Beyond venomous fangs: Uloboridae spiders have lost their venom apparatus but not their toxins

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Abstract

Venom is one of the most potent chemical secretions in nature which has played a significant role in the evolutionary success of many animal groups, including spiders. However, the family Uloboridae has seemingly lost the venom-producing organs, leaving the presence and function of toxins in question. Uloborids employ a distinct hunting method involving extensive silk-wrapping and regurgitation of digestive fluids onto the whole prey, yet the precise mechanism by which prey is immobilized remains unknown. One hypothesis is that toxins may have shifted from the venom to other secretions that come into contact with prey, such as silk or digestive fluids. Here, we use a combination of histology and multi-tissue transcriptomics, to 1) investigate the absence of venom glands, and 2) examine the expression of venom toxins in different body parts of *Uluborus plumipes*. Our findings indicate that *U. plumipes* effectively does not possess venom glands, nor the duct opening in the fangs. However, we identified putative neurotoxins that are highly expressed in the digestive gland, suggesting that these may contribute to prey immobilization. Among the most highly expressed were U3-aranetoxins, U24-ctenitoxins, and a defensin, although the latter was not predicted to have neurotoxic activity. These results support our hypothesis that Uloboridae spiders have lost their venom apparatus, but not their toxins. These "digestive toxins" might be responsible for complete prey immobilisation and represent an alternative hunting strategy that replaced the loss of a frontal venom system.

Introduction

The arms race between predators and their prey is a fundamental driving force of animal evolution. Accordingly, animals have developed various mechanisms to succeed in this ongoing battle for survival and to gain advantages over their opponents. One of nature's most effective weapons is venom - a potent blend of proteins and peptides that can incapacitate prey or serve as a means of self-defence. Approximately 15% of extant animal species are venomous (Modica et al. 2021); this success can be attributed to the unique ability of venom to shift the battleground from the physical to the chemical level, enabling smaller and slower creatures to prevail over larger and faster ones. Moreover, venom has been linked to adaptive radiations in several animal clades, such as cone snails (Puillandre et al. 2017) and spiders (Vassilevski et al. 2009; King and Hardy 2013; Lüddecke et al. 2021), with diversification rates twice as high in venomous compared to non-venomous animal families (Arbuckle and Harris 2021).

Given these advantages, it may seem counterintuitive for an animal to relinquish a powerful weapon such as venom. However, venom production and expenditure are linked to significant metabolic costs due to its high protein content. Consequently, venom systems compete with other physiological processes for resources (Nisani and Hayes 2011; Nelsen et al. 2014; Cooper et al. 2017; Lüddecke et al. 2021). In ancient lineages, whose venoms are governed by negative selection (Sunagar and Moran 2015), this economic dilemma may cause the loss of venom when its use becomes nonessential for survival. Certain ecological and life history traits may relax selective pressures related to the maintenance of the specialised glands dedicated to venom production, leading to their eventual loss (Morgenstern and King 2013). This phenomenon has been observed in some species of catfish, where increased body sizes effectively protect them from predators, as well as in sea snakes feeding on eggs (Li et al. 2005), rendering their venom unnecessary (Wright 2017). One case of secondary loss of venom glands has also been reported in spiders, although with limited evidence. According to a hand-drawn illustration from 1931 (Millot 1931), members of the Uloboridae family do not carry a functional venom system. Instead, they possess only muscle bundles in the frontal part of their prosoma, where venom glands of araneomorph spiders are typically located. Similarly to sea snakes, which have shifted their trophic behaviour, uloborid spiders may have evolved alternative strategies for hunting prey, making venom glands redundant. Unlike other spiders, uloborids wrap their prey excessively in silk (up to hundreds of metres/prey) (Lubin 1986; Opell 1988; Eberhard et al. 2006a; Eberhard et al. 2006b). However, the precise mechanism by which the prey is killed is not well understood (Weng et al. 2006). Neurotoxins have been found enclosed in viscous droplets and deposited on the silk strands of foraging webs of *Nephila clavipes* to intoxicate insect prey upon contact (Marques et al. 2005; Esteves et al. 2020). It is plausible that uloborids may have shifted the secretion of toxins from venom to other secretions that come into contact with prey, such as silk. If we assume that uloborids do not longer have venom-secreting glands, it remains unclear whether they have completely lost their venom components too.

Here, we used a combination of histology and multi-tissue transcriptomics to confirm the absence of specialised venom-secreting glands in the prosoma of *Uloborus plumipes*, and to investigate whether this spider species might produce toxins elsewhere to incapacitate prey. Specifically, we tested the hypothesis that uloborids may have shifted the secretion of

toxins from venom to the silk, making venom redundant and leading to the loss of venom-secreting glands.

Our findings indicate that uloborids have indeed lost their venom glands, as confirmed by our histological examination that only revealed muscle bundles in the frontal part of the prosoma. However, our transcriptomic analysis revealed the presence of several transcripts encoding venom toxin homologs across different body tissues, particularly in the midgut gland. This discovery suggests an adaptive shift in the production of lethal toxins from the venom glands to the digestive system, with the consequent secondary loss of the frontal venom apparatus. However, the secretion of toxins in the digestive fluid of other spider species hints a deeper evolutionary link between venom and digestive system, and a conserved functional role, either in spider physiology or as a hunting strategy for effective prey immobilization in uloborids.

Results

Histology

Based on visual examination of dissected individuals and histological semi-thin sections across the chelicerae and anterior part of the prosoma, we confirmed the absence of venom glands in *U. plumipes*. As shown in Fig. 1, the chelicerae have a prominent epithelium and are equipped with large muscles, but lack any kind of duct known for species with venom glands (see for comparison Fig. 2 of (Schmidtberg et al. 2021).

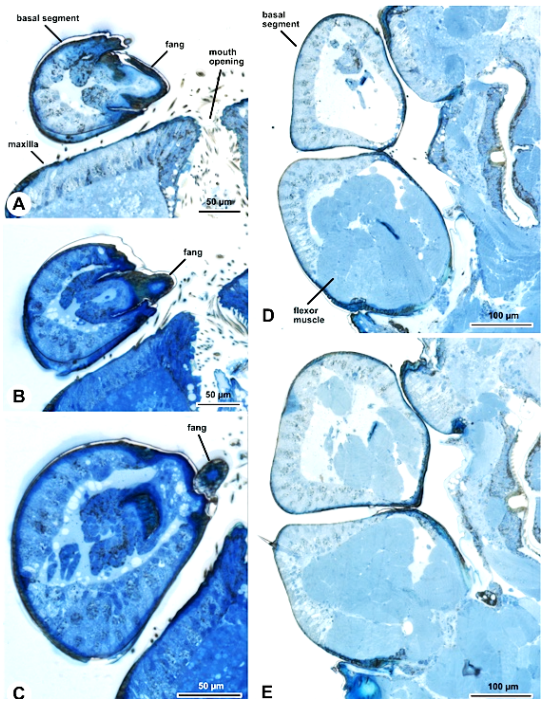

Figure 1: Transversal sections of the chelicerae of *Uloborus plumipes* in different planes (A-C, distal region of left chelicera; D-E, proximal region of both chelicerae). A, B: intersection of the basal segment and fang. C. Section through the area just above the fang base. Note that the fang does not contain any kind of canal. D, E: Section through the basal segments with the left chelicerae close to the transition to the prosoma. Note the very large flexor muscle in the basal segment.

Scanning electron microscopy

As we did not find venom glands, we sought to investigate the presence of venom duct openings in the fangs. Electron microscopy revealed the presence of three small pores at the tip of the fangs (Fig. 2A); however, due to their size, it is unlikely that any of them is connected to a venom duct which is generally much larger (Fig. 2B). Similar small cuticular pits have been observed at the fang's tip of the orb-web spider *Nephila clavata*, although their function remains unknown (Moon and Yu 2007).

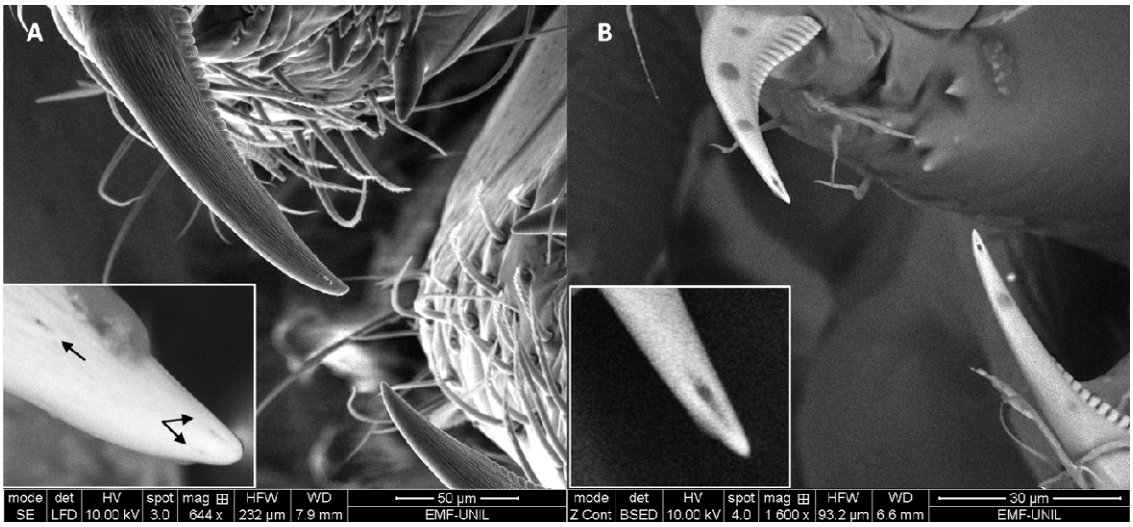

Figure 2: **Fangs of *Uloborus plumipes* and *Parasteatoda tepidariorum*.** *U. plumipes* (A) possesses small pits at the tip of the fangs (black arrows in the zoomed insert). As a comparison, a juvenile of *P. tepidariorum* (B) possesses a needle-like opening of the venom duct at the tip of the fangs.

Transcriptomics

Although our morphological analysis confirmed the absence of a specialized venom producing and delivery system in the anterior part of the spider body, it is important to note that these spiders are tiny, and it is possible that small venom glands could have been missed by histological observations. Furthermore, uloborid spiders might have shifted the production of venom components in other organs, even if specialized venom-secreting glands have been lost. To investigate this further, we performed RNA sequencing of 13 libraries from different tissues including chelicerae, prosoma, midgut gland, silk glands and gonads. The libraries ranged in size from 53 to 107 million reads (Supplementary Table S1).

The resulting *de novo* transcriptome contained 302'504 assembled transcripts, of which 20'243 (6.7%) passed our annotation pipeline (Table S2, Dataset 1). Our final assembly was 83.5% complete according to OMArk (Nevers et al. 2022), and contained 2'997 genes from the Arthropod Hierarchical Orthologous Groups, with 2'335 single genes and 662 duplicated genes (Fig. S1). OrthoVenn3 (Sun et al. 2023) identified 18'258 transcripts (90% of the total) as homologous to the closely related *Uloborus diversus* genome (GCF_026930045), and these were then clustered into 10'518 orthogroups. Among these orthogroups, 9'817 were shared with *U. diversus*.

The distribution of transcript counts was similar among libraries from the same tissue, except the midgut sample "UM1", which had a smaller median value than the others (Fig S2, S3). Principal component analysis showed that samples clustered by tissue type, with the first two components explaining 60% of the total variance (Fig S4).

Classification of toxin-like transcripts

Our manual screening of the final assembly for venom components revealed that 1'368 translated sequences had at least one hit to the UniProt-ToxProt database (Jungo et al. 2012). We then investigated their tissue-specific expression patterns. Specifically, we sought to determine whether putative toxins were expressed in the anterior part of the spider body, where venom glands are typically found, or if they were expressed in other organs, suggesting either a potential shift in the production of venom, or another role than toxins.

Differential expression analysis revealed 256 putative toxins with biased expression across tissues (Dataset 2), predominantly upregulated in the midgut gland samples (Fig. S5). We divided the differentially expressed putative toxins into two groups: group 1, consisting of 142 sequences with the highest Blast bit score to a toxin, hence more likely belonging to venom protein families; group 2, consisting of 114 sequences with a Blast hit to a venom protein but not the one with highest bit score, therefore less likely to be true toxins. In group 1, 107 transcripts (75%) were upregulated in the midgut gland, while 15 were upregulated in the silk glands (10%). Similarly, in group 2, most toxin-like transcripts were expressed in the midgut gland (42%) and 9% in the silk glands.

The putative toxins were classified into their corresponding protein families based on their Blast hits and protein domain identified by InterProScan (Quevillon et al. 2005). In group 1, the most common family was astacin-like metalloprotease with 41 sequences (28%), followed by cysteine-rich peptides including atracotoxins, prokineticins, kunitz inhibitors and thyroglobulin type-1, with a total of 40 sequences (28%) (Fig. 3). Serine protease transcripts accounted for 14%, followed by acetylcholinesterases (10%). In group 2, the most dominant transcripts were related to serine proteases (21%), followed by metalloproteases (11%), lipases (8.8%) and acetylcholinesterases (6%) (Fig. S6).

Although we identified multiple sequences that had Blast hits with protein families commonly found in animal venoms, it is uncertain if these sequences correspond to proteins with toxic effects. Differentiating between toxins and their non-toxic paralogs can be challenging because many venom molecules rely on the same mechanisms as physiological proteins, such as digestive enzymes involved in proteolysis or matrix dissolution. Additionally, venom contains enzymes that act as spreading factors, such as phospholipases, which facilitate the movement of toxins in prey (Langenegger et al. 2019). Nevertheless, ultimately, it is neuropeptides that typically paralyze and kill spiders' prey. If uloborids have retained some toxins despite losing their venom system, it is likely that these molecules play a fundamental role in immobilizing prey. As a result, we specifically searched for peptides that might have neurotoxic activity. Nonetheless, it is important to note that some peptides which are minimally toxic on their own, become up to 65% toxic in the presence of other venom components (Lüddecke et al. 2021).

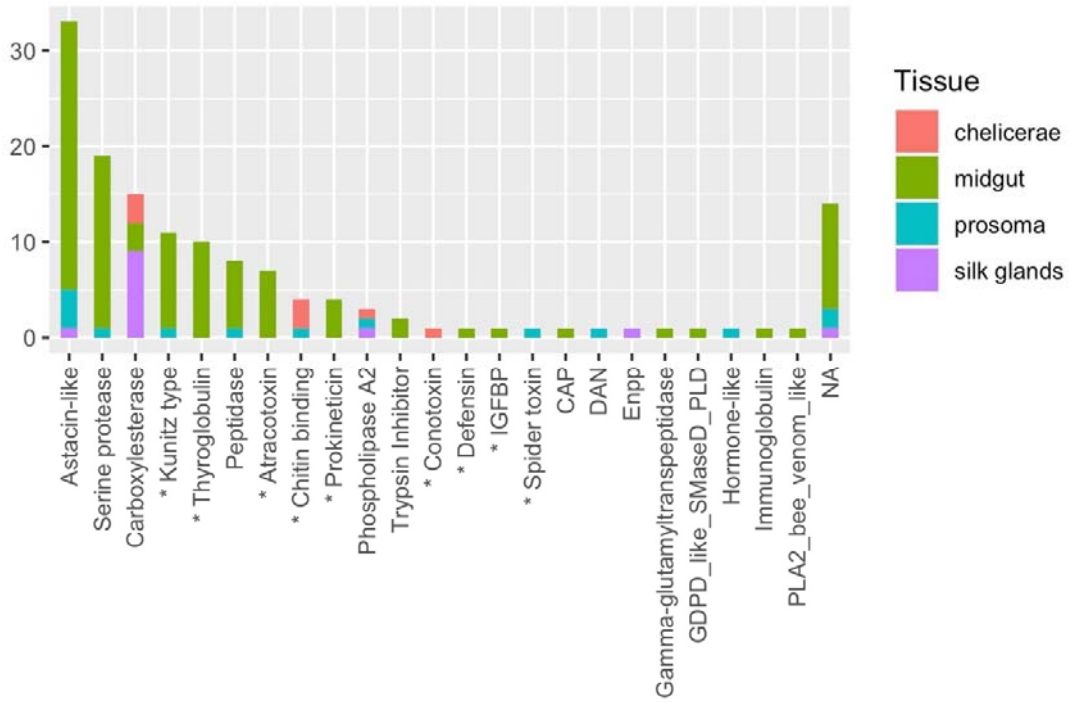

Figure 3: Classification and tissue distribution of putative toxic transcripts (group 1). The y axis corresponds to the number of sequences assigned to a protein family, and the colour corresponds to the body tissue where they are differentially expressed. 'NA' includes sequences without InterProScan annotation but with a positive Blast hit.

Few putative toxins in prosoma and chelicerae

High expression of toxin genes in the anterior part of the spider body would support the presence of venom glands that could have been missed by histology. However, we found only a few toxins-like in the prosoma and chelicerae, and none of them were the expected 'classic' spider toxins.

In the chelicerae, we observed three acetylcholinesterases (Carboxylesterase family), one phospholipase A2 (PLA2), and three scoloptoxins which were significantly upregulated.
Acetylcholinesterase is an enzyme primarily found at postsynaptic neuromuscular junctions, where it breaks down the neurotransmitter acetylcholine. This action terminates the signal transmission and allows for the nerve cell to reset and prepare for the next signal. Acetylcholinesterase has been reported in the venom of *Trittame loki* at quite high expression levels, although its role is unknown (Undheim et al. 2013). As chelicerae and prosoma are full of muscle fibres (Fig. 1), we could expect an abundance of these enzymes even in the absence of venom.
The PLA2 that we identified was predicted to have neurotoxic activity. However, the homolog with the highest identity (65%) was a peroxisomal membrane protein PMP34 like from *Argiope bruennichi* (KAF8793880.1), therefore it is more likely that this PLA2 is a moonlighting peroxisomal protein with phospholipase activity.
Similarly, the scoloptoxins showed homology to chitin-binding domain containing proteins (90% identity), in agreement with the domain prediction of a chitin-binding domain, therefore it is unlikely that they are toxins.

In the prosoma, we identified significant upregulation of one cysteine-rich peptide, two scoloptoxins, one PLA2, five metalloproteinases, one serine proteinases, and three hormones.
The cysteine-rich peptide (NODE_38334) showed high expression levels and homology to U8-agatoxin-Ao1a (UniProt: Q5Y4U4). It was the only sequence predicted to have neurotoxic properties and featured a knottin domain. However, upon alignment, we observed a disruption in the classic cysteine framework (C-CXC-CC-CXC-CXC-C), with the absence of the second and tenth cysteine residues (Fig. 4). This could indicate a shift in function from U8-agatoxin-Ao1a.
Similar to the scoloptoxins found in the chelicerae, the upregulated scoloptoxins in the prosoma possessed chitin-binding domains, suggesting it is unlikely that they are toxins.
The other upregulated transcripts in the prosoma belong to abundant and common protein classes that perform various physiological functions in the organism. It is plausible that these are regular body proteins. However, their specific roles in spider venom remain poorly studied, making it challenging to assess their function solely through computational analysis. Considering that the prosoma contains multiple organs, such as excretory coxal glands, brain, and stomach, it is highly likely that the identified toxin-like sequences are physiological body proteins. However, the high and selective expression of U8-agatoxin in the prosoma is intriguing and it raises questions about its specific organ of expression.

Few putative toxins in silk glands

In the silk glands, we identified nine acetylcholinesterases, one metalloproteinase, one PLA2, one phosphodiesterase, one phospholipase D (PLD), and one insulin-like growth factor binding protein (IGFBP). None of these were predicted to be neurotoxic peptides by NT_estimation.
The abundance of acetylcholinesterases is interesting. Exploratory Blast searches against various SRA libraries revealed differential expression of some acetylcholinesterase-coding genes in silk glands of *P. tepidariorum* and *U. diversus*, suggesting a common expression of these proteins in spider silk glands.
PLD, also known as Sphingomyelinase D, is a potent dermonecrotic toxin predominantly synthetized in the venom of Sicariidae spiders (e.g., *Loxosceles* and *Sicarius*). However, its low expression level in the silk glands suggests that it is unlikely to be a crucial toxic component in the silk.
In summary, our findings indicate the absence of neurotoxins as well as other potential venom components secreted in the silk, with the exception of acetylcholinesterases, which is intriguing and warrants further investigation.

Several putative toxins in the midgut gland

The midgut gland exhibited the highest number of significantly upregulated toxin-like transcripts. While no peptides with a knottin domain, typical of spider venom, were found, a total of 26 sequences, primarily aranetoxins and ctenitoxins, were predicted to have neurotoxic activity by the NT-estimation model. The most diverse family was the astacin-like metalloprotease, with 35 significantly upregulated transcripts. However, despite their abundance, they were not the most highly expressed. A substantial number of astacins in the digestive fluids of *N. cruentata* (Fuzita et al. 2016), *Stegodyphus mimosarum* and *Acanthoscurria geniculata* (Walter et al. 2017) suggests their important role in breaking

down proteinaceous prey in spiders. Phylogenetic analysis revealed that Araneae spiders had the highest number of duplications within this protein family compared to other groups such as scorpions and ticks (Fuzita et al. 2016). While the presence of metalloproteases in the midgut fluids of araneomorph and mygalomorph spiders suggests a conserved role in digestion, it does not exclude the possibility of their involvement as spreading factors for toxins.

The second most abundant group consisted of cysteine-rich peptides, with a total of 31 transcripts, 21 of which had a framework of 10 cysteine residues (C-C-CC-C-C-CXC-C-C).
Eleven transcripts were identified as U3-aranetoxins (Uniprot: Q8MTX1.1) with either a prokineticin domain, MIT-like atracotoxin, or no predicted domain. NODE_12247, with a prokineticin domain, stood out as the second most highly expressed transcript in the entire midgut transcriptome with transcripts per million (TPM) > 33'000. It is orthologous to XP_054706256.1 in *U. diversus* (86% identity), which is annotated as U1-hexatoxin-lw1e-like with a MIT-like atracotoxin family structure, but it was not predicted to be a neurotoxin.
Out of the nine U3-aranetoxins predicted as neuropeptides by the NT_estimation model, three had expression levels > 2'000 TPM. NODE_77354 is homologous to U8-theraphotoxin-Hhn1c-like (XP_054706263.1) of *U. diversus*, but no domain was identified by InterProScan. However, a Blast search against the NCBI transcriptome database TSA revealed a broad distribution of its homologs across the spider phylogeny. The other two highly expressed U3-araneotoxins included NODE_51429 with a MIT-atracotoxin-like domain, and NODE_47918 with a prokineticin domain.
Other abundant cysteine-rich peptides were identified as U24-ctenitoxins (Uniprot: P84032) with ten transcripts possessing a thyroglobulin domain, six of which were predicted to be neurotoxins. NODE_57518 was the third most highly expressed putative toxin in the midgut gland.
Additional predicted neurotoxic peptides included U9-ctenitoxins with a prokineticin domain, U19-ctenitoxins with no predicted domain, and U1-hexatoxin with a MIT-like atracotoxin domain.

Apart from the 'classic' spider venom peptides, we discovered a highly expressed defensin (NODE_62030) ranking as the second most abundant putative toxin with TPM values > 6'000. This defensin showed homology to the scorpion BmKDfsin3. Although we did not find annotated defensins in the *U. diversus* genome, our sequence aligned to a specific position on chromosome 4, revealing the presence of an unannotated gene (Fig. 4). Furthermore, we identified several homologous sequences in other spider transcriptomes in the TSA database.

Other intriguing highly expressed putative venom components included an Insulin-like growth factor-binding protein (IGFBP) predicted as neurotoxic, but lacking a signal peptide, and a PLD. Similarly to the isoform found in the silk gland, this PLD belongs to the beta type, rather than the necrotic alpha type. Recently, a PLD was discovered in the digestive fluids of a *Uloborus* spider (Valladão et al. 2023), and the authors suggested its potential digestive role, although its insecticidal and necrotic activities cannot be ruled out without functional tests.

Finally, several ferritin transcripts were observed, ranking among the most highly expressed in the entire transcriptome. Ferritin has been commonly reported as a component in cone snail venom gland transcriptomes (Hu et al. 2011; Abalde et al. 2018), and has also been detected in digestive fluids (Fuzita et al. 2016).

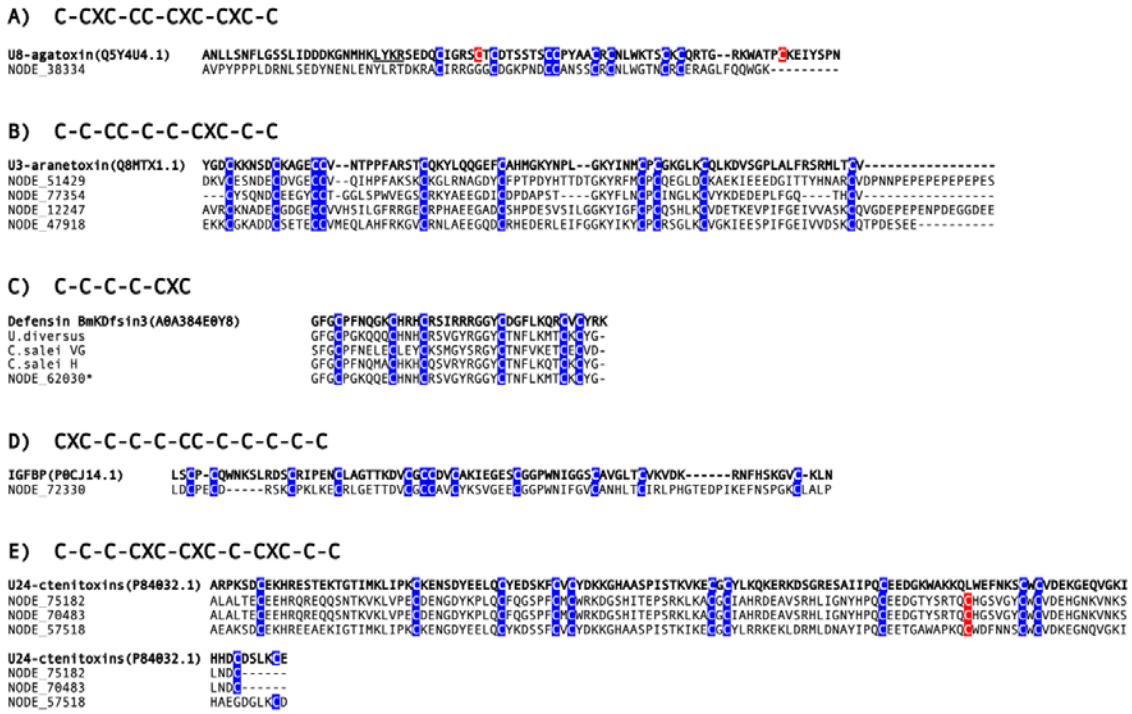

Figure 4: Alignment of putative neurotoxins. VG: contig expressed in venom glands; H: transcript expressed in haemocytes (Kuhn-Nentwig et al. 2019). *Predicted non-neurotoxic.

Discussion

Spiders are widely successful predators that rely on their foraging webs and potent venoms to capture and subdue prey. While many spider clades have lost their ability to use silk for hunting, virtually all spiders still depend on venom to subdue their prey – with one exception. Uloboridae spiders seem to lack venom glands accordingly to a 1931 hand-drawing (Millot 1931) depicting three uloborid species with muscle bundles in the area where typically venom glands are positioned. As a result, numerous studies (e.g., (Weng et al. 2006; Langenegger et al. 2019; Lüddecke et al. 2021) have suggested the secondary loss of venom in uloborids. This raises the question of how uloborids have adapted to effectively kill prey and ensure their survival. In light of this, we investigated whether these spiders have lost the entire venom system, including their associated venom toxins. We proposed the hypothesis that uloborids may have undergone a shift in toxin secretion from their venom glands to their silk glands, leading to the redundancy and eventual loss of the venom system.

Our histological analysis confirmed the absence of venom glands in the prosoma of *U. plumipes*. Notably, we found that, not only were the venom glands absent, but the opening of the venom duct in the tip of the fangs was also not visible, suggesting a complete loss of the venom apparatus. Nevertheless, we observed the presence of small pores on the fangs,

346 which seem commonly found in spiders (Moon and Yu 2007), but whose function remains
347 unknown.
348 In contrast to the histology results, transcriptomics analysis revealed the expression of
349 genes related to venom components in the prosoma, where venom glands are typically
350 found. However, these transcripts are not typical spider venom peptides, but more likely
351 physiological body proteins such as phospholipases, hormones and metalloproteinases.
352 With the exception of one ICK-predicted peptide with a disrupted cysteine framework, no
353 neurotoxin was detected. Overall, our molecular analysis supports the morphological
354 observation of a lack of venom glands in *U. plumipes*. Nonetheless, the expression of a few
355 genes related to venom protein families, in particular the ICK peptide, is intriguing and
356 warrants further investigation. Techniques such as whole-mount RNA *in situ* hybridization or
357 spatial transcriptomics could be used to localize the organ secreting the ICK-peptide, as well
358 as functional tests to verify its toxicity.
359
360 After confirming the absence of venom-producing glands in the prosoma, we investigated
361 the expression of potential toxins in other body parts. For instance, neurotoxins have been
362 found in the foraging webs of *N. clavipes* to intoxicate insect prey upon contact (Marques et
363 al. 2005; Esteves et al. 2020). These silk toxins are similar to neuropeptides found in spider
364 venoms (Esteves et al. 2020). Uloborids have an unusual way of feeding: they wrap prey
365 with excessive amounts of silk, a unique behaviour among spiders (Lubin 1986; Opell 1988;
366 Eberhard et al. 2006a; Eberhard et al. 2006b). Because of the extensive use of silk for prey
367 capture, we hypothesized that toxins might be secreted into the silk. However, our RNA-seq
368 investigation revealed the expression of only a few toxin-like genes in the silk, and none of
369 them were predicted to have neurotoxic properties. Intriguingly, we did find several
370 acetylcholinesterases upregulated in silk glands. Their role in silk production or silk gland
371 functioning warrants further investigation.
372
373 After wrapping their prey in silk, uloborids cover the entire surface of the prey with
374 digestive fluids, in contrast to other spiders which typically regurgitated the fluids only on
375 the area near their own mouthparts (Lubin 1986; Opell 1988; Eberhard et al. 2006a;
376 Eberhard et al. 2006b). This broad application of digestive fluids onto the prey appears to be
377 lethal. Weng et al (2006) found that most ants in silk packages exposed to digestive fluids of
378 the uloborid *Philoponella vicina* were dead (91%), suggesting the presence of molecules that
379 can kill the prey. Only 18% of unwetted ants and 27% of ants wetted with water died,
380 showing that they are able to survive the compression produced by wrapping silk lines.
381 Examination of wrapped prey revealed disarticulated segments of legs and a lack of
382 intersegmental membranes, suggesting the presence of enzymes that disrupt the
383 extracellular matrix and digest membranes, possibly facilitating the diffusion of toxins.
384 These observations brought us to examine the midgut gland, the organ producing the
385 putatively lethal digestive fluids. Here, we identified several typical spider venom cysteine-
386 rich peptides (e.g., ctenitoxins) which were strongly upregulated and predicted to have
387 neurotoxic activity. Among the most highly expressed and diverse were U3-aranetoxins of
388 the MIT-like atracotoxin family, and U24-ctenitoxins with thyroglobulin domain. The MIT-
389 like atracotoxin family includes homologs to atracotoxin-Hvf17 which lacks both insecticidal
390 activity, and effect on smooth muscle contractility (Szeto et al. 2000; Wen et al. 2005).
391 However, the role of this peptide family remains unknown (Wen et al. 2005). U24-ctenitoxin
392 from the venom of the black widow, *Latrodectus geometricus*, was recently shown to have

neurotoxic activity by slowing down the inactivation of insect sodium channels (Khamtorn et al. 2022).

Besides cysteine-rich peptides, we found a highly expressed small defensin peptide homologous to the scorpion BmKDfsin3. Defensins are small peptides of the immune system produced by a variety of organisms, including plants, insects, and vertebrates. Specifically, they can target and kill a wide range of microorganisms, including bacteria, fungi, and viruses, by disrupting their membranes or interfering with their cellular processes. In scorpions, defensins have innate antimicrobial properties, and they are found in the haemolymph, as well as in venom glands (Meng et al. 2020). They can block the potassium channels Kv1.1, Kv1.2, Kv1.3 and SK3 using the same mechanism as classic venom neurotoxins such as OSK1 and ScyTx (Meng et al. 2020). Phylogenetic analysis showed that BmKDfsin defensins and neurotoxins may derive from the same ancestral gene, and while neurotoxins target specifically either Kv1.x or SK channels, defensins can affect both types of channels. A defensin was detected specifically in the venom glands of *Cupiennius salei* (Kuhn-Nentwig et al. 2019), as well as in the midgut gland of *N. cruentata* at the transcriptome level but not in the proteome (Fuzita et al. 2016). Similarly, defensins were not found in the digestive fluids of other spiders (Walter et al. 2017), including *Uloborus* sp. (Valladão et al. 2023). The presence of defensin might be explained by contamination of the midgut sample with haemolymph, which is generally rich in antimicrobial peptides. However, this is very unlikely. First, if it was contamination, it would not have such high TPM values only in one tissue type across all three replicates, but it would be found in all tissue samples as the organs were dissected within the same dish. Second, considering that spiders have an open circulatory system, the haemolymph would have contaminated all harvested organs, and not only certain tissues. The occurrence of defensin at the protein level and its functional role in the digestive fluids therefore warrants further investigation.

The secretion of venom components in the midgut gland appears to be a common trait among spiders, as similar proteins have been detected in *N. cruentata* (Fuzita et al. 2016), *Acanthoscurria geniculata* and *Stegodyphus mimosarum* (Walter et al. 2017), although in varying proportions. For example, in *S. mimosarum*, 66% of the components isolated in the venom were also found in the digestive fluids, whereas this proportion was only 9% in *A. geniculata* (Walter et al. 2017). Notably, several digestive components were identified in their venoms. While the presence of digestive enzymes in venom may serve as an initial step in extra-oral digestion (Walter et al. 2017), the interpretation of toxins in digestive fluids poses a greater challenge, and no hypotheses have been put forward thus far. Further investigation is needed to determine if the venom-related proteins found in digestive fluids exhibit effective insecticidal activity, and to elucidate their potential function. A recent study reported that the liquid content of the abdomen, including digestive fluids, from the garden orb-web spider *Argiope australis* is utilized by southern Africa Bushman to enhance hunting arrows (Bird et al. 2023). Apparently, the abdomen is the sole ingredient of the arrow poison, while the prosoma containing venom glands is discarded. The "spider poison" exhibited lethal effects on various animals, including large ones like eland and impala, resulting in death within two hours (Bird et al. 2023). These findings strongly suggest the presence of potent toxins in the spider's abdomen where the midgut is located. Furthermore, as mentioned earlier, when digestive fluids were applied on silk-wrapped ants, it caused their death, also indicating the presence of lethal components (Weng et al. 2006).

One possible explanation for the occurrence of toxins in digestive fluid is that the feeding process in spiders is quite slow; as a result, if the prey remains unparalyzed, it may have the ability to break free from the silk wrapping and escape. In fact, thin silk lines can be quickly dissolved after just five minutes of exposure to the digestive fluid (Weng et al. 2006). Over time, the silk wrap may weaken, creating an opportunity for the prey to flee. Therefore, the inclusion of neurotoxins in the digestive fluid could serve to prevent potential escape attempts. Further functional studies are anticipated to examine the toxicity of digestive fluid in *U. plumipes* and validate this hypothesis.

Conclusions

Based on our findings, it is evident that spider toxins are not exclusively confined to specialized venom-secreting glands, but they are secreted also in the midgut. This suggests a plausible evolutionary link between venom and the digestive system, with toxin expression potentially shifting from the digestive gland to the venom system over time. The persistent expression of toxins in the midgut gland suggests a preserved functional role, either in the spider physiology or as a hunting strategy for effective prey immobilization. Overall, our study illuminates the intricate biology of spiders and their toxins, underscoring the significance of conducting comprehensive investigations into toxin expression across multiple tissues whenever possible.

Material and Methods

Specimen

Individuals of *Uloborus plumipes* were collected from greenhouses in Viernheim and Merzig, Germany.

Histology

To verify the absence of venom-producing glands in *U. plumipes* we performed a histological analysis of the chelicerae and anterior part of the prosoma. The apparent lack of a venom system in Uloboridae spiders is widely found in the literature; however, this is based on a drawing (Millot 1931), and to our knowledge no further investigation has been carried out to verify this finding. We dissected five individuals directly in the fixative, Karnovsky´s solution (Karnovsky 1965), and fixed the chelicerae with the part of the prosoma overnight. After washing in 0.1M phosphate buffer the samples were post-fixed in 2% osmium tetroxide solution for two hours and dehydrated using a graded series of ethanol. Embedding was carried out using Embed812 resin embedding kit (Science Services GmbH, München, Germany). During the final embedding step, samples were transferred into a "VacuTherm" vacuum heating cabinet (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated at 40 °C and 100 mbar for 3 × 30 min. Polymerization of the resin blocks was carried out in a heating cabinet at 60 °C for a minimum of 24 h.
Semi-thin sections were obtained with a Leica UC6 ultra-microtome (Leica Microsystems GmbH, Wetzlar, Germany), with a DiATOME histo Jumbo diamond knife (Diatome Ltd., Nidau, Switzerland) at a thickness of 700 nm. Staining was done with toluidine blue at 70 °C. Images of sections were obtained with a Fritz Slide Scanner (PreciPoint GmbH, Freising, Germany).

Scanning electron microscopy

485 We used three individuals to investigate the fang morphology. In particular, we aimed at
486 investigating whether the opening of the venom duct at the tip of the fangs were present,
487 or whether they disappeared as a consequence of the loss of the venom-secreting
488 apparatus.
489 The spiders were euthanized and fixed in liquid nitrogen and then placed on an aluminium
490 holder with double-sided carbon adhesive. Samples were examined with a scanning electron
491 microscope (Quanta FEG 250, TFS) with the environmental mode (partial pressure 80Pa).
492 The detectors used were the Large Field Detector and the Backscattered electron detector
493 (BSED) at 10kV spot 4, working distance between 7.9 and 6.6mm.
494
495 **RNA-seq**
496 Even though uloborids effectively lost their venom glands, it may be that some of the venom
497 components are expressed elsewhere to aid with prey immobilisation and killing. In
498 particular, we hypothesised that neurotoxins might be found in other secretions that come
499 into contact with the prey, such as the silk or the digestive fluids (or both). To test this
500 hypothesis, we performed RNA-seq. Tissue samples of chelicerae, prosoma, midgut gland,
501 silk gland and gonads (ovaries) were dissected from approximately 12 adult individuals. To
502 obtain enough RNA, for each tissue, we pooled multiple samples for a total of three
503 replicates except the ovaries for which we had only one. Total RNA was isolated by
504 homogenising tissues in TRIzol (Invitrogen, USA) following manufacturer's instructions, with
505 an additional on-column DNA purification step. Thirteen cDNA libraries were generated with
506 the TruSeq RNA Sample Preparation kit (Illumina) with 150 read length, followed by pair-end
507 sequencing on an Illumina NovaSeq at the Genomic Technologies Facility of the University of
508 Lausanne, Switzerland.
509
510 **Assembly and annotation**
511 Raw reads were assessed with FastQC v0.11.9 (Andrews 2010) and quality-filtered with
512 Fastp v0.22.0 (Chen et al. 2018). Reads shorter than 30bp were discarded. As no genome for
513 Uloboridae spiders was available at time of the project, all reads were concatenated and
514 used for *de novo* assembly with SPAdes v3.15.3 (Bushmanova et al. 2019). We adopted a
515 multi-step quality filtering approach to prune the raw transcriptome assembly. First, we
516 used the program Borf v1.2 (Signal and Kahlke 2021) to predict open reading frames (ORFs)
517 and we retained only sequences with a minimum length of 20 amino acids and a complete
518 ORF. Next, we aligned the remaining amino acid sequences with BLASTP (Camacho et al.
519 2009) against multiple databases, including the NCBI non-redundant pre-formatted Refseq
520 database, UniProt/SwissProt (The UniProt Consortium 2023), UniProt-ToxProt (Jungo et al.
521 2012), Arachnoserver (Pineda et al. 2018), and a customised database consisting of 11
522 spider genomes (Supplementary Table S3). All databases were downloaded on 4.7.2022.
523 Protein families and domains were predicted with InterProScan v5.51.85.0 (Quevillon et al.
524 2005), and signal peptides were detected using SignalP-6.0 (Teufel et al. 2022). Only
525 transcripts with a blast hit to at least one database and e-value < 1e-5 were retained.
526 To maximise the chance of detecting potential transcripts encoding venom components, we
527 assigned a transcript as a putative toxin if one of its 100 hits to the UniProt/SwissProt
528 database was a protein listed in UniProt-ToxProt. We then classified putative toxins as
529 'group 1' if the best hit was a protein in UniProt-ToxProt, 'group 2' if the top hit was not to a
530 toxin sequence. A best hit was defined by the highest bit score. We also scanned sequences
531 with incomplete ORFs by searches against UniProt/SwissProt, Arachnoserver, and

532 InterProScan. Presence of a knottin domain was tested with the tool Knotter 1D from the
533 Knottin database website (Postic et al. 2018).
534 Neurotoxicity of the identified potential toxins was predicted using NT_estimation, a deep
535 learning approach which uses a peptide data augmentation method to improves the
536 recognition of spider neurotoxic peptides via a convolutional neural network model (Lee et
537 al. 2021).
538
539 We retained only transcripts with a hit to at least one database and reduced redundancy by
540 clustering all sequences with > 99% identity using CD-HIT v4.8.1 (Fu et al. 2012). The
541 completeness of the final assembled transcriptome was assessed by OMArk (Nevers et al.
542 2022) by calculating the overlap of the non-redundant annotated genes and conserved
543 ancestral gene set of the phylum Arthropod.
544
545 **Expression levels**
546 Transcript abundances were quantified using Kallisto v0.48.0 (Bray et al., 2016) with default
547 parameters for paired-end reads.
548 All analyses were conducted in R version 4.1.3 (R Core Team 2019). Count distribution
549 across libraries was inspected with the package *vioplot* v 0.3.7 (Adler et al., 2021) and genes
550 with TPM value >= 1 in at least one library were kept for further analysis. Consistency of
551 expression patterns between samples from the same tissue was assessed by means of
552 principal component analysis, and the percentage of explained variance by each component
553 was calculated using the function fviz_eig in *Ade4* v1.7.19 (Dray and Dufour, 2007).
554 To further narrow down the toxin candidates, we performed differential expression analysis
555 based on the assumption that toxins should have bias expression specifically in one tissue as
556 their production is physiologically expensive. Differential expression analysis was performed
557 in *Sleuth* v0.30.0 (Pimentel et al., 2017) and the fold change (FC) of transcripts was
558 calculated as the ratio between the highest and the second highest expression values. Only
559 putative toxin transcripts with fold change >= 2 and q-val < 0.05 were retained.
560 Because *Sleuth* automatically filter out genes which are exclusively expressed in one tissue
561 type, we checked also those which did not passed *Sleuth* filter and kept all the sequences
562 with FC >= 2. Finally, we removed the transcripts with the highest expression levels in the
563 gonads as highly unlikely to be true toxins.
564
565 **Orthology assignment**
566 We identified orthologous gene clusters with the recently published genome of *Uloborus*
567 *diversus* (Miller et al 2023) using the web platform OrthoVenn3 (Sun et al. 2023).
568

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575

576 Author contributions

577 T.L. and G.Z. conceived the study. X.P. performed the transcriptomic analysis and
578 contributed to the writing. T.D. performed the histology experiments. P.M. dissected the

animals and contributed to the histology. A.M. performed the electron microscopy experiments. M.R-R. contributed to the transcriptomic analysis. G.Z. contributed to the transcriptomic analysis and wrote the manuscript, with input from all authors.

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