

1 **Title: Expression of squid iridescence depends on environmental luminance and peripheral**
2 **ganglion control.**

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9 **Short title:** Circuit controlling squid iridescence

10 **Keywords:** Structural coloration, neural control, visual, behaviour, extracellular stimulation, iridophore.

11 **SUMMARY**

12 Squids display impressive changes in body coloration that are afforded by two types of dynamic skin
13 elements: structural iridophores (which produce iridescence) and pigmented chromatophores. Both color
14 elements are neurally controlled, but nothing is known about the iridescence circuit, or the environmental
15 cues, that elicit iridescence expression. To tackle this knowledge gap, we performed denervation,
16 electrical stimulation and behavioral experiments using the long-fin squid, *Doryteuthis pealeii*. We show
17 that while the pigmentary and iridescence circuits originate in the brain, they are wired differently in the
18 periphery: (i) the iridescence signals are routed through a peripheral center called the stellate ganglion and
19 (ii) the iridescence motoneurons likely originate within this ganglion (as revealed by nerve fluorescence
20 dye fills). Cutting the inputs to the stellate ganglion that descend from the brain shifts highly reflective
21 iridophores into a transparent state. Taken together, these findings suggest that although brain commands
22 are necessary for expression of iridescence, integration with peripheral information in the stellate
23 ganglion could modulate the final output. We also demonstrate that squids change their iridescence
24 brightness in response to environmental luminance; such changes are robust but slow (minutes to hours).
25 The squid's ability to alter its iridescence levels may improve camouflage under different lighting
26 intensities.

27 **INTRODUCTION**

28 Eyes evolved as early as 515 Ma ago, and thereafter the evolutionary pressure to deceive the vision of
29 predatory animals resulted in the refinement of reflective body elements (Parker, 2005). A myriad of
30 biological reflective elements exists today that produce color through the use of repeated nanostructures,

31 and not pigments. Iridescence is one such type of structural coloration, characterized by a high spectral
32 purity and intensity that is angle-dependent (Vukusic et al., 2002). Because iridescence can be produced
33 at any visible wavelength, it confers some advantages over pigment-based coloration for purposes such as
34 signaling or camouflage (Meadows et al., 2009). For example, butterflies and damselflies use blue
35 iridescence for mate recognition and as a badge of fitness (Fitzstephens and Getty, 2000; Kemp, 2007).
36 Given that iridescence is angle-dependent and that most animals cannot alter the nanostructures that
37 produce the color, their placement on the body (e.g. butterflies; Rutowski et al., 2007; Vukusic et al.,
38 2002) and the animal movements during courtship (e.g. peacock spiders; Girard et al., 2011) become
39 crucial when producing signals of ecological relevance. Only a few species have evolved ways to quickly
40 alter their iridescent nanostructures. Of those, only a few teleost fish species (Iga et al., 1987; Kasukawa
41 et al., 1986; Muske and Fernald, 1987) and the Atlantic longfin squid *Doryteuthis pealeii* (Wardill et al.,
42 2012) are known to control their iridescence neurally. These two iridescence systems evolved
43 independently, and have different activation mechanisms. In squids, the iridescent cells, called iridocytes,
44 contain platelets made of reflectin (Crookes et al., 2004), a protein that is reversibly condensed upon ACh
45 application (Cooper and Hanlon, 1986; DeMartini et al., 2013; Izumi et al., 2010). Reflectin platelets
46 form stacks called iridosomes (Arnold, 1967). As the reflectin refractive index is higher than that of the
47 inter-platelet space, which is made up of cytoplasm and extracellular fluids (Kramer et al., 2007),
48 iridosomes act as biological Bragg stacks (Holt et al., 2011; Wu et al., 2007), interfering with different
49 light wavelengths and producing iridescence. Higher ACh concentrations induce thinner and denser
50 platelets (Cooper and Hanlon, 1986; Cooper et al., 1990; Mähnger et al., 2004) resulting in a greater color
51 shift towards the blue wavelengths (Mähnger et al., 2009; Sutherland et al., 2008; Tao et al., 2010) and an
52 increased refractive index, respectively. In addition, the increase in intracellular calcium elicited by
53 exposure to ACh also causes water to be expelled from the cell, reducing the inter-platelet space further
54 and contributing to the color shift (DeMartini et al., 2013). In contrast, the platelets present in fish
55 iridocytes are made of guanine crystals (Clothier and Lythgoe, 1987). These platelets disperse (Iga et al.,
56 1987) upon norepinephrine exposure (Muske and Fernald, 1987; Oshima and Fujii, 1987), which
57 increases the inter-platelet distance and shifts the reflected coloration from blue towards red wavelengths
58 (Clothier and Lythgoe, 1987; Nagaishi and Oshima, 1989) .

59

60 The advantages of neurally controlled tunable iridescence are self-evident and our understanding of these
61 dynamic nanostructures has expanded dramatically in recent years (DeMartini et al., 2013; Tao et al.,
62 2010). For instance, the endogenous source of ACh remained controversial until Wardill *et al.* (2012)
63 showed that the site of neurotransmitter released takes place at the iridophore layer. These macroscopic
64 iridescent splotches are created by aggregations of 10 to 100s of iridocytes. However, much remains to be

65 clarified about this system. For example, boutons indicating synaptic contacts between iridocytes and
66 axons are yet to be reported. Thus, the current hypothesis is that within the iridophores, the ACh release
67 may be *en passant* in nature (Wardill et al., 2012). In addition to this lack of information about the neural/
68 iridocyte interface, every other detail of this neural circuit has remained obscure. This is significant
69 because evolution of iridescence may be linked to phylogenetic relationships in species that are visually
70 guided (Parker, 2005). Moreover, although previous studies have proposed that dynamic iridescence may
71 act as a private intraspecific communication channel (Chiou et al., 2007; Hanlon et al., 1990; Mäthger and
72 Hanlon, 2006) or as an aid in camouflage (Hanlon et al., 1999; Mäthger et al., 2009), direct evidence on
73 the function of cephalopod dynamic iridescence is still lacking. In this study, we used
74 electrophysiological, morphological and behavioral approaches to investigate the neural circuit that
75 controls iridescence in the squid *Doryteuthis pealeii*. We provide unexpected findings on the neural
76 wiring of the circuit that controls tunable iridescence and the light conditions that elicit changes in
77 reflectivity.

78 **MATERIALS AND METHODS**

79 **Animals.**

80 Squids were collected by trawling outside of Woods Hole, MA. For more details see (Wardill et al., 2012)
81 and [supplementary material](#). All procedures carried out in this study comply with institutional
82 recommendations for cephalopods. To minimize stress, squids were held in low density (1-5 squids) per
83 holding pens, made by using divisions within large tanks. The tanks had a continuous high flow supply of
84 seawater at 22°C. The squids were fed twice daily with live fish (*Fundulus* sp.). For animal transfer,
85 squids were caught with a net and carefully moved by hand between enclosures. Any signs of stress
86 displayed by the squid did not last more than a few seconds. For the experiments involving nerve cuts, the
87 animals were held for brief periods of time (15 seconds), from which they recovered almost immediately.
88 However, if an animal was restless when first held, it was lightly anesthetized by submersion for <30
89 seconds in 1.5% EtOH in sea water before denervation. For the experiments that required dissection of the
90 fin tissues and nerves, the animals were first deeply anesthetized with 3% EtOH, and their
91 unresponsiveness tested before proceeding with decapitation and decerebration.

92

93 **Denervation**

94 Young (1936b) used the terms pallial nerve and mantle connective interchangeably to describe all the
95 axons that leave the brain and travel towards the mantle, some of which enter the stellate ganglion. In this
96 study more precise naming was necessary for clarity. Hence, we have reserved the name "*pallial nerve*"
97 for the bundle that contains all the axons which descend from the brain. We refer to the branch of the

98 pallial nerve that travels into the stellate ganglion as the "*stellate connective*" and to the branch that
99 proceeds towards the fin as the "*fin nerve*" (Fig.1A,B). Squids were held briefly and their nerves or
100 connectives cut with a micro-scissor. All squids were denervated on the same side, the other half of the
101 animal was left intact and served as internal control.

102 **Nerve fills in the stellate ganglion**

103 The stellate ganglia were removed and bathed in Ca²⁺-free solution for 10 minutes (recipe from
104 Strathmann, 1987). Nerves were filled (methods from Chrachri, 1995) with Lucifer yellow and
105 MicroRuby and tissue prepared as shown in Gonzalez-Bellido & Wardill (2012). For more details, see
106 supplementary material.

107

108 **Assessing iridescence output and neural excitability 7 and 15 days after denervation**

109 In squid, the name iridophore refers to a group of iridescent cells (named iridocytes) that form an
110 iridescent "splotch" (Wardill et al., 2012). We recorded the changes of reflectivity from single
111 iridophores, in response to neural stimulation. The animals were tested either 7 (n=9) or 15 (n=8) days
112 post-denervation. The same protocol as that of Wardill *et al.* (2012) was used, so that results between the
113 two studies could be compared. Briefly, nerve fascicles named dermal nerves, which radiate from the base
114 of the large fin nerve and innervate the skin, were exposed and stimulated via suction electrode. Electrical
115 pulses of 7 volts and 0.6 ms in duration, were delivered at a frequency of 10 Hz for 15 seconds while
116 iridescence was monitored with a spectrometer. The spectrometer was calibrated with a white standard,
117 and the spectral reflectance recorded every 0.1 s over 3 – 8 minutes. For analysis, the background
118 reflectance was subtracted and the data were normalized and then smoothed with a Savitzky-Golay filter
119 using Matlab. Normalized reflected intensity could be read directly after smoothing and the change in
120 color over time was determined by finding the peak reflecting wavelength at each time point from the
121 smoothed data.

122

123 **Assessing time required for drop in iridescence**

124 After the stellate connective was cut, each animal (n=15) was placed back into a small round arena
125 (25 cm diameter), with black felt lined walls, smooth grey substrate and a continuous seawater supply.
126 The animal was videoed for 60 minutes. To assess iridescence change over time, we extracted images
127 from the video at 5, 10, 20, 30, 40, 50 and 60 min. At each time point, the iridescence was scored as one
128 of the three options: no change, substantial decline or completely absent.

129

130 **Behavioral assessment of iridescence decrease due to dark adaptation**

131 Squids were placed in an arena with black lined walls and black pebbled substrate, 24 hours after
132 denervation. The whole ensemble was placed inside a blackened tent surrounded with galvanized sheet
133 metal (0.4 nW) and the animal left to dark adapt for either 1 (n=17) or 2 (n=6) hours. After that time, a
134 picture was taken with a remote trigger using the Canon EOS 5D MarkII camera with flash to capture the
135 body patterning of the animal.

136

137 **Behavioral assessment of iridescence increase due to light exposure**

138 To assess iridescence changes due to light exposure, animals (n=4) were dark adapted as explained above.
139 The arena was covered with a glass panel to prevent ripples on the water surface reflecting light into the
140 camera. Animals were imaged at 300 frames per second with a Casio Exilim Pro EX-F1 camera as they
141 were exposed to continuous light.

142

143 **RESULTS**

144 **Cutting the stellate connective abolishes iridescence**

145 To begin, we re-confirmed that cutting the pallial nerve, just prior to the stellate connective branching
146 point, resulted in immediate skin blanching (due to cessation of chromatophore neural control; Sanders
147 and Young, 1974), lack of respiratory mantle contractions and a deficiency of undulatory fin movements
148 in the denervated side of the animal (**Fig.1C, pallial nerve**). Accordingly, cutting the stellate connective
149 resulted in absence of respiratory movements and a lack of mantle chromatophore activity (**Fig.1C,**
150 **stellate connective**) and cutting the fin nerve caused cessation of undulatory fin movements and
151 chromatophore activity from the base of the fins to the posterior tip of the animal (**Fig.1C, fin nerve**). We
152 did not observe an immediate decrease in iridescence in any of the three treatments. This was not
153 surprising, since in our recent experience (Wardill et al., 2012), iridescence decline does not take place
154 immediately after the last neural stimulation. Hence, we returned the animals to their holding tanks. After
155 24 hours, all the animals with a stellate connective cut (n=10) exhibited a complete lack of skin
156 iridescence on the side ipsilateral to the cut (**Fig.2A**). However, these animals retained complete control
157 of their fin chromatophores, including expansion of a single color type of chromatophores (yellow;
158 **Fig.2B**), full red flash warning display (**Fig.2C; supplementary material, Movie 1**) and mottled
159 camouflage patterns (**Fig.2D**). Moreover, fin undulatory movements remained intact (**supplementary**
160 **material, Movie 2**). Iridescence was also abolished ipsilaterally in the animals with a pallial nerve cut,
161 but remained bright in the animals with a fin nerve cut. Thus, activation of fin and mantle iridescence
162 requires descending inputs from the brain that enter the stellate ganglion.

163

164 **Fin iridescence motorneurons leave the stellate ganglion and join the fin nerve**

165 The results above were contradictory to the prevailing dogma, which stated that all the motorneurons
166 innervating the fin descend directly from the brain through the fin nerve (Hofmann, 1907; Young, 1932).
167 To solve this conundrum, we removed the sheath surrounding the fin nerve and revealed that some of its
168 fibers form a fascicle that originates in the 1st stellar nerve (**Fig.3A**). By electrically stimulating this
169 fascicle, we recovered fin iridescence in the animals whose stellate connective had been severed (**Fig. 3B**;
170 **supplementary material Movie 3**). Importantly, this fascicle activated not just the iridophores, but also
171 the scattered iridocytes (**supplementary material, Fig.S1**). Because the fin chromatophores remained
172 relaxed during electrical stimulation of this fascicle, we have named it the “*fin iridescence nerve*” or “*FI*
173 *nerve*.” These findings confirm that the first stellar nerve carries the iridescence motorneurons that
174 innervate the fin. We also found that electrical stimulation of the FI nerve resulted in fin contractions that
175 reached tetanus at frequencies >20 Hz (**supplementary material, start of stimulation, Movie 3**).

176

177 **The majority of the FI nerve fibers originate in the stellate ventral wall**

178 To elucidate if the motor fibers forming the FI nerve originate in the stellate ganglion, or simply travel
179 through it, we backfilled the FI nerve with Lucifer yellow. The labeling revealed a large group of cell
180 bodies in the stellate ganglion and a few fibers (6 at most) that traveled farther and became part of the
181 stellate connective (**Fig.3C**). The labeled cell bodies were large (up to 100 μm), located in the most
182 medial part of the ventral cell wall (adjacent to the giant lobe; **Fig.3C**), with a single neurite sporting thin
183 branches that resembled dendritic spines (**Fig.3C**). These neurites form part of the ventral root that exits
184 the ganglion through the 1st stellar nerve. Due to the complexity of the neuropile, it was not possible to
185 resolve if the fibers that continued to the stellate connective also had cells bodies in the ganglion, or if
186 they extended dendritic trees or terminals within it. To clarify this point, we carried out double fills of the
187 FI nerve and the stellate connective, with Lucifer yellow and MicroRuby (**Fig.3D**). No fibers were doubly
188 labeled, most likely due to the nerve fills being incomplete (occasionally, axons remained unfilled) and
189 the low number of fibers that travel directly from the FI nerve to the stellate connective. However, the
190 labeling of the stellate connective did fill some blood vessels that surrounded the FI nerve cell bodies
191 (**Fig.3D**). In addition, the stellate connective fill also labeled a cell body located between the cell bodies
192 labeled from the FI nerve (**Fig.3D**). Thus, in addition to the possibility that these fibers are continuous
193 neurites that descend directly from the brain, or sensory afferents ascending towards the brain, we must
194 add the following two possibilities: they could be (i) monopolar cells extending neurites towards the brain
195 and the periphery, but with cell bodies in the stellate ganglion or (ii) blood vessels supplying the stellate
196 ganglion.

197

198 **Iridescence motorneurons remain excitable 7 and 15 days after the stellate connective is cut**

199 To elucidate if the iridescence motorneurons belonged to the group of fibers that originated in the stellate
200 ganglion, we used a paradigm based on Young's (1972) denervation experiments with cuttlefish (*Sepia*
201 *officinalis*). Namely, degeneration of the distal stumps of the chromatophore axons is observed 4-7 days
202 after the *Sepia* pallial nerve is severed (animals are kept at 17-23°C) (Young, 1972). This is because the
203 axons of the chromatophore motorneurons descend directly from the brain (Dubas et al., 1986a;
204 Hofmann, 1907; Young, 1932). Messenger *et al.* (1997) further confirmed the validity of this paradigm
205 in squid. Accordingly, we cut the squid stellate connective and waited 7 and 15 days before testing if the
206 iridescent motorneurons innervating the fin would respond to electrical stimulation (for full protocol see
207 Wardill et al., 2012). At maximum stimulation, the color of the iridophores was not significantly different
208 between the animals that had been denervated 7 (n=9 iridophores from 4 animals) and 15 (n=8
209 iridophores from 3 animals) days previous to the experiment (evidenced by the standard deviation overlap
210 throughout the response; [supplementary material, Fig.S2](#)). Hence, we pooled these results from here
211 onwards and refer to it as the denervated group. Likewise, the color obtained in intact (Wardill et al.,
212 2012) and denervated (this study) groups were not significantly different (evidenced by the standard
213 deviation overlap throughout the rising phase of the response; [Fig.3E](#)). The one striking difference
214 between these two groups was the reflectance change, which was significantly higher in the denervated
215 group ($p > 1.428 \times 10^{-30}$; Denervated = 313.94 ± 164.43 S.D, n=15; Intact = 245.17 ± 119.56 S.D,
216 n=13). Moreover, the maximum iridophore reflectance change also belonged to a denervated animal
217 (Denervated max = 624.46%, Intact max = 440.43%. See example of a 491% change from a denervated
218 animal in [supplementary material Fig.S3](#)). This is because the baseline iridescence in denervated
219 animals was extremely low. Taken together, these findings indicate that a stellate connective cut
220 extinguishes iridescence because the descending inputs to the motorneurons are severed, and not the
221 motorneurons themselves.

222

223 **Severing the stellate connective substantially reduces iridescence within 30 min**

224 Elucidating the rise/decay rates for dynamic iridescence may help us understand its behavioral role. In
225 this experiment we recorded the iridescence decay following the last neural impulse i.e. the time elapsed
226 until iridophores become transparent following a cut of the stellate connective. Of the 15 animals tested, 8
227 (>50%) exhibited fin iridophores that were transparent within 30 minutes and 12 (80%) displayed a
228 substantial decline in iridescence within 60 minutes ([Fig.4A; supplementary material, Fig.S4](#)). Three
229 animals (20%) had little or no change in iridescence at 60 minutes post denervation. Thus, the passive
230 decline of iridescence is slow, taking >30 min to complete. This experiment does not address the
231 possibility that an active process may exist to reduce iridescence in a shorter time.

232

233 **Squids reduce their iridescence in response to dark adaptation**

234 Iridophores are efficient light reflectors, hence their reflectivity may increase the risk of predation, both
235 day and night. To test if squids would reduce their iridescence in response to darkness, we dark-adapted
236 the animals for 1 or 2 hours. The side of the animal with a stellate connective cut served as an internal
237 control for no iridescence display. After 1 or 2 hours in the dark (0.4 nW), 12 of 18 animals (66%) and 5
238 of 6 (83%), respectively, showed a substantial decline in iridescence (**Fig.4B**).

239

240 **Squids increase their iridescence in response to light exposure**

241 Given the drop of iridescence after dark adaptation, we tested if squids would increase their iridescence if
242 exposed to light. We used high-speed videography in case this response was rapid, as it is in
243 chromatophores. Moreover, high speed allows short exposure times, which is crucial in the case that the
244 animal jetted in response to illumination due to being startled. However, our high-speed video showed
245 that although squids did increase their iridescence expression, the change was slow; after 90 seconds of
246 light exposure, iridescence was detectable and continued to increase during the four minutes that followed
247 (n=4)(**Fig.4C**).

248

249 **DISCUSSION**

250 **Squids have a dedicated neural circuit for iridescence control**

251 Dynamic coloration and patterning on the mantle of cephalopods have long been known to be influenced
252 by motorneurons emanating from the posterior chromatophore lobes (PCL) in the central nervous system
253 (CNS)(Sereni and Young, 1932). In the loliginid squid *Lolliguncula brevis*, those neurons travel without
254 synapse from the PCL to the chromatophore organs in the skin (Dubas et al., 1986a; Dubas et al., 1986b).
255 Similar investigations focusing on iridescence were lacking, and here we discover in the loliginid
256 *Doryteuthis pealeii* that iridescent structural coloration control is routed through the peripheral stellate
257 ganglion. The stellate ganglion contains one of the best-studied synapses known to neuroscience – the
258 giant synapse that mediates jet escape via the 3rd order giant axons (Otis and Gilly, 1990; Young, 1936a;
259 Young, 1936b; Young, 1937; Young, 1939). It is surprising that the anatomical and neurophysiological
260 features of the iridescence circuit of this large ganglion have not been noted or studied to date.

261

262 We have demonstrated, through targeted denervations, that the neural circuits that control iridescence and
263 pigmentary coloration are wired differently. Both circuits originate in the brain, but the iridescence
264 signals are routed through the stellate ganglion while pigmentary signals descend through the fin nerve.

265 Thus, pigmentary and structural skin elements are controlled by different motorneurons. Importantly, our
266 denervation experiments have revealed that iridophores remain visually transparent when neural signals
267 are absent. Furthermore, previous studies showed that not all squid iridescent cells are active; for
268 example, those on the lateral and ventral surfaces of squids such as *Lolliguncula brevis* are considered
269 passive as they do not alter their reflectance to bath application of ACh (Cooper et al., 1990; Hanlon et
270 al., 1990). In this study we have shown that the scattered iridocytes of the dorsal fin that do not form
271 aggregations into an iridophore also respond to neural stimulation. Color fluctuations from single
272 iridocytes are too small to be detected by the naked eye. Thus, it is possible that they too are involved in
273 producing background coloration that is tunable. It remains to be clarified if the iridescent cells of the
274 ventral skin respond to neural stimulation.

275

276 **Iridescence motorneurons are most likely located in the stellate ganglion**

277 Our fluorescent dye fills showed that the cell bodies of the fin iridescence nerve motorneurons arise from
278 the ventral root of the last stellar nerve. This is important because Young (1972) showed that all the fibers
279 that pass through the stellate ganglion on their way to/from the brain, such as the chromatophore
280 motorneurons, do so as part of the dorsal roots. Taken together, these observations support the premise
281 that FI nerve fibers are unlikely to descend directly from the brain. Moreover, we have shown that the
282 fibers responsible for iridescence activation, which travel via the fin iridescence nerve, are silenced but
283 not ablated when the stellate connective is severed. All such results are supported by those of Young
284 (1972), who confirmed that cutting the pallial nerve resulted in degeneration of the inputs to the ventral
285 neuropile of the ganglion, but that the postsynaptic cells, whose cell bodies are located in the ventral wall,
286 remained intact. Therefore, although intracellular recording/stimulation is needed for direct proof, thus far
287 all results indicate that the cell bodies of the iridescence motorneurons are located in the ventral wall of
288 the stellate ganglion.

289

290 **Role of the stellate ganglion in iridescence control**

291 The stellate ganglion has long been recognized as a center for signal integration (Wilson, 1960) and reflex
292 coordination (Gray, 1960), but only for the purposes of locomotion and respiration (Young, 1972). We
293 have shown that the stellate ganglion does not act as a simple peripheral reflex in the control of
294 iridescence, because descending brain inputs are needed for iridescence expression. However, it is
295 plausible that within the stellate ganglion, the integration of peripheral information modulates the level of
296 iridescence expression. Whether afferent signals from peripheral senses, such as mechanosensory "lateral
297 line" (Mackie, 2008; Preuss and Budelmann, 1995) and nociception (Crook et al., 2011), relay
298 information in the stellate ganglion remains controversial (e.g. Gray, 1960; Wilson, 1960; Young, 1972).

299 If so, modulation of iridescence motor neural signal may be inhibitory or excitatory. In this regard, it is
300 noteworthy that Miledi (1972) recorded intracellularly from ventral cells of the stellate ganglion and
301 showed that they receive excitatory and inhibitory inputs from both the stellate connective and stellar
302 nerves. Such findings highlight the integrative role of the ventral neuropile cells. Unfortunately, Miledi
303 (1972) did not suggest a role for the cells that he recorded from.

304

305 **Role of the stellate ganglion in fin motor control**

306 In addition to activating fin iridescence, electrical stimulation of the FI nerve at 20 Hz produced a tonic
307 contraction of the fin. Such contraction is not produced by giant axons, because we did not see any large
308 axons in our fluorescent fills of the FI nerve and because none of the cell bodies filled were located within
309 the giant axon lobe. Moreover, we also recognize that such contraction is not produced by units that
310 control the undulatory fin motion in a live animal, because our denervations and behavioral results
311 evidenced that such control is wired directly through the fin nerve. Thus, the function of FI nerve fibers
312 that drive fin muscles remains to be elucidated.

313

314 At the current time, we are unable to elucidate what percentage of the cell bodies labeled through the Fin
315 Iridescence nerve and located in the stellate ganglion, innervate the fin musculature and what proportion
316 target the iridophore layer. Alternatively, it is possible that each of the labeled cells branch at the base of
317 the fin and innervate both iridophores and fin muscles, such that an increase in iridescence would always
318 be coupled to a fin contraction.

319

320 **Neural control of iridescence: possible innervation mechanism and timing dynamics.**

321 Throughout this manuscript we have used the term motorneuron to refer to neurons that convey impulses
322 to an effector tissue; the iridophore layer in this case. Indeed, within the iridophore layer it remains to be
323 elucidated if iridocytes are activated via classical synapses, *en passant* delivery or muscles. Wardill *et al.*
324 (2012) suggested that muscles may be involved in the activation of iridescence because single iridocytes
325 can be seen to “flicker” (Wardill et al. 2012) and because muscles have been found closely associated to
326 iridescent cells (Mirow, 1972). Currently, a correlation between muscle activity and iridophore
327 iridescence is still lacking. Moreover, the speed of iridescence activation reported here and in Wardill *et*
328 *al.* (2012) is not comparable to that of the squid muscular system (See supplementary material, Movie 3).
329 Nonetheless this does not constitute evidence against some (yet unknown) involvement of muscles in the
330 control of iridescence. For an iridocyte to be noticeable, reflectin must first be sufficiently condensed.
331 This intracellular process is likely to be the time-limiting step for the speed of iridescence expression,
332 even if a muscle mechanism with an active role in the iridescence process was activated at the same time.

333 Wardill *et al.* (2012) concluded that a process different to the reflectin condensation must cause the large
334 and fast color shift seen upon iridophore activation, and suggested that a muscle mechanism may play this
335 role. Shortly after, DeMartini *et al.* (2013) reported that activated iridocytes expel water, lowering the
336 inter-platelet distance and producing a color shift. Thus, it remains to be clarified if muscles play a role in
337 iridophore activation; either through the previously observed rapid iridocyte flicker or from fin muscles
338 activated by motorneurons that travel through fin iridescence nerve. Another possibility is that fin
339 musculature activation by fin iridescence nerve fibers could modulate the control of iridescence via the
340 release of fin muscle neurotransmitter. This case is unlikely because we did not observe a difference
341 between denervated and wild type animals upon stimulation of their FI nerve.

342

343 **Squid iridescence changes in response to environmental light intensity**

344 Unlike pigmentary chromatophore changes, the passive decline of iridescence is slow; the decline was
345 substantial within 30 min, but some iridescence was still visible in nearly half of the tested animals after 1
346 hour. Since squid in these experiments reduced their iridescence in response to dark adaptation and
347 increased reflectance upon light exposure, we interpret this to mean that the slow iridescence changes are
348 related to different light levels experienced in the wild. It is probable that squids tailor their iridescence
349 expression to improve their camouflage depending on the amount of light present during the day.
350 Moreover, a substantial iridescence reduction in low lighting conditions or at nighttime may also reduce
351 the risk of predation. Squids are likely to detect such overall environmental luminance through their eyes,
352 but neurons in the photosensitive vesicles (Young, 1978) or rhodopsin located in the skin (Mäthger *et al.*,
353 2010), could also be employed. Squids such as *Doryteuthis pealeii* are found nearshore at depths from 3-
354 30 m when inshore during the summer (Shashar and Hanlon, 2013; Summers, 1983) and often forage near
355 surface waters of 1-10 m depth at night time (Serchuk and Rathjen, 1974). In winter, *D. pealeii* lives
356 offshore in canyons on the continental slope at depths up to 170 m (Summers, 1967) where light is dim
357 even during the day. Even so, we recognize that in our testing set up the lighting was extremely low
358 (0.4 nW) and such luminance may not be encountered by this squid species in the wild, especially since
359 calculated luminance at 100 m depth of clear ocean water is 18 mW/m/m (calculated using water type
360 values from Jerlov (1968) and light penetration values from Gordon & McCluney (1975)). To clarify this
361 point, it would be necessary to test iridescence output in a variety of low light intensities, as well as the
362 outcome in the presence of conspecifics.

363

364 **Evolution of neural control of iridescence and the stellate ganglion**

365 The only extant cephalopod species known to lack a stellate ganglion is *Nautilus* (Young, 1972), which is
366 not a coleoid and does not possess neural control of its skin (Crook and Basil, 2008). The extinct

367 ancestors of current coleoid cephalopods, named belemnoids, had elongated bodies and dorsal fins (Lewy,
368 2009), but it is not know if they had evolved a stellate ganglion. Moreover, a comparative demonstration
369 in sepioids and octopods of neural stimulation activating iridescence is currently lacking. Hence a
370 comprehensive comparative approach among extant coleoids will be the most efficient route for
371 understanding the evolution of iridescence control among coleoid groups.

372

373 **Summary**

374 This is the first study to describe part of the neural circuit controlling iridescence in a cephalopod. We
375 have shown that (1) iridescence is controlled independently from pigmentary elements - chromatophores,
376 (2) fin iridescence neural signals are routed through the stellate ganglion (instead of descending directly
377 through the fin nerve) and the iridescent motoneurons likely originate within it, (3) iridescence
378 expression requires the input of neurons descending from the brain , (4) passive decline of iridescence is
379 slow and (5) squids turn their iridescence off and on in response to ambient darkness and light,
380 respectively. At present, the brain area where the iridescence signals are computed remains to be located.
381 In addition, single iridescence motoneurons will need to be stimulated to fully understand the role of
382 each iridescence motor unit. Further behavioral studies are necessary to test if squids can tune their
383 iridescence output depending on its role for either camouflage or signaling. Our current research
384 directions focus on these questions.

385

386 **ACKNOWLEDGMENTS**

387 We thank fellow lab members for their support and discussion of this study. We thank MBL Equipment
388 Resources, MBL Apparatus Department and Zeiss Microscopes for assistance with equipment. We also
389 thank Nathan Boor (Aimed Research, Burghill, OH) for his generosity in lending us the High-Speed
390 video camera. We thank the MBL Central Microscopy facility for providing imaging resources and the
391 Aquatic Resources Division of MBL for supplying squid.

392 **AUTHOR CONTRIBUTION**

393 PTGB and TJW had the initial idea, designed and performed the denervation, electrophysiological and
394 imaging experiments. PTGB and TJW also analyzed the data for such sections. KCB and KMU
395 performed the dark adaptations experiments while TJW performed the light adaptation experiments. RTH
396 helped refine the initial ideas and oversaw the multiple objectives. PTGB wrote the initial manuscript. All
397 authors contributed to the interpretations of the results and revisions of the manuscript.

398

400 **FUNDING**

401 This research was supported by the ONR Basic Research Challenge grant no. N00014-10-1-0989 and by
402 the AFOSR grant FA9950090346.

403

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560

561

562 **Figure Legends:**

563 **Fig.1. Neural control of the squid mantle and fins.** (A) The bright splotches of structural coloration,
564 named iridophores, can be seen among the reddish pigmentary color on the squid skin. The undulatory
565 motion of the fins is also evident in this picture. The axons, descending from the brain through the pallial
566 nerve, control the movement and skin coloration of the mantle and the fins. (B) This diagram shows the
567 neural wiring of descending pathways (known to date) along with an image of the stellate ganglion; the
568 pallial nerve (red) splits into the stellate connective (purple) and the fin nerve (green). The stellate
569 connective travels into the stellate ganglion (orange), whereas the fin nerve proceeds towards the fin. (C)
570 Skin coloration in an intact animal and immediately after severing the pallial nerve, the stellate connective
571 and the fin nerve, respectively. Instantly after severing the nerves, iridescence remained unchanged, but
572 chromatophores relaxed, which resulted in a ghostly appearance.

573
574 **Fig.2. Severing the stellate connective abolishes iridescence.** (A) Iridescence was absent in the cut side
575 of the animal 24 hours after the stellate connective was severed. (B) The only noticeable difference
576 between the two fins of an animal with a stellate connective cut is the lack of iridescence on the cut side.
577 Despite the stellate connective cut and the subsequent loss of iridescence, the animal maintained the
578 following abilities across the full surface of both fins: (C) full chromatophore expansion, demonstrated
579 here with a red warning flash (these three sequential frames were obtained from supplementary Movie 1)
580 and (D) localized patches of chromatophore expansion for the purpose of camouflage, in addition to intact
581 undulatory fin movements ([supplementary material, Movie 2](#)),

582 **Fig.3. The putative cell bodies for the motoneurons controlling fin iridescence are located in the**
583 **stellate ganglion.** (A) Removal of the fin nerve sheath reveals a fascicle that originates in the 1st stellar
584 nerve and joins the fin nerve. (B) Electrical stimulation of the newly described fascicle recovers fin
585 iridescence, which was absent due to a stellate connective cut 24 h prior. Arrows indicate the same skin
586 location in both photographs. We named this fascicle the Fin Iridescence (FI) nerve. (C) Backfilling the
587 FI nerve reveals that the majority of the fibers have cell bodies located medially in the ventral wall of the
588 stellate ganglion. Each cell body extends a single neurite bearing fine branches. In addition, a few fibers
589 continue and join the stellate connective. Images shown are maximum intensity projections of the stellate
590 ganglion ventral side. (D) Double fill of the FI nerve (blue) and the stellate connective (red) and close up
591 showing overlapping area. (E) At maximum excitation, the iridophore color in animals whose stellate
592 connective was severed 7 and 15 days previous to the experiment (data for both groups pooled; [electronic](#)
593 [supplementary material, Fig. S2](#)) was not significantly different to that obtained in intact animals (dark
594 solid line, data from Wardill et al., 2012). Grey shading represents the standard deviation. Note how the
595 standard deviations overlap along the rising phase of the response.

596 **Fig. 4. Timing of reflectance change in live animals kept in laboratory arena.** (A) Example of
597 iridescence decline after denervation (stellate connective cut). A substantial drop in iridescence is
598 observed 10 min after the cut. The iridophores are almost transparent at 30 min and not expressed at 50
599 min. (B) Pictures of a squid before and after 2 hours of dark adaptation, showing the decline in
600 iridescence elicited by the dark conditions (the intact side now matches the denervated side, which serves
601 as an internal non-iridescent control). (C) After dark adaptation, squids increase their iridescence in
602 response to light exposure. The iridescence increase is seen only in the intact side of the animal, further

603 demonstrating that this process requires descending inputs from the brain. Note that the increase in
604 iridescence due to light adaptation is faster than the decline due to denervation.

605