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Exocuticular hyaline layer of sea scorpions and horseshoe crabs suggests cuticular fluorescence is plesiomorphic in chelicerates

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Abstract

The cuticle of scorpions (Chelicerata: Arachnida) fluoresces under long-wave ultraviolet (UV) light due to the presence of beta-carboline and 7-hydroxy-4-methylcoumarin in the hyaline layer of the exocuticle. The adaptive significance of cuticular UV fluorescence in scorpions is debated. Although several other chelicerate orders (e.g. Opiliones and Solifugae) have been reported to fluoresce on exposure to UV light, the prevalence of cuticular UV fluorescence has not been confirmed beyond scorpions. A systematic study of living chelicerates revealed that UV fluorescence of the unsclerotized integument is ubiquitous across Chelicerata, whereas only scorpions and horseshoe crabs (*Xiphosura*) exhibit cuticular UV fluorescence. Scanning electron microscopy and histological sectioning confirmed the presence of a hyaline layer in taxa exhibiting cuticular fluorescence. The hyaline layer is absent in all other chelicerates except sea scorpions (Eurypterida) in which a taphonomically altered hyaline layer, that may have fluoresced under UV light, was observed in exceptionally preserved cuticle. Cuticular UV fluorescence appears to be associated with the presence of a hyaline layer, as has long been recognized in scorpions, and may be plesiomorphic among chelicerates. The presence of a hyaline layer in horseshoe crabs and sea scorpions suggests that several putative functions for cuticular UV fluorescence in scorpions can be discounted.

Introduction

It has been known for more than half a century that scorpions fluoresce under long-wave ultraviolet (UV) light (Lawrence, 1954; Pavan, 1954a; Pavan & Vachon, 1954). Several theories have been advanced to explain the function of UV fluorescence in scorpions including protection against UV light (Lourenço & Cloudsley-Thompson, 1996; Frost *et al.*, 2001), attracting prey (Kloock, 2005), and mating strategies (Fasel *et al.*, 1997), but none has been conclusively demonstrated (Brownell & Polis, 2001).

The thin, outermost layers of the epicuticle were originally thought to cause the fluorescence (Pavan, 1954b,c) but later research identified a hyaline layer in the exocuticle (Kennaugh, 1959). Two compounds (beta-carboline and 7-hydroxy-4-methylcoumarin), present in the hyaline layer, are responsible for the cuticular UV fluorescence of scorpions (Frost *et al.*, 2001). Cuticular UV fluorescence is associated with cuticular sclerotization and increases in intensity with successive instars.

Several other chelicerate orders, e.g., harvestmen (Opiliones) and camel spiders (Solifugae), have also been observed to fluoresce on exposure to long-wave UV light, but the extent of fluorescence was noted to be variable and whether it was cuticular or integumentary in origin was not ascertained (Lawrence, 1954). Scorpion hemolymph also fluoresces when exposed to UV light due to the presence of tyrosol in the hemocyanin (Klarman, Shakhai & Daniel, 1977). However, it is unlikely that cuticular fluorescence is caused by the uptake of fluorophores in the blood, as the fluorescent compounds in the cuticle differ from those in the hemolymph (Klarman *et al.*, 1977; Frost *et al.*, 2001), and cuticular fluorophore concentration is greatest in the hyaline layer with limited interchange with the upper endocuticle (Wankhede, 2004). Cuticular UV fluorescence differs from the integumentary UV fluorescence documented, e.g., in spiders of the suborder Araneomorphae (Andrews, Reed & Masta, 2007). Loss of the tergites in araneomorph spiders (Dunlop & Lamsdell, 2017) permits UV light to penetrate through the reinforced integument to stimulate the

hemolymph fluorophores, causing the opisthosoma to fluoresce (unlike the prosoma or legs, which are covered by cuticle). Furthermore, the fluorescent compounds in spider hemolymph differ from those in scorpion cuticle (Reed, Do & Masta, 2008).

The prevalence of cuticular UV fluorescence has not been explored among chelicerate orders other than scorpions, and its phylogenetic significance is unknown. Chelicerate phylogeny is controversial (Jones *et al.*, 2007; Shultz, 2007; Dunlop, 2010; Pepato, da Rocha & Dunlop, 2010; Arabi *et al.*, 2012; Legg, Sutton & Edgecombe, 2013; Dunlop, Borner & Burmester, 2014; Garwood & Dunlop, 2014; Sharma *et al.*, 2014; Lamsdell *et al.*, 2015a; Selden, Lamsdell & Qi, 2015; Lamsdell, 2016). Nevertheless, sea scorpions (Eurypterida), an extinct chelicerate order that appeared in the Ordovician and disappeared with the Permian extinction (Lamsdell *et al.*, 2015b), are consistently retrieved as the sister group to Arachnida, with horseshoe crabs (Xiphosura) forming the sister group to this clade (Shultz, 2007; Lamsdell *et al.*, 2015a; Selden *et al.*, 2015; Lamsdell, 2016). The hyaline layer is commonly preserved in the cuticle of fossil scorpions (Bartram, Jeram & Selden, 1987). However, previous scanning electron microscopy (SEM) studies of Silurian and Carboniferous eurypterid cuticle only documented the presence of the laminate endocuticle and

provided no indication of a hyaline layer (Dalingwater, 1973, 1975).

To determine the prevalence of cuticular UV fluorescence among chelicerates, exemplars of all living chelicerate orders, along with the extinct sea scorpions, were systematically surveyed for UV fluorescence. A subsample of these specimens were then dissected, examined with SEM, thin sectioned and stained to verify the presence or absence of a hyaline layer in the exocuticle.

Materials and methods

Exemplars representing all living orders of Chelicerata were assessed for cuticular UV fluorescence (Table 1) by scanning material in the Arachnida Collections of the American Museum of Natural History, with a 395 nm wavelength light-emitting diode (LED) UV flashlight. The 395 nm wavelength for the UV light was selected as it straddled the fluorophore excitation ranges observed experimentally in scorpions (Fasel *et al.*, 1997; Stachel, Stockwell & Van Vranken, 1999; Frost *et al.*, 2001; Wankhede, 2004; Kloock, 2008; Gaffin *et al.*, 2012; Lourenço, 2012), spiders (Andrews *et al.*, 2007; Reed *et al.*, 2008), and more distantly related arthropods such as diplopods (Kuse *et al.*, 2001, 2010). Klarman *et al.* (1977) also noted

Table 1 Distribution of integumentary and cuticular fluorescence among the chelicerates surveyed in this study

				Cuticular	Integumentary
Arachnida	Acari	Acariformes	Anystidae		X
		Parasitiformes	Opilioacaridae		X
			Ixodidae		X
	Amblypygi	Euamblypygi	Charinidae		X
		Phrynoidea	Phrynidae		X
	Araneae	Araneomorphae	Tetragnathidae		X
		Mesothelae	Liphistiidae		X
		Mygalomorphae	Dipluridae		X
	<i>Opiliones</i>	Dyspnoi	Sabaconidae		X
		Eupnoi	Phalangidae		X
		Laniatores	Cranidae		X
			Gonyleptidae		X
			Stygidae		X
			Eukoeneniidae		X
	Palpigradi				X
	Pseudoscorpiones	Epiochierata	Chthoniidae		X
		Ioichierata	Cheliferidae		X
			Neobisiidae		X
	Ricinulei	Neoricinulei	Ricinoididae		X
	Schizomida		Hubbardiidae		X
	Scorpiones	Buthoidea	Buthidae	X	X
		Scorpionoidea	Scorpionidae	X	X
	Solifugae		Galeodidae		X
	Thelyphonida		Thelyphonidae		X
					X
Eurypterida [†]		Eurypterina [†]	Eurypteridae [†]	?	?
			<i>Incertae sedis</i> [†]	?	?
Pycnogonida		Pantopoda	Colossendeidae		?
			Nymphonidae		?
Xiphosura			Limulidae		X
			Limulinae	X	X
			Tachypleinae	X	X

Extinct taxa are denoted by[†]

that all fluorophores in scorpions fluoresced equally above an excitation spectrum of 370 nm. Therefore, a wavelength of 395 nm was likely to elicit a fluorescent response from any fluorophores present in the cuticle.

All scanned specimens were stored in ethanol. Although the compounds responsible for fluorescence in scorpions are reportedly insoluble in water and other solvents at temperatures below 100°C (Pavan & Vachon, 1954), these compounds are partly soluble in the ethanol in which scorpion specimens are preserved (Wankhede, 2004), rendering it fluorescent with time (Lawrence, 1954). Scorpion fluorescence is known to persist long after death, and has been reported from specimens preserved in ethanol for over 100 years (Stahnke, 1972). Therefore, no meaningful loss of fluorescence was expected among the specimens surveyed. In addition, multiple specimens with a range of accession dates were assessed per taxon. Loss of cuticular fluorescence through solution in ethanol is therefore unlikely to have impacted the results of this study.

Five specimens were selected to represent taxa that might exhibit fluorescence under UV light: two scorpions, a buthid *Centruroides insulanus* (Thorell, 1876) and a scorpionid, *Heterometrus petersii* (Thorell, 1876); a horseshoe crab (Xiphosura), *Limulus polyphemus* (Linnaeus, 1758); a galeodid camel spider (Solifugae), *Galeodes turkestanus* (Kraepelin, 1899); and an unidentified gonyleptid harvestman (Opiliones). Two specimens were selected to represent taxa that did not exhibit UV fluorescence: a thelyphonid whip scorpion (Thelyphonida), *Typopeltis* sp.; and a phalangiid harvestman, *Leiobunum* sp. All specimens were photographed using a Microptics digital photomicrography system with Infinity optics, and a Nikon D300 DSLR camera with a Micro Nikkor 60 mm lens. Photography was performed in a dark room where ambient light was kept to a minimum. Specimens were lit solely by directed full-spectrum (white) light or 395 nm UV light (filtered) using a fiber optics system. Fossilized cuticle of two sea scorpions, *Eurypterus tetragonophthalmus* Fischer de Waldheim, 1839, from Estonia (Tuuling & Flodén, 2013), and an undescribed species from the Ordovician Big Hill Formation in Michigan, USA (Lamsdell *et al.*, 2017), was photographed to permit comparison between extant and extinct chelicerate taxa. Degree of fluorescence per unit area was quantified using ImageJ (Burgess *et al.*, 2010).

Parts of the prosomal carapace were subsequently removed from each specimen, gold-coated and examined using a Hitachi S4700 Field Emission SEM. Additional samples were embedded in LR White acrylic resin and thin sectioned using a Sorvall MT-2 Porter-Blum ultra-microtome. Exceptionally preserved sea scorpion cuticle from the Big Hill Formation (Lamsdell *et al.*, 2017) was sectioned with a glass knife at a thickness of 3–4 µm. Thin sections were stained using Mallory's Triple Stain technique (acid fuchsin, orange G, and aniline blue) replacing the phosphomolybdic acid with phosphotungstic acid. Ethanol was excluded from the process to avoid detachment of samples from the coverslips. This staining technique allows the hyaline layer to be easily identified as a clear, unstained region of the cuticle. Thin sections were examined using a Leica DM 2500 microscope and photographed using a Leica DFC 500 camera.

There is some confusion in the literature regarding the terminology of the layers of chelicerate cuticle. The terminology of Dalingwater (1987) is applied in the present contribution.

Results

Integumentary UV fluorescence was ubiquitous among representatives of the living orders of Chelicerata surveyed, whereas cuticular UV fluorescence was observed only in horseshoe crabs and scorpions (Table 1; Fig. 1). Although the camel spider (Fig. 1d) and the gonyleptid harvestman (Fig. 1e) both appeared brighter than other non-fluorescing taxa under UV light, their fluorescence per unit area is in the 100–200 range rather than the 1000 range observed in the scorpions and horseshoe crab (Table 2), indicating that the camel spider and gonyleptid fluorescence is due to excitement of hemolymph fluorophores (supported by the gonyleptid and camel spider having thinner cuticle than the other sectioned chelicerates) and not the result of cuticular fluorescence as in the scorpions and horseshoe crab. The fossil sea scorpion (Fig. 1h,i) did not fluoresce on exposure to long-wave UV light.

The presence of a hyaline exocuticle as a clear-staining region above the dark-stained inner exocuticle was apparent in histological sections of the scorpions (Fig. 2a,b) and horseshoe crab (Fig. 2g). The outer epicuticle occurs above the hyaline exocuticle as a pale layer with a dark blue to black horizon at the cuticle surface. The hyaline exocuticle was most obvious in the buthid scorpion *Centruroides* (Fig. 2b) and the horseshoe crab *Limulus* (Fig. 2g). The whip scorpion possessed a stained inner exocuticle but no evidence of a hyaline exocuticle in histological section, with the epicuticle directly overlying the inner exocuticle (Fig. 2f).

The thinness of the cuticle in the camel spider and both harvestmen prevented determination of its microstructure in section, with a thin laminate endocuticle overlain by an apparently homogenous cuticle layer. In the camel spider, this upper layer was almost equal in thickness to the endocuticle (Fig. 2e), whereas the upper layer was much thinner than the endocuticle in the harvestmen (Fig. 2c, d). Staining suggests that these upper layers are distinct from the endocuticle, but their thinness in the harvestmen makes it impossible to discern whether the upper layers include a hyaline layer or comprise a single layer of cuticle (i.e., only the epicuticle). The brown staining of the layer in the camel spider suggests it may be homologous to the inner exocuticle. The fossilized cuticle of the sea scorpion did not stain, as expected for a specimen having undergone diagenesis, and there was no evidence of the preservation of an endocuticle (Fig. 2h).

Scanning electron microscopy confirmed the presence of the hyaline exocuticle in the scorpions (Fig. 2a,b) and the horseshoe crab (Fig. 2g). No hyaline layer was evident in the SEM of the whip scorpion cuticle (Fig. 2f). The thin upper cuticle layer, visible in histological section of the camel spider and harvestmen, was evident as a single layer with no laminations under SEM (Fig. 2c–e), confirming its identity as the epicuticle. The fossil eurypterid cuticle, however, revealed a faint margin of separation under SEM (Fig. 2h), suggesting that the preserved cuticle comprises both the hyaline exocuticle and the inner exocuticle, both of which both lack large-scale laminations.

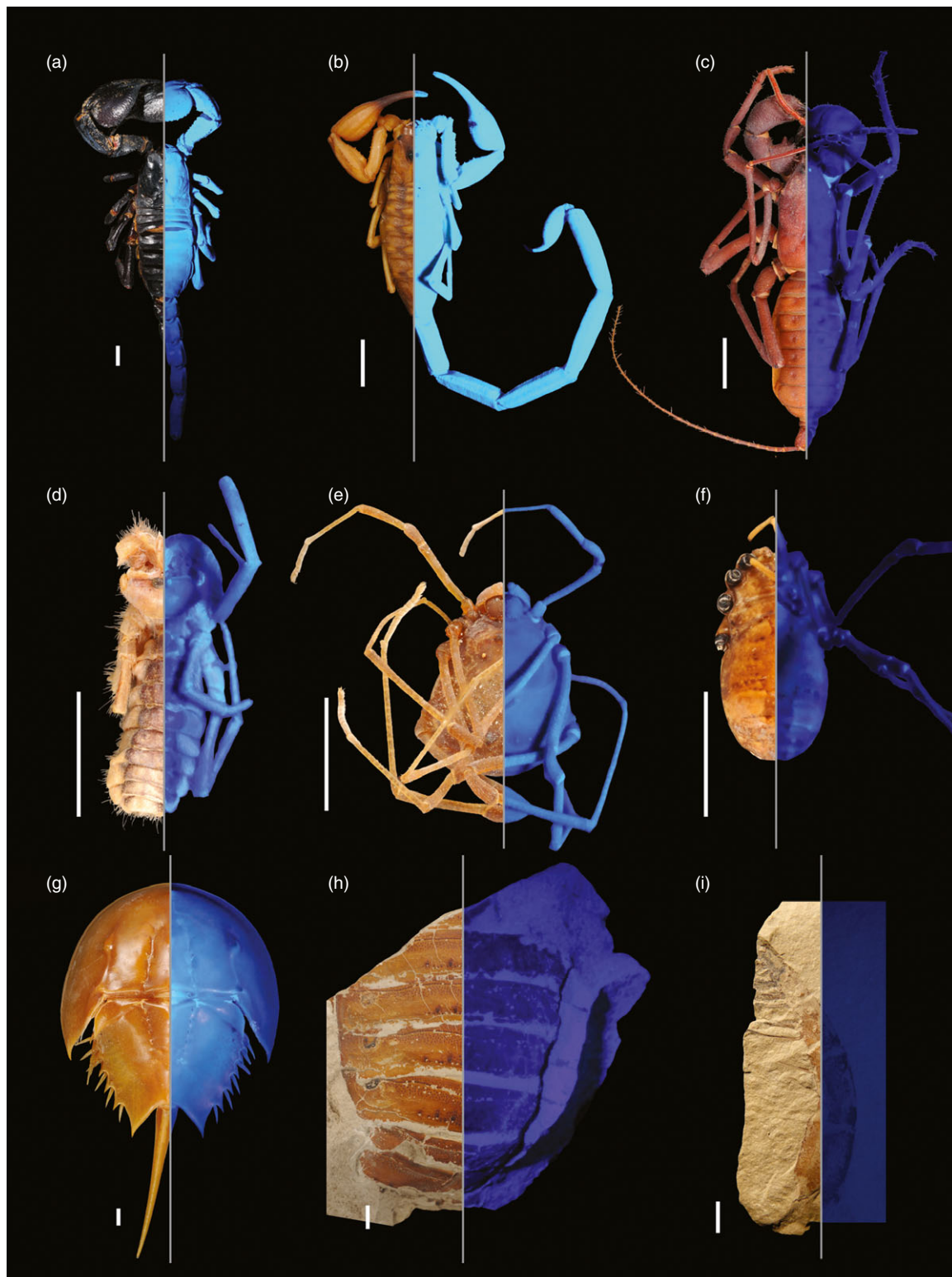


Figure 1 Fluorescence in extant and extinct chelicerates. (a) Scorpionid scorpion, *Heterometrus petersii* (Thorell, 1876). (b) Buthid scorpion, *Centruroides insulanus* (Thorell, 1876). (c) Thelyphonid whip scorpion, *Typopeltis* sp. (d) Galeodid camel spider, *Galeodes turkestanus* Kraepelin, 1899;. (e) Undetermined gonyleptid harvestman. (f) Phalangiid harvestman, *Leiobunum* sp. (g) Horseshoe crab, *Limulus polyphemus* (Linnaeus, 1758). (h) Sea scorpion, *Eurypterus* sp. (i) Undescribed sea scorpion. Scale bars = 10 mm.

Table 2 Fluorescence of the specimens shown in Fig. 1 under ultraviolet light. Values shown are fluorescence per unit area; those in boldface denote specimens that exhibit fluorescence

Eurypterida	<i>Eurypterus</i>	46
	Undet. eurypterid	49
Opiliones	Undet. gonyleptid	221
	<i>Leiobunum</i>	34
Scorpiones	<i>Heterometrus</i>	1023
	<i>Centruroides</i>	1070
Solifugae	<i>Galeodes</i>	101
Thelyphonida	<i>Typopeltis</i>	50
Xiphosura	<i>Limulus</i>	1072

Discussion

Although previously suggested that chelicerates besides scorpions, such as camel spiders, fluoresce when exposed to long-wave UV light (Lawrence, 1954), this is to our knowledge the first time cuticular UV fluorescence has been investigated experimentally across all extant chelicerates. Aside from scorpions, previous reports of UV fluorescence among chelicerates are shown here to be the result of fluorescence of the hemo-coel through thin cuticle. For the first time, horseshoe crabs (*Xiphosura*) are shown to fluoresce under UV light, due to compounds within the sclerotized cuticle, as in scorpions. The hyaline layer has been traced as the origin of cuticular fluorescence in arachnids in previous histological studies (Frost *et al.*, 2001; Wankhede, 2004) and these results are supported here. Taxa that do not fluoresce lack the hyaline layer altogether. Furthermore, it was previously noted that the earliest (non-fluorescing) instars of scorpions lack the exocuticle (Kennaugh, 1959). The SEM and histological examination of chelicerate cuticle corroborate previous studies concerning the cuticle of scorpions, harvestmen, whip scorpions, camel spiders, and horseshoe crabs. Krishnakumaran (1962) demonstrated that whip scorpions possess a sclerotized cuticle comprising an epicuticle, an exocuticle without a hyaline layer, and an endocuticle, whereas camel spiders apparently lack an epicuticle and possess only an exocuticle (again without a hyaline layer) and an endocuticle. Both observations are supported by the data presented here. Grainge & Pearson (1966) studied the cuticle of phalangiid harvestmen, including *Leiobunum*, and reported that the sclerite cuticle was thin (14 µm) and comprised a thin epicuticle and a thicker laminate endocuticle, as shown here. Scorpions, meanwhile, are well known to possess the epicuticle, hyaline exocuticle, inner exocuticle, and endocuticle (Mutvei, 1977; Filshie & Hadley, 1979; Hadley & Filshie, 1979), as confirmed here. Horseshoe crabs are also known to possess an epicuticle, exocuticle, and endocuticle (Krishnakumaran, 1962; Dalingwater, 1975). Two layers had previously been recognized within the exocuticle of horseshoe crabs, with the upper layer compared to the hyaline layer (Mutvei, 1977), an observation also confirmed here. Dalingwater (1980) noted that the endocuticle may consist of vertical rather than horizontal laminations around the carapace margins and telson, again confirmed here.

Among the chelicerate groups that were not sectioned for this study, ticks possess an epicuticle, exocuticle, and

endocuticle, but no hyaline layer is present within the exocuticle (Hackman, 1982). This is also true of the prosomal cuticle of spiders (Hadley, 1981). Ricinuleids exhibit a very different cuticular structure, however, apparently lacking an endocuticle, the cuticle instead being composed solely of epicuticle and exocuticle (Kennaugh, 1968). The presence of a hyaline layer within ricinuleids, although tentatively suggested (Kennaugh, 1968), could not be confirmed in the present study. As such, it appears that the hyaline exocuticle layer is directly responsible for cuticular UV fluorescence in chelicerates and that, among the extant chelicerate orders, only scorpions and horseshoe crabs possess a hyaline layer. It is therefore of interest that a hyaline layer was identified (Fig. 2h) in a sea scorpion (Eurypterida), suggesting these extinct chelicerates may have fluoresced if exposed to long-wave UV light. Previous studies of eurypterid cuticle identified the laminate endocuticle and inner exocuticle (Dalingwater, 1973, 1975) but preserved no epicuticle or hyaline exocuticle. The hyaline and inner exocuticles were observed in fossil scorpions (Bartram *et al.*, 1987), however. The fossilized cuticle studied here preserves no epicuticle or endocuticle and so appears to exhibit similar preservation to the Carboniferous scorpion cuticle that also lacks the endocuticle and the epicuticle (Bartram *et al.*, 1987). The lack of cuticular UV fluorescence in the fossil material (Fig. 1h,i) is therefore likely due to diagenetic changes caused by the fossilization process and the degradation of the fluorescent compounds.

The presence of the hyaline layer within horseshoe crabs and sea scorpions suggests further that cuticular UV fluorescence evolved in the common ancestor of chelicerates or possibly even earlier. Chelicerate phylogeny is controversial (Jones *et al.*, 2007; Shultz, 2007; Dunlop, 2010; Pepato *et al.*, 2010; Arabi *et al.*, 2012; Legg *et al.*, 2013; Garwood & Dunlop, 2014; Sharma *et al.*, 2014; Lamsdell *et al.*, 2015b; Selden *et al.*, 2015; Lamsdell, 2016). Although there is broad agreement that Eurypterida are the sister group to Arachnida and that Xiphosura are the sister group to euchelicerates, the position of scorpions is debatable, with analyses retrieving them either as the sister group of all other arachnids (Jones *et al.*, 2007; Legg *et al.*, 2013; Garwood & Dunlop, 2014; Lamsdell *et al.*, 2015b; Selden *et al.*, 2015; Lamsdell, 2016), as part of a Dromopoda clade, comprising camel spiders, harvestmen and pseudoscorpions, nested within Arachnida (Pepato *et al.*, 2010; Sharma *et al.*, 2014), or in an unresolved position (Shultz, 2007; Dunlop, 2010; Arabi *et al.*, 2012). If scorpions are part of Dromopoda, the hyaline exocuticle layer would have evolved independently in basal euchelicerates and scorpions. However, if scorpions are the sister group of all other arachnids, cuticular fluorescence would be plesiomorphic. In the latter scenario, the hyaline layer (and its associated fluorescence under long-wave UV light) would have no adaptive significance for scorpions (Gould & Lewontin, 1979); rather it would be a relict trait (Frost *et al.*, 2001) or serve a function common to scorpions, horseshoe crabs and eurypterids.

Although it is not currently possible to speculate about the function of the hyaline exocuticle and its associated fluorescent compounds, it is possible to discount some previous hypotheses based in its occurrence in aquatic taxa that trace their

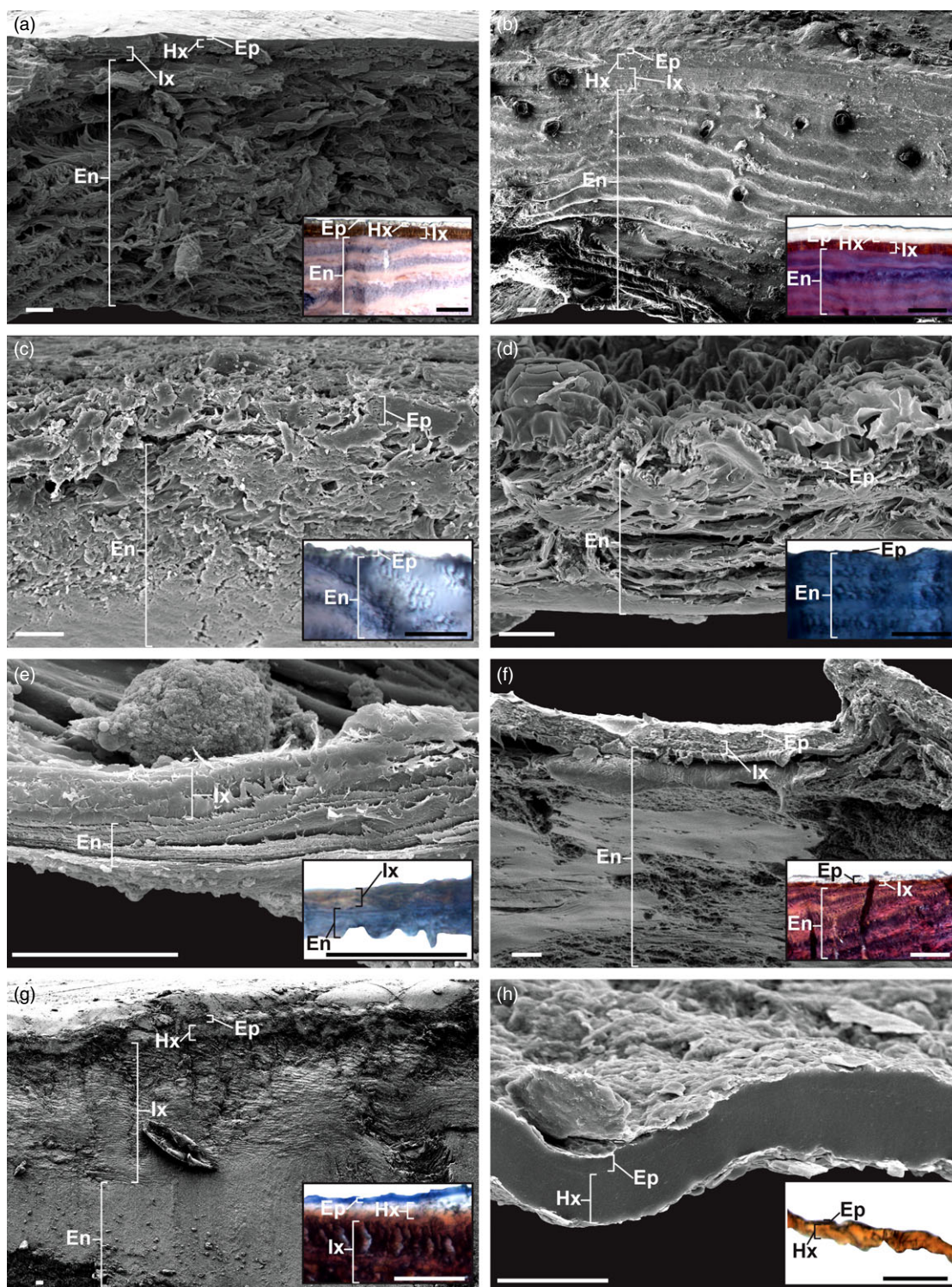


Figure 2 Cuticular structure of chelicerates. Main image shows cuticle under SEM, insets show histological section of cuticle stained with Mallory's Triple Stain. (a) Scorpionid scorpion, *Heterometrus petersii* (Thorell, 1876). (b) Buthid scorpion, *Centruroides insulanus* (Thorell, 1876). (c) Thelyphonid whip scorpion, *Typopeltis* sp. (d) Galeodid camel spider, *Galeodes turkestanus* Kraepelin, 1899;. (e) Undetermined gonyleptid harvestman. (f) Phalangiid harvestman, *Leiobunum* sp. (g) Horseshoe crab, *Limulus polyphemus* (Linnaeus, 1758). (h) Undescribed sea scorpion. Abbreviations: En, endocuticle; Ep, epicuticle; Hx, hyaline exocuticle; Lx, inner exocuticle. Scale bars on scanning electron micrographs = 10 µm, scale bars on histological section insets = 30 µm.

origins to the Ordovician (Rudkin, Young & Nowlan, 2008; Van Roy *et al.*, 2010; Lamsdell *et al.*, 2015a,b). Suggestions that cuticular fluorescence evolved as a prey lure to attract insects can be discounted as insects had not yet evolved when the hyaline layer was first expressed in chelicerates, and insects have been shown to actually avoid fluorescing scorpions (Kloock, 2005). Similarly, the expression of the hyaline exocuticle in aquatic taxa would suggest that it did not develop as a protection against UV light during terrestrialization (Lourenço & Cloudsley-Thompson, 1996). Its occurrence among taxa with a wide range of reproductive modes makes the use of UV fluorescence in mate determination unlikely (Fasel *et al.*, 1997). The occurrence of a hyaline layer among eurypterids, a morphologically diverse group (Lamsdell & Selden, 2017), also makes it unlikely that difference in fluorescent wavelength acts as a species-specific identifier (Kloock, 2008). One hypothesis that has received a degree of experimental support and is not impacted by the occurrence of the hyaline layer in aquatic taxa is the role of the hyaline layer in detection of UV light (Blass & Gaffin, 2008; Kloock, Kubli & Reynolds, 2010; Gaffin *et al.*, 2012). Horseshoe crab reproduction is linked to the tidal/lunar cycle (Brockmann, 1990), and UV light detection could conceivably play a role in gauging the timing of these events.

The current research confirms the correlation between the occurrence of the hyaline exocuticle and UV fluorescence, and extends the recorded occurrence of the hyaline layer beyond scorpions to horseshoe crabs and eurypterids. Future investigations should assess the chemical composition of the hyaline exocuticle across the diversity of scorpions and horseshoe crabs. The source of the fluorescent compounds within the cuticle, specifically whether they are formed within the hyaline exocuticle itself or transported and stored within the hyaline layer from the hemolymph, should also be investigated. Further study of chelicerate cuticle through histology and SEM are also warranted, and may provide context as to whether the hyaline exocuticle plays a structural function within the sclerotized cuticle.

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