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RESEARCH ARTICLE

The protamines of the spider *Steatoda* sp. provide an example of liquid–liquid phase separation chromatin transitions during spermiogenesis

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ABSTRACT

Although there is extensive information about sperm nuclear basic proteins (SNBP) in vertebrates, there is, by comparison, very little information in Arthropoda. This study aims to contribute to filling this gap by analyzing these proteins in the sperm of the noble false widow spider *Steatoda nobilis* (order Araneae, family Theridiidae). To this end, we have developed a protein extraction method that allows the extraction of both cysteine-containing and non-cysteine-containing protamines that is suitable for the preparation and analysis of SNBPs from samples in which the amount of starting tissue material is limited. We carried out top-down mass spectrometry sequencing and molecular phylogenetic analyses to characterize the protamines of *S. nobilis* and other spiders. We also used electron microscopy to analyze the chromatin organization of the *Steatoda* sperm and we found it to exhibit liquid–liquid phase spinodal decomposition during the late stages of spermiogenesis. These experiments further our knowledge on the distribution of SNBPs within the animal kingdom and provide additional support for a proposed evolutionary origin of many protamines from a histone H1 (H5) replication-independent precursor.

KEY WORDS: Sperm nuclear basic proteins (SNBPs), Protamines, Spider, Mass spectrometry, Liquid–liquid phase separation, Phylogeny

INTRODUCTION

Sexual reproduction in metazoan organisms involves two highly specialized cell types (gametes: sperm and oocytes) with important functional and epigenetic differences from their germ cell progenitors (Kota and Feil, 2010; Feng and Chen, 2015). Sperm exhibits important structural (Baccetti and Afzelius, 1976; Pitnick et al., 2009) and chromosomal protein composition characteristics (Eirin-Lopez and Ausio, 2009), which involves three main groups of sperm nuclear basic proteins (SNBPs): histone (H); protamine-like (PL); and protamine (P) types (Ausió, 1999). Histones (H type proteins) have a composition rich in both lysine and arginine, vary in their mass between 10 and 25 kDa, and are the constituents of nucleosomes, the basic subunit of somatic chromatin (van Holde, 1988). The H group encompasses those organisms in which histones are retained without any apparent replacement by any other SNBPs (Muñoz-Guerra et al., 1982a, 1982b) and organisms such as echinoderms (Poccia, 1995), in which highly specialized sperm-specific histone variants replace the somatic histones. During spermiogenesis, SNBPs of the PL and P types replace the somatic histones of the sperm progenitor cells that are present at the onset of spermatogenesis (Oliva and Dixon, 1991; Govin et al., 2004; Rathke et al., 2014; Moritz and Hammoud, 2022). Protamine-like proteins (Lewis and Ausió, 2002) are also rich in arginine and lysine but they exhibit much more structural variability than histones and they can range in mass between 6 and 40 kDa. Finally, protamines (Kasinsky et al., 2012) are highly arginine rich. They can have up to 80% arginine, as in the case in the California market squid (*Loligo opalescens*) (Lewis et al., 2004b). They can range in mass from 3 to 10 kDa.

SNBPs have been characterized in the phylum Porifera (Ausió et al., 1997), and in the classes Schiphozoa (jellyfish; *Aurelia aurita* and *Thaumatoscyphus hexaradiatus*), Hydrozoa [*Catablema* sp.; *Mitrocoma cellularia* (Rocchini et al., 1996), *Hydractinia echinata* (Török et al., 2023)] and Anthozoa [anemones; *Urticina* (=*Tealia*) *crassicornis*, *Anthopleura xanthogrammica* and *Metridium senile*] of the phylum Cnidaria (Rocchini et al., 1996, 1995b). SNBPs have also been analyzed in the phylum Annelida (*Chaetopterus varipedatus*) (Fioretti et al., 2012) and extensively in the phylum Mollusca (Subirana et al., 1973; Casas et al., 1993) and in the phylum Chordata (Oliva and Dixon, 1991; Lewis et al., 2003; Saperas and Ausio, 2013). However, despite arthropods representing 80% of all animals (Zhang, 2011) and the order Araneae consisting of approximately 48,500 species (Dimitrov and Hormiga, 2021), information about the SNBPs in the phylum Arthropoda has been very limited (Leyden et al., 2024).

Within the SNBP types, the replacement of histones by protamines in vertebrate taxa was initially hypothesized to fulfill two main functions: (1) compaction of the DNA to streamline the mobility of sperm and to provide protection against damage during its journey in search of the egg and (2) to assist the erasure of transcriptional and epigenetic marks (Oliva and Dixon, 1991). However, as variation in the H, PL, and P SNBP types across the tree of life shows (Bloch, 1969; Eirin-Lopez and Ausio, 2009), compaction of DNA is not essential for fertilization. Nevertheless, Kasinsky hypothesized in this regard that internal fertilization might

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have operated as a constraint on the range of SNBPs to protamines in amniotes (Kasinsky, 1989, 1995). Also, protamine amino acid composition, in particular arginine content, has been shown to affect sperm head shape, and a possible involvement in sperm competition for the mammalian P1 protamine (Lüke et al., 2016). In the functional regard, protamines have recently gained a lot of attention. In the case of invertebrate protamines, the protamine-mediated removal of histones in the sperm of *Drosophila* (Rathke et al., 2014) has been shown to protect paternal chromosomes from premature division at fertilization (Dubruille et al., 2023). In mice, it has been shown that, despite the seemingly monotonous arginine composition of these protamines, substitution of a single lysine in protamine 1 in this vertebrate results in sperm chromatin and reproductive fitness alterations (Moritz et al., 2023).

Here, we report for the first time the characterization of the SNBPs of a spider, the noble false widow species *S. nobilis* (Araneae, Theridiidae). We show that the SNBPs correspond to the P type, discuss their occurrence in the context of arthropod phylogeny, and analyze their amino acid sequences. We also describe their involvement in spermiogenic chromatin condensation mediated by the spinodal decomposition (SD) and nucleation (Nc) dynamic mechanisms of liquid–liquid phase separation (LLPS) (Harrison et al., 2005; Kasinsky et al., 2021).

RESULTS

SNBPs of *S. nobilis* within the context of arthropod phylogeny

Cysteine occurs sporadically in several SNBPs and has been described to be present in the protamines and other SNBPs of marine invertebrates (Zhang et al., 1999; Giménez-Bonafé et al., 2002), insects and vertebrates (Gusse and Chevaillier, 1978; Retief et al., 1995), where it is ubiquitously present in eutherian mammals (Oliva and Dixon, 1991; Balhorn, 2007). Therefore, and given our access to only a limited amount of material, we decided to modify our SNBP extraction method (Leyden et al., 2024) to make it more inclusive of both non-cysteine-containing and cysteine-containing protamines. To this end, we added a cysteine alkylation by pyridyl ethylation step prior to the HCl protein solubilization, and we optimized our methods for small amounts of starting material (see Materials and Methods and Fig. S1). This method allows for the extraction of both cysteine-containing and non-cysteine-containing protamines.

Fig. 1A shows the electrophoretic pattern of the proteins extracted from the sperm from 54 *S. nobilis* pedipalps (SN), using this method, in comparison with the SNBP protamines from the California mussel (MC) and salmon (SL) as well as the histones from chicken erythrocytes (CM) that are used here as markers for protamines and somatic histones, respectively. The markers CM and SL provide a relative estimate of the molecular mass (Mr) associated with the electrophoretic mobility, as in AU-PAGE, in contrast to SDS-PAGE, proteins do not run corresponding to their Mr. The electrophoretic pattern of *S. nobilis* SNBPS observed in Fig. 1A displays a complex mixture of bands with two predominant groups with relatively low and high electrophoretic mobility within the areas corresponding to histones and protamines, respectively. The former presumably arise from the contamination with the somatic tissue from the male pedipalp structures sampled here (in spiders, the sperm is stored in pedipalps prior to copulation; Zhang et al., 2022). Within the protamine region, an intense double band (Fig. 1A, SN/P) exhibits an electrophoretic mobility similar to that of the PL-IV SNBP of the mussel *Mytilus californianus* (Fig. 1A, MC), which has an Mr of 6.5 kDa (Carlos et al., 1993a).

Although this work analyzes only one spider species, and despite the large number of species and high complexity of the order Araneae (Lipke and Michalik, 2015; Kulkarni et al., 2023), the occurrence of protamines in spiders documented here is of interest in terms of the SNBP phylogenetic distribution in the phylum Arthropoda (Fig. 1B). Unfortunately, the amount of information available for this phylum, which encompasses by far the largest number of existing species within the animal kingdom, is very limited. Fig. 1C summarizes some of this information. Within the order Xiphosura, the only SNBP information available is that of the horseshoe crab *Limulus polyphemus*, which, as shown in Fig. 1C (LP), belongs to the H type. This is similar to what is observed in the brown crab *Cancer pagurus* (Fig. 1C, CP) (Kurtz et al., 2008), which appears to be a common occurrence in decapod crustaceans (shrimp and crabs) (Wu et al., 2015; Chen et al., 2019). In insects, a protamine-like SNBP has been described in *Drosophila* (Jayaramaiah Raja and Renkawitz-Pohl, 2005), and a protamine is present in the sperm of the honey bee (*Apis mellifera*) (Fig. 1C, AM; Leyden et al., 2024). Therefore, the information available so far suggests that, as in vertebrates (Eirin-Lopez and Ausio, 2009) and plants (Borg and Berger, 2015; D'Ippolito et al., 2019), SNBPs are sporadically distributed in arthropods and that a transition from the H to the P SNBP types might have occurred several times (Ausió, 1999) throughout the evolution of this group.

Characterization of *S. nobilis* protamines

We recently published the amino acid sequences of SNBPs from several insect species (Leyden et al., 2024). Following a similar mass spectrometry (MS) approach to that described by Leyden et al. (2024), two partial closely related sequences of proteins (P1, P2) with molecular masses 6538.8 and 6395.9 were obtained (Fig. 2A) corresponding to the protamines shown in Fig. 1A. A genome BLAST search using these sequences identified a hypothetical histone H1-H5 protein, AVEN_19411.1, from *Araneus ventricosus* (family Araneidae) (Kono et al., 2019) (Fig. 2B, 1), which encompassed a highly homologous protein of a very similar size (Mr ∼6200) at its C-terminal domain (Fig. 2B, 1,2). Further searching using the *A. ventricosus* sequence allowed us to obtain an alignment of more spider sequences (Arakawa et al., 2022) (Fig. 2C) and identify a putative consensus protamine sequence for spiders of the Araneidae and Theridiidae families (Fig. 2D). Unfortunately, we were not able to retrieve sequences from any other families. It is interesting to note that the two partial P1 and P2 amino acid sequences from *S. nobilis* (Fig. 2A) indicate that, as in insects (Leyden et al., 2024), the spider protamines also exhibit sequence microheterogenity, likely as a result of the existence of more than one encoding gene. The isoelectric points of all these proteins (12.2-12.5) are well within the range of what was observed for insect protamines.

Of interest, in addition to the protamine sequences, the high sensitivity of our MS analyses allowed us to determine the presence in our sample (Fig. 1A) of peptides such as: DEKKKDDKKSGTG-KPQQKPEEKKPEKGGKKDEKKPEKKPEQKKK (∼5101 Da), MKEKPDDKGKPGEKKPEGPKPGEKKPEPGKPGEKKPGEP-KPGEKKPEEKGPKK (∼5810 Da) and MDKKPDDKGKPGEK-KPELGKPGEKKPDDKggKPGEKKPEEKGPKK (∼4953 Da). In the last two instances, the sequences contained oxidized methionines and acetylated N termini with the lower case residues not having been confirmed. A BLAST search of these proteins against the NR database (with no organism chosen) revealed some extent of homology to proteins of bacterial origin. This is not surprising as the sperm used in our analysis had been obtained from the spider

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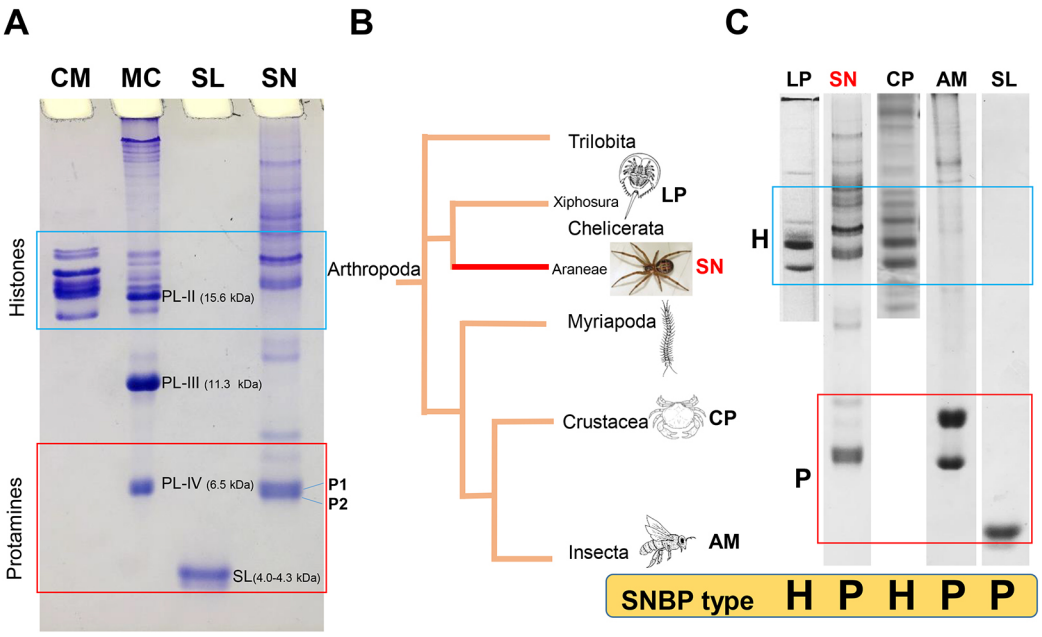

Fig. 1. SNBPs of *S. nobilis* in comparison to other groups of arthropods. (A) AU-PAGE of *S. nobilis* SNBPs (SN) in relation to the chromosomal proteins of other organisms used as protein markers: CM, chicken erythrocyte nuclear histones; MC, *M. californianus* (California mussel) SNBPs indicating its major PL-II (Mr=15.6 kDa) (Carlos et al., 1993b), PL-III (11.3 kDa) (Rocchini et al., 1995a) and PL-IV (6.5 kDa) components (Carlos et al., 1993a); and SL, salmine protamine (4.0-4.3 kDa) (Ando et al., 1973) from the salmon *Onchorhyncus keta*. (B) Simplified phylogeny of Arthropods adapted from Giribet and Edgecombe (2019). (C) Electrophoretic analysis of the SNBPs of several Arthropod representative species in comparison to the protamine of the salmon (SL, salmine). AM, *Apis mellifera* (honey bee) (Leyden et al., 2024); CP, *Cancer pagurus* (brown crab) (Kurtz et al., 2008); H, histones; LP, *Limulus polyphemus* (horse shoe crab) (Munoz Guerra et al., 1982b); P, protamines; SN, *S. nobilis*.

pedipalps (see Materials and Methods) and contained a significant amount of bacteria. The relevance of this to our protamine analysis will be discussed below.

LLPS chromatin transition during *S. nobilis* spermiogenesis

To visualize the process of spermiogenesis in *S. nobilis*, transmission electron microscopy was performed of *Steatoda grossa*, a closely related species. Fig. 3 shows the ultrastructural changes undergone by the nucleus and chromatin during the differentiation process. Interestingly, late spermatids undergo SD (Fig. 3, 2a,2b) and Nc chromatin condensation (Fig. 3, 2c,2d) LLPS transitions. This is similar to what was initially described for the mollusk *Murex brandaris* (Harrison et al., 2005) and to what has been extensively observed during insect spermiogenesis (Kasinsky et al., 2021). Yet this is far from being an invertebrate chromatin condensation phenomenon; previous evidence indicates this also takes place in condrychtian fish spermiogenesis (Gusse and Chevaillier, 1978), and somatic chromatin has also been described to involve LLPS (Gibson et al., 2019).

SD is a physicochemical model involving kinetic, equilibrium and structural aspects of a system en route to equilibrium (Harrison et al., 2005). It is a mechanism for the separation of two liquid phases with transient patterns being produced in the unstable thermodynamic state by gradually growing concentration inhomogeneity (see Fig. 3, 2b). Nc is the first step representing the transition to a metastable state of the phase separation diagram, involving a self-organization of the mixture of the two liquid phases (see Fig. 3, 2d). These are two different dynamic mechanisms of LLPS that in several instances, such as in the cuttlefish *Eledone cirrhosa* (Giménez-Bonafé et al., 2002) or in the insect *Anurida maritima* (Kasinsky et al., 2021) and in *S. grossa* analyzed here (Fig. 3) might co-exist during the chromatin condensation during spermiogenesis. This transition from SD to Nc can be seen as an inversion from chromatin as a dispersed phase (Fig. 3, 2b) to nucleoplasm as a dispersed phase (Fig. 3, 2d) (Harrison et al., 2005; Kasinsky et al., 2012) that precedes the tight chromatin compaction observed in mature sperm (Fig. 4).

Analysis of the pattern in Fig. 3, 2b yielded a value of 170±20 Å for the distance between the chromatin lamellae (which corresponds to the lambda parameter used in the SD calculations; Harrison et al., 2005) and a chromatin lamellae thickness of approximately 50 Å (Fig. 4A, 3). The chromatin in the mature sperm found in the spermatheca is completely condensed, and glycogen is present in substantial amounts (Fig. 3, 3,4). The co-existence of glycogen with mature sperm in spiders has been well documented (Coyle et al., 1983; Michalik et al., 2004, 2005). The presence of glycogen is widespread in both invertebrate and vertebrate species (Anderson and Personne, 1970) and although its function is still controversial it appears to have an important, yet overlooked, contribution to different aspects of spermatogenesis (Silva et al., 2022).

DISCUSSION

Of clams, chickens, and spiders

An early analysis of the SNBPs from mollusks revealed extensive protein heterogeneity in both size and composition between the species analyzed (Subirana et al., 1973), with those with Mr generally exhibiting a higher arginine content. A hypothetical evolutionary pathway was proposed at the time whereby the arginine-rich protamine-like proteins and protamines might have been derived from a ‘strongly basic fragment’ of a histone precursor (Subirana et al., 1973). However, the precise molecular details remain unknown. When in 1987 we determined the amino acid sequence of the globular domain of the PL protein from the sperm of the surf clam *Spisula solidissima*, a high extent of homology was

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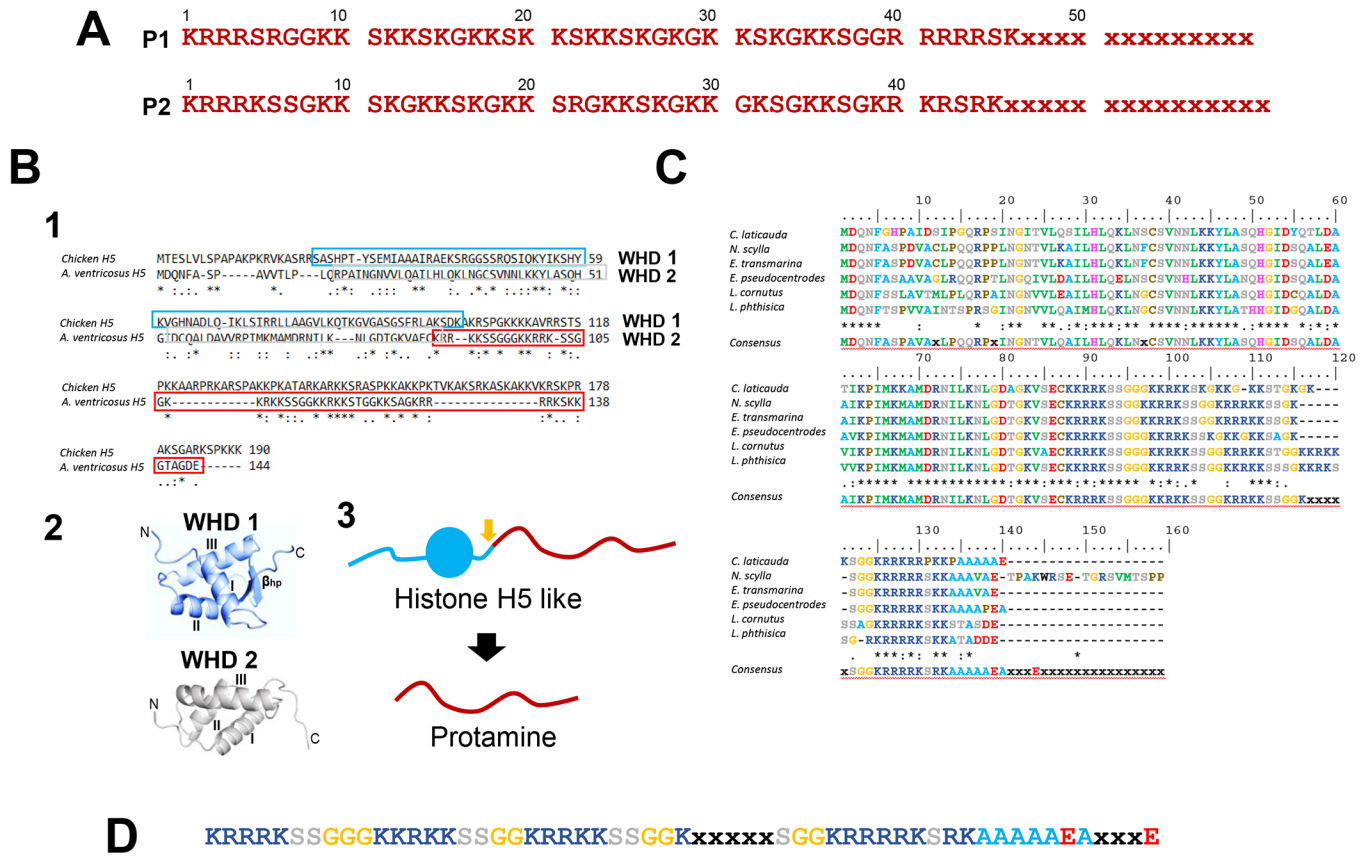

Fig. 2. Protein sequences of spider protamines. (A) Partial amino acid sequences of the P1 (~6359 Da) and P2 (~6396 Da) protamines from *S. nobilis* determined by LC-MS. (B) (1) Amino acid sequence alignment of chicken histone H5 (AAA48798.1) and *A. ventricosus* H1-H5 like protein (GBL99906). The amino acid sequence regions corresponding to the WHD of histone H5 (WHD1) and *A. ventricosius* (WHD2) are indicated by blue and gray margin boxes, respectively. (2) Tertiary structure of WHD1 and WHD2 determined by Neurosnap AlphaFold 2 (Jumper et al., 2021). (3) Diagram of the *A. ventricosous* protein shown in 1. The protamine region (red) is part of the C-terminal region of a histone H5-lke protein (blue). (C) Sequence alignments of the histone H5-like proteins from different spiders: *Cyclosa laticauda* (IAWK01030102.1); *Neoscona scylla* (ICBS01001118.1); *Eriophora transmarina* (IBPW01032809.1); *Eriovixia pseudocentrodes* (ICEC01024091.1); *Larinoides cornutus* (IBUI01019351.1) and *Larinia phthisica* (IAJV01034118.1). All these species belong to the family Araneidae. In these alignments, asterisks indicate that the amino acid is the same for all the sequences at that position and colons indicate that some of the sequences have different amino acids at that position, but that the chemical properties of the different amino acids are similar. (D) Consensus sequence of spider protamines.

found with that of the winged helix domain (WHD) (Ramakrishnan et al., 1993) of the histone H5 of chicken erythrocytes (Ausió et al., 1987) as is the case for the histone H5-like protein identified in *A. ventricosus* (Fig. 2B, 1,2). Histone H5 is a replication-independent member of the histone H1.0 family of histone H1 linker proteins, which are often present in terminally differentiated cells (Cheema and Ausio, 2015), such as the nucleated erythrocytes of fish, amphibians, reptiles and birds (Shay et al., 1988). Such an observation provided the first hint that sperm protamines and PLs might have evolved from a common ancestral histone H1 (Ausió, 1995, 1999). Since then, information has been accumulating in support of this proposal in such diverse organisms as a marine annelid *Chaetopterus variopedatus* (Fioretti et al., 2012), tunicates (Lewis et al., 2004a; Saperas and Ausio, 2013) and more recently in the liverwort *Marchantia polymorpha* (D'Ippolito et al., 2019). The relationship of protamines to histone H1 is not surprising as the presence of arginine in both protein types contribute to their cooperative binding to DNA (Piscopo et al., 2010). The protamines of *S. nobilis* and those of the superfamily Araneoidea (Fig. 2) provide further and unequivocal support to the early histone H1 hypothesis (Ausió, 1999). Interestingly, like in other PL precursors, the *A. ventricososus* WHD 2 (Fig. 2B, 2) domain lacks the C-terminal hairpin (Cheema and Ausio, 2015).

In most of the organisms in which the final SNBP at the end of spermiogenesis was a protamine arising from a histone H1-H5, the precursor was clearly identified in the samples analyzed. Yet this is not the case here, despite the high sensitivity of the MS approach used. This may be because, in contrast to previous studies, in the case of *S. nobilis* only the protein products present in the mature sperm obtained from the spider pedipalps were used in the analysis. The detection of bacterial protein/peptides in this study is consistent with this, as spiders such as *S. nobilis* use intromittent organs to transfer sperm to females, and environmental microbes are introduced into females during mating (Spicer et al., 2019).

SNBP distribution in Arthropoda

The occurrence of an arginine-containing protamine-like SNBP in lycosid wolf spiders was described earlier using cytochemical staining (Rasch and Connelly, 2006), yet no information on the molecular details have been reported. The presence of protamines in the order Aranea (Fig. 1B,C) is interesting when considered in the context of Arthropoda evolution. As in fishes (Saperas et al., 1997) and amphibians (Ausio et al., 2007) within the phylum Chordata,

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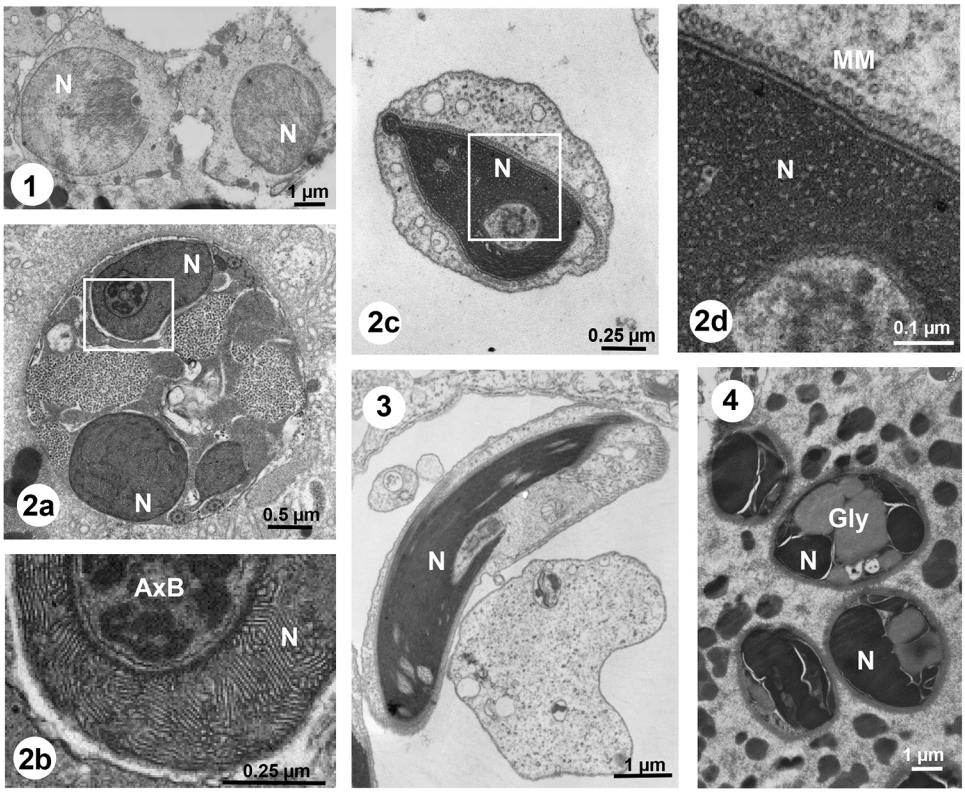

Fig. 3. Chromatin transitions during *Steatoda grossa* spermiogenesis. Transmission electron microscopy of: (1) early spermatids with beginning of chromatin condensation; (2a-2d) cross-sections of late spermatids at different stages of chromatin condensation undergoing SD (2a-2b) and Nc (2c-2d); (3) longitudinal section of a late spermatid; and (4) mature sperm. AxB, axonemal basis; Gly, glycogen; N, nucleus; MM, manchette of microtubules.

the SNBPs of the phylum Arthropoda also appear to exhibit a sporadic distribution (Saperas et al., 1994) (Fig. 1C).

Although it would be tempting to correlate the SNBP types to the living environment with the H type SNBP occurring in the aquatic environment versus the P type corresponding to the terrestrial species, this is unlikely to be the case. In this regard, the three main SNBP types (H, PL and P) are present in fish. The same is also true if the internal versus external fertilization type is considered. While *L. polyphemus* (Fig. 1C, LP) has external fertilization (Brockmann et al., 1994), crustaceans have both external and internal fertilization (Aiken et al., 2004), but the crab *C. pagurus* (Fig. 1C, CP) has internal fertilization (Edwards, 1966). In contrast, it appears that at least in the spider *S. nobilis* and in insects such as in *A. mellifera*, both with internal fertilization, protamines constitute the prevalent type (Fig. 1C, SN, AM). Hence, as Chordata, the SNBP types in Arthropoda also exhibit a sporadic distribution, and, in both instances, the evolutionary cause remains unclear. As we had previously proposed, such heterogeneous distribution could be possibly explained by a repeated and independent loss of the expression of the protamine gene (or loss of the gene itself) (Saperas et al., 1994) in the course of Metazoan evolution.

Ubiquitous occurrence of LLPS by SD during metazoan spermiogenesis

When the mechanism of protamine-driven LLPS underlying sperm chromatin condensation by SD and Nc was initially described (Harrison et al., 2005), it might have appeared to be a rare phenomenon sporadically distributed. However, upon closer inspection it shows persistent occurrence in insects (Kasinsky et al., 2021) and we show here it also occurs in spiders, suggesting this mechanism is by far more widespread than originally anticipated.

One of the first detailed studies describing the lamellar organization, which is characteristic of SD during spermiogenesis, was carried out in the condrichthyan lesser spotted fish *Scylliorhinus canicula* (Gusse and Chevaillier, 1978). The authors were able to characterize the different protein transitions undergone during spermiogenesis, taking advantage of the testicular zonal organization consisting of different cell type layers corresponding to the stages of differentiation (Gusse and Chevaillier, 1981). We now know that such peculiar chromatin lamellar organization is the result of a highly dynamic process of LLPS known as SD (Harrison et al., 2005; Harrison, 2010). This is a dynamic process that, in the species in which this has been observed so far, at the biochemical level involves a massive degradation of most of the genomic histones and post-translational processing of both chromosomal proteins involved (histone acetylation and protamine phosphorylation and cleavage) (Fig. 4). Hence, this process is not only highly dynamic but it is also very complex (Kasinsky et al., 2012). The difficulty in studying the biochemical events involved in the chromatin transitions at the different stages of spermiogenesis, particularly in invertebrates, in which only low amounts of sample are usually available, arises from the difficulty in fractionating the different cell types at each stage. Hence, Fig. 4B is not based on any experimental work carried out in this study but on previously published papers reviewed by Kasinsky et al. (2012).

Whether additional proteins are involved, such as coiled-coil glutamate-rich protein 1 (CCER1), recently described in mice, which participates in the histone-to-protamine transition during post-meiotic spermatid differentiation through formation of a liquid–liquid phase-separated condensate (Qin et al., 2023), is not known. Regardless, all these biochemical events leading to the particular chromatin patterning observed in SD (Fig. 4) occur through reactions that take place in the interchromatin compartment (Cremer et al., 2020), which is the main player in the different chromatin transitions observed.

Little is known about lamellar nucleoprotamine organization (Fig. 4A, 3). Whereas chromosomal proteins that bind to DNA in a

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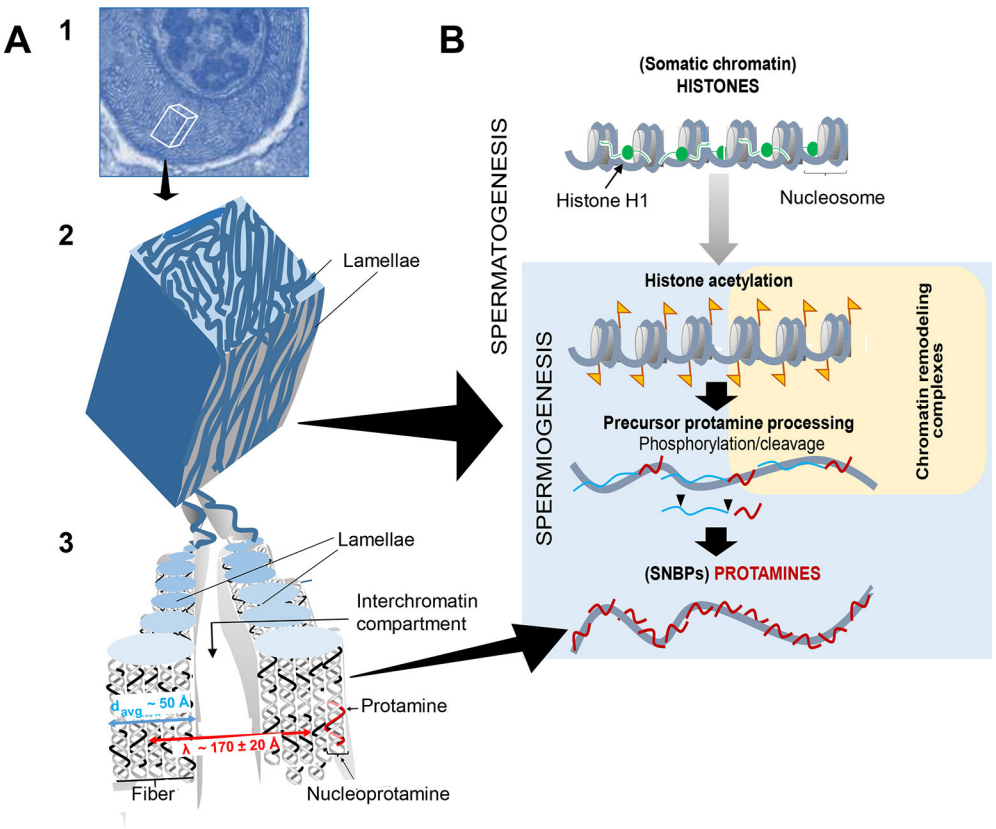

Fig. 4. Model for chromatin transitions during spermatogenesis. (A) (1) Example image (also shown in Fig. 3, 2b) showing spermatid chromatin undergoing SD in *S. grossa*. (2) 3D schematic of the cube section indicated in 1. (3) Representation of the dynamic lamellae in 2. (B) Schematic of the major chromatin changes during *S. nobilis* spermatogenesis. During post-meiotic spermiogenesis, histones of the nucleosomal chromatin fiber become hyperacetylated (yellow flags) in preparation for histone eviction and replacement by protamine precursors. This is a highly dynamic complex process that involves histone degradation and several precursor protamine post-translational modifications (cleavage and phosphorylation), presumably assisted by chromatin remodeling complexes and chaperone proteins, that takes place at the interchromatin compartment (Cremer et al., 2020). By the late stages of spermiogenesis, protamines replace most of the germ cell progenitors.

sequence-specific way preferentially bind to the major groove (Garvie and Wolberger, 2001), those that bind in a non-sequence specific way, such as histones (Luger et al., 1997), usually do it through interactions with the minor groove (Murphy and Churchill, 2000). Despite this, it has long been known that protamines, which also bind DNA with non-sequence specificity, bind to the major groove (Fita et al., 1983; Puigjaner et al., 1986; Hud et al., 1994; Mukherjee et al., 2021). Recent work solved this apparent puzzle by showing that phosphorylation reduces the binding affinity of the minor groove by introducing an important deformation that eliminates the minor groove preference of protamines (Chhetri et al., 2023). Hence, serine phosphorylation at the time of protamine deposition onto DNA during the replacement of histones by protamines (Willmitzer and Wagner, 1980; Oliva and Dixon, 1991; Balhorn, 2007) (Fig. 4B) appears to play a crucial role in SD and in the resulting organization of the nucleoprotamine complexes along the lamellae (Fig. 4A, 3). Importantly, *S. nobilis* protamines (Fig. 2A) contain several serines at positions similar to the RRRS amino acid motifs of the peptides used in the Chhetri study (Chhetri et al., 2023).

Limitations of the work and future perspectives

The current work was limited by the amount of material and time available to perform all the experiments as well as by our inability to identify the last C-terminal amino acids (Fig. 2A). Time also played an important role in preventing electron microscopy from being carried out in the same *Steatoda* species and from performing a sequential analysis of the SNBP variation during spermiogenesis in testes rather than just in the mature sperm of the pedipalps.

Notwithstanding, it is hoped that the modified protamine extraction described in this work from very low amounts of material, in combination with the increasing sensitivity of the MS technology, will incentivize further biochemical analysis in many species of organisms for which this type of information is still missing.

MATERIALS AND METHODS

Biological material

S. nobilis (Thorell, 1875), the noble false widow, is a cobweb-building spider species in the Theridiidae family. It is an invasive and synanthropic species (Dunbar et al., 2020) native to the Canary Islands or Madeira that has been spread by human activity to almost every continent (Bauer et al., 2019). Females and males can be found living in urban areas around human-made structures (Dunbar et al., 2020). The *S. nobilis* males used in this experiment were the laboratory F1 offspring of field-mated females collected in 2022 from a population in Nottingham, UK (Cullen, 2017). Males were grown in temperature and light-controlled rooms (21-26°C, 12 h:12 h light:dark) at the Andrade Laboratory at the University of Toronto in Scarborough, ON, Canada. Similar to other spider species (Zhang, 2011), *S. nobilis* males do not have a direct connection between their gonads (glands responsible for sperm production) and their paired intromittent organs (pedipalps, structures responsible for carrying and transferring the sperm during mating (Foelix, 2011). Therefore, after reaching adulthood, males eject sperm from their genital opening onto a small web and take it up into their pedipalps during a process called sperm induction (Foelix, 2011). In spiders, sperm cells are encapsulated with a protein sheath and can be transferred from the pedipalps to the female's genital duct via a sclerotized structure on the palp (the embolus; Foelix, 2011). *S. grossa* (Koch, 1838), the cupboard spider, of the Theridiidae family was used for electron microscopy work.

Sperm collection for protein extraction

Unmated adult males anesthetized with CO_2 were transferred to a Petri dish under a dissecting microscope. Their pedipalps were removed using iridectomy microdissection scissors and placed in a LoBind 1.5 ml micro test-tube (Eppendorf) containing 40 µl of sperm solution: [220 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 7 mM KCl, 0.1% Triton X-100, Tris-HCl (pH 8.2)] (Snow and Abndrade, 2004; Modanu et al., 2013), and the pedipalps were crushed with a micropestle (Argos Technologies, Inc.) to

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release the encapsulated sperm cells. Thereafter, another 35 µl of sperm solution was added to rinse the remaining sperm cells from the micropestle into the test-tube, for a total of 75 µl in each tube. The tube containing the sperm sample was then vortexed for approximately 30 s and centrifuged at 1000 ***g*** for 10 min, and this process was repeated a total of three times (Snow and Abndrade, 2004). After the last round of centrifugation, the tube was filled (close to their maximum volume) with 80% ethanol. The same procedure was conducted for a total of 54 adult unmated males. The samples were shipped to the University of Victoria, BC, Canada.

Protein extraction

Proteins were extracted from 54 pedipalps prepared as described above. At the University of Victoria, ethanol was removed by centrifugation (10 min at 4°C and 7850 ***g***), and the pellet was vacuum-dried and Dounce-homogenized in 2 ml oh 0.6 N HCl. The homogenate was centrifuged in an Eppendorf microfuge at 16,000 ***g***, and the supernatant was precipitated with six volumes of acetone and overnight incubation at −20°C. The next day, the sample was centrifuged at 7850 ***g*** and the pellet was vacuum-dried and dissolved in 1.5 ml of ddH_2O.

Method to extract cysteine-containing SNBPs from small amounts of sample using pyridylethylation

We used mouse testes to optimize a method for the extraction of cysteine-containing protamines (Fig. S1). To this end, mouse testes were homogenized in buffer [0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM MES (pH 6.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton X-100 containing 1:100 protease inhibitor cocktail (Roche; 5056489001)] using a Dounce homogenizer with ten strokes on ice. Approximately 1.5 ml of buffer per one testis was used. The purpose of this step was to remove the fat and part of the connective tissue. The homogenate thus obtained was centrifuged at 2000 ***g***, and the pellet thus obtained will be referred to as mouse testes. Approximately 20 mg of mouse testes (this amount would be equivalent to the 15-20 mg of spider/insect testes we currently use for the extraction of protamines from these organisms) were re-suspended in 50 µl of 4 M guanidinium chloride, 50 mM Tris-HCl (pH7.5), 1.25 mM EDTA and thoroughly vortexed. Upon addition of 1 µl of β-mercaptoethanol, the sample was homogenized with a microcentrifuge polypropylene pellet pestle and incubated for 90 min in the dark. Next, 2 µl vinyl pyridine were added to the mixture and the sample was further incubated in the dark for an additional 30 min with brief vortexing at 5 min intervals. After incubation, 400 µl 0.6 N HCl were added to the mixture followed by homogenization using a 2 ml Wheaton glass Dounce homogenizer on ice with ten strokes. The amount of 0.6 N HCl was optimized in order to dilute the concentration of the guanidinium chloride in the starting solution without compromising the precipitation of the protein in the HCl extract. This dilution importantly eliminated the previous dilutions of the pyridilethylated sample (testes or sperm) with a large volume of distilled water immediately after pyridylethylation. This step in the initial procedure was used in order to rebind the proteins to the DNA while removing the large amount of guanidiniuum chloride before pelleting the sample for subsequent HCl extraction (Ausio et al., 2014). The 0.6 N HCl homogenate was centrifuged at 16,000 ***g*** at 4°C in an Eppendorf microfuge. The supernatant was split into two 220 µl aliquots (in Eppendorf tubes) and precipitated with by mixing it with 6 volumes of acetone and allowing it to stand overnight at −20°C. The precipitate thus obtained was centrifuged at 16,000 ***g*** at 4°C. The supernatant was discarded and the pellet was speed-vacuumed for 10 min at room temperature and used for further analysis.

Gel electrophoresis

Acetic acid (5%)–urea (2.5 M) polyacrylamide gel electrophoresis (AU-PAGE) was carried out according to Hurley (1977) and as described elsewhere (Ausió, 1992).

Reversed-phase HPLC (RP-HPLC)

HPLC was performed as described (Ausió and Moore, 1998; Cheema and Ausio, 2017). In brief, a 1000 µl aqueous solution of the protein extract from the sperm of 54 HCl-extracted pedipalps were injected onto a C_{18} column (Vydac) (4.6×250 mm; particle size: 5 µm; pore size: 300 Å) and eluted at 1 ml/min using a mobile phase consisting of (0.1% trifluoroacetic acid) and acetonitrile gradient. Samples were fractionated on a Beckman Coulter SYSTEM GOLD® 126 Solvent Module equipped with SYSTEM GOLD® 168 Detector.

Electron microscopy

Male specimens were dissected in 0.1 M phosphate buffer (PB) to which 1.8% sucrose was added. The reproductive system was fixed for 2 h in 2.5% glutaraldehyde in PB and postfixed for 2 h in PB-buffered 2% OsO_4. After being washed in PB, samples were dehydrated in graded ethanols and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections (60 nm) were obtained using a Diatome Ultra 45° diamond knife on a Leica ultramicrotome UCT. Sections were stained with uranyl acetate and lead citrate following Reynolds (1963) and examined with a JEOL TEM 1011 electron microscope at 80 kV. Images were captured with an Olympus Mega View III digital camera using iTEM software.

MS

Pierce LC-MS grade water and formic acid were purchased from Thermo Scientific. LC-MS grade acetonitrile and 2-propanol were purchased from Honeywell. Acetic acid was purchased from Sigma-Aldrich.

For hydrophilic interaction chromatography (HILIC), 1 µl of extracted protein solution in 0.5% acetic acid was diluted tenfold with acetonitrile. Approximately 1 µl (95%) of the dilution, corresponding to ∼5% of the total suspension of the unfractionated samples, was pressure-loaded onto in-house prepared pre-columns (360 µm OD×100 µm ID) (Udeshi et al., 2008). Fractionated samples were reconstituted in 1% acetic acid in water and diluted with acetonitrile to 80% acetonitrile. Of the original fractionated solution, 10% was pressure loaded onto pre-columns. Both analytical and pre-columns had a 2 mm Kasil 1624 frit, and the analytical column (360 µm OD×75 µm ID) had a laser-pulled electrospray tip (Ficarro et al., 2009). The HILIC pre-column was packed to 7 cm with 12 µm diameter, 300 Å PolyHYDROXYETHYL A (PHEA) packing material from PolyLC Inc. and was connected to an analytical column packed to 10 cm with 5 µm diameter, 300 Å PHEA packing material. For reverse-phase chromatography, 5% of the extracted protein solution was pressure loaded onto a reverse-phase column in 0.5% acetic acid. The reverse-phase pre-column was packed to 7 cm with 10 µm diameter, 300 Å PLRP-S packing material from Agilent and connected to an analytical column packed to 10 cm with 3 µm diameter, 300 Å PLRP-S packing material.

An Agilent Technologies 1100 Series Binary HPLC system coupled to a Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer operated in low pressure Intact Protein Mode was used to analyze the proteins in each sample.

The PLRP-S pre-column was rinsed with 100% solvent A (0.3% formic acid in water) for 20 min at a flow rate of ∼3 µl/min and then connected to the PLRP-S analytical column. Proteins were eluted using a gradient of 0-60-100% solvent B (72% acetonitrile, 18% 2-propanol, 10% water, and 0.3% formic acid) in 0-60-70 min at a flow of ∼100 nl/min. Highly basic proteins are not retained well on reverse-phase columns, and, for this reason, HILIC was used to retain the highly hydrophilic proteins in the samples. The HILIC PHEA-packed pre-column was washed with solvent B (95% acetonitrile, 15% water, 0.2% acetic acid) for 20 min at a flow rate of ∼3 µl/min and then connected to a PHEA-packed analytical column. Proteins were eluted using a gradient of 100-0% solvent B for 60 min with a 10-min hold of 100% solvent A (0.5% acetic acid in water) before re-equilibrating the column back to 100% solvent B at a flow rate of ∼100 nl/min (Buszewski and Noga, 2012).

Proteins were selected for fragmentation from a 60,000 resolution Orbitrap MS1 scan. Using a 3-s cycle time, proteins with a charge state ≥3 were isolated by the quadrupole with an isolation window of 2 m/z and fragmented by electron transfer dissociation (ETD) for 5 ms and collisional dissociation (Mikesh et al., 2006; Compton et al., 2012). MS2 scans were acquired in the Orbitrap at 120,000 resolution with an automatic gain control target of 1e5.

MS1 and MS2 spectra were manually inspected using Qual Browser (Thermo Scientific). MS2 ETD spectra were deconvolved using the Xtract algorithm (Thermo Scientific) (Senko et al., 1995). The protein sequences

were determined by manual *de novo* analysis of MS2 spectra. Identified proteins were searched by BLAST to identify potential protein matches in bacterial and spider species (Boratyn et al., 2013) (see Table S1, Figs S2, S3).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.C.B.A., J.A.; Methodology: M.R.L., P.M., L.B., S.M., L.K., D.F.H., J.M.E.-L., J.S., J.A.; Formal analysis: J.M.E.-L.; Data curation: D.F.H.; Writing - original draft: J.A.; Writing - review & editing: M.R.L., L.B., M.C.B.A., J.A.; Funding acquisition: M.C.B.A.

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Data availability
All relevant data can be found within the article and its supplementary information.

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