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

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SUMMARY

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Animals.

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

Denervation

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

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

Nerve fills in the stellate ganglion

The stellate ganglia were removed and bathed in Ca^{2+}-free solution for 10 minutes (recipe from Strathmann, 1987). Nerves were filled (methods from Chrachri, 1995) with Lucifer yellow and MicroRuby and tissue prepared as shown in Gonzalez-Bellido & Wardill (2012). For more details, see supplementary material.

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

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

Assessing time required for drop in iridescence

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

Behavioral assessment of iridescence decrease due to dark adaptation

Squids were placed in an arena with black lined walls and black pebbled substrate, 24 hours after denervation. The whole ensemble was placed inside a blackened tent surrounded with galvanized sheet 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 picture was taken with a remote trigger using the Canon EOS 5D MarkII camera with flash to capture the body patterning of the animal.

Behavioral assessment of iridescence increase due to light exposure

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

RESULTS

Cutting the stellate connective abolishes iridescence

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

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

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

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

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

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

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

Severing the stellate connective substantially reduces iridescence within 30 min

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

Squids reduce their iridescence in response to dark adaptation

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

Squids increase their iridescence in response to light exposure

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

DISCUSSION

Squids have a dedicated neural circuit for iridescence control

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

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

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

Iridescence motorneurons are most likely located in the stellate ganglion

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

Role of the stellate ganglion in iridescence control

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

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

Role of the stellate ganglion in fin motor control

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

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

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

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

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

Squid iridescence changes in response to environmental light intensity

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

Evolution of neural control of iridescence and the stellate ganglion

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

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

Summary

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

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AUTHOR CONTRIBUTION

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

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Figure Legends:

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

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

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

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

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