic stress tolerance (**Adeneji et al., 2019; Koza et al., 2022; Omotayo and Babalola 2021**). The putative disruption of the soil surrounding the roots might have led a generalized increase in benzoxazinoid content that overshadows the impact of (bio)luminescent exposure. In the distant experimental setup, the (bio)luminescent sources were placed 3-4 cm apart from the plant stem, ensuring that the soil in direct contact with the roots remained undisturbed. This hypothesis is supported by the fact that the total benzoxazinoid content is twofold higher in both leaves and roots of plants in the close experimental setup compared to the distant experimental setup. It could be argued that such stress would likely trigger an increase in phytohormonal levels involved in roots-microbes interactions, including JA and SA, in the plants of the close experimental setup (**Egamberdieva et al., 2017**). We did not observe this increase in the quantification of phytohormones we performed six days after the beginning of the treatments. However, phytohormones typically return quickly to basal levels after a stress, while glycolyzed benzoxazinoids, serving as a storage form, could have remained at a relatively high concentration. Another possibility is that microbes present in the rhizosphere might mediate the plant responses to *Photorhabdus*

bioluminescence. Under this scenario, the potential disruption of microbial communities in the close experimental setup might have rendered the plants unable to fully integrate *Photorhabdus* bioluminescence.

Our experimental approach aimed to specifically test the effect of *Photorhadbus* bioluminescence. To this end, we used *G. mellonella* larvae infected with either naturally bioluminescent or aluminescent mutant *Photorhabdus* strains enclosed in transparent polystyrene tubes. *Galleria mellonella* is a model organism used in parasitology known for its high susceptibility to infection by the *Heterorhabditis-Photorhabdus* pair and for its conveniency in laboratory (e.g. ease of rearing, cost effectivity). While this organism was ideal for our investigation, we acknowledge that *G. mellonella* is not a natural host for EPNs as this organism parasitizes beehives and do not typically live belowground. Entomopathogenic nematodes are parasitoids that kill a wide range of soil-dwelling insects, including herbivores. Under natural conditions, plants likely perceive additional cues, such as volatiles emitted by EPN-infected insects, herbivory feeding, or volatile organic compounds emitted by surrounding attacked plants, alongside exposure to *Photorhabdus* bioluminescence. It is tempting to posit that these cues might interact and be integrated by plants, each playing a role in the modulation of plant responses. Future investigations exploring how additional cues influence plant responses to *Photorhabdus* bioluminescence would provide valuable insights. A way to explore our hypothesis would be to test whether there is a synergistic effect of both root feeding by the WCR and *Photorhabdus* bioluminescence exposure on the accumulation of benzoxazinoids in maize shoots.

Our results indicate that in maize, root exposure to *Photorhabdus* bioluminescence triggers weak transcriptomic, phytohormonal and metabolomic responses but induces an accumulation in leaves of DIMBOA-Glc and HMBOA-Glc, two defensive secondary metabolites, in an intensity-dependent manner. Taken together our results suggest that *Photorhabdus* bioluminescence might inform plants about the presence of both herbivorous insects and their natural enemies, EPNs, in the rhizosphere, which in turn, initiate plant systemic defensive responses.

Conclusions

We tested whether plants respond to *Photorhabdus* bioluminescence exposure belowground. To do so, we assessed transcriptomic, phytohormonal and metabolomic changes in plants whose roots were exposed to (bio)luminescent sources compared to control treatments. Our results reveal that plants exhibit weak transcriptomic, phytohormonal, and metabolomic responses to (bio)luminescence exposure. Interestingly, we detected, using the distant experimental setup, an increase of total benzoxazinoid content in the leaves of plants whose roots were exposed to (bio)luminescence. This increase resulted from the accumulation of two glycosided benzoxazinoids, DIMBOA-Glc and HMBOA-Glc, and is dependent on the intensity of (bio)luminescence. Altogether, our findings suggest that plant are capable of sensing *Photorhabdus* bioluminescence belowground and responding to it by activating defense response aboveground in an intensity-dependent manner. This bioluminescence might inform plants about the presence of both herbivorous insects and their natural enemies, EPNs in the rhizosphere. This work provides the first insight about the potential role of *Photorhabdus* bioluminescence as a cross-kingdom signal, modulating defense responses in plants.

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Supplementary materials

Table S1. Characteristics of the genes used for transcriptomic responses analyses.

Figure	Pathway	Gene symbol	Gene name	NCBI identifier
Figure 3	Light signaling	PHYA1	PhytochromeA1	LOC115101004
		PHYA2	PhytochromeA2	LOC103643916
		PHYB1	PhytochromeB1	LOC100381811
		PHYB2	PhytochromeB2	LOC100383702
		PHYC1	PhytochromeC1	LOC103644014
		PHYC2	PhytochromeC2	LOC100192976
		CRY1	Chryptochrome1	LOC100502533
		CRY2	Chryptochrome2	LOC100277533
		CRY-DASH	Chryptochrome3	LOC100280309
		CIB1	Cryptochrome-interaction bHLH1	LOC100383089
		COP1	Constitutively photomorphogenic 1	LOC100286122
		SPA1	Suppressor of phyA	LOC103635349
		HY5	Hypocotyl5	LOC100286123
		GLK2	Golden2-like2	LOC541882
Figure 4A	Salicylic acid signaling	PAL	Phenylalanine ammonia lyase homolog1	LOC542258
		PAL1	Phenylalanine ammonia-lyase1	LOC109941607
		PAL1	Phenylalanine ammonia-lyase1	LOC103655990
		PAL2	Phenylalanine ammonia lyase2	LOC100384215
		PAL4	Phenylalanine ammonia lyase4	LOC100281532
		PAL5	Phenylalanine ammonia lyase5	LOC100285115
		PAL6	Phenylalanine ammonia lyase6	LOC100281042
		PAL7	Phenylalanine ammonia lyase7	LOC100381820
		PAL8	Phenylalanine ammonia lyase8	LOC103627433
		PAL9	Phenylalanine ammonia lyase9	LOC100273579
		ICS	Isochorismate synthase 2 chloroplastic	LOC100275708
		EDS1	Enhanced disease susceptibility protein	LOC100281696
Figure 4B	Jasmonic acid signaling	LOX1	Lipoxygenase	lox1

Figure 4B	Jasmonic acid signaling	LOX2	Lipoxygenase	lox2
		LOX3	Lipoxygenase	lox3
		LOX4	Lipoxygenase	lox4
		LOX5	Lipoxygenase	lox5
		LOX6	Lipoxygenase	lox6
		LOX7	Lipoxygenase	lox7
		LOX8	Lipoxygenase	ts1
		LOX9	Lipoxygenase	lox9
		LOX10	Lipoxygenase	lox10
		LOX11	Lipoxygenase	lox11
		LOX12	Lipoxygenase	lox12
		LOX13	Lipoxygenase	LOC103642039
		AOS1	Allene oxide synthase	aos1
		AOS1c	Allene oxide synthase chloroplastic	LOC103625850
		AOS2	Cytochrome P450 CYP74A19	aos2
		AOS3	Allene oxide synthase	aos3
		AOS4	Allene oxide synthase	aos4
		AOC1	Allene oxide cyclase1	aoc1
		AOC2	Allene oxide cyclase 3 chloroplastic	aoc2
		OPR1	12-oxo-phytodienoic acid reductase1	opr1
		OPR2	12-oxo-phytodienoic acid reductase2	opr2
		OPR3	12-oxo-phytodienoic acid reductase3	opr3
		OPR4	12-oxophytodienoate reductase 1	opr4
		OPR5	12-oxo-phytodienoic acid reductase5	opr5
		OPR6	12-oxo-phytodienoic acid reductase6	opr6
		OPR7	12-oxo-phytodienoic acid reductase7	opr7
		OPR8	12-oxo-phytodienoic acid reductase8	opr8
		JAR1a	Jasmonic acid-amido synthetase JAR1	LOC100381512
		JAR1b	Jasmonic acid-amido synthetase JAR1	LOC100280367

Figure 4C	Ethylene signaling	ZmACS2	1-aminocyclopropane-1-carboxylate synthase2	LOC103646045
		ZmACS6	1-aminocyclopropane-1-carboxylate synthase6	LOC100217270
		ZmACS7	1-aminocyclopropane-1-carboxylate synthase7	LOC100279981
		ZmACO15	1-aminocyclopropane-1-carboxylate oxidase15	LOC100273458
		ZmACO20	1-aminocyclopropane-1-carboxylate oxidase20	LOC542137
		ZmACO31	1-aminocyclopropane-1-carboxylate oxidase15	LOC542136
		ZmACO35	1-aminocyclopropane-1-carboxylate oxidase35	LOC100191321
		ZmETR2	Ethylene receptor homolog2	LOC541627
		ZmEIN2	Ethylene insensitive 2	LOC103649560
		EIL-transcription factor 2	Ethylene insensitive 3-like 2 protein	LOC103647946
		EIL-transcription factor 3	Ethylene insensitive 3-like 3 protein	LOC100285594
		EIL-transcription factor 4	Ethylene insensitive 3-like 3 protein	LOC100216639
		EIL-transcription factor 5	Ethylene insensitive 3-like 5 protein	LOC103653667
		EIL-transcription factor 6	Ethylene insensitive 3-like 2 protein	LOC103633580
		EIL-transcription factor 7	Ethylene insensitive 3-like 5 protein	LOC100274454
		EIL-transcription factor 8	Ethylene insensitive 3-like 5 protein	LOC103627330
		ethylene insensitive-like1	Ethylene insensitive-like1	LOC100279789

Figure 4C	Ethylene signaling	ethylene insensitive-like3	Ethylene insensitive-like3	LOC100285672
Figure 4D	Abscisic acid signaling	ZEPc1	Zeaxanthin epoxidase chloroplastic	LOC103641704
		ZEPc3	Zeaxanthin epoxidase chloroplastic	LOC100285076
		ZEP1	Zeaxanthin epoxidase1	LOC100285076
		ZEP2	Zeaxanthin epoxidase2	LOC100285076
		VP14	Viviparous14	LOC732819
		NCED	9-cis-epoxycarotenoid dioxygenase9	LOC103626043
		SDR	Retinol dehydrogenase 12	LOC100285880
		AO1	Aldehyde oxidase1	LOC542228
		AO2	Aldehyde oxidase2	LOC542229
Figure 5	Terpene biosynthesis	TPS1	Terpene synthase 1	LOC541974
		TPS2	Terpene synthase 2	LOC732758
		TPS3	Terpene synthase 3	LOC732759
		TPS4	Terpene synthase 4	LOC542754
		TPS5	Terpene synthase 5	LOC103641226
		TPS6	Terpene synthase 6	LOC542688
		TPS7	Terpene synthase 7	LOC542156
		TPS8	Terpene synthase 8	LOC732834
		TPS9	Terpene synthase 9	LOC542689
		TPS10	Terpene synthase 10	LOC732751
		TPS11	Terpene synthase 11	LOC103641097
		TPS17	Terpene synthase 17	LOC100281754
		TPS21	Terpene synthase 21	LOC103640304
		TPS23	Terpene synthase 23	LOC103641105
		TPS26	Terpene synthase 26	LOC100125649
		CYP92C5	Cytochrome P450 19	LOC103631958
		CYP92C6	Cytochrome P450 20	CYP92C6
Figure 6	Benzoxazinoid biosynthesis	IGPS	Indole-3-glycerol phosphate synthase chloroplastic	LOC100286258
		BX1-igl1	Tryptophan synthase A homolog1	LOC541855

Figure 6	Ethylene signaling	BX1-igl2	Tryptophan synthase alpha chain chloroplastic	LOC100273163
		BX1	Benzoxazinless1	LOC542117
		BX2	Benzoxazinone synthesis2	LOC100192631
		BX3	Benzoxazinone synthesis3	LOC103652724
		BX4	Benzoxazinone synthesis4	LOC100382554
		BX5	Benzoxazinone synthesis5	LOC103652726
		BX6	DIBOA-glucoside dioxygenase6	LOC541977
		BX7	O-methyltransferase ZRP4	LOC100147731
		BX8	Benzoxazinone synthesis8	LOC100277344
		BX9	Benzoxazinone synthesis9	LOC100274317
		BX10	Benzoxazinone synthesis10	LOC103635822
		BX11	Benzoxazinone synthesis11	LOC100272836
		BX12	Benzoxazinone synthesis12	LOC103635831
		BX13	Benzoxazinone synthesis13	LOC100282831
		BX14	Benzoxazinone synthesis14	LOC100281319
Figure 7	Citric acid cycle	ACO	Aconitase1	LOC100216599
		ACO	Aconitase2	LOC100281040
		ACO	Aconitate hydratase1	LOC100304277
		ACO	Aconitase3	LOC100304315
		IDH	Isocitrate dehydrogenase	LOC100191657
		IDH	Isocitrate dehydrogenase	LOC100217128
		IDH	Isocitrate dehydrogenase	LOC100272371
		IDH	Isocitrate dehydrogenase	LOC100274592
		IDH	Isocitrate dehydrogenase	LOC103633296
		IDH	Isocitrate dehydrogenase	LOC100191841
		IDH	Isocitrate dehydrogenase	LOC100272953
		IDH	Isocitrate dehydrogenase	LOC100274026
		IDH	Isocitrate dehydrogenase	LOC100283574
		IDH	Isocitrate dehydrogenase	LOC542698
		DLD	Dihydrolipoamide dehydrogenase	LOC100381818

		DLD	Dihydrolipoamide dehydrogenase	LOC100383599
		DLD	Dihydrolipoamide dehydrogenase	LOC100501719
		DLD	Dihydrolipoamide dehydrogenase	LOC103650140
		OGDC	2-oxoglutarate dehydrogenase	LOC100280624
		OGDC	2-oxoglutarate dehydrogenase	LOC100284269
		SDS	Succinyl-CoA synthetase	LOC100283329
		SDH	Succinate dehydrogenase	LOC100216648
		SDH	Succinate dehydrogenase	LOC100274754
		SDH	Succinate dehydrogenase	LOC100277027
		SDH	Succinate dehydrogenase	LOC100279930
		SDH	Succinate dehydrogenase	LOC100280324
		SDH	Succinate dehydrogenase	LOC100502233
		SDH	Succinate dehydrogenase	LOC103653238
		SDH	Succinate dehydrogenase	LOC109944584
		SDH	Succinate dehydrogenase	LOC115874042
Figure 7	Citric acid cycle	FH	Fumarate hydratase	LOC100279871
		CS	Citrate synthase	LOC100279573
		CS	Citrate synthase	LOC100280203
		PCK	Phosphoenolpyruvate carboxykinase	LOC100279748
		PCK	Phosphoenolpyruvate carboxykinase	LOC541622
		MDH	Malate dehydrogenase	LOC100193491
		MDH	Malate dehydrogenase	LOC100193663
		MDH	Malate dehydrogenase	LOC100193743
		MDH	Malate dehydrogenase	LOC100272900
		MDH	Malate dehydrogenase	LOC100273428
		MDH	Malate dehydrogenase	LOC100280767
		MDH	Malate dehydrogenase	LOC100282134
		MDH	Malate dehydrogenase	LOC103633793
		MDH	Malate dehydrogenase	LOC103648465
		MDH	Malate dehydrogenase	LOC107305678
		MDH	Malate dehydrogenase	LOC542598

Figure 7	Citric acid cycle	CS	Citrate synthase	LOC100194338
		CS	Citrate synthase	LOC100275174
		CS	Citrate synthase	LOC100279573
		CS	Citrate synthase	LOC100280203
		PCK	Phosphoenolpyruvate carboxykinase	LOC100279748
		PCK	Phosphoenolpyruvate carboxykinase	LOC541622

Table S2. Characteristics of the plant metabolites responding to root (bio)luminescent exposure treatements. For information on experimental procedure carried out, refer to Fig. 10.

(Bio)luminescent source	Tissue	Compound	m/z	RT (min)	Putative idenfication	Response to (bio)luminescence
Distant	Leaves	57	577.15	5.44	Flavonoid-glycoside	Down
Distant	Leaves	86	356.10	3.75	HMBOA-Gluc	Up
Distant	Leaves	89	372.09	3.84	DIMBOA-Gluc	Up
Distant	Leaves	92	401.01	2.53	Unidentified	Down
Distant	Leaves	95	431.19	4.48	Unidentified	Up
Distant	Leaves	96	433.20	4.31	Unidentified	Down
Distant	Leaves	99	449.10	4.64	Unidentified	Down
Distant	Leaves	105	535.19	4.15	Unidentified	Down
Distant	Leaves	107	549.16	1.47	Unidentified	Down
Distant	Leaves	115	589.15	5.85	Unidentified	Down
Distant	Leaves	119	607.16	5.85	Flavonoid-glycoside	Down
Distant	Leaves	130	861.24	7.92	Oligosaccharide	Down
Distant	Roots	98	435.95	1.81	Unidentified	Up
Distant	Roots	110	566.15	5.22	Unidentified	Down
Distant	Roots	114	582.14	5.26	Unidentified	Down
Close	Leaves	62	623.16	4.85	Unidentified	Down
Close	Leaves	64	803.36	6.78	Unidentified	Up

(Bio)luminescent source	Tissue	Compound	m/z	RT (min)	Putative idenfication	Response to (bio)luminescence
Close	Roots	2	85.02	2.01	Unidentified	Up
Close	Roots	6	129.02	2.00	Citraconic acid	Up
Close	Roots	11	173.01	2.00	Aconitic acid	Up
Close	Roots	40	385.05	8.08	Unidentified	Up

General conclusions and perspectives

Photorhabdus bacteria possess the fascinating capacity to produce bioluminescence belowground and infection by the *Heterorhabditis/Photorhabdus* pair leads to the occurrence of bioluminescent infected organisms in the soil. While bioluminescence has been extensively studied in marine environments, little is known about the evolution, the regulation and the biological functions of the bioluminescence emitted by *Photorhabdus,* the only terrestrial bioluminescent bacteria. The aim of this thesis is to provide insights into these aspects. In this last section I briefly discuss the main findings from this research and propose several experiments to guide future studies.

Using a panel of strains representing all species and subspecies of *Photorhabdus*, we showed that bioluminescence displays an important intra- and inter-specific variability across the genus. Based on an ancestral state reconstruction of *Photorhabdus* bioluminescence and our characterization of its production *in vivo* and *in vitro*, we demonstrated that this trait is neither disappearing nor decreasing through subsequent speciation events. We performed experimental evolution experiments and obtained lab-evolved strains exhibiting changes in the levels of bioluminescence produced. Thanks to the identification of mutations involved in these changes, we demonstrated that *Photorhabdus* bioluminescence production is tightly controlled by a multicomponent network of genes. Our results suggest that genes involved in stress perception pathways play significant roles in this process. The following investigations would contribute to deepen our understanding of the molecular mechanisms underpinning the regulation of *Photorhabdus* bioluminescence in the future: (i) test the effect of abiotic stresses, including nutrient limitations and oxidative stress, on the regulation of the aforementioned genes and bioluminescence production; (ii) perform genetic manipulation of these genes (e.g. knock out mutations, overexpression) and characterized the resulting changes in bioluminescence production. Results from our experimental evolution experiments indicate that *Photorhabdus* bioluminescence production can rapidly evolve under laboratory settings in strain-specific. The controlled conditions we used allowed us to track the mutations acquired by *Photorhabdus* bacteria affecting their bioluminescence production. Whether mutations in the same genes will occur under natural conditions during the course of evolution is unknown, but possible.

We demonstrated that bioluminescence plays important biological function throughout the lifecycle of *Photorhabdus*. Under natural conditions, rapid changes in the levels of bioluminescence production, such as the ones observed during our experimental evolution experiments, would likely affect the symbiosis with *Heterorhabditis* nematodes and might alter the fitness of the nematodes. The results from our nematodes choice assays in chapter 2 suggest that host-seeking entomopathogenic nematodes (EPNs) might be able to sense and respond to physiologically high levels of *Photorhabdus* bioluminescence in a species-dependent manner to avoid competition. A decrease in bioluminescence production might impair this behavior and thus be counter-selected. This interpretation relies heavily on the results from nematode choice assays conducted with luminescent beads. To strengthen our interpretation, I propose the following experiments: (i) generate aluminescent mutants from highly bioluminescent *Photorhabdus* strains and use these pairs of bioluminescent wild type and aluminescent mutant strains to perform nematode choice assays; (ii) conduct nematode choice assays with *Heterorhabditis* nematodes wherein the expression of the GuR-3 homolog has been knocked down to test whether it abolishes the avoidance of bioluminescence; (iii) adapt the choice arena to use conditions closer to the natural ones by using sand instead of phytagel, and/or natural hosts of EPNs such as *Diabrotica virgifera virgifera* or *D. balteata* larvae instead of *Galleria mellonella* larvae.

The results from the third chapter of this thesis highlight that in addition to its modulating role on host-seeking EPNs behavior, *Photorhabdus* bioluminescence is required for symbiosis with *Heterorhabditis* nematodes. Quantification performed on infected insects revealed that aluminescent *Photorhabdus* mutant strains are impaired in the production of 3,5-dihydroxy-4-isopropyl-trans-stilbene (IPS), which has been shown to be essential for the normal development of *Heterorhabditis* nematodes. This crucial finding opens up new avenues for research aimed at unraveling the mechanisms involved in the regulation of symbiosis by *Photorhabdus* bioluminescence. The following experiments would provide information in that direction: (i) test if bioluminescence exposure and/or addition of IPS restore the symbiotic capability of aluminescent *Photorhabdus* mutant strains; (ii) test if bioluminescence exposure restores the production of IPS in *Photorhabdus* mutant strains impaired in alarmone production; (iii) test if bioluminescence exposure restores the symbiotic capability of *Photorhabdus* mutant strains impaired in alarmone production; (iv) test if *Heterorhabditis*

nematodes wherein the expression of the GuR-3 homolog has been knocked down are able to undergo successful development.

In the last chapter, we showed that plants exhibit weak transcriptomic, phytohormonal and metabolomic responses upon exposure to *Photorhabdus* bioluminescence. Nevertheless, we detected an increase of total benzoxazinoid content in leaves of plants whose roots were exposed to bioluminescence, and this accumulation was shown to be dependent on the intensity of the bioluminescence. While our experiments were specifically designed to test the effect of bioluminescence, plants likely perceive multiple cues during events where their roots are infested by herbivores infected with EPNs. These additional cues include mechanical damage caused by root feeding, chemicals present in the herbivores saliva, volatiles emitted by EPN-infected herbivores, and herbivory-induced plant volatiles emitted by neighboring plants. To investigate whether plants integrate multiple interacting cues in their responses, future research could implement our method with the following modifications. Firstly, I propose incorporating mechanical damage with and without the application of root herbivore-derived cues, in addition to *Photorhabdus* bioluminescence exposure, to test for a synergistic effect of these treatments on plant responses. Secondly, we previously enclosed *Photorhabdus*-infected insects in transparent polystyrene tubes to expose plant roots to bioluminescence specifically. I propose placing infected insects directly in the rhizosphere, without using tubes, to determine if plant responses are stronger when they perceive all cues derived from *Photorhabdus*-infected insects.

This thesis highlights the crucial roles of bioluminescence produced by *Photorhabdus* bacteria in their interactions with other soil-dwelling organisms, such as EPNs and plants. It demonstrates that *Photorhabdus* bioluminescence is not a disappearing trait but is essential for establishing successful symbiosis with *Heterorhabditis* nematodes. By contributing to a better understanding of the biological and ecological roles of bioluminescence in soil ecosystems, this work addresses an area that has been relatively understudied. Altogether, this research deepens our knowledge about the lifecycle of *Photorhabdus* bacteria and *Heterorhabditis* nematodes.

Publications

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