Das Reproduktionssystem der Lanzenfliegen (Diptera: Lonchopteridae)

Dissertation an der Fakultät für Biologie der
Ludwig-Maximilians-Universität München

vorgelegt von

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München, 2024

Diese Dissertation wurde unter der Leitung von Prof. Dr. Martin Heß am Department Biologie II der Ludwig-Maximilians-Universität München und mit der Unterstützung von Dr. Marion Kotrba von der SNSB-Zoologischen Staatssammlung München angefertigt.

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Tag der Abgabe:	31.10.2024
Tag der mündlichen Prüfung:	24.04.2025

Für Marion.

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Abstract

Diese aus insgesamt drei Publikationen bzw. Kapiteln bestehende Dissertation soll zum allgemeinen Verständnis der Mechanismen der postkopulatorischen sexuellen Selektion beitragen, die zur Entstehung von Riesenspermien geführt haben. Zu diesem Zweck wurde das Fortpflanzungssystem der Lanzenfliegen (Diptera: Lonchopteridae) eingehender untersucht. Bei den Vertretern dieser Familie gibt es hierbei bemerkenswerte Unterschiede, wobei einige Arten Riesenspermien produzieren und die entsprechenden Weibchen extrem lange Spermatheken besitzen. Mit einer Länge von 7.500 µm und einer Dicke von 1,3 µm gehört das Spermium von *Lonchoptera fallax* zu den größten bisher bekannten überhaupt. Die Weibchen dieser Art besitzen Spermatheken mit einer Gesamtlänge von etwa 14.000 µm, womit diese etwa viermal so lang wie deren Körper sind.

Anhand von 3D-Rekonstruktionen und Erkenntnissen aus der Elektronenmikroskopie wird in Kapitel I die Morphologie und Ultrastruktur der Spermien der nahe verwandten Art *Lonchoptera lutea* näher beschrieben. Mit einer Länge von 2.200 µm und einer Dicke von 1,4 µm sind diese zwar deutlich kleiner als die von *L. fallax*, können aber dennoch als

This dissertation combines three separate publications (structured in three chapters) and aims to contribute to the understanding of the mechanisms of postcopulatory sexual selection leading to the evolution of giant spermatozoa. In this respect, the reproductive system of spear-winged flies (Diptera: Lonchopteridae) was studied in detail. Among species of this family, there are remarkable variations in the reproductive system, with some species producing giant spermatozoa and the respective females possessing extremely long spermathecae. With a length of 7,500 µm and a width of 1.3 µm, the spermatozoon of *Lonchoptera fallax* ranks among the largest known to date and with a total length of about 14,000 µm, the female spermathecae of this species are more than four times longer than their body.

Based on data from 3D reconstructions combined with insights from electron microscopy, the morphology and ultrastructure of the spermatozoa of the closely related species *Lonchoptera lutea* are described in Chapter I. With a length of 2,200 µm and a width of 1.4 µm, their dimensions are far smaller than those of *L. fallax*, but they can

riesig bezeichnet werden. Anders als die typischen Spermien anderer Fliegen haben sie einen stark asymmetrischen Querschnitt mit nur einem, wenn auch sehr großen Mitochondrienderivat und einem Paar massiver akzessorischer Körper, von denen sich aber nur einer über die gesamte Länge des Flagellums erstreckt.

Um die Entstehung von Riesenspermien in dieser Fliegen-Familie näher zu erforschen, wurden für Kapitel II die Körpergröße, die Hodengröße, die Spermiengröße und die Anzahl der Spermien pro Bündel und pro Hoden bei insgesamt elf *Lonchoptera*-Arten untersucht. Anhand dieser Ergebnisse wird diskutiert, wie diese Merkmale miteinander in Beziehung stehen und wie ihre Entwicklung die Ressourcenverteilung unter den Spermien beeinflusst. Auf Grundlage der Spermienmerkmale und unterstützt durch einen aus DNA-Barcodes abgeleiteten molekularen Stammbaum wird zudem eine phylogenetische Hypothese für die Gattung *Lonchoptera* formuliert.

Um die postkopulatorischen Prozesse zu verstehen, die zur Entstehung von riesigen Spermien bei Lonchopteridae geführt haben, wurden für Kapitel III die Abmessungen der Spermatheken, die wiederum in vier morphologisch, histologisch und funktionell unterschiedliche Abschnitte unterteilt werden können, bei

nevertheless be considered as giant. Unlike the typical brachyceran spermatozoon, they have a highly asymmetrical cross-section with only one single, albeit very large, mitochondrial derivative and a pair of massive accessory bodies, however, only one of which extends throughout the entire length of the sperm tail.

To extend these findings and to address the evolution of giant spermatozoa within this brachyceran family, in Chapter II, body size, testis size, sperm size and the spermatid number per bundle and per testis were examined across eleven *Lonchoptera* species. Results are discussed in terms of how these characters are related to each other and how their evolution affects the resource allocation amongst the spermatozoa. Additionally, based on some discrete spermatozoal characters as well as a molecular tree derived from DNA barcodes a phylogenetical hypothesis for the genus *Lonchoptera* is proposed.

To understand the processes of postcopulatory sexual selection having led to the evolution of giant spermatozoa among Lonchopteridae, in Chapter III the dimensions of the spermathecae, which can be divided into four morphologically, histologically and functionally distinct sections were examined across

elf *Lonchoptera*-Arten untersucht und mit den Maßen der entsprechenden Spermien ins Verhältnis gesetzt. 3D-Rekonstruktionen machten es zudem möglich, das Volumen in diese Überlegungen mit einzubeziehen, was einen neuen Ansatz in diesem Kontext darstellt. Die Ergebnisse zeigen, dass die Spermatheken immer deutlich länger sind als die entsprechenden Spermien und dass ein hochsignifikanter, positiv linearer Zusammenhang zwischen beiden Größen besteht, was auf einen zugrundeliegenden koevolutiven Prozess hindeutet. Auf Grundlage aller Ergebnisse werden einige evolutive Szenarien inklusive eines neuen Ansatzes zu den selektiven Vorteilen längerer Spermien aufgezeigt, um zu diskutieren, wie Spermien- und Spermathekenlänge bei Lonchopteridae abhängig voneinander entstanden sein könnten.

eleven *Lonchoptera* species and related to the dimensions of the respective spermatozoa. Besides, 3D reconstructions made it possible to include the volume in these considerations, which is a new approach in this context. Results show that the spermathecae are per se far longer than the respective spermatozoa and that there is a highly significant positive linear correlation between these two characters, suggesting an underlying coevolutive process. Based on the whole scope of this research some evolutionary scenarios, including a new hypothesis on the selective advantage of increased spermatozoon length, are presented to discuss possible explanations concerning the extant coevolution[1] of spermatozoon and spermathecal length among Lonchopteridae.

[1] Der Begriff „Koevolution“ wird im deutschen Sprachgebrauch definitionsgemäß nur für zwischenartliche Evolutionsprozesse verwendet (z. B. Nabors, 2007), die englischsprachige Literatur verwendet ihn jedoch landläufig auch für evolutive Anpassungsprozesse zwischen Männchen und Weibchen einer Art (z. B. Kotrba et al., 2014; Dallai et al., 2021).

Allgemeine Einleitung

1 Vielfalt der Insektenspermien

Mit mehr als einer Million beschriebenen Arten sind die Insekten die mit Abstand artenreichste Gruppe aller Organismen. Vorsichtige Schätzungen gehen davon aus, dass weltweit sogar bis zu 5,5 Millionen Insektenarten existieren; folglich sind mehr als 80% der Wissenschaft noch nicht bekannt (Stork, 2018). Dallai (2014) führt diesen enormen Artenreichtum und damit den hohen evolutiven Erfolg der Insekten auf ein Zusammenspiel mehrerer Faktoren zurück: (1) Sie haben bereits eine verhältnismäßig lange Evolutions- und Stammesgeschichte, da ihr Ursprung im unteren Devon (vor 411-407 Millionen Jahren) liegt (Engel und Grimaldi, 2004). (2) Sie besitzen einige morphologische Besonderheiten wie Flügel sowie stark modifizierbare Beine und Mundwerkzeuge, die ihnen eine Vielzahl an ökologischen Nischen eröffnen (Mayhew, 2007). (3) Sie durchlaufen eine teils vollständige Metamorphose, die vielgestaltige Umwandlungen erlaubt und die Möglichkeit bietet, dass Larven und Imagines unterschiedliche Ernährungsweisen nutzen und dadurch innerartliche Konkurrenz reduziert wird. Und, (4) sie zeichnen sich durch eine hohe Fortpflanzungsrate aus, die meist auf sexueller Fortpflanzung mit innerer Befruchtung basiert. Gerade die sexuelle Fortpflanzung hat sich als Grundlage und/oder Triebkraft evolutiver Prozesse herausgestellt und ist daher auch die häufigste Fortpflanzungsstrategie vielzelliger Eukaryoten (Nieuwenhuis und James, 2016).

Bei einer Mehrheit der vielzelligen Tiere (Metazoa), bei denen sexuelle Fortpflanzung auftritt, umfasst diese die Kopulation (Übertragung der Spermien des Männchens auf das Weibchen) und die Befruchtung (Verschmelzung eines Spermiums mit einer Eizelle) (Boulton et al., 2023). Zusammen sind sie von fundamentaler Bedeutung für die Weitergabe von Erbinformationen und für die Aufrechterhaltung der genetischen Vielfalt in Populationen. Gerade den länglichen und meist sehr beweglichen Spermien kommt hierbei eine besondere Rolle zu, da sie in vielen Fällen den Weg zur Eizelle finden und meistern müssen, sei es innerhalb oder, bei äußerer Befruchtung, außerhalb des Weibchens. Spermien sind daher die vielgestaltigsten Zelltypen überhaupt (z. B. Pitnick et al. 2009; Schärer et al., 2011) und in keiner Tiergruppe ist die Diversität der Spermien größer als bei den Insekten, obwohl der Grundbauplan jedes Insektenspermiums grundsätzlich gleich ist (Jamieson et al., 1999; Dallai, 2014). Die länglichen Zellen bestehen aus einem apikalen Akrosom, einem meist verlängerten Zellkern, der fast den gesamten vorderen Bereich des Spermiums einnimmt und einem posterior orientierten, länglichen Flagellum (Geißel). Dieses besteht wiederum aus einer mikrotubulären Struktur, dem Axonem, meist

zwei Mitochondrienderivaten und meist zwei akzessorischen Körpern (Dallai, 2014). Die trotzdem schier unzähligen bekannten Größen und Formen der Insektenspermien können durch vielgestaltige Variationen im Grundplan zustande kommen. So macht allein der Zellkern 95% des gesamten Spermienvolumens bei der Termitenart *Reticulitermes lucifuges* aus (Baccetti et al., 1981), fehlt jedoch vollständig bei den apyrenen Spermien von Schmetterlingen (Phillips, 1971). Auch das Akrosom kann ausgesprochen vielgestaltig sein, so fehlt es zum Beispiel gänzlich bei einigen Gallmücken- (Dallai, 1988) oder Köcherfliegenarten (Dallai und Afzelius, 1995; Dallai et al., 1995a) oder es ist über 2.500 µm lang wie bei der Wasserläuferart *Gerris remigis* (Tandler und Moriber, 1966). Die Spermien der Gespenstschrecken besitzen keine Mitochondrien (Baccetti et al., 1973a), wohingegen diese bei dem Gemeinen Rückenschwimmer *Notonecta glauca* über 80% des Spermienvolumens ausmachen (Afzelius et al., 1976). Auch kennt man Insektenspermien gänzlich ohne ein Flagellum, zum Beispiel bei einigen Köcherfliegenarten (Dallai et al., 1975; Dallai und Afzelius, 1994) und solche mit hunderten, wie bei der Termitenart *Mastotermes darwiniensis* (Baccetti und Dallai, 1978). Am augenscheinlichsten wird die Vielfalt des Formenreichtums der Insektenspermien jedoch im Hinblick auf die Spermienlänge. So gibt es sehr kurze Spermien von ca. 1,7 µm Länge bei der oben genannten Termitenart *R. lucifuges* (Baccetti et al., 1981), aber auch Riesenspermien (laut Dallai (2014) alle Spermien, die eine Länge von 1.000 µm und/oder einen Durchmesser von 0,7 µm übertreffen) mit einer Länge von bis zu 58.000 µm bei der Fruchtfliegenart *Drosophila bifurca* (Pitnick et al., 1995). Schon Sivinski (1980) hat diesen enormen Formenreichtum der Insektenspermien treffend als „the bizarre menagerie of insect sperm types“ beschrieben und ist der Meinung, dass die Voraussetzungen und Bedingungen innerhalb der Weibchen möglicherweise die Ursachen für diese Vielfalt sein könnten.

Nicht selten wurde diese enorme Vielfalt der Insektenspermien bereits dazu genutzt, um phylogenetische Fragestellungen anhand von vergleichenden Spermienuntersuchungen zu klären. So postulierte zum Beispiel bereits Baccetti (1979), dass die Ordnung Strepsiptera aufgrund ihrer Spermienultrastruktur nicht zusammen mit der Ordnung Diptera als Gruppe „Halteria“ zusammengefasst werden darf, wie es zum Beispiel noch von Whiting et al. (1997) oder von Wheeler et al. (2001) getan wurde. Vielmehr stellte er sie in die Nähe der Ordnung Coleoptera, wo sie aktuell aufgrund morphologischer und genetischer Untersuchungen auch gesehen wird (Beutel et al., 2019).

So unterschiedlich die Insektenspermien hinsichtlich Morphologie und Ultrastruktur auch sein mögen, sie werden alle zunächst durch den Prozess der Spermatogenese in den Hoden der Männchen gebildet.

2 Spermatogenese

Männliche Insekten besitzen meist zwei Hoden. Jeder besteht aus mehreren, röhrenförmigen Follikeln, an deren Spitze sich „Hub"-Zellen, Urkeimzellen und somatische Stammzellen befinden (Jamieson et al., 1999; Dallai, 2014). Wenn sich eine Urkeimzelle teilt, bleibt eine der Tochterzellen mit den „Hub"-Zellen verbunden und die andere wird zu einem Gonioblasten (Yamashita et al., 2003). Dieser wiederum durchläuft mehrere mitotische Zellteilungen und eine entsprechende Anzahl an Spermatogonien entsteht, die meist zusätzlich von somatischen Zellen umgeben sind (Gorgoń und Świątek, 2021). Bei der Fruchtfliege *Drosophila melanogaster*, bei der die Phasen der Spermatogenese umfassend erforscht sind, entstehen auf diese Weise 16 Spermatogonien, die von zwei somatischen Zellen begleitet werden (Fuller, 1998). Nach und nach werden die Spermatogonien von der Spitze des Follikels verdrängt, beginnen mit synchronen, meiotischen Teilungen und werden zu einer Zyste, einer von einem Epithel aus somatischen Zellen umgebenen Gruppe von Keimzellen. Während dieses Prozesses werden sie als primäre und sekundäre Spermatozyten bezeichnet, je nachdem, welchen Schritt der Meiose sie bereits durchlaufen haben (z. B. Cruz-Landim, 2001). Nach Abschluss der Spermatogenese befinden sich in den Zysten von *D. melanogaster* 64 haploide Spermatiden im selben Stadium der Entwicklung. Bei anderen Taxa kann durch weniger oder mehr mitotische Teilungen vor der Meiose eine andere, wenn auch artspezifische Anzahl an Spermatiden entstehen, die meist jedoch ein Vielfaches von zwei (2^n) darstellt (Jamieson et al., 1999). Es wird vermutet, dass es innerhalb der Insekten einen Zusammenhang zwischen der Anzahl der Spermatiden in einer Zyste und der systematischen Position der betrachteten Gruppe gibt. Nach Virkki (1973) und Lachaise und Joly (1991) haben die basalen Insektenordnungen in der Regel viele Keimzellen pro Zyste, während die weiter abgeleiteten Ordnungen tendenziell weniger haben. So sind es bei manchen Libellenarten bis zu 65.536 (2^{16}) Spermatiden pro Zyste (Virkki, 1973), bei einigen Käferarten jedoch nur 16 (2^4). Nichtsdestotrotz lassen sich immer wieder auch innerhalb einzelner Ordnungen (Dallai, 2014) oder sogar zwischen den verschiedenen Arten einer Gattung Unterschiede in der Anzahl der Keimzellen pro Zyste feststellen. Anschließend durchlaufen die Spermatiden einen Reifungsprozess, die Spermiogenese (Miao et al., 2019), zu dessen Beginn die

Zyste „polarisiert“ wird, sodass alle Zellkerne zum einen Ende und die wachsenden Flagella zum anderen Ende hin orientiert werden (Fabian und Brill, 2012).

Das Akrosom entsteht während der Spermiogenese in den Spermatiden durch die Aktivität eines speziellen Golgi-Apparats, welcher als Akroblast bezeichnet wird. Dieser erreicht später eine apikale Position über dem Zellkern und wird zum Akrosom umgewandelt (Gassner et al., 1972; Farí et al., 2016). Dieses ist im Wesentlichen ein mit hydrolysierenden Enzymen gefülltes Bläschen, mit deren Hilfe die Schutzhülle der Eizelle aufgelöst und durchdrungen werden kann (Dallai, 2014). Es sitzt in seiner endgültigen Form häufig als Kappe über dem vorderen Ende des Zellkerns, also an der Spitze des Spermienkopfes, und besitzt eine konische oder stabförmige Form. Man unterscheidet hiervon grundsätzlich drei morphologische Typen: (1) Ein Akrosom, das unterhalb des akrosomalen Bläschens ein Perforatorium besitzt, einen mit dichtem filamentösem Material gefüllten Raum, wird als „bi-layered“ bezeichnet und stellt die häufigste akrosomale Form bei Insekten dar. (2) Bei einigen Käferarten wie zum Beispiel *Tenebrio molitor* (Baccetti et al., 1973b) besitzt das Akrosom am Ende der Spermiogenese noch Rückstände zwischen dessen Spitze und der Plasmamembran und wird deshalb als „three-layered“ bezeichnet. (3) Das „mono-layered“ Akrosom besteht am Ende der Spermiogenese nur noch aus dem akrosomalen Bläschen. Diese eher seltenere Form des Akrosoms kommt zum Beispiel bei der Eintagsfliegengattung *Cloeon* (Lupetti et al., 2011), bei der Fächerflüglergattung *Mengenilla* (Pohl et al., 2013) oder bei einigen Mückenarten (Dallai et al., 1984) vor.

Die Zellkernmorphogenese während der Spermiogenese umfasst zwei Hauptaspekte: Die Reorganisation des Chromatins (Kondensation) und die Verlängerung des Kerns (Kernformung). In den Spermatozyten ähnelt der Zellkern noch dem somatischer Zellen mit Bereichen an Heterochromatin und Euchromatin. Während der Spermatidenreifung verdichtet sich das Chromatin, bis es in den reifen Spermien so dicht gepackt ist, dass seine Anordnung homogen wirkt. Diese Reorganisation des Chromatins geht vor allem mit dem Austausch der Histone gegen Protamine einher und sorgt mit dafür, dass in reifen Spermien keine Transkription mehr stattfindet (Fabian und Brill, 2012). Die Kernformung wird durch einzelne Mikrotubuli bewirkt, die um den Kern herum angeordnet sind und so dessen Durchmesser reduzieren und ihn bei manchen Arten auch in eine schraubige Form bringen (Schrankel und Schwalm, 1974; Dallai, 2014). Am kaudalen Ende des Kerns kann sich zusätzlich noch eine Ansammlung an dichtem Material von granulärer oder fibröser Struktur finden, die als „centriole adjunct“ bezeichnet wird. Auch wenn das „centriole adjunct“ einen festen Bestandteil der meisten Insektenspermien darstellt und

es wohl hauptsächlich aus Proteinen, Ribonukleoproteinen und Spuren von RNA besteht (Dallai et al., 2016), ist dessen Funktion noch immer unklar. Da bei manchen Taxa der reife Kern zusätzlich eine axiale Einstülpung besitzt, aus der das Axonem des Flagellums entspringt (Dallai et al., 1995b), könnte das „centriole adjunct“ zur Stabilisierung eben dieses Übergangs von Kopf und Schwanz des Spermiums dienen, was von manchen Autoren aber angezweifelt wird (Dallai, 2014; Dallai et al., 2016).

Das Axonem der Insektenspermien ist eine auf Mikrotubuli basierende Zytoskelettstruktur, die sich im Laufe der Evolution stabil erhalten hat und die den Kern des Flagellums bildet (Mencarelli et al., 2008). Es dient als dessen Stützstruktur und sorgt durch die Fähigkeit, sich zu biegen, für dessen Beweglichkeit (Werner und Simmons, 2008). Es entsteht aus einem einzelnen Zentriol, welches bereits in den frühen Spermatiden vorhanden ist. Dieses spielt eine Schlüsselrolle bei der Ausbildung des Basalkörpers, der die Grundstruktur des Axonems bildet. Die meisten Insekten weisen eine 9+9+2-Anordnung der Mikrotubuli auf, was bedeutet, dass es neun einfache äußere Mikrotubuli und neun innere Doppelmikrotubuli gibt, die von zwei einzelnen Mikrotubuli im Zentrum unterstützt werden. Ein Doppelmikrotubuli besteht aus einem vollständigen A-Tubulus und einem „unvollständigen“ B-Tubulus. Ersterer besteht wie die beiden zentralen Mikrotubuli aus 13 Protofilamenten, letzterer nur aus 10. Die einfachen äußeren Mikrotubuli entstehen während der Spermiogenese aus den B-Tubuli. Ihre Anzahl an Protofilamenten kann jedoch je nach Taxa von 13 bis 20 variieren (Dallai und Afzelius, 1990; Dallai und Afzelius, 1991). Ein Dyneinprotein, welches wie Ärmchen an den A-Tubuli ansetzt, wird durch die Möglichkeit der ATP-abhängigen Konformationsänderung für die Biegbarkeit des Axonems verantwortlich gemacht (Werner und Simmons, 2008). Untersuchungen an reifen Spermien deuten zudem darauf hin, dass die Anzahl und Anordnung der Mikrotubuli eine entscheidende Rolle bei der Motilität der Spermien spielt. So können die 9+2 Spermien des Hundeflohs *Ctenocephalus canis* lediglich eine planare Wellenbewegung vollführen, wohingegen die typischen 9+9+2 Spermien anderer Insekten eine komplexe, dreidimensionale, schraubige Schwanzbewegung zeigen (Dallai et al., 2006) – sowohl bei der Vorwärts- als auch bei der Rückwärtsbewegung (Baccetti et al., 1989; Curtis und Benner, 1991). Während der weiteren Differenzierung verlängern sich die Mikrotubuli entlang der gesamten Länge des Flagellums und tragen damit zu dessen Verlängerung bei. Die abschließende Phase der Axonementwicklung beinhaltet die Bildung einer äußeren Hülle, die das Axonem umgibt und es vor äußeren Einflüssen schützt. Diese Hülle besteht aus

verschiedenen Proteinen und trägt zur Stabilität und Funktionsfähigkeit des Axonems bei (Jamieson et al., 1999; Dallai, 2014).

Schon zu Beginn der Spermiogenese bei *D. melanogaster* kommt es zu einer Agglomeration und anschließenden Fusion der Mitochondrien an einer Seite des Zellkerns. Hierbei entstehen zunächst der sogenannte „Nebenkern“ und daraus später die Mitochondrienderivate (Fabian und Brill, 2012). Die zwei Mitochondrienderivate in den frühen Spermatiden sind grundsätzlich von gleicher Größe. Anschließend strecken sie sich während der weiteren Reifung und tragen dadurch maßgeblich zur Verlängerung des Spermiums bei (Noguchi et al., 2012). Durch diesen Differenzierungsprozess unterscheiden sie sich von normalen Mitochondrien in dreierlei Hinsicht: (1) Ihre Größe kann enorme Dimensionen annehmen und in manchen Arten sogar den größten Volumenanteil des Spermiums ausmachen. (2) Die mitochondrialen Cristae sind regelmäßig angeordnet und senkrecht zur Längsachse ausgerichtet. (3) Die mitochondriale Matrix beinhaltet ein auffälliges, parakristallines Material, größtenteils bestehend aus dem Protein „Crystallomitin“, welches einen Großteil des gesamten Mitochondrienderivats einnehmen kann (Jamieson et al., 1999). Bei einigen Arten sind die Mitochondrienderivate in den reifen Spermien in ihrer Größe und Form unterschiedlich, wobei eines fast den gesamten Bereich des Flagellums einnimmt, wohingegen das andere nach einem gewissen Abschnitt endet oder stark reduziert ist (Dallai, 2014). Einige Autoren vermuten, dass die elastischen Eigenschaften der Mitochondrienderivate mit ihren parakristallinen Einschlüssen wahrscheinlich eine Rolle bei der Motilität der Spermien spielen, während ihre Rolle als energieproduzierende Organellen in Frage gestellt wird (Werner und Simmons, 2008). So zeigte eine Studie von Perotti (1973), dass die Aktivität der Cytochrome-c-Oxidase (COX) in den Spermienschwänzen von *D. melanogaster* sehr niedrig ist. Zudem wurde hierbei festgestellt, dass die Mitochondrienderivate intakt und mit unveränderten parakristallinen Strukturen ins Zytoplasma der Eizelle eintreten. Daher geht Perotti (1973) davon aus, dass die Mitochondrienderivate keine intrazellulären Reserven für den Spermienstoffwechsel während des Spermientransfers und des Durchgangs durch den weiblichen Fortpflanzungstrakt sind. Stattdessen könnten sie eine Rolle bei den Prozessen nach der Befruchtung spielen und als Anreicherung von väterlichem Material in der Eizelle, das nicht aus dem Spermienkern stammt, betrachtet werden. Bei Arten der Gattung *Drosophila* bleiben die Mitochondrienderivate sogar während der gesamten Embryonalentwicklung in ihrer Struktur nahezu unverändert bestehen und werden von der Larve nach dem Schlüpfen ausgeschieden (Pitnick und Karr, 1998). Letztlich ist die funktionelle Bedeutung der

Mitochondrienderivate aber noch nicht hinreichend geklärt (Jamieson et al., 1999; Kotrba et al., 2016).

Zusätzlich zu den Mitochondrienderivaten besitzen die Flagella vieler Insektenspermien noch meist zwei akzessorische Körper, die in den verschiedenen Taxa jedoch in Größe und Struktur stark variieren können (Dallai, 2014). Bereits Jamieson (1987) stellte fest, dass nicht alle akzessorischen Körper homolog sind und man nur solche als „wahre" akzessorische Körper bezeichnen sollte, die als Verlängerung des „centriole adjunct" entstehen. Obwohl man noch wenig über deren biochemische Natur weiß, scheinen die akzessorischen Körper, wie das Mitochondrienderivat, stabile Strukturen zu sein (Baccetti et al., 1977; Mencarelli et al., 2000), weshalb Mencarelli et al. (2008) davon ausgehen, dass beide Strukturen dazu beitragen, das Axonem zu stabilisieren, es vor mechanischem Schaden zu schützen und/oder dessen Bewegungsenergie als zusätzliche elastische Komponente zu verstärken. Letztlich ist deren genaue Funktion aber auch noch nicht hinreichend geklärt (Dallai, 2014).

Normalerweise werden Insektenspermien vor deren Freisetzung aus den Hoden in Bündeln zusammengehalten. Innerhalb jedes Bündels sind sie mit großer Regelmäßigkeit parallel zueinander angeordnet. Bei *D. melanogaster* sind die letzten Schritte der Spermiogenese jedoch bereits die Individualisierung und das Aufrollen der reifen Spermien. Die Individualisierung erfordert zuvor die Bildung eines Individualisierungskomplexes, der aus 64 Aktinzapfen besteht, die sich um die 64 nadelförmigen Kerne in einer reifen Spermatidenzyste bilden. Der Individualisierungskomplex bewegt sich fortlaufend entlang der Zyste hinunter, entfernt dabei nicht mehr benötigte Organellen und Zytoplasma und löst interzelluläre Brücken auf. Nach der Individualisierung wickeln sich die Gruppen reifer Spermien umeinander und ziehen sich zur Basis des Hodens zurück. Von hier werden sie anschließend entlassen und zur Samenblase transportiert, wo sie bis zur Kopulation gespeichert werden (Fabian und Brill, 2012).

3 Spermienübertragung und -speicherung

Ein auffälliges Merkmal des megadiversen Stammbaums der Insekten ist die enorme morphologische Vielfalt des Genitalapparats, vor allem bei den Männchen. Es ist wahrscheinlich, dass das Potenzial zur Entwicklung einer extremen Vielfalt von Strukturen und Verhaltensweisen im Zusammenhang mit der Spermienübertragung eng mit der beispiellosen Diversifizierung der Insekten zusammenhängt (Dallai et al., 2013). Bei vielen apterygoten Insekten ist die Übertragung von Spermien mit Hilfe einer Spermatophore, einem

Spermienpaket, die vom Männchen abgesetzt oder übergeben werden kann, üblich. Die Weibchen nehmen diese zur Besamung auf (Proctor, 1998). Alle Männchen pterygoter Insekten, die Spermatophoren produzieren, übertragen diese während der Kopulation direkt in das Weibchen. Dieser interne Spermientransfer stellt eine evolutionäre Neuheit der Pterygota dar. Er erhöht die Wahrscheinlichkeit einer erfolgreichen Befruchtung und mag damit zum evolutionären Erfolg der geflügelten Insekten beigetragen haben (Dallai et al., 2013).

Bei *D. melanogaster* werden die Spermien zwar nicht als Spermatophore, aber trotzdem vom Männchen auf das Weibchen in großer Masse übertragen, die anschließend die Vagina vollständig ausfüllt. Binnen weniger Minuten finden die ersten Spermien ihren Weg in die Spermienspeicherorgane. Davon gibt es bei dieser Fliegenart zwei unterschiedliche Typen (z. B. Miller, 1965), die jedoch nicht repräsentativ für alle Dipteren zu sehen sind: Das ventrale Rezeptakulum und die Spermatheken. Das ventrale Rezeptakulum ist eine lange, spiralige Röhre, die ventral, knapp unterhalb des Eileiters, in die Vagina einmündet. Bei *D. melanogaster* beträgt dessen Gesamtlänge etwa 2 mm, was in etwa auch der Länge eines Spermiums bei dieser Art entspricht (Pitnick et al., 1999). Im Gegensatz dazu sind die beiden Spermatheken pilzförmig und über jeweils einen etwa 0,1 mm langen Gang mit der Vagina verbunden, der dorsal in diese einmündet (Middleton et al., 2006; Hopkins et al., 2020). Die Speicherung einer großen Menge an Spermien in den Spermienspeicherorganen erlaubt es den weiblichen Fliegen, noch bis zu zwei Wochen nach der Kopulation fertile Eier zu produzieren, indem sie die Spermien bis zu deren Verwendung vital und am Leben erhalten (Lefevre und Jonsson, 1962). Diese grundsätzliche Fähigkeit, die bei den meisten Insekten, aber auch bei vielen anderen Taxa vorkommt, kann als Schlüsselmechanismus betrachtet werden, der es den Weibchen erlaubt, sich nicht regelmäßig paaren zu müssen und dadurch reproduktiv unabhängig zu sein (Pascini und Martins, 2017).

4 Riesenspermien und sexuelle Selektion

Nun könnte man vermuten, dass es für die Männchen einen evolutiven Vorteil hat, möglichst viele Spermien pro Kopulation auf das Weibchen zu übertragen, da diese dort über einen gewissen Zeitraum zur Befruchtung der Eier verwendet werden können und somit der Fortpflanzungserfolg des Männchens längerfristig erhöht wird. Bei sehr kleinen Spermien ist diese Methode durchaus denkbar und findet auch bei zahlreichen Insekten Anwendung (z. B. Gilbert, 1981; Pitnick, 1996; Hosken und Ward, 2001; Gage und Morrow,

2003; Kraus et al., 2004; García-González und Simmons, 2007; Sturm, 2014; Esfandi et al., 2019; Strobl et al., 2019). Auch große oder gar riesige Spermien wie die von *D. melanogaster* oder *D. bifurca* werden in den Spermienspeicherorganen der Weibchen gespeichert, jedoch können diese nur in deutlich geringerer Zahl übertragen werden. Zum einen, da der verfügbare Speicherplatz im Weibchen begrenzt ist und zum anderen, da das materielle und energetische Investment des Männchens in ein einzelnes Spermium deutlich größer ist und daher in Summe auch deutlich weniger Spermien produziert werden können (bei vergleichbarer Körper- und/oder Hodengröße). Dass Riesenspermien trotzdem evolutionsbiologisch stabil sind und sich in diversen Taxa unabhängig voneinander entwickelt haben (Fitzpatrick et al., 2022), lässt sich nur durch Prozesse der sexuellen Selektion erklären. Sie ist die treibende Kraft für die Evolution von Geschlechtsmerkmalen und fördert in erster Linie Merkmale, die die Attraktivität eines Individuums für das andere Geschlecht erhöhen und/oder den Zugang zum anderen Geschlecht ermöglichen, also allgemein Merkmale, die den Fortpflanzungserfolg eines Individuums vergrößern. Damit unterscheidet sie sich von der natürlichen Selektion, bei der Merkmale aufgrund ihres Überlebensvorteils in einer bestimmten Umwelt gefördert werden (Witte, 2009).

Es gibt zwei Hauptformen der sexuellen Selektion: Intrasexuelle und intersexuelle Selektion (Campbell et al., 2011). Bei der intrasexuellen Selektion konkurrieren Mitglieder des gleichen Geschlechts miteinander um den Zugang zu Partnern des anderen Geschlechts. Dies führt oft zu geschlechtsspezifischen Merkmalen und Verhaltensweisen, die es den konkurrierenden Tieren ermöglichen, sich gegenüber Rivalen durchzusetzen. Ein bekanntes Beispiel ist der Wettbewerb von Hirschen um die Gunst von Hirschkühen während der Paarungszeit, der häufig durch die Größe des Geweihs entschieden wird. Die intersexuelle Selektion fördert Merkmale, die die Attraktivität eines Individuums für das andere Geschlecht erhöht. Ein klassisches Beispiel ist die Präferenz weiblicher Vögel für Männchen mit lebhaften Federkleidern wie den Schwanzfedern von Pfauen, die auf gute Gene und Überlebensfähigkeiten hinweisen. Bereits Charles Darwin (1871) beschrieb die Idee der sexuellen Selektion in seinem Buch "The Descent of Man, and Selection in Relation to Sex" und führte die Entwicklung vieler ausgefeilter männlicher Merkmale auf die Selektion durch die weibliche Partnerwahl zurück. Die Auswahl durch das Weibchen ist nach wie vor eine Grundlage der Theorie der sexuellen Selektion, wobei die meisten neueren Modelle ein Muster der wechselseitigen Anpassung zwischen weiblicher Präferenz und männlichen Merkmalen vorhersagen (Andersson, 1994). Darüber hinaus ist es wichtig zu betonen, dass sexuelle Selektion nicht immer so offensichtlich ist wie in den oben

genannten Beispielen. In einigen Fällen ist sie eher subtil und manifestiert sich in feinen Verhaltensweisen oder Geruchsstoffen, die für die Partnerwahl von entscheidender Bedeutung sind oder in Merkmalen, die erst nach der Kopulation zum Tragen kommen und die biologische Fitness eines oder beider Geschlechter erhöhen können (Birkhead, 2000; Birkhead und Pizzari, 2002; Firman et al., 2017). Hierzu zählt auch die Ausbildung von Riesenspermien, wie sie zum Beispiel innerhalb der Familie der Lanzenfliegen (Lonchopteridae) auftritt.

5 Lonchopteridae

Zu den Lanzenfliegen (Diptera: Lonchopteridae) gehören kleine (2-5 mm), schlanke, gelb bis braunschwarze Fliegen, deren deutscher Name sich auf ihre lanzettförmig zugespitzten Flügel bezieht. Die Larven sind saprophag und leben meist in der Laubstreu in feuchten oder nassen Umgebungen. Die erwachsenen Tiere sind teils in großer Zahl in feuchten, grasbewachsenen Lebensräumen und in offenen Wäldern anzutreffen, wo man sie oft im Unterholz über Blätter krabbeln sieht (Bährmann und Bellstedt, 1988; Ebejer, 2012). Arten dieser Familie sind aus der Paläarktis, der Orientalis, der Afrotropis und der Nearktis beschrieben. Einzig die weltweit vorkommende Art *Lonchoptera bifurcata* kennt man aus der Australis und der Neotropis (Klymko und Marshall, 2008).

Frühe Untersuchungen (vgl. Anhang A) zeigten, dass die Lanzenfliegen durchaus vielversprechend im Hinblick auf die Untersuchung der evolutiven Prozesse bei der Entstehung von Riesenspermien sein könnten. Zum einen, da in Totalpräparaten der inneren weiblichen Geschlechtsorgane von *L. lutea* bereits lichtmikroskopisch deutlich erkennbare Strukturen auf das Vorhandensein von Riesenspermien hindeuteten und zum anderen, da es sich hierbei um eine recht kleine, taxonomisch gut etablierte Familie innerhalb der Fliegen (Brachycera) handelt, in der nur eine einzige rezente Gattung und 69 Arten (Whittington und Beuk, 2022) beschrieben sind. Sie gehört zu den Phoroidea, die wiederum zu den Aschiza gezählt und in der Nähe der Basis der Cyclorrhapha eingeordnet werden (Wiegmann und Yeates, 2017). Damit stehen die Lonchopteridae phylogenetisch im „Mittelbau“ der Dipteren, was die Vergleichbarkeit mit nah verwandten Taxa grundsätzlich erleichtert. Wie sich im Laufe dieser Dissertation bestätigt hat, weist diese Fliegenfamilie tatsächlich eine enorme Diversität in ihrem Reproduktionssystem auf, vor allem im Hinblick auf deren Spermien und deren Spermienspeicherorgane. So gibt es Arten mit kurzen (ca. 200 µm) und solche mit extrem langen Spermien (ca. 7.500 µm). Die entsprechenden Weibchen haben verhältnismäßig kurze (ca. 1.100 µm) oder extrem lange (ca.

14.000 µm) Spermatheken. Dies deutet auf eine wechselseitige Anpassung dieser Strukturen und damit auf sexuelle Selektion hin. Von der sich wohl hauptsächlich parthenogenetisch fortpflanzenden Art *L. bifurcata* sind die Spermien bisher noch nicht bekannt, auch wenn von unterschiedlichen Autoren immer wieder das Vorkommen vereinzelter Männchen beschrieben wurde (Collin, 1938; Andersson, 1970; Drake, 1983). Im Verlauf dieser Studie hat sich die gezielte Beschaffung von geeignetem Material teilweise jedoch als Schwierigkeit erwiesen (vgl. Anhang B), denn obwohl vor allem die Arten *L. lutea* und *L. bifurcata* in Mitteleuropa noch recht häufig anzutreffen und daher einfach zu beschaffen sind, sind andere Arten selten oder auf bestimmte Habitate und/oder Regionen beschränkt. Um jedoch die Evolution des Reproduktionssystems innerhalb der Familie umfassend zu erfassen und zu verstehen, war es unabdingbar, möglichst viele Arten in diese Überlegungen mit einzubeziehen, weshalb größtenteils auf Sammlungsmaterial zurückgegriffen wurde. Auch das teils unausgeglichene Geschlechterverhältnis in der freien Natur, wie es schon von Bährmann und Bellstedt (1988) beschrieben wurde, verzögerte und erschwerte stellenweise die laufenden Untersuchungen, da gerade von *L. lutea* häufig nur Weibchen und kaum Männchen gefangen werden konnten. Darüber hinaus stellte auch die Bestimmung der Arten teilweise ein Hindernis dar. De Meijere (1906) beschrieb bereits in der Einleitung seines umfassenden Werks über „Die Lonchopteren des palaearktischen Gebietes“, dass die Bestimmung der *Lonchoptera*-Arten mit bedeutenden Schwierigkeiten verbunden ist. Dies führt er vor allem auf die große Variabilität der einzelnen Arten hinsichtlich Färbung und Struktur der Flügeladerung zurück. Als verlässlicher zur Determination der Arten nennt er die Beborstung der Beine und des Scheitels. Beide Merkmale werden maßgeblich auch in heute noch gängigen Bestimmungsschlüsseln der europäischen Arten verwendet (Smith, 1969; Bährman und Bellstedt, 1988), was eine sichere Bestimmung der Arten „im Feld“ aber schier unmöglich machte und daher der Sammlungserfolg oder -misserfolg erst im Labor erkennbar wurde.

Nichtsdestotrotz gelang es im Rahmen dieser Studie elf verschiedene *Lonchoptera*-Arten zu untersuchen und dadurch interessante Einblicke in das Reproduktionssystem der Lanzenfliegen zu gewinnen, die das allgemeine Verständnis über die Prozesse der sexuellen Selektion hinsichtlich der Entstehung von Riesenspermien erweitert haben. Dies war auch das übergreifende Ziel dieser Dissertation, die insgesamt aus drei Kapiteln besteht. Kapitel I zeigt, dass die Spermien von *L. lutea* eine Länge von über 2.200 µm und eine Dicke von 1,4 µm haben, deshalb nach Dallai (2014) als Riesenspermien kategorisiert werden können und dass deren Ultrastruktur von der typischen Spermienultrastruktur anderer

Fliegen (Jamieson, 1987; Jamieson et al., 1999) abweicht, da sie über weite Teile nur ein Axonem, ein einzelnes massives Mitochondrienderivat und einen, strukturell außergewöhnlichen, akzessorischen Körper aufweist. Basierend auf ausgewählten morphologischen Merkmalen der Spermien der verschiedenen Arten der Gattung *Lonchoptera* stellt Kapitel II eine durch DNA Barcoding gestützte Hypothese zur internen Phylogenie der Lanzenfliegen auf, schlussfolgert daraus, dass Riesenspermien ein abgeleitetes Merkmal innerhalb dieser Fliegengattung darstellen und zeigt Zusammenhänge zwischen Spermienanzahl, Spermiengröße und weiteren morphologischen Merkmalen auf. Abschließend zeigt Kapitel III, dass über die untersuchten Arten der Gattung *Lonchoptera* hinweg eine hochsignifikante Korrelation zwischen der Spermienlänge und der Spermathekenlänge besteht, was auf Prozesse der sexuellen Selektion und der Koadaptation hindeutet. Zusätzlich werden abschließend und auf der Basis aller Untersuchungsergebnisse mehrere evolutive Szenarien formuliert, die die Entstehung von Riesenspermien innerhalb der Gattung *Lonchoptera* zu erklären versuchen.

Kapitel I

Morphology and ultrastructure of the spermatozoa of *Lonchoptera lutea* Panzer, 1809 (Diptera: Lonchopteridae)

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Abstract

Lonchoptera lutea males produce giant spermatozoa that are more than 2000 µm long and 1.4 µm wide. Unlike the typical brachyceran spermatozoon, they have a highly asymmetrical cross-section with only a single, albeit very large, mitochondrial derivative and a pair of massive accessory bodies, one of which extends throughout the entire length of the sperm tail. The accessory bodies consist of an electron-dense matrix in which numerous peculiar electron-lucid substructures are embedded. In the mated female, the giant spermatozoa are found inside two tubular spermathecae which are also extremely long, measuring 4000 µm or more.

Veröffentlicht als:

Kotrba, M., Tröster, M., Gensler, H., Ruthensteiner, B., Heß, M., 2021. Morphology and ultrastructure of the spermatozoa of *Lonchoptera lutea* Panzer, 1809 (Diptera: Lonchopteridae). Arthropod Structure & Development 60, 101004.

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Arthropod Structure & Development 60 (2021) 101004

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Morphology and ultrastructure of the spermatozoa of *Lonchoptera lutea* Panzer, 1809 (Diptera: Lonchopteridae)

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ARTICLE INFO

Article history:
Received 28 September 2020
Accepted 29 October 2020
Available online xxx

Keywords:
Giant spermatozoa
Accessory body
Mitochondrial derivative
Spermatheca
Reproduction

ABSTRACT

Lonchoptera lutea males produce giant spermatozoa that are more than 2000 μm long and 1.4 μm wide. Unlike the typical brachyceran spermatozoon, they have a highly asymmetrical cross-section with only a single, albeit very large, mitochondrial derivative and a pair of massive accessory bodies, one of which extends throughout the entire length of the sperm tail. The accessory bodies consist of an electron-dense matrix in which numerous peculiar electron-lucid substructures are embedded. In the mated female, the giant spermatozoa are found inside two tubular spermathecae which are also extremely long, measuring 4000 μm or more.

1. Introduction

In his comprehensive overview on spermatogenesis and sperm structure of Hexapoda, Dallai (2014) states that "spermatozoa, being under a constant evolutionary pressure, exhibit a rapid and often bizarre diversification". This particularly applies to the order Diptera, where the diversity in sperm structure is said to be greater than in all other insect groups taken together (Jamieson et al., 1999). Species within the suborder Brachycera, however, are held to have a rather uniform sperm structure (Jamieson et al., 1999; Dallai 2014), with the exception of those few species provided with giant spermatozoa (Pitnick et al., 1995; Kotrba and Heß 2013). The typical, plesiomorphic condition deduced for Brachycera comprises a filamentous spermatozoon with an apical monolayered acrosome, a compact nucleus, two mitochondrial derivatives and a 9 + 9 + 2 axonemal complex (Jamieson 1987). But detailed knowledge on sperm morphology is available only for very few brachyceran taxa. When structures that appeared to be very large spermatozoa were noticed in the spermathecal ducts of *Lonchoptera lutea* Panzer, 1809 females (Fig. 1), a literature search yielded nothing with respect to the spermatozoa of Lonchopteridae.

Lonchopteridae are a small family of Diptera within the Phoroidea, which belongs to the Aschiza and is placed near the base of the Cyclorrhapha (Wiegmann and Yeates, 2017). Members of this family are small (2–5 mm), slender, yellow to brownish-black flies. The common name spear-winged flies refers to their pointed wings. Of the approximately 63 species worldwide at least 13 species occur in Europe (Pape et al., 2011). Apart from descriptions of the male external genitalia, very little information on the reproductive system of Lonchopteridae is available. De Meijere (1906) describes and illustrates the spermathecae of *L. lutea* as two very long tubular structures of 4 mm length in which spermatozoa are frequently observed. He finds that in *L. bifurcata* (Fallén, 1810; senior synonym of *L. furcata* (Fallén, 1823)) the spermathecae are of similar shape but only 0.7–1 mm long and have never been observed to contain spermatozoa. Sinclair and Cumming (2006) describe and illustrate the spermathecae of an undetermined *Lonchoptera* species from Japan as two sausage-shaped unpigmented receptacles on very long and often tightly coiled ducts.

Because sperm shape often coevolves with the female genital apparatus, Dallai (2014) suggests to extend studies of sperm structure to include an analysis of the respective female genitalia

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https://doi.org/10.1016/j.asd.2020.101004
1467-8039/

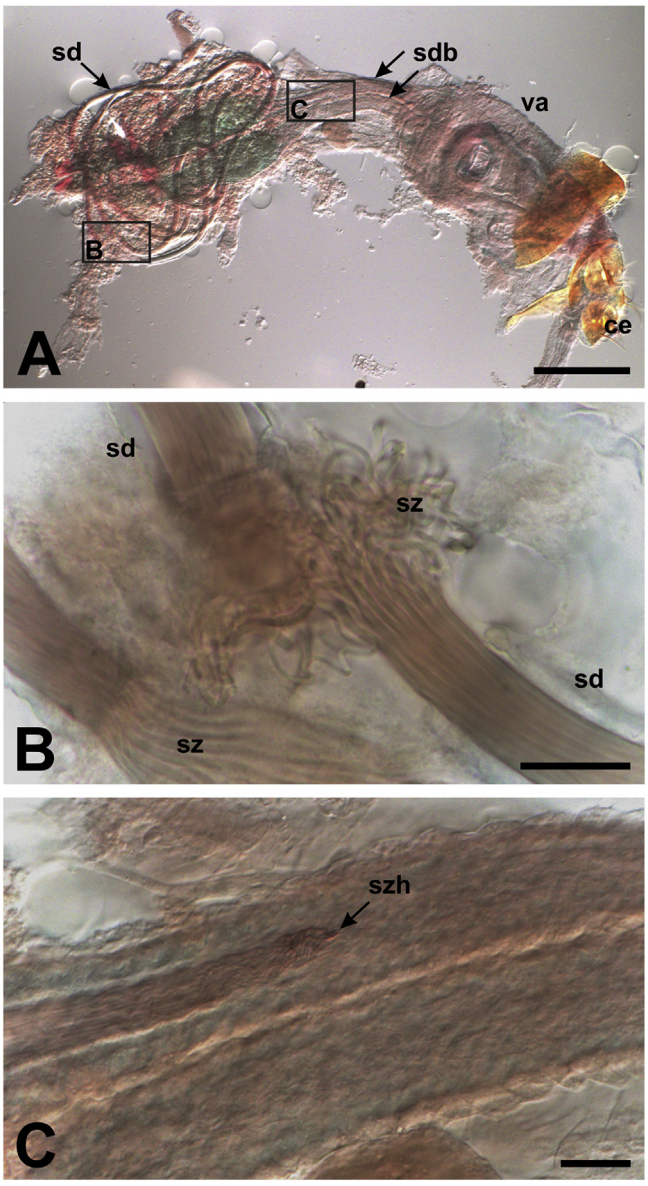

Fig. 1. Female genitalia of *Lonchoptera lutea,* whole mount. **A:** Overview, **B:** Spermatozoa in parallel arrangement inside spermathecal ducts and projecting where ducts are ruptured (magnification from A); **C:** Spermatozoa heads in basal part of spermathecal ducts (magnification from A). ce, cerci; sd, spermathecal ducts; sdb, wide base of spermathecal ducts; sz, spermatozoa; szh, spermatozoa heads; va, vagina. Scale bars: A = 200 μm; B, C = 20 μm.

regarding structural modifications that may occur in the female genital ducts. To resolve the nature of the presumably gigantic spermatozoa in *L. lutea*, the reproductive system of this species was studied with a focus on the dimensions and ultrastructure of the spermatozoa and the dimensions of the spermathecal ducts.

2. Materials and methods

2.1. Animals

L. lutea and *L. bifurcata* adult specimens were collected by sweeping vegetation at the Botanical Garden Munich, Germany, in the summers of 2017 and 2019 and at Andechs, Germany, in the summer of 2020. The specimens were determined according to Bährmann and Bellstedt (1988).

2.2. Preparation/processing

The flies were killed by freezing and the reproductive tracts of males and females were removed in their entirety by dissection of the animals in a droplet of water. For studying the gross morphology, these structures were mounted on microscopic slides in polyvinyl lactophenol with an admixture of chlorazol black E.

Some freshly dissected *L. lutea* testes were fixed in 2.5% glutaraldehyde buffered in 0.1 M phosphate buffer at pH 7.4 for several hours at room temperature, post-fixed in 1% osmium tetroxide for 2 h, and dehydrated in a graded acetone series. The testes were then embedded in Epon resin for micro-CT imaging or in Spurr's resin for semithin and ultrathin sectioning. Serial sections were cut on a Leica UC6 Ultramicrotome using a diamond knife, continuously alternating between 10 consecutive semithin sections (1.5 μm) and 5–10 ultrathin sections (50–70 nm). The semithin sections were mounted on glass slides and stained with Richardson's reagent (Richardson et al., 1960). The ultrathin sections were mounted on formvar coated copper grids and contrasted with uranyl acetate and lead citrate.

2.3. Examination and imaging

Whole mounts and semithin sections were investigated with bright field illumination and/or differential interference contrast (DIC) using a Zeiss Axioskop 2 equipped with Plan-Apochromat 20×, Plan-Apochromat 40× and Plan-Neofluar 100× oil objectives as well as a drawing tube and a Jenoptic Progres Gryphax Subra digital camera. Photographs included a digital scale bar calibrated with a stage micrometer. Some of the photos were stacked by hand from photos at different focal planes using Corel PHOTO-PAINT X5. The dimensions of the spermathecae of four females were measured in sketches done with the help of the drawing tube using a stage micrometer.

Photos of a complete series of semithin sections of one testis were imported into Amira software (version 6.5) to obtain a 3D reconstruction of the testis and the spermatid bundles therein. This method allows measurement of the length of the spermatid bundles and to determine the exact position of the corresponding ultrathin sections with respect to the total length of the spermatozoon. Ultrathin sections were observed with a FEI Morgagni transmission electron microscope operating at 80 kV.

Micro-CT scanning was performed with a Phoenix Nanotom m (GE Measurement & Control, Wunstorf, Germany) CT scanner at a voltage of 80 kV and a current of 325 μA using a tungsten ("standard") target. 1441 projection images were taken during a 360° rotation at a total duration of 120 min. The 3D volume (voxel size 0.6999 μm) was converted to 8-bit and exported using VG Studio Max 2.2 (Volume Graphics GmbH, Heidelberg, Germany). Further visualization procedures were carried out with Amira 6.5 software. The figure plates were created using CorelDRAW X5 and Corel PHOTO-PAINT X5.

3. Results

3.1. Gross morphology

The male internal reproductive system of *L. lutea* comprises two ovoid testes, paired vasa deferentia, one seminal vesicle and an ejaculatory duct with a sperm pump (Fig. 2A). The testes are roughly 500 μm wide and coated with a brown pigment layer. They open into extremely narrow vasa deferentia, which unite after roughly 1300 μm and connect with the seminal vesicle after another 1300 μm. The seminal vesicle has a gastric shape, is about 300–500 μm long and lined by a thick layer of gland cells. No

accessory glands were found. The ejaculatory duct is about twice as wide as the vasa deferentia and roughly 2000 μm long, leading from the seminal vesicle to the aedeagus. It is lined by a thick epithelium and a thin layer of muscles. A muscular sperm pump with a small club-shaped ejaculatory apodeme (Fig. 2B) is inserted near the transition to the hypopygium. Some spermatozoa were dislodged from the male genitalia in the dissection, thus lying separate and allowing a first assessment of the sperm morphology. They are filiform, roughly 2000 μm long and 1.4 μm wide, and arranged in helical waves, which become smaller posteriorly. The head region is much narrower, comparatively straight, and about 20 μm long (Fig. 3).

The spermathecae of *L. lutea* have a wide base and a very long tubular part (Fig. 1A), terminating in short end sections which are surrounded by gland cells. They are unpigmented. The length of the spermathecae ranges from 4.1 to 6.1 mm (average 5.2 mm ± 0.7 s.d., N = 8). In mated females, spermatozoa were found in the spermathecae. They lie densely packed in parallel arrangement and may take up the entire width of the spermathecal duct lumen (Fig. 1B). Their heads, stained dark by chlorazol black E, are closely aligned with each other in the basal part of the spermathecal duct, directed towards the vagina (Fig. 1C).

3.2. Micro-CT

The micro-CT rendering of the testis shows a roundish shape, reminiscent of a lemon (Fig. 4). A small hump at the apex corresponds to the germarium. Inside the apical part of the testis no distinct structures stand out. Towards the middle, long strands of compact material gradually emerge, corresponding to bundles of maturing spermatids (below). They are arranged in a circular fashion along the testis perimeter, sparing the centre. With increasing distance from the germarium, these strands become more and more sharply defined and acquire an undulating shape. The funnel-shaped base of the testis, where the vas deferens originates, does not contain spermatid bundles. Instead, this part is occupied by a patty-shaped plug of dense material.

3.3. Histology

In longitudinal semithin sections of the testis, the described zonation is clearly evident (Fig. 5A). The apical germarium contains an irregular arrangement of spermatogonia and spermatocytes, evident as cells with large nuclei and prominent nucleoli. The adjacent area appears as a more homogenous, generally less strongly stained mass, in which the faint outlines of large cysts can be discerned. Next to that, bundles of early spermatids begin to emerge within the cysts. The spermatid bundles correspond to the strands of compact material observed by micro-CT (above). In the apical part of the testis the bundles are wider and the contained spermatids are less densely packed. Here, each spermatid cross-section consists of 2–3 clearly separated, strongly stained subunits (Fig. 5B). Towards the base of the testis the spermatid bundles and also the individual spermatids become more and more condensed. Here, the spermatid cross-sections appear as solid, strongly stained discs (Fig. 5C). Inside each bundle, the spermatids are aligned with each other and in the same stage of maturation. In the studied testis, the number of spermatids per bundle ranges from 62 to 89 (average 76 ± 6 s.d., N = 75). A large, clearly delimited space next to the origin of the vas deferens does not contain any spermatid bundles. Instead it is occupied by a compact lump of material in which individual spermatozoa are embedded. The vas deferens also contains some individual spermatozoa.

3.4. 3D reconstruction

The reconstruction of one testis from a complete series of serial sections showed the presence of roughly 50 spermatid bundles. The supposedly mature or nearly mature spermatid bundles in the basal part of the testis are arranged in roughly 2 1/2 large circular loops following the curvature of the testis wall, their tapering ends spiraling towards the center (Fig. 6). Additionally, each bundle is twisted in shallow helical turns. The length of the bundles is approximately 2200 μm (±60 s.d., N = 13) on average, maximally 2320 μm.

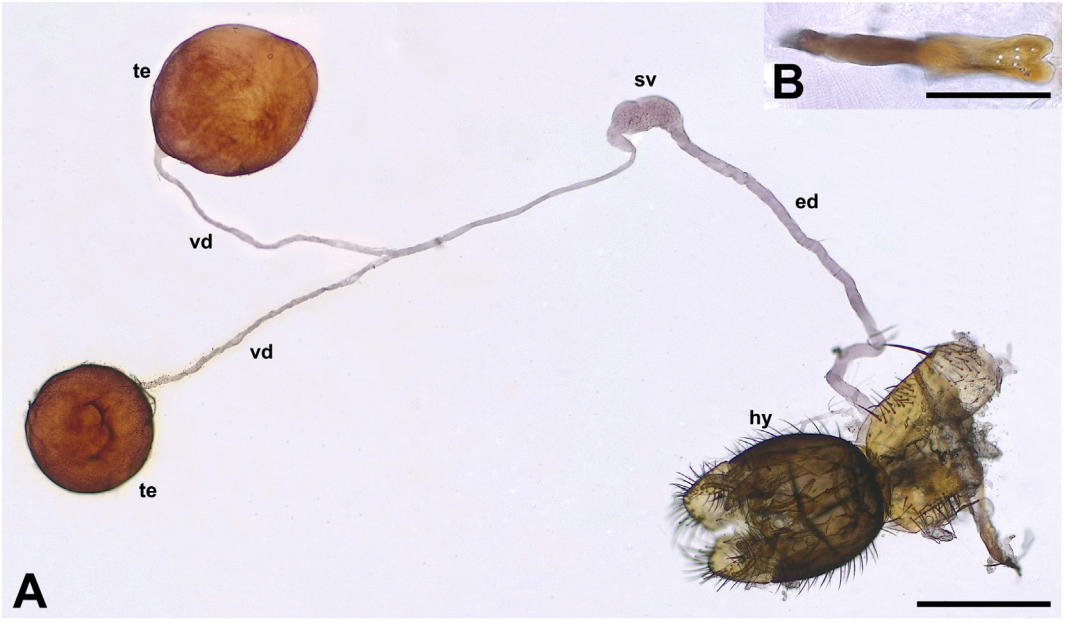

Fig. 2. Male genitalia of *Lonchoptera lutea,* whole mount. **A:** Overview, **B:** Ejaculatory apodeme. ed, ejaculatory duct; hy, hypopygium; sv, seminal vesicle; te, testis; vd, vas deferens. Scale bars: A = 500 μm; B = 100 μm.

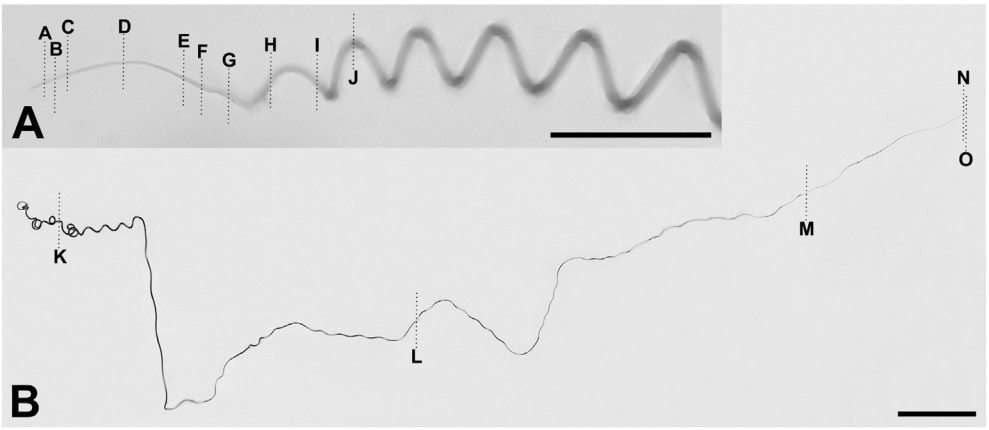

Fig. 3. Spermatozoa of *Lonchoptera lutea,* whole mount. **A:** Head region, overlap region and beginning of flagellar region. **B:** Another spermatozoon in its entire length. Lines and letters in A and B indicate the approximate position of the respective TEM sections in Fig. 7. Scale bars: A = 20 μm; B = 100 μm.

3.5. Ultrastructure

Fig. 7 illustrates the ultrastructure of the spermatozoon. The relative positions of the TEM cross-sections along the length of the sperm cell, as established by the 3D reconstruction of the bundles, are indicated by lines and letters in Fig. 3. Subsequently, the spermatozoon is subdivided into head, overlap, and flagellar region following Curtis et al. (1989) and other authors.

3.5.1. Head region

The head region is about 20 μm long and 0.3 μm wide (Fig. 7A–D). Its apex is constituted by the acrosome, which consists of moderately electron-dense material and is in tight contact with the plasma membrane (Fig. 7A–C). In some cross-sections it contains a small, electron-lucent core (Fig. 7C). The base of the acrosome is tapered, roughly triangular in cross-section, and tightly fitted into a corresponding lateral furrow in the anterior portion of the nucleus. Posterior to this, the nucleus is round in cross-section, with two narrow lateral incisions that start behind the tip and become deeper posteriorly (Fig. 7D). These incisions form a blunt angle of roughly 150°, thus defining a larger "dorsal" and a smaller "ventral" subdivision of the nucleus. The nucleus contains electron-dense, finely granular material that is generally somewhat more condensed near the incisions.

3.5.2. Overlap region

The appearance of a round area in the nucleus marks the beginning of the peg region (Fig. 7E). In subsequent cross-sections the peg regions widens within the dorsal section of the nucleus (Fig. 7F) and the root of the axoneme emerges inside the peg. The axoneme shows the typical brachyceran 9 + 9 + 2 pattern and was not further investigated here. The dorsal part of the nucleus narrows and splits, so that the axoneme comes to lie next to the plasma membrane (Fig. 7G). The split lies off the center line, resulting in a longer and a shorter dorsal nuclear lobe and establishing a fundamental asymmetry that persists throughout almost the entire rest of the spermatozoon. Electron-dense areas, here interpreted as centriole adjunct material, appear between the ends of the dorsal nuclear lobes and the axoneme (Fig. 7G). Moreover, a glob of moderately electron-dense material between the axoneme and the

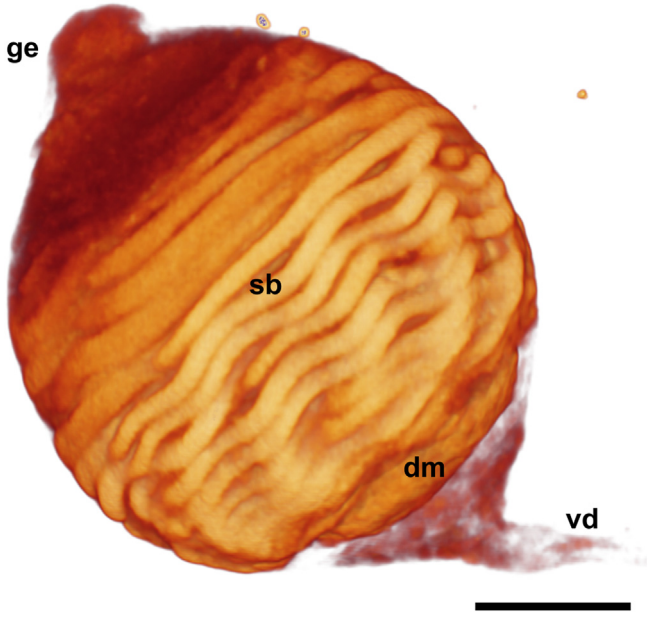

Fig. 4. Testis of *Lonchoptera lutea,* volume rendering of micro-CT data. dm, plug of dense material; ge, germarium; sb, spermatid bundles; vd, vas deferens. Scale bar: 100 μm.

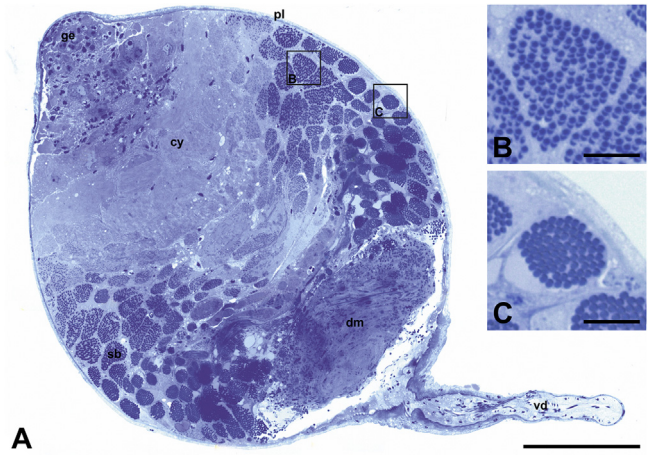

Fig. 5. Testis of *Lonchoptera lutea,* semithin section. **A:** Overview, **B:** spermatid bundle, strongly stained subunits visible (magnification from A), **C:** more mature and condensed spermatid bundle (magnification from A). cy, cysts with early spermatids; dm, plug of dense material with individual spermatozoa embedded; ge, germarium; pl, peritoneal sheath with pigment layer; sb, spermatid bundles; vd, vas deferens. Scale bars: A = 100 μm; B, C = 10 μm.

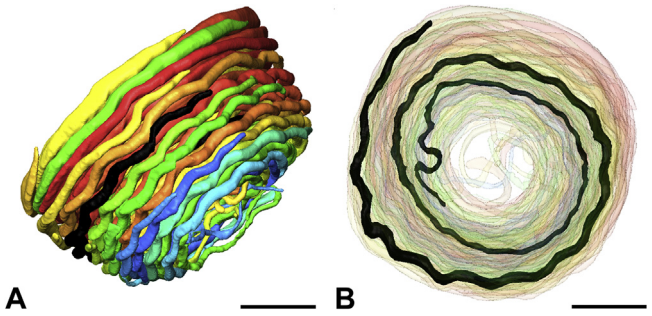

Fig. 6. Individual spermatid bundles within testis of *Lonchoptera lutea,* 3D surface rendering from serial semithin sections. **A:** All reconstructed bundles. **B:** Same reconstruction, rotated to display an entire idividual spermatid bundle (black in both figures), all other bundles rendered transparent. Scale bars: 100 μm.

ventral part of the nucleus constitutes the beginning of the mitochondrial derivative.

Further posterior, the dorsal lobes of the nucleus decline while the adjacent electron-dense areas grow. Subsequently this space is occupied by the massive, curved accessory bodies. They consist of an electron-dense matrix in which numerous substructures are embedded (Fig. 7H–M). The substructures are elongate in cross-section, suggesting that their three-dimensional shape is band-like. At higher magnification they appear as regularly spaced rows of electron-lucent spots, somewhat reminiscent of a cross-section through a strip of corrugated cardboard (Fig. 8D). These rows are about 20 nm wide and 100 nm long on average. They are embedded in the matrix with an average distance of 14 nm, not less than about 6 nm. In early stages of spermatogenesis, i. e. in spermatid bundles that are located closer to the germarium, the substructures appear as larger electron-lucent round dots with an electron-dense spot in the center (Fig. 8A). These dots are about 60 nm wide and embedded in the matrix with an average distance of about 24 nm, not less than 15 nm. Stages with mixtures of both forms of substructures occur (Fig. 8B), suggesting that the dots mature into the rows during spermatogenesis.

The ventral part of the nucleus projects posteriorly in the shape of a rapidly shrinking band, which is embraced between the ventral margin of one accessory body (ab1) and the ventral inner margin of the other (ab2) (Fig. 7H). This second accessory body, which emerges as the smaller one in anterior cross-sections, posteriorly increases in size and replaces the end of the nucleus (Fig. 7I). The complete zone of overlap, from the beginning of the peg region to the posterior end of the nucleus, has a total length of approximately 30 μm. Its width increases to about 0.9 μm posteriorly.

3.5.3. Flagellar region

For a short sector posterior to the end of the nucleus, the two accessory bodies are about equal in size and shape, and the cross-section of the spermatozoon is symmetrical once more (Fig. 7I). But while the first accessory body (ab1) rapidly tapers and ends, the second one (ab2) continues to increase (Fig. 7J). From here on posteriorly, with the exception of the very tip of the tail, the spermatozoon shows a rather uniform cross-section, comprising the axoneme and a large accessory body that curves around the side of a single, large mitochondrial derivative (Fig. 7K–M). Within one spermatid bundle all cross-sections have the same orientation and across all spermatid bundles in the testis the accessory body is positioned to the same side of the axoneme.

The width of the flagellum increases, reaching its maximum of about 1.4 μm after about 500 μm of the total sperm length and then gradually decreasing again. The mitochondrial derivative occupies the largest share of the flagellum cross-section, thus largely defining the shape of the sperm tail. The principal accessory body (ab2) forms a large C-shaped band that is fitted against the axoneme at one end and curves around the mitochondrial derivative, tapering into a blunt edge. At the widest part of the sperm tail the accessory body contains up to 55 of the electron-lucent substructures described above.

Nearing the tip of the sperm tail the accessory body (ab2) is the first to disappear from the cross-sections, shortly followed by the mitochondrial derivative (Fig. 7N). Finally the arrangement of the microtubules within the axoneme becomes irregular and then disappears (Fig. 7O).

4. Discussion

4.1. Size

According to Dallai (2014), insect spermatozoa exceeding 1000 μm in length and/or 0.7 μm in width are to be considered exceptional. With a length of about 2200 μm and a width of 1.4 μm the spermatozoa of *L. lutea* can therefore justly be classified as giants. Their extraordinary length concurs with the likewise extraordinary length of the females' spermathecae, which exceed 4 mm in *L. lutea* and were frequently observed to contain spermatozoa throughout their entire length (De Meijere 1906). Notably, in the congeneric species *L. bifurcata,* the spermathecae are of similar shape but only 0.7–1 mm long and have never been observed to contain spermatozoa (De Meijere 1906). De Meijere (1906) interprets this as an adaptation to parthenogenesis. Without more information on the conditions in other Lonchopteridae, however, speculations on the potential coevolution of the male and female structures and on the phylogenetic origin of the giant spermatozoa in this family must remain vague and are not further addressed here. A comparative study across a larger range of *Lonchoptera* species is in preparation to address these issues.

4.2. Morphology

Apart from their extraordinary size, the spermatozoa of *L. lutea* also have an unusual internal structure, involving a highly asymmetrical cross-section with a single, very large mitochondrial derivative and a very prominent accessory body with peculiar ultrastructure.

4.2.1. Mitochondrial derivative

Two mitochondrial derivatives are present in the ground plan of Brachycera (Jamieson 1987). They are sometimes asymmetrical, e. g. in Empididae, Drosophilidae and Muscidae (Jamieson et al., 1999). The complete reduction of one of the mitochondrial derivatives, as observed in *L. lutea*, constitutes a rare derived condition. It has been reported to occur e. g. in some Calliphoridae (Name et al., 2012a). In *L. lutea*, the single mitochondrial derivative is extremely large in terms of both width and length, constituting the largest volume of all cell components. Similarly large mitochondrial derivatives that are many times wider than the axoneme have been found as a derived condition in *Drosophila kanekoi* Watabe and Higuchi, 1979 (Dallai et al., 2008). Moreover Kotrba et al. (2013, 2016) described their occurrence in *Diasemopsis comoroensis* Carr et al., 2006. The functional significance of the mitochondrial derivatives, specifically such gigantic ones, is unresolved (Jamieson et al., 1999; Kotrba et al., 2016).

4.2.2. Accessory bodies

The most enigmatic component of the *L. lutea* spermatozoon are the large accessory bodies with their peculiar substructure.

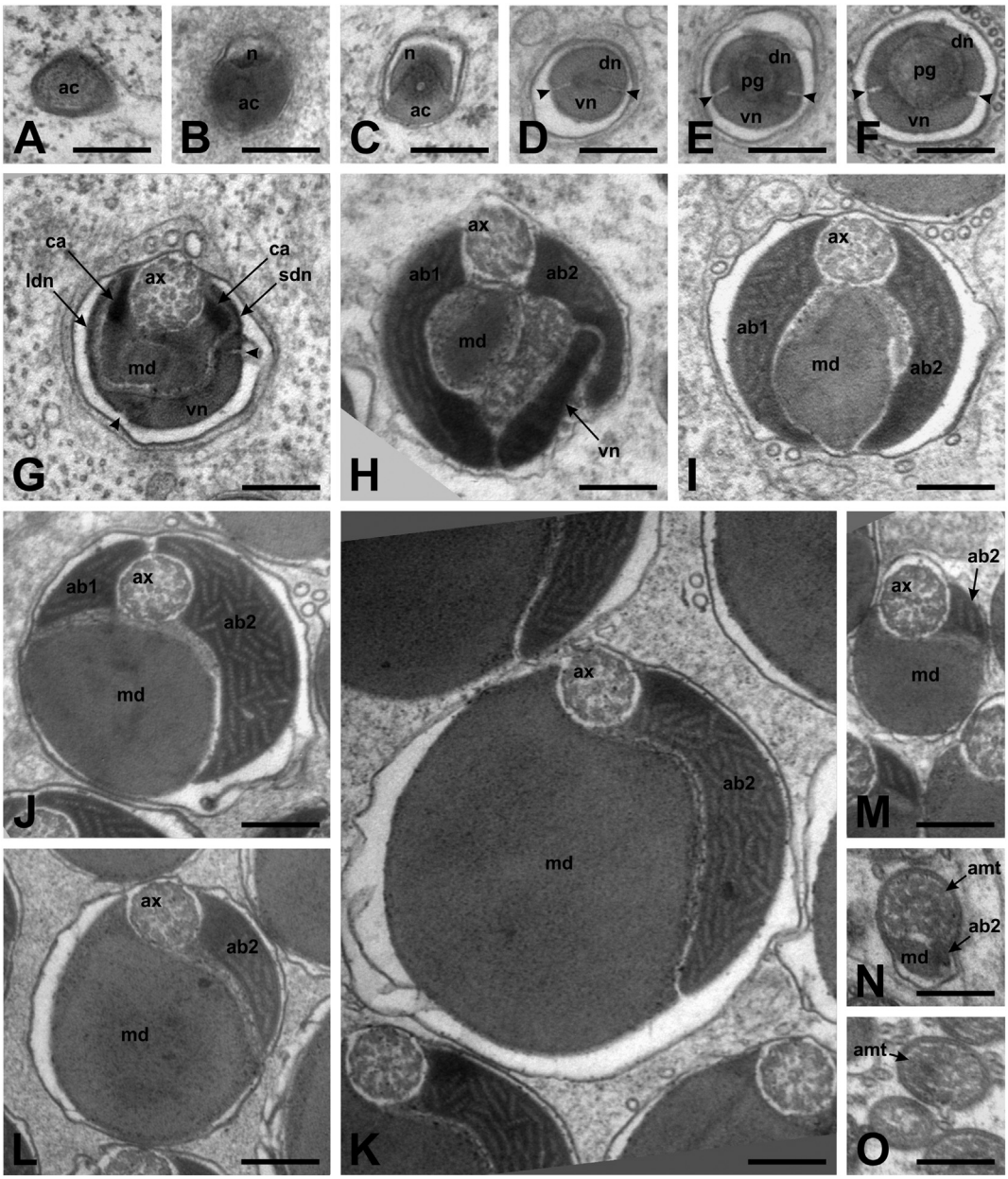

Fig. 7. Spermatozoon of *Lonchoptera lutea*, TEM cross-sections. Parts A-O positioned along the length of the spermatozoon as indicated by the respective letters and lines in Fig. 3. ab1, smaller accessory body; ab2, principal accessory body; ac, acrosome; amt, axonemal microtubuli in loose arrangement; ax, axoneme; ca, centriole adjunct material; dn, dorsal part of nucleus; ldn, larger dorsal lobe of nucleus; md, mitochondrial derivative; n, nucleus; pg, peg-like nuclear insertion of axoneme; sdn, smaller dorsal lobe of nucleus; vn, ventral part of nucleus; arrowheads, nuclear incisions. Scale bars: 0.3 μm.

Although accessory bodies are constant components of insect spermatozoa (Dallai 2014), the nature of these structures is generally not well understood. Jamieson (1987) established that "it is doubtful that all accessory bodies are homologous but we may define as 'true' accessory bodies those, constituting the most common type, which are derived as extensions of the centriole adjunct from which they may or not become separated". Not much substantial insight on these structures has accrued since. Dallai et al. (2016) summarized that in advanced spermatogenesis the dense and compact material that is deposited beneath the nucleus constitutes what has been described as centriole adjunct, and this in many insect orders gives rise to two prolongations, or accessory bodies, of variable length that accompany the axoneme for most of its length.

In the branch leading to the Diptera, accessory bodies are purportedly lost (Jamieson 1987). But various scattered occurrences of accessory bodies or centriole adjuncts have been reported within that order (Curtis et al., 1989; Jamieson et al., 1999; Dallai et al., 2016). For the suborder Brachycera, to which *L. lutea* belongs, the respective information is briefly reviewed here.

The closest relative of *L. lutea*, in which the sperm ultrastructure has been studied, is the phorid *Megaselia scalaris* (Loew, 1866). Like Lonchopteridae, Phoridae are placed in the superfamily Phoroidea, a basal branch of the Cyclorrhapha: Aschiza (Wiegman and Yeates, 2017). Curtis et al. (1989) described and illustrated the accessory body in the overlap region of the spermatozoon of *M. scalaris* as a massive C-shaped structure, in contact with the axoneme at one end, and curving around the lateral side of one of the mitochondrial

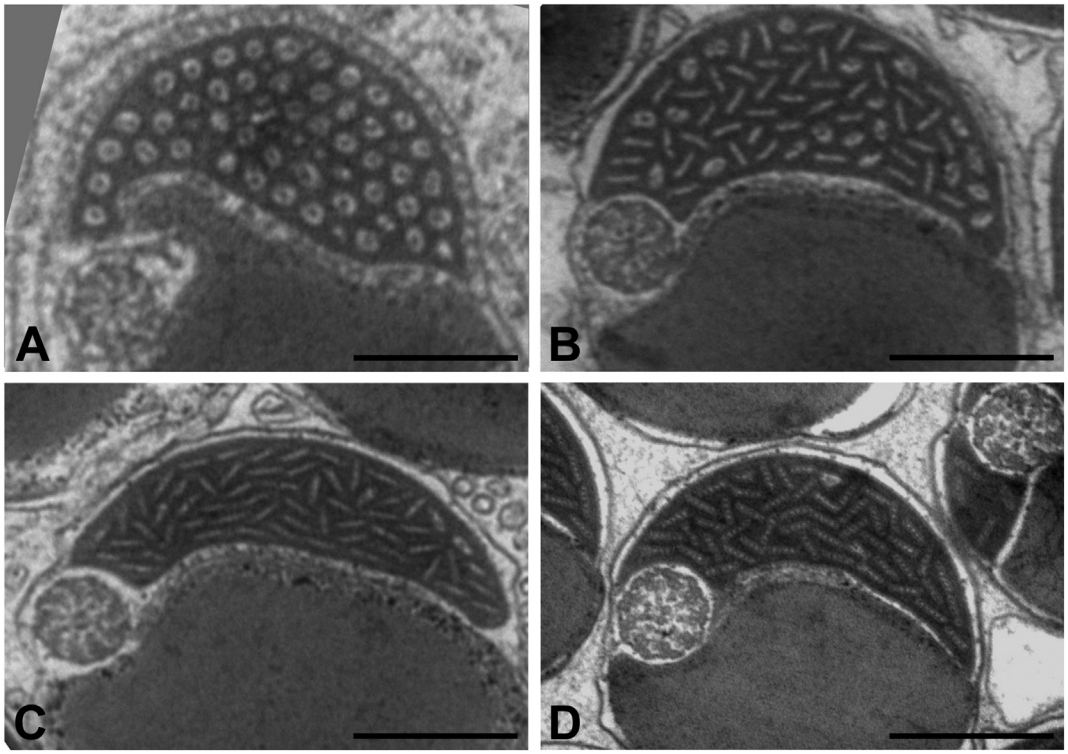

Fig. 8. Accessory body of *Lonchoptera lutea* spermatids with electron-lucent substructures, TEM cross-sections showing various stages of maturation/development. **A:** Early spermatid with substructures appearing as larger electron-lucent round dots with an electron-dense spot in the center, **B:** Later stage with some of the substructures narrower and elongated, **C:** Stage with all substructures narrow and elongated, **D:** Mature spermatozoon with substructures consisting of rows of regularly spaced electron-lucent spots. Scale bars: 0.5 μm.

derivatives. It consists of an electron-dense matrix with a vaguely lamellated appearance. Posteriorly, the accessory body rapidly narrows and is absent in the second half of the flagellum.

The only other Aschiza taxon, in which the sperm ultrastructure has been studied, is the syrphid *Eristalinus tarsalis* (Macquart, 1855). For this species Wu and Zhou (1986) report a big centriole adjunct located in a short neck, i. e. overlap region, between the head and flagellum of the mature spermatozoon, but very little detail is visible in the respective photograph (Wu and Zhou 1986: Figs. 5–7). In the flagellum a centriole adjunct or accessory body are lacking. From this it appears that the centriole adjunct is relatively short and does not give rise to an accessory body.

For the Orthorrhapha, which occupy a more basal position within the Brachycera than the Cyclorrhapha: Aschiza, two species have been studied. For the bombyliid *Anthrax* sp. Jamieson et al. (1999) report an exceptionally long, single, approximately semicircular accessory body, which symmetrically embraces the two mitochondrial derivatives opposite of the axoneme. In the respective figures it appears as a solid, homogenously electron-dense structure. Anteriorly it originates from two lateral bodies posterior of the nucleus which "comprise the centriole adjunct or, alternatively, may be regarded as a paired commencement of the accessory body". Posteriorly it persists for a large portion of the length of the axoneme.

The spermatozoa of the stratiomyiid *Hermetia illucens* (Linnaeus, 1758) were independently described by Kotzé et al. (2019) and Malawey et al. (2019). In the published cross-sections various structures are interpreted as centriole adjunct material by one and/or the other author. From anterior to posterior these are specifically (1) various structures of different densities between the axoneme and the nucleus in the overlap region (Kotzé et al., 2019: Fig. 3A and C; Malawey et al., 2019: Fig. 4C–F). (2) A single, asymmetrically arranged, very electron-dense U-shaped structure (Kotzé et al., 2019: Fig. 3B and C; Malawey et al., 2019: Fig. 4F). Malawey et al. interpret this as centriole adjunct material "same as the accessory body of *M. scalaris*", whereas Kotzé et al. label it as nucleus. (3) A single body positioned between the axoneme and the two mitochondrial derivatives (Kotzé et al., 2019: Fig. 3E-G; Malawey et al., 2019: Fig. 4 G). This structure is filled with microtubules flanked by a thin electron dense U-shaped lamina. (4) A symmetrical two-winged structure posterior of this (Kotzé et al., 2019: Fig. 4A and B; Malawey et al., 2019: Fig. 4H and I), which according to Kotzé et al. (2019) gives rise to two accessory bodies posteriorly. The relations between these various structures remain unclear, not least because of their controversial interpretation by the respective authors.

In the Cyclorrhapha: Schizophora, which occupy a more derived position than the Cyclorrhapha: Aschiza, a number of taxa has been studied with respect to the spermatozoa ultrastructure. For the tephritid *Bactrocera oleae* (Rossi, 1790), Dallai and Afzelius (1991) report and illustrate a short centriole adjunct. It forms a homogenous electron-dense bar embedded between the two mitochondrial derivatives, present in the overlap region immediately behind the centriolar region, but absent in the main portion of the sperm tail. Very similar conditions are found in a number of calyptrate taxa, such as the scathophagid *Scathophaga* sp. (Dallai and Afzelius 1990), the muscid *Musca domestica* Linnaeus, 1758 (Gassner et al., 1972), and the calliphorids *Chrysomya megacephala* (Fabricius, 1794) (Name et al., 2010), *Lucilia cuprina* (Wiedemann, 1830) (Name et al., 2012a), and *Cochliomyia hominivorax* (Coquerel, 1858) (Name et al., 2012b), but there the centriole adjunct may extend further into the region of the flagellum. In the diopsid *Teleopsis whitei* (Curran, 1936) the electron-dense central bar is more substantial (Kotrba 1993), and in the confamilial *D. comoroensis*, which

produces giant spermatozoa (Kotrba and Heß 2013; Kotrba et al., 2016) it is very greatly enlarged and extends through the entire length of the sperm tail. Various drosophilid species have been studied in detail (e. g. Dallai and Afzelius 1991; Dallai et al., 2008; Fabian and Brill 2012, Gracielle et al., 2016, Rego et al., 2013), including the giant spermatozoa of *Drosophila bifurca* (Patterson and Wheeler, 1942), which represent the longest sperm in the animal kingdom, reaching a length of 58,000 μm (Pitnick et al., 1995). But for this family no centriole adjunct or accessory body is reported for the mature spermatozoon.

In all likelyhood, the electron-dense bar, which is embedded between the two mitochondrial derivatives in the median axis of the sperm tail, is homologous across all Schizophoran taxa where it occurs. Beyond this clade, the spacial arrangement is very different and the homology relations are uncertain. Compared with the accessory body here described for *L. lutea*, the accessory body in *M. scalaris* is arguably the most similar regarding its shape and asymmetrical arrangement. This matches the fact that Phoridae and Lonchopteridae are quite closely related. The similarity could therefore possibly be based on homology, but in *M. scalaris* the accessory body occupies a much shorter portion of the sperm tail and lacks the characteristic electron-lucent substructures. The electron-dense U-shaped structure in the spermatozoon overlap region of the orthorrhaphan *H. illucens* is also reminiscent of the accessory body in *L. lutea*. But because the true identity of the U-shaped structure in *H. illucens* is controversial, and also because this taxon is phylogenetically remote from *L. lutea*, the structures can not be homologized with certainty.

The few available records on the presence, morphology, and ultrastructure of accessory bodies in brachycerous Diptera regard phylogenetically remote taxa and are very different in appearance. Therefore, although the C-shaped structure in the spermatozoa of *L. lutea* is here classified as accessory body, its relationship with the respective structures in other Brachycera cannot be established yet.

5. Conclusions

The new results add to the growing body of evidence that brachyceran spermatozoa are not as uniform as once believed. Particularly, the occurrence of gigantic mitochondrial derivatives and of sometimes very substantial accessory bodies, which constitute an enigmatic type of cell organelles, deserves further study. This will undoubtedly be costly, requiring technologies such as TEM and laser scanning microscopy, the application of a range of molecular markers, etc. But it will also be rewarding, as it can hardly fail to produce new and important insights, regarding, e. g., (1) the ultrastructural and biochemical properties and the cellular origin of these structures, (2) their questionable homology across taxa and thus the potential phylogenetic signal, and (3) their function with respect to sperm motility and/or longevity, various kinds of sexual selection, and/or male investment.

Author contributions

MK and MT designed the study, obtained and dissected specimens, acquired light microscope images, evaluated the results, and wrote the manuscript. BR acquired Micro-CT data and provided support in 3D procedures. HG performed semithin and ultrathin sectioning. HG and MT acquired TEM images. MT carried out 3D data processing and designed the figures. MH supervised the TEM study. All authors revised the manuscript and read and approved the final version.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

The authors would like to thank Eva Lodde-Bensch (SNSB-ZSM, Munich, Germany) for helping with semi-thin sectioning, Romano Dallai (Department of Life Sciences, University of Siena, Italy) for helpful comments on the manuscript, and Eva Facher (Department of Biology, LMU, Germany) for her efforts in trying to produce SEM images of spermatozoa of *L. lutea*.

References

Bährmann, R., Bellstedt, R., 1988. Beobachtungen und Untersuchungen zum Vorkommen der Lonchopteriden auf dem Gebiet der DDR, mit einer Bestimmungstabelle der Arten. (Dipt., Lonchopteridae). Deut Entomol. Z. 35, 265–279.

Curtis, S.K., Benner, D.B., Musil, G., 1989. Ultrastructure of the spermatozoon of *Megaselia scalaris* Loew (Diptera: Brachycera: Cyclorrhapha: Phoridae). J. Morphol. 200, 47–61.

Dallai, R., 2014. Overview on spermatogenesis and sperm structure of Hexapoda. Arthropod Struct. Dev. 43, 257–290.

Dallai, R., Afzelius, B.A., 1990. Microtubular diversity in insect spermatozoa: results obtained with a new fixative. J. Struct. Biol. 103, 164–179.

Dallai, R., Afzelius, B.A., 1991. Sperm flagellum of *Dacus oleae* (Gmelin) (Tephritidae) and *Drosophila melanogaster* Meigen (Drosophilidae) (Diptera). Int. J. Insect Morphol. Embryol. 20, 215–222.

Dallai, R., Mercati, D., Giusti, F., 2008. Structural organization of the "zipper line" in *Drosophila* species with giant spermatozoa. J. Struct. Biol. 161, 43–54.

Dallai, R., Paoli, F., Mercati, D., Lupetti, P., 2016. The centriole adjunct of insects: need to update the definition. Tissue Cell 48, 104–113.

De Meijere, J.C.H., 1906. Die Lonchopteren des palaearktischen Gebietes. Tijdschr. Entomol. 49, 44–98.

Fabian, L., Brill, J.A., 2012. *Drosophila* spermiogenesis: big things come from little packages. Spermatogenesis 2, 197–212.

Gassner, G., Klemetson, D.J., Richard, R.D., 1972. Spermiogenesis in house fly, *Musca domestica* L. (Diptera: Muscidae): a transmission electron microscope study. Int. J. Insect Morphol. Embryol. 1, 105–120.

Gracielle, I.M.S., Tidon, R., Bao, S.N., 2016. Structure and ultrastructure of spermatozoon in six species of Drosophilidae (Diptera). Tissue Cell 48, 596–604.

Jamieson, B.G., 1987. The Ultrastructure and Phylogeny of Insect Spermatozoa. Cambridge Univ. Press, Cambridge, UK.

Jamieson, B.G.M., Dallai, R., Afzelius, B.A., 1999. Insects: Their Spermatozoa and Phylogeny. Science Publishers, Enfield, New Hampshire, USA.

Kotrba, M., 1993. Das Reproduktionssystem von *Cyrtodiopsis whitei* Curran 1936 (Diopsidae, Diptera) unter besonderer Berücksichtigung der inneren weiblichen Geschlechtsorgane. Bonn. Zool. Monogr. 33, 1–115.

Kotrba, M., Heß, M., 2013. Giant spiral shaped spermatozoa of *Diasemopsis comoroensis* (Diptera, Diopsidae) with a unique ultrastructural component. Tissue Cell 45, 443–445.

Kotrba, M., Heß, M., Dallai, R., 2016. Giant spermatozoa of *Diasemopsis* (Diopsidae, Diptera) – structural, ultrastructural and functional aspects. Arthropod Struct. Dev. 45, 42–56.

Kotzé, R.C.M., Muller, N., du Plessis, L., van der Horst, G., 2019. The importance of insect sperm: sperm ultrastructure of *Hermetia illucens* (black soldier fly). Tissue Cell 59, 44–50.

Malawey, A.S., Mercati, D., Love, C.C., Tomberlin, J.K., 2019. Adult reproductive tract morphology and spermatogenesis in the black soldier fly (Diptera: Stratiomyidae). Ann. Entomol. Soc. Am. 112, 576–586.

Name, K.P.O., Barros-Cordeiro, K.B., Filho, J.B.G., Wolff, M., Pujol-Luz, J.R., Báo, S.N., 2012a. Structure and ultrastructure of spermatozoa and spermiogenesis in three species of *Lucilia* Robineau-Desvoidy, 1830 (Diptera: Calliphoridae). J. Morphol. 273, 160–172.

Name, K.P.O., Barros-Cordeiro, K.B., Filho, G., Wolff, M., Pujol-Luz, J.R., Báo, S.N., 2012b. Morphological and cytochemical aspects of spermatozoa in the genus *Cochliomyia* (Diptera: Calliphoridae). J. Electron. Microsc. 61, 415–422.

Name, K.P.O., Pujol-Luz, J.R., Báo, S.N., 2010. Structure and ultrastructure of spermatozoa of *Chrysomya megacephala* (Diptera: Calliphoridae). Micron 41, 853–860.

Pape, T., Blagoderov, V., Mostovski, M.B., 2011. Order Diptera Linnaeus, 1758. In: Zhang, Z.-Q. (Ed.), Animal Biodiversity: an Outline of Higher-Level Classification and Survey of Taxonomic Richness, 3148. Zootaxa, pp. 222–229.

Pitnick, S., Spicer, G.S., Markow, T.A., 1995. How long is a giant sperm? Nature 375, 109.

Rego, L.N.A.A., Silistino-Souza, R., Azeredo-Oliveira, M.T.V.D., Madi-Ravazzi, L., 2013. Spermatogenesis of *Zaprionus indianus* and *Zaprionus sepsoides* (Diptera,

Drosophilidae): cytochemical, structural and ultrastructural characterization. Genet. Mol. Biol. 36, 50–60.
Richardson, K.C., Jarret, L., Finke, E.H., 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol. 35, 313–323.
Sinclair, B.J., Cumming, J.M., 2006. The morphology, higher-level phylogeny and classification of the Empidoidea (Diptera). Zootaxa 1180, 1–172.
Wiegmann, B.M., Yeates, D.K., 2017. Phylogeny of Diptera. In: Kirk-Spriggs, A.H., Sinclair, B.J. (Eds.), Manual of Afrotropical Diptera, Suricata 4, vol. 1. South African National Biodiversity Institute, Pretoria, pp. 253–265.
Wu, D.-S., Zhou, H.-X., 1986. Studies on ultrastructure of spermatozoa from flies: *Eristalinus tarsalis* (Maq.). Acta Entomol. Sin. 29, 25–28.

Kapitel II

Variation of sperm size and evolution of giant spermatozoa in Lonchopteridae (Diptera)

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Abstract

Among species of the spear-winged flies (Lonchopteridae) there is remarkable variation in sperm size, with some species producing giant spermatozoa. With a length of 7500 µm and a width of 1.3 µm the spermatozoon of *Lonchoptera fallax* ranks among the largest known to date. In the present study body size, testis size, sperm size, and spermatid number per bundle and per testis were examined across 11 *Lonchoptera* species. Results are discussed in terms of how these characters are related with each other and how their evolution affects the resource allocation amongst spermatozoa. Based on some discrete morphological characters and a molecular tree derived from DNA barcodes a phylogenetic hypothesis of the genus *Lonchoptera* is proposed. The occurrence of giant spermatozoa in Lonchopteridae is compared to convergent occurrences reported in other taxa.

Veröffentlicht als:

Tröster, M., Kotrba, M., Heß, M., 2023. Variation of sperm size and evolution of giant spermatozoa in Lonchopteridae (Diptera). Arthropod Structure & Development 75, 101285.

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Arthropod Structure & Development 75 (2023) 101285

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

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ARTICLE INFO

Article history:
Received 25 April 2023
Received in revised form
31 May 2023
Accepted 6 June 2023
Available online xxx

Handling Editor: Dr G. Scholtz

Keywords:
Giant spermatozoa
Reproduction
Phylogeny of Lonchopteridae

ABSTRACT

Among species of the spear-winged flies (Lonchopteridae) there is remarkable variation in sperm size, with some species producing giant spermatozoa. With a length of 7500 μm and a width of 1.3 μm the spermatozoon of *Lonchoptera fallax* ranks among the largest known to date. In the present study body size, testis size, sperm size, and spermatid number per bundle and per testis were examined across 11 *Lonchoptera* species. Results are discussed in terms of how these characters are related with each other and how their evolution affects the resource allocation amongst spermatozoa. Based on some discrete morphological characters and a molecular tree derived from DNA barcodes a phylogenetic hypothesis of the genus *Lonchoptera* is proposed. The occurrence of giant spermatozoa in Lonchopteridae is compared to convergent occurrences reported in other taxa.

1. Introduction

Spermatozoa are under constant evolutionary pressure and frequently show signs of a rapid, oftentimes bizarre diversification (e.g., Sivinski, 1980; Jamieson et al., 1999; Dallai 2014). Giant spermatozoa independently evolved multiple times even within genera (Pitnick et al., 1999, 2009; Fitzpatrick et al., 2022), constituting a significant divergence from the common scheme of producing as many small spermatozoa as possible (Parker, 1970, 1982). Within Diptera giant spermatozoa are known to occur in very few families such as Cecidomyiidae, Drosophilidae and Diopsidae, likely as a consequence of postcopulatory sexual selection by sperm competition and/or changes in female reproductive tract design (Hihara and Kurokawa, 1987; Kotrba, 1995; Pitnick et al., 1995a, 1999, 2009; Presgraves et al., 1999; Kotrba et al., 2016). Recently Kotrba et al. (2021) described the giant spermatozoa of a species of Lonchopteridae, a small family of Diptera also known as spear-winged flies. Lonchopteridae are a family of Phoroidea, which belong to the Aschiza and are placed near the base of the Cyclorrhapha (Wiegmann and Yeates, 2017). They contain only one recent genus which occurs worldwide. Of the approximately 68 species at least 15 occur in Europe (Whittington and Beuk, 2022). The common palearctic species *Lonchoptera lutea* Panzer, 1809 has spermatozoa which are not only very long, but also much wider than the usual dipteran spermatozoon. Using 3D reconstruction, it was possible for Kotrba et al. (2021) to measure the total length of the spermatozoa and to assign ultrastructural details in cross-sections to their corresponding positions in the mature spermatozoon. This elaborate method also allows to determine the volume of a spermatid bundle and calculate the approximate volume of a single spermatozoon. Through the comparative study of several species the present study aims to resolve whether the conditions found in *L. lutea* apply to Lonchopteridae in general or whether they vary within this taxon. To elucidate the underlying evolutionary trends, the data are analyzed in two approaches. (1) Analysis with respect to the existence and shape of mathematical correlations between various numerical characters of the male reproductive system allows to construct hypotheses on likely functional associations and/or operative reproductive strategies. Correlations alone, however, do not indicate the direction of change. Therefore, (2) the variation of the analyzed characters is discussed in the light of possible evolutionary pathways. Because no information on the intrafamilial phylogeny of Lonchopteridae is available from the literature, an evolutionary hypothesis is constructed based on the studied characters combined with the results of a DNA barcode-based neighbor-joining tree.

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https://doi.org/10.1016/j.asd.2023.101285
1467-8039/

2. Materials and methods

2.1. Material

The study is based on alcohol preserved material (Table 1). *Lonchoptera nigrociliata* Duda, 1927, *Lonchoptera nitidifrons* Strobl, 1898, *Lonchoptera scutellata* Stein, 1890, *Lonchoptera tristis* Meigen, 1824, *Lonchoptera spec. T1* and *Lonchoptera spec. T2* specimens were acquired from the SNSB Bavarian State Collection of Zoology wet collection. *Lonchoptera strobli* De Meijere, 1906 and *Lonchoptera fallax* De Meijere, 1906 specimens were provided by P. Zwick and *Lonchoptera spec. R1* specimens were provided by M. von Tschirnhaus from their respective collections. Specimens of *Lonchoptera barberi* Klymko, 2008 were freshly collected and identified by K. Barber in the summer of 2021. The 7 European species were identified on the basis of morphological characters using Bährmann and Bellstedt (1988). Data for *L. lutea* are from Kotrba et al. (2021).

2.2. Preparation/processing

One male per species was studied by serial sectioning. After assessing the body length (of those species for which no literature data on the average body length were available), abdomina were detached, dried in a graded alcohol series and embedded in Spurr's resin. Serial semithin sections of 1.0 μm were cut with a RMC MTXL ultra-microtome using a diamond blade, mounted on glass slides and stained with Richardson's reagent (Richardson et al., 1960).

2.3. Examination and imaging

Body length and morphological characteristics for species identification were examined using a Leica MZ 8 dissecting microscope. To assess the number of spermatids per bundle and the width of the spermatozoa, semithin sections were investigated with a Zeiss Axioskop2 equipped with a Jenoptic Progres Gryphax Subra digital camera at maximal magnification using a Plan-Neofluar 100 × oil objective and 1.6 × additional magnification. Photographs included a digital scale bar calibrated with a stage micrometer. Measurements in the photos were taken using CorelDRAW Home and Student 2018. The maximal width of the spermatozoa was measured in semithin sections through the widest part of the bundle as assessed by digital 3D reconstruction (below).

For digital 3D reconstruction, the series of semithin sections were photographed with an automated Olympus BX61VS microscope with 40 × objective and DotSlide software using an Olympus XC10 digital camera. The photos were imported into Amira software (version 5.4.5) to obtain volume rendering depictions of the testis and the spermatid bundles therein. For detailed 3D reconstructions of the testes and individual spermatid bundles therein, the circumference of these structures was digitized by hand for each individual section. Due to the laboriousness of this method only one testis per species and 3–4 supposedly mature spermatid bundles from the basal part of this testis were reconstructed. Lengths and widths were assessed with the Amira 3D length measurement tool and volumes were calculated with the Amira material statistics tool. Because the spermatids are arranged in parallel longitudinally within the bundles, the length of the mature bundles was used as an approximation for the length of the contained spermatozoa. Because the width of the bundles changes along their length, the maximal width of the reconstructed spermatid bundles was chosen for comparison across the species. The volume of the individual spermatozoon was estimated by dividing the reconstructed volume of the bundle by the number of contained spermatids. The potential total number of spermatozoa per testis was estimated by dividing the testicular volume by the spermatozoon volume. Both assessments are overestimations because small gaps occur between the densely packed spermatozoa in the bundles and because parts of the testis are occupied by earlier stages of spermatogenesis. Because the same restrictions apply to all studied species, these estimates can still be used for comparative aspects.

The figure plates were created using CorelDRAW Home and Student 2018 and Corel PHOTO-PAINT Home and Student 2018. Statistical analyses and scatter plots were computed with MS Excel 2019. For each character pairing the mathematical model that provided the most plausible approach in terms of trendline fit and the highest r^2 (linear or inverse proportional) was selected. In case of inverse proportionality, the data were reciprocally transformed into a linear relationship before running the statistical analysis (the respective r^2 and P values are marked by *).

2.4. DNA barcoding

All vouchers were sent to AIM - Advanced Identification Methods GmbH in Leipzig, Germany for DNA barcoding using a DNeasy kit (QIAGEN, Valencia, CA). Laboratory operations were carried out following standardized protocols for COI amplification and sequencing. DNA barcode sequences, primer pairs and trace files are publicly accessible in the dataset 'DS-BALONCH' (Dataset ID: dx.doi.org/10.5883/DS-BALONCH) on the Barcode of Life Data System (BOLD; www.boldsystems.org (Ratnasingham and Hebert, 2007)). Although fast evolving protein coding genes such as COI cannot be used for phylogenetic analyses that focus on deep and old branches, they can be useful for the study of more recent phylogenetic events on species level (Raupach et al., 2019). To obtain a visualization of putative species clusters based on DNA barcode distance divergences, a neighbor-joining (NJ) tree was calculated with the analysis tools of BOLD following alignment based on Kimura-2-parameter (K2P) distances (Kimura, 1980). To add to the support and root the resulting cladogram, publicly available

Table 1
Collection data.

Species	Date	Location	Collector
L. barberi	2021-08-14	Canada, Ontario, Sault Ste. Marie	K. Barber
L. fallax	2003-06-11	Austria, Vorarlberg, Silbertal	P. Zwick
L. lutea	2019-08-26	Germany, Bavaria, Munich	M. Tröster
L. nigrociliata	1985-08-12	France, Alpes-de-Haute-Provence, Montlaux	W. Schacht
L. nitidifrons	1988-09-11	Germany, Bavaria, Ottmaring	W. Schacht
L. scutellata	2003-06-14	Germany, Mecklenburg-Western Pomerania, Gützkow	M. Kotrba
L. spec. R1	1999-08-09	Russia, Kamchatka, Delta of river Avacha	M. v. Tschirnhaus
L. spec. T1	2002-12-09	Taiwan, Nantou County, Puli	W. Schacht
L. spec. T2	2000-07-01	Taiwan, Nantou County, Puli	W. Schacht
L. strobli	1983-10-14	Austria, Lower Austria, Lunz	P. Zwick
L. tristis	1991-09-06	Germany, Bavaria, Schöngeising	W. Schacht

sequences from the BOLD database were incorporated into the analyzed dataset, including not only longer sequences for the species studied here, but also some additional lonchopterids, namely *Lonchoptera bifurcata* (Fallen, 1810), *Lonchoptera uniseta* Curran, 1934, *Lonchoptera impicta* Zetterstedt, 1848 and *Lonchoptera occidentalis* Curran, 1934, as well as representatives of other Phoroidea (Ironomyidae, Phoridae, Platypezidae and Opetiidae) as outgroups. The sequences were aligned using ClustalW and analyzed using a neighbor-joining cluster analysis based on K2P distances with MEGA X (Kumar et al., 2018) in order to obtain non-parametric bootstrap support values by resampling and analyzing 1000 replicates (Felsenstein, 1985).

3. Results

In Fig. 1 the testes and the reconstructed spermatid bundles therein are illustrated comparatively for all studied species. All numerical characters assessed are shown in Table 2 and their correlations are shown in Table 3. Fig. 2 illustrates all significant correlations from Table 3 in terms of a network. Relationships indicated in bold will be addressed below. Correlations indicated by narrow lines are not specifically addressed. They are likely secondary effects, as evident from the fact that they can be traced back to a combination of two or three of the relationships indicated in bold.

3.1. Testes

The testes are elongate to roundish (Fig. 1). Their volume is biggest in *L. nitidifrons* and more than seven times smaller in *L. tristis* (Table 2). This is the only one of the assessed characters that shows a significant correlation with the body length ($r^2 = 0.47$, $P = 0.02$) (Fig. 2). The relationship is positive and seems to be linear (Fig. 3A). However, the data points are widely spread, only a single testis per species was reconstructed, and variation due to different states of maturity or depletion after mating cannot be excluded. Larger sample sizes would be needed to corroborate the finding. The volume rendering reveals the internal organization of the testes (Fig. 1). Basal of the germarium strands of compact material emerge, corresponding to bundles of maturing spermatids. Towards the base of the testis these bundles and the spermatids therein become more and more condensed and mature. In species with very long spermatid bundles, these are arranged in large transversal coils within the testes. The otherwise elongate testes are correspondingly distended into a roundish shape. The distortion is reflected in a positive correlation between testes width and bundle length ($r^2 = 0.36$, $P = 0.05$) and a negative correlation between testes length and bundle length ($r^2 = 0.56$, $P < 0.01$). The testis width shows a significant correlation with the testis volume across the species ($r^2 = 0.66$, $P < 0.01$), whereas the testis length does not.

3.2. Spermatid bundles and spermatozoa

The length of the mature spermatid bundles varies greatly across the species (Fig. 1, Table 2). They are short in *L. barberi*, *L. nigrociliata* and *L. scutellata* (190 μm in *L. scutellata*) and up to 40 times longer in *L. fallax* and *L. spec. R1* (7500 μm in *L. fallax*) (Fig. 1). In the remaining species their length ranges from about 1000 μm to about 2000 μm. Within the bundles all spermatids are in an equal stage of maturation and arranged longitudinally in parallel. The length of the spermatozoa therefore can be directly inferred from the length of the reconstructed mature spermatid bundles with the same dramatic variation across the species. Within the scope of this study no evidence for sperm polymorphism was found. As opposed to the variable length of the mature spermatid bundles, their maximal width is rather similar in all studied species (11.5–14.5 μm). Correspondingly, the reconstructed volume of the mature spermatid bundles increases with their length in terms of a positive linear correlation ($r^2 = 0.90$, $P < 0.01$).

In spite of the small variation in bundle width, the number of spermatids per bundle varies greatly across the species, generally amounting to a multiple of 2 or slightly less (Table 2, Fig. 4, Fig. 5). It is 64 (2^6) or slightly less in *L. fallax*, *L. nitidifrons* and *L. spec. R1*, 128 (2^7) or slightly less in *L. spec. T1*, *L. spec. T2* and *L. tristis*, and 256 (2^8) or slightly less in *L. barberi*, *L. nigrociliata* and *L. scutellata*. Only *L. lutea* and *L. strobli* deviate from this pattern, as their number of spermatids per bundle ranges above 64, but also significantly below 128 (Fig. 5). Because across the species a similar spermatid bundle cross section area is apportioned to a variable number of spermatozoa, it is no surprise that the width of the individual spermatozoa shows an inverse proportional correlation with their number per bundle ($r^2 = 0.85$*, $P < 0.01$*) (Fig. 3B). But there is also an inverse proportional correlation between spermatozoon length and spermatid number per bundle ($r^2 = 0.57$*, $P < 0.01$*) (Fig. 3C). The width and length of the spermatozoa are positively related, with the short spermatozoa of *L. barberi*, *L. nigrociliata* and *L. scutellata* being only about 0.4 μm wide, whereas the spermatozoa of *L. lutea* and *L. nitidifrons* reach a maximum diameter of 1.4 μm (Fig. 3D). The relationship is significant under the hypothesis of a linear correlation ($r^2 = 0.45$, $P = 0.02$). However, the data points for the longest spermatozoa are widely spread. The significance would be much increased by excluding either *L. fallax* and *L. spec. R1* ($r^2 = 0.81$, $P < 0.01$) or *L. lutea* and *L. nitidifrons* ($r^2 = 0.83$, $P < 0.01$) as outliers. This would also strongly affect the slope of the respective trendline (alternative trendlines in Fig. 3D). Because the width and the length of the spermatozoa are related with each other and also both are related with the spermatid number per bundle, it can not be decided, which part of these effects is a secondary result of the others. The highly variable and positively correlated spermatozoon length and width both contribute to the spermatozoon volume in terms of a positive linear correlation (length $r^2 = 0.93$, $P < 0.01$, width $r^2 = 0.54$, $P < 0.01$) (Fig. 3E and F). Accordingly, the reconstructed spermatozoon volume varies by a factor of up to 150. In Fig. 3F the data points for the wider spermatozoa show a large vertical spread, because in these taxa the volume is much affected by an excessive variation in length. After exclusion of the species pair with the longest spermatozoa (*L. fallax* and *L. spec. R1*) the data for the remaining species closely fit a linear trendline ($r^2 = 0.80$, $P < 0.01$).

3.3. Sperm number per testis

The potential number of spermatozoa per testis is related with the volume of the individual spermatozoon in terms of an inverse proportional correlation (Fig. 3G), i.e., the testes may hold many small spermatozoa or fewer large ones ($r^2 = 0.89$*, $P < 0.01$*). In all species producing large numbers of (comparatively small) spermatozoa the testes are rather small, but in only about half of the species with small testes these contain large sperm numbers (Fig. 3H). Similarly, all species with large testes produce small numbers of (comparatively large) spermatozoa but in only about half of the species with small sperm numbers the testes are large. Basically, large testes and large sperm numbers are not related in terms of a significant correlation, but instead mutually exclusive, leaving the upper right sector of the scatter plot empty (Fig. 3H).

3.4. Analysis of character evolution

Four of the numeric characters show discrete character states

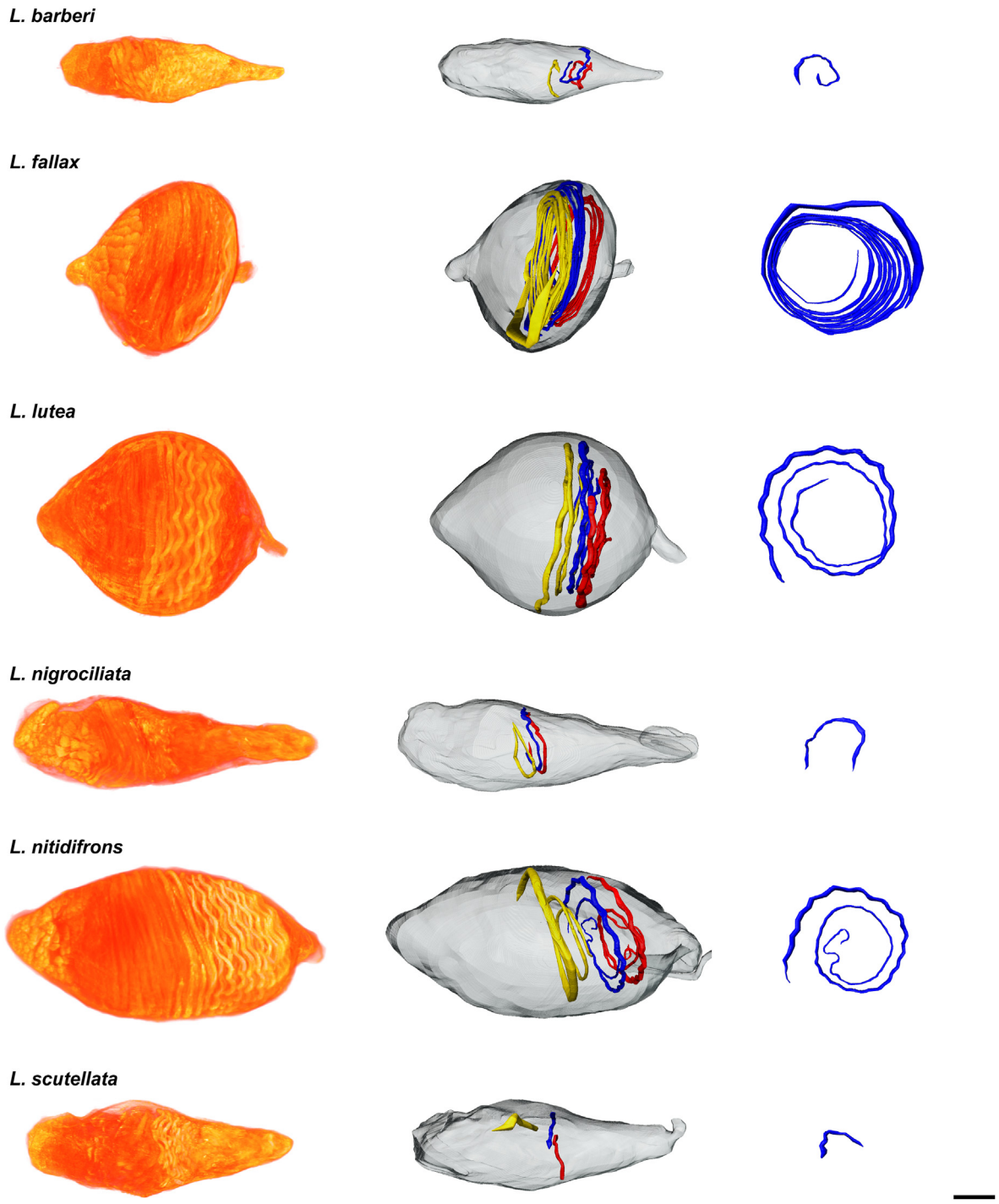

Fig. 1. 3D reconstruction of the testis and three individual spermatid bundles from semithin sections in *L. barberi*, *L. fallax*, *L. lutea*, *L. nigrociliata*, *L. nitidifrons*, and *L. scutellata*. Left, volume rendering; middle, surface rendering of the testes and three spermatid bundles therein; right, one of the reconstructed spermatid bundles. Scale bar = 100 μm.

and were evaluated with respect to the contained phylogenetic information. These are the length and the width of the spermatozoa, the number of spermatids per bundle, and the estimated number of spermatozoa per testis (Table 4).

Unfortunately, very little information from potential outgroups is available to determine the polarity of these characters. Phoridae is the only closely related aschizan family for which some detailed information on the sperm morphology has been published. Curtis et al. (1989) described the spermatozoon of *Megaselia scalaris* (Loew, 1866) as "threadlike, lacking distinct head and tail areas" with a total length of 150 μm. The respective illustrations show a spermatozoon with a maximal width of about 0.5 μm (estimated there in Figs. 18–20). Other than that, some information on the sperm morphology of the more distantly related Syrphidae has been published by Wu and Zhou (1986). The authors described the flagellum axoneme of *Eristalinus tarsalis* (Macquart, 1855) with a total length of approximately 500 μm, which is here used as an approximation for the length of the entire spermatozoon. The

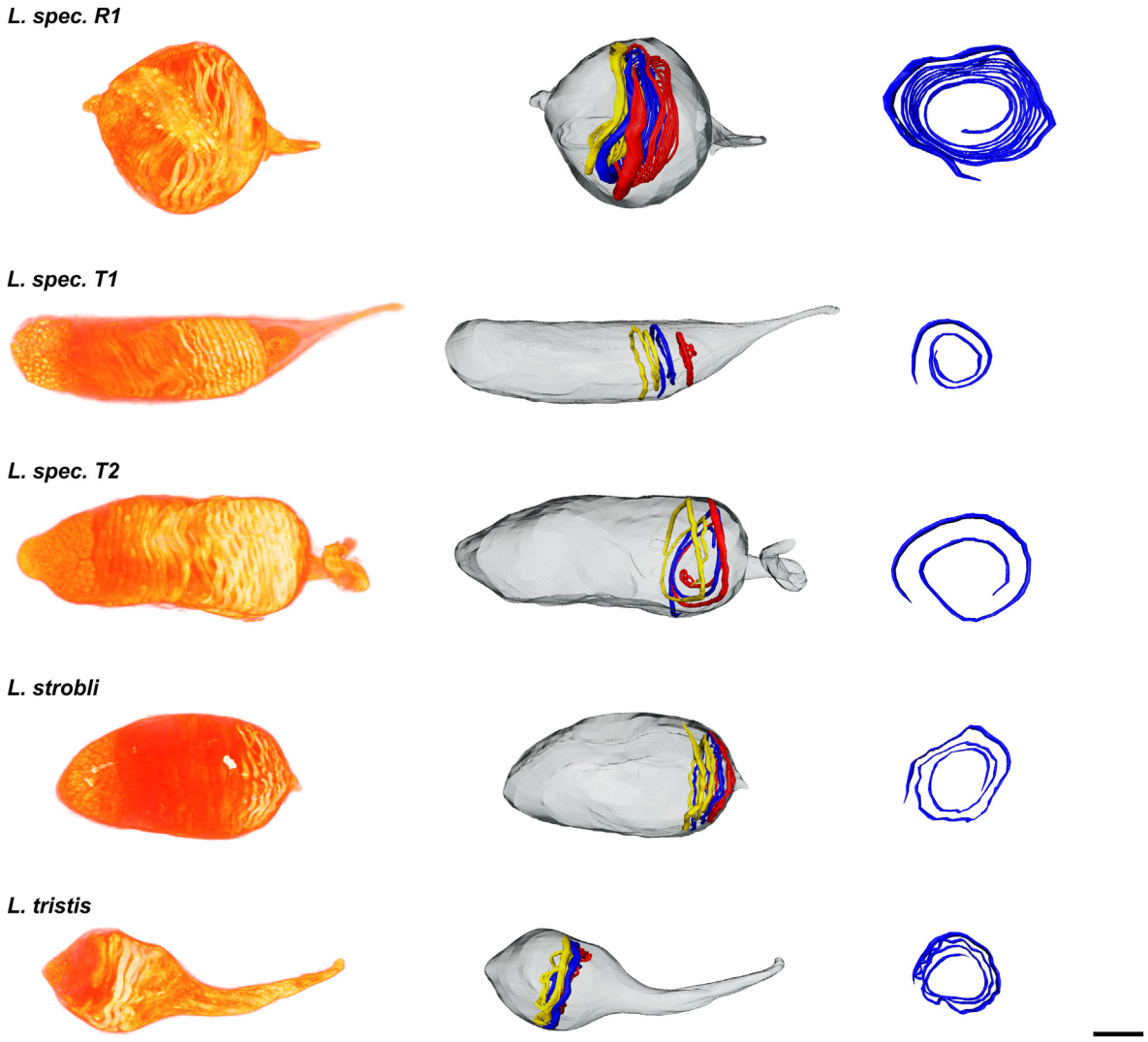

Fig. 1. (continued): 3D reconstruction of the testis and three individual spermatid bundles from semithin sections in *L. spec. R1*, *L. spec. T1*, *L. spec. T2*, *L. strobli*, and *L. tristis*. Left, volume rendering; middle, surface rendering of the testes and three spermatid bundles therein; right, one of the reconstructed spermatid bundles. Scale bar = 100 μm.

Table 2
Body length; testis length, width and volume; spermatid bundle average length, maximal width and volume; spermatozoon average length (equal to average spermatid bundle length), maximal width and approximate volume; average number of spermatids per bundle; estimated number of spermatozoa per testis; ratio of maximum and minimum values per column. Body length data taken from the literature: [1]Klymko and Mashall (2008); [2]Czerny (1934); [3]De Meijere (1906).

Species	Body	Testis			Spermatid bundle			Spermatozoa				
	Length [mm]	Length [μm]	Width [μm]	Volume [x 10^6 $μm^3$]	Length [μm] ± s.d. (N)	Width [μm]	Volume [x 10^3 $μm^3$]	Length [μm] ± s.d. (N)	Width [μm]	Volume [$μm^3$]	Number per bundle ± s.d. (N)	Number per testis [x 10^3]
L. barberi	2.7[1]	550	175	6.3	280 ± 25 (4)	12.5	10 ± 0.9 (4)	280 ± 25 (5)	0.4	40	250 ± 2.6 (6)	157.5
L. fallax	3.0[2]	400	370	21.4	7500 ± 157 (3)	12.5	370 ± 13.7 (3)	7500 ± 157 (3)	1.3	5970	62 ± 1.7 (8)	3.6
L. lutea	3.0[2] 3.0[3]	500	450	38.5	2200 ± 60 (13)	14.5	190 ± 13.5 (10)	2200 ± 60 (13)	1.4	2560	74 ± 7.1 (47)	15.0
L. nigrociliata	2.5[2] 2.6[1]	730	210	13.0	370 ± 13 (3)	12.5	14 ± 0.5 (4)	370 ± 13 (3)	0.4	61	235 ± 13 (5)	213.6
L. nitidifrons	3.5	780	350	41.0	1700 ± 120 (3)	12.5	82 ± 5.3 (3)	1700 ± 120 (3)	1.4	1300	63 ± 0.7 (11)	32.0
L. scutellata	2.8[2]	650	230	15.5	190 ± 13 (4)	14.5	14 ± 0.8 (4)	190 ± 13 (4)	0.4	55	255 ± 0.5 (3)	282.3
L. spec. R1	2.8	380	350	18.7	7150 ± 193 (3)	12.5	270 ± 31 (3)	7150 ± 193 (3)	1.3	4290	63 ± 1.3 (7)	4.4
L. spec. T1	2.5	700	180	12.4	970 ± 50 (3)	13.5	40 ± 3.6 (2)	970 ± 50 (3)	0.7	320	125 ± 3 (5)	38.8
L. spec. T2	3.4	630	260	26.8	1150 ± 20 (3)	14.0	80 ± 4.4 (3)	1150 ± 20 (3)	0.9	655	122 ± 6.3 (6)	40.9
L. strobli	3.0	490	270	16.0	1500 ± 26 (3)	11.5	38 ± 3 (3)	1500 ± 26 (4)	0.7	430	81 ± 7 (23)	37.0
L. tristis	3.0[2]	650	200	5.5	1730 ± 26 (4)	14.0	66 ± 6.3 (4)	1730 ± 26 (4)	0.9	540	122 ± 2.8 (5)	10.2
max./min.	1.4	2.1	2.5	7.5	40	1.3	37	40	3.5	149	4	78

Table 3
Correlations between all assessed numerical characters. For all significant correlations the slope is indicated by arrows (↗ linear, positive linear; ↘ linear, negative linear; ↓ inv. prop., inverse proportional). n.s., not significant; r^2, coefficient of determination; *P*, probability of error.

	Testis length	Testis width	Testis volume	Bundle length = Spermatozoon length	Bundle width	Bundle volume	Spermatozoon width	Spermatozoon volume	Number per bundle	Number per testis
Body length	n.s. $r^2 < 0.01$ $P = 0.77$	n.s. $r^2 = 0.22$ $P = 0.15$	↗ linear $r^2 = 0.47$ $P = 0.02$	n.s. $r^2 < 0.01$ $P = 0.80$	n.s. $r^2 < 0.01$ $P = 0.89$	n.s. $r^2 = 0.03$ $P = 0.62$	n.s. $r^2 = 0.33$ $P = 0.07$	n.s. $r^2 = 0.02$ $P = 0.67$	n.s. $r^2 = 0.26$ $P = 0.11$	n.s. $r^2 = 0.18$ $P = 0.20$
Testis length		n.s. $r^2 = 0.23$ $P = 0.13$	n.s. $r^2 < 0.01$ $P = 0.91$	↘ linear $r^2 = 0.56$ $P < 0.01$	n.s. $r^2 = 0.06$ $P = 0.48$	↘ linear $r^2 = 0.53$ $P = 0.01$	n.s. $r^2 = 0.13$ $P = 0.28$	↘ linear $r^2 = 0.53$ $P = 0.01$	n.s. $r^2 = 0.15$ $P = 0.23$	n.s. $r^2 = 0.15$ $P = 0.25$
Testis width			↗ linear $r^2 = 0.66$ $P < 0.01$	↗ linear $r^2 = 0.36$ $P = 0.05$	n.s. $r^2 < 0.01$ $P = 0.94$	↗ linear $r^2 = 0.55$ $P < 0.01$	↗ linear $r^2 = 0.75$ $P < 0.01$	↗ linear $r^2 = 0.53$ $P = 0.01$	↓ inv. prop. $r^2 = 0.68$* $P < 0.01$*	n.s. $r^2 = 0.27$* $P = 0.10$*
Testis volume				n.s. $r^2 = 0.03$ $P = 0.62$	n.s. $r^2 = 0.02$ $P = 0.69$	n.s. $r^2 = 0.12$ $P = 0.30$	↗ linear $r^2 = 0.53$ $P = 0.01$	n.s. $r^2 = 0.11$ $P = 0.33$	n.s. $r^2 = 0.31$ $P = 0.07$	n.s. $r^2 = 0.12$ $P = 0.30$
Bundle length = Spermatozoon length					n.s. $r^2 = 0.08$ $P = 0.41$	↗ linear $r^2 = 0.91$ $P < 0.01$	↗ linear $r^2 = 0.46$ $P = 0.02$	↗ linear $r^2 = 0.93$ $P < 0.01$	↓ inv. prop. $r^2 = 0.57$* $P < 0.01$*	↓ inv. prop. $r^2 = 0.96$* $P < 0.01$*
Bundle width						n.s. $r^2 < 0.01$ $P = 0.77$	n.s. $r^2 < 0.01$ $P = 0.96$	n.s. $r^2 = 0.03$ $P = 0.61$	n.s. $r^2 = 0.03$ $P = 0.59$	n.s. $r^2 = 0.02$ $P = 0.67$
Bundle volume							↗ linear $r^2 = 0.58$ $P < 0.01$	↗ linear $r^2 = 0.99$ $P < 0.01$	↓ inv. prop. $r^2 = 0.59$* $P < 0.01$*	↓ inv. prop. $r^2 = 0.88$* $P < 0.01$*
Spermatozoon width								↗ linear $r^2 = 0.55$ $P < 0.01$	↓ inv. prop. $r^2 = 0.85$* $P < 0.01$*	↓ inv. prop. $r^2 = 0.39$* $P = 0.04$*
Spermatozoon volume									↓ inv. prop. $r^2 = 0.60$* $P < 0.01$*	↓ inv. prop. $r^2 = 0.89$* $P < 0.01$*
Number per bundle										↗ linear $r^2 = 0.85$ $P < 0.01$

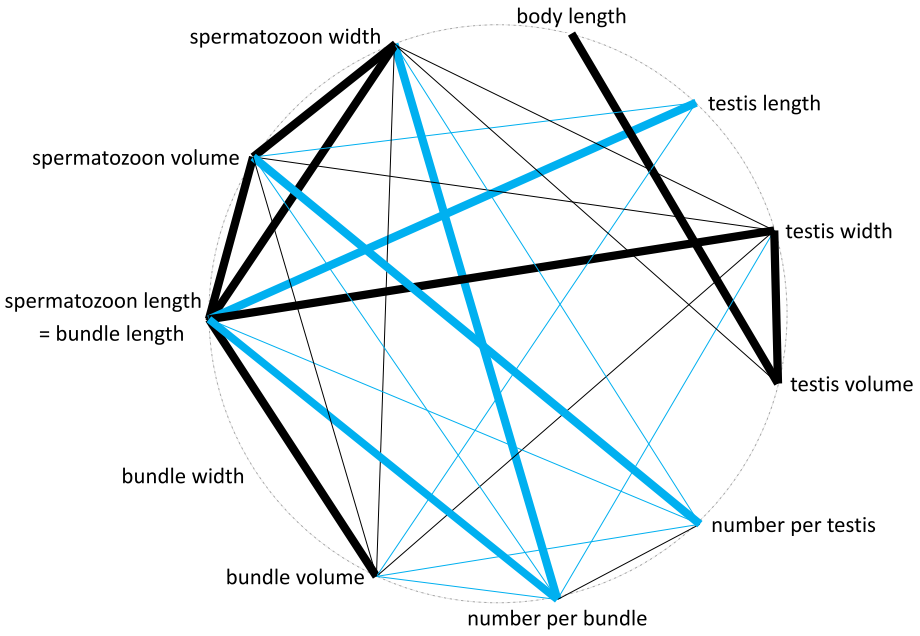

Fig. 2. Graphical representation of correlations between the assessed numerical characters (Tables 2 and 3); bold lines, associations that are specifically adressed in the results; black, positive correlation; blue, negative correlation.

respective illustrations show a spermatozoon with a maximal width of about 0.5 μm (estimated there in Figs. 6–7). Short and narrow spermatozoa can thus be identified as plesiomorphic conditions for the Lonchopteridae. Moreover, it is plausible that these characters evolved progressively and are to be treated as ordered. This allows to arrange the studied lonchopterid species into a hierarchy of three intrafamilial clades each set against a paraphyletic group of two or more species (Fig. 6). The most basal paraphyletic species group comprises *L. scutellata*, *L. barberi* and *L. nigrociliata* (group I). These species are characterized by short (190–370 μm)

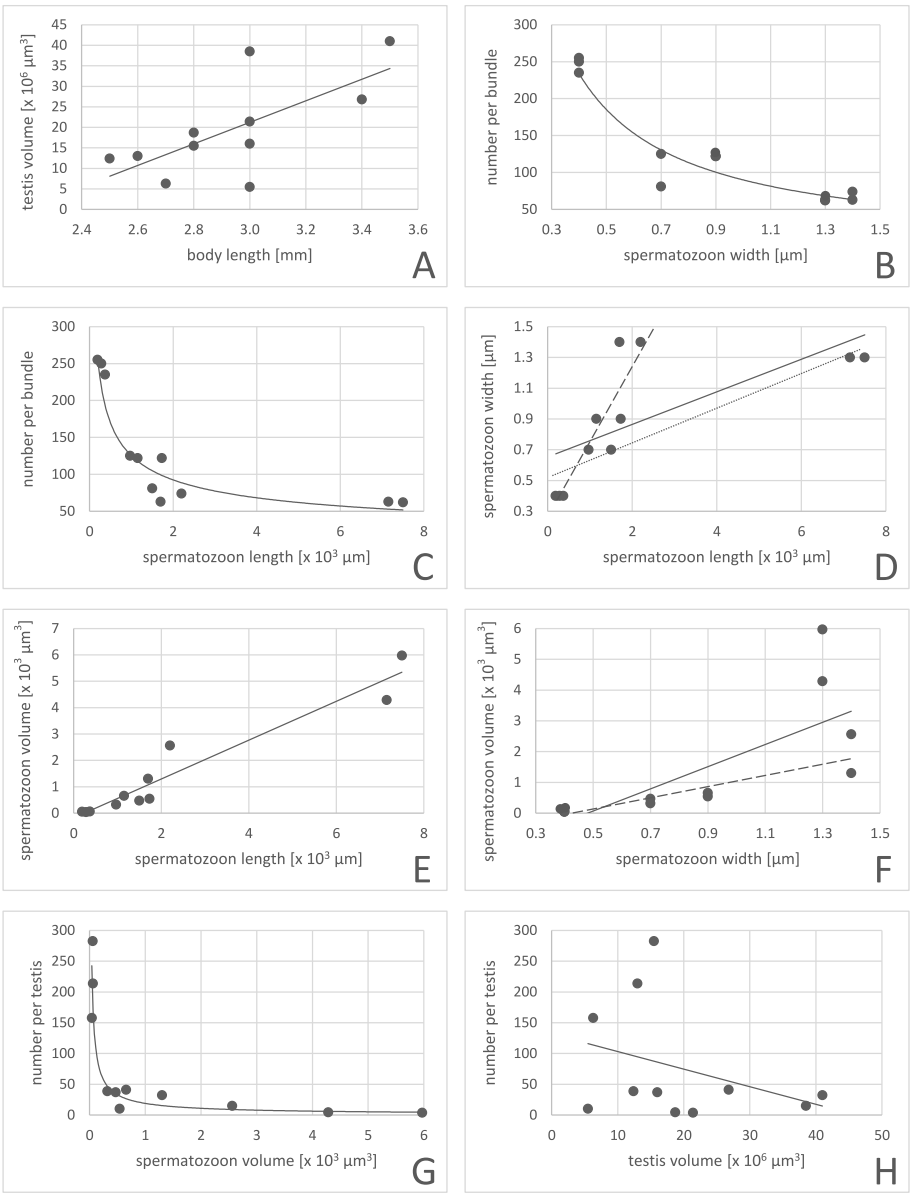

Fig. 3. Interspecific correlations between selected characters across the 11 investigated *Lonchoptera* species with trendlines for the most plausible mathematical model (linear or inverse proportional). **A:** Testis volume and body length, **B:** Spermatid number per bundle and spermatozoon width, **C:** Spermatid number per bundle and spermatozoon length, **D:** Spermatozoon width and spermatozoon length (dashed trendline, *L. fallax* and *L. spec. R1* excluded; dotted trendline, *L. lutea* and *L. nitidifrons* excluded), **E:** Spermatozoon volume and spermatozoon length, **F:** Spermatozoon volume and spermatozoon width (dashed trendline, *L. fallax* and *L. spec. R1* excluded), **G:** Sperm number per testis and spermatozoon volume, **H:** Sperm number per testis and testis volume.

and narrow (0.4 μm) spermatozoa, approximately 256 (2^8) spermatids per bundle and elongate testes containing about 150 thousand or more spermatozoa. Unfortunately, the numbers of spermatids per bundles and per testes are not available for *M. scalaris* and *E. tarsalis*, so the status of these characters as plesiomorph or apomorph cannot be resolved at this level. The first intrafamilial clade (groups II-IV) is characterized by spermatozoa about 1000 μm long or more and 0.7 μm wide or more, about 128 (2^7) or less spermatids per bundle, and not more than about 50 thousand spermatozoa per testis. Within this first clade, a second clade (groups III-IV) is characterized by spermatozoa 1.3–1.4 μm wide and approximately 2^6 spermatids per bundle. The paraphyletic species group set against this clade comprises *L. tristis*, *L. strobli*, *L. spec. T1* and *L. spec. T2* (group II). These species have an intermediary spermatozoon width (0.7–0.9 μm) and approximately 2^7 spermatids per bundle. Only *L. strobli* constitutes an outlier with only about 81 spermatids per bundle. Within the second clade *L. fallax* and *L. spec. R1* form a small clade (group IV) characterized by extremely long spermatozoa (more than 7000 μm) and only about 4 thousand spermatozoa per testis. The paraphyletic species group set against this third clade comprises *L. lutea* and *L. nitidifrons* (group III) with 1700–2200 μm long spermatozoa and 15–32 thousand spermatozoa per testis. *L. lutea* shares with the third clade the shape of the testis which is distinctly roundish instead of elongate.

3.5. DNA barcode analysis

DNA barcode sequences were recovered for all vouchers except *L. nitidifrons*, ranging from 227 to 658 base pairs (bp), but mostly

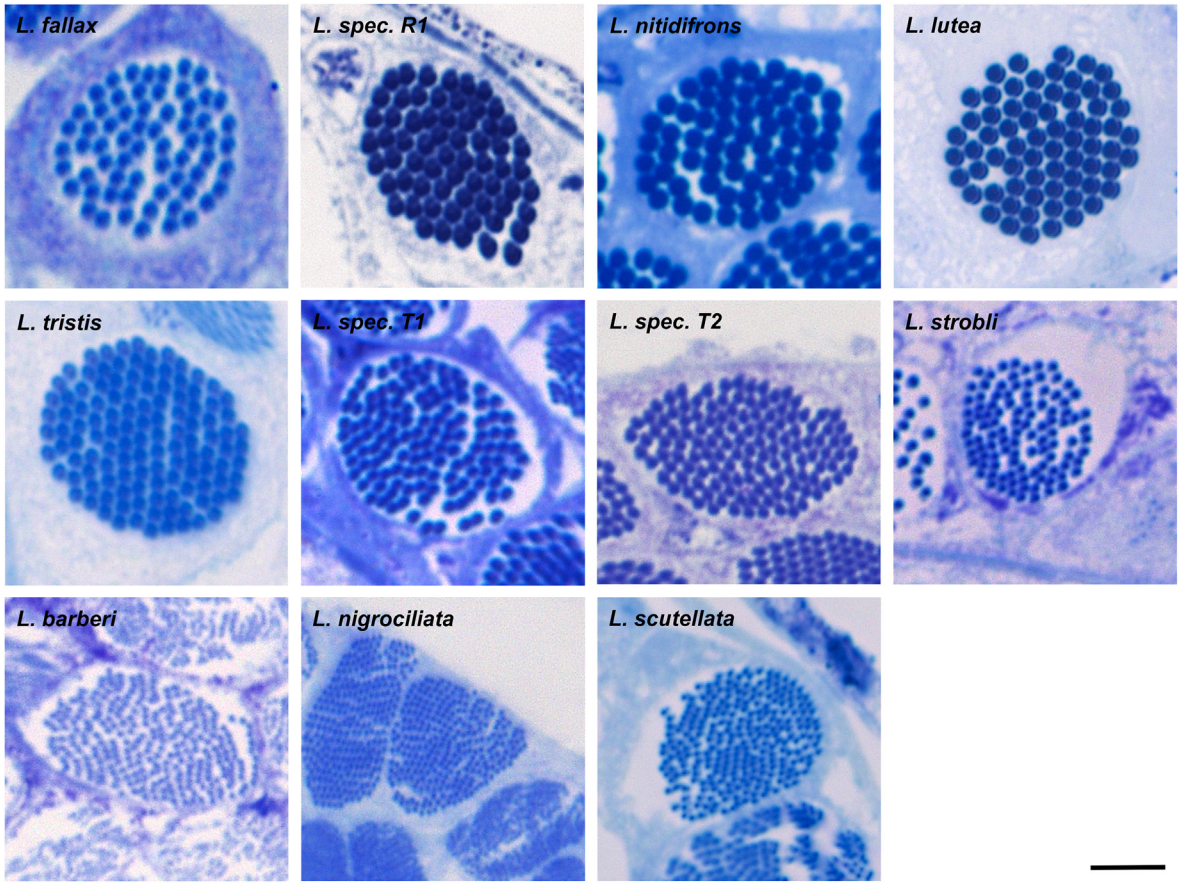

Fig. 4. Cross-sections of spermatid bundles of the investigated *Lonchoptera* species. Semithin sections, not necessarily at the widest part of the spermatid bundle. Scale bar = 5 μm.

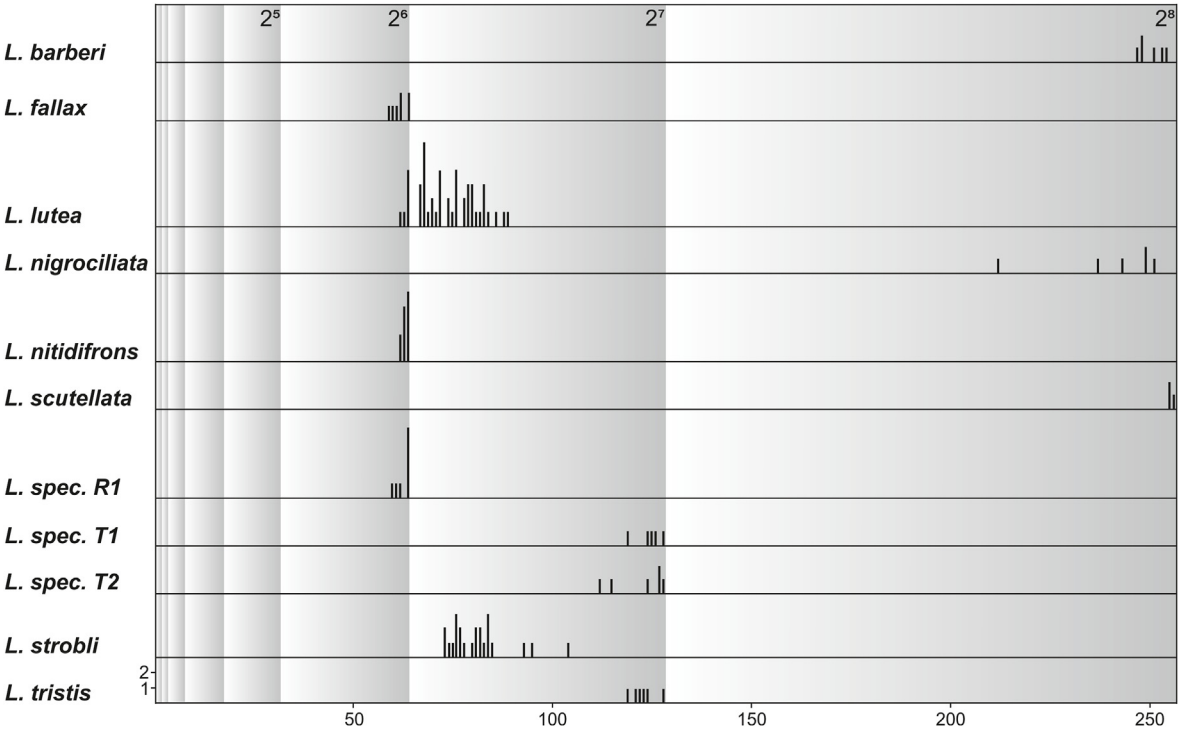

Fig. 5. Frequency of spermatid number per bundle across the 11 investigated *Lonchoptera* species.

Table 4
Character coding for the characters evaluated with respect to their phylogenetic information (Fig. 6).

	Character	Character states
1	Spermatid number per bundle	(1) 235–255, (2) 81–125, (3) 62-74
2	Spermatozoon width	(1) 0.4–0.5 μm, (2) 0.7–0.9 μm, (3) 1.3–1.4 μm
3	Spermatozoon length	(1) 150–500 μm, (2) 970–2200 μm, (3) 7150–7500 μm
4	Estimated number of spermatozoa per testis [x 10^3]:	(1) 157.5–282.3, (2) 10.2–40.9, (3) 3.6–4.4

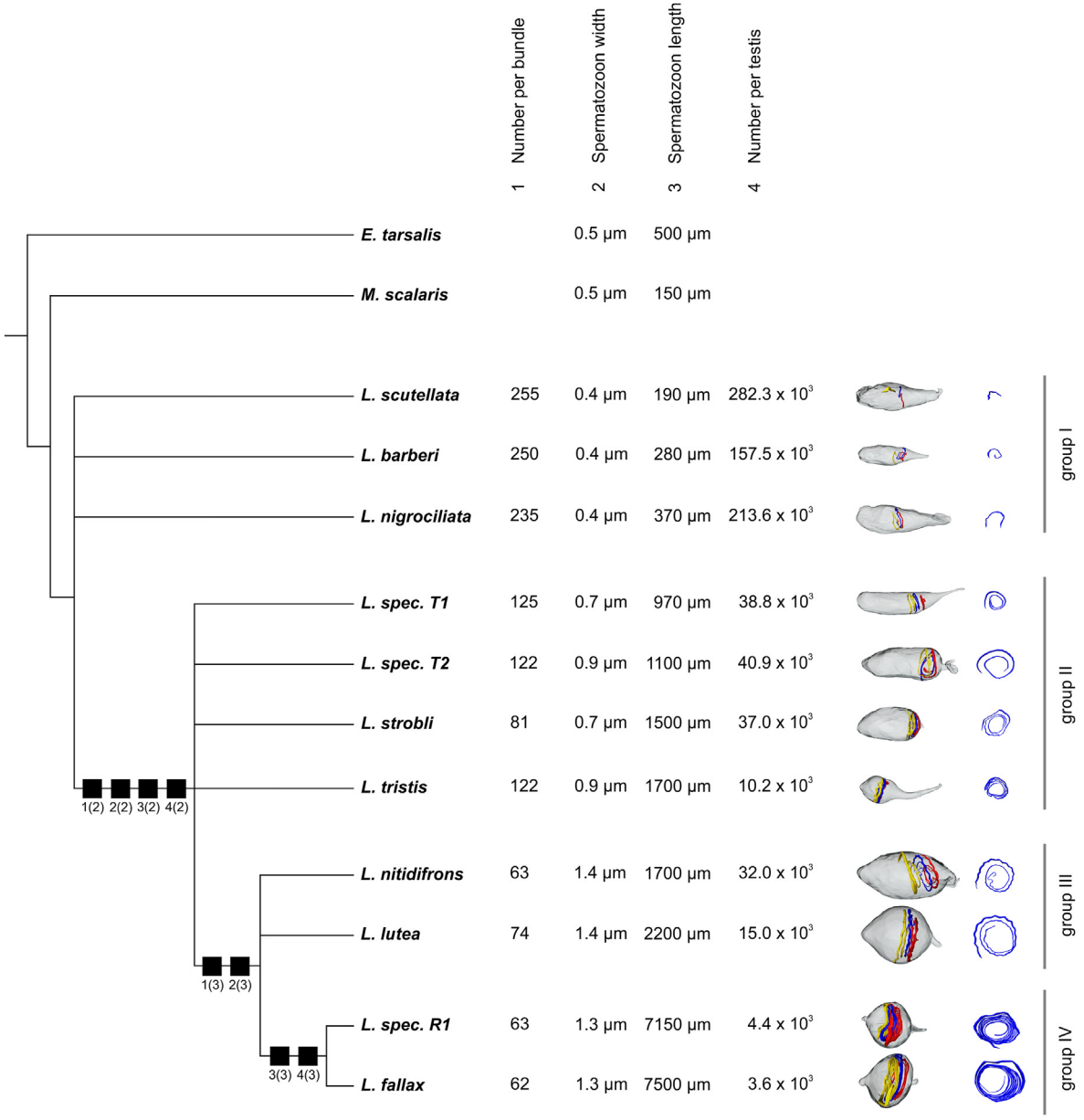

Fig. 6. Most parsimonious hypothesis for the evolution of sperm morphology and numbers in *Lonchoptera*. Characters states and species groups defined in chapter 3.4. Character changes constituting synapomorphies are indicated by black squares. Values for *E. tarsalis* from Wu and Zhou (1986) and for *M. scalaris* from Curtis et al. (1989).

below 350 bp (Fig. 7). Where longer conspecific sequences (>500 bp) were publicly available, the voucher sequences invariably cluster with these, corroborating the species identification and considerably adding to the support of the topology. Overall, 31 DNA barcode sequences of 15 *Lonchoptera* species were analyzed. Moreover, 13 datasets for other families of the Phoroidea were added as outgroups. The NJ analysis invariably groups the specimens belonging to one species or to closely related sister species (i.e., *L. barberi*/*L. occidentalis*, *L. lutea*/*L. nitidifrons*) in non-overlapping clusters with very strong support (95–100%) (Fig. 7). For all other groupings above the species level the support values are not significant. It is noteworthy, however, that the found topology is mostly compatible with the results of the character analysis above. *Lonchoptera* is retrieved as a monophyletic clade.

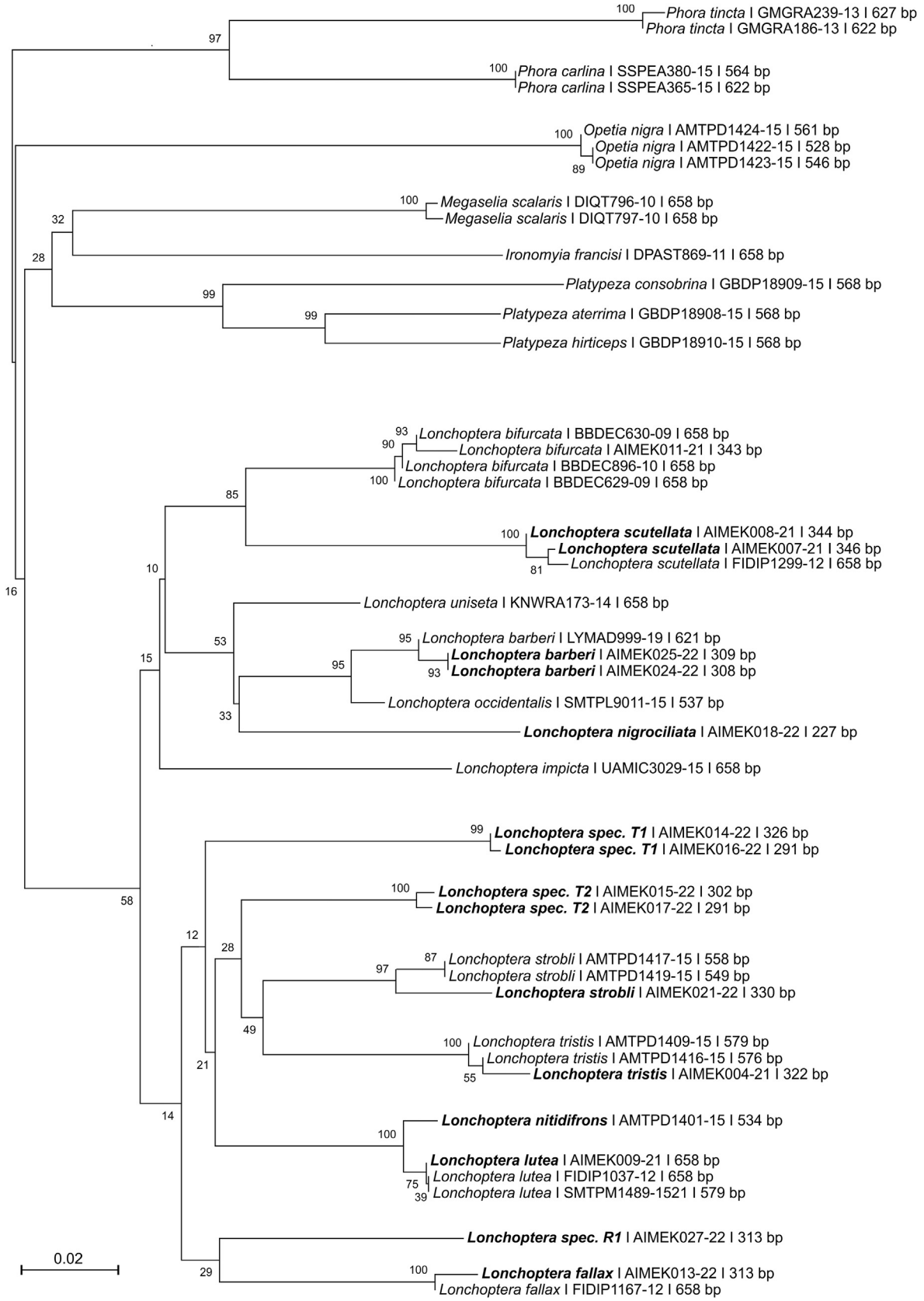

Fig. 7. Neighbor-joining tree of the analyzed *Lonchoptera* and outgroup species based on Kimura-2-parameter distances. Species names followed by BOLD sequence/process ID and length of sequence. Specimens from this study are indicated in bold. Numbers next to nodes represent non-parametric bootstrap values (1000 replicates). Scale bar = 0.02 substitutions/site.

Within the genus a basal bifurcation separates two major clades. One comprises *L. barberi*, *L. scutellata* and *L. nigrociliata* but also the parthenogenetic *L. bifurcata* and the other three additional species from the BOLD database, i.e., *L. uniseta*, *L. impicta* and *L. occidentalis*. Therein two species pairs emerge with strong support, i.e., *L. barberi*/*L. occidentalis* (95%) and *L. bifurcata*/*L. scutellata* (85%). In the other clade the species pair *L. fallax*/*L. spec. R1* emerges as a weakly supported (29%) sister clade to the remaining 6 species, i.e., *L. strobli*, *L. tristis*, *L. nitidifrons*, *L. lutea*, *L. spec. T1* and *L. spec. T2*, among which *L. lutea*/*L. nitidifrons* form a very strongly supported clade (100%). The only inconsistency with respect to the results of the character analysis is that *L. fallax*/*L. spec. R1.* do not emerge next to *L. lutea*/*L. nitidifrons.*

4. Discussion

The giant spermatozoa described for *L. lutea* by Kotrba et al. (2021) are not unique to this species, but also not a general feature of the family Lonchopteridae. Instead, the comparative study of 11 species of *Lonchoptera* revealed considerable variation across the species, not only with respect to the length of the spermatozoa, but also with respect to their width and their numbers per spermatid bundle and per testis. These four numeric characters show discrete character states. Although they are not independent of each other and by far not sufficient to construct a well-founded phylogenetic hypothesis, an evolutionary scenario can be drafted based on the assumption of a progressive development (Fig. 6). The topology receives some support from the fact that it is mostly compatible with the topology of a NJ tree based on a DNA barcode analysis (Fig. 7). However, the latter has weak support for most of the nodes.

4.1. Plesiomophic conditions in the basal taxa

The most basal taxa in the family are characterized by comparatively short (190–370 μm) and narrow (0.4 μm) spermatozoa, which are most similar to the conditions in the outgroup taxa *M. scalaris* and *E. tarsalis* and quite typical for Diptera in general (according to data in Fitzpatrick et al., 2022). The respective species *L. scutellata*, *L. nigrociliata* and *L. barberi* are further characterized by a large number of spermatids per bundle (2^8), as well as club-shaped testes, which accommodate large numbers of spermatozoa. The molecular NJ tree combines these species in one clade together with *L. impicta, L. uniseta, L. occidentalis* and *L. bifurcata*, of which no male specimens were available for study. It is therefore very likely that numerous small spermatozoa are a common feature for these species as well.

4.2. Trends within the Lonchopteridae

The remaining eight species comprise a clade characterized by a number of trends which occur in parallel and are more or less related to each other (Fig. 2): (1) The number of spermatids per bundle is reduced. (2) The spermatozoon width increases. (3) The spermatozoon length increases. (4) The number of spermatozoa per testis (and the number of spermatid bundles per testis) decreases.

4.2.1. Reduction of number of spermatids per bundle

The number of spermatids per bundle results from a variable but typically species-specific number of spermatogonial premeiotic divisions preceding the final two meiotic divisions (Virkki, 1969; Cruz-Landim, 2001). For example, 64 (2^6) spermatids per bundle are the result of four premeiotic multiplication divisions of the primary spermatogonia in the germarium followed by two meiotic divisions of the secondary spermatogonia. In Lonchopteridae, the numbers of spermatids per bundle show some intraindividual variation, usually ranging slightly below the respective (2^n) value. This very likely results from a small loss of spermatids during spermiogenesis. Only *L. lutea* and *L. strobli* do not fit this pattern. In these species the numbers range between the values (2^6 and 2^7) which could be due to either a major loss of spermatids during spermiogenesis or nonsynchronous divisions. Deviations from the 2^n pattern were also described in various studies for *Drosophila* (Liebrich et al., 1982; Hanna et al., 1982; Schärer et al., 2008). Schärer et al. (2008) note that some heritable variation for a certain trait is the necessary precondition for selection to act and evolution to occur. Evolution generally proceeds towards a reduced number of divisions so that “specialized insects tend to have less spermatozoa per bundle than more primitive insects” (Virkki, 1973). This pattern was corroborated for Drosophilidae by Schärer et al. (2008). It is also evident in *Lonchoptera*, where the plesiomorphic condition of roughly 256 (2^8) spermatids per bundle in the basal taxa (group I) is reduced to roughly 128 (2^7) at the base of the first major clade and roughly 64 (2^6) in the next subordinate clade therein (Fig. 6). Because the number of mitotic divisions stands ontogenetically at the beginning of spermatogenesis, its variation is most likely at least partially causal to the consecutive changes in sperm morphology. The first and most immediate effect is a respective change in spermatozoon width, which appears to result from a redistribution of material within the individual cyst.

4.2.2. Increase in spermatozoon width

The width of the spermatid bundles is the only one of the assessed characters which shows no obvious correlation with any of the other characters. Its superficial constancy conceals the substantial changes occurring within. Comparison of histological sections of mature spermatid bundles across the species shows that a rather consistent bundle cross section area is apportioned to a variable number of spermatozoa, thereby directly affecting the width of the individual spermatozoa (Table 2, Figs. 3B, Fig. 4). The close relationship between spermatid number per bundle and spermatozoon width is reflected in a highly significant inverse proportional correlation of the two characters (Fig. 3D) and in their simultaneous progressive changes on the family tree (Fig. 6). With a diameter of about 0.4 μm the spermatozoa of the basal taxa (group I) roughly match those of the outgroups. In the derived clades, the spermatozoa are about twice (0.7–0.9 μm) and in a second step even more than three times as wide (up to 1.4 μm). According to a comprehensive review on insect spermatozoa by Dallai (2014), spermatozoa exceeding a width of 0.7 μm are exceptional. In Diptera extraordinarily wide spermatozoa have been found in four very distantly related families, i.e., Cecidomyiidae, Drosophilidae, Diopsidae, and Lonchopteridae. In each case, the width increase has been achieved by different ultrastructural changes. In *L. lutea* there is a very largely increased mitochondrial derivative which is accompanied by an also very large and unusual accessory body (Kotrba et al., 2021). These subcellular components are so enormous that they even show up in light microscopic images of *L. spec. R1* and *L. lutea* (Fig. 4). In *Diasemopsis comoroensis* Carr and Foeldvari, 2006 a very large accessory body (“central band”) is flanked by two large mitochondrial derivatives, which are particularly wide at the very tip of the sperm tail where the diameter reaches its maximum of up to 3 μm (Kotrba et al., 2016). In *Drosophila kanekoi* Watabe and Higuchi, 1979 (Dallai et al., 2008) and *Drosophila hydei* Sturtevant, 1921 (Jamieson et al., 1999) there are one or two comparatively large mitochondrial derivatives respectively, but no conspicuous accessory bodies. Only in Cecidomyiidae the axoneme itself is modified, involving a multiplication of the axonemal doublets to several thousands (Dallai, 1988;

Dallai et al., 1996). The fundamental differences in ultrastructural architecture reflect the independent evolution of exceptionally wide spermatozoa in these distantly related taxa.

4.2.3. Increase in spermatozoon length

During the elongation period, the sperm cysts and the spermatids within attain their final length which varies by a factor of up to 40 across the studied species. It ranges at a few hundred micrometers in the basal taxa (group I), increases to about 1000–2000 μm at the base of the first major clade and in a second step soars to over 7000 μm in the species pair *L. fallax* and *L. spec. R1*. The spermatozoa of the latter species range among the longest spermatozoa known to date. Out of the 1667 species recorded in the sperm tree data base (Fitzpatrick et al., 2022), they are surmounted only by several *Drosophila* species (Diptera), three *Notonecta* species (Hemiptera), and one species each in Coleoptera, Lepidoptera and Ostracoda. Phylogenetic analysis of the data shows that extraordinarily long spermatozoa arose repeatedly in comparatively small and derived clades within distantly related taxa. In almost every case, for which comparative data for several species within the respective genus are available, the spermatozoon length varies strongly on the subgeneric level, as it does in *Lonchoptera*, indicating a fast and recent evolution of the spermatozoon length. In fact, the intrageneric variation in *Drosophila* alone ranges from 139 μm to 58360 μm, covering 99.8% of the known variation in the entire animal kingdom.

If the increase in length of the spermatids was mainly achieved by a redistribution of material within the spermatids, it would be accompanied by a respective decrease in diameter. This is not the case. To the contrary, in *Lonchoptera* spermatozoon length and width are positively correlated (Table 3, Fig. 3D) and on the proposed cladogram the increase in spermatozoon length either concurs with or follows an increase in spermatozoon width (Fig. 6). In the species pair *L. fallax*/*L. spec. R1*, however, further investment in spermatozoon width is abandoned in favor of an escalating increase in length. This species pair is placed as sister clade to the first major clade in the DNA barcode NJ tree (Fig. 7), but according to the evolutionary scenario based on the morphological characters arises from the subordinate clade therein (Fig. 6). An increase in width is apparently not, or only to a limited extent, an obligatory precondition for the evolution of longer spermatozoa. Most notably the spermatozoon of *Drosophila bifurca* Patterson and Wheeler, 1942, the longest spermatozoon known to date (58 mm, Pitnick et al., 1995b), has an only moderately increased diameter of approximately 0.6 μm (measured in Dallai, 2014 in Fig. 1). On the other hand, the spermatozoa of the closely related *D. kanekoi*, which ranges second in terms of length (24.29 mm, Pitnick et al., 1999), has a much larger diameter of at least 1.2 μm (estimated in Dallai et al., 2008 in Figs. 5a, e) due to the presence of a very wide mitochondrial derivative. Findings in Zoraptera suggest a positive relationship between spermatozoon length and width in that taxon (Dallai et al., 2014). Unfortunately, literature data on spermatozoon width and ultrastructure are much less common than those on spermatozoon length, so there is not enough substantiation for speculation about the general relationship of spermatozoon width and length across the taxa.

In *Lonchoptera* the positively correlated spermatozoon length and width both have a positive effect on the spermatozoon volume (Fig. 2) resulting in an overproportional spermatozoon volume variation by a factor of up to 150. Compared with the spermatozoa of *L. scutellata*, those of the outgroup taxa *M. scalaris*, *E. tarsalis,* and, in fact, the vast majority of the animal kingdom (Fitzpatrick et al., 2022), the spermatozoa of *L. fallax* are veritable giants in terms of length, width and especially volume (almost 6000 μm^3). This obviously implies huge differences in the male investment per spermatozoon.

4.2.4. Decrease in the number of spermatozoa per testis

The evolutionary increase of the volume of the spermatid bundles and the individual spermatozoa is not matched by a respective inflation of the testis size (Fig. 1). Therefore, the projected number of spermatozoa per testis decreases dramatically. This corroborates respective predictions by Lüpold et al. (2016). It seems likely that fewer spermatozoa will be transferred per mating and that in case of multiple matings competition among individual spermatozoa and/or female choice are involved rather that dilution effects in terms of a raffle system.

4.3. General considerations regarding the evolution of sperm number and size

In the numerous comparative studies dealing with the evolution of sperm number and/or dimensions two fundamental questions frequently arise.

(1) Is testis size an indicator of the contained number of spermatozoa with the respective implications regarding the underlying reproductive strategies?
(2) What is the benefit of investing in larger individual spermatozoa at the expense of sperm numbers?

(1) The relationship between body size and testis size was not in the focus of the present study and the assessed data are not sufficient for a conclusive analysis in this respect. Still, the finding of a positive linear relationship between these parameters across the lonchopterid species matches earlier results in other insects (e.g., Gage, 1994; Pitnick, 1996). That larger species have the option to, and in fact do, assign more resources to sperm production is plausible and was extensively discussed in a study by Pitnick (1996) on the investment in the testes in *Drosophila*. However, testis size does not necessarily translate into sperm numbers. In *Lonchoptera* both the testis volume and the projected number of spermatozoa per testis vary strongly across the species with a factor of up to 7.5 and 80 respectively. In this taxon the testis size is no straightforward indicator of the contained sperm number (Fig. 3H), mainly because the spermatozoon volume varies strongly (by a factor of up to 150). In some of the species with small testes these can theoretically accommodate the highest numbers of spermatozoa, whereas in the five species with the largest testes these can accommodate only comparatively small numbers of (larger) spermatozoa. Very similar results can be deduced from the respective values reported for *Drosophila* (Pitnick, 1996). In both taxa resources are redistributed within the testes to produce either many small or fewer larger spermatozoa. And in both taxa the redistribution in favor of large spermatozoa seems to occur especially in species with larger testes, i.e., species that allot more resources to the testes in general.

The results highlight, that the superficial dimensions of the testes alone cannot be used as an indicator of the contained sperm numbers. Neither can they be used to gauge the dimensions of the individual spermatozoa, at least not without detailed information on the internal organization of the testes. Other than in Drosophilidae (Pitnick, 1996; Lowe and Montell, 2022) and Diopsidae (Kotrba et al., 2016), in Lonchopteridae the spermatid bundles are arranged in coils which are oriented transversally inside the testes (Fig. 1). In species with very long spermatozoa and respectively larger coils, the otherwise club-shaped testes are distended into a roundish shape and thus get wider and shorter. In consequence, neither the volume nor the length of the testes show a positive correlation with the length of the spermatozoa, as is the case in *Drosophila* (Pitnick, 1996).

(2) That the development of giant spermatozoa comes at the

expense of spermatid number per bundle and per testis is evident not only in *Lonchoptera* but also in *Drosophila* (Pitnick, 1996; Schärer et al., 2008). Moreover, a tradeoff between spermatozoon length and spermatid number per bundle can be deduced from values published for Tenebrionoidea (values in Dias et al., 2021; Dias et al., 2022). So why has the widespread strategy of producing as many small spermatozoa as possible (Parker, 1970, 1982) in these taxa been replaced by its opposite, which involves a highly increased paternal investment per individual spermatozoon?

Parker's predictions that under sperm competition males are selected to produce numerous, tiny spermatozoa mainly apply to taxa with external fertilization and to large organisms with internal fertilization such as vertebrates, where the female reproductive organs are very large in comparison to the spermatozoa. It has received widespread support that under such conditions males transferring more sperm gain a numerical advantage, particularly when fertilization approximates a random process (Parker, 1982; Pizzari, 2006; Lüpold et al., 2020). Subsequent models, however, have suggested that in small organisms such as many insects sperm competition occurs in the form of displacing rival sperm from densely packed sperm-storage organs and this can lead to stronger selection on spermatozoon length than on sperm number (Parker, 1993; Pizzari, 2006; Lüpold et al., 2020). This argument can be broadened to include spermatozoon width and/or volume. The long and wide spermatozoa of *L. lutea* seem perfectly suited to displace rival spermatozoa from the long and narrow tubular spermathecae described for this species (Kotrba et al., 2021) or to resist such displacement. Sperm competition might therefore be the driving factor in the evolution of giant spermatozoa in Lonchopteridae. But other selective forces such as cryptic female choice or paternal investment may also be involved. The very long spermathecae in *L. lutea* constitute a suggestive clue in this respect. Coevolution of sperm length and the morphology of the female sperm-storage organs has been documented in a wide range of taxa (Lüpold et al., 2020; Dallai et al., 2021). That this is driven by female choice has been shown experimentally in *Drosophila* (Miller and Pitnick, 2002). Female choice could select for longer spermatozoa as an indicator of male genetic fitness, but also as an indicator of more immediate benefits, e.g., in terms of zygote provisioning (Perotti, 1973; Bressac et al., 1994; Kotrba et al., 2016).

Clues regarding the functional particularities of the giant spermatozoa could be expected from their ultrastructural and biochemical properties. Remarkably, across the taxa sperm gigantism is achieved by the enlargement of different cell components (above). This may still serve a common purpose such as expanding the spermatozoon volume, thereby facilitating sperm displacement and/or the resistance against such displacement or meeting the requirements of some kind of cryptic female choice. Alternatively the different forms of sperm gigantism serve likewise different purposes such as improved motility or stability, prolonged survival, or zygote provisioning. Unfortunately, next to nothing is known about the properties of the accessory body and little is known about the potential function of enlarged mitochondrial derivatives (Baccetti et al., 1977; Perotti, 1973; Kotrba et al., 2016).

Which process of selection is crucial for the evolution of giant spermatozoa in Lonchopteridae cannot yet be determined, but it is clear which issues need to be addressed next to resolve this question: (1) The precise phylogeny of Lonchopteridae; (2) the morphology of the internal female reproductive system with respect to its potential coevolution with the sperm morphology and also with respect to the number and arrangement of the stored spermatozoa; (3) the mating behavior, especially with respect to the levels of promiscuity, but also with respect to the evolution of parthenogenesis in *L. bifurcata*, which belongs to group I (Figs. 6 and 7), i.e., the clade in which the many small sperm strategy prevails; (4) the mechanical and biochemical properties of the spermatozoon's giant mitochondrial derivative and the peculiar accessory body (Kotrba et al., 2021); (5) the oviposition, especially with respect to the fate of the spermatozoon in the egg and also with respect to the use of ephemeral substrates, which might select for extra egg provisioning, giving the larvae a head start over their competitors, similar to vivipary (Meier et al., 1999). Each of these approaches may contribute to the understanding of the advantages of giant spermatozoa in lonchopterids, but also in general. A detailed comparative study of the female reproductive system of the species included in this study is already in progress.

5. Conclusions

This study corroborates that sperm gigantism is a derived feature which evolves rapidly within small clades, generally below the genus level. It also corroborates that sperm gigantism comes at the cost of reduced sperm numbers per bundle and per testis. Lonchopteridae represent a promising taxon for the study of such evolutionary processes because they are highly diverse, especially with respect to sperm morphology, with some species matching the common scheme of producing many small spermatozoa and others producing very few giant spermatozoa. The study of these tiny nondescript flies may thus contribute to the resolution of big questions such as how reproductive strategies change and why "exaggerated traits" such as giant spermatozoa generally only occur in small and derived clades.

Author contributions

MT and MK designed the study, obtained and dissected specimens, acquired light microscope images, evaluated the results, and wrote the manuscript. MT performed semithin sectioning, carried out 3D data processing and designed the figures. MH supervised the study. All authors revised the manuscript, read and approved the final version.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

The authors would like to thank Michael von Tschirnhaus (Faculty of Biology, University of Bielefeld, Germany), Peter Zwick and Kevin Barber for providing alcohol preserved material of Lonchopteridae, Michael Raupach (SNSB-ZSM, Munich, Germany) for helping with calculating the bootstrap values, Heidemarie Gensler (Department of Biology, LMU, Germany) for advising on semi-thin sectioning and Alexander Rachel (Department of Mathematics and Statistics, LMU, Germany) for mathematical advice.

References

Baccetti, B., Dallai, R., Pallini, V., Rosati, F., Afzelius, B.A., 1977. Protein of insect sperm mitochondrial crystals. J. Cell Biol. 73, 594–600.

Bährmann, R., Bellstedt, R., 1988. Beobachtungen und Untersuchungen zum Vorkommen der Lonchopteriden auf dem Gebiet der DDR, mit einer Bestimmungstabelle der Arten. (Dipt., Lonchopteridae). Mitt Mus. Natur. Be 35, 265–279.

Bressac, C., Fleury, A., Lachaise, D., 1994. Another way of being anisogamous in *Drosophila* subgenus species: giant sperm, one-to-one gamete ratio, and high zygote provisioning. Proc. Natl. Acad. Sci. USA 91, 10399–10402.

Cruz-Landim, C., 2001. Organization of the cysts in bee (Hymenoptera, Apidae) testis: number of spermatozoa per cyst. Iheringia Ser. Zool. 91, 183–189.

Curtis, S.K., Benner, D.B., Musil, G., 1989. Ultrastructure of the spermatozoon of *Megaselia scalaris* Loew (Diptera: Brachycera: Cyclorrhapha: Phoridae).

J. Morphol. 200, 47–61.
Czerny, L., 1934. Familie 30: Musidoridae (Lonchopteridae). In: Lindner, E. (Ed.), Die Fliegen der palaearktischen Region lfg 83. E. Schweitzerbart'sche Verlagsbuchhandlung Erwin Nägele GmbH, Stuttgart.
Dallai, R., 1988. The spermatozoon of Asphondyliidi (Diptera, Cecidomyiidae). J. Ultra. Mol. Struct. Res. 101, 98–107.
Dallai, R., 2014. Overview on spermatogenesis and sperm structure of Hexapoda. Arthropod Struct. Dev. 43, 257–290.
Dallai, R., Gottardo, M., Mercati, D., Machida, R., Mashimo, Y., Matsumura, Y., Beutel, R.G., 2014. Giant spermatozoa and a huge spermatheca: a case of coevolution of male and female reproductive organs in the ground louse *Zorotypus impolitus* (Insecta, Zoraptera). Arthropod Struct. Dev. 43, 135–151.
Dallai, R., Lupetti, P., Afzelius, B.A., Mamaev, B.M., 1996. The sperm structure of the gall-midges *Anaretella* and *Lestremia* (Insecta, Diptera, Cecidomyiidae). Tissue Cell 28, 331–338.
Dallai, R., Mercati, D., Giusti, F., 2008. Structural organization of the "zipper line" in *Drosophila* species with giant spermatozoa. J. Struct. Biol. 161, 43–54.
Dallai, R., Fanciulli, P.P., Mercati, D., Lupetti, P., 2021. Coevolution between female seminal receptacle and sperm morphology in the semiaquatic measurer bug *Hydrometra stagnorum* L. (Heteroptera, Hydrometridae). Arthropod Struct. Dev. 60, 101001.
Dias, G., Lino-Neto, J., Mercati, D., Fanciulli, P.P., Lupetti, P., Dallai, R., 2021. The sperm ultrastructure of members of basal Tenebrionoidea (Coleoptera). Arthropod Struct. Dev. 66, 101129.
Dias, G., Mercati, D., Rezende, P.H., Lino-Neto, J., Fanciulli, P.P., Lupetti, P., Dallai, R., 2022. New findings on the sperm structure of Tenebrionoidea (Insecta, Coleoptera). Insects 13, 485.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
Fitzpatrick, J.L., Kahrl, A.F., Snook, R.R., 2022. SpermTree, a species-level database of sperm morphology spanning the animal tree of life. Sci. Data 9, 1–6.
Gage, M.J.G., 1994. Associations between body size, mating pattern, testis size and sperm lengths across butterflies. Proc. Roy. Soc. Lond. B 258, 247–254.
Hanna, P.J., Liebrich, W., Hess, O., 1982. Evidence against a $(2)^n$ synchronous increase of spermatogonia to produce spermatocytes in *Drosophila hydei*. Gamete Res. 6, 365–370.
Hihara, F., Kurokawa, H., 1987. The sperm length and the internal reproductive organs of Drosophila with special references to phyogenetic relationships. Zool. Sci. 4, 167–174.
Jamieson, B.G.M., Dallai, R., Afzelius, B.A., 1999. Insects: Their Spermatozoa and Phylogeny. Science Publishers, Enfield, New Hampshire, USA.
Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
Klymko, J., Marshall, S.A., 2008. Review of the Nearctic Lonchopteridae (Diptera), including descriptions of three new species. Can. Entomol. 140, 649–673.
Kotrba, M., 1995. The internal female genital organs of *Chaetodiopsis* and *Diasemopsis* (Diptera: Diopsidae) and their systematic relevance. Ann. Natal. Mus. 36, 147–159.
Kotrba, M., Heß, M., Dallai, R., 2016. Giant spermatozoa of *Diasemopsis* (Diopsidae, Diptera) – structural, ultrastructural and functional aspects. Arthropod Struct. Dev. 45, 42–56.
Kotrba, M., Tröster, M., Gensler, H., Ruthensteiner, B., Heß, M., 2021. Morphology and ultrastructure of the spermatozoa of *Lonchoptera lutea* Panzer, 1809 (Diptera: Lonchopteridae). Arthropod Struct. Dev. 60, 101004.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.
Liebrich, W., Hanna, P.J., Hess, O., 1982. Evidence for asynchronous mitotic cell divisions in secondary spermatogonia of *Drosophila*. Int. J. Invertebr. Reprod. 5, 305–310.
Lowe, D.D., Montell, D.J., 2022. Unconventional translation initiation factor EIF2A is required for *Drosophila* spermatogenesis. Dev. Dynam. 251, 377–389.
Lüpold, S., de Boer, R.A., Evans, J.P., Tomkins, J.L., Fitzpatrick, J.L., 2020. How sperm competition shapes the evolution of testes and sperm: a meta-analysis. Phil. Trans. R. Soc. B 375, 20200064.
Lüpold, S., Manier, M.K., Puniamoorthy, N., Schoff, C., Starmer, W.T., Luepold, S.H.B., Belote, J.M., Pitnick, S., 2016. How sexual selection can drive the evolution of costly sperm ornamentation. Nature 533, 535–538.
Meier, R., Kotrba, M., Ferrar, P., 1999. Ovoviviparity and viviparity in the Diptera. Biol. Rev. 74, 199–258.
Meijere, J.C.H. De, 1906. Die Lonchopteren des palaearktischen Gebietes. Tijdschr Entomol. 49, 44–98.
Miller, G.T., Pitnick, S., 2002. Sperm-female coevolution in *Drosophila*. Science 298, 1230–1233.
Parker, G.A., 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45, 526–567.
Parker, G.A., 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J. Theor. Biol. 96, 281–29.
Parker, G.A., 1993. Sperm competition games: sperm size and sperm number under adult control. Proc. Roy. Soc. Lond. B 253, 245–254.
Perotti, M.E., 1973. The mitochondrial derivate of the spermatozoon of *Drosophila* before and after fertilization. J. Ultra. Res. 44, 181–198.
Pitnick, S., 1996. Investment in testes and the cost of making long sperm in *Drosophila*. Am. Nat. 148, 57–80.
Pitnick, S., Hosken, D.J., Birkhead, T.R., 2009. Sperm morphological diversity. In: Birkhead, T.R., Hosken, D.J., Pitnick, S. (Eds.), Sperm Biology. Academic Press (Elsevier), London, pp. 69–149.
Pitnick, S., Markow, T.A., Spicer, G.S., 1995a. Delayed male maturity is a cost of producing large sperm in *Drosophila*. Proc. Natl. Acad. Sci. USA 92, 10614–10618.
Pitnick, S., Marrow, T., Spicer, G.S., 1999. Evolution of multiple kinds of female sperm-storage organs in Drosophila. Evolution 53, 1804–1822.
Pitnick, S., Spicer, G.S., Markow, T.A., 1995b. How long is a giant sperm? Nature 375, 109.
Pizzari, T., 2006. Evolution: the paradox of sperm leviathans. Curr. Biol. 16, R462–R464.
Presgraves, D.C., Baker, R.H., Wilkinson, G.S., 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. Proc. Roy. Soc. Lond. B 266, 1041–1047.
Ratnasingham, S., Hebert, P.D.N., 2007. BOLD: the barcode of life data systems. Mol. Ecol. Notes 7, 355–364.
Raupach, M.J., Hanning, K., Morinière, J., Hendrich, L., 2019. About *Notiophilus* Duméril, 1806 (Coleoptera, Carabidae): species delineation and phylogeny using DNA barcodes. Dtsch. Entomol. Z. 66, 63–73.
Richardson, K.C., Jarret, L., Finke, E.H., 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol. 35, 313–323.
Schärer, L., Da Lage, J.-L., Joly, D., 2008. Evolution of testicular architecture in the Drosophilidae: a role for sperm length. BMC Evol. Biol. 8, 143.
Sivinski, J., 1980. Sexual selection and insect sperm. Fla. Entomol. 63, 99–111.
Virkki, N., 1969. Sperm bundles and phylogenesis. Z. Zellforschung 101, 13–27.
Virkki, N., 1973. Evolution of sperm cell number per bundle in insects. An. Esc. nac. Cienc. biol. Mex. 20, 23–34.
Whittington, A.E., Beuk, P.L.T., 2022. A description of a new species of Western Palaearctic *Lonchoptera* Meigen (Diptera, Lonchopteridae) from Georgia. ZooNova 20, 1–18.
Wiegmann, B.M., Yeates, D.K., 2017. Phylogeny of Diptera. In: Kirk-Spriggs, A.H., Sinclair, B.J. (Eds.), Manual of Afrotropical Diptera, Suricata 4, vol. 1. South African National Biodiversity Institute, Pretoria, pp. 253–265.
Wu, D.-S., Zhou, H.-X., 1986. Studies on ultrastructure of spermatozoa from flies: *Eristalinus tarsalis* (Maq.). Acta Entomol. Sin. 29, 25–28.

Kapitel III

Coevolution of spermatozoa and spermathecae in Lonchopteridae (Diptera)

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Abstract

Across the species of spear-winged flies (Diptera: Lonchopteridae) there is a remarkable variation in size of the female reproductive tract, especially of the spermathecae. In this family there are two tubular spermathecae, which are divided into four morphologically and histologically distinct sections of different lengths and functions. The dimensions of the spermathecae and their individual sections were examined across 11 *Lonchoptera* species and related to the dimensions of the respective spermatozoa. 3D reconstructions from serial sectioning made it possible to include the volume in these considerations, which is a new approach in this context. Results show that the spermathecae are always longer than the respective spermatozoa. There is a highly significant positive linear correlation between the length of the spermatozoa and the length of the spermathecae in total as well as some of the individual spermathecal sections, suggesting a coevolution of these characters. Moreover, the volume of the spermathecae is much larger in those species with longer and more voluminous spermatozoa, but the volume increase is not sufficient to keep constant the number of spermatozoa that fit within. The observed patterns are discussed with respect to their functional and evolutionary implications, including a new hypothesis on the possible selective advantage of increased spermatozoon length.

Veröffentlicht als:

Tröster, M., Kotrba, M., Heß, M., 2024. Coevolution of spermatozoa and spermathecae in Lonchopteridae (Diptera). Arthropod Structure & Development 82, 101385.

Arthropod Structure & Development 82 (2024) 101385

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

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ARTICLE INFO

Article history:
Received 25 June 2024
Received in revised form
29 August 2024
Accepted 2 September 2024
Available online xxx

Handling Editor: Dr G. Scholtz

Keywords:
Lonchopteridae
Spermatheca
Coevolution
Sexual selection
Sperm competition
Reproduction

ABSTRACT

Across the species of spear-winged flies (Diptera: Lonchopteridae) there is a remarkable variation in size of the female reproductive tract, especially of the spermathecae. In this family there are two tubular spermathecae, which are divided into four morphologically and histologically distinct sections of different lengths and functions. The dimensions of the spermathecae and their individual sections were examined across 11 *Lonchoptera* species and related to the dimensions of the respective spermatozoa. 3D reconstructions from serial sectioning made it possible to include the volume in these considerations, which is a new approach in this context. Results show that the spermathecae are always longer than the respective spermatozoa. There is a highly significant positive linear correlation between the length of the spermatozoa and the length of the spermathecae in total as well as some of the individual spermathecal sections, suggesting a coevolution of these characters. Moreover, the volume of the spermathecae is much larger in those species with longer and more voluminous spermatozoa, but the volume increase is not sufficient to keep constant the number of spermatozoa that fit within. The observed patterns are discussed with respect to their functional and evolutionary implications, including a new hypothesis on the possible selective advantage of increased spermatozoon length.

1. Introduction

The occurrence of giant spermatozoa in the spear-winged fly *Lonchoptera lutea* Panzer, 1809 was first reported by Kotrba et al. (2021). In a subsequent comparative study of the male reproductive system of 11 *Lonchoptera* species, Tröster et al. (2023) found a dramatic variation in their sperm dimensions. Spermatozoon length and width both progressively increase within part of the genus at the expense of the sperm number per bundle and per testis. With a length of 7500 μm and a width of 1.3 μm the spermatozoon of *Lonchoptera fallax* De Meijere (1906) ranks among the largest in the animal kingdom. The authors discuss that the driving factor in the evolution of giant spermatozoa in Lonchopteridae might be sperm competition, but that other selective forces such as cryptic female choice may also be involved and that the very long spermathecae of *L. lutea* constitute a suggestive clue in this respect. Coevolution of spermatozoon length and the morphology of the female sperm storage organs has been documented in a wide range of insect orders such as Zoraptera (Dallai et al., 2014), Coleoptera (Ptiliidae: Dybas and Dybas, 1981; Bruchidae: Rugman-Jones and Eady, 2008; Dytiscidae: Higginson et al., 2012), Heteroptera (Gerridae: Dallai et al., 2021a; Hydrometridae: Dallai et al., 2021b), Lepidoptera (Gage, 1994; Morrow and Gage, 2000) and especially Diptera (Diopsidae: Presgraves et al., 1999; Scatophagidae: Minder et al., 2005; Drosophilidae: Pitnick et al., 1999), but also in other taxa such as birds (Briskie and Montgomerie, 1992; Briskie et al., 1997). For Drosophilidae, Miller and Pitnick (2002) have shown experimentally that the length of the female's sperm storage organ (the seminal receptacle) represents the mechanical determinant of postcopulatory female “sperm choice” and this is the driving factor for the evolution of spermatozoon length. Nevertheless, the convergent evolution of giant spermatozoa in a number of phylogenetically unrelated taxa (Fitzpatrick et al., 2022) constitutes a puzzling phenomenon. In spite of a number of studies in that respect, the underlying selective mechanisms have not been resolved. Based on their studies in Lonchopteridae Tröster et al. (2023) suggested a number of potential approaches to address this problem. One of them is the (comparative) investigation of the

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https://doi.org/10.1016/j.asd.2024.101385

morphology of the internal female reproductive system with respect to its potential coevolution with the sperm morphology. This approach is pursued in the present study by studying the same set of lonchopterid species as in Tröster et al. (2023) with a focus on the dimensions of the spermathecae and their four morphologically and histologically distinct sections. Some evolutionary scenarios are discussed as possible explanations for the observed patterns.

2. Materials and methods

2.1. Material

The study is based on alcohol preserved material (Table 1). The species list follows that of Tröster et al. (2023) with the addition of *Lonchoptera bifurcata* (Fallén, 1810), but lacking *Lonchoptera spec. R1* of which no female material was available. *L. bifurcata* and *L. lutea* specimens were collected by sweeping at the Botanical Garden Munich, Germany, in the summer of 2017, killed by freezing and immediately preserved in 80 % alcohol. *L. fallax*, *Lonchoptera nigrociliata* Duda, 1927, *Lonchoptera nitidifrons* Strobl, 1898, *Lonchoptera scutellata* Stein, 1890, *Lonchoptera tristis* Meigen, 1824, *Lonchoptera spec. T1* and *Lonchoptera spec. T2* females were acquired from the SNSB Bavarian State Collection of Zoology wet collection. Females of *L. strobli* De Meijere (1906) were provided by P. Zwick from his respective collection. Females of *L. barberi* Klymko, 2008 were freshly collected and identified by K. Barber in the summer of 2021. The 8 European species were identified on the basis of external morphological characters using Bährmann and Bellstedt (1988).

2.2. Preparation/Processing

For studying the gross morphology, freshly collected females of *L. bifurcata* and *L. lutea* were killed by freezing and their reproductive tract was extracted by dissection in a droplet of water using a Leica MZ 8 dissecting microscope. The extracted structures were mounted on microscopic slides in polyvinyl lactophenol with an admixture of chlorazol black E. Moreover, one female of each of the 11 species (Table 1) was studied by serial sectioning (two of *L. fallax*). For this, the abdomina of the alcohol preserved specimens were detached, dried in a graded alcohol series and embedded in Spurr's resin. Serial semithin sections were cut on an RMC MTXL ultra-microtome using a diamond knife, mounted on glass slides and stained with Richardson's reagent (Richardson et al., 1960).

2.3. Examination and imaging

The external morphological characters for species identification were examined using a Leica MZ 8 dissecting microscope. To assess the details of the internal female reproductive tract, semithin sections were investigated with a Zeiss Axioskop2 equipped with a Jenoptic Progres Gryphax Subra digital camera at maximal magnification using a Plan-Neofluar 100 × oil objective and 1.6 × additional magnification. Photographs included a digital scale bar calibrated with a stage micrometer. Measurements in the photos were taken using CorelDRAW Home and Student 2018.

For digital 3D reconstruction, the series of semithin sections were photographed with an automated Olympus BX61VS microscope with 40 × objective and DotSlide software using an Olympus XC10 digital camera. The photos were imported into Amira software (version 5.4.5) and aligned to obtain volume rendering depictions of the internal female reproductive tract. For detailed 3D reconstructions of the spermathecae, the circumference of these structure was digitized by hand for each individual section. Due to the laboriousness of this method only the two spermathecae of one specimen per species were reconstructed. Lengths and widths were assessed with the Amira 3D length measurement tool and volumes were calculated with the Amira material statistics tool. For some of the specimens retrieved from museum collections the quality of preservation is uncertain. This has to be considered as source of error especially regarding the volume renderings, but also with respect to the fact that the position of the spermatozoa within the reproductive tract may have shifted.

Statistical analyses and scatter diagrams were computed with MS Excel 2019. Because L. *fallax* often constitutes an outlier due to its extraordinarily long spermatozoa and was treated as such by Tröster et al. (2023), some of the regression analyses were calculated alternatively with and without including this species. The values for spermatozoon length and volume are taken from Tröster et al. (2023). Because body size does not vary greatly across the investigated species and no correlation between body size and characters of the male reproductive tract was found by Tröster et al. (2023), this character was not included in the present study. In the scatter diagrams the published sperm dimensions are consistently displayed on the abscissa, while the newly assessed spermathecal dimensions are displayed on the ordinate. This is not meant to imply an a priori hypothesis regarding a direction of dependence.

The figure plates were created using CorelDRAW Home and Student 2018 and Corel PHOTO-PAINT Home and Student 2018.

3. Results

3.1. Female reproductive tract of Lonchopteridae

The inner reproductive tract, as exemplified by *L. lutea* and *L. barberi* in Figs. 1–3, comprises paired ovaries and lateral oviducts, which are connected to the anterior end of the vagina by the common oviduct. The number of ovarioles per ovary varies from species to species. It is 5–7 in *L. barberi* (Fig. 2), *L. bifurcata* and *L. scutellata*, 4–5 in *L. lutea*, *L. nitidifrons*, *L. spec. T1*, *L. spec. T2*,

Table 1
Collection data.

Species	Date	Location	Collector
L. barberi	2021-08-14	Canada, Ontario, Sault Ste. Marie	K. Barber
L. bifurcata	2017-08-07	Germany, Bavaria, Munich	M. Tröster
L. fallax	1992-05-23	Germany, Bavaria, Schöngeising	W. Schacht
L. lutea	2017-08-07	Germany, Bavaria, Munich	M. Tröster
L. nigrociliata	1985-08-12	France, Alpes-de-Haute-Provence, Montlaux	W. Schacht
L. nitidifrons	1988-09-11	Germany, Bavaria, Ottmaring	W. Schacht
L. scutellata	2003-06-14	Germany, Mecklenburg-Western Pomerania, Gützkow	M. Kotrba
L. spec. T1	2002-12-09	Taiwan, Nantou County, Puli	W. Schacht
L. spec. T2	2000-07-01	Taiwan, Nantou County, Puli	W. Schacht
L. strobli	1983-10-14	Austria, Lower Austria, Lunz	P. Zwick
L. tristis	1991-09-06	Germany, Bavaria, Schöngeising	W. Schacht

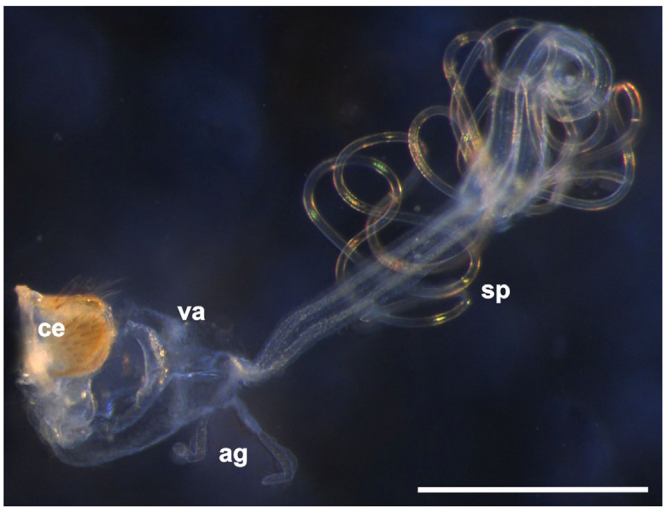

Fig. 1. Female reproductive tract of *L. lutea,* fresh dissection, without ovaries. ag, accessory glands; ce, cerci; sp, spermathecae; va, vagina. Scale bar: 500 μm.

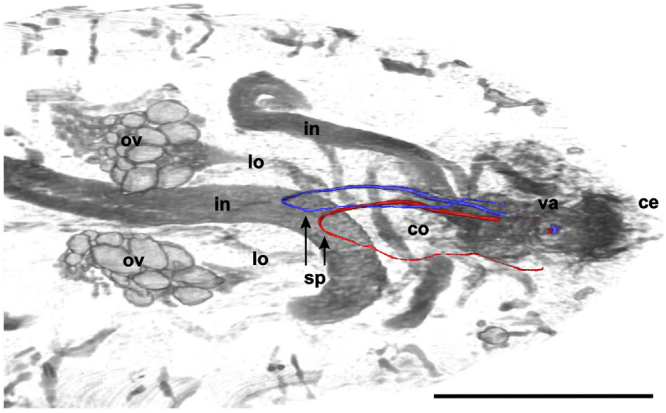

Fig. 2. Abdomen of *L. barberi* in ventral view, volume rendering from serial semithin sections with surface rendering of the spermathecae (red and blue). ce, cerci; co, common oviduct; in, intestine; lo, lateral oviduct; ov, ovary; sp, spermathecae; va, vagina. Scale bar: 400 μm.

L. strobli and *L. tristis*, and 2–3 in *L. fallax*. For *L. nigrociliata* the number could not be determined. No discrete structure resembling the ventral receptacle or the fertilization chamber of higher Diptera was identified. Only in some of the *L. lutea* whole mounts a sack-like membranous evagination of the anterior vagina was detected (Fig. 3), which never contained spermatozoa. Into the dorsal vagina wall open two unpigmented tubular spermathecae, each arising from an individual evagination of the vagina wall and, slightly posterior to these, two very delicate accessory glands (Fig. 1). Along the length of the spermathecae, four sections can be distinguished based on morphological and histological differences (Figs. 1 and 3). Their differentiation into spermathecal ducts and spermathecal reservoirs in terms of homology with the respective organs of other Diptera is not obvious from the results.

3.2. Morphology and histology of the spermathecae

The first section (S1), i.e., the basal part of the spermathecae, is lined by thin cuticle born on a layer of cuboidal epithelium. In most species a surrounding layer of circular musculature was detected (Fig. 4A, B, E, G) and this is likely present in all species. In L. *bifurcata*, *L. barberi, L. nigrociliata* and *L. scutellata* the lumen of S1 is narrow throughout with a smooth internal surface. In the other species the lumen widens apically. Here the cuticular lining is thicker, mostly due to a prominent endocuticular component, and the internal surface is adorned with transverse fringes of very fine, apically directed bristles (Fig. 4E–G).

The transition to the second section (S2) is defined by an abrupt widening of the lumen and a distinct change in the cuticular lining. The cuticle of S2 is denser and stains more strongly than that of S1 (Figs. 4 and 5). In the majority of the species, the inner surface is smooth throughout S2. Only in L. *barberi*, *L. bifurcata*, *L. nigrociliata* and *L. scutellata* the area apically adjacent to the transition from S1 to S2 is internally adorned with fringes of fine, apically directed bristles (Fig. 4A–D). The beginning of S2 is moreover generally defined by a surrounding layer of longitudinal musculature, which runs along its entire length and can be quite prominent. In *L. nigrociliata*, which shows an intermediary condition, the part marked by the internal cuticular ornamentation is similar to that in *L. barberi*, *L. bifurcata* and *L. scutellata*, but lacks the surrounding longitudinal musculature, which inserts apically of it (Fig. 4D), as in the remaining species. Apically the lumen of S2 tapers off gradually and the longitudinal muscle fibres terminate (Fig. 5A–C). Only in *L. strobli* the apical change in diameter appears as abrupt as the basal one (Fig. 5C).

The third section (S3) constitutes the longest of the four sections. It is a narrow tube lined by thin smooth cuticle born on a delicate epithelial layer and devoid of any prominent musculature. The lumen is widest near the transition from S2 and narrows apically. It is particularly narrow in *L. strobli* (Fig. 5C), *L. tristis* and *L. fallax* (Fig. 5E) with only 2–3 μm diameter along much of its length.

The fourth section (S4) is slightly wider than S3 and rather short. It is lined by thin smooth cuticle surrounded by glandular epithelium (Fig. 5F).

3.3. Spermatozoa within the reproductive tract

Matings were never observed in the wild or in the collecting cages. But in most of the dissections, with exception of *L. barberi* and the supposedly parthenogenetic species *L. bifurcata*, spermatozoa were detected within the female reproductive tract (Figs. 3–5). These were mostly located in S2 and often also in the basal part of S3, but not beyond the basal two-thirds. Inside S4 none were ever observed. Within the wide lumen of S2 the spermatozoa are usually arranged in a dense tangled mass (Fig. 4B and F, 5A-C) with sometimes some spermatozoa protruding a short way into S1 (Fig. 3B, also Fig. 1C in Kotrba et al., 2021). Near the transition to S3 and in the basal part of S3 itself, the spermatozoa lie stretched out in parallel, often very densely packed (Fig. 3C and 5D). Judged by their width, they are mostly oriented with their head regions pointing basally (Fig. 3C and D).

Very rarely, spermatozoa were observed in other regions of the reproductive tract. In some L. *lutea* specimens, masses of spermatozoa were observed in the posterior part of the vagina and once in the anterior region near the oviduct, but not within the sack-like membranous evagination. In one specimen of *L. fallax* spermatozoa were found transiting between the vagina and S1. These cases are taken to show transitory states shortly after mating or possibly preceding oviposition. From these results it appears that in Lonchopteridae spermatozoa are stored exclusively in the

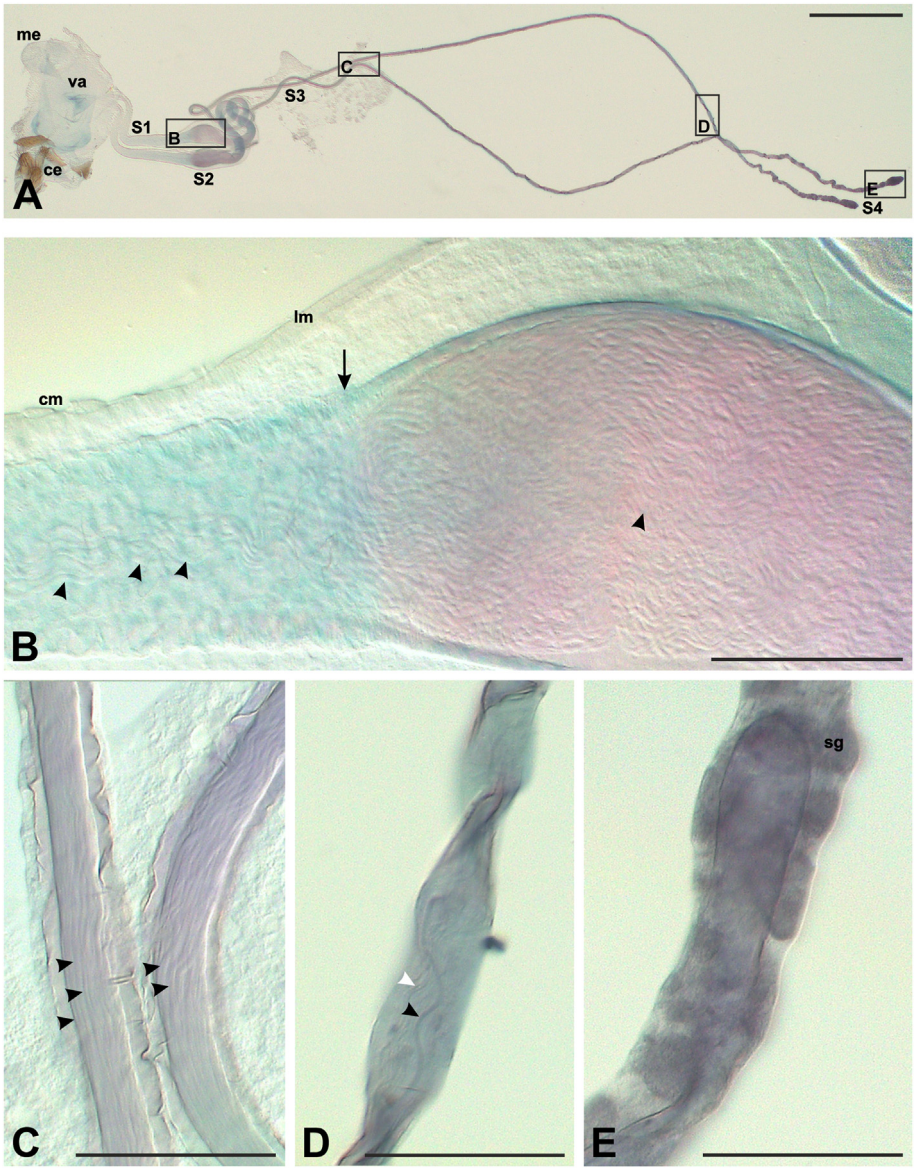

Fig. 3. Female reproductive tract of *L. lutea*, whole mount, without ovaries. **A:** Total, **B-E:** Magnifications from A, **B:** Detail of the transition between section 1 and section 2 of the spermatheca, **C, D:** Details of section 3 of the spermatheca, **E:** Detail of section 4 of the spermatheca. ce, cerci; me, membranous evagination; S1-4, sections 1-4 of the spermatheca; sg, glandular epithelium surrounding section 4 of the spermatheca; cm, circular musculature; lm, longitudinal musculature; va, vagina; arrow, transition from section 1 to section 2 of the spermatheca; black arrowhead, spermatozoon, wide anterior region; white arrowhead, spermatozoon, narrow tail region. Scale bars: A = 500 μm; B, C, D, E = 50 μm.

spermathecae, and the morphometric investigations below focus on the dimensions of these.

3.4. Morphometry of spermathecae and correlation with spermatozoon size

The numerical characters used for the statistical analysis are compiled in Table 2. These are the length and volume of the spermathecae and their individual sections as assessed from the 3D reconstructions (Fig. 6), as well as the length and volume of the respective spermatozoa from Tröster et al. (2023). Fig. 7 comparatively illustrates the length of the spermathecae and their four sections as well as the respective spermatozoon length across the studied species, which are arranged in four groups according to the phylogenetic hypothesis proposed by Tröster et al. (2023). The width of the spermathecae changes along their length, even within the individual sections. Thus, no representative value for the width of the spermathecae could be assessed and this character was not encompassed in the statistical analysis, but exemplary values are included in Fig. 7.

The length of the spermathecae varies dramatically across the species. With up to 1300 μm it is shortest in group I, which comprises *L. barberi*, *L. nigrociliata* and *L. scutellata*. *L. bifurcata* also falls into this category. In *L. fallax*, which belongs to the most derived group IV, the spermathecae are more than ten times longer, measuring about 13700 μm. In the species of groups II and III the length of the spermathecae is intermediary, ranging from about 3100 μm to about 5600 μm. From the exemplary values for the width, it is evident, that the width of S2 follows a similar pattern. It is smallest in group I, intermediary in group II, and largest in groups III-IV. The width of S3 shows no such consistency. Particularly wide forms occur in groups II and III, and particularly narrow forms with diameters of only 2–3 μm occur in groups II and IV. Comparison of the length of the spermathecae with the length of the respective

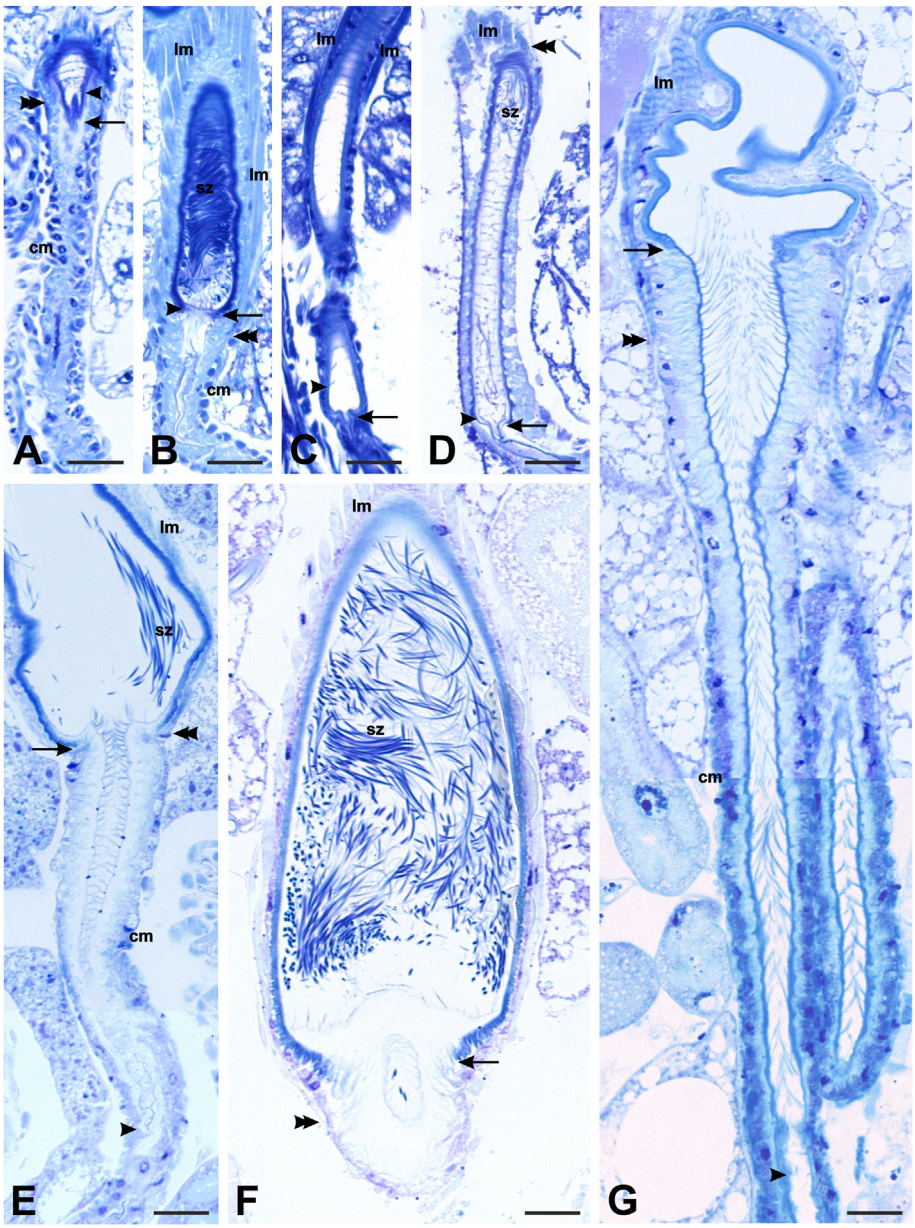

Fig. 4. Semithin sections of section 1 and section 2 of the spermatheca of some *Lonchoptera* species. **A:** *L. bifurcata*, **B:** *L. scutellata*, **C:** *L. barberi*, **D:** *L. nigrociliata*, **E:** *L. tristis*, **F:** *L. fallax*, **G:** *L. lutea*. cm, circular musculature; lm, longitudinal musculature; sz, spermatozoa; arrow, transition from section 1 (below) to section 2 (above) of the spermatheca indicated by a distinct change in the cuticle; arrowhead, basal end of the area internally adorned with transverse fringes of bristles; double arrowhead, basal end of the longitudinal musculature. Scale bars: 20 μm.

spermatozoa shows that the spermathecae are always much longer. The individual length variation of the four sections is not proportional to the variation of the total length. Instead, S3 stands out as the most variable section in this respect.

3.5. Statistical analysis

3.5.1. Length

The statistical analysis reveals a highly significant positive linear correlation between the total length of the spermathecae and the length of the spermatozoa across the species ($r^2 = 0.95$, $P < 0.01$) (Fig. 8A). All values lie well above the 1:1 line, indicating that the spermathecae are always longer than the spermatozoa. The slope of the trendline is 1.7. Its intercept is at 1500 μm. The statistical correlation remains strong when the outlier *L. fallax* is excluded ($r^2 = 0.83$, $P < 0.01$), albeit with a steeper slope of 2.3 and smaller intercept of 900 μm. The length ratio between spermathecae and spermatozoa decreases as their length increases. Thus, the spermathecae are about twice as long as the spermatozoa in those species with the longest spermatozoa, i.e., *L. fallax* and *L. lutea*, and up to 6 times as long in the other species (Fig. 8B).

Analysis of the individual sections (Fig. 8C–F) identifies S3 as the main contributor to the described correlation. This is the longest of the four sections, and it is the only part of the spermathecae that is always longer than the respective spermatozoa, with all values lying above the 1:1 line (Fig. 8E). The highly significant positive correlation with the length of the spermatozoa is very similar to that of the total spermathecal length ($r^2 = 0.93$, $P < 0.01$). The slope of the trendline is 1.5, its intercept at 900 μm. Again, the statistical correlation remains strong when the outlier *L. fallax* is

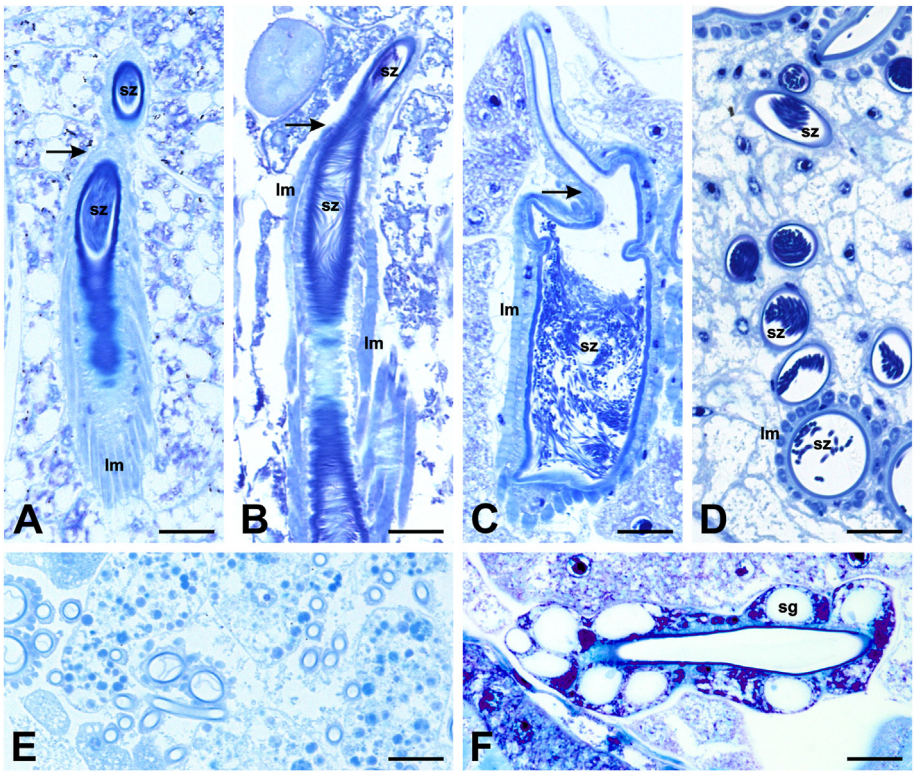

Fig. 5. Semithin sections of sections 2-4 of the spermatheca of some *Lonchoptera* species. **A:** *L. scutellata*, **B:** *L. nigrociliata*, **C:** *L. strobli*, **D:** *L. nitidifrons*, cross sections of section 2 of the spermatheca containing some spermatozoa and section 3 densely packed with spermatozoa, **E:** *L. fallax*, extremely long and convoluted section 3 of the spermatheca seen in a large number of oblique cross sections, **F:** *L. strobli*, oblique section through section 4 of the spermatheca. lm, longitudinal musculature; sg, spermathecal gland cells; sz, spermatozoa; arrow, transition from section 2 (below) to section 3 (above) of the spermatheca indicated by the apical end of the longitudinal musculature. Scale bars: 20 μm.

Table 2
Average length and volume of the spermathecae in total and the individual sections as assessed from the 3D reconstructions as well as the respective values for the spermatozoa from Tröster et al. (2023).[1] The reconstructed length in *L. lutea* falls in the range described by De Meijere (1906) and Kotrba et al. (2021).

Species	Length spermathecae [μm] ± s.d. (N)	Length S1 [μm] ± s.d. (N)	Length S2 [μm] ± s.d. (N)	Length S3 [μm] ± s.d. (N)	Length 4 [μm] ± s.d. (N)	Volume spermathecae [x 10^3 $μm^3$]	Volume S2 [x 10^3 $μm^3$]	Volume S3 [x 10^3 $μm^3$]	Volume S4 [x 10^3 $μm^3$]	Length spermatozoa [μm] ± s.d. (N)	Volume spermatozoa [x 10^3 $μm^3$]
L. barberi	1183 ± 16 (2)	191 ± 4 (2)	258 ± 6 (2)	542 ± 15 (2)	192 ± 1 (2)	58	31	24	4	280 ± 25 (5)	0.04
L. bifurcata	1134 ±15 (2)	244 ± 6 (2)	216 ± 2 (2)	511 ± 12 (2)	162 ± 7 (2)	122	70	49	4	–	–
L. fallax	13705 ±384 (4)	496 ± 3 (4)	860 ± 37 (4)	11945 ± 419 (4)	403 ± 4 (4)	723	458	230	35	7500 ± 157 (3)	5.97
L. lutea	4678 ± 41[1] (2)	635 ± 24 (2)	397 ± 6 (2)	3289 ± 75 (2)	358 ± 4 (2)	760	190	545	25	2200 ± 60 (13)	2.56
L. nigrociliata	1278 ± 6 (2)	261 ± 9 (2)	340 ± 0 (2)	464 ± 6 (2)	214 ± 10 (2)	90	65	16	8	370 ± 13 (3)	0.06
L. nitidifrons	4896 ±2 (2)	550 ± 11 (2)	759 ± 47 (2)	3398 ± 50 (2)	190 ± 10 (2)	754	142	594	18	1700 ± 120 (3)	1.30
L. scutellata	1133 ±11 (2)	230 ± 7 (2)	193 ± 3 (2)	527 ± 16 (2)	183 ± 9 (2)	70	50	15	6	190 ± 13 (4)	0.05
L. spec. T1	4378 ± 71 (2)	401 ± 3 (2)	267 ± 3 (2)	3596 ± 76 (2)	115 ± 4 (2)	173	56	123	4	970 ± 50 (3)	0.32
L. spec. T2	3143 ±125 (2)	540 ± 32 (2)	903 ± 9 (2)	1505 ± 81 (2)	196 ± 3 (2)	950	703	238	8	1150 ± 20 (3)	0.66
L. strobli	4845 ±25 (2)	495 ± 37 (2)	148 ± 10 (2)	4004 ± 63 (2)	198 ± 11 (2)	218	134	75	9	1500 ± 26 (4)	0.47
L. tristis	5597 ±108 (2)	519 ± 41 (2)	478 ± 10 (2)	4398 ± 60 (2)	203 ± 18 (2)	665	373	279	13	1730 ± 26 (4)	0.54

excluded ($r^2 = 0.70$, $P < 0.01$) with a steeper slope of 1.9.

The other sections are much shorter than S3 (Table 2). In the species belonging to group I, i.e., *L. barberi*, *L. nigrociliata* and *L. scutellata* (Fig. 7), each of these sections are about as long as the respective spermatozoa, as evident from the fact that the values lie close to the 1:1 line (Fig. 8C, D, F). In the other species, which are characterized by longer spermatozoa, the values lie below the 1:1 line, indicating that S1, S2 and S4 are each distinctly shorter than

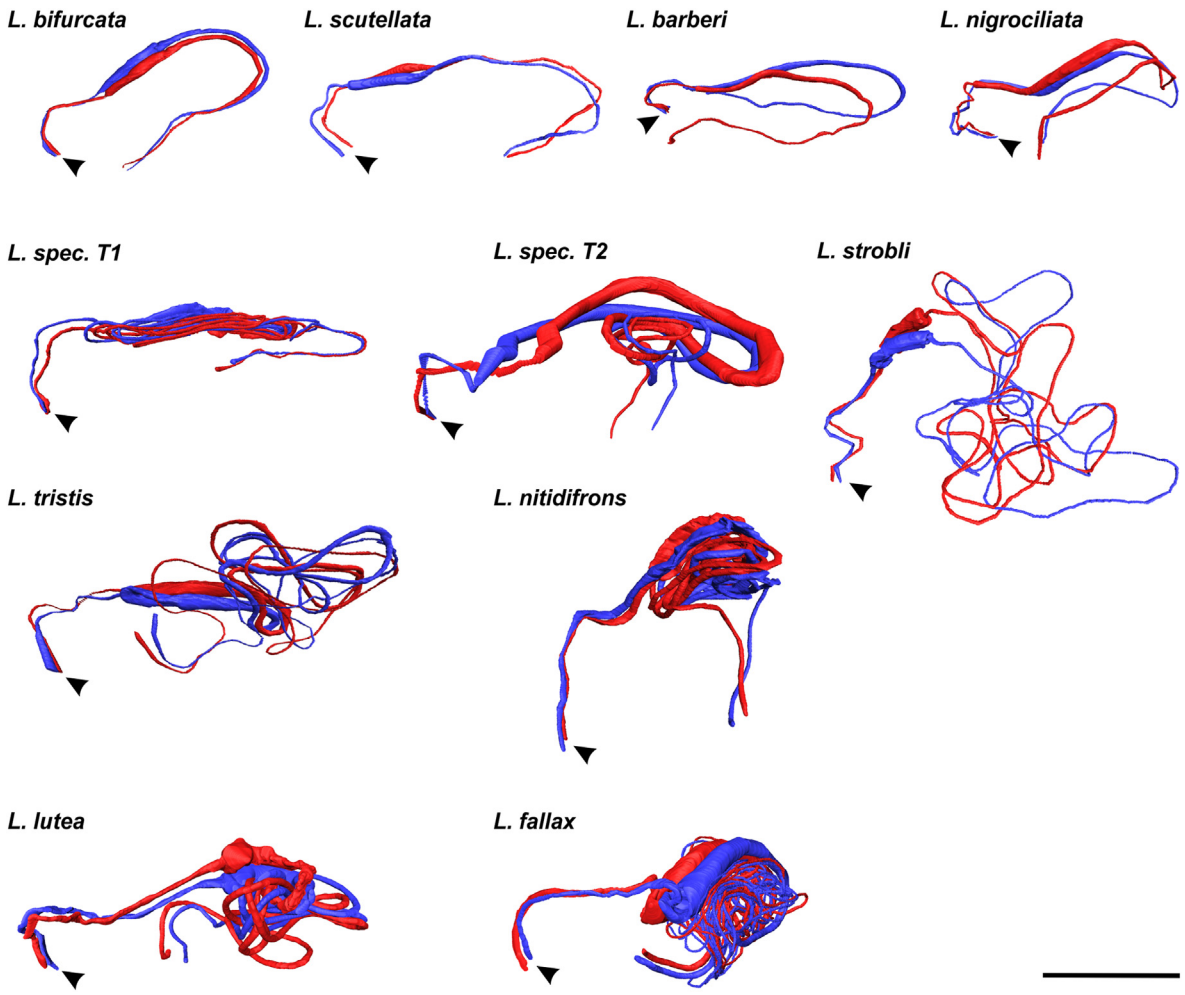

Fig. 6. 3D reconstructions of the paired spermathecae of the 11 investigated *Lonchoptera* species, surface rendering from serial semithin sections. Arrowhead, basal end of section 1 of the spermatheca. Scale bar: 300 μm.

the spermatozoa.

A significant positive correlation with the length of the spermatozoa is evident for S1 when the outlier *L. fallax* is excluded ($r^2 = 0.92$, $P < 0.01$) (Fig. 8C), but with a slope of only 0.2 the respective trendline is very shallow. Moreover, there is a similarly shallow positive correlation between the length of the spermatozoa and S4 ($r^2 = 0.64$, $P < 0.01$) (Fig. 8F) with a slope of 0.3, which turns insignificant upon the exclusion of *L. fallax*. The length of S2 shows no significant correlation with the length of the respective spermatozoa at all (Fig. 8D).

3.5.2. Volume

The observed changes in volume are not strictly proportional to those in length, because the widths of the spermathecae and their individual sections also varies across the species. The volume of the spermathecae is much larger in those species with longer and more voluminous spermatozoa, but not in terms of a strict linear correlation across all species. Instead, the spermathecal volume increases with the spermatozoon volume across the species of groups I-II ($r^2 = 0.76$, $P = 0.01$), but levels off in groups III-IV (Fig. 9G), i.e., in the three species with the most voluminous spermatozoa *L. fallax*, *L. lutea* and *L. nitidifrons*. The volume increase does not suffice to keep constant the number of spermatozoa that would theoretically fit into the spermathecae (spermathecal volume/spermatozoon volume), but it considerably reduces its decline (Fig. 9H). Thus, while the spermatozoon volume varies by a factor of 150, the number of spermatozoa that theoretically fit into the spermathecae only varies by a factor of 12.

Like the total spermathecal volume (above), there is a positive correlation between the spermatozoon volume and the volume of S2 across the species of groups I-II ($r^2 = 0.68$, $P = 0.02$), but the values for groups III-IV fall behind (Fig. 9I). The impression that these latter values might lie on a second, much shallower trendline would need to be substantiated by studying more species. The volume of S3 shows a significant positive correlation with the volume of the respective spermatozoa when the outlier *L. fallax* is excluded ($r^2 = 0.15$, $P = 0.26$, respectively $r^2 = 0.77$, $P < 0.01$ with *L. fallax* excluded) (Fig. 9J).

S4 is the smallest part or the spermathecae. It never contributes more than 8 % and often much less to the total volume of the spermathecal complex. But this is the section which shows the most striking correlation with the volume of the spermatozoa ($r^2 = 0.89$, $P < 0.01$) (Fig. 9K). The trendline is steeper when the outlier *L. fallax* is excluded ($r^2 = 0.88$, $P < 0.01$). Because this section is glandular and most likely produces a secretion which is discharged into the spermathecal tract, the relation of its volume with that of the entire spermathecae was also computed. The analysis shows a positive correlation ($r^2 = 0.39$, $P = 0.05$, respectively $r^2 = 0.45$, $P = 0.05$ with *L. fallax* excluded) (Fig. 9L), but the significance is much lower than that for the correlation with the spermatozoon volume.

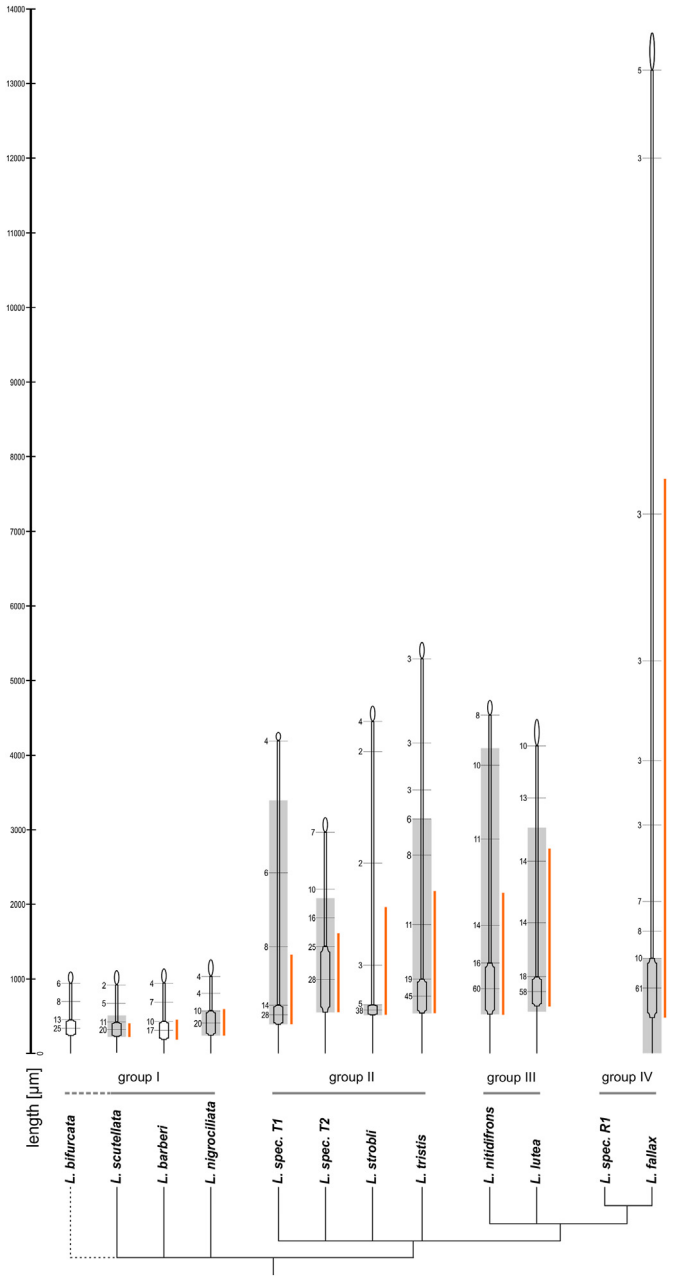

Fig. 7. Comparative schematic representation of the assessed numerical characters (Table 2) of the spermathecae and their four morphologically distinct sections combined with the length of the spermatozoa (red line) and the cladogram presented in Tröster et al. (2023). The lengths of the spermathecae and spermatozoa refer to the scale bar on the left. Exemplary local values for the width (in µm) are indicated left of the horizontal lines in the graphical representation. Gray blocks mark those parts of the spermathecae in which spermatozoa were observed.

4. Discussion

4.1. Evolutionary trajectories

The found changes in the morphology and the dimensions of the spermathecae are consistent with the phylogenetic hypothesis proposed by Tröster et al. (2023) based on their study of the dimensions of the spermatozoa of Lonchopteridae (cladogram included in Fig. 7). The results reflect the correspondence of these two structures and possibly lend further support to that hypothesis. It has to be considered, however, that some of the characters are not independent. The shift of the location of the internal ornamentation with fringes of cuticular bristles between the apical region of S1 and the basal region of S2 separates group I from the clade which comprises groups II-IV. Group I is further characterized by short spermatozoa and also short spermathecae, both characters showing little variation within this group. The spermathecae of *L. bifurcata* very closely match those of the species of group I in terms of both morphology and dimensions. This suggests the inclusion of *L. bifurcata* in this group. It contradicts the assumption of De Meijere (1906) that the short spermathecae of *L. bifurcata* represent a specific adaptation to parthenogenesis. His assumption was based on the comparison with *L. lutea* only. In comparison with the species of group I no reduction is evident. This might suggest that either the shift to parthenogenesis is only partial or it occurred very recently which, in turn, is consistent with isolated evidence of *L. bifurcata* males from the last century (Collin, 1938; Smith, 1969; Andersson, 1970). If males of *L. bifurcata* do exist, they likely produce short spermatozoa, like *L. barberi* and *L. scutellata*, not only because of the close relationship with these species, but also because the spermatozoon length correlates with the length of the spermathecae.

The clade comprising groups II-IV is characterized by an increase in both spermatozoon length and spermathecal length and also by a reduction in the number of ovarioles per ovary. These trends escalate in the most derived group IV, i.e., *L. fallax*, which emerged as an outlier in the preceding study regarding the dimensions of the spermatozoa (Tröster et al., 2023) and again in the present study regarding the length of S3.

4.2. Coevolution of spermathecae and spermatozoa

The evolutionary increase in length and volume of the spermatozoa in the genus *Lonchoptera* (Tröster et al., 2023) is matched by an increase in the length and, to some degree, also the volume of the female sperm storage organs, i.e., the spermathecae. This finding suggests that the two structures coevolved. The finding does not come as a surprise. A positive correlation between the spermatozoa and the female sperm storage organs has been reported in several other nonrelated taxa, sometimes involving the evolution of extraordinarily long forms (introduction). Various hypotheses regarding the adaptive significance of unusually large spermatozoa and a female selection for such giants have been proposed (e.g., Perotti, 1973; Dybas and Dybas, 1981; Parker, 1993; Bressac et al., 1994; Miller and Pitnick, 2002; Pizzari, 2006; Kotrba et al., 2016; Tröster et al., 2023). Many of these address the resulting benefits in terms of overall fitness for the male and female side. But tangible evidence on the underlying selective processes on the morphological and physiological level, regarding the interactions either among competing spermatozoa and/or between the spermatozoa and the female structures, remains scarce. The topic is extremely multifaceted and challenging to analyze in detail. For example, although changes in the size of the spermatozoa are most often regarded in terms of their length, they also affect a number of closely related components such as the width and volume (Tröster et al., 2023), but also the surface area. These components are difficult to discriminate from each other in an analysis and each offer their own range of implications for selective processes. The length might have effects on the propulsion, lends itself for some kind of direct competition in lengthwise arrangement with rivals, but might also serve to bridge a given distance, allowing the spermatozoon to be near to two distant points of special interest at the same time (below). The width might have effects regarding the rigidity of the sperm tail, but also on blocking a narrow duct or passage. The volume might signify some kind of paternal

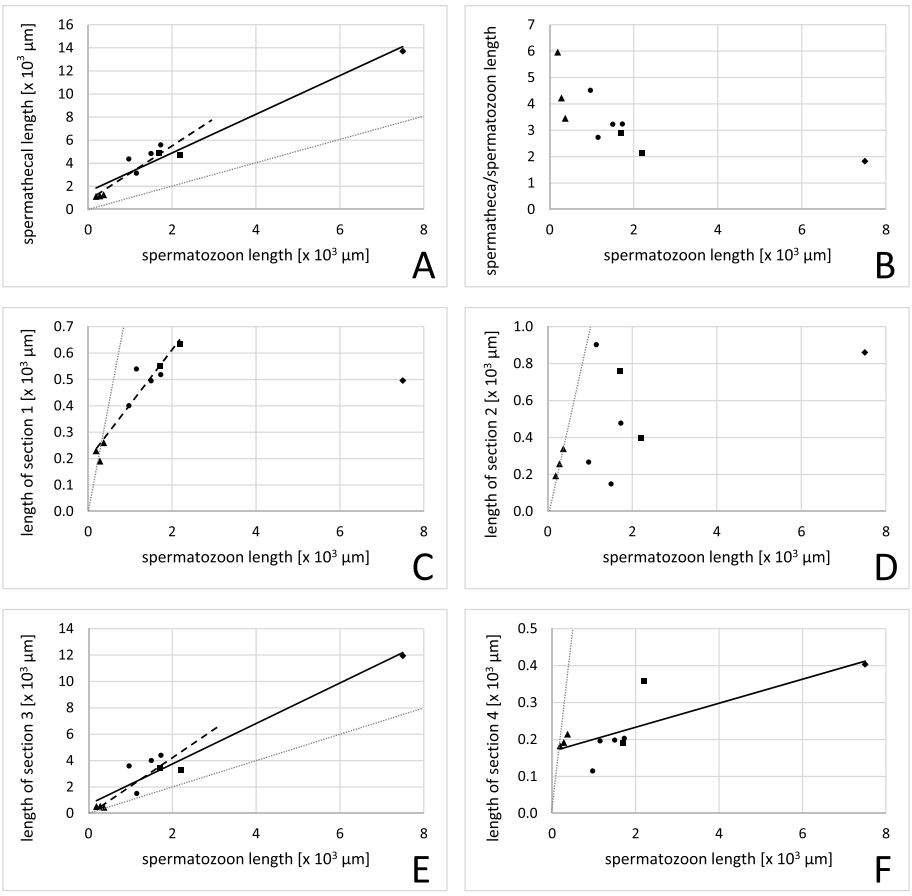

Fig. 8. Interspecific correlations between selected length characters of the spermathecae and the spermatozoon length across the investigated *Lonchoptera* species, only significant trendlines shown. **A:** Spermathecal length in total, **B:** Spermatheca length/spermatozoon length, **C:** Length of section 1 of the spermatheca, **D:** Length of section 2 of the spermatheca, **E:** Length of section 3 of the spermatheca, **F:** Length of section 4 of the spermatheca. Triangles, species of group I; dots, species of group II; squares, species of group III; diamonds, species of group 4 (*L. fallax*). Solid line, trendline across all species; dashed line, trendline with the outlier *L. fallax* excluded; dotted line, 1:1 line.

investment, but may also have effects regarding sperm displacement. The surface area might affect the number of present receptor molecules, but also the friction. The respective selective processes might take place during sperm transfer, storage and release from the female storage organs, but also on the way to, and directly at, the fertilization site. Observed changes of the female morphology might be secondary adaptations to changes in the spermatozoa. For example, the storage volume might be increased to ensure the necessary number of stored spermatozoa for egg fertilization, or some glandular tissue might be enlarged to support the survival of larger and more demanding spermatozoa. But the female morphology might also evolve to set the stage for enhanced sperm competition or to actively select for sperm of some superior quality.

This is just a selection of the possible aspects and it is very challenging to consider them all. Regarding the female morphology, the results of this study have revealed a considerable diversity. The limited amount of data is not sufficient for a proper multifactorial analysis. But even ordinary regression analyses already allow some preliminary speculations on the significance of the observed evolutionary changes.

4.2.1. Volume

One of the first obvious connections that springs to mind is the fact that any increase in spermatozoon volume will reduce the number of spermatozoa that can be stored, unless the female sperm storage organs adapt accordingly. Such a reduction is not necessarily a selective disadvantage for the female, especially if the fewer spermatozoa that are stored are of superior quality. But this applies only as long as their number is sufficient to fertilize all eggs to be oviposited. Hence, the first and most proximate reason for an increase in size/volume of the sperm storage organs might be to ensure that the number of stored spermatozoa is sufficient for egg fertilization. Beyond that, a secondary reason might be to ensure a surplus in sperm number and thus allow for enhanced sperm competition and/or female sperm selection.

The volume relations between spermatozoa and sperm storage organs have rarely been addressed in previous publications, probably due to the fact that they are difficult to assess. The present studies of the genus *Lonchoptera* with 3D reconstructions make such considerations possible. The results, particularly those regarding S2, have to be considered with a caveat, however, because here the volume is probably much dependent on the degree of filling and the tension of the surrounding tissues. Some of the reconstructed shapes of S2 appear to be considerably distorted (Fig. 6). The analysis suggests that the effect of the increased spermatozoon volume is partially neutralized by a concurrent increase in spermathecal volume, but not in terms of a strict linear correlation (Fig. 8G). The volume increase is kept up throughout the species of groups I-II, but discontinued in the derived groups III-IV. Whereas the spermatozoon volume varies by a factor of 150, the number of spermatozoa theoretically fitting into the spermathecae only varies by a factor of 12, i.e., more than ten times less (Fig. 8H). At the same time the number of ovarioles is smaller in species with larger spermatozoa. In a conservative calculation for the species

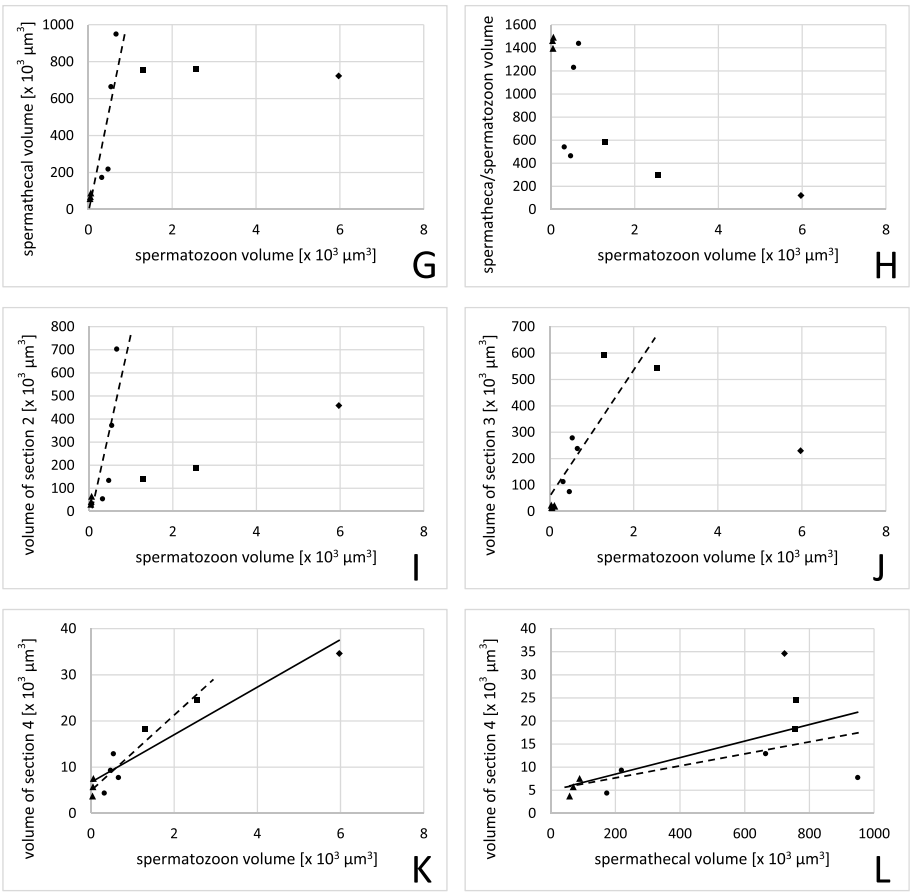

Fig. 9. Interspecific correlations between selected volume characters of the spermathecae and the spermatozoa across the investigated Lonchoptera species. G: Spermathecal volume in total, H: Spermatheca volume/spermatozoon volume, i.e. theoretical storage capacity, I: Volume of section 2 of the spermatheca, J: Volume of section 3 of the spermatheca, K: Volume of section 4 of the spermatheca, L: Volume of section 4 of the spermatheca and spermathecal volume in total. Triangles, species of group I; dots, species of group II; squares, species of group III; diamonds, species of group 4 (L. fallax). Solid line, trendline across all species; dashed line in J and L, trendline with the outlier L. fallax excluded; dashed line in G and I, trendline with L. fallax, L. lutea and L. nitidifrons excluded; dotted line, 1:1 line.

with the most voluminous spermatozoa *L. fallax*, taking into account only the storage capacity of S2 alone (77 per spermatheca) and the maximal observed number of ovarioles (3 per ovary), the ratio of stored spermatozoa per ovariole would still be about 25 as opposed to more than 100 in the group I species with small spermatozoa. Because within the ovarioles the eggs mature consecutively, this should still suffice for many oviposition events. Moreover, the female has time to remate while new eggs are maturing in the ovaries. The evolutionary increase in spermathecal volume together with a reduced number of ovarioles thus ensures that the number of stored spermatozoa does not become a limiting factor for the number of eggs that can be fertilized in species with larger spermatozoa. But the number of stored spermatozoa is still much reduced. There is no indication that the observed volume increase of the spermathecae per se enhances sperm competition or selection for more voluminous spermatozoa.

4.2.2. Length

As opposed to the volume, the length of the spermatozoa and their coevolution with the female sperm storage organs has been addressed in a large number of previous publications. Dybas and Dybas, 1981 were the first to describe the complementary morphological relationship between large spermatozoa and corresponding female structures in featherwing beetles (Ptiliidae). They attributed this condition to the ensurance of sperm precedence by the first male to mate with the female and to the preclusion of displacement by spermatozoa from subsequent matings, but provided no experimental evidence for this hypothesis. By now, the best studied taxon in this respect is the dipteran genus *Drosophila*. In this very speciose genus, the spermatozoon length is highly variable (e.g., Hatsumi and Wakahama, 1986; Hihara and Kurokawa, 1987; Joly et al., 1989; Joly and Bressac, 1994; Pitnick et al., 1995a), including the longest spermatozoon known to date (Pitnick et al., 1995b). The length of the tubular seminal receptacle of the female is likewise highly variable and strongly correlated with the spermatozoon length across the species (Hihara and Kurokawa, 1987; Joly and Bressac, 1994; Yang and Lu, 2011). Miller and Pitnick (2002) demonstrated experimentally that the length of the seminal receptacle in *Drosophila melanogaster* Meigen, 1830 constitutes a mechanical determinant of postcopulatory female "sperm choice". They speculate that this could be affected by providing an environment in which "being the right size" offers some advantage to spermatozoa in occupying a superior position, e.g., with their heads located near the exit of that organ and thus closest to the site of egg fertilization (Miller and Pitnick, 2002). Pattarini et al. (2006) showed that longer spermatozoa are better at displacing and resisting being displaced by shorter spermatozoa from that position and Manier et al. (2010) corroborated that this physical displacement by competitor spermatozoa is a critical determinant of competitive fertilization success. But even after decades of intense studies in this exemplary taxon a functional explanation as to how the selection on spermatozoon length is

exerted is still lacking.

The ventrally positioned seminal receptacle of *Drosophila* is not homologous with the dorsally positioned spermathecae, but an entirely different organ that has secondarily assumed the function of sperm storage (Nonidez, 1920; Pitnick et al., 1999). However, in several aspects the spermathecae of *Lonchoptera* species seem quite comparable. Both structures constitute narrow tubular ducts where large numbers of filamentous spermatozoa are stored prior to fertilization. And in both cases the length of the sperm storage organ is significantly correlated with the length of the spermatozoa across the species. Therefore, it seems plausible to expect similar selective regimes in both systems. However, there are also some notable differences. The seminal receptacle of *Drosophila* is not conspicuously differentiated along its length and has no distinct glandular section at its distal end. It is generally only little longer than the spermatozoa, which are predominantly arranged longitudinally within. In contrast, the spermathecae of *Lonchoptera* species are differentiated into four morphologically and histologically distinct sections, including a specific glandular section at their distal end (Figs. 3A and E, 5F). They are always much longer than the spermatozoa, which lay in a tangled mass within the voluminous S2, while only those (parts of the) spermatozoa extending into S3 are arranged longitudinally. Thus, in *Lonchoptera* it can be excluded, that the observed coevolution of spermatozoon length and spermathecal length is driven by a straightforward mechanical fit of the two structures in total. Instead, the potential interactions of the spermatozoa with the four individual sections should to be scrutinized separately.

The basal section S1, which directly connects to the vagina, most likely has the function of a duct, allowing passage in and out of storage, rather than being a storage site itself. It was only rarely found to contain any sperm. The surrounding circular musculature probably allows to constrict or seal off this passage. Moreover, the transition into S2 is marked by a sharp increase in diameter and adjacent areas are lined by fringes of fine, apically directed bristles. These fringes may obstruct the exit of spermatozoa from S2 or alternatively help their orientation into and/or out of S2. In some dissections a cluster of sperm heads was found protruding from S2 into this bristly area. These morphological details suggest that the female can exert some control over the release of spermatozoa from S2 into S1. The length of S1 is significantly correlated with the spermatozoon length across the majority of the species, albeit with a very shallow slope (Fig. 8C). Only in the species of group I this section is long enough to accommodate the entire length of the respective spermatozoa; in all other species it is much too short. Thus, it is hard to conceive a selective mechanism that could affect the coevolution of the lengths of S1 and the respective spermatozoa.

S2 is always the widest, and in the majority of the species also the most voluminous part of the spermathecae, where usually the bulk of the spermatozoa is found in a coiled or apparently unordered tangled mass (Fig. 4B andF). This suggests that S2 is the predominant site for sperm storage. It is surrounded by a prominent layer of longitudinal musculature (Fig. 3B and 4B-G, 5A-D) which likely plays a part in actively discharging its contents. The length of S2 shows no significant correlation with the spermatozoon length. Only in the species of group I it is long enough to accommodate the entire length of the respective spermatozoa; in all other species it is much too short. All evidence considered, S2 seems not to be the relevant section of the spermathecae for the length coevolution between spermatozoa and spermathecae.

S3 is the most interesting component in this respect. It constitutes the longest and most variable part of the spermathecae and this is the only section that is always more than long enough to accommodate the entire length of the spermatozoa in an outstretched fashion. Moreover, its length is strongly correlated with the spermatozoon length across all species. Within the (sometimes extremely) long and narrow lumen of S3, whose perfectly round diameter is stabilized by a solid cuticular lining (Fig. 5D and E), the contained spermatozoa are found stretched out in parallel and sometimes very densely packed (Figs. 3D and 5D). Thus, it is plausible that the lengths of the spermatozoa and S3 directly interact in some way. However, the selective advantage for the spermatozoa of entering S3 instead of mixing with the bulk of spermatozoa in S2 is not obvious. One possible reason is that spermatozoa lodged in S3 are isolated from any kind of sperm displacement occurring in S2, be it by rival spermatozoa or by the female itself. Moreover, the longitudinal arrangement within the narrow lumen of S3 provides for increased contact between the spermatozoa and the spermathecal wall which may facilitate sperm locomotion (Morrow and Gage, 2000; Werner et al., 2007) and/or improve the ability to displace other spermatozoa or resist such displacement (Dybas and Dybas, 1981; Presgraves et al., 1999; Pattarini et al., 2006). However, S3 is not merely a tubular extension of S2. This narrow duct connects the site of glandular discharge with the site of bulk sperm storage. Spermatozoa residing in S3 will have better and faster access to any discharge generated in S4 and at the same time may effectively reduce access to this secretion for other spermatozoa by blocking the lumen of S3. Although nothing is known about the biochemistry of the spermathecal glands in *Lonchoptera*, their secretion is likely to constitute an important resource for the spermatozoa, possibly fostering their survival, performance or release. In *Drosophila* the survival of spermatozoa in the seminal receptacle depends on the presence of functional spermathecal glands (Anderson, 1945; Boulétreau-Merle, 1977; Pitnick et al., 1999). A similar dependency could exist in *Lonchoptera*.

S4, which is the smallest of the four sections and the apical end of the spermathecae, is obviously itself no major player in driving the coevolution of length of spermatozoa and spermathecae. This section constitutes the glandular part of the complex, as evident from the surrounding glandular epithelium. Spermatozoa were never observed in this section and, if they ever enter it at all, are definitely not stored there. Nevertheless, the length and especially the volume of this section are significantly correlated with the respective sperm dimensions (Fig. 8F and K). One possible explanation for this relation is that more secretion has to be produced to meet some needs of the larger spermatozoa. Alternatively, more secretion might be required to fill the more voluminous spermathecae of those species with larger spermatozoa. The fact, that the correlation with the volume of the spermatozoa (Fig. 8K) is stricter than that with the volume of the spermathecae (Fig. 8L), presently lends more support to the first scenario.

The preceding considerations suggest that the female structures most relevant for a coevolution with the spermatozoa in terms of length are likely either S3 or S2 and S3 combined. Moreover, they allow to identify two positions that might infer a particular advantage for spermatozoa residing there. The first one is near the basal exit of S2, which might provide a head start at the time of sperm release for egg fertilization. In the dissected specimens, spermatozoa were found in S2 and in the basal part of S3 with their head portions predominantly oriented towards or inside S2 and their tails extending towards S4. In some specimens, a cluster of sperm heads was found embedded in the bristly area at the apical end of S1. Of course, it cannot be dismissed that spermatozoa may have shifted their position during the process of killing and fixing the specimen. But the latter arrangement is very much reminiscent of a similar finding described for *Drosophila*, where “a small and well-organized group of sperm heads can be observed near the proximal end of the SR [seminal receptacle] with their tails

extending distally" (Miller and Pitnick, 2002). The authors propose that these by virtue of their location take precedence over sperm residing more distally within the seminal receptacle. In *Lonchoptera*, a second location of potentially high advantage is the position near the distal end of the spermathecae next to S4, which provides priority access to the secretions of that section. If, for example, at the time of oviposition a chemical signal secreted in S4 triggered the departure of the spermatozoa from the spermathecae towards the fertilization site, then spermatozoa residing next to this section might be the first if not the only ones to receive this signal, as opposed to their rivals further down the spermathecal complex. The spermatozoa – long as they may be – are still always much shorter than the distance between the two advantageous positions, which implies that they cannot partake in both advantages at the same time. In this scenario any increase in spermatozoon length should translate into a melioration of that dilemma and thus to an immediate gain in fitness for the respective male. At the same time, the female fitness might be advanced by further extending the distance and thereby directly selecting for increased spermatozoon length as a sign of superior male genetic quality or of more immediate benefits e.g., in terms of zygote provisioning (Perotti, 1973; Bressac et al., 1994; Kotrba et al., 2016).

For now, it remains unresolved which of the sexes actually takes the leading part. On the one hand, the excessive length of the spermathecae and also of S3 alone, which is always much longer than necessary to accommodate the length of the spermatozoa (Figs. 7 and 8A, B, E), suggests that the female structures might constitute the driving factor. On the other hand, the fact that the length ratio between spermathecae and spermatozoa decreases as their length increases (Fig. 8B), i.e., the length of the spermatozoa increases faster than that of the spermathecae, seems to indicate that the spermatozoon size is the driving factor. In total, the described system seems predestined for entering an evolutionary arms race (Dawkins and Krebs, 1979; Parker, 1983) and the extremely long spermatozoa and spermathecae found in *L. fallax* appear to be a result of just that.

5. Conclusions

The findings of this study show that the spermatozoa and the female sperm storage organs coevolved in Lonchopteridae and add to the notion that such coevolution constitutes a widespread pattern. Some functional implications of the observed morphological structures and dimensional correlations are elucidated, contributing to the general understanding of the covert interactions taking place within the female reproductive tract. But ultimately, it can not be discriminated which form of sexual selection occurs in Lonchopteridae and, most importantly, whether male or female aspects constitute the driving factor in this respect. In order to understand the processes in mechanistic and functional terms, it could be rewarding to study more taxa with a record of extremely long sperm storage organs for a correlated response in the sperm morphology. In this respect, Diptera offer a wide array of distantly related taxa, such as Asilidae (Artigas et al., 1997), Dolichopodidae (Sturtevant, 1925, 1926), Chloropidae and Milichiidae (Sturtevant, 1925, 1926; Brake, 2000) for extremely long spermathecae, and Agromyzidae (Sasakawa, 1958), Clusiidae (Sturtevant, 1925, 1926; Lonsdale and Marshall, 2006), Anthomyzidae (Roháček, 1999, 2006) and Micropezidae (Kotrba, 2016) for long tubular ventral receptacles, just to name a few. But merely finding more examples for the established pattern will not add to the understanding of the underlying mechanisms. This will only be achieved by extending the detailed studies with the more sophisticated methods available today, e.g., fluorescent markers and/or specific antibodies to directly observe the interactions of spermatozoa and female sperm storage organs in vivo or by the precise analysis of the biochemical composition and physiological role of the spermathecal secretion. Ultimately, it must be decided in case-by-case studies which processes of postcopulatory sexual selection led to the coevolution of spermatozoa and sperm storage organs in each respective taxon, but the study of tiny nondescript flies such as Lonchopteridae can certainly contribute to the resolution of such big questions.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Michael Tröster: Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Marion Kotrba:** Writing – review & editing, Writing – original draft, Conceptualization. **Martin Heß:** Supervision.

Declaration of competing interest

We have no relevant interest to disclose.

Acknowledgements

The authors would like to thank Michael von Tschirnhaus (Faculty of Biology, University of Bielefeld, Germany), Peter Zwick and Kevin Barber for providing alcohol preserved material of Lonchopteridae and Heidemarie Gensler (Department of Biology, LMU, Germany) for advising on semi-thin sectioning.

References

Anderson, R.C., 1945. A study of the factors affecting fertility of lozenge females of *Drosophila melanogaster*. Genetics 30, 280–296.
Andersson, H., 1970. Notes on north european Lonchoptera (Dipt., Lonchopteridae) with lectotype designations. Entomol. Ts. Arg. 91, 42–45.
Artigas, J.N., Papavero, N., da Costa, N.C., 1997. The American genera of Asilidae (Diptera): Keys for identification with an atlas of female spermathecae and other morphological details. VIII. Subfamily Laphystiinae GH Hardy, with descriptions of five new genera and species and a catalogue of the Neotropical species. Arq. Zool. (Sao Paulo) 34, 1–55.
Bährmann, R., Bellstedt, R., 1988. Beobachtungen und Untersuchungen zum Vorkommen der Lonchopteriden auf dem Gebiet der DDR, mit einer Bestimmungstabelle der Arten. (Dipt., Lonchopteridae). Dtsch. Entomol. Z. 35, 265–279.
Boulétreau-Merle, J., 1977. Role des spermatheques dans l'utilisation du sperme et la stimulation de l'ovogenese chez *Drosophila melanogaster*. J. Insect Physiol. 23, 1099–1104.
Brake, I., 2000. Phylogenetic systematics of the Milichiidae (Diptera, Schizophora). Entomologica scandinavica, Supplements No. 57, 1–120.
Bressac, C., Fleury, A., Lachaise, D., 1994. Another way of being anisogamous in Drosophila subgenus species: Giant sperm, one-to-one gamete ratio, and high zygote provisioning. Proc. Natl. Acad. Sci. USA 91, 10399–10402.
Briskie, J.V., Montgomerie, R., 1992. Sperm size and sperm competition in birds. Proc. Roy. Soc. Lond. B 247, 89–95.
Briskie, J.V., Montgomerie, R., Birkhead, T.R., 1997. The evolution of sperm size in birds. Evolution 51, 937–945.
Collin, J.E., 1938. The British species of *Lonchoptera*. Ent. Mon. Mag. 74, 60–65.
Dallai, R., Gottardo, M., Mercati, D., Machida, R., Mashimo, Y., Matsumura, Y., Beutel, R.G., 2014. Giant spermatozoa and a huge spermatheca: A case of coevolution of male and female reproductive organs in the ground louse *Zorotypus impolitus* (Insecta, Zoraptera). Arthropod Struct. Dev. 43, 135–151.
Dallai, R., Fanciulli, P.P., Lupetti, P., Mercati, D., 2021a. The ultrastructure of sperm and female sperm storage organs in the water strider *Gerris lacustris* L. (Heteroptera) and a possible example of genital coevolution. Arthropod Struct. Dev. 61, 101043.
Dallai, R., Fanciulli, P.P., Mercati, D., Lupetti, P., 2021b. Coevolution between female seminal receptacle and sperm morphology in the semiaquatic measurer bug *Hydrometra stagnorum* L. (Heteroptera, Hydrometridae). Arthropod Struct. Dev. 60, 101001.
Dawkins, R., Krebs, J.R., 1979. Arms races between and within species. Proc. Roy. Soc.

Lond. B 205, 489–511.
De Meijere, J.C.H., 1906. Die Lonchopteren des palaearktischen Gebietes. Tijdschr. Entomology (Tokyo) 49, 44–98.
Dybas, L.K., Dybas, H.S., 1981. Coadaptation and taxonomic differentiation of sperm and spermathecae in featherwing beetles. Evolution 35, 168–174.
Fitzpatrick, J.L., Kahrl, A.F., Snook, R.R., 2022. SpermTree, a species-level database of sperm morphology spanning the animal tree of life. Sci. Data 9, 1–6.
Gage, M.J.G., 1994. Associations between body size, mating pattern, testis size and sperm lengths across butterflies. Proc. Roy. Soc. Lond. B 258, 247–254.
Hatsumi, M., Wakahama, K.-I., 1986. The sperm length in *Drosophila nasuta* subgroup. Jpn. J. Genet. 61, 241–244.
Higginson, D.M., Miller, K.B., Segraves, K.A., Pitnick, S., 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. USA 109, 4538–4543.
Hihara, F., Kurokawa, H., 1987. The sperm length and the internal reproductive organs of *Drosophila* with special references to phyogenetic relationships. Zool. Sci. 4, 167–174.
Joly, D., Bressac, C., 1994. Sperm length in Drosophilidae (Diptera): Estimation by testis and receptacle lengths. Int. J. Morphol. and Embryol. 23, 85–92.
Joly, D., Cariou, M.-L., Lachaise, D., David, J.R., 1989. Variation of sperm length and heteromorphism in drosophilid species. Genet. Sel. Evol. 21, 283–293.
Kotrba, M., 2016. The female reproductive tract of *Grallipeza* sp. A (Diptera: Micropezidae) – large ventral receptacle substitutes for spermathecae convergently in small and widely separated dipteran clades. Org. Divers. Evol. 16, 525–532.
Kotrba, M., Heß, M., Dallai, R., 2016. Giant spermatozoa of *Diasemopsis* (Diopsidae, Diptera) – structural, ultrastructural and functional aspects. Arthropod Struct. Dev. 45, 42–56.
Kotrba, M., Tröster, M., Gensler, H., Ruthensteiner, B., Heß, M., 2021. Morphology and ultrastructure of the spermatozoa of *Lonchoptera lutea* Panzer, 1809 (Diptera: Lonchopteridae). Arthropod Struct. Dev. 60, 101004.
Lonsdale, O., Marshall, S.A., 2006. Revision of the new world species of *Craspedochaeta* (Diptera: Clusiidae). Zootaxa 1291, 1–101.
Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., Pitnick, S., 2010. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science 323, 354–357.
Miller, G.T., Pitnick, S., 2002. Sperm-female coevolution in *Drosophila*. Science 298, 1230–1233.
Minder, A.M., Hosken, D.J., Ward, P.I., 2005. Co-evolution of male and female reproductive characters across the Scatophagidae (Diptera). J. Evol. Biol. 18, 60–69.
Morrow, E.H., Gage, M.J.G., 2000. The evolution of sperm length in moths. Proc. Roy. Soc. Lond. B 267, 307–313.
Nonidez, J.F., 1920. The internal phenomena of reproduction in *Drosophila*. Biol. Bull. 39, 207–230.
Parker, G.A., 1983. Arms races in evolution – an ESS to opponent-independent costs game. J. Theor. Biol. 101, 619–648.
Parker, G.A., 1993. Sperm competition games: Sperm size and sperm number under adult control. Proc. Roy. Soc. Lond. B 253, 245–254.
Pattarini, J.M., Starmer, W.T., Bjork, A., Pitnick, S., 2006. Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. Evolution 60, 2064–2080.
Perotti, M.E., 1973. The mitochondrial derivative of the spermatozoon of *Drosophila* before and after fertilization. J. Ultra. Res. 44, 181–198.
Pitnick, S., Markow, T.A., Spicer, G.S., 1995a. Delayed male maturity is a cost of producing large sperm in *Drosophila*. Proc. Natl. Acad. Sci. USA 92, 10614–10618.
Pitnick, S., Marrow, T., Spicer, G.S., 1999. Evolution of multiple kinds of female sperm-storage organs in Drosophila. Evolution 53, 1804–1822.
Pitnick, S., Spicer, G.S., Markow, T.A., 1995b. How long is a giant sperm? Nature 375, 109.
Pizzari, T., 2006. Evolution: The paradox of sperm leviathans. Curr. Biol. 16, R462–R464.
Presgraves, D.C., Baker, R.H., Wilkinson, G.S., 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. Proc. Roy. Soc. Lond. B 266, 1041–1047.
Roháček, J., 1999. Taxonomy and distribution of West Palaearctic Anthomyzidae (Diptera), with special regard to the Mediterranean and Macaronesian faunas. Bolletino Del Museo Regionale di Scienze Naturali di Torino 16, 189–224.
Roháček, J., 2006. A monograph of Palaearctic Anthomyzidae (Diptera), Part 1. Čas. Slez. Muz. Opava (A) 55, 1–328.
Richardson, K.C., Jarret, L., Finke, E.H., 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol. 35, 313–323.
Rugman-Jones, P.F., Eady, P.E., 2008. Co-evolution of male and female reproductive traits across the Bruchidae (Coleoptera). Funct. Ecol. 22, 880–886.
Sasakawa, M., 1958. The female terminalia of the Agromyzidae, with description of a new genus (I). Scientific Reports of the Saikyo University, Agriculture 10, 133–150.
Smith, K.G.V., 1969. Diptera, Lonchopteridae. Handb. Identif. Br. Insects 10, 1–9.
Sturtevant, A.H., 1925. The seminal receptacles and accessory glands of the Diptera, with special reference to the Acalypterae. J. N. Y. Entomol. Soc. 33, 195–215.
Sturtevant, A.H., 1926. The seminal receptacles and accessory glands of the Diptera, with special reference to the Acalypterae (continued). J. N. Y. Entomol. Soc. 34, 1–21.
Tröster, M., Kotrba, M., Heß, M., 2023. Variation of sperm size and evolution of giant spermatozoa in Lonchopteridae (Diptera). Arthropod Struct. Dev. 75, 101285.
Werner, M., Gack, C., Speck, T., Peschke, K., 2007. Queue up, please! Spermathecal filling in the rove beetle *Drusilla canaliculate* (Coleoptera, Staphylinidae). Naturwissenschaften 94, 837–841.
Yang, Y., Lu, X., 2011. *Drosophila* sperm motility in the reproductive tract. Biol. Reprod. 84, 1005–1015.

Allgemeine Diskussion

1 Postkopulatorische sexuelle Selektion und die Entstehung von Riesenspermien

Die Idee der sexuellen Selektion besteht seit Darwins (1871) Veröffentlichung "The Descent of Man, and Selection in Relation to Sex", aber erst einhundert Jahre später kam Parker (1970) zu dem Schluss, dass sexuelle Selektion nicht mit der Paarung enden muss, sondern auch danach noch Einfluss nehmen kann. Die unabhängige Entstehung von Riesenspermien innerhalb der Familie Lonchopteridae und in diversen anderen Taxa (Fitzpatrick et al., 2022) lässt sich nur aus eben diesem Blickwinkel nachvollziehen und erklären. Eberhard (2015) unterscheidet hierbei drei unterschiedliche Formen der postkopulatorischen sexuellen Selektion: (1) „Cryptic Female Choice“, (2) „Sexually Antagonistic Coevolution“ und (3) „Sperm Competition“.

1.1 „Cryptic Female Choice“

Der erstmals von Thornhill (1983) geprägte Begriff „Cryptic Female Choice“ beschreibt eine Form der intersexuellen Selektion, die durch mit dem Weibchen assoziierte Prozesse oder Mechanismen während oder nach der Paarung entsteht. Sie kann dazu führen, dass bestimmte Spermien mit höherer Wahrscheinlichkeit zur Befruchtung genutzt werden und wirkt sich damit direkt auf den Fortpflanzungserfolg der entsprechenden Männchen aus. Eberhard (1996) hat diese Idee in seinem Buch „Female Control: Sexual Selection by Cryptic Female Choice” weiter ausgearbeitet und von den Weibchen vermittelte morphologische, verhaltensbedingte oder physiologische Mechanismen beschrieben, die die Befruchtungwahrscheinlichkeit der Spermien bestimmter Männchen beeinflussen können. Dazu zählen zum Beispiel die Kontrolle der aufgenommenen Spermienmenge und die damit unter Umständen verbundene, systematische Ausscheidung von Spermien oder deren Neutralisation, die Spermienversorgung und -lagerung in speziellen Spermienspeicherorganen, die Beeinflussung der Spermienleistung (vor allem bezogen auf deren Schwimmfähigkeit) oder deren gezielte Aktivierung (Firman et al., 2017). Männchen wiederum, die „Cryptic Female Choice“ zu ihren Gunsten beeinflussen können, besitzen einen Vorteil im Wettbewerb mit anderen Männchen und damit eine erhöhte biologische Fitness. So können die Verhinderung der Neutralisation der Spermien nach der Paarung durch einen schützenden Schleimbeutel, wie es zum Beispiel bei dem Plattwurm *Dugesia gonocephala* (Vreys et al., 1997) und der Schnecke *Helix pomatia* (Lind, 1973) vorkommt oder die zusätzliche Übertragung von nicht-befruchtenden Pseudospermien bei der Fruchtfliege *Drosophila pseudoobscura*, die der spermiziden Wirkung des weiblichen

Genitaltrakts entgegenwirken können (Holman und Snook, 2008), den Fortpflanzungserfolg der entsprechenden Männchen steigern. Auch ein Allomon, welches bei Landschnecken mit dem Liebespfeil übertragen wird und die rasche Einlagerung der Spermien in den Spermienspeicherorganen begünstigt, dient einem vergleichbaren Zweck (Chase und Blanchard, 2006). Umgekehrt und unter der Annahme, dass „Cryptic Female Choice" vor allem der biologischen Fitness des Weibchens zugutekommt, ist ein sexueller Konflikt zwischen dem Weibchen und seinen Geschlechtspartnern, deren Spermien durch „Cryptic Female Choice" unter Umständen nicht zur Befruchtung genutzt werden würden, unausweichlich (Eberhard, 2015). Aus diesem Grund kann beispielsweise bereits im Vorfeld durch das Männchen eine weitere Kopulation des Weibchens mit anderen Männchen verhindert oder verzögert werden, indem die Partnerin vor Rivalen „geschützt" oder ein Kopulationspfropfen eingesetzt wird. Auch durch die Übertragung akzessorischer Drüsenproteine während der Kopulation, die das Paarungsverhalten des Weibchens beeinflussen, können weitere Kopulationen und/oder für das Männchen negative „Cryptic Female Choice" umgangen werden (Firman et al., 2017).

Das Wissen über die Existenz der nicht zufälligen Nutzung von Spermien durch „Cryptic Female Choice" und die damit einhergehenden erhöhten Kosten (in erster Linie für das Weibchen) erforderte passende evolutionsbiologische Erklärungsansätze, von denen in den Jahren nach Eberhards (1996) Veröffentlichung zahlreiche etabliert wurden: (1) Verhinderung von Polyspermie (z. B. Levitan et al., 2007; Firman et al., 2014), (2) Steigerung der Lebensfähigkeit der Nachkommen („Good genes") (z. B. Fisher et al., 2006), (3) Steigerung des Reproduktionserfolges der Nachkommen („Fisherian runaway selection") (z. B. Lüpold et al., 2016), (4) Optimierung der Geschlechterverteilung (z. B. Calsbeek und Bonneaud, 2008), (5) Vermeidung von Inzucht (z. B. Bretman et al., 2009; Tregenza und Wedell, 2002; Welke und Schneider, 2009; Firman und Simmons, 2015) oder (6) Vermeidung von Hybridisierung (z. B. Manier et al., 2013; Tyler et al., 2013; Yeates et al., 2013).

Unabhängig von Grund und Mechanismus treiben „Cryptic Female Choice" und die entsprechenden „Reaktionen" der Männchen darauf intersexuelle koevolutive Prozesse voran, was sich bei Insekten zum Beispiel nicht selten in einem direkten Zusammenhang zwischen Spermienlänge und der Größe der Spermienspeicherorgane widerspiegelt (z. B. Dybas und Dybas, 1981; Gage, 1994; Morrow und Gage, 2000; Minder et al., 2005; Higginson et al., 2012; Joly und Schiffer, 2010; Rugman-Jones und Eady, 2008; Dallai et al., 2014, 2021; Presgraves et al., 1999; Pitnick et al., 1999). Auch Diversifizierung und

Artbildung können gefördert werden, weshalb „Cryptic Female Choice“ einen wichtigen postkopulatorischen Prozess der sexuellen Selektion darstellt und daher taxonomische Hinweise liefern kann, die zur Bestimmung bzw. Zuordnung von Arten oder Artengruppen genutzt werden können (Firman et al., 2017).

1.2 „Sexually Antagonistic Coevolution“

Koevolutive Prozesse zwischen zwei Arten, die zu einer (teilweise sehr engen) wechselseitigen Anpassung geführt haben, sind in vielgestaltiger Form weithin bekannt (z. B. Ehrlich und Raven, 1964; Anderson und May, 1982; Rothstein, 1990; Dawkins und Krebs, 1979; Woolhouse et al., 2002; Weiblen, 2003; Nabors, 2007; Netz und Renner, 2017). Als „Sexually Antagonistic Coevolution“ bezeichnet man hingegen den koevolutionären Wettstreit zwischen Männchen und Weibchen einer Art, bei dem sich Merkmale des Reproduktionssystems und/oder des Fortpflanzungsverhaltens im Laufe der Evolution verändern, um denen des anderen Geschlechts entgegenzuwirken und so einen maximalen Fortpflanzungserfolg zu gewährleisten (Eberhard, 1985, 1996; Arnqvist und Rowe, 2002, 2005; Rowe und Arnqvist, 2002; Sirot, 2003; Hosken und Stockley, 2004). Diese Form der intersexuellen Selektion beruht auf der Tatsache, dass die Fortpflanzungsinteressen eines Weibchens (primäres Investment in den Nachwuchs) selten vollständig mit denen der Männchen (primäres Investment in die Maximierung der Fortpflanzungsmöglichkeiten) übereinstimmen (Holland und Rice, 1998; Pizzari und Birkhead, 2002; Miller und Pitnick, 2003). So können sich bei Insekten Verhaltensweisen oder morphologische Eigenschaften der Männchen vor, während oder nach der Paarung nachteilig auf die biologische Fitness der Weibchen auswirken (Eberhard, 2006). Beispielsweise könnte ein Weibchen vom Männchen dazu veranlasst werden, seine Eier früher nach der Kopulation zu legen (bevor ein anderes Männchen sich mit ihm paaren kann), obwohl der optimale Eiablageplatz noch nicht gefunden ist (Eberhard, 2015). Dies reduziert die Überlebenswahrscheinlichkeit der Nachkommen und damit die Gesamtfitness des Weibchens. In solchen Fällen begünstigt die Selektion vornehmlich Merkmale des Weibchens, welche diese schädlichen Einflüsse durch das Männchen verringern. Ein Beispiel für solch eine Veränderung auf verhaltensbiologischer Ebene könnte sein, dass das Weibchen grundsätzlich länger mit der Eiablage wartet oder bei der Suche nach Eiablageplätzen unmittelbar nach der Kopulation wählerischer ist. Eine dergestalte Verhaltensänderung des Weibchens verringert wiederum die Fähigkeit des Männchens, das Weibchen zu beeinflussen und erhöht damit dessen adaptiven Selektionsdruck (Eberhard, 2015). Wenn bei

solch wechselseitigen Prozessen weder männliche noch weibliche Merkmale zu einer stabilen „Lösung“ für die vom anderen Geschlecht aufgeworfenen „Probleme“ führen, kann sich „Sexually Antagonistic Coevolution“ zu einem fortwährenden evolutiven Wettlauf ausdehnen, der die rasch divergierende Entwicklung von morphologischen oder Verhaltensmerkmalen erklären kann, die im Zusammenhang mit intersexuellen Interaktionen häufig auftritt (Pizzari und Birkhead, 2002). Da die in einer bestimmten evolutionären Linie bevorzugten männlichen oder weiblichen Merkmale von den Merkmalen des anderen Geschlechts in derselben Linie abhängen, liefert „Sexually Antagonistic Coevolution“ somit einen möglichen Ansatz, der die oft deutlichen Unterschiede im Reproduktionssystem und/oder im Fortpflanzungsverhalten zwischen eng verwandten Arten erklären kann. Ein typisches Beispiel in diesem Zusammenhang ist die rasche und stark divergierende Evolution der männlichen Genitalien (z. B., Eberhard, 1985, 2009; Sirot, 2003; Hosken und Stockley, 2004; Joly und Schiffer, 2010; Kotrba et al., 2014). Ursprünglich diente zu deren Erklärung die klassische Schlüssel-Schloss-Hypothese, die besagt, dass sich diese Vielfalt entwickelt hat, um eine artspezifische Passung zwischen männlichen und weiblichen Genitalien herzustellen und so interspezifische Paarung und Hybridisierung zu vermeiden (Eberhard, 1985; Mikkola, 2008). Da aber in vielen Fällen die Ausprägung der männlichen Geschlechtsmerkmale das Maß, das notwendig wäre, um Hybridisierung durch nahverwandte Arten auszuschließen, erheblich übersteigt, reicht das ursprüngliche Erklärungsmodell nicht mehr aus (Kotrba et al., 2014), weshalb auch Mechanismen wie „Sexually Antagonistic Coevolution“ in Betracht gezogen werden müssen.

1.3 „Sperm Competition“

Paarungen eines Weibchens mit mehreren Männchen führen unweigerlich zu einem Wettstreit unter den Spermien der verschiedenen Männchen (Spermienkonkurrenz) und damit zu einer Form der intrasexuellen Selektion. Laut Eberhard (2015) wird der Begriff „Sperm Competition“ heute sogar noch weiter gefasst und beinhaltet zusätzlich Prozesse zur direkten Beeinflussung der Befruchtungswahrscheinlichkeit von konkurrierenden Spermien, die auf Aktivitäten des Männchens zurückzuführen sind. Dazu gehören zum Beispiel das aktive Entfernen der Spermien eines anderen Männchens aus dem Weibchen, deren Neutralisation oder der Aufbau von Barrieren, die künftige Besamungen verhindern sollen (z. B. Danielsson, 1998). In den über 50 Jahren seit Parkers (1970) Erkenntnissen zeigte sich, wie außerordentlich weit verbreitet Mehrfachpaarungen im Tierreich tatsächlich sind (Birkhead und Møller, 1998; Birkhead et al., 2009), was einen ersten

Erklärungsansatz für die vielgestaltigen Formen der „Sperm Competition“ liefert. Daher ist es nur folgerichtig, davon auszugehen, dass Männchen ihr grundlegendes Investment in die Spermienproduktion steigern (müssen), sobald Spermienkonkurrenz auftritt. So geht die Grundannahme der Theorie zur „Sperm Competition“ (Parker, 1970) davon aus, dass Männchen, die in solchen Fällen mehr Spermien produzieren und übertragen, einen numerischen Vorteil erlangen, der ihnen einen höheren Fortpflanzungserfolg gewährleistet. In diesem Kontext gibt es grundsätzlich zwei Modelle, die Erhöhung der produzierten Spermienmenge zu erklären: (1) Die Ausbildung größerer und effektiverer Hoden und (2) die Produktion kleinerer Spermien in größerer Anzahl.

(1) Die erste Annahme hat große Unterstützung durch empirische Studien erfahren. Übereinstimmend mit den modernen Modellen zur „Sperm Competition“ (Parker und Pizzari, 2010) fanden Lüpold et al. (2020) in einer großangelegten Metaanalyse über verschiedenste Taxa hinweg heraus, dass Männchen grundsätzlich mehr in ihre Hoden investieren, wenn sie einem erhöhten Maß an Spermienkonkurrenz ausgesetzt sind. Daher wird heutzutage häufig die relative Hodengröße als Index für den Grad der Spermienkonkurrenz verwendet (Lüpold et al., 2020). Dass dieses Vorgehen ohne quantitative Überprüfung zu enormen Fehlschlüssen führen kann, haben jedoch mehrere experimentelle Studien an der Gattung *Drosophila* (Crudgington et al., 2009; Wigby und Chapman, 2004; Chechi et al., 2017) und einer Vielzahl anderer Taxa, zum Beispiel Fischen (Pyron, 2000), Fröschen (Byrne et al., 2002), Robben (Fitzpatrick et al., 2012) oder Fasanen (Liao et al., 2019) gezeigt. Hier konnte kein Anstieg der Hodengröße bei erhöhter Spermienkonkurrenz festgestellt werden, was die Frage aufwirft, warum es scheinbar nicht selten zu Abweichungen von diesem allgemein akzeptierten Modell kommt. In diesem Kontext ist zu berücksichtigen, dass auch andere Faktoren einen Einfluss auf die Hodengröße haben können, die nicht notwendigerweise etwas mit Spermienkonkurrenz zu tun haben müssen. So kann die Selektion auf größere Hoden zum Beispiel aus einer höheren Fortpflanzungsrate der Männchen resultieren (Vahed und Parker, 2012; Crudgington et al., 2009; Reuter et al., 2008). Auch spiegelt die Hodengröße nicht immer nur die tatsächliche Spermienproduktion wider, da vor allem bei Vertebraten ein Teil des Hodens zur Hormonproduktion dient, wodurch die tatsächliche Menge an spermienproduzierendem Zellgewebe nur bis zu einem Drittel der Hodengröße betragen kann (Russell et al., 1990; Moreira et al., 1997). Schließlich spielt auch die Effizienz des spermienproduzierenden Zellgewebes eine Rolle und kann damit die tatsächliche Korrelation zwischen Hodengröße und „Sperm Competition“ verfälschen (Wistuba et al., 2003; Lüpold et al., 2011; Ramm und Stockley,

2010). All das verdeutlicht, dass die bloßen Abmessungen der Hoden alleine nicht als Indikatoren für das tatsächliche Maß an Spermienkonkurrenz dienen können.
(2) Parkers (1970, 1982) ursprüngliche Modelle sagten einen Selektionsvorteil für eine große Anzahl an kleinen Spermien beim Auftreten von „Sperm Competition“ voraus. Auch diese Annahme hat bereits mehrfach Unterstützung durch empirische Studien erfahren (Martin et al., 1974; Gage und Morrow, 2003; García-González und Simmons, 2007). Jedoch längst nicht alle Männchen übertragen in solchen Fällen eine große Anzahl kleiner Spermien, was auch hier die Frage nach den Gründen für die Abweichung von diesem Modell aufwirft. Darüber hinaus kann in manchen Taxa und unter bestimmten Bedingungen immer wieder ein Trend hin zu wenigen, aber größeren Spermien festgestellt werden, was scheinbar der Grundannahme, insgesamt mehr Spermien zu produzieren, um einen numerischen Vorteil zu erhalten, gänzlich widerspricht. Spätere Überlegungen von Parker (1993) schlossen die Möglichkeiten der Vergrößerung der Spermien mit ein, wenn größere Spermien zum Beispiel eine höhere Überlebenswahrscheinlichkeit haben, es eine Beschränkung der Spermienanzahl gibt oder die Größe einen kompetitiven Vorteil bei hohem Spermienaufkommen bringt. Wieder zeigten Lüpold et al. (2020) in ihrer Metaanalyse, dass diese Modelle grundsätzlich zutreffen und daher in verschiedenen Taxa und unabhängig voneinander immer wieder große oder sogar Riesenspermien auftreten, wenn entsprechende Selektionsfaktoren eine Rolle spielen. Vor allem bei Arthropoden, in deren verhältnismäßig kleinen Spermienspeicherorganen die Spermiengröße zum Beispiel ein Vorteil bei der optimalen Positionierung gegenüber konkurrierenden Spermien sein kann (Pattarini et al., 2006), tritt dieses, von Parkers (1970) ursprünglicher Annahme signifikant abweichende Phänomen, häufiger auf. Bei Vertebraten wird dagegen eher davon ausgegangen, dass der kompetitive Vorteil größerer bzw. längerer Spermien eher darin besteht, effektiver schwimmen und dadurch die Strecke zur Eizelle schneller überwinden zu können (Gomendio und Roldan, 1991; Fitzpatrick et al., 2009; Lüpold et al., 2009; Gómez Montoto et al., 2011). So oder so, ohne einen wie auch immer gearteten Selektionsvorteil lässt sich die Evolution von größeren oder sogar Riesenspermien und das damit verbundene erhöhte Investment des Männchens in ein einzelnes Spermium nicht erklären.

2 Riesenspermien bei Lonchopteridae

Kapitel I zeigt, dass die Männchen von *L. lutea* Spermien produzieren, die eine Länge von 2.200 µm und eine Dicke von 1,4 µm haben und damit nach Dallai (2014) als

Riesenspermien klassifiziert werden können. Auch bei weiteren Arten der Lonchopteridae treten Riesenspermien auf, aber längst nicht bei allen (Kapitel II). Welche Evolutionsprozesse bei dieser Fliegenfamilie zur Entstehung von Riesenspermien und damit zu einer signifikanten Abweichung der sonst für Zweiflügler üblichen Spermienmorphologie geführt haben, war eine zentrale Fragestellung dieser Dissertation. Zunächst galt es daher, die Morphologie und Ultrastruktur der extrem dicken und langen Spermien von *L. lutea* aufzuklären und die Zellkomponenten zu identifizieren, die zu deren Vergrößerung beitragen. Dies gelang durch eine Kombination von 3D-Rekonstruktion und Transmissionselektronenmikroskopie (Kapitel I). Bei *L. lutea* dominiert über den Großteil der Gesamtlänge des Spermiums ein stark vergrößertes Mitochondrienderivat, welches von einem großen, ungewöhnlich gestalteten akzessorischen Körper flankiert wird. Beide tragen maßgeblich zur Größe und zum Gesamtvolumen des Spermiums bei. Innerhalb der Ordnung Diptera wurden Riesenspermien bisher erst bei drei weiteren, nicht näher verwandten Familien beschrieben (Diopsidae, Drosophilidae, Cecidomyiidae). Hier lässt sich die Vergrößerung der Spermien jedoch auf ultrastrukturelle Besonderheiten zurückführen, die von denen der Lonchopteridae klar abzugrenzen sind. Bei der Stielaugenfliege *Diasemopsis comoroensis* sind dies ein großer akzessorischer Körper („central band“) und zwei Mitochondrienderivate, die ihrerseits eine besondere Größe erreichen (Kotrba und Heß, 2013; Kotrba et al., 2016). Bei den Riesenspermien einiger Vertreter der Taufliegen (Drosophilidae) sind es zwei stark vergrößerte Mitochondrienderivate ohne einen akzessorischen Körper (Jamieson et al., 1999; Dallai et al., 2008) und bei manchen Vertretern der Gallmücken (Cecidomyiidae) erreicht das Axonem durch mehr als tausendfache Vervielfältigung der Mikrotubuluspaare eine außergewöhnliche Größe (Dallai, 1988). Diese grundlegenden Unterschiede in der ultrastrukturellen Architektur der Riesenspermien innerhalb der Ordnung Diptera spiegeln die unabhängige Entwicklung voneinander wider und könnten auf einen Selektionsdruck hindeuten, der die schiere Größe der Spermien begünstigt und nicht die Ausgestaltung einzelner Zellkomponenten. In Kapitel II wird zudem klar, dass dieser, wie auch immer geartete Selektionsdruck, vermutlich nicht auf alle Arten der Lonchopteridae gleichermaßen wirkt oder wirken kann, da innerhalb der Familie deutliche Unterschiede in der Spermiengröße bestehen. Mit einer Länge von etwa 190-370 µm weisen einige Arten (*Lonchoptera barberi*, *Lonchoptera nigrociliata*, *Lonchoptera scutellata*) für Fliegen völlig unauffällig große Spermien auf, während *L. fallax* mit einer Gesamtlänge von etwa 7.500 µm eines der längsten bekannten Spermien im Tierreich besitzt (Fitzpatrick et al., 2022). Wie bereits beschrieben, kommen zur

Erklärung dieses schwer greifbaren Phänomens nach Eberhard (2015) drei Formen postkopulatorischer sexueller Selektion in Frage.

(1) „Sperm Competition“: Zwar ist bei Lonchopteridae nichts Näheres über eine aktive Entfernung der Spermien eines anderen Männchens aus dem Weibchen, deren Neutralisation oder den Aufbau von Barrieren, die künftige Besamungen verhindern sollen, bekannt, aber wenn man Form und Funktionalität der Spermatheken in die Überlegungen mit einbezieht (Kapitel III), dann ist „Sperm Competition“ zunächst der naheliegendste Erklärungsansatz für die Entstehung von Riesenspermien in dieser Fliegenfamilie. Die Spermatheken der Lonchopteridae lassen sich in vier morphologisch und histologisch verschiedene Abschnitte einteilen, wobei gerade dem zweiten Abschnitt maßgeblich die Aufgabe der Spermienspeicherung zukommt. Dieser zeichnet sich durch eine vergleichsweise bauchige Form aus, in der die Spermien scheinbar willkürlich verknäult aufbewahrt werden. Lediglich in dessen basalem Bereich, am Übergang zum ersten Abschnitt, finden sich eindeutige Anzeichen dafür, dass sich die Köpfe einzelner Spermien in Richtung Vagina ausrichten und sogar in den distalen Bereich des ersten Abschnitts vordringen (Kapitel I und Kapitel III). Eine ähnliche Orientierung der Spermien in den Spermienspeicherorgangen kennt man zum Beispiel von *Drosophila* (Miller und Pitnick, 2002). Das weitere Vordringen der Spermien in den ersten Abschnitt wird bei den untersuchten *Lonchoptera*-Arten durch einen von Ringmuskulatur verschlossenen Übergang sowie durch reusenartig angeordnete Borsten erschwert oder verhindert. Nichtsdestotrotz bleibt die Positionierung der Spermienköpfe in diesem speziellen Bereich der Spermathek oder möglichst nahe daran ein selektiver Vorteil, da diejenigen Spermien, die beim Öffnen der Ringmuskulatur als erste in Richtung Vagina und Ei starten können, eine größere Wahrscheinlichkeit auf Befruchtung haben. Diesen Vorteil haben folglich vor allem Spermien, die sich aufgrund ihrer Größe/Dicke diese Position sichern können, indem sie die eventuell etwas kleineren Spermien anderer Männchen verdrängen können und/oder vor solch einer physischen Verdrängung geschützt sind (Displacement-Hypothese). Diese Hypothese wurde bei anderen Taxa schon vielfach formuliert (z. B. Lefevre und Jonsson, 1962; Dybas und Dybas, 1981; Presgraves et al., 1999; Miller und Pitnick, 2002; Minder et al., 2005; Rugman-Jones und Eady, 2008; Higginson et al., 2012; Dallai et al., 2021) und erfährt durch die Untersuchungen von Pattarini et al. (2006) starke Unterstützung, die zeigten, dass längere Spermien besser in der Lage sind, kürzere Spermien aus einer günstigen Position zu verdrängen und/oder vor eben solch einer Verdrängung besser geschützt sind. Auch Manier et al. (2010) bestätigten experimentell, dass diese physische

Verdrängung durch konkurrierende Spermien ein entscheidender Faktor für den Befruchtungserfolg einzelner Spermien ist. Bei den Lonchopteridae sprechen jedoch zwei Untersuchungsergebnisse dieser Dissertation eher gegen die Displacement-Hypothese oder zumindest dafür, dass sie nicht der einzige Faktor postkopulatorischer sexueller Selektion sein kann, der zur Entwicklung von Riesenspermien in dieser Fliegenfamilie geführt hat. (1) Eine extreme Länge, wie die der Spermien von *L. fallax* (7.500 µm), erscheint doch etwas unverhältnismäßig für die bloße Verdrängung rivalisierender Spermien in einem vergleichsweise winzigen Abschnitt der Spermathek, selbst wenn man die gesamte Länge des zweiten Abschnitts (etwa 860 µm bei *L. fallax*) als „Schauplatz" der Selektion in Betracht zieht. (2) Die Spermatheken aller untersuchten *Lonchoptera*-Arten weisen im entsprechenden Bereich einen sehr ähnlichen Grundbauplan auf, das heißt, einen sehr engen Übergang vom zweiten Abschnitt in den ersten, der zusätzlich durch Ringmuskulatur noch verjüngt/verschlossen wird, sowie reusenartig angeordnete Borsten (Kapitel III). Folglich sollte der Selektionsdruck, der nach der Displacement-Hypothese auf die Spermien wirkt, auf die Spermien aller Arten in ähnlicher Weise wirken und daher bei allen Arten zu vergrößerten oder Riesenspermien führen. Da dies nicht der Fall ist, im Gegenteil, Riesenspermien bei den untersuchten *Lonchoptera*-Arten ein abgeleitetes Merkmal darstellen und eher die Ausnahme bilden (Kapitel II), kann „Sperm Competition" zumindest nicht der alleinige Faktor sexueller Selektion sein, der für eine derart extreme Verlängerung der Spermien verantwortlich ist.

(2) „Sexually Antagonistic Coevolution": Auch Prozesse der „Sexually Antagonistic Coevolution" können grundsätzlich die Entstehung von Riesenspermien verursachen oder zumindest begünstigen. Geht man davon aus, dass ein Weibchen sich mit mehreren Männchen paart, um die genetische Variabilität ihrer Nachkommen zu erhöhen, würde es für das Weibchen einen evolutiven Nachteil bedeuten, wenn das erste Männchen, mit dem es sich paart, bei der Paarung die Spermienspeicherorgane komplett auffüllen würde, so dass keine Spermien anderer Männchen mehr aufgenommen werden könnten. Für das erste Männchen wäre dies jedoch ein entscheidender Selektionsvorteil, da auf diese Weise seine Vaterschaft für alle Nachkommen dieses Weibchens sichergestellt wäre (Miller und Pitnick, 2003; Minder et al., 2005). Um dies zu erreichen, gibt es grundsätzlich zwei Möglichkeiten: Pro Kopulation könnten entweder eine größere Anzahl vergleichbar großer Spermien oder insgesamt deutlich größere Spermien auf das Weibchen übertragen werden. So könnte ein evolutiver Wettstreit zwischen Männchen und Weibchen entstehen, der sukzessive zu voluminöseren Spermienspeicherorgangen und, wäre vor allem

letzteres der Fall, zu größeren Spermien führen würde. Da bisher weder in freier Wildbahn noch in Gefangenschaft Paarungen beobachtet werden konnten, ist über Menge und Anzahl der pro Kopulation übertragenen Spermien bei Lonchopteridae nichts bekannt. Auch nicht darüber, ob tatsächlich Mehrfachpaarungen in dieser Fliegenfamilie vorkommen. In Kapitel II konnte jedoch gezeigt werden, dass es weder einen Zusammenhang zwischen der Hodengröße und der produzierten Spermienanzahl pro Hoden, noch zwischen der Hoden- und der Spermiengröße gibt. Sollte „Sexually Antagonistic Coevolution“ maßgeblich für die Evolution der Riesenspermien bei Lonchopteridae verantwortlich sein, so wäre zumindest zu erwarten, dass Arten mit sehr voluminösen Spermatheken (entspricht den Arten mit langen Spermien) auch größere Hoden haben sollten, da Riesenspermien zwar ein erhöhtes Investment des Männchens in ein einzelnes Spermium bedeuten, bei vergleichbarer Hodengröße jedoch nur eine Umverteilung des Materials darstellen. Ein wie oben beschriebener Prozess würde aber einen verstärkten Selektionsdruck auf das Gesamthodenmaterial bedeuten und dadurch zu insgesamt größeren Hoden führen. Auch von der Morphologie der Spermatheken der untersuchten *Lonchoptera*-Arten wäre zu erwarten, dass die Selektion vor allem Spermatheken mit größeren Volumina hervorbringt, vor allem der Abschnitte zwei und drei, in denen die Spermien maßgeblich gespeichert werden. Eine überproportionale Verlängerung des dritten Abschnitts (Kapitel III), wie es vor allem bei *L. fallax* der Fall ist, ließe sich mit dieser Hypothese nicht hinreichend erklären. Daher sprechen die Untersuchungsergebnisse dieser Dissertation eher gegen den vorherrschenden Einfluss von „Sexually Antagonistic Coevolution“ bei der Entstehung von Riesenspermien innerhalb der Lonchopteridae.

(3) „Cryptic Female Choice“: Bei *Lonchoptera* sind viele Prozesse der „Cryptic Female Choice“ nicht bekannt. So weiß man bisher noch nichts darüber, ob Weibchen aktiv die Ausscheidung oder Neutralisation bestimmter Spermien herbeiführen oder ob sie deren Aktivierung oder Leistung steuern können. Hinweise darauf gibt jedoch die Morphologie der Spermatheken (Kapitel III). Deren vierter Abschnitt ist bei allen untersuchten Arten von einem Drüsengewebe umgeben, das Sekrete produziert, die in die Spermathek abgegeben werden. Aus Untersuchungen bei *Drosophila* weiß man, dass das Überleben der Spermien im ventralen Rezeptakulum vom Vorhandensein funktionsfähiger Spermathekendrüsen abhängt (Anderson, 1945; Boulétreau-Merle, 1977; Pitnick et al., 1999). Zwar besitzen Lonchopteridae kein ventrales Rezeptakulum und die Spermien werden, anders als bei *Drosophila*, direkt in den Spermatheken gespeichert, aber eine ähnliche Funktion der Spermathekensekrete ist naheliegend. Auch eine zusätzliche Aktivierung oder

gezielte Freisetzung der Spermien aus der Spermathek, die über solcherlei Sekrete vom Weibchen gesteuert werden, sind vorstellbar. Über die Biochemie dieser Sekrete und deren Funktion bei Lonchopteridae ist bisher nichts bekannt, aber Spermien, die durch eine außergewöhnliche Länge mit ihrem Schwanz möglichst nah an den vierten Abschnitt der Spermathek heranreichen, erhalten früher Zugang zu den entsprechenden Sekreten und damit zu etwaigen Signalen des Weibchens. Dies könnte in Kombination mit der Displacement-Hypothese zur Selektion auf kräftigere und längere Spermien hinweisen, um einerseits die Positionierung nahe dem Ausgang des zweiten Abschnitts zu sichern, und andererseits, um möglichst nah an den vierten Abschnitt heranzureichen. Über den entscheidenden Selektionsdruck, der auf die Morphologie des weiblichen Fortpflanzungstrakts wirkt und damit „Cryptic Female Choice" ermöglicht, kann nur spekuliert werden und auch die Untersuchungen an *Lonchoptera* lieferten diesbezüglich keine eindeutigen Hinweise, aber eine weit verbreitete Theorie ist, dass die Weibchen von einer stärkeren Kontrolle über den Befruchtungsprozess grundsätzlich profitieren (Eberhard, 1996; Hellriegel und Ward, 1998; Pitnick et al., 1999).

Zusammenfassend ist zu sagen, dass die Ergebnisse dieser Studie keine eindeutige Aussage darüber zulassen, welcher konkrete Prozess der postkopulatorischen sexuellen Selektion bei Lonchopteridae zur Entstehung von Riesenspermien geführt hat und es kann auch keiner kategorisch ausgeschlossen werden. Eine Kombination aus „Sperm Competition" und „Cryptic Female Choice" scheint hinsichtlich der vorliegenden Daten jedoch den plausibelsten Erklärungsansatz zu liefern.

3 Revision der Gattung *Spilolonchoptera*

In dieser Dissertation wurde für die umfassendere Untersuchung der Morphologie und der Evolution des Reproduktionssystems der Lonchopteridae auch auf nicht näher determiniertes Sammlungsmaterial der SNSB-Zoologischen Staatssammlung München zurückgegriffen, das von einem langjährigen Mitarbeiter der Sektion Diptera in den Jahren 1997-2002 auf mehreren Forschungsreisen in Taiwan gesammelt wurde. Die zwei Arten wurden in Kapitel II und Kapitel III zunächst als *Lonchoptera spec. T1* und *Lonchoptera spec. T2* bezeichnet. Auf der Basis vergleichender Spermienmorphologie und mit Hilfe von Daten aus dem DNA Barcoding konnten beide dem „Mittelbau" der in Kapitel II aufgestellten phylogenetischen Hypothese und damit zweifelsfrei der Gattung *Lonchoptera* zugeordnet werden. Erst mit Unterstützung eines Dipterologen aus Wales konnten diese beiden Arten kurz vor Abschluss dieser Arbeit als *Lonchoptera zhejiangensis* (T1)

und *Lonchoptera malaisei* (T2) determiniert werden. Erstere wurde ursprünglich von Gao et al. (2021) als *Spilolonchoptera zhejiangensis* und damit in einer Schwesterngattung zu *Lonchoptera* beschrieben. Diese, ausschließlich in China vorkommende, Gattung wurde von Yang (1998) etabliert, galt seit jeher als umstritten und wurde von etlichen „westlichen" Autoren nicht anerkannt (z. B. Sinclair und Cumming, 2006; Klymko und Marshall, 2008; Kahanpää, 2014; Whittington und Beuk, 2022). Mit Hilfe der Daten und des Materials aus dieser Dissertation gelang Whittington (2024) die Revision dieser Fliegengruppe und die Umgruppierung der in der Gattung *Spilolonchoptera* beschriebenen Arten in die Gattung *Lonchoptera*. Dies ist ein gutes Beispiel dafür, wie hilfreich und wichtig reproduktionsmorphologische Merkmale bei der Bestimmung von Arten und bei der Aufstellung einer gesicherten Phylogenie sind.

4 Zusammenfassung und Ausblick

Im Rahmen dieser Dissertation gelang es, das Reproduktionssystem der Lanzenfliegen (Lonchopteridae) näher zu untersuchen und zu beleuchten. So konnte zum einen die grundlegende Morphologie der inneren männlichen und weiblichen Geschlechtsorgane näher beschrieben werden, deren letzte Beschreibung bereits über 100 Jahre alt und eher oberflächlich ist (De Meijere, 1906). Zum anderen stellte sich heraus, dass bei einigen Arten Riesenspermien auftreten, dass diese eine von den herkömmlichen Spermien der Zweiflügler abweichende Morphologie und Ultrastruktur aufweisen und dass wohl koevolutive Prozesse in Zusammenhang mit den Spermienspeicherorganen der Weibchen für deren außergewöhnliche Größe verantwortlich sind. Solcherlei Prozesse, vor allem bezogen auf die Länge der Spermien und der Spermienspeicherorgane, sind für andere Taxa bereits mehrfach beschrieben. Die aufwändige 3D-Rekonstruktion dieser Komponenten des Reproduktionssystems der Lonchopteridae ermöglichte es jedoch zusätzlich, die entsprechenden Volumina in die Überlegungen mit einzubeziehen, was bisher von keinen anderen Autoren gemacht wurde. Obwohl in Kapitel III eine vielversprechende neue Hypothese zur Entstehung von Riesenspermien vorgestellt wird, gibt auch diese Arbeit keine finale Antwort auf die Frage, welche konkreten Evolutionsprozesse bei *Lonchoptera* zur Entstehung von Riesenspermien geführt haben. Die Bandbreite der hier dargestellten Forschungsergebnisse lieferte dennoch wichtige Anhaltspunkte, die das allgemeine Verständnis dieses sehr vielschichtigen Themas erweitert und vertieft haben. Um dieses zusätzlich auszuweiten und zu festigen, sollte in anschließenden Untersuchungen (1) die genaue Phylogenie der Lonchopteridae geklärt, (2) deren Paarungsverhalten, vor

allem im Hinblick auf Mehrfachpaarungen geprüft, (3) die mechanischen und biochemischen Eigenschaften des riesigen Mitochondrienderivats und des strukturell außergewöhnlichen akzessorischen Körpers untersucht und (4) die biochemische und funktionelle Natur der Spermathekensekrete aufgeklärt werden. Erst dann werden sich eindeutige Aussagen über das Reproduktionssystem und die Prozesse postkopulatorischer sexueller Selektion in dieser spannenden und außergewöhnlichen Fliegenfamilie treffen lassen.

Literaturverzeichnis

Afzelius, B.A., Baccetti, B., Dallai, R., 1976. The giant sperm of *Notonecta*. J. Submicros. Cytol. 8, 149-161.

Anderson, R.C., 1945. A study of the factors affecting fertility of lozenge females of *Drosophila melanogaster*. Genetics 30, 280-296.

Anderson, R.M., May, R., 1982. Coevolution of hosts and parasites. Parasitology 85, 411-426.

Andersson, H., 1970. Notes on north european *Lonchoptera* (Dipt., Lonchopteridae) with lectotype designations. Entomol. Ts. Arg. 91, 42-45.

Andersson, M., 1994. Sexual selection. Princeton University Press, New Jersey.

Arnqvist, G., Rowe, L., 2002. Antagonistic coevolution between the sexes in a group of insects. Nature 415, 787-789.

Arnqvist, G., Rowe, L., 2005. Sexual conflict. Princeton University Press, New Jersey.

Baccetti, B., 1979. Ultrastructure of sperm and its bearing on arthropod phylogeny. In: Gupta, A.P. (Ed.), Arthropod Phylogeny. Van Nostrand Reinhold, New York, pp. 609-644.

Baccetti, B., Dallai. R., 1978. The first multiflagellate animal spermatozoon in *Mastotermes darwiniensis*. J. Submicros. Cytol. 10, 107.

Baccetti, B., Burrini, A.G., Dallai, R., Pallini, V., Periti, P., Piantelli, F., Rosati, F., Selmi, G., 1973a. The spermatozoon of Arthropoda. XIX. Structure and function in the spermatozoon of *Bacillus rossius*. J. Ultrastruct. Res. 44, 5-73.

Baccetti, B., Burrini, A.G., Dallai, R., Giusti, F., Mazzini, M., Renieri, T., Rosati, F., Selmi, G., 1973b. The spermatozoon of Arthropoda XX. Structure and function in the spermatozoon of *Tenebrio molitor*. J. Mechanochem. Cell Motil 2, 149-161.

Baccetti, B., Dallai, R., Pallini, V., Rosati, F., Afzelius, B.A., 1977. Protein of insect sperm mitochondrial crystals. Crystallomitin. J. Cell Biol. 73, 594-600.

Baccetti, B., Dallai, R., Callaini, G., 1981. The spermatozoon of Arthropoda: *Zootermopsis nevadensis* and isopteran sperm phylogeny. Int. J. Invertebr. Reproduction 3, 87-99.

Baccetti, B., Gibbons, B.H., Gibbons, B.R., 1989. Bidirectional swimming in spermatozoa of tephritid flies. J. Submicrosc. Cytol. Pathol. 21, 619-625.

Bährmann, R., Bellstedt, R., 1988. Beobachtungen und Untersuchungen zum Vorkommen der Lonchopteriden auf dem Gebiet der DDR, mit einer Bestimmungstabelle der Arten (Dipt., Lonchopteridae). Deut. Entomol. Z. 35, 265-279.

Beutel, R.G., Pohl, H., Yan, E.V., Anton, E., Liu, S.-P., Ślipiński, A., McKenna, D., Friedrich, F., 2019. The phylogeny of Coleopterida (Hexapoda) – morphological characters and molecular phylogenies. Syst. Entomol. 44, 75-102.

Birkhead, T.R., 2000. Defining and demonstrating postcopulatory female choice – again. Evolution 54, 1057-1060.

Birkhead, T.R., Møller, A.P., 1998. Sperm competition and sexual selection. Academic Press, San Diego.

Birkhead, T.R., Pizzari, T., 2002. Postcopulatory sexual selection. Nature reviews genetics 3, 262-273.

Birkhead, T.R., Hosken, D.J., Pitnick, S., 2009. Sperm biology: an evolutionary perspective. Academic Press, San Diego.

Boulétreau-Merle, J., 1977. Role des spermatheques dans l'utilisation du sperme et la stimulation de l'ovogenese chez Drosophila melanogaster. J. Ins. Phys. 23, 1099-1104.

Boulton, R.A., Hardy, I.C.W., Siva-Jothy, M.T., Ode, P.J., 2023. Mating behavior. In: Hardy, I.C.W., Wajnberg, E. (Eds.), Jervis´s insects as natural enemies: practical perspectives. Springer International Publishing, Cham, pp. 295-355.

Bretman, A., Newcombe, D., Tregenza, T.O.M., 2009. Promiscuous females avoid inbreeding by controlling sperm storage. Mol. Ecol. 18, 3340-3345.

Byrne, P.G., Roberts, J.D., Simmons, L.W., 2002. Sperm competition selects for increased testes mass in Australian frogs. J. Evol. Biol. 15, 347-355.

Calsbeek, R., Bonneaud, C., 2008. Postcopulatory fertilization bias as a form of cryptic sexual selection. Evolution 62, 1137-1148.

Campbell, N.A., Reece, J.B., Urry, L.A., Cain, M.L., Wasserman, S.A., Minorsky, P.V., Jackson, R.B., 2011. Campbell Biologie (8. aktualisierte Auflage). Pearson Studium, München, pp. 599-676.

Chase, R., Blanchard, K.C., 2006. The snail´s love-dart delivers mucus to increase paternity. Proc. R. Soc. Lond. B 273, 1471-1475.

Chechi, T.S., Ali Syed, Z., Prasad, N.G., 2017. Virility does not imply immensity: testis size, accessory gland size and ejaculate depletion pattern do not evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. J. Insect Physiol. 98, 67-73.

Collin, J.E., 1938. The British species of *Lonchoptera*. Ent. Mon. Mag. 74, 60-65.

Crudgington, H.S., Fellows, S., Badcock, N.S., Snook, R.R., 2009. Experimental manipulation of sexual selection promotes greater male mating capacity but does not alter sperm investment. Evolution 63, 926-938.

Cruz-Landim, C., 2001. Organization of the cysts in bee (Hymenoptera, Apidae) testis: number of spermatozoa per cyst. Iheringia Ser. Zool. 91, 183-189.

Curtis, S.K., Benner, D.B., 1991. Movement of spermatozoa of *Megaselia scalaris* (Diptera: Brachycera: Cyclorrhapha: Phoridae) in artificial and natural fluids. J. Morphol. 210, 85-99.

Dallai, R., 1988. The spermatozoon of Asphondyliidi (Diptera, Cecidomyiidae). J. Ultrastruct. Mol. Struct. Res. 101, 98-107.

Dallai, R., 2014. Overview on spermatogenesis and sperm structure of Hexapoda. Arthropod Struct. Dev. 43, 257-290.

Dallai, R., Afzelius, B.A., 1990. Microtubular diversity in insect spermatozoa. Results obtained with a new fixative. J. Struct. Biol. 103, 164-179.

Dallai, R., Afzelius, B.A., 1991. Sperm flagellum of *Dacus oleae* (Gmelin) (Tephritidae) and *Drosophila melanogaster* Meigen (Drosophilidae) (Diptera). Int. J. Insect Morphol. Embryol. 20, 215-222.

Dallai, R., Afzelius, B.A., 1994. Sperm structure of Trichoptera. I. Integripalpia: Limnephiloidea. Int. J. Insect Morphol. Embryol. 23, 197-209.

Dallai, R., Afzelius, B.A., 1995. Sperm structure in Trichoptera. II. The aflagellate spermatozoa of *Hydroptila*, *Orthotrichia* and *Stactobia* (Hydroptilidae). Int. J. Insect Morphol. Embryol. 24, 161-170.

Dallai, R., Baccetti, B., Bernini, F., Bigliardi, E., Burrini, A.G., Giusti, F., Mazzini, M., Pallini, V., Renieri, T., Rosati, F., Selmi, G., Vegni, M., 1975. New models of aflagellate arthropod spermatozoa. In: Afzelius, B.A. (Ed.), The functional anatomy of the spermatozoon. Pergamon Press, Oxford, pp. 278-287.

Dallai, R., Baccetti, B., Mazzini, M., Sabatinelli, G., 1984. The spermatozoon of three species of Phlebotomus (Phlebotomidae) and the acrosomal evolution in nematoceran dipterans. Int. J. Insect Morphol. Embryol. 13, 1-10.

Dallai, R., Lupetti, P., Afzelius, B.A., 1995a. Sperm structure of Trichoptera. III. Hydropsychidae, Polycentropodidae and Philopotamidae (Annulipalpia). Int. J. Insect Morphol. Embryol. 24, 171-183.

Dallai, R., Lupetti, P., Afzelius, B.A., Mamaev, B.M., 1995b. Characteristics of the sperm flagellum in fungus gnats (Mycetophiloidea, Diptera, Insecta). Zoomorphology 115, 213-219.

Dallai, R., Lupetti, P., Mencarelli, C., 2006. Unusual axonemes of hexapod spermatozoa. Int. Rev. Cytol. 254, 45-99.

Dallai, R., Mercati, D., Giusti, F., 2008. Structural organization of the "zipper line" in *Drosophila* species with giant spermatozoa. J. Struct. Biol. 161, 43-54.

Dallai, R., Gottardo, M., Mercati, D., Machida, R., Mashimo, Y., Matsumura, Y., Beutel, R.G., 2013. Divergent mating patterns and a unique mode of external sperm transfer in Zoraptera: an enigmatic group of pterygote insects. Naturwissenschaften 100, 581-594.

Dallai, R., Gottardo, M., Mercati, D., Machida, R., Mashimo, Y., Matsumura, Y., Beutel, R.G., 2014. Giant spermatozoa and a huge spermatheca: a case of coevolution of male and female reproductive organs in the ground louse Zorotypus impolitus (Insecta, Zoraptera). Arthropod Struct. Dev. 43, 135-151.

Dallai, R., Paoli, F., Mercati, D., Lupetti, P., 2016. The centriole adjunct of insects: need to update the definition. Tissue and Cell 48, 104-113.

Dallai, R., Fanciulli, P.P., Mercati, D., Lupetti, P., 2021. Coevolution between female seminal receptacle and sperm morphology in the semiaquatic measurer bug Hydrometra stagnorum L. (Heteroptera, Hydrometridae). Arthropod Struct. Dev. 60, 101001.

Danielsson, I., 1998. Mechanisms of sperm competition in insects. Ann. Zool. Fennici 35, 241-257.

Darwin, C., 1871. The descent of man, and selection in relation to sex. Murray, London.

Dawkins, R., Krebs, J.R., 1979. Arms reces between and within species. Proc. R. Soc. Lond. B 205, 489-511.

De Meijere, J.C.H., 1906. Die Lonchopteren des palaearktischen Gebietes. Tijdschr Entomol. 49, 44-98.

Drake, C.M., 1983. *Lonchoptera meijeri* Collin in Cumbria, and mating in *L. furcata* Fallén (Dipt., Lonchopteridae). In: Entomologist´s Monthly Magazine 119, p. 12.

Dybas, L.K., Dybas, H.S., 1981. Coadaptation and taxonomic differentiation of sperm and spermathecae in featherwing beetles. Evolution 35, 168-174.

Ebejer, M.J., 2012. The families of Lonchopteridae, Opetiidae and Pipunculidae of Malta (Diptera, Aschiza). Bul. Entomol. Soc. Malta 5, 77-79.

Eberhard, W.G., 1985. Sexual selection and animal genitalia. Harvard University Press, Cambridge.

Eberhard, W.G., 1996. Female control: sexual selection by cryptic female choice. Princeton University Press, New Jersey.

Eberhard, W.G., 2006. Sexually antagonistic coevolution in insects is associated with only limited morphological diversity. J. Evol. Biol. 19, 657-681.

Eberhard, W.G., 2009. Static allometry and animal genitalia. Evolution 63, 48-66.

Eberhard, W.G., 2015. Cryptic female choice and other types of post-copulatory sexual selection. In: Peretti, A.V., Aisenberg, A. (Eds.), Cryptic female choice in arthropods: patterns, mechanisms and prospects. Springer International Publishing, Cham, pp. 1-23.

Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants: a study in coevolution. Evolution 18, 586-608.

Engel, M.S., Grimaldi, D., 2004. New light shed on the oldest insect. Nature 427, 627-630.

Esfandi, K., He, X.Z., Wang, Q., 2019. Sperm allocation strategies in a sperm heteromorphic insect. Curr. Zool. 66, 285-292.

Fabian, L., Brill, J.A., 2012. *Drosophila* spermiogenesis: big things come from little packages. Spermatogenesis 2, 197-212.

Farí, K., Takács, Ś., Ungár, D., Sinka, R., 2016. The role of acroblast formation during Drosophila spermatogenesis. Biology Open 5, 1102-1110.

Firman, R.C., Simmons, L.W., 2015. Gametic interactions promote inbreeding avoidance in house mice. Ecol. Lett. 18, 937-943.

Firman, R.C., Gomendio, M., Roldan, E.R.S., Simmons, L.W., 2014. The coevolution of ova defensiveness with sperm competitiveness in house mice. Am. Nat. 183, 565-572.

Firman, R.C., Gasparini, C., Manier, M.K., Pizzari, T., 2017. Postmating female control: 20 years of cryptic female choice. Trends Ecol. Evol. 32, 368-382.

Fisher, D.O., Double, M.C., Blomberg, S.P., Jennions, M.D., Cockburn, A., 2006. Post-mating sexual selection increase lifetime fitness of polyandrous females in the wild. Nature 444, 89-92.

Fitzpatrick, J.L., Montgomerie, R., Desjardins, J.K., Stiver, K.A., Kolm, N., Balshine, S., 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc. Natl. Acad. Sci. USA 106, 1128-1132.

Fitzpatrick, J.L., Almbro, M., Gonzalez-Voyer, A., Kolm, N., Simmons, L.W., 2012. Male contest competition and the coevolution of weaponry and testes in pinnipeds. Evolution 66, 3595-3604.

Fitzpatrick, J.L., Kahrl, A.F., Snook, R.R., 2022. SpermTree: a species-level database of sperm morphology spanning the animal tree of life. Scientific Data 9, 1-6.

Fuller, M.T., 1998. Genetic control of cell proliferation and differentiation in *Drosophila* spermatogenesis. Cell. Dev. Biol. 9, 433-444.

Gage, M.J.G., 1994. Association between body size, mating pattern, testis size, and sperm length across butterflies. Proc. R. Soc. Lond. B. 258, 247-254.

Gage, M.J.G., Morrow, E.H., 2003. Experimental evidence for the evolution of numerous, tiny, sperm via sperm competition. Curr. Biol. 13, 754-757.

Gao, S., Zhang, B., Yang, D., 2021. Two new species of genus *Spilolonchoptera* Yang, 1998 from China (Diptera, Lonchopteridae). Zootaxa 4980, 389-394.

García-González, F., Simmons, L.W., 2007. Shorter sperm confer higher competitive fertilization success. Evolution 61, 816-824.

Gassner, G., Klemetson, D.J., Richard, R.D., 1972. Spermiogenesis in house fly, *Musca domestica* L. (Diptera: Muscidae): a transmission electron microscope study. Int. J. Insect Morphol. Embryol. 1, 105-120.

Gilbert, D.G., 1981. Ejaculate esterase 6 and initial sperm use by female *Drosophila melanogaster*. J. Insect Physiol. 27, 641-650.

Gomendio, M., Roldan, E.R.S., 1991. Sperm competition influences sperm size in mammals. Proc. R. Soc. Lond. B 243, 181-185.

Gómez Montoto, L., Sánchez, M.V., Tourmente, M., Martín-Coello, J., Luque-Larena, J.J., Gomendio, M., Roldan, E.R.S., 2011. Sperm competition differentially affects swimming velocity and size of spermatozoa from closely related muroid rodents: head first. Reproduction 142, 819-830.

Gorgoń, S., Świątek, P., 2021. The apical cell – an enigmatic somatic cell in leech ovaries – structure and putative functions. Develop. Biol. 469, 111-124.

Hellriegel, B., Ward, P.I., 1998. Complex female reproductive tract morphology: its possible use in postcopulatory female choice. J. Theor. Biol. 190, 179-186.

Higginson, D.M., Miller, K.B., Segraves, K.A., Pitnick, S., 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. USA 109, 4538-4543.

Holland, B., Rice, W.R., 1998. Perspective: chase-away sexual selection: antagonistic seduction versus resistance. Evolution 52, 1-7.

Holman, L., Snook, R.R., 2008. A sterile sperm caste protects brother fertile sperm from female-mediated death in *Drosophila pseudoobscura*. Curr. Biol. 18, 292-296.

Hopkins, B.R., Sepil, I., Wigby, S., 2020. Structural variation in *Drosophila melanogaster* spermathecal ducts and its association with sperm competition dynamics. R. Soc. Open Sci. 7, 200130.

Hosken, D.J., Ward, P.I., 2001. Experimental evidence for testis size evolution via sperm competition. Ecology Letters 4, 10-13.

Hosken, D.J., Stockley, P., 2004. Sexual selection and genital evolution. Trends Ecol. Evol. 19, 87-93.

Jamieson, B.G.M., 1987. The Ultrastructure and phylogeny of insect spermatozoa. Cambridge University Press, Cambridge.

Jamieson, B.G.M., Dallai, R., Afzelius, B.A., 1999. Insects: their spermatozoa and phylogeny. Science Publishers, New Hampshire.

Joly, D., Schiffer, M., 2010. Coevolution of male and female reproductive structures in *Drosophila*. Genetica 138, 105-118.

Kahanpää, J., 2014. Checklist of the families Lonchopteridae and Phoridae of Finland (Insecta, Diptera). ZooKeys 441, 213-223.

Klymko, J., Marshall, S.A., 2008. Review of the Nearctic Lonchopteridae (Diptera), including descriptions of three new species. Canad. Entomol. 140, 649-673.

Kotrba, M., Heß, M., 2013. Giant spiral shaped spermatozoa of *Diasemopsis comoroensis* (Diptera, Diopsidae) with a unique ultrastructural component. Tissue and Cell 45, 443-445.

Kotrba, M., Huber, J., Feijen, H.R., 2014. Coevolution of male and female genitalia in stalk-eyed flies (Diptera: Diopsidae). Org. Divers. Evol. 14, 187-201.

Kotrba, M., Heß, M., Dallai, R., 2016. Giant spermatozoa of *Diasemopsis* (Diopsidae, Diptera) – Structural, ultrastructural and functional aspects. Arthropod Struct. Dev. 45, 42-56.

Kraus, F.B., Neumann, P., van Praagh, J., Moritz, R.F.A., 2004. Sperm limitation and the evolution of extreme polyandry in honeybees (*Apis mellifera* L.). Behav. Ecol. Sociobiol. 55, 494-501.

Lachaise, D., Joly, D., 1991. Sperm and evolution in *Drosophila*. In: Hewitt, G.M., Johnston, A.W.B., Young, J.P.W. (Eds.), Molecular Technique in Taxonomy. Springer Verlag, Berlin, pp. 201-216.

Lefevre, G., Jonsson, U.B., 1962. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. Genetics 47, 1719-1736.

Levitan, D.R., TerHorst, C.P., Fogarty, N.D., 2007. The risk of polyspermy in three congeneric sea urchins and its implications for gametic incompatibility and reproductive isolation. Evolution 61, 2007-2014.

Liao, W.B., Zhong, M.J., Lüpold, S., 2019. Sperm quality and quantity evolve through different selective processes in the Phasianidae. Sci. Rep. 9, 19278.

Lind, H., 1973. The functional significance of the spermatophore and the fate of spermatozoa in the genital tract of *Helix pomatia* (Gastropoda: Stylommatophora). J. Zool. 169, 39-64.

Lupetti, P., Mencarelli, C., Mercati, D., Gaino, E., Dallai, R., 2011. The spermatodesm of *Cloeon dipterum* (L.): fine structure and sperm movement. Tissue Cell 43, 157-164.

Lüpold, S., Calhim, S., Immler, S., Birkhead, T.R., 2009. Sperm morphology and sperm velocity in passerine birds. Proc. R. Soc. Lond. B 276, 1175-1181.

Lüpold, S., Wistuba, J., Damm, O.S., Rivers, J.W., Birkhead, T.R., 2011. Sperm competition leads to functional adaptations in avian testes to maximize sperm quantity and quality. Reproduction 141, 595-605.

Lüpold, S., Manier, M.K., Puniamoorthy, N., Schoff, C., Starmer, W.T., Buckley Luepold, S.H., Belote, J.M., Pitnick, S., 2016. How sexual selection can drive the evolution of costly sperm ornamentation. Nature 533, 535-538.

Lüpold, S., de Boer, R.A., Evans, J.P., Tomkins, J.L., Fitzpatrick, J.L., 2020. How sperm competition shapes the evolution of testes and sperm: a meta-analysis. Phil. Trans. R. Soc. B 375, 20200064.

Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., Pitnick, S., 2010. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science 328, 354-357.

Manier, M.K., Lüpold, S., Starmer, W.T., Berben, K.S., Ala-Honkola, O., Collins, W.F., Pitnick, S., 2013. Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. Curr. Biol. 23, 1853-1862.

Martin, P.A., Reimers, T.J., Lodge, J.R., Dziuk, P.J., 1974. Effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. J. Reprod. Fertil. 39, 251-258.

Mayhew, P.J., 2007. Why are there so many insect species? Perspectives from fossils and phylogenies. Biol. Rev. 82, 425-454.

Mencarelli, C., Lupetti, P., Rosetto, M., Dallai, R., 2000. Morphogenesis of the giant sperm axoneme in *Asphondylia ruebsaameni* Kertesz (Diptera, Cecidomyiidae). Tissue Cell. 32, 188-197.

Mencarelli, C., Lupetti, P., Dallai, R., 2008. New insights into the cell biology of insect axonemes. Int. Rev. Cell. Mol. Biol. 268, 95-145.

Miao, Y., Liu, B.P., Hua, B.Z., 2019. Spermiogenesis of the hangingfly *Terrobittacus implicatus* (Huang and Hua) (Mecoptera: Bittacidae). Protoplasma 256, 1695-1703.

Middleton, C.A., Nongthomba, U., Parry, K., Sweeney, S.T., Sparrow, J.C., Elliott, C.J.H., 2006. Neuromuscular organization and aminergic modulation of contractions in the *Drosophila* ovary. BMC Biol. 4, 1-14.

Mikkola, K., 2008. The lock-and-key mechanisms of the internal genitalia of the Noctuidae (Lepidoptera): how are they selected for? Euro. J. Entomol. 105, 13-25.

Miller, A., 1965. The internal anatomy and histology of the imago of *Drosophila melanogaster*. In: Demerec, M. (Ed.), Biology of *Drosophila*. Hafner Publishing Company, New York, pp. 420-534.

Miller, G.T., Pitnick, S., 2002. Sperm-female coevolution in Drosophila. Science 298, 1230-1233.

Miller, G.T., Pitnick, S., 2003. Functional significance of seminal receptacle length in *Drosophila melanogaster*. J. Evol. Biol. 16, 114-126.

Minder, A.M., Hosken, D.J., Ward, P.I., 2005. Co-evolution of male and female reproductive characters across the Scathophagidae (Diptera). J. Evol. Biol. 18, 60-69.

Moreira, J.R., Clarke, J.R., Macdonald, D.W., 1997. The testis of capybaras (*Hydrochoerus hydrochaeris*). J. Mammal. 78, 1096-1100.

Morrow, E.H., Gage, J.G., 2000. The evolution of sperm length in moths. Proc. Royal Soc. Lond. B 267, 307-313.

Nabors, M.W., 2007. Botanik. Pearson Studium, München, pp. 674.

Netz, C., Renner, S.S., 2017. Long-spurred *Angraecum* orchids and long-tongued sphingid moths on Madagascar: a time frame for Darwin´s predicted *Xanthopan*/*Angraecum* coevolution. Biol. J. Linn. Soc. 122, 469-478.

Nieuwenhuis, B.P.S., James, T.Y., 2016. The frequency of sex in fungi. Phil. Trans. R. Soc. B 371, 20150540.

Noguchi, T., Koizumi, M., Hayashi, S., 2012. Mitochondria-driven cell elongation mechanism for competing sperms. Fly 6, 113-116.

Parker, G.A., 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45, 526-567.

Parker, G.A., 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J. Theor. Biol. 96, 281-294.

Parker, G.A., 1993. Sperm competition games: sperm size and sperm number under adult control. Proc. R. Soc. Lond. B 253, 245-254.

Parker, G.A., Pizzari, T., 2010. Sperm competition and ejaculate economics. Biol. Rev. 85, 897-934.

Pascini, T.V., Martins, G.F., 2017. The insect spermatheca: an overview. Zoology 121, 56-71.

Pattarini, J.M., Starmer, W.T., Bjork, A., Pitnick, S., 2006. Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. Evolution 60, 2064-2080.

Perotti, M.E., 1973. The mitochondrial derivative of the spermatozoon of *Drosophila* before and after fertilization. J. Ultrastruct. Res. 44, 181-198.

Phillips, D.M., 1971. Morphogenesis of the lacinate appendages of lepidopteran spermatozoa. J. Ultrastruct. Res. 34, 567-585.

Pitnick, S., 1996. Investment in testis and the cost of making long sperm in *Drosophila*. Am. Nat. 148, 57-80.

Pitnick, S., Karr, T.L., 1998. Paternal products and by-products in *Drosophila* development. Proc. R. Soc. Lond. B 265, 821-826.

Pitnick, S., Spicer, G.S., Markow, T., 1995. How long is a giant sperm? Nature 375, 109.

Pitnick, S., Markow, T.A., Spicer, G.S., 1999. Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. Evolution 53, 1804-1822.

Pitnick, S., Hosken, D.J., Birkhead, T.R., 2009. Sperm morphological diversity. In: Birkhead, T.R., Hosken, D.J., Pitnick, S. (Eds.), Sperm biology: an evolutionary perspective. Academic Press, Oxford, pp. 69-149.

Pizzari, T., Birkhead, T.R., 2002. The sexually-selected sperm hypothesis: sex-biased inheritance and sexual antagonism. Biol. Rev. 77, 183-209.

Pohl, H., Dallai, R., Gottardo, M., Beutel, R.G., 2013. The spermatozoon of *Mengenilla moldrzyki* (Strepsiptera, Mengenillidae): ultrastructure and phylogenetic considerations. Tissue Cell. 45, 397-401.

Presgraves, D.C., Baker, R.H., Wilkinson, G.S., 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. Proc. R. Soc. Lond. B 266, 1041-1047.

Proctor, H.C., 1998. Indirect sperm transfer in Arthropods: behavioral and evolutionary trends. Annu. Rev. Entomol. 43, 153-174.

Pyron, M., 2000. Testes mass and reproductive mode of minnows. Behav. Ecol. Sociobiol. 48, 132-136.

Ramm, S.A., Stockley, P., 2010. Sperm competition and sperm length influence the rate of mammalian spermatogenesis. Biol. Lett. 6, 219-221.

Reuter, M., Linklater, J.R., Lehmann, L., Fowler, K., Chapman, T., Hurst, G.D.D., 2008. Adaptation to experimental alterations of the operational sex ratio in populations of *Drosophila melanogaster*. Evolution 62, 401-412.

Rothstein, S.I., 1990. A model system for coevolution: avian brood parasitism. Annu. Rev. Ecol. Syst. 21, 481-508.

Rowe, L., Arnqvist, G., 2002. Sexually antagonistic coevolution in a mating system: combining experimental and comparative approaches to address evolutionary processes. Evolution 56, 754-767.

Rugman-Jones, P.F., Eady, P.E., 2008. Co-evolution of male and female reproductive tract across the Bruchidae (Coleoptera). Funct. Ecol. 22, 880-886.

Russell, L.D., Ren, H.P., Hikim, I.S., Schulze, W., Hikim, A.P.S., 1990. A comparative study in twelve mammalian species of volume densities, volumes, and numerical densities of selected testis components, emphasizing those related to the Sertoli cell. Am. J. Anat. 188, 21-30.

Schärer, L., Littlewood, D.T.J., Waeschenbach, A., Yoshida, W., Vizoso, D.B., 2011. Mating behavior and the evolution of sperm design. Proc. Natl. Acad. Sci. USA 108, 1490-1495.

Schrankel, K.R., Schwalm, F.E., 1974. Structures associated with the nucleus during chromatin condensation in *Coelopa frigida* (Diptera) spermiogenesis. Cell Tiss. Res. 153, 45-53.

Sinclair, B.J., Cumming, J.M., 2006. The morphology, higher-level phylogeny and classification of Empidoidea (Diptera). Zootaxa 1180, 1-172.

Sirot, L.K., 2003. The evolution of insect mating structures through sexual selection. Florida Entomologist 86, 124-133.

Sivinski, J., 1980. Sexual selection and insect sperm. Fla. Entomol. 63, 99-111.

Smith, K.G.V., 1969. Diptera, Lonchopteridae. Handbooks for the identification of British Insects 10, 1-9.

Stork, N.E., 2018. How many species of insects and other terrestrial arthropods are there on earth?. Annu. Rev. Entomol. 63, 31-45.

Strobl, V., Straub, L., Bruckner, S., Albrecht, M., Maitip, J., Kolari, E., Chantawannakul, P., Williams, R., Neumann, P., 2019. Not every sperm counts: male fertility in solitary bees, *Osmia cornuta*. PLoS ONE 14, e0214597.

Sturm, R., 2014. Comparison of sperm number, spermatophore size, and body size in four cricket species. J. Orthoptera Res. 23, 39-47.

Tandler, B., Moriber, L.G., 1966. Microtubular structures associated with the acrosome during spermiogenesis in water-strider, *Gerris remiges* (Say). J. Ultrastruct. Res. 14, 391-404.

Thornhill, R., 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122, 765-788.

Tregenza, T., Wedell, N., 2002. Polyandrous females avoid costs of inbreeding. Nature 415, 71-73.

Tyler, F., Harrison, X.A., Bretman, A., Veen, T., Rodríguez-Muñoz, R., Tregenza, T., 2013. Multiple post-mating barriers to hybridization in field crickets. Mol. Ecol. 22, 1640-1649.

Vahed, K., Parker, D.J., 2012. The evolution of large testes: sperm competition or male mating rate? Ethology 118, 107-117.

Virkki, N., 1973. Evolution of sperm cell number per bundle in insects. An. Esc. nac. Cienc. biol. Mex. 20, 23-34.

Vreys, C., Schockaert, E.R., Michiels, N.K., 1997. Formation, transfer and assimilation of the spermatophore of the hermaphroditic flatworm *Dugesia gonocephala* (Tricladida, Paludicola). Can. J. Zool. 75, 1479-1486.

Weiblen, G.D., 2003. Interspecific coevolution. Encyclopedia of Life Sciences, 1-12.

Welke, K., Schneider, J.M., 2009. Inbreeding avoidance through cryptic female choice in the cannibalistic orb-web spider *Argiope lobata*. Behav. Ecol. 20, 1056-1062.

Werner, M., Simmons, L.W., 2008. Insect sperm motility. Biol. Rev. 83, 191-208.

Wheeler, W.C., Whiting, M.F., Wheeler, Q.D., Carpenter, J.M., 2001. The phylogeny of the extant hexapod orders. Cladistics 17, 113-169.

Whiting, M.F., Carpenter, J.C., Wheeler, Q.D., Wheeler, W.C., 1997. The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Syst. Biol. 46, 1-68.

Whittington, A.E., Beuk, P.L.T., 2022. A description of a new species of western palaearctic *Lonchoptera* Meigen (Diptera, Lonchopteridae) from Georgia. ZooNova 20, 1-18.

Whittington, A.E., 2024. The status of genera in the Lonchopteridae (Diptera), and new records of *Lonchoptera* from Taiwan. ZooNova 38, 1-11.

Wiegmann, B.M., Yeates, D.K., 2017. Phylogeny of Diptera. Suricata 4, 253-265.

Wigby, S., Chapman, T., 2004. Sperm competition. Curr. Biol. 14, 100-103.

Wistuba, J., Schrod, A., Greve, B., Hodges, J.K., Aslam, H., Weinbauer, G.F., Luetjens, C.M., 2003. Organization of seminiferous epithelium in primates: relationship to spermatogenic efficiency, phylogeny, and mating system. Biol. Reprod. 69, 582-591.

Witte, K., 2009. Sexuelle Selektion: Die Bedeutung genetischer und sozialer Faktoren für die weibliche Partnerwahl. PdN-BioS 3, 18-22.

Woolhouse, M.E.J., Webster, J.P., Domingo, E., Charlesworth, B., Levin, B.R., 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nature Genetics 32, 569-577.

Yamashita, Y.M., Jones, D.L., Fuller, M.T., 2003. Orientation of asymmetric stem cell division by APC tumor suppressor and centrosome. Science 301, 1547-1550.

Yang, C.K., 1998. Diptera: Lonchopteridae. In: Xue, W.Q., Chao, C.M. (Eds.), Flies of China. Vol 1. Liaoning Science and Technology Press, Shenyang, pp. 49-59.

Yeates, S.E., Diamond, S.E., Einum, S., Emerson, B.C., Holt, W.V., Gage, M.J.G., 2013. Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. Evolution 67, 3523-3536.

Danksagung

Zunächst möchte ich meinem Doktorvater Prof. Dr. Martin Heß (LMU) danken, der das Wagnis eingegangen ist, mich trotz meines eher ungewöhnlichen Werdegangs als Doktoranden anzunehmen, mir einen Arbeitsplatz und Arbeitsmaterialen zur Verfügung gestellt und mich jederzeit inhaltlich und methodisch unterstützt hat.

Dr. Marion Kotrba hat mich bereits im Jahr 2009 bei meiner Zulassungsarbeit an der SNSB-Zoologischen Staatssammlung betreut und war ohne zu zögern wieder bereit, mich zu unterstützen, als ich zunächst mit einer vorsichtigen Forschungsanfrage an sie herangetreten bin. Letztlich wurde daraus für uns beide ein Herzensprojekt, das uns thematisch und auch privat verbunden hat und so ist es nicht verwunderlich, dass sie zu meiner „Doktormutter im Geiste“ geworden ist. Danke, Marion, für deine Unterstützung, deinen Enthusiasmus, deine Ratschläge und deine Freundschaft. Danke, dass du mir beigebracht hast, wie man wissenschaftlich arbeitet, denkt und schreibt und dass du nie müde geworden bist, das Beste aus mir herausholen zu wollen.

Mein Dank gilt auch denjenigen, die mir das Vertrauen entgegengebracht und sich die Zeit genommen haben, mich bei den unterschiedlichsten Methoden und Labortechniken zu unterstützen oder mich in diese einzuarbeiten: Dr. Bernhard Ruthensteiner (Amira), Dr. Eva Facher (REM), PD Dr. Michael Raupach (DNA Barcoding) und Eva Lodde-Bensch (Mikrotom). Mein ganz besonderer Dank gilt in dieser Hinsicht zudem Heidemarie Gensler, die mir alles beigebracht hat, was nötig ist, um gute Transmissionselektronenmikroskopie zu betreiben. Danke, Heidi, für deinen pragmatischen Blick auf die Dinge und für deine Fähigkeit, meinen Fokus immer wieder richtig auszurichten.

Nicht zuletzt möchte ich mich für die redaktionelle Unterstützung von Andrea Reichart, Kirsten Kollers, Ursula Böhm und Marion Zinner bedanken.

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Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass meine Dissertation selbstständig und ohne unerlaubte Hilfsmittel angefertigt worden ist.

Die vorliegende Dissertation wurde weder ganz noch teilweise bei einer anderen Prüfungskommission vorgelegt.

Ich habe noch zu keinem früheren Zeitpunkt versucht, eine Dissertation einzureichen oder an einer Doktorprüfung teilzunehmen.

München, den 20.10.2024

Michael Tröster

Anhang

A) Poster: Giant spermatozoa of *Lonchoptera lutea* (Lonchopteridae, Diptera) – Structural and ultrastructural aspects

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Veröffentlicht als:

Tröster, M., Kotrba, M., 2019. Giant spermatozoa of *Lonchoptera lutea* (Lonchopteridae, Diptera) – Structural and ultrastructural aspects. 20. Jahrestagung der Gesellschaft für Biologische Systematik, GfBS (München, Germany).

Giant spermatozoa of *Lonchoptera lutea* (Lonchopteridae, Diptera) – Structural and ultrastructural aspects

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Introduction:

Light microscopic dissections of the female reproductive tract of Lonchopteridae (Diptera, Brachycera) revealed spermatozoa apparently much wider and much longer than the average dipteran spermatozoon. Subsequent studies in the species *Lonchoptera lutea* Panzer, 1809 with micro-CT, serial sectioning, and TEM substantiate this finding and reveal new and interesting details.

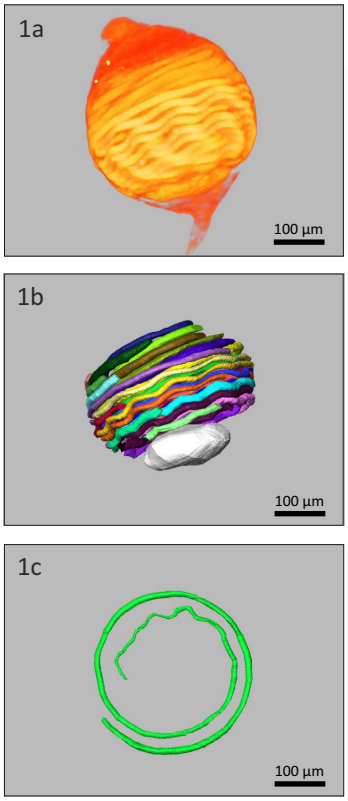

Fig. 1. Micro-CT scan, visualization with Amira. **a:** Testis with spermatocyte bundles. **b:** Partial reconstruction of spermatocyte bundles. **c:** Partial reconstruction of single spermatocyte bundle.

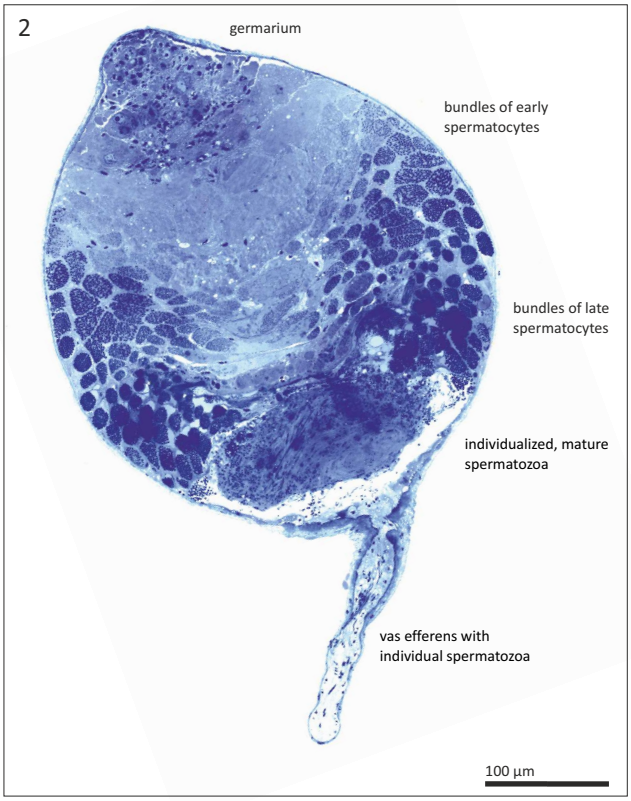

Fig. 2. Histological semi-thin sections. Longitudinal section through testis of *Lonchoptera lutea* shows different stages of spermatogenesis. Stiched image from 17 light microscopic photos at 100x magnification.

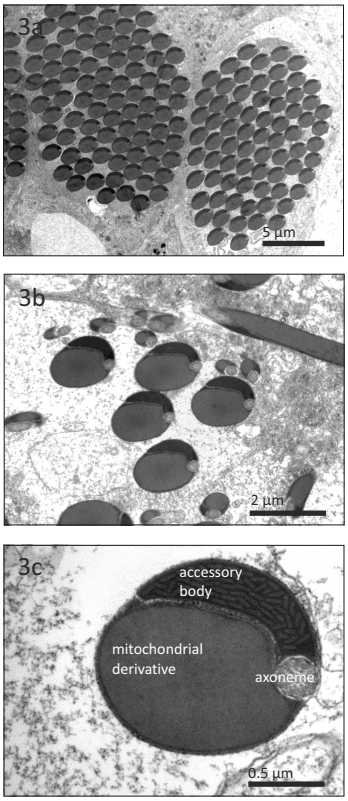

Fig. 3. TEM images. **a:** Cross section of bundles of late spermatocytes. **b:** Wide mid region and narrower end region of mature spermatozoa. **c:** Mature spermatozoon.

Results:

Partial reconstructions of bundles of maturing spermatocytes show that some of them exceed 1800 µm in length (Figs. 1a-c). The bundles comprise 62 to 89 (average 76) spermatocytes, while in other Diptera the number is generally 64 (Figs. 2, 3a). Mature spermatozoa have a width of about 1.4 µm along much of their length (Figs. 3b-c). The commonplace axoneme (0.2 µm wide) is accompanied by a single large pseudocrystalline mitochondrial derivative (1.2 µm wide) and a large band-shaped accessory body (1.3 µm x 0.2 µm). The ultrastructure of the accessory body shows up to 50 evenly spaced electron-light tubules embedded in an otherwise homogenous electron-dense matrix. This condition is unique among all Diptera studied to date.

Outlook:

Apart from their large size, the most striking feature of the studied spermatozoa is the enigmatic accessory body (Figs. 3c, 4). Investigating the early stages of spermatogenesis could shed light on its cellular origin and molecular composition. The ongoing study will add considerably to the hitherto scarce information on the diversity of spermatozoa structure and ultrastructure in the Aschiza section of higher Diptera.

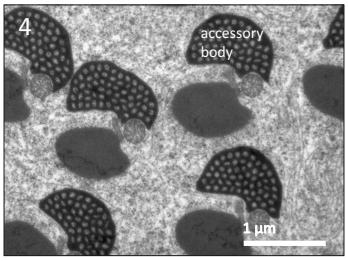

Fig. 4. TEM images. Stage from early spermatogenesis showing accessory body with peculiar ultrastructure.

Acknowledgements:

We thank E. Lodde-Bensch, B. Ruthensteiner, H. Gensler for technical assistance.

staatliche naturwissenschaftliche sammlungen bayerns

Anhang

B) Wanted: Fresh or alcohol material of Lonchopteridae

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Veröffentlicht als:

Tröster, M., Kotrba, M., 2020. Wanted: Fresh or alcohol material of Lonchopteridae. Fly Times 65, 45-46.

The North American Dipterists Society and their Editor-in-Chief Stephen Gaimari are acknowledged for granting permission to reproduce this article in the present dissertation.

Wanted: Fresh or alcohol material of Lonchopteridae

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We just published a paper (Kotrba et al. 2020) on the morphology and ultrastructure of the spermatozoa of *Lonchoptera lutea* Panzer, 1809 (Diptera: Lonchopteridae). Therein we show that *L. lutea*, which is very common in Europe, has exceptionally large spermatozoa. with a length of 2,200 µm and a width of 1.4 µm. Furthermore, females of this species have very long, tubular spermathecae (Fig. 1), which can exceed a length of 4.1 mm and often contain densely packed bundles of spermatozoa.

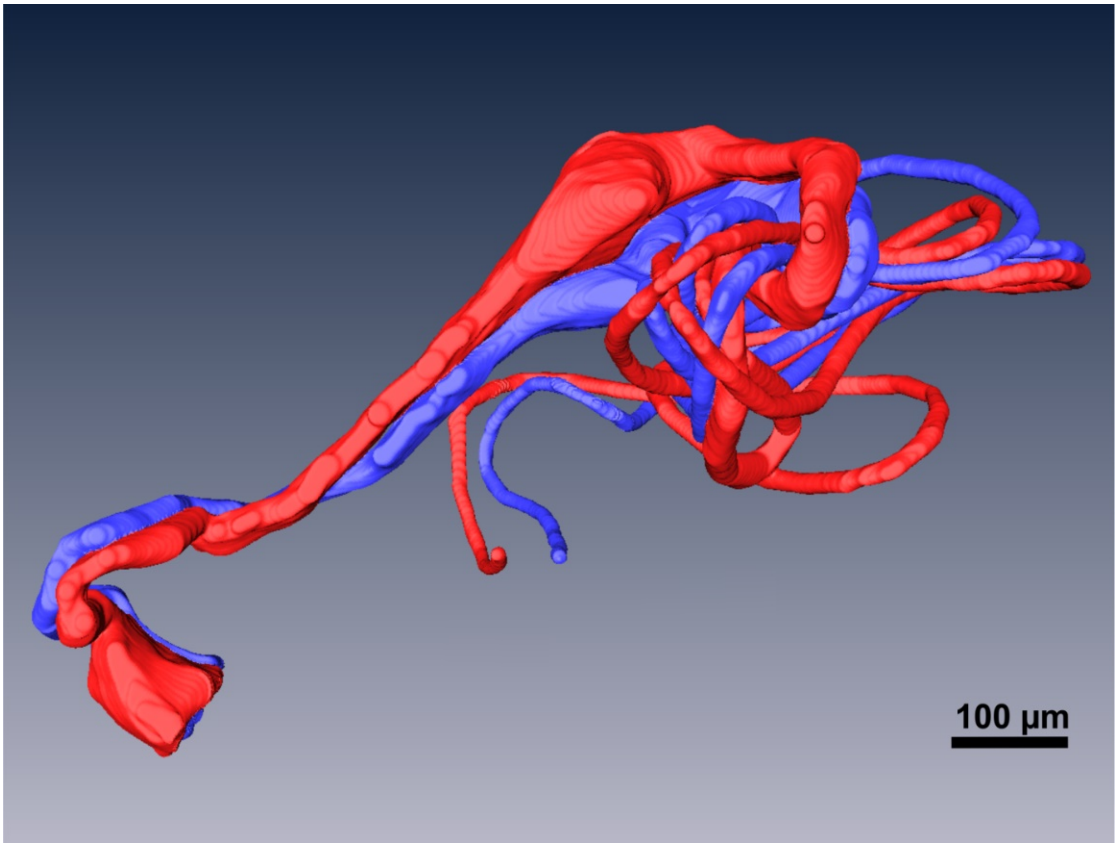

Fig. 1. Spermathecal ducts of *Lonchoptera lutea* Panzer, 3D-reconstruction from serial sections

Another very common species in Europe is *Lonchoptera bifurcata* (Fallén, 1810, senior synonym of *Lonchoptera furcata* (Fallén, 1823)). Its spermathecae are of similar shape but only 0.7–1 mm long and never contain spermatozoa. DeMeijere (1906) interprets this as an adaptation to parthenogenesis, especially because males of this species are extremely scarce. This hypothesis is plausible but requires substantiation by outgroup comparison.

To resolve the evolutionary trajectories of the length of the spermathecae and the length of the spermatozoa and the potential relationship between these two traits in Lonchopteridae, it is necessary

to study other species of this family. From the Bavarian State Collection of Zoology we could already acquire alcohol material of the species *L. fallax* DeMeijere, 1906, *L. tristis* Meigen, 1824, *L. scutellata* Stein, 1890 and *L. nitidifrons* Strobl, 1899 for further investigations. However, in order to cover the phylogenetic range of the family, it is essential to study more European species, such as *L. meijerei* Collin, 1938, *L. impicta* Zetterstedt, 1848, *L. nigrociliata* Duda, 1927, *L. strobli* DeMeijere, 1906 or *L. pictipennis* Bezzi, 1899, but also species from other regions of the world. Therefore we would be very thankful, if you could provide fresh or alcohol material of male and/or female adults of the family Lonchopteridae for serial sectioning.

Reference

Kotrba M., Tröster M., Gensler H., Ruthensteiner B. & Heß, M. 2020. Morphology and ultrastructure of the spermatozoa of *Lonchoptera lutea* Panzer, 1809 (Diptera: Lonchopteridae). Arthropod Structure & Development 2021, 60:101004. doi: 10.1016/j.asd.2020.101004 (open access at https://www.zsm.mwn.de/publikation/marion-kotrba/?lang=en until 19 January 2021)

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