1   **Towards the identification of the molecular toolkit involved in scale worm bioluminescence**

2   **(Polinoidae, Annelida).**

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14 **Abstract**

15 **Background:** Bioluminescence, or the ability of a living organism to produce light, has evolved

16 independently in numerous taxa inhabiting a panoply of ecosystems, although it is more frequent

17 among marine animals. Scale worms are a group of marine polynoid annelids characterized by

18 having dorsal scales, known as elytra, capable of emitting bioluminescent light by a mostly

19 unknown molecular mechanism that may involve a photoprotein called polynoidin. Here, we used

20 RNA-seq data to characterize the expression of genes potentially involved in light production in

21 the polynoid species *Harmothoe imbricata* (Linnaeus, 1767) and *Harmothoe areolata* (Grube,

22 1860) across tissues of the specimens. We also compared the transcriptomes of the selected

23 species with other bioluminescent and non-bioluminescent polynoids, to identify shared

24 orthologous genes potentially involved in light production. In addition, we investigated the

25 disposition of the photocytes on the elytra using confocal microscopy and histological analyses.

26   **Results:** Our results showed a total of 16 candidate genes, 15 orthologous genes and 12 enriched

27   GO terms potentially involved in bioluminescence, including genes related with oxidative stress,

28   cytoskeleton, nervous system, stress response, wounding response, eye constituents and metabolic

29   pathways. We also confirmed the presence of photocytes in both species, which appeared

30   distributed around the elytrophore.

31   **Conclusions:** Among the genes found potentially implicated in bioluminescence we suggest that

32   the oxidoreductase protein, peroxidasin, could be a polynoidin candidate since it appears

33   overexpressed in the elytra of both species and it is located in the endoplasmic reticulum, where

34   this photoprotein has been described to be found.

35   **Keywords:** bioluminescence, oxidoreductase, photocyte, polynoids, polynoidin.

36

37   **Background**

38   Bioluminescence, the ability to produce light by a living organism, is widespread through the Tree

39   of Life. The production of bioluminescence normally involves the oxidation of a light emitting

40   photoprotein, that in some organisms is called luciferin, in a chemical reaction catalysed by an

41   enzyme, called luciferase, resulting in the emission of a photon [1, 2]. Biological mechanisms

42   behind light emission have evolved independently in both terrestrial and aquatic organisms from

43   polar to tropical regions and from coastal waters to the open ocean [3], being more frequent among

44   marine dwellers [4]. There are more than 10,000 species that have disparate mechanisms for the

45   production of light [5], the majority of which are still unknown. The ability to produce light has

46   evolved independently in the different organism lineages, resulting unique mechanisms through

47   which the different taxa produce light. This has led to diversity not only in how each taxon

48   produces light but also in the wavelengths emitted and the specific functions associated with light

49   emission [4–11]. Although there is diversity in the chemical nature of the light-emitting proteins

50   responsible for the production of light, the generic terms of luciferase for the enzymes involved

51   and luciferin for the substrate have become widely accepted [3]. The most studied luciferin was

52   originally described from fireflies, and since then only a few have been characterized, some of

53   which are shared by different lineages [1, 12, 13]. The chemical reaction involved in

54   bioluminescence should be sufficiently energetic to produce an excited molecule that will

55   generate a visible photon when it relaxes, and oxidation reactions fit well into this description,

56   with the breakdown of peroxide bonds among the most widespread mechanism [3]. In this sense,

57   most animal lineages possess different kinds of luciferases for light production [5].

58   Only in the phylum Annelida, there are more than 100 bioluminescent species described

59   to date [11]. Among these, there are important differences in the chemistry of the bioluminescence

60   systems used by marine and terrestrial taxa, which suggests the independent origin of

61   bioluminescence [14–18]. Polynoids, also known as scale worms, are a group of marine annelids

62   with several bioluminescent species reported, including members of the genera *Gattyana*,

63   *Malmgrenia*, *Polynoe* and *Harmothoe* [11, 16, 19–21] and possibly *Neopolynoe* [10]. Polynoids

64   are characterized by a series of paired structures denominated scales or elytra which are arranged

65   in two rows covering entirely or partially the length of the dorsum [22] (Fig. 1). The elytra are

66   dorsal cirri modified into flat, circular disc-like plates that are attached to the body by a thin

67   layered structure called elytrophore (Fig. 1). The scales serve a wide variety of functions,

68   including egg brooding, facilitating water circulation and also enabling defensive behaviors [23,

69   24]. When threatened, luminous polynoids emit flashes of light to startle predators that come from

70   the ventral epithelium of the elytra, which has a layer of bioluminescent cells called photocytes

71   not found in non-luminous species [19, 25]. If the stimulus is strong, one or more glowing elytra

72   might be detached from the insertion zone of the elytrophore while the animal swims away [16,

73   19, 24–26]. The elytra, despite not being attached to the body anymore, continue producing

74   flashes of light for some time, catching the attention of the predator that follows the light source,

75   allowing the polynoid time to escape [24]. Under extreme danger, polynoids might autotomize

76   the entire posterior end of the body. When this happens, the elytra of the posterior end emits light

77   that attracts the predator while the rest of the animal remains dark and escapes to later regenerate

78   the missing segments [19, 27]. Therefore, bioluminescence in scale worms is thought to serve

79   mainly as a warning or distracting mechanism for defense, as elytra are easily autotomized and

80   emit bright flashes of light for some time, acting as a sacrificial lure and allowing the animal to

81   escape avoiding potential threats [1, 16, 19, 24, 28].

82   Morphologically, elytra are composed of a unicellular epidermis, which is interrupted by

83   the insertion of a muscular elytrophore, covered by a cuticular layer. A main nerve trunk traverses

84   the elytrophore branches into nerves that cover the whole structure. The efferent nerve fibres from

85   the main nerve trunk are connect to the photogenic cells, known as photocytes [19] located

86   ventrally, around the elytrophore zone. Each photocyte contains 30 to 50 fluorescent photosomes,

87   the bioluminescent organelles, arranged around the nucleus of the photocytes [29] and linked by

88   various junctions [30]. The polygonal-shaped photocytes exhibit a granular structure composed

89   of undulating microtubules (Fig. 1d), considered to produce secretions implicated in

90   bioluminescence production [29].
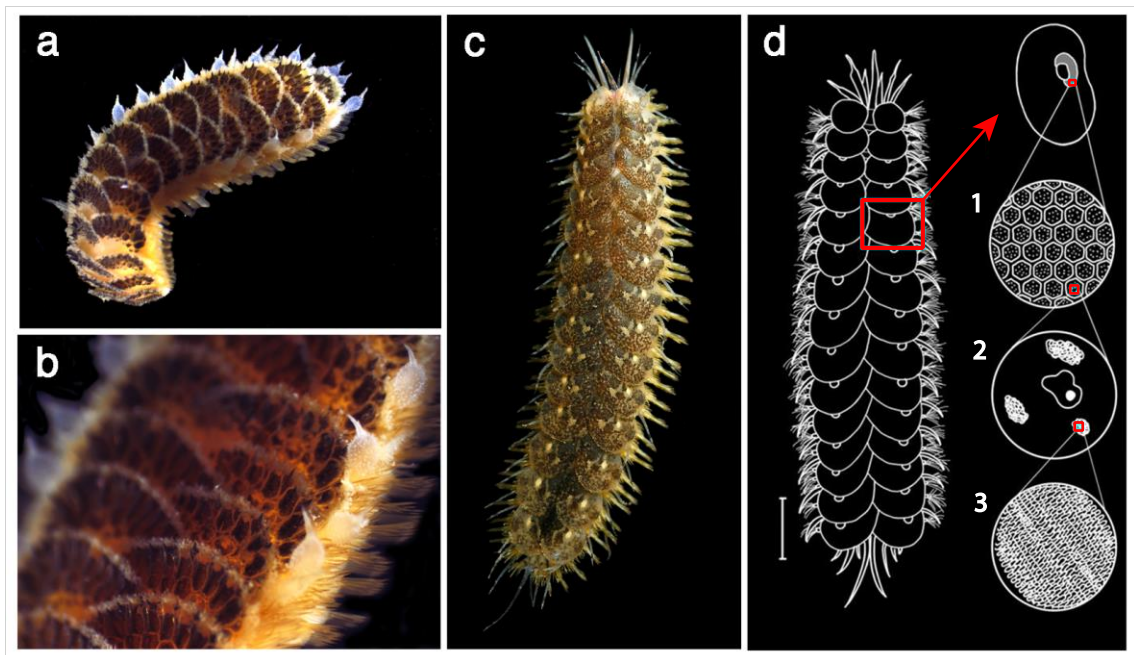
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92   **Figure 1. Photographs of *H. areolata* and *H. imbricata* with a diagram representing the**

93   **bioluminescence system.** (**a-b**). Images of *Harmothoe areolata* with a close-up of the dorsum

94   with the elytra in detail. (**c**). Dorsal view of *Harmothoe imbricata*. (**d**). Diagram representing an

95   individual of the polynoidae family showing the disposition of the bioluminescent system. The

96   shaded zone of the elytra corresponds to the bioluminescent area. (**1**). Zoom of this area where

97   the photocytes are arranged in a mosaic pattern. (**2**). A magnified photocyte with three

4

98 photosomes and a nucleus. (**3**). The paracrystalline structure of the endoplasmic reticulum of the

99 photosomes. (Scale = 5 mm). Modified from *Ouldali et al., 2018* [31].

100

101   Light production in scale worms involves a membrane photoprotein of about 65 kDa

102 called polynoidin, located in the membrane of the photosomes [32] and triggered by superoxide

103 or hydroxyl radicals in the presence of Ca^{2+} and Fe^{2+}[30, 32]. The oxidation of the reduced flavin

104 present in the photosomes is hypothesized to produce the superoxide or hydroxyl radicals [16, 29]

105 to trigger light production. Interestingly, polynoidin is also present in non-luminescent scale

106 worms suggesting that bioluminescence might have originated from a mechanism able to quench

107 superoxide radicals [25]. Previous studies of polynoid bioluminescence have been mostly focused

108 on the morphology and ultrastructure of the elytra, the biochemistry of light emission by

109 polynoidin, and the electrophysiology of the photogenic epithelium [19, 25, 30, 32–35]. However,

110 the genetic machinery behind bioluminescence in this group of marine annelids is currently

111 unknown. Here, we take advantage of RNA-Seq based differential gene expression analysis to

112 identify genes potentially involved in scale worm bioluminescence and investigate the

113 morphology of the elytra and the structure of the bioluminescent system in two polynoid species:

114 *Harmothoe imbricata* (Linnaeus, 1767) [36] and *Harmothoe areolata* (Grube, 1860) [37]. For this

115 purpose, we generated RNA-Seq libraries of both elytra and the posterior end of the body and

116 performed differential expression analyses to identify candidate genes potentially involved in

117 light production. Our results suggest that a potential candidate for polynoidin could be a

118 peroxidasin homolog that has been found overexpressed in the elytra of both studied

119 bioluminescent species. Furthermore, numerous transcripts were identified in connection with

120 secondary aspects of the bioluminescent mechanism, encompassing those linked to a response to

121 injury and stress, as well as others correlated with the composition of the photosomes.

122

123 **Results**

124 **Transcriptomic and differential expression analyses**

125 The sequencing of the six libraries prepared for *Harmothoe imbricata* resulted in a total of 96.9

126 million raw reads. The filtered reads examined with FastQC were assembled *de novo* producing

127 a reference transcriptome of 384,174 transcripts including 303,091,081 assembled base pairs (bp)

128 and an N50 value of 1,439 [see Additional file 1]. The sequencing of the six libraries prepared for

129 *H. areolata* resulted in a total of 153.4 million raw reads. The filtered reads examined with FastQC

130 were assembled *de novo* producing a reference transcriptome of 513,553 transcripts including

131 377,970,784 assembled base pairs (bp) and a N50 value of 807 [see Additional file 1]. The

132 completeness of the transcriptomes based on BUSCO values was high compared to that of other

133 transcriptomic analyses of annelids [38].

134 The differential gene expression (DGE) analysis for *H. imbricata* resulted in a total of

135 61,785 transcripts differentially expressed, of which 331 were upregulated in the elytra (Fig. 2a).

136 Of the upregulated transcripts, only 111 could be functionally annotated [see Additional file 2].

137 To identify genes potentially involved in bioluminescence production, we focused our

138 downstream analyses only on the annotated transcripts differentially expressed in the elytra. The

139 DGE analysis for *H. areolata* resulted in a total of 72,161 transcripts differentially expressed [see

140 Additional file 2], of which 475 were upregulated in the elytra (Fig. 2b). Of the upregulated

141 transcripts, only 65 could be functionally annotated, and again, we focused on those to identify

142 transcripts potentially involved in bioluminescence.

143 Of the 331 transcripts upregulated in the elytra of *H. imbricata*, only 3 were identified as

144 potentially involved in the bioluminescence production. The annotation of two of these transcripts

145 was related with the cytoskeleton: TACC3 (transforming acidic coiled-coil-containing protein 3)

146 [39] and FLNA (filamin-A) [40]. The remaining transcript blasted against a gene with functions

147 associated with the nervous system, NF60 (neurofilament protein)[41]. In *H. areolata* 475

148 transcripts were upregulated in the elytra and we selected 11 as possible candidates involved in

149 the bioluminescence process. Several of these transcripts had annotations related to

150 oxidoreductase function: AOC1(amiloride-sensitive amine oxidase) [42], DHGL (Glucose

151 dehydrogenase) [43], PERC (Chorion peroxidase)[44], PERO (Peroxidase) [45], PXDN2

152 (Peroxidasin homolog) [46], S5A1 (3-oxo-5-alpha-steroid 4-dehydrogenase) [47] and SDH (L-

153   sorbose 1-dehydrogenase) [48]. Another transcript was related with oxygen radical production,

154   KPCD (Protein kinase C delta type) [49]. We also found transcripts annotated to functions in the

155   nervous system, NF60 (neurofilament protein), shared by both species, and FAT3 (Protocadherin

156   Fat 3) [50]. Finally, we found a cytoskeleton related gene, FLNA, which also appeared

157   upregulated in *H. imbricata*.

158   Heat maps in Figure 2 (c–d) illustrate the expression levels of the transcripts selected in

159   the elytra and the body of both species. We found small differences in the expression profiles of

160   the replicates of each tissue but large differences between tissues, with higher levels of expression

161   in the elytra. For *imbricata*, 6 transcripts were additionally included, given their high expression

162   in the elytra (although they were not differentially expressed). These additional transcripts were

163   annotated to oxidoreductase function: AMO (Putative amine oxidase) [51], KMO (Kynurenine 3-

164   monooxygenase) [52], NOX5 (NADPH oxidase 5) [53] and CP2J6 (Cytochrome P450) [54].

165   Inside this group we include PERO and PXDN which are also found upregulated in the elytra of

166   *areolata.*

167   Besides oxidation-reduction processes, in our analyses, we retrieved functional

168   information from 1,149 transcripts of those found upregulated in the elytra in *H. imbricata* and

169   594 in *H. areolata*, which allowed us to identify various enriched GO terms potentially relevant

170   for bioluminescence (Fig. 2e). The shared pathways with a possible relation with bioluminescence

171   between the two species were: tryptophan metabolism, response to wounding, response to stress,

172   regulation of serotonin biosynthetic process, oxidoreductase activity, oxidative stress response,

173   glycolisys/gluconeogenesis and dopamine metabolic process (Fig. 2e). For *H. imbricata* we found

174   four additional pathways that could be involved in bioluminescence: valine, leucine and

175   isoleucine degradation, peroxidase activity, pentose phosphate pathway and microtubule

176   cytoskeleton organization. For more information of the enriched GO terms [see Additional file

177   3].

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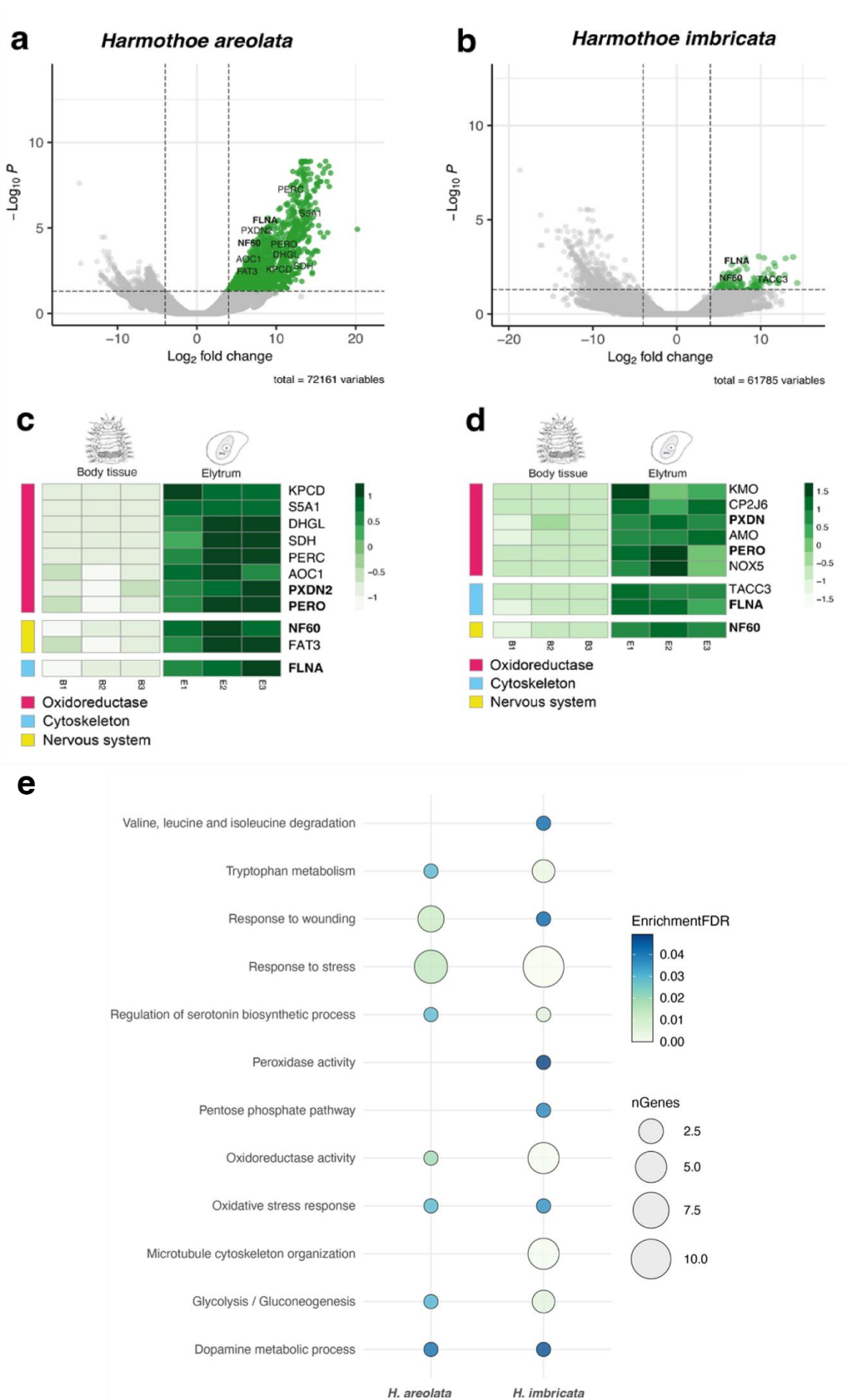
179   **Figure 2. Differential expression and gene enrichment in the elytra of *Harmothoe areolata***

180   **and *H. imbricata*.** (**a–b**). Volcano plots displaying the –log10 p value (false discover rate [FDR])

181   as a function of fold change in the scale and the body of *H. areolata* (**a**) and in *H. imbricata* (**b**).

182   Grey dots in the left represent upregulated genes in the body while green dots at the right

183   correspond to upregulated genes in the elytrum. (**c–d**). Heatmaps showing selected upregulated

184   genes in the elytrum with their associated functions in *H. areolata* (**c**) and in *H. imbricata* (**d**).

185   (**e**). Gene Ontology enrichment analysis of the upregulated annotated genes in the elytra of both

186   species.

187

188   **Identification of shared orthologs between bioluminescent species**

189   The reference transcriptomes of the elytra of *H. imbricata* and *H. areolata* were compared with

190   other bioluminescent and non-bioluminescent polynoid species. With this analysis, we identified

191   orthologous genes shared by bioluminescent species, and present in the elytra of the selected

192   species, but not present in non-bioluminescent species. We focused on the clusters of orthologous

193   genes shared only between the bioluminescent species to identify genes potentially involved in

194   bioluminescence.

195   All the polynoid species included in the analysis shared a total of 2,994 clusters while *H.*

196   *extenuata, H. imbricata* (GenBank), and the elytra of *H. imbricata* and *H. areolata* shared 400

197   clusters of orthologous genes [see Additional file 4]. Of those 400 shared clusters, we identified

198   15 that could be potentially related to bioluminescence and are listed in Table 1. Interestingly, the

199   Orthovenn analyses revealed a total of 7 clusters related with an oxidoreductase function shared

200   among the bioluminescent polynoids (i.e. Amine metabolic process, hydroquinone, 3-

201   hydroxybutyrate dehydrogenase activity, positive regulation of lipid biosynthetic process, l-

202   ascorbic acid biosynthetic process, thioredoxin-disulfide reductase activity and octopamine

203   biosynthetic process). One cluster exhibited a connection to microtubule organization (i.e. Actin

204   binding), while two clusters were linked to the nervous system, sharing the same GO annotation

205   (i.e. Synaptic transmission, cholinergic), and another two were associated with response to

206   oxidative stress (i.e. Sulfur amino acid metabolic process and regulation of heme biosynthetic

207 process). Furthermore, three clusters demonstrated similarities to genes involved in visual sense

208 (i.e. Estructural constituent of eye lens, retinol metabolic process and visual perception).

209

210 **Histology and tissue organization of elytra**

211 The excitation light used to image the fluorescent photocytes was 405 nm and the fluorescent

212 structures of the elytra emitted within a range spectrum of 517 nm and 570 nm. Figure 3 shows

213 an image of the complete structure of the elytra in *H. imbricata* (Fig. 3a) and *H. areolata* (Fig.

214 3d) taken from a ventral position. It is known that tubercules covering the surface are

215 autofluorescent, as well as the photocytes [25]. Although the tubercles were located in the dorsal

216 part of the elytra, their autofluorescence was visible from the ventral part. The tubercules covering

217 the dorsum in *H. areolata* were much more conspicuous and with spines, while in *H. imbricata*

218 are rounded (Fig. 3a–f). In figures the ventral side, surrounding the elytrophore we observed a

219 dense mass of brighter fluorescent cells (Fig. 3b–c, e–f). These cells corresponded to the

220 fluorescent organelles of the photocytes, the photosomes, which are distributed within the

221 photogenic area (Fig. 3b–c, e–f). Normally there are 30-50 photosomes per photocyte, with a

222 diameter of 1 - 5 micrometres [29].

223 In the transversal histological sections of the elytra of *H. imbricata* (Fig. 3) we observed

224 several tubercules in the dorsal side (Fig. 3g). Beneath the cuticle we found a continuous upper

225 and lower monostratified epithelium. On the upper layer of the epithelium, we found cell bodies,

226 some of which contained pigment granules (Fig. 3i), nerves (*Fb*) connecting the cuticle with the

227 lower epithelium in a ganglion near the elytral stalk [55], although we could not identify the

228 ganglia in our sections. The lower epithelium was similar to the upper one, with the exception of

229 the presence of photocytes, which were modified epithelial cells, arranged near the elytrophore

230 (Fig. 3i). The insertion of the elytrophore was not clearly identified, although we observed

231 photocytes not following a continuous pattern (Fig. 3h). Photocytes appeared as calyx-shaped

232 cells (Fig. 3i) similar to what has been observed in other species [10].

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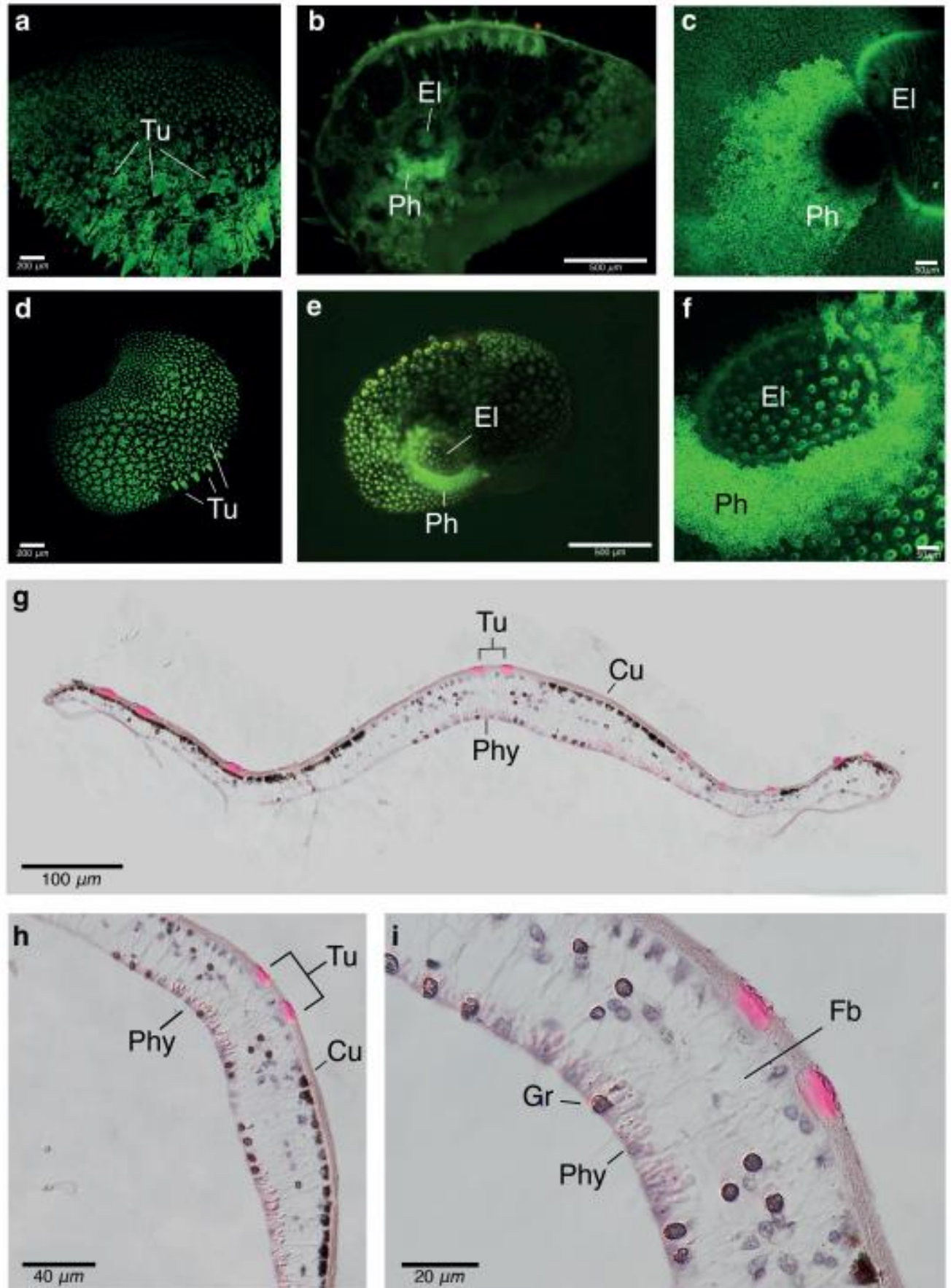
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236 **Figure 3. Confocal and light microscopy images of the elytra of *H. imbricata* (a–c) and *H.**

237 *areolata* (d–f).** Absorption spectrum between 517 and 570 nm. (**a**). Dorsal picture of the whole

238 elytrum of *H. areolata* where dorsal tubercules (Tu) are observed as spines. (**b**). Ventral picture

239 of the whole elytrum of *H. areolata* with photosomes (Ph) arranged concentrically around the

240 elytrophore (El). (**c**). Detail of the photogenic area with the photosomes (Ph) in *H. areolata*

241 surrounding the elytrophore (EI). (**d**). Dorsal picture of the whole elytrum of *H. imbricata* where

242 dorsal tubercules (Tu) are observed as small circles. (**e**). Ventral picture of the whole elytrum of

243 *H. imbricata* with photosomes (Ph) arranged concentrically around the elytrophore (El). (**f**).

244 Detail of the photogenic area with the photosomes (Ph) surrounding the elytrophore (El). (**g-i**).

245 Transversal Histological section of an elytrum of H. imbricata stained with haematoxylin-eosin

246 and mounted in DPX. (**g**). Picture of the elytrum the cuticle (Cu) and tubercules (Tu) appearing

247 dorsally and with photocytes (Phy) appearing ventrally. (**h**). Same section at a higher

248 magnification showing photocytes (Phy) at the ventral side breaking their continuity due to the

249 elytrophore. (**i**). Detail of the photocytes (Phy) where the calix shape of these cells is observed.

250 Pigmented granules (Gr) and fibres nerves (Fb).

251

252 **Discussion**

253 Our study aimed to identify the genetic machinery involved in the bioluminescence of the

254 polynoid worms *Harmothoe imbricata* and *Harmothoe areolata*. The search of candidate genes

255 with the different analyses led us to the identification of various transcripts in both species that

256 could potentially be involved in light production and additional processes associated with

257 bioluminescence. The DGE analysis allowed us to identify genes that were upregulated in the

258 elytra, the structures where the photogenic cells are located, and thus may be related to

259 bioluminescence production. Additionally, our comparative transcriptomic analysis allowed us to

260 identify orthologous genes shared only by bioluminescent species, which might also be involved

261 in light production. Finally, the morphological analyses with light and confocal microscopy

262 revealed the location and structure of the bioluminescent system within the elytra of both polynoid

263 species.

264 **Genes potentially involved in the bioluminescence chemical reaction**

265 Bioluminescence in polynoid worms takes place in the elytra. More specifically, this process

266 happens in the photosomes of the bioluminescent cells present in the photogenic area, thanks to

267 the photoprotein polynoidin which is activated by the emission of superoxide or hydroxyl radicals,

268 Ca2+ and Fe2+ [30, 32]. We identified several candidate transcripts and pathways involved in

269 bioluminescence in *H. imbricata* and *H. areolata*, sharing a total of 9 pathways between the two

270 species (Fig. 2e). A total of 11 genes were found differentially expressed and upregulated in the

271 elytra of both species involved in an **oxidoreductase** function. These were identified as the most

272 plausible candidates for luciferase function, given that the primary mechanism operating in

273 bioluminescence involves the breakdown of a peroxide bond [11]. In this sense, one of the most

274 accepted theories of the origin of bioluminescence is the antioxidative hypothesis, and it refers to

275 the evolutionary history of the bioluminescent substrate. Since luciferin has antioxidative

276 properties and high reactivity with reactive oxygen species (ROS), the antioxidative hypothesis

277 suggests that originally, the luciferin was a molecule implicated in reducing oxidative stress [56].

278 In marine ecosystems, organisms living in shallow waters attempting to escape from predators,

279 might have migrated to the deep ocean where oxidative stress is reduced, leading to a functional

280 change from the antioxidative to the chemiluminescent properties of this molecule [56]. These

281 proteins involved in oxidoreductase processes included Putative Amine Oxidase, Kynurenine 3-

282 monooxygenase, NADPH Oxidase 5 and Cytochrome P450 for *H. imbricata*, while for *H.*

283 *areolata* these were Amiloride-sensitive Amine Oxidase, Glucose dehydrogenase, Chorion

284 peroxidase, 3-oxo-5-alpha-steroid 4-dehydrogenase, L-sorbose 1-dehydrogenase and Protein

285 Kinase C (Table 2, Additional file 2). Interestingly, both species shared two genes, a peroxidase

286 and a peroxidasin homolog. Oxidoreductase enzymes are involved in controlling the amount of

287 oxygen radicals or reduced compounds that appear in the cells [57]. It is known that the

288 bioluminescent reaction in polynoids is triggered by superoxide radicals that result from the

289 oxidation of riboflavin [16]. Many of the genes selected have NAPH oxidase/dehydrogenase

290 function which are considered one of the major sources of reactive oxygen species [53].

291 Oxidoreductases are also involved in many other bioluminescent systems including fireflies [58],

13

292   bacteria [59], fungi [60] and other annelids [61]. Oxygen has been proved to be essential for the

293   luminescent reaction in polynoids [32], therefore, we hypothesize that the oxidoreductase

294   homologs identified here represent candidate polynoidins, the protein responsible of catalyzing

295   the bioluminescent reaction in polynoids. The two only genes that appeared shared by both species

296   are peroxidase (PERO) and peroxidasin (PXDN). The appearance of these genes in the two

297   bioluminescent species studied reinforces the possibility that one of them may be the elusive

298   polynoidin. Moreover, given that the peroxidasin homolog shared is found in endoplasmic

299   reticulum, where polynoidin has been described to be found, this gene appears as the most

300   possible candidate to catalyze the light production in *Harmothoe imbricata* and *H. areolata* [32].

301   Interestingly, two of the orthologous clusters shared by bioluminescent species and one

302   of the upregulated genes in the elytra of both species (NADPH oxidase 5) are involved in

303   **oxidative stress responses**. Oxidative stress is generated by reactive oxygen species [62],

304   between others, which could finally produce bioluminescence in polynoids. It is is possible that

305   initially, bioluminescence occurred as an antioxidant mechanism in order to avoid oxidative stress

306   [56]. This response appearing in the genes overexpressed in the elytra confirms the idea that there

307   are products in the cells of the polynoids capable of acting against this oxidative stress and thus,

308   potentially leading to the production of light.

309

310   **Genes potentially involved in additional aspects of the bioluminescence process**

311   Besides the potentially direct production of light of the genes commented above, we identified a

312   battery of genes indirectly related to the bioluminescence process, including those potentially

313   involved in the formation of bioluminescent structures, or with the nervous signal triggering the

314   bioluminescence. Among them, we found two genes differentially expressed in the elytra of the

315   two worms and one shared orthologous cluster associated with the **cytoskeleton.** For both species

316   Filamin-A was upregulated in the elytra. But also, *H. imbricata* showed another gene upregulated

317   in their elytra, Transforming Acidic coiled-coil-containing Protein 3. The cytoskeleton plays an

318   important role in the formation of the photosomes as it is its main structural component. The

319   granules of the photosomes are composed of undulating microtubules that appear regularly

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320 arranged in a paracrystalline organization continuous with the endoplasmic reticulum [29]. It has

321 been described that the photocytes of fireflies are also connected to the endoplasmic reticulum,

322 suggesting that this organelle plays a role in the light emission [63, 64]. The task carried out by

323 the gene found homolog to the Transforming Acidic coiled-coil-containing Protein 3 consists in

324 stabilizing the microtubules in the kinetochore fibers of the mitotic spindle [65]. This gene,

325 therefore, could be related with the formation of the paracrystalline organization which conforms

326 the photosomes where the bioluminescent reaction takes part. Filamin-A and the Actin biding

327 cluster, which are shared by both polynoids, are not related with the microtubule organization,

328 but with the actin cytoskeleton. Although the main structural component of the photosomes is

329 indeed the microtubule cytoskeleton, the intermediate filaments of the cytoskeleton, that are

330 formed of actin, are one of the principal elements of the desmosome junctions that join the

331 photosomes inside the photocytes [30]. It is also remarkable that in dinoflagellates, filaments of

332 the actin cytoskeleton are involved in the mechanotransduction of bioluminescence [66].

333 Consequently, these genes could potentially play an important role in the formation of the

334 photosomes as they have been proved to have relationships with their main structure and with the

335 junctions between them.

336 One of the most enriched GO categories in both species was **stress response**. The

337 mechanism of bioluminescence in polynoids has hypothesized to be a defensive strategy to escape

338 from predators [67]. It seems obvious that there must be a toolkit to perceive stress and respond

339 creating an impulse ending in light emission to scape. In other studies, similar analyses have also

340 revealed genes overexpressed in the luminal tissue with stress response functions in Terebellidae

341 species [68]. Our study suggests that these genes present upregulated in the elytra of the polynoid

342 worms might work under stress conditions when the animal is trying to escape from its predators.

343 [16][31, 33] We also found several genes related to the **nervous system** that are potentially

344 involved in the bioluminescence process. Bioluminescence in many taxa is controlled by the

345 nervous system, and this includes polynoids. Each elytrum has a central ganglion located near the

346 elytrophore from which nerves branch into nerve fibers in various directions covering the

347 periphery of the elytra [19]. In its upper part, sensory fibers connect with the tubercles or papillae

15

348   of the cuticle and in its lower part, efferent fibers travel until they reach the photocytes at the base

349   of the elytrum. The stimulus, thus, starts at the upper part and it is transmitted to the central

350   ganglion to reach the photocytes [69]. We identified two upregulated genes in the elytra,

351   Protocadherin Fat 3 and Neurofilament protein and as well as several shared orthologus clusters

352   involved in cholinergic synaptic transmission. Protocadherins homolog, present only in *areolata*,

353   are transmembrane proteins specialized in signaling. Specifically, fat cadherins are defined to

354   enable influence multiple features of neuronal development [50]. This Protocadherin may be

355   involved in the establishment of neuronal connections related to the bioluminescence signal, as

356   these proteins normally binds calcium. Calcium is one of the ions starting the action potential of

357   the luminous process [70] and it could be related to the transmission of the nerve impulse

358   triggering bioluminescence in *H. areolata*. The neurofilament protein, found upregulated in the

359   elytra of both species, is commonly present in neuronal tissue as it is the cytoskeletal constituent

360   of neurons [71]. As it appears overexpressed in both species, this gene may be in fact, one of the

361   genes being responsible of the formation of neurons performing in the elytra. A cholinergic

362   synapse is a chemical synapse that use acetylcholine molecules as neurotransmitter. Nicolas and

363   collaborators in 1978 specified that a cholinergic mechanism was indirectly involved in the

364   luminescence control, and previously it was confirmed a positive acetylcholinesterase

365   histochemical reaction of the elytral ganglion and nerves [72]. All the above mentioned genes

366   may be involved in the neurotransmission necessary for emission of light in polynoids.

367   Another function indirectly related with the process of bioluminescence is wounding,

368   which its related GO term, **wounding response,** was enriched in the upregulated component of

369   the elytra. When light is being produced, the polynoids detach their luminescent elytra while they

370   are glowing. When this occurs, the luminescent elytra keep on glowing as the same time that the

371   animal tries to scape [24]. The autotomization of the elytra leaves a wound in the worms, that is

372   repaired in the next 15 days, when a new elytrum is grown [73]. Genes capable of responding to

373   this loss of the elytra must be in the elytrum itself, as it must be repaired in order to serve the

374   polychaeta a defensive function.

375   Three orthologous clusters appeared shared by the bioluminescent species related to

376   **visual perception**. At first it seems strange that genes related to vision appear in the elytra of the

377   polynoids. We must remember that orthologous genes were obtained with the transcriptome of

378   the elytra of our target species and the whole transcriptome of *H. extenuata*. These results suggest

379   that there are homologous genes that are expressed both in the body of the bioluminescent species

380   and in their elytra. With these results we can verify genetically the study carried out by Bassot

381   and Nicolas in 1978 where they confirm that the paracrystalline endoplasmic reticulum

382   characteristic of the photogenic cells in polynoids appears also in the eyes, where it constitutes a

383   greater part of the lens. The relationship that exists in the appearance of the same structures in the

384   photoreceptors as in the photosomes of polinoid species is a mystery to be studied.

385   Two metabolic pathways related with glycolysis and tryptophan were enriched in the

386   elytra, and even though they have not been reported in bioluminescent systems of polynoids, they

387   have been proved to be involved in other bioluminescent species. The process of obtaining energy

388   out of the glycolysis metabolism, provides energy to an inhibitory mechanism that maintains the

389   photophores in a non-luminescent state in the teleost fish *Porichthys* [74]. In a more closely

390   related species, the earthworm *Lampito mauritii* [75], this metabolic pathway has also been shown

391   to activate a greenish light emission, when the celomic cells where exposed to stimulants and

392   some products of gluconeogenesis [76]. This could indicate that the glucose pathway may

393   interfere in the system of activation and deactivation of light production by polynoids. Also,

394   tryptophan metabolism is central to produce tryptophan compounds, which are one of the main

395   elements of the sea firefly luciferin, cypridinid luciferin [77, 78]. Polynoid's luciferin has not

396   been discovered yet and since the tryptophan metabolic pathway has been found to be

397   overexpressed in the elytra of polynoids, we propose that tryptophan or its derivatives may be

398   part of the composition of polynoid luciferin.

399   Finally, we retrieved two more enriched GO categories that were only present in *H.*

400   *imbricata*: valine, leucine and isoleucine degradation and pentose phosphate metabolism. The

401   generation of NADPH molecules is essential for the bioluminescent process in Polynoidae, as

402   these coenzymes provide reactive oxygen species [16]. Earlier, we indicated the importance of

17

403 oxidative stress in the reaction of luminescence. Pentose phosphate pathway and valine, leucine

404 and isoleucine degradation are both metabolic pathways in which NADPH is generated [79, 80].

405 Our study suggests that these pathways may be functioning to generate compounds necessary for

406 beginning the bioluminescent reaction in *H. imbricata*.

407

408 **Morphology of the bioluminescent system**

409 Light production in polynoid worms takes place in the photosomes, organelles located in

410 specialized cells called photocytes and found in the elytra. The photosomes are autofluorescent

411 and thus are responsible for the fluorescent properties of elytra, having the ability of emit light

412 under specific excitation wavelengths. In addition, the tubercules also show this autofluorescence.

413 First, it was thought that this property was only activated after the bioluminescent reaction was

414 triggered [81] but it has been proved that this fluorescence is permanently present in these

415 structures [25, 35]. Figure 3 shows images taken from individuals preserved in KINFix after three

416 months, confirming therefore that they effectively do not lose their autofluorescence and that it is

417 present independently of bioluminescence light emission. We confirmed that the excitation

418 wavelength that works best to see the arrangement of bioluminescent structures is UV light, as

419 suggested in previous studies [25, 82]. The green light emission spectrum (517-570 nm) is

420 consistent with that reported for this 20 and other species in previous articles (510-525 nm) [10,

421 16, 32, 83]. We observed two distinctive green autofluorescent zones: the papilla and tubercules

422 and the area around the insertion zone of the elytrophore. The disposition of the photosomes can

423 be observed more accurately on figure 3c and 3f, where they appear as a dense, tangled mass,

424 which may be due to the paracrystalline form of the endoplasmic reticulum from which they are

425 formed. Our histological sections show the disposition and structure of the photosomes, arranged

426 in the centre of the ventral side of the elytra (Fig. 3g, h, i). They are elongated cells that appear

427 together and close to one another. Each photocyte is connected by efferent fibres to the central

428 ganglion of the elytra, responsible for the transmission of the impulse that triggers

429 bioluminescence. These nerves are visualized as the thin transversal lines connecting both

430 epithelia (Fig. 3i). This innervation can also be seen in non-bioluminescent species, although they

18

431 lack photosomes [84]. In general, our morphological study is consistent with the organization of

432 light producing organs in other polynoids [10, 19, 25, 35, 82, 85].

433   Although the light production ability of the polynoid photoprotein system could not be

434 assessed, we identified an array of proteins involved in oxidoreductase processes that point to

435 both luciferase and luciferin proteins. Peroxidasin might correspond to the elusive polynoidin, the

436 photoprotein responsible of catalyzing bioluminescence reaction in polynoids. We have seen that

437 the oxidoreductase most likely to be polynoidin is a **peroxidasin homolog**, since it is

438 overexpressed in the elytra of both species and is found in the endoplasmic reticulum, which is

439 an essential part of the structure of photocytes and where this photoprotein has been described to

440 be found. Functional validation analysis must be done to confirm the role of this protein in the

441 bioluminescent system of *Harmothoe*. It will be necessary to search for this supposed polynoidin

442 in other bioluminescent polynoids to verify its role in the bioluminescence process within this

443 genus.

444

445 **Methods**

446 **Sample collection and preservation**

447 Six specimens of *Harmothoe imbricata* and six of *Harmothoe areolata* were collected by SCUBA

448 diving in O'Grove (42.498448, -8.865719, Galicia, Spain; 12 m depth) and near Blanes

449 (41.673245, 2.802646, NW Mediterranean Sea; 10 m depth) respectively, and kept alive in

450 seawater for transport to the laboratory. Taxonomic identification was done following the original

451 description of the species and relevant additional literature [36, 86] under a Zeiss Stemi 2000

452 stereoscope up to 35x magnification by experts on annelid taxonomy. Before sample preservation,

453 bioluminescence emission was confirmed in a dark room by disturbing the individual with

454 tweezers. For transcriptomic analyses, we preserved the specimens in RNAlater (Life

455 Technologies), during 24h at 4 ºC, replaced it once, and stored samples at -80 ºC until further

456 processing. For histological analyses, samples were preserved in 2.5% glutaraldehyde in 1M PBS

457 and 0.34M NaCl and KINFix, which allows the sample to be used in examination by histological

458 or immunohistochemical techniques [87].

459

RNA extraction, cDNA library preparation, and sequencing

For RNA extraction, dorsal elytra and a portion of the posterior end of the body were dissected from three specimens of each of the two species, totalling three replicates per tissue and per species. Total RNA was extracted from the elytra and from the posterior end of the body with TRIzol (Invitrogen), following the manufacturer's instructions. Total RNA from each of the six biological replicates of *H. areolata* was sent to New York University Center for Genomics and Systems Biology (New York, USA) for cDNA library preparation and sequencing. A total of twelve cDNA libraries were generated with Illumina TruSeq library prep kit v2, corresponding to the three biological replicates of each tissue type. We verified the quality and integrity of the total RNA and the cDNA library with an Agilent BioAnalyzer (RNA and high sensitivity DNA assay respectively). Libraries were then sequenced using Illumina HiSeq 2500 v4 technology, at 150 base-pairs (bp) paired-end reads.

472

De novo transcriptome assembly, annotation, and differential expression analysis

The quality of the raw reads generated from each of the six libraries was evaluated using FastQC v0.11.5 [88] and then, adapter sequences and low-quality bases (phred score < 30) were removed using Trimmomatic [89] Processed reads of all replicates were then pooled together and assembled *de novo* using the software Trinity v2.4.0 ([90, 91] to generate a reference transcriptome. Functional annotation of the reference transcriptomes was performed first by blasting the transcripts with DIAMOND [92] against the UniprotKB database [93, 94] with a cut-off E value of 1e-5. Transcripts with blast hits were further annotated using Gene Ontology (GO) terms with BLAST2GO PRO [95].

To identify the genes significantly upregulated in the elytra and the posterior end of the body, a differential gene expression (DGE) analysis was carried out with the Trinity module [90], which incorporates Bowtie and RSEM [96], to map and estimate transcript abundance, and edgeR [97] to identify differentially expressed transcripts [91] in both species. We later selected only those genes showing a minimum of 2-fold change, and *p*-value cut off for FDR of 0.01. A GO

487   enrichment analysis was performed using the GO annotations of the significant differentially

488   expressed transcripts using gProfiler [98] with a FDR cutoff 0.05.

489

490   **Comparative transcriptomics of bioluminescent and non-bioluminescent polynoid species.**

491   To assess whether bioluminescent polynoids possess a unique set of proteins shared across the

492   family, we compared the transcriptomes of bioluminescent (including our reference

493   transcriptomes) and non-bioluminescent species using OrthoVenn2 [99]. For this, we assembled

494   the reference transcriptomes of the non-bioluminescent polynoids *Branchipolynoe pettibone*

495   (Miura & Hashimoto, 1991) [100] and *Lepidonotopodium* sp. [100, 101] and the bioluminescent

496   *Harmothoe extenuata* (Grube, 1840) [102], using the available reads in SRA (Table 2) and

497   applying the same bioinformatic pipeline as for our reference transcriptomes. Predicted protein

498   datasets from the transcriptomes were obtained with TransDecoder [90] and implemented in

499   OrthoVenn2 where we set a cut-off E value of 1e-5 for the comparisons. This served us to select

500   only those orthologous gene clusters shared by the luminous polynoid species ("bioluminescent

501   clusters") for downstream analyses. Functional annotation of the shared bioluminescents clusters

502   was automatically performed in OrthoVenn2 against UniProtKB [93, 94] as well an additional

503   GO enrichment analysis.

504

505   **Histology analysis of elytra**

506   To identify the presence of photocytes, histological analyses were performed on the preserved

507   elytra of *H. imbricata.* Briefly, samples were rinsed in distilled water, dehydrated through an

508   ascending series of ethanol, bathed in xylene and embedded in paraffin overnight at 60ºC. Serial

509   7µm-thick sections were then made with a Leitz 1512 microtome and stained with a standard

510   haematoxylin - eosin protocol and mounted with DPX. The resulting preparations were scanned

511   using an Olympus© BX51-P microscope and photographed with an Olympus DP-23 camera.

512   Confocal and light microscopy of elytra preserved in formalin was also performed taking

513   advantage of the autofluorescence of the photosomes present in the photocytes of the elytra of

514   both species. The excitation light used was of 405 nm in order to observe the disposition and

21

515 arrangement of the photocytes within the elytra. The confocal microscope used was an LSCM

516 Olympus FV1200 at Universidad Complutense de Madrid and the light microscope was a

517 Olympus BX53 at Museo Nacional de Ciencias Naturales (MNCN).

518

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529

530 **References**

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771

772   **Tables**

773   **Table 1: List of clusters shared between the bioluminescent species**

774

Cluster	Protein ID	Go Annotation	Relation with bioluminescence
cluster13850	Q9C0H6	Actin binding	Microtubule organization
cluster10051	P17644	Synaptic transmission, cholinergic	Nervous system
cluster11826	Q5IS76	Synaptic transmission, cholinergic	
cluster3310	H2A0M3	Amine metabolic process	Oxidorreductase
cluster5878	J9VQZ4	Hydroquinone:oxygen oxidoreductase activity	
cluster6532	O86034	3-hydroxybutyrate dehydrogenase activity	
cluster8278	Q7Z5P4	Positive regulation of lipid biosynthetic process	
cluster16223	Q8HXW0	L-ascorbic acid biosynthetic process	

cluster5790	Q6PHY8	Thioredoxin-disulfide reductase activity	
cluster9970	Q6UVY6	Octopamine biosynthetic process	
cluster5785	P08761	Sulfur amino acid metabolic process	Response to oxidative stress
cluster4890	Q9CQN6	Regulation of heme biosynthetic process	
cluster2261	Q9EPF3	Estructural constituent of eye lens	
cluster10748	Q9QYF1	Retinol metabolic process	Visual sense
cluster11855	Q9GLM3	Visual perception	

775

776 The table shows the identification protein label (Protein ID), the annotation made by Orthovenn

777 (Go Annotation), and the corresponding function related with bioluminescence

778 (Bioluminescence).

779

780 **Table 2 Acession numbers**.

Species	Accesion number NCBI
Harmothoe areolata	SAMN38153224
Harmothoe imbricata	SAMN38153218
Harmothoe imbricata elytra	SRR4841788
Harmothoe extenuata	SRR1237766
Branchipolynoe pettiboneae	SRR4419842
Lepidonotopodium sp.	SRR4419843

781

782 NCBI Reference numbers of the transcriptomes of the species used in the Orthovenn analyses.

783

784 **Ethics declarations**

785

786 - Ethics approval and consent to participate: Not applicable.

787 - Consent for publication: Not applicable.

788   -   Competing interests: The authors declare no conflict of interests.

789

Availability of data and materials

791   The datasets generated and/or analyzed during the current study are available in the [Additional

792   files] repository,

793   [https://www.dropbox.com/sh/rqx5l06cglk7ojp/AAAdKFH_i9xSo_2_yeYJNpFQa?dl=0]

794

Additional files:

796   **Additional file 1 Sequencing information.** *De novo* transcriptome assembly metrics and

797   transcriptome completeness statistics based on BUSCO analysis with the Metazoa gene set.

798

799   **Additional file 2 Scale up subset.** Table showing the results of the Differential Expression

800   Analyses performed for both species (one species per sheet).

801

802   **Additional file 3 GO terms.** Results of the Gen Ontology analyses performed with the whole

803   transcriptome of the selected species as background. The results for each species are found on

804   separate sheets.

805

806   **Additional file 4 Orthovenn results.** Orthologous genes shared by the selected bioluminescent

807   species.