1   **Novelty and emergent patterns in sperm: morphological diversity and evolution**
2   **of spermatozoa and sperm conjugation in ground beetles (Coleoptera:**
3   **Carabidae)**

4   **Running title: Diversity and evolution of ground beetle sperm**

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9

10 1. Abstract

11   The beetle family Carabidae, with about 40,000 species, exhibits enough
12   diversity in sperm structure and behavior to be an excellent model system for
13   studying patterns and processes of sperm evolution. We explore their potential,
14   documenting sperm form in 177 species of ground beetles and collecting data on 1
15   qualitative and 7 quantitative sperm phenotypic traits. Our sampling captures 61% of
16   the tribal-level diversity of ground beetles. These data highlight the notable
17   morphological diversity of sperm in ground beetles and suggest that sperm in the
18   group have dynamic evolutionary histories with much morphological innovation and
19   convergence. Sperm vary among species in total length from 48–3,400µm and in
20   length and width of the sperm head. Most ground beetles make filamentous sperm
21   with visually indistinct heads, but some or all studied members of the genus
22   *Omophron*, genus *Trachypachus*, and tribe Dyschiriini make broad-headed sperm that
23   show morphological differences between species. Most ground beetles package their
24   sperm into groups of sperm, termed conjugates, and ground beetles show variation in
25   conjugate form and in the number and arrangement of sperm in a conjugate. Most
26   ground beetles make sperm conjugates by embedding their sperm in a non-cellular
27   rod or spermatostyle, but some Trechitae make conjugates without a spermatostyle.
28   The spermatostyle is remarkably variable among species and varies in length from
29   17–41,000µm. Several unrelated groups of ground beetles make only singleton sperm,
30   including Nebriinae, Cicindelinae, many Trechinae, and the tribe Paussini. Given
31   current views about ground beetle relationships, we propose preliminary hypotheses
32   on ground beetle sperm diversification. We hypothesize that spermatostyle and
33   conjugate traits evolve faster than sperm traits and that head width evolves more
34   slowly than head length and sperm length. We propose that conjugation with a
35   spermatostyle evolved early within the history of Carabidae and that it has been lost
36   independently at least three times.

37

38 **Key Words**

39 Post-mating sexual selection, morphological evolution, sperm form, emergence

40

41 **Research highlights**

42 • **Ground beetle sperm is morphologically diverse.**
43 • **Most species make sperm conjugates with a spermatostyle, and there is**
44 **variation in sperm, spermatostyles, and conjugates.**
45 • **Sperm have dynamic evolutionary histories.**
46

47 **2. Introduction**

48   Animal sperm are among the most morphologically diverse cell type known.
49 Although sperm have been described from thousands of species, patterns in sperm
50 evolution remain largely unexplored (Birkhead and Montgomerie, 2009). Despite
51 their few constituent parts, almost every part of a sperm cell that could be altered has
52 been altered over evolutionary time, including the loss of cellular structures typical of
53 sperm such as flagella and nuclei (see review by Pitnick et al., 2009a). Sperm live
54 particularly odd "lives", being launched away from the soma to face a variety of
55 challenges unique among animal cells (Sivinski, 1984); variation in the environments
56 sperm encounter is thought to account for their diversity of form.

57   There is also variation in how sperm travel upon leaving the male soma; some
58 travel as singletons, but others travel in groups (Fig. 1), called conjugates. In sperm
59 conjugates, two or more sperm cells join or are joined together for motility or
60 transport through the female reproductive tract (see review by Higginson and Pitnick,
61 2011). Individual sperm in a conjugate frequently swim in a highly coordinated
62 fashion (Taggart et al., 1993), and there is some evidence that an individual sperm's
63 form can be adaptive for conjugation (Immler et al., 2007; Taggart et al., 1993). Sperm
64 conjugation is thought to be present in a small fraction of animal species but be
65 taxonomically widespread. Given current phylogenetic hypotheses for relationships
66 among animals (Hinchliff et al., 2015), it is likely that conjugation has evolved multiple
67 times independently (Higginson and Pitnick, 2011).

68   In numerous animal clades, the striking variation of animal sperm form and
69 function suggests that sperm are evolving rapidly and divergently. Rapid
70 morphological divergence is common to reproductive traits and is a core prediction of
71 evolution by sexual selection (Arnqvist and Rowe, 2005; Darwin, 1871; Eberhard,
72 1985; 1996; Holman and Snook, 2006; Hosken and Stockley, 2004; Miller and Pitnick,
73 2002; Parker, 1970; 1979; 2005; Pitnick and Hosken, 2010; Thornhill and Alcock,
74 1983). Although post-mating sexual selection is widely considered to be the
75 mechanism driving sperm morphological variation, the adaptive function of most

76   sperm traits is not known (Lüpold and Pitnick, 2018). Some sperm traits are
77   recognized as exaggerated ornaments evolving under female choice or male
78   persistence traits in sexual conflicts (e.g., Lupöld et al., 2016; Schärer et al., 2011).

79   The joining of sperm into groups of cells poses interesting broad scale
80   evolutionary questions regarding how individual traits and group traits coevolve
81   (Higginson and Pitnick, 2011). Do changes in sperm form drive changes in sperm
82   conjugates such as the number of sperm in a group? Do the different components of a
83   sperm conjugate show variation between species and if so, do they evolve at different
84   rates? How do conjugates evolve? Is it the parts that change, or the arrangement of
85   parts? Do females make decisions based upon a male's sperm as well as his sperm
86   conjugate? Do sperm cooperate (Fisher et al., 2014; Immler et al., 2007; Moore et al.,
87   2002; Pizzari and Foster, 2008)? Although it is too early to draw conclusions about
88   general processes from the available literature on the topic, early signs indicate that
89   evolution of sperm conjugation is a fertile topic of investigation (e.g., Ferraguti et al.,
90   1989; Fisher et al., 2014; Higginson et al., 2012a,b; Immler et al., 2007; Moore et al.,
91   2002; Sasakawa, 2007).

92   Ground beetles (family Carabidae) are a large clade suitable as a study system
93   for understanding the evolutionary patterns and processes of sexual trait evolution, as
94   previous studies hint at diverse sperm forms. Carabid beetles are an old, varied family
95   of terrestrial insects with nearly 40,000 described species (Lorenz, 2005; 2018). They
96   reproduce sexually and have internal fertilization (Crowson, 1981). During
97   copulation, males inseminate females, and females store sperm prior to fertilization
98   (Crowson, 1981). Female reproductive tracts are morphologically diverse across the
99   family, but all are of the "cul-de-sac" type with one duct leading to and away from the
100   sperm storage organ (Liebherr and Will, 1998). Previous studies report variation in
101   sperm across the species that have been studied (Supporting Information Table S1
102   and references therein). Ground beetle sperm vary in length from 68μm to 700μm
103   (Takami and Sota, 2007; Sasakawa, 2009), and both sperm dimorphism and sperm
104   conjugation are known to occur in the group (Supporting Information Table S1).

105   Although carabid beetles are a promising group in which to study sperm
106   phenotypic evolution, essential data are lacking for most of the group's diversity
107   (Supporting Information Table S1). For example, most of the data (54 of the 69
108   studied species) come from only two genera, *Carabus* and *Pterostichus*, which are on
109   widely separated branches of the tree of Carabidae. The near relatives of carabids, the
110   diving beetles (Dytiscidae), are advancing as a system for studying sexual trait
111   evolution (see review by Miller and Bergsten, 2014 and references therein). Diving
112   beetles are known for their complex female reproductive tracts, diverse sperm forms
113   with sperm length ranging from 128μm to 4493μm, three different qualitative types
114   of sperm conjugation, and several, independently derived instances of dimorphism
115   and/or conjugates that include more than one sperm morph (Higginson et al.,
116   2012a,b). Carabid beetles are ten times as diverse as diving beetles, and if their sperm

117   are variable like their near relatives, carabids are likely to provide numerous
118   opportunities for studying the evolution of complex sperm traits.

119   The primary goal of the present study is to document sperm morphological
120   diversity in ground beetles, making an effort to sample broadly across this diverse
121   radiation of terrestrial insects by gathering data from as many lineages as possible
122   within the family. We examine patterns and trends in sperm evolution in light of our
123   results.

124

125   **3. Materials & Methods**

126   *Taxon sampling*

127   Our study focused on identifying broad-scale patterns in sperm form across
128   carabid beetles, and we prioritized capturing morphological variation of sperm across
129   subfamilies and tribes within Carabidae. In total we studied 177 species of carabid
130   beetle classified in 121 genera across 61 tribes or approximately 0.44%, 5.8%, and
131   61% of the known global diversity of carabid species, genera, and tribes (Table 1;
132   Lorenz, 2005; 2018). Our attempt to sample different higher-level groups of ground
133   beetles was guided by current classification of carabid beetles (e.g., Bousquet, 2012;
134   Lorenz, 2005; 2018), current views about carabid relationships (e.g., Arndt et al.,
135   2005; Figs. 3–4), and recent molecular phylogenetic studies of the group (Maddison et
136   al., 1999; 2009; 2019; Ober, 2002; Ober and Maddison, 2008). Table 1 summarizes our
137   sampling and includes the number of specimens studied per species by sex. We
138   attempted to study multiple specimens per species in order to understand the
139   stability of sperm traits within a species, and we averaged about two specimens per
140   species (range = 1–8 specimens/species; Table 1).

141

142   *Specimens*

143   Our study is based on a total of 397 specimens (Tables 1, 2, S2). We collected
144   live beetles for sperm morphology in the United States, Mexico, the Republic of South
145   Africa, and Mozambique. We also studied additional specimens preserved in 10%
146   neutral-buffered formalin from Germany and Guatemala (Supporting Information
147   Table S2). 10% neutral-buffered formalin has a long history of use in sperm
148   morphology, and recent evidence from passerine birds suggests that it does not alter
149   the form of sperm (Schmoll et al., 2016).

150   We kept beetles alive in small containers separated by collection locality and
151   species prior to dissection or preservation in neutral-buffered formalin. When
152   possible we stored the beetles in a refrigerator or cooler to limit movement and
153   increase longevity. Following dissection and slide preparation, we associated slides

154 with their parent specimens with the use of unique alphanumeric codes given to each
155 specimen. We attempted to identify all of our specimens to species with the aid of
156 taxonomic literature or help from taxonomic specialists (see Acknowledgements). If
157 we were unable to identify a specimen to species because it represents an
158 undescribed species or is part of group in need of revision, the specimen was only
159 identified to genus.

160

161 *Phylogeny*

162 The phylogenetic relationships of most ground beetles sampled for sperm data
163 are not well understood, which limits insights into carabid sperm evolution. We use a
164 low-resolution phylogenetic hypothesis of ground beetles to guide our interpretation
165 of sperm data (Figs. 3–4). This phylogenetic hypothesis is based on published
166 phylogenies with minor contributions from traditional classifications of ground
167 beetles. The tree's shape is predominately derived from large-scale molecular studies
168 of ground beetle phylogeny (Maddison et al., 1999; 2009; Ober, 2002; Ober and
169 Maddison, 2008). Additional molecular phylogenetic studies provided support for
170 relationships in the following clades: Carabinae (Osawa et al., 2004), Cicindelinae
171 (Vogler and Pearson, 1996; Gough et al., 2018), Harpalini (Martínez-Navarro et al.,
172 2005), Paussinae (Moore, 2008; Robertson and Moore, 2016), Pterostichini and allies
173 (Will and Gill, 2008), and Trechinae (Maddison and Ober, 2011; Maddison et al.,
174 2019).

175

176 *Use of terms in sperm conjugation*

177 The study of sperm conjugation has been complicated by the variation in
178 sperm conjugation across animals and the historical lack of standard terms to refer to
179 these structures and their method of development (Higginson and Pitnick, 2011).
180 Previous workers have referred to the sperm conjugates of carabid beetles by a
181 variety of terms such as sperm bundles (e.g., Hodgson et al., 2013), spermatodesms
182 (Sasakawa, 2007; Sasakawa and Toki, 2008), or spermiozeugmata or similar (e.g.,
183 Ferenz, 1986; Schubert et al., 2017). Higginson and Pitnick (2011) suggest restricting
184 the use of terms like these to particular morphological and developmental patterns.
185 Higginson and Pitnick (2011) identified two major types of conjugation: primary and
186 secondary. Primary conjugates like spermatodesms result from the products of a
187 single spermatogonium remaining grouped together following spermiogenesis
188 (Higginson and Pitnick, 2011). Secondary conjugates like sperm bundles result from
189 sperm becoming joined together after individualization with sperm that are not
190 necessarily from the same cyst (Higginson and Pitnick, 2011).

191 Data are still lacking regarding whether the sperm conjugates of carabid
192 beetles are primary or secondary conjugates. Evidence from beetles in the closely

193 related families Haliplidae and Gyrinidae that make conjugates that look similar to
194 those in many carabids (Breland and Simmons, 1970; Higginson and Pitnick, 2011)
195 suggests that they are spermatodesms. Schubert et al., (2017) studied the
196 reproductive tract and spermatogenesis in a carabid beetle, *Limodromus assimilis*, and
197 came to a different conclusion. They examined various sections of the male internal
198 tract in this beetle and found that sperm individualize prior to becoming joined
199 together with a hyaline rod (Schubert et al., 2017). It is still unclear whether the
200 sperm conjugates of *L. assimilis* are composed of sperm derived from a single
201 spermatogonium, which is necessary for it to be considered to be primary
202 conjugation. The form of the male internal tract of *L. assimilis* is also highly similar to
203 whirligig beetles in the genus *Dineutus*, which are known to make spermatodesms
204 (Pitnick, unpublished data). Because of the uncertainty in conjugate type in carabid
205 beetles studied thus far and the lack of data for the overwhelming majority of species
206 in the family, we choose to refer to these multi-sperm forms by the neutral term
207 conjugate (Fig. 1).

208   We classified the variation in conjugation we observed into different
209 qualitative discrete types (Figs. 2–3). If we did not observe any physical association
210 between two or more sperm, we considered those species to lack conjugation. The
211 conjugates of species that make sperm that are physically associated via their heads
212 with a hyaline rod, or spermatostyle, with unbounded flagella were considered rod
213 conjugates. Conjugates characterized by sperm with flagella that are bounded to a
214 spermatostyle were considered sheet conjugates following Sasakawa, (2007). Those
215 with sperm joined together via their heads and cementing material but without a
216 spermatostyle were considered aggregate conjugates (Higginson et al., 2012a). In
217 some rare cases, we observed species that make singleton sperm bounded to a
218 spermatostyle with a 1-to-1 match between sperm and spermatostyle. We did not
219 consider this to be an example of sperm conjugation. Conjugates that form as a result
220 of sperm grappling onto one another in a seemingly imprecise location were
221 considered mechanical conjugates reminiscent of the sperm trains of muroid rodents
222 (Higginson and Pitnick, 2011).

223

224 *Sperm and tissue preparation for light microscopy*

225   Our survey largely focused on the form of mature spermatozoa of different
226 species of carabid beetles. We dissected both males and females and extracted sperm
227 from either the seminal vesicle of males or the sperm storage organ (spermatheca) of
228 females, respectively. Our sampling (Table 1) is biased towards male beetles because
229 aspects of our sampling were largely opportunistic, the probability of collecting
230 mature sperm is high in males whereas in females it requires their having been
231 inseminated, and we found consistent evidence that sperm, particularly sperm
232 conjugates, undergo changes in the female reproductive tract, posing challenges for

233 documenting sperm form prior to their exposure to the environment of the female
234 reproductive tract.

235   Our sperm preparation methods largely followed those of Higginson et al.,
236 (2012a; 2015). We removed the external and internal genitalia from live beetles or,
237 rarely, beetles preserved in 10% neutral buffered formalin, and placed them in a small
238 drop of 1X Phosphate Buffered Saline (PBS) prior to further dissection. For small-
239 bodied beetles (5mm and smaller), we frequently removed the entire abdomen and
240 placed it in 1X PBS prior to isolating portions of the reproductive tract and collecting
241 sperm. We reassociated dissected tissues with the specimen either by placing them on
242 the slide alongside the sperm or, more commonly, by placing them in a micro vial with
243 glycerin stored beneath the pinned specimen.

244   For males, we first isolated the accessory glands and testes from the aedeagus.
245 We then used an insect pin and fine forceps to gently loosen the testes and male
246 internal tract. We identified the vas deferens where it meets the accessory glands and
247 severed a portion of it. We transferred the severed portion of the male's tract to a
248 small drop of 1X PBS on a clean gelatin-coated or charged slide. We gently shook the
249 tissue to release sperm into the saline or held the tissue with forceps and ran fine
250 scissors along the length of the tract to extract sperm. For females, we generally
251 isolated the spermatheca and its subtending duct from the bursa copulatrix (Liebherr
252 and Will, 1998). We transferred the severed tract to a drop of 1X PBS on a clean
253 subbed slide. We gently shook the tissue to release sperm or made a longitudinal
254 incision along the outer wall of the spermatheca and compressed it to release stored
255 sperm. After collecting sperm, the slides were allowed to air dry and were stored in
256 slide boxes prior to fixation, staining, and mounting.

257   The majority of our sampling is based on sperm preparations made using a
258 portion of the male's seminal vesicle or female's spermatheca. In a few cases, however,
259 we also made observations from slides of testes, additional female reproductive tract
260 structures, and spermatophores by placing the tissues or spermatophores in saline on
261 a subbed slide and allowing the slide to air dry.

262   Once dry, we fixed and stained the slides using two different protocols. For 21
263 of our earliest samples (up to specimen RAGspcmn0000000134), we simultaneously
264 fixed and stained sperm using SpermBlue and the manufacturer's standard protocol
265 (van der Horst and Maree, 2010) followed by mounting in Euparal. Sperm heads in
266 carabid beetle sperm were not easily visible with SpermBlue and brightfield
267 microscopy, and we switched to viewing heads using DAPI and fluorescence. For DAPI
268 staining, we first placed slides in Coplin jars with a 3:1 mixture of methanol and acetic
269 acid for 1 minute. After fixation, we rinsed the slides in 1X PBS for 1 minute and then
270 removed the slides from buffer to dry briefly. Once partly dry, we placed a 2µl drop of
271 ProLong Diamond Antifade Mountant with DAPI on top of our sample along with a
272 clean cover slip and left the mountant to cure for at least 24 hours.

273

Light microscopy, imaging, and image analysis

275   For eight species we recorded videos of live sperm *in vitro* using a Leica Z6 lens
276 and JVC KY-F75U camera in conjuction with Microvision's Archimed software. Sperm
277 were removed from beetles using our standard dissection procedure and placed on a
278 slide in 1X PBS under a coverslip. We recorded a total of 16 short movies of live
279 ground beetle sperm conjugates from eight species at ambient temperature
280 (Supporting Information MV1–MV16). Although the videos are low quality, they give a
281 coarse-grained view of how the conjugates of these species move and perhaps insight
282 into how morphologically similar conjugates might move.

283   We visualized dead sperm using brightfield, darkfield, and fluorescence
284 microscopy and differential inference contrast (DIC) on a Leica DM5500 compound
285 microscope. We observed sperm and sperm conjugates at magnifications ranging
286 between 100–400x depending on the size of the subject. Sperm heads were most
287 easily visualized with fluorescence at 1000x as they are regularly about 1μm in length.

288   We used a Leica C425 camera paired with the Leica LAS software package to
289 image our samples on a Leica DM5500 microscope. We chose to photograph sperm
290 and sperm conjugates that were relatively isolated, in good condition, and easy to
291 image or measure. We took a variable number of photographs per specimen and/or
292 sperm preparation depending on the complexity and size of the subject matter, the
293 quality of the preparation, and the sex of the beetle. For instance, sperm longer than
294 1mm frequently required taking more than one photo and stitching them together
295 afterwards to fully capture the entire cell in a single image. We attempted to image at
296 least five individual sperm cells and at least five sperm conjugates per preparation.
297 We did not take measurements of sperm conjugates from females as the conjugates
298 are modified by the female's reproductive tract. We made qualitative observations of
299 sperm conjugates from our female preparations and categorized conjugates by type.

300   We gathered morphometric data on sperm morphological variation in carabid
301 beetles from these photographs using ImageJ (Rasband, 2012). We recorded data on
302 the physical dimensions of individual sperm and the resulting conjugate when
303 present. We studied 5.4 sperm on average per preparation across all preparations
304 (n=397). Sperm conjugation was observed in 147 of 177 species, and we studied 4.56
305 sperm conjugates on average per preparation among the 212 male preparations with
306 conjugates. We gathered linear morphometric data for the following six traits: sperm
307 length, head length, head width, spermatostyle length, spermatostyle width, conjugate
308 length, and the length of the spermatostyle that is bare apically. We also directly
309 counted or estimated the number of sperm found within a given conjugate. Carabid
310 beetle sperm conjugates can frequently include hundreds to thousands of sperm, and
311 when a direct count of sperm number was not an option, we used ImageJ and
312 calculated the corrected total cell fluorescence of sperm heads or mitochondrial

313 derivatives following McCloy et al., (2014) to estimate the number of sperm in a
314 conjugate from our DAPI-stained sperm preparations.

315   We investigated the precision of our instruments and workflow in order to
316 determine the number of significant figures we can reliably report in our
317 measurements. To do this, we repeatedly measured identical subjects from
318 photographs obtained from several rounds of imaging and microscope recalibration at
319 different magnifications. Results from our test showed that we could reliably measure
320 quantitative sperm traits down to two significant figures. For example, our
321 instruments and workflow were precise to the nearest 1μm when measuring sperm
322 heads between 10-20μm in length and were precise to the nearest 0.1μm for sperm
323 heads about 1μm or less in width. Based on the results of our investigation, we
324 rounded off our measurements to two significant figures.

325

326 *Data accessibility*

327   Specimens dissected for this study and all resulting slides are stored in the
328 personal research collection of RA Gomez and are available for examination upon
329 request. The 6,499 light microscope images we captured are all available online
330 through Morphobank (at http://morphobank.org/permalink/?P3123) and are
331 organized by species and specimen code. We took a dorsal habitus photograph of one
332 specimen of each species we studied for this project excluding *Pseudaptinus*
333 *tenuicollis*. These photos are available online through Morphobank.

334

335 **4. Results**

336 *Overview of sperm form in carabid beetles*

337   Our dataset includes new sperm data for 177 species of carabid beetle from
338 throughout the group's taxonomic breadth (Fig. 2) and reveals notable variation in
339 ground beetle sperm, sperm conjugates, and sperm storage (Fig. 5–12). These data are
340 summarized by species in Table 2 and by specimen in Supporting Information
341 Spreadsheet S1. In advance of presenting taxon-by-taxon results (next section), we
342 provide here an overview of our findings.

343   New discoveries from our study include new instances of sperm conjugation,
344 types of sperm conjugation previously unknown for the family, new occurrences of
345 singleton sperm, newly documented sperm phenotypic variation, and the discovery
346 that some female ground beetles store different parts of the sperm conjugate in
347 different organs.

348 Sperm length frequently varies from one lineage to another, but sperm heads
349 are almost always slender and narrow (Fig. 2B–C). Carabid beetle sperm range in total
350 length from 48–3400μm whereas head width ranges from 0.2–6.3μm. Very few
351 lineages of carabid beetles possess broad-headed sperm (Fig. 2C), but those that do,
352 such as the genus *Omophron* (Fig. 6F–J) and many Dyschiriini, have very distinctive
353 sperm that may be species-specific (Fig. 9I–N). Sperm head length varies much less
354 than sperm total length and ranges from 0.5–270μm. Most ground beetles make
355 sperm with heads that are shorter than 20μm in length. The sperm head is generally
356 conspicuous as a single region of fluorescence following DAPI staining, but sperm in
357 several lineages show two regions of fluorescence following DAPI staining: one faint
358 region anteriorly approximately 1–2μm in length and a second prominent filament of
359 much higher intensity fluorescence (Fig. 7B; Schubert et al., 2017). We attributed the
360 short, faint region of fluorescence to the sperm's nuclear DNA and the second,
361 prominent region of fluorescence to the sperm's mitochondrial DNA based on TEM
362 observations of ground beetle sperm ultrastructure (Dallai et al., 2019; Witz, 1990;
363 Gomez, unpublished data) and a recent study of carabid sperm with a similar staining
364 pattern (Schubert et al., 2017). We considered the short, faint region of fluorescence
365 the head because of its compact size, which is typical of heads of ground beetle sperm
366 with this staining pattern (Schubert et al., 2017) and its location, as it is consistently
367 located on the end of the sperm that is embedded in the spermatostyle.

368 Some male carabid beetles make singleton sperm, but the vast majority instead
369 make sperm conjugates (Figs. 2–4), usually by joining variable numbers of sperm to a
370 non-living structure called a spermatostyle. Sperm conjugation without a
371 spermatostyle can be found in some trechite carabid beetles such as some Bembidiini,
372 which make aggregate conjugates with few sperm (Figs. 7D–E). It is also found in
373 some tachyine trechites such as *Tachyta inornata* and *Tachyura rapax*, whose sperm
374 form haphazard groupings by grappling onto one another (Figs. 7–J). Singleton sperm
375 were observed in several unrelated carabid lineages, including *Nebria* and near
376 relatives (subfamily Nebriinae), *Gehringia olympica* (tribe Gehringiini), *Apotomus* sp.
377 (tribe Apotomini), *Eucamaragnathus oxygonus* (tribe Hiletini), various tribes in the
378 large clade Trechitae, tiger beetles (subfamily Cicindelinae), the ant parasite genus
379 *Paussus* and near relatives (tribe Paussini), and the subfamily Broscinae (Fig. 3).

380 The spermatostyle is present in nearly all ground beetles that make conjugates
381 (Figs. 3–4). It is absent in all examined species that make singleton sperm except for
382 two instances in the tribe Broscini indicating that sperm conjugation does not always
383 follow spermatostyle production (Figs. 8A–C). We studied two Broscini, *Zacotus*
384 *matthewsii* and *Broscodera insignis*, and both make singleton sperm joined to
385 individual spermatostyles.

386 Several different aspects of the spermatostyle have been modified through
387 evolutionary time, including size, overall shape, shape of the apex, placement or
388 location of sperm on the spermatostyle, rigidity, thickness, and texture. Some carabid

389   beetles make spermatostyles of varying sizes or two different size classes of similarly
390   shaped spermatostyles (Takami and Sota, 2007). Males of the bombardier beetle
391   *Brachinus elongatulus* make two distinct conjugates using two very different
392   spermatostyles (Fig 11A–B).

393   The spermatostyle displays remarkable variation in length with relatively little
394   variation observed in its width (Fig. 2D,E). As with sperm, the width of the
395   spermatostyle tends to be fairly conserved across Carabidae. The widest
396   spermatostyle we observed measured on average 140μm at its broadest. Most species,
397   however, make spermatostyles that are much narrower in width, usually measuring
398   between 2–20μm. The longest individual spermatostyle we observed was 5.8cm
399   whereas the shortest individual spermatostyle we observed measured only 13μm, a
400   more than 4,000-fold difference in length (Supporting Information Spreadsheet S1).
401   The spermatostyle frequently varies in length between related species within major
402   carabid lineages suggesting that spermatostyle length evolves rapidly and
403   convergently (Figs. 2–4).

404   Spermatostyles are frequently rod-shaped, fusiform, or comet-shaped (broader
405   anteriorly and attenuating to a narrow point posteriorly), but there are many
406   exceptions. Some spermatostyles maintain this general shape but are helically shaped
407   and rigid like a corkscrew (e.g., *Promecognathus laevissimus* spermatostyles; Fig. 8D–
408   F) or compacted like a slinky (e.g., *Chlaenius ruficauda* spermatostyles; Fig. 11G).
409   Others are cap-like and gelatinous (e.g., *Chlaenius prasinus* spermatostyles) or thin
410   and ribbon-like (e.g., *Stenocrepis elegans* spermatostyles; Fig. 11I). Sperm can also be
411   distributed along the spermatostyle in a variety of ways and in varying numbers. We
412   recorded as few as five sperm in a conjugate to as many as a few thousand. The
413   spermatostyle can include hyaline flanges (Figs. 6E, 8D) and channels or grooves that
414   appear to be associated with sperm attachment or storage (Fig. 12D). The material
415   surrounding sperm at their attachment point to the rod can have a different
416   appearance compared to the remainder of the rod (Dallai et al., 2019; Hodgson et al.,
417   2013). *Clivina* species make a capsule-like spermatostyle with a large sealed cavity
418   that contains a mass of sperm (Fig. 9A). Sperm are usually densely distributed on all
419   sides of a spermatostyle, but this trait is also variable. There are frequently extensive
420   bare regions without attached sperm on the spermatostyles of many ground beetle
421   conjugates (e.g., Figs. 6F, 9B, 9F, 12E). These bare regions are frequently found on the
422   anterior end, but they commonly occur medially (Fig. 11K) or, in a few species,
423   posteriorly. In *Aspidoglossa subangulata* sperm are attached to only one side of the
424   spermatostyle, and although these large spermatostyles measure 6.6mm, less than
425   1mm of their length bear sperm (Fig. 9B). In *Dyschirius tridentatus*, the sperm are
426   embedded in the spermatostyles via their heads in a single row with regular intervals
427   between sperm (Figs. 9F–G). The spermatostyle commonly appears to be designed to
428   accommodate sperm, particularly when the sperm heads are broad. Sperm will
429   frequently be placed parallel to the longitudinal axis of the spermatostyle, but this is
430   less common when sperm are broad-headed. For example, the broad-headed sperm of

431 *Trachypachus* are placed within diagonally arranged slots on one side of the
432 spermatostyle (Fig. 5G).

433   We dissected several female carabid beetles (Table 1), and we were usually
434 able to recover sperm, indicating that most wild-caught females had been mated at
435 least once. Of those preparations that were successful, we found that females stored
436 sperm in their cul-de-sac type spermatheca and its adjoining duct. Conjugated sperm
437 appear to become disassociated from each other in the spermatheca, and we
438 consistently observed morphological differences in the spermatostyles recovered
439 from our male and female slide preparations with spermatostyles recovered from
440 female reproductive tracts appearing thinner, compressed, possibly digested, or
441 completely absent in some of our female preparations (Figs. 12E–J). We frequently
442 recovered some intact conjugates from our female preparations, but the spermathecae
443 typically contained mostly individual sperm.

444   An unexpected sperm-female interaction from our study is the discovery that
445 some females store different components of the male's sperm conjugate in different
446 storage organs (Figs. 12A–D). Females in the genus *Galerita* appear to store large
447 quantities of spermatostyles in a balloon-shaped storage organ and individual sperm
448 in a physically removed small spherical organ that had been thought to be glandular
449 by Liebherr and Will, (1998). We surmise that female *Galerita* and relatives may have
450 partially decoupled spermatostyle morphological evolution from sperm evolution by
451 storing sperm and spermatostyles in different organs. It is clear from our study that
452 sperm conjugation in Carabidae is widespread and variable, and females are
453 interacting with conjugates, sperm, and spermatostyles. However, much research
454 remains to be done to tease apart the nature and consequences of these sperm-female
455 and conjugate-female interactions.

456

457 *Sperm form across major groups of carabid beetles*

458 Subfamily Carabinae (Fig. 1A–E; 5A–E; 12I–J)

459 *Species examined.* (Table 1). Tribe Carabini: *Carabus nemoralis*, *Carabus taedatus*, and
460 *Calosoma peregrinator*. Tribe Cychrini: *Cychrus tuberculatus*, *Scaphinotus marginatus*,
461 *Sphaeroderus schaumii*, and *Sphaeroderus stenostomus*.

462 *Sperm overview.* The sperm of carabines tend to be among the shortest known sperm
463 in carabid beetles (Table 2; Fig. 4A). The sperm thus far known are filamentous with
464 slender heads that are visually indistinguishable from the rest of the cells (Fig. 5C).
465 The sperm heads are obvious with DAPI staining (Figs. 5A, C).

466   All examined species of carabines make sperm conjugates with a spermatostyle
467 (Table 2). The sperm conjugates of carabines all feature a spermatostyle that is either
468 cap-like, short, and gelatinous in appearance (Figs. 5A–B) or rod-like, elongate, and

469   stiff (Figs. 1A–E; 5D). Sperm are embedded in the spermatostyle via their heads
470   although their flagella are unbounded (Figs. 1A–E). Species of *Calosoma* and *Carabus*
471   (tribe Carabini) make sperm conjugates that are reminiscent of shuttlecocks with a
472   short oblong spermatostyle. Longer spermatostyles with likely more sperm have been
473   previously recorded from species of *Carabus* subgenus *Ohomoperus* (Takami and Sota,
474   2007). Species of *Sphaeroderus* and *Scaphinotus* (tribe Cychrini) make elongate
475   conjugates composed of lengthy spermatostyles that include a large number of
476   associated sperm.

477   *Within-species variation.* Carabines, particularly members of the genus *Carabus*, are
478   among the best-studied carabid beetles for sperm morphology. Takami and Sota,
479   (2007) studied several species of *Carabus* in the subgenus *Ohomopterus* and observed
480   conjugate size polymorphism between specimens; many *Ohomopterus* make a single
481   sperm form that is packaged into different size classes of conjugates (Takami and Sota,
482   2007). Takami and Sota, (2007) also found evidence for a positive correlation
483   between risk of sperm competition and sperm conjugate polymorphism. If conjugates
484   perform different roles depending on their size, different size classes of sperm
485   conjugates would be expected.

486   Among the carabines we examined for this study, we found significant within-
487   male conjugate size variation (Fig. 5E) in the species of Cychrini we studied, and
488   minimal variation in *Carabus* and *Calosoma* (tribe Carabini). *Scaphinotus marginatus*
489   and the two *Sphaeroderus* species we studied make a single short sperm morph, but
490   males package their sperm into conjugates of different sizes (Table 2; Fig. 5E). The
491   *Carabus* and *Calosoma* species we studied all make sperm conjugates with
492   spermatostyles that vary less dramatically in length within males (Table 2; Fig. 5E).

493   *Within-genera variation.* Our sampling included two different species of the large
494   cosmopolitan genus *Carabus*, which is split into numerous subgenera and two species
495   of the eastern North American genus *Sphaeroderus*. We observed distinct differences
496   in sperm length, placement of sperm in their conjugates, and number of included
497   sperm between *C. (Tanaocarabus) taedatus* and *C. (Archicarabus) nemoralis*. We note
498   that these species are likely not particularly closely related. Within *Carabus* subgenus
499   *Ohomopterus*, Takami and Sota, (2007) observed variation in conjugate size
500   polymorphism as well as minor variation in sperm length among several closely
501   related species. Two *Sphaeroderus*, *S. schaumii* and *S. stenostomus,* possess
502   morphologically similar sperm.

503   *Reproductive tract observations*. Male carabines tend to devote a considerable amount
504   of intra-abdominal space to their testes and accessory glands. Perhaps because of the
505   small size of their sperm and their generally large bodies, carabines consistently
506   appear to make very large quantities of sperm.

507   We recovered partly bare spermatostyles from the spermathecae of female
508   specimens of *Cychrus tuberculatus*, *Scaphinotus marginatus*, and *Sphaeroderus*

509 *stenostomus* (Figs. 12I–J), confirming that the conjugates of conspecific males travel to
510 the spermatheca.

511 *Comments*. Bouix, (1961; 1963) studied the sperm of several species of *Carabus* and
512 reported finding dramatic instances of sperm polymorphism in DNA complement with
513 some beetles having macrocephalic sperm with multiple sets of chromosomes. We did
514 not investigate this topic systematically, but we found no instances of sperm
515 polymorphism in DNA content in our samples. DAPI stained sperm heads within a
516 species gave consistent fluorescent signals from cell to cell, which would not be
517 expected if some sperm had more DNA. We suspect that Bouix, (1961; 1963) may
518 have mistaken sperm conjugates for individual sperm cells, but this topic awaits
519 further inquiry.

520

521 Subfamily Elaphrinae (Figs. 5; 12G–H)

522 *Species examined.* (Table 1). Tribe Elaphrini: *Elaphrus purpurans* and *Blethisa*
523 *oregonensis*.

524 *Sperm overview*. The sperm of elaphrines are short and filamentous (Table 2). The
525 sperm heads are thin, tapered anteriorly, and are visually indistinguishable from the
526 flagella (Fig. 5F). The heads are conspicuous with DAPI staining.

527   Both *Elaphrus* and *Blethisa* make sperm conjugates with moderately long rod-
528 like spermatostyles (Table 2; Fig. 5F). The sperm are embedded in the spermatostyles
529 via their heads with unbounded flagella, and the sperm are distributed more or less
530 equally on all sides of the spermatostyles except for a short region anteriorly without
531 attached sperm (Fig. 5F). The spermatostyles differ in size between *E. purpurans* and
532 *B. oregonensis* but are similar in overall shape. The spermatostyles are narrowly
533 rounded anteriorly and tapered posteriorly and resemble comets.

534 *Within-species variation.* Both male *B. oregonensis* studied showed high levels of size
535 variation in spermatostyle length and the number of sperm in a conjugate with almost
536 no variation between specimens in sperm size or variation in the density of sperm
537 placement along the spermatostyle (Supporting Information Spreadsheet S1). The
538 form of *B. oregonensis* sperm conjugates appears stable within males and within the
539 species.

540 *Reproductive tract observations*. We recovered several largely intact conjugates from
541 the spermatheca of one female *E. purpurans* and several completely bare
542 spermatostyles from the spermatheca of a second female (Fig. 12G–H).

543 *Sperm motility observations*. The conjugates of *E. purpurans* move in the direction of
544 the spermatosyle's tapered slender end, which we considered posterior based on the
545 anterior orientation of the sperm heads in the spermatostyle and histological studies

546 of carabid conjugates (Hodgson et al., 2013; Schubert et al., 2017). It appears as
547 though the sperm do not helically beat their flagella along their longitudinal axis but
548 instead maintain a regular stroke pattern. The resulting movement of the conjugate is
549 directional, similar to a rowboat (Supporting Information movies MV5–MV8). There
550 does not appear to be any difference in swimming patterns between conjugates
551 recovered from a female's spermatheca and those found in a spermatophore.

552

553 Subfamily Trachypachinae (Fig. 5G–H)

554 *Species examined.* (Table 1). Tribe Trachypachini: *Trachypachus inermis* and
555 *Trachypachus slevini*.

556 *Sperm overview*. The sperm of *Trachypachus* is moderately long and filamentous
557 (Table 2). Our measurements of sperm length in *T. slevini* vary somewhat across
558 specimens (Supporting Information Spreadsheet S1) suggesting that sperm length in
559 *T. slevini* is variable or that this variation is an artifact of our slide preparations for
560 these samples. The sperm heads are slightly broader than the remainder of the cells
561 (Fig. G). The heads are rod-shaped and appear narrowly rounded anteriorly (Fig. 5G).

562 The conjugates of *Trachypachus* are distinctive because of the asymmetrical
563 arrangement of sperm in a conjugate, the small number of sperm in a conjugate, and
564 the small size of the spermatostyle (Table 2; Fig. 5G). The spermatostyle is narrowly
565 rounded anteriorly and attenuated posteriorly to a thin point. Sperm are located on
566 only one side of the spermatostyle, and the heads are arranged diagonally relative to
567 the longitudinal axis of the spermatostyle.

568 *Within-genera variation. Trachypachus inermis* sperm and their heads are slightly
569 shorter than the sperm and heads of *T. slevini*, respectively.

570 *Reproductive tract observations*. We recovered two mostly complete conjugates from
571 the spermatheca of a female *T. slevini* (Fig. 5H). The spermatostyles are asymmetrical
572 with diagonally arranged slots and are similar to the spermatostyles of *T. inermis*
573 sperm conjugates.

574 *Sperm motility observations. Trachypachus slevini* conjugates appear to move faster
575 than individual sperm and seem able to change direction readily (Supporting
576 Information MV15–MV16).

577

578 Subfamily Loricerinae (Figs. 6A, E)

579 *Species examined.* (Table 1). Tribe Loricerini: *Loricera foveata* and *Loricera*
580 *decempunctata*.

581   *Sperm overview. Loricera* sperm are short and filamentous (Table 2). The sperm heads
582   are thin, tapered anteriorly, and are visually indistinguishable from the flagella (Fig.
583   6E). The heads are conspicuous with DAPI staining (Fig. 6E).

584   Gilson, (1884) first viewed the large conjugates of *Loricera* but mistook them
585   for spermatophores. The sperm conjugates of *Loricera* include a long and thin
586   spermatostyle with numerous sperm embedded via their heads with unbounded
587   flagella (Fig. 6A). The sperm are distributed more or less equally on all sides of the
588   spermatostyle along its entire length apart from a short region anteriorly (Fig. 6A).
589   The spermatostyle is rod-like and narrows to a sharp point anteriorly and posteriorly.
590   It is crescent-shaped and curved.

591   *Within-genera variation.* Sperm differ slightly in total length between *L. foveata* and *L.*
592   *decempuncata*, but our data are limited.

593   *Reproductive tract observations*. The spermatheca of *Loricera* resembles a Gordian
594   knot, and we found several sets of spermatostyles within the spermathecae of our
595   specimens of *L. foveata* (Supporting Information MV9).

596

597   Subfamily Nebriinae (Figs. 6B–D)

598   *Species examined.* (Table 1) Tribe Nebriini: *Nebria brevicollis*. Tribe Opisthiini:
599   *Opisthius richardsoni*. Tribe Notiophilini: *Notiophilus sylvaticus*.

600   *Sperm overview.* Sperm in Nebriinae are generally long and filamentous (Table 2).
601   Sperm heads in nebriines are either thin, tapered apically, and visually indistinct as in
602   many other early-diverging carabid groups or rod-like and slightly broader than the
603   remaining portions of the cells. *Opisthius richardsoni* sperm heads are rod-like and are
604   slightly thickened (Fig. 6C–D). The sperm of *N. brevicollis* and *Notiophilus* are notable
605   for having rather long heads that are visually indistinct when unstained (Fig. 6B).

606   All nebriines studied to date only make singleton sperm with no evidence of a
607   spermatostyle (Table 2). Depending on the phylogenetic position of nebriines, this
608   could represent an early loss of conjugation and the spermatostyle in the tree of
609   Carabidae (Fig. 2) or singleton sperm could be the ancestral state of Carabidae.

610

611   Subfamily Omophroninae (Figs. 6F–J)

612   *Species examined.* (Table 1). Tribe Omophronini: *Omophron americanum* and
613   *Omophron ovale*.

614   *Sperm overview.* The sperm of *Omophron* are among the most distinctive sperm in
615   carabid beetles (Table 2). The sperm heads are broad, asymmetrical and

616 approximately V-shaped (Figs. 6G, I), and the flagellum joins the head asymmetrically
617 on one of its sides (Fig. 6J).

618   The sperm of *Omophron* conjugates are arranged in a highly organized fashion
619 inside of a rod-like spermatostyle (Fig. 6F–G,I). The sperm heads are paired together
620 such that the side of the head bearing the flagellum is lateral (Fig. 6G,I). These pairs of
621 sperm are radially stacked one inside of another in a row that is very reminiscent of
622 the rouleaux stacking in diving beetle sperm (Higginson et al., 2012a; Pitnick et al.,
623 2009a). Unlike the rouleaux stacking of diving beetles, the stacked grouping of
624 *Omophron* sperm heads are embedded in a rod-like spermatostyle (Table 2). The
625 spermatostyle is bare for approximately 80% of its length in *O. americanum* (Fig. 6H)
626 and about 50% of its length in *O. ovale* with sperm located only in the posterior part of
627 the conjugate (Fig. 6F).

628 *Within-genera variation.* Sperm are notably different between *O. americanum* and *O.*
629 *ovale* with numerous morphological differences in their sperm. The sperm differ in
630 total length, head size and shape, spermatostyle length, and the extent to which the
631 spermatostyle lacks sperm.

632 *Reproductive tract observations.* We have been unable to recover sperm from the
633 female reproductive tract of field-collected *Omophron* females despite at least six
634 attempts to do so. In contrast, in other carabid genera we typically found sperm in a
635 female's spermatheca. *Omophron* is unusual in this regard, and we speculate that
636 either our timing was bad or females are storing sperm in another location or using it
637 in a non-typical way.

638

639 Subfamily Trechinae, Tribe Patrobini (Figs. 7A–B)

640 *Species examined.* (Table 1). *Diplous filicornis* and *Patrobus longicornis*.

641 *Sperm overview.* Patrobine sperm are short and filamentous (Table 2; Figs. 7A–B). The
642 heads are short and compact and visually indistinct. When stained with DAPI,
643 patrobine sperm show two regions of fluorescence.

644   The sperm conjugates of patrobines include a simple rod-like spermatostyle
645 with sperm embedded via their heads with unbounded flagella (Table 2; Figs. 7A–B).
646 We did not gather morphometric data from the sperm conjugates recovered from our
647 female specimen of *D. filicornis*, but it is clear that *D. filicornis* males make rod
648 conjugates with generally 50 sperm or less embedded in a short and slender
649 spermatostyle.

650 *Reproductive tract observations.* We recovered several seemingly intact sperm
651 conjugates from the spermatheca of a female *D. filicornis*.

652

653   Subfamily Trechinae, Supertribe Trechitae (Figs. 7C–J)

654   *Species examined.* (Table 1). Tribe Trechini, subtribe Trechodina: *Pachydesus* sp.,
655   *Perileptus* sp., *Trechodes* sp., *Trechosiella scotti*. Tribe Trechini, subtribe Trechina:
656   *Trechus humboldti*. Tribe Anillini: an undescribed form from Oregon, USA. Tribe
657   Bembidiini: *Bembidion incrematum*, *Bembidion iridescens*, one of two species under
658   the name *Bembidion kuprianovi*, *Bembidion* sp. nr. *transversale*, *Bembidion sejunctum*,
659   *Bembidion zephyrum*, and an undescribed species of *Lionepha*. Tribe Pogonini:
660   *Diplochaetus planatus*. Tribe Tachyini: *Mioptachys flauvicauda*, *Paratachys* sp. 1,
661   *Paratachys* sp. 2, *Tachyta inornata*, and *Tachyura rapax.*

662   *Sperm overview.* Trechitae sperm vary dramatically in length, and this variation
663   appears to depend on conjugation state (Table 2; Fig. 2). Trechitae sperm tend to be
664   short to very long when singletons, moderately long generally when part of a
665   conjugate with cementing material, or very long when involved in mechanical
666   conjugation. Sperm total length ranges from as short as 100µm in an unidentified
667   species of *Trechodes* to the longest known sperm in Carabidae found in the tachyine
668   *Tachyta inornata* with its 3400µm-long sperm. Sperm length across Trechitae tends to
669   be shorter than 1mm, and sperm seem to have increased in length in the tribe
670   Pogonini and some members of the tribe Tachyini. Sperm heads in Trechitae are
671   generally thin, tapered anteriorly and filamentous (e.g., Fig. 7C). We were unable to
672   consistently observe or confidently identify the heads of some trechite sperm
673   following DAPI staining. Some trechite sperm show only one large region of
674   fluorescence removed from either end of the sperm (e.g., *Mioptachys flauvicauda* and
675   all studied *Bembidion* species), which we suspect corresponds to their mitochondrial
676   derivatives (Fig. 7C). Based on the sperm heads that we could visualize, head length
677   ranges from very short and patrobine-like in *Trechus* and trechodine trechines to long
678   or very elongate in *Diplochaetus* and tachyines (Figs. 7G–H). The heads of *Tachyta*
679   *inornata*, *Tachyura rapax*, and *Paratachys* spp. are unusual for their elongate size and
680   zig-zag shape (Figs. 7G–H).

681   Sperm conjugation is either absent or present in Trechitae (Table 2). Singleton
682   sperm are found in some *Bembidion*, an undescribed anilline, trechodine trechines,
683   *Trechus humboldti*, *D. planatus*, *M. flauvicauda*, and the two *Paratachys* species we
684   studied. The species that do make conjugates do so without an apparent
685   spermatostyle. Conjugated sperm in the subfamily are either aggregates (Figs. 7D, E)
686   or mechanical conjugates (Figs. 7G–J). Aggregate conjugates are found in some
687   *Bembidion* and *Lionepha*. Within one species, *Bembidion* sp. nr. *transversale*, we had
688   one specimen with evident aggregate conjugates, but in the other specimens we found
689   only singleton sperm; the cause of these differences is not known (Supporting
690   Information Spreadsheet S1). The heads of aggregate conjugates appear to be aligned
691   in register and presumably are joined together via cementing material. Because these
692   sperm are aligned parallel to one another and are joined together without a
693   spermatostyle, the conjugate is approximately the same length as an individual sperm

694   cell. Mechanically conjugated sperm were observed in *Tachyta inornata* and *Tachyura*
695   *rapax*, whose sperm form conjugates haphazardly via grappling onto one another (see
696   Sperm motility observations).

697   *Within-genera variation.* We studied several different species of the large and complex
698   genus *Bembidion* and found that sperm differ in total length and, perhaps, presence or
699   frequency of conjugation. Because we did not focus on a group of closely related
700   *Bembidion*, our data cannot speak to the usefulness of sperm-level variation in species
701   delimitation in *Bembidion*.

702   *Reproductive tract observations*. The spermatheca of many trechites is small, compact,
703   and frequently well sclerotized unlike most other ground beetles (Liebherr and Will,
704   1998).

705   *Sperm motility observations*. The *Bembidion* sperm that we observed consisted of a mix
706   of singleton sperm and conjugated sperm (Supporting Information MV3). *Bembidion*
707   sperm move via helical klinotaxis, and their sperm conjugates swim notably faster
708   than singleton sperm though we did not quantify this apparent difference in speed
709   (Supporting Information MV3–MV4).

710   *Tachyta inornata* and *Tachyura rapax* sperm are singletons, but we observed
711   them forming haphazard groups when released from the spermatheca or the male
712   internal tract (Supporting Information MV12–MV14 for *T. inornata*). We observed the
713   sperm of these beetles forming hairpin loops with their flagella (Figs. 7I–J) while
714   undulating up and down and beating their flagella. Because of this motion and their
715   long length, these sperm became net-like, and they began grappling onto adjacent
716   sperm as they moved. It is difficult to fully characterize their behavior from our
717   videos, but it appears as though sperm latch onto adjacent sperm and slide up their
718   neighbor sperm. We observed live sperm of three male and one female *Tachyta*
719   *inornata* and one male *Tachyura rapax*. Sperm in these species consistently formed
720   hairpin loops leading to the formation of groups of sperm of varying size. Although the
721   data are limited, we think that this is an example of secondary conjugation in
722   Carabidae and a novel example of mechanical conjugation in animals. Mechanical
723   conjugation is defined as a grouping of sperm that results from sperm haphazardly
724   grappling onto one another and forming groups of variable size (Higginson and
725   Pitnick, 2011), which is in keeping with our observations of sperm in these tachyines.
726   Mechanical conjugation has been previously reported only from muroid rodents.
727   Rodent sperm conjugates or trains have been the topic of much active research on the
728   biomechanics of sperm (Fisher et al., 2014), and they possibly represent a case of
729   sperm cooperation (e.g., Higginson and Pitnick, 2011; Immler et al., 2007; Moore,
730   2002; Pizzari and Foster, 2008).

731

732   Subfamily Broscinae (Figs. 8A–C)

733   *Species examined.* (Table 1). *Broscodera insignis* and *Zacotus matthewsii.*

734   *Sperm overview.* Broscinae sperm are moderately long to long (Table 2). The sperm
735   heads are visually indistinct from the remainder of the cell, but they are obvious with
736   DAPI staining (Fig. 8C). The heads are filamentous and rod-like in *Z. matthewsii* (Fig.
737   8C) and slender, elongate, and wavy in *Broscodera insignis* (Fig. 8A).

738   Broscinae are notable for making a spermatostyle without conjugation (Table
739   2; Figs. 8A–C), which is a character combination that we have not observed outside of
740   these two species. Broscinae make singleton sperm that are filamentous and are
741   individually embedded in a cap-like or sleeve-like spermatostyle. The spermatostyle
742   of *Z. matthewsii* is short, broad, and sperm-like in form (Fig. 8C) such that when sperm
743   are joined to these spermatostyles, they resemble broad-headed sperm. The
744   spermatostyle of *B. insignis* is sleeve-like and elongate (Fig. 8A–B).

745   *Reproductive tract observations.* The sperm of both species appear to become easily
746   separated from their spermatostyles, and we were generally unable to find sperm
747   joined to spermatostyles in our female preparations. We collected sperm from
748   spermathecae of several female *Z. matthewsii* and found mostly sperm without
749   spermatostyles.

750

751   Non-Harpalinae Carabidae *incertae sedis* (Figs. 8D–H)

752   *Species examined.* (Table 1). Tribe Gehringiini: *Gehringia olympica.* Tribe Hiletini:
753   *Eucamaragnathus oxygonus.* Tribe Apotomini: *Apotomus* sp. Tribe Promecognathini:
754   *Promecognathus laevissimus.* Tribe Psydrini: *Psydrus piceus.*

755   *Sperm overview.* Sperm in these beetles are filamentous and variable in length (Table
756   2). We were unable to visualize the sperm heads of our *G. olympica* and *Apotomus* sp.
757   sperm preparations. The heads of the remaining beetles are thin, tapered anteriorly
758   and more-or-less indistinct from the rest of the cells (e.g., Figs. 8F–H). They are
759   conspicuous with DAPI staining.

760   *Gehringia olympica, Eucamaragnathus oxygonus*, and *Apotomus* sp. all make
761   singleton sperm (Table 2). We were unable to study the sperm of male *P. piceus*, and
762   we found no evidence for conjugation in our preparation of a female *P. piceus.*
763   *Promecognathus laevissimus* makes large conjugates by joining hundreds of sperm to a
764   large corkscrew-shaped spermatostyle (Figs. 8D–E). The anterior end of the
765   spermatostyle is spoon-shaped and without sperm (Fig. 8D). The spermatostyle
766   appears to be composed of two parts: a central opaque rod with attached hyaline
767   flanks (Fig. 8D). The sperm are more heavily distributed laterally on the hyaline flanks
768   of the spermatostyle.

769   *Reproductive tract observations*. We recovered several intact and motile sperm
770   conjugates from the spermatheca of our studied female *P. laevissimus* specimen.

771   *Sperm motility observations*. *Promecognathus laevissimus* conjugates move in the
772   direction of their anterior end and spin in a helical fashion as they swim perhaps due
773   to the shape of the corkscrew-shaped spermatostyle and the action of its hundreds of
774   attached sperm (Supporting Information MV10–MV11).

775

776   Tribes Clivinini and Dyschiriini (Fig. 9)

777   *Species examined.* (Table 1). Tribe Clivinini: *Ardistomis obliquata, Ardistomis schaumii,*
778   *Aspidoglossa subangulata, Paraclivina bipustulata, Clivina fossor, Schizogenius*
779   *litigiosus,* and *Semiardistomis viridis.* Tribe Dyschiriini: *Akephorus obesus, Dyschirius*
780   *thoracicus, Dyschirius dejeanii, Dyschirius globosus, Dyschirius haemorrhoidalis,*
781   *Dyschirius pacificus,* and *Dyschirius tridentatus.*

782   *Sperm overview*. Sperm in Clivinini and Dyschiriini are diverse (Table 2). Sperm length
783   varies from moderately short to long. Sperm heads are filamentous (tribe Clivinini) or
784   short and generally broad and distinctively shaped (tribe Dyschiriini). We were
785   consistently unable to identify the heads of clivinine sperm, and we did not collect any
786   morphometric data on their sperm heads. Sperm heads in Dyschiriini are typically
787   broad and asymmetrical and possibly species- or lineage-specific in shape (Figs. 9I–N).
788   The putative mitochondrial derivatives of Clivinini but not Dyschiriini are conspicuous
789   with DAPI staining and frequently form complex loops that can be mistaken for sperm
790   heads.

791   All clivinines and dyschiriines studied to date make sperm conjugates (Table
792   2). The sperm conjugates all include a spermatostyle, but there is notable variation
793   within these groups at the level of the conjugate. The sperm conjugates of Clivinini
794   tend to be either rod conjugates (Fig. 9C) or sheet conjugates (*cf.* Sasakawa 2007; Fig.
795   9B). The spermatostyle varies substantially in length between Clivinini species as does
796   the number of sperm in a conjugate (Table 2). The rod conjugates of Dyschiriini
797   generally include less than 35 embedded sperm paired to spermatostyles of varying
798   lengths (Figs. 9E–H). The sperm conjugates of Dyschiriini are unusual among carabids
799   because they include so few sperm in a conjugate that the sperm can be easily counted
800   (e.g., Figs. 9E–H). Perhaps because of their typically broad size, the sperm heads of
801   Dyschiriini are arranged in a neat row within the spermatostyle (Fig. 9E) and are
802   never placed parallel to one another.

803   *Unusual conjugate-level variation*. The sperm conjugates of some Clivinini and
804   Dyschiriini are particularly unusual in that they include more spermatostyle than
805   sperm. For example, the sperm conjugate of *A. subangulata* includes a large 6600μm
806   spermatostyle, of which less than 1/6th of its length bears sperm; the rest of the
807   spermatostyle is completely bare. Sperm in *A. subangulata* conjugates are distributed

808   on only one side of the spermatostyle further biasing the conjugate towards
809   spermatostyle and less towards sperm. The sperm conjugates of *Clivina* are unusual in
810   that their spermatostyles feature an expanded cavity where the sperm are sealed (Fig.
811   9A), and based upon observations during dissections, it appears as though the
812   conjugates are not motile. Upon rupturing the apical portion of the spermatostyle of
813   *Clivina* conjugates, sperm were released via a narrow internal duct subtending the
814   cavity.

815   *Within-species variation.* Many of the sperm traits we recorded for Clivinini and
816   Dyschiriini sperm show high degrees of variance between preparations. We suspect
817   that these large variances are largely symptomatic of our preparation of the sperm
818   and sperm conjugates of these beetles, which are lengthy and easily damaged.

819   *Within-genera variation.* We studied more than one species of *Dyschirius*, *Clivina*, and
820   *Ardistomis*. Species of *Clivina* and *Ardistomis* have largely similar sperm conjugates
821   that differ slightly in the size and shape of the spermatostyle as well as in the lengths
822   of their sperm and mitochondrial derivatives. *Dyschirius*, however, appears to be an
823   especially interesting group of Carabidae in which to study the evolution of sperm
824   form, sperm-female morphological coevolution, and in which to explore the possibility
825   of using sperm form for species delimitation. We have studied a handful of different
826   species of *Dyschirius*, and it is clear that sperm, particularly head shape, evolves
827   rapidly within this group. The sperm heads are frequently complex in shape and
828   notably different from one lineage to the next. Understanding the extent to which
829   sperm head shape varies within *Dyschirius* species was not a goal of this study, and we
830   note that these data are still preliminary.

831   *Reproductive tract observations.* The large sheet conjugates of some clivinines such as
832   *A. subangulata* appear to occupy a large amount of space in the male reproductive
833   tract and are relatively few in number. These large conjugates can be particularly
834   difficult to extract undamaged.

835

836   Subfamily Scaritinae excl. the tribes Clivinini and Dyschiriini (Figs. 10A–D)

837   *Species examined.* (Table 1). Tribe Scaritini: *Haplotrachelus atropsis, Haplotrachelus cf.*
838   *latesulcatus, Haplotrachelus* sp.*, Scarites marinus, Scarites (Distichu*s) sp., and *Scarites*
839   *(Parallelomorphus) sp.* Tribe Pasimachini: *Pasimachus californicus.*

840   *Sperm overview.* Sperm in these groups are moderately short in total length (Table 2).
841   The sperm are filamentous, and the heads are visually indistinct. This grouping of
842   beetles make sperm with two regions of fluorescence following DAPI staining similar
843   to Patrobini sperm and the sperm of the vast majority of Harpalinae that we studied
844   (Fig. 10B–D). The sperm heads are inconspicuous and weakly fluorescent compared to
845   the intensely fluorescent mitochondrial derivatives. The heads are small and compact
846   (Figs. 10B–C), and the mitochondrial derivatives are significantly longer than the

847 sperm heads and average between 70–78μm in *P. californicus* and *S. marinus* (Fig.
848 10D).

849   The species we studied in *Haplotrachelus*, *Pasimachus,* and *Scarites* all make
850 sperm conjugates with small, cap-like spermatostyles or short rod-like spermatostyles
851 (Table 2; Figs. 10A, D). Sperm are embedded in the spermatostyle via their small,
852 compact heads; their flagella are unbounded. The spermatostyle is short and cap-like
853 in *S. marinus* and in an unidentified species of *Scarites* subgenus *Parallelomorphus.*
854 The remaining species that we studied all make rod spermatostyles that are more
855 elongate. *Haplotrachelus* males make spermatostyles that are noticeably less rigid
856 than the spermatostyles of other beetles in this group and their apices are flattened
857 and spatulate.

858 *Within-species variation.* We found notable variation in sperm conjugate size between
859 specimens of *P. californicus* (Supporting Information Spreadsheet S1). *Pasimachus*
860 *californicus* sperm appear to be monomorphic, but their spermatostyles differ in
861 average length between specimens. The spermatostyles also differ in shape with some
862 spermatostyles appearing short and oblong or stretched posteriorly and elongated.
863 The differences in spermatostyle size and shape influence the average number of
864 sperm in a conjugate, and males show large variances in the average number of
865 embedded sperm in their conjugates.

866   Sasakawa, (2009) studied the sperm of a Japanese species of *Scarites*, *S.*
867 *terricola,* and found an unusual example of within-male variation in sperm. *Scarites*
868 *terricola* males makes a short filamentous sperm morph that looks similar to the
869 sperm of close relatives and is involved in conjugation, and a second sperm morph
870 that is large and macrocephalic and always present as singletons. These sperm traits
871 are distinct from other cases of sperm dimorphism in adephagan beetles like those
872 seen in many diving beetles (Higginson et al., 2012a) because the two different sperm
873 forms of *S. terricola* do not combine to make a conjugate.

874 *Within-genera variation.* Large-bodied Scaritinae are frequently known to be
875 morphologically homogenous and taxonomically challenging (e.g., Jeannel, 1941;
876 Nichols, 1988). We studied three different species of the Old World scaritine genus
877 *Haplotrachelus* and three likely distantly related species of the cosmopolitan genus
878 *Scarites*. We observed minor differences in sperm length between these species
879 compared to their congeners. *Haplotrachelus* species all make remarkably similar
880 spermatostyles that differ slightly in size. The conjugates of the *Scarites* species we
881 examined differ in shape, size, and number of included sperm.

882 *Sperm ultrastructure.* Witz, (1990) studied the sperm form of two species of
883 *Pasimachus*, *P. strenuus* and *P. subsulcatus*. He found that *Pasimachus* sperm include a
884 small, electron dense nucleus with two adjacent large mitochondrial derivatives with
885 a herringbone pattern of paracrystaline material in cross section (Witz, 1990). Their
886 sperm have a typical 9+9+2 arrangement of microtubules in the axoneme, and he was

887 unable to discern an acrosome in the mature sperm of *P. subsulcatus*. Witz, (1990) also
888 found a series of small microtubules adjacent to the nucleus and developing
889 mitochondria in *Pasimachus* spermatids and hypothesized that these are involved in
890 organelle elongation in the mature sperm.

891 *Reproductive tract observations*. Males in these genera of Carabidae all have a blind sac
892 termed a vesicula seminalis (Will et al., 2005) that branches off of the vas deferens
893 prior to meeting the accessory glands. We ruptured the vesicula seminalis of our
894 studied male beetles and consistently found it to contain numerous sperm conjugates.

895

896 Subfamily Rhysodinae (Fig. 10E)

897 *Species examined.* (Table 1). Tribe Clinidiini: *Clinidium* sp. nr. *guatemalenum*. Tribe
898 Omoglymmiini: *Omoglymmius hamatus*.

899 *Sperm overview*. Rhysodinae sperm are moderately short and filamentous (Table 2;
900 Fig. 10E). The sperm heads are thin and filamentous and visually indistinct from the
901 rest of the cells. The heads are conspicuous with DAPI, and the mitochondrial
902 derivatives of rhysodine sperm are not visible following DAPI staining.

903 Rhysodines make sperm conjugates with a relatively short and oblong rod-like
904 spermatostyle (Table 2; Fig. 10E). The sperm are embedded in the spermatostyle via
905 their heads with unbounded flagella.

906 *Reproductive tract observations*. Male rhysodines also have a vesicula seminalis (Will
907 et al., 2005). We ruptured the vesicula seminalis of our studied male beetles and
908 recovered numerous sperm conjugates. The accessory glands of rhysodines are
909 unusual among carabids for their very elongate tips that are compacted inside their
910 bodies (Will et al., 2005).

911

912 Subfamily Cicindelinae (Figs. 10F–G)

913 *Species examined.* (Table 1). Tribe Amblycheilini: *Omus audouini* and *Omus dejeanii*.
914 Tribe Cicindelini: *Brasiella wickhami* and *Cicindela haemorrhagica*. Tribe
915 Megacephalini: *Tetracha carolina*.

916 *Sperm overview*. Sperm in Cicindelinae are short and filamentous with little variation
917 in length across the group (Table 2; Fig. 4A). The heads are filamentous, tapered
918 anteriorly, and visually indistinct from the remainder of the cells. DAPI staining
919 typically reveals one large region of fluorescence nearly two-thirds of the length of the
920 sperm or more, sometimes with a more or less isolated small, lanceolate region of
921 weak fluorescence apically (Fig. 10F–G). Werner, (1965) studied the sperm of a
922 European tiger beetle, *Cicindela campestris*, using TEM and discovered that the

923 nucleus of their sperm runs parallel to the axoneme and the mitochondrial derivatives
924 similar to some Harpalinae (Dallai et al., 2019). Werner, (1965) also found that the
925 nucleus ends before the mitochondrial derivatives and other axonemal structures. We
926 suspect that with DAPI staining and light microscopy, we are visualizing both the
927 mitochondrial derivatives and the nucleus of tiger beetle sperm (Fig. 10F–G). Because
928 tiger beetles may all possess a nucleus that runs parallel to their axoneme similar to *C.*
929 *campestris* and because we could not easily identify a gap in fluorescence between the
930 mitochondrial derivates and the nucleus, we did not record sperm head
931 measurements of our tiger beetle sperm preparations. The mitochondrial derivatives
932 are filamentous and intensely fluorescent following DAPI staining. They range in
933 length from 71μm in *C. haemorrhagica* to 110μm in *O. dejeanii.*

934   All Cicindelinae studied to date make only singleton sperm, and we have seen
935 no evidence of spermatostyle production in any cicindeline preparation (Table 2).

936 *Within-genera variation.* We studied two species of the North American genus *Omus*
937 from the Pacific Northwest, *O. dejeanii* and *O. audouini*. The sperm of the two species
938 differ very slightly in total length and length of their mitochondrial derivates.

939 *Reproductive tract observations*. Perhaps because of their small sperm and lack of
940 conjugation, we consistently found the seminal vesicles of male Cicindelinae to be
941 filled with very large quantities of individual sperm.

942

943 Subfamily Paussinae (Figs. 10H–K)

944 *Species examined.* (Table 1). Tribe Metriini: *Metrius contractus*. Tribe Ozaenini:
945 *Goniotropis parca*, *Ozaena* sp., and *Pachyteles* sp. Tribe Paussini: *Cerapterus* sp.,
946 *Paussus cucullatus*, and an unidentified species of *Paussus (Bathypaussus)*.

947 *Sperm overview*. Sperm in Paussinae (Table 2) are filamentous and short when
948 conjugated (Fig. 10H–J) or filamentous and moderately long when singletons (Fig.
949 10K). The sperm of *Goniotropis parca* is currently the shortest known sperm for the
950 family Carabidae (Table 2). The heads of paussine sperm are thread-like and tapered
951 anteriorly or slightly thickened and relatively short as in *Metrius contractus* (Figs.
952 10H–K). The heads are conspicuous with DAPI staining as a single region of
953 fluorescence. The mitochondrial derivatives are not obvious following DAPI staining.

954   Paussines either make sperm conjugates or singleton sperm (Table 2). *Metrius*
955 *contractus* and *Goniotropis parca* package their sperm into sperm conjugates with a
956 moderately short rod-like spermatostyle (Figs. 10H–J). Species of the tribe Paussini,
957 which include many obligate ant nest parasites, were found to make only singleton
958 sperm (Fig. 10K). We only sampled a single female of *Pachyteles* and *Ozaena,* and we
959 were unable to determine if they make conjugates. The sperm of *M. contractus* and *G.*
960 *parca* are embedded in the spermatostyle via their heads with their flagella

961   unbounded. Sperm appear to be distributed on all sides of the spermatostyle in the
962   conjugates of *M. contractus* (Fig. 10H), but in *Goniotropis parca*, the sperm are
963   generally located laterally on the spermatostyle with a prominent bare region
964   medially (Fig. 10J).

965   *Within-species variation.* The sperm conjugates of *Metrius contractus* show high levels
966   of polymorphism in size between specimens (Fig. 10I), but their sperm appear to be
967   monomorphic. Because sperm are distributed throughout the vast majority of the
968   length of the spermatostyle, these conjugates also vary in the number of included
969   sperm. *Goniotropis parca* males, similarly, have variation between specimens in
970   conjugate size with monomorphic sperm, but this variation is smaller than what we
971   have observed in *M. contractus*.

972   *Within-genera variation.* We studied two species of the obligate ant-parasite genus
973   *Paussus*, *P. cucullatus* (subgenus *Hylotorus*) and an unidentified species of the
974   subgenus *Bathypaussus*. The sperm of these two species differ slightly in total length
975   and head length. We also observed rather different sperm lengths but not head
976   lengths between our specimens of *P. cucullatus* from two different populations
977   (Supporting Information Table S2).

978   *Reproductive tract observations*. Male paussines all have a blind sac termed a vesicula
979   seminalis (Will et al., 2005) that joins their vas deferens prior to its meeting with the
980   accessory glands. We ruptured the vesicula seminalis of our studied male beetles and
981   recovered numerous sperm conjugates.

982

983   Subfamily Brachininae (Figs. 11A–D)

984   *Species examined.* Tribe Brachinini: *Brachinus elongatulus*, *Brachinus ichabodopsis*,
985   *Mastax* sp., *Pheropsophus* sp. 1, *Pheropsophus* sp. 2.

986   *Sperm overview*. Brachininae sperm are filamentous and short with little variation in
987   sperm total length (Table 2). The sperm heads are generally short, tapered anteriorly,
988   and visually indistinct from the rest of the cells. Following DAPI staining, Brachininae
989   sperm show two regions of fluorescence: the large and intensely fluorescent
990   mitochondrial derivatives and the notably fainter, small and compact sperm heads
991   (Fig. 11B). The sperm heads are short and narrow, under 1μm in length and width.
992   The mitochondrial derivatives vary slightly in length between species, but they are
993   generally conspicuous following DAPI staining and measure two-thirds of the total
994   length of the sperm or more (Fig. 11B).

995   Brachininae all make sperm conjugates with either a short, cap-like
996   spermatostyle and/or a slender and elongate rod-like spermatostyle (Figs. 11A–D).
997   The sperm are embedded in the spermatostyle via their heads with unbounded
998   flagella. *Brachinus elongatulus* is unusual because males make two distinct sperm

999   conjugate morphs (Table 2; Fig. 11A). One of the sperm conjugate morphs of *B.*
1000   *elongatulus* includes between 30–70 sperm joined to a small, cap-like spermatostyle.
1001   The second conjugate morph of *B. elongatulus* is composed of hundreds of sperm,
1002   which we were unable to estimate accurately, joined to an elongate, ribbon-like
1003   spermatostyle. *Mastax* males make small conjugates with a cap-like spermatostyle
1004   that resemble the small conjugate morph of *B. elongatulus*. The remaining brachinines
1005   we studied make larger spermatostyles including the giant 41mm-long
1006   spermatostyles of the large-bodied bombardier genus *Pheropsophus*. *Pheropsophus*
1007   sperm conjugates currently hold the record for largest sperm conjugates in Carabidae
1008   and are likely among the largest sperm conjugates known. The giant spermatostyles of
1009   *Pheropsophus* are flexible and ribbon-like, forming numerous loops of varying sizes on
1010   the slide (Figs. 11C–D). The sperm of *Pheropsophus* conjugates are regularly
1011   distributed throughout the length of the spermatostyle. Although we cannot
1012   accurately estimate the number of sperm in these giant conjugates, they likely include
1013   thousands of sperm given the dense packing of sperm and the giant size of the
1014   spermatostyle.

1015   *Within-species variation.* Our measurements of *Pheropsophus* sperm conjugates
1016   include a high amount of variance, between 4–5mm, in spermatostyle length between
1017   specimens. This variation in conjugate size may be accurate, but it may be an artifact
1018   of our preparations caused by the large size of these otherwise thin structures.

1019   *Within-genera variation.* We studied two different, likely distantly related species of
1020   the large complex genus *Brachinus* and found obvious differences in sperm form.
1021   *Brachinus elongatulus* sperm differ from sperm in *B. ichabodopsis* in total length and
1022   the size of their mitochondrial derivatives. *Brachinus elongatulus* make two distinct
1023   sperm conjugates whereas *B. ichabodopsis* makes a single conjugate morph with a
1024   very slender elongate spermatostyle that does not resemble the spermatostyle of
1025   either conjugate morph in *B. elongatulus*.

1026

1027   Subfamily Harpalinae (Figs. 11E–K; 12B–F)

1028   *Species examined.* Tribe Abacetini: *Abacetus* sp., *Stolonis intercepta*, and *Stolonis* sp.
1029   Tribe Anthiini: *Anthia (Termophilum)* sp. Tribe Catapiesisini: *Catapiesis* sp. Tribe
1030   Chlaeniini: *Chlaenius cumatilis, Chlaenius glaucus, Chlaenius harpalinus, Chlaenius*
1031   *leuscoscelis, Chlaenius prasinus, Chlaenius ruficauda, Chlaenius sericeus,* and *Chlaenius*
1032   *tricolor.* Tribe Ctenodactylini: *Leptotrachelus sp.* Tribe Cyclosomini: *Tetragonoderus*
1033   *fasciatus* and *Tetragonoderus* sp. nr *latipennis.* Tribe Dryptini: *Drypta* sp. Tribe
1034   Galeritini: *Galerita atripes, Galerita bicolor, Galerita forreri*, and *Galerita lecontei.* Tribe
1035   Graphipterini: *Cycloloba* sp. and *Graphipterus* sp. Tribe Harpalini: *Anisodactylus*
1036   *alternans, Anisodactylus anthracinus, Anisodactylus similis, Bradycellus* sp. 1,
1037   *Bradycellus* sp. 2, *Discoderus* sp., *Euryderus grossus, Harpalus affinis, Polpochila erro,*
1038   *Selenophorus* sp., *Stenolophus* sp., and *Stenomorphus convexior.* Tribe Helluonini:

1039   *Helluomorphoides papago* and *Macrocheilus* sp. Tribe Lachnophorini: *Ega sallei,*
1040   *Lachnophorus* sp. nr. *elegantulus,* and *Lachnophorus elegantulus.* Tribe Lebiini: *Agra*
1041   sp. 1, *Agra* sp. 2, *Apenes lucidula, Calleida bella, Calleida jansoni, Calleida decora,*
1042   *Cymindis punctifera, Cymindis punctigera,* an unidentified member of the *basipunctata-*
1043   group of *Cymindis* subgenus *Pinacodera, Lebia deceptrix, Lebia subgrandis, Lebia*
1044   *viridis, Phloeoxena nigricollis, Stenognathus quadricollis, Syntomus americanus,* and
1045   *Thyreopterus flavosignatus.* Tribe Licinini: *Badister ferrugineus, Dicaelus suffusus,* and
1046   *Diplocheila nupera.* Tribe Morionini: *Morion* sp. Tribe Odacanthini: *Colliuris*
1047   *pensylvanica.* Tribe Oodini: *Anatrichis minuta, Oodes fluvialis,* and *Stenocrepis elegans.*
1048   Tribe Panagaeini: *Panagaeus sallei.* Tribe Peleciini: *Disphaericus* sp. Tribe Perigonini:
1049   *Perigona nigriceps.* Tribe Pentagonicini: *Pentagonica sp.* Tribe Platynini: *Agonum*
1050   *piceolum, Agonum muelleri,* an unidentified species of *Rhadine dissecta*-group, and
1051   *Sericoda bembidioides.* Tribe Pseudomorphini: *Pseudomorpha* sp. Tribe Pterostichini:
1052   *Abaris splendidula, Hybothecus flohri, Cyclotrachelus dejeanellus, Cyrtomoscelis* cf.
1053   *dwesana, Pterostichus (Morphnosoma) melanarius, Pterostichus (Hypherpes) lama,*
1054   *Pterostichus (Leptoferonia) infernalis, Poecilus laetulus,* and *Poecilus scitulus.* Tribe
1055   Sphodrini: *Calathus peropacus* and *Synuchus dubius.* Tribe Zabrini: *Amara aenea* and
1056   *Amara farcta.* Tribe Zuphiini: *Pseudaptinus horni, Pseudaptinus simplex,* and
1057   *Pseudaptinus tenuicollis.*

1058   *Sperm overview.* The large subfamily Harpalinae, containing half of all carabid species,
1059   have sperm that are filamentous and vary widely in length from short to long (Table
1060   2). Instances of both short and long sperm occur repeatedly throughout the subfamily.
1061   The sperm heads are typically inconspicuous and visually indistinct from the
1062   remainder of the cells. Following DAPI staining, Harpalinae sperm show one or two
1063   regions of fluorescence. The mitochondrial derivates are large and fluoresce intensely
1064   with DAPI staining (Fig. 11H) whereas the nuclei are weakly fluorescent, making
1065   morphological observation of the heads difficult. There are several specimens for
1066   which we were unable to clearly discern the head (41.5% of all Harpalinae
1067   preparations studied). Of the sperm heads that we could observe, our data show that
1068   Harpalinae sperm heads are short, commonly between 0.5–5.0µm in length (Table 2;
1069   Fig. 11H). The heads are generally tapered anteriorly or weakly asymmetrical and
1070   narrow, varying minimally in width.

1071   Sperm conjugation seems to be the rule across Harpalinae with only few
1072   ambiguous exceptions (Table 2; Figs 3,11E–K). Sperm conjugation involves a
1073   spermatostyle, and the variation in conjugate shape and size across Harpalinae is
1074   striking, with numerous species making particularly elongate spermatostyles. The
1075   spermatostyle varies in total length from the short and cap-like spermatostyle of
1076   *Chlaenius prasinus* (Chlaeniini) to the enormous rod-like spermatostyles of
1077   *Pterostichus lama* (Pterostichini) and *Diplocheila nupera* (Licinini) that are among the
1078   largest spermatostyles in Carabidae (Figs. 3–4). The spermatostyle varies dramatically
1079   in shape with some species making corkscrew-shaped or spiral spermatostyles (e.g.,
1080   *Anisodactylus alternans, Tetragonoderus fasciatus;* Fig. 11K), flat and ribbon-like

1081 spermatostyles (e.g., *Stenocrepis elegans*; Fig. 11I), slinky-shaped spermatostyles (e.g.,
1082 *Chlaenius ruficauda*; Fig. 11G), or small and cap-like spermatostyles, in addition to
1083 variations on the more common simple rod-like spermatostyle (Fig. 11E). The apex of
1084 the spermatostyle shows a lot of variation and is frequently distinct in shape and/or
1085 width from the remainder of the spermatostyle. The apex is frequently simply tapered
1086 or gently expanded, but we have seen species with spermatostyles that are spoon-
1087 shaped or spatulate apically (e.g., *Pterostichus nigrita* Hodgson et al., 2013, *A.*
1088 *alternans*, *Cymindis punctigera*) or jagged and knife-like (*Poecilus* species).

1089 Harpalinae make either rod or sheet conjugates (Fig. 4). In sheet conjugates,
1090 the sperm, including their flagella, are joined to the spermatostyle by hyaline material
1091 (Figs. 11F, J–K; Dallai et al., 2019; Sasakawa, 2007). We have observed sheet
1092 conjugates in various unrelated groups of ground beetles. Sheet conjugates occur in
1093 members of the following tribes: Abacetini (e.g., *Abacetus* sp.), Chlaeniini (e.g., some
1094 but not all *Chlaenius* we studied), Ctenodactylini (e.g., *Leptotrachelus* sp.), Cyclosomini
1095 (e.g., *Tetragonoderus* spp.), Lebiini (e.g., *Calleida jansoni, Lebia* spp., and *Syntomus*
1096 *americanus*), Lachnophorini (e.g., a Mexican species of *Lachnophorus*), and Harpalini
1097 (e.g., *Discoderus* sp., *Stenolophus* sp., and *Stenomorphus convexior*). Most of the
1098 Pterostichini we studied also make sheet conjugates. Males of the genus *Galerita* make
1099 sperm conjugates that we scored as sheet conjugates. *Galerita* conjugates are unusual
1100 in that the spermatostyles include a long groove that seems to be associated with
1101 sperm placement (Fig. 12D), reminiscent of the sperm conjugates of some *Clivina*.
1102 Typically sperm are distributed more or less evenly along the entire length of the
1103 spermatostyle in Harpalinae. Sometimes sperm are more densely distributed along
1104 the sides of the spermatostyle (common in sheet conjugates (Hodgson et al., 2013)) or
1105 along particular stretches of the spermatostyle resulting in prominent bare regions
1106 that are common posteriorly (e.g., the spermatostyle of *Euryderus grossus* is 2900μm
1107 long but only 200–300μm of its length bears sperm). The number of sperm in a
1108 conjugate varies dramatically within Harpalinae. Most of the Harpalinae we studied
1109 make conjugates with between 30–1000 sperm in a conjugate. We were not always
1110 able to estimate the number of sperm in Harpalinae conjugates particularly when the
1111 conjugates were very large (e.g., the 9mm conjugates of *P. lama*).

1112 We found no unambiguous evidence that conjugation is missing in any of the
1113 Harpalinae we studied. However, we were unable to discern some morphological
1114 details of conjugation in some of our lower quality preparations. In our *Anthia* and
1115 *Galerita* preparations, for example, it was difficult to identify whether sperm had
1116 simply become detached from the spermatostyle or were not physically associated
1117 with it in the first place.

1118 *Within-species variation.* Male Harpalinae frequently make large conjugates with large
1119 spermatostyles. The spermatostyles are typically wider anteriorly than posteriorly
1120 and frequently possess a long and thin tail that is easily broken. In several of our
1121 harpaline preparations, we recorded large spermatostyle length variances between

1122   specimens of a given species. We suspect that some of the variation that we have
1123   observed is due to our damaging these spermatostyles during slide preparation.

1124   *Within-genera variation.* We studied two or more species in several widely related
1125   Harpalinae genera. Harpalinae sperm conjugates tend to be more morphologically
1126   variable than sperm between species. Sperm frequently will differ slightly in total
1127   length between species. For example, *Agonum piceolum* and *Agonum muelleri* make
1128   sperm that differ slightly in length by nearly 20μm, but their conjugates are notably
1129   different. The sperm conjugates of *A. muelleri* include a longer spermatostyle that is
1130   straight and rigid and includes a small bare region apically. We found a similar pattern
1131   in sperm and sperm conjugate variation among species of *Anisodactylus*, *Bradycellus*,
1132   *Calleida*, *Galerita*, *Cymindis*, and *Lebia*.

1133   Conjugate type is typically stable within a genus, but some harpaline genera
1134   include species that make either rod or sheet conjugates (e.g., *Chlaenius*, present
1135   study; *Pterostichus*, Sasakawa, 2007). We had the opportunity to study several
1136   different North American species of the large cosmopolitan ground beetle genus
1137   *Chlaenius*. We studied eight different species of *Chlaenius* classified in 5 different
1138   subgeneric groupings. Most studied species of *Chlaenius* make rod conjugates, but
1139   *Chlaenius ruficauda* makes sheet conjugates. The spermatostyle is notably variable in
1140   size and shape between species, and the range of variation observed in spermatostyle
1141   length across *Chlaenius* is almost as extensive as the variation observed across
1142   Carabidae as a whole (Table 2). The sperm of *Chlaenius* varies significantly in total
1143   length and, to a lesser degree, in sperm head length. The monophyly of *Chlaenius*
1144   subgeneric groups remains an open question, but it appears that sperm, particularly
1145   sperm conjugates, evolve rapidly in *Chlaenius*.

1146   *Sperm ultrastructure.* Recently, Dallai et al., (2019) studied the sperm ultrastructure of
1147   several *Pterostichus* species, *Amara aulica*, and *Demetrias atricapillus*. The sperm
1148   nuclei of these species are long, thin, and parallel to their axonemes like the nucleus of
1149   *Cicindela campestris* sperm (Dallai et al., 2019). If most Harpalinae sperm possess long
1150   and thin nuclei like these species, then perhaps it is the shape and size of their nuclei
1151   that explains the difficulty we had observing the sperm heads in many of our
1152   Harpalinae preparations (Fig. 11H). Their sperm have a typical 9+9+2 axoneme
1153   flanked by mitochondrial derivatives and small accessory bodies; their heads bear
1154   small, flat acrosomes (Dallai et al., 2019). Sperm in these species are packaged into
1155   sheet conjugates and are embedded laterally into the sidewall of the spermatostyle via
1156   their heads; their flagella are located in chambers that are joined to the spermatostyle
1157   by laminar extensions (Dallai et al., 2019).

1158   *Reproductive tract observations.* We collected sperm from female Harpalinae for
1159   several of our slide preparations. We almost always found individual sperm and
1160   collections of spermatostyles or intact conjugates in the spermathecae of females from
1161   throughout our sampling. The spermatostyles of Harpalinae males are generally long,
1162   and they tend to be compacted within the female's sperm storage organ. For example,

1163   male *Harpalus affinis* sperm conjugates include a long, slender, sickle-shaped
1164   spermatostyle (Fig. 12E), and we recovered large haphazard spermatostyle masses
1165   from the spermathecae of female *H. affinis* (Fig. 12F). Compacted masses of
1166   spermatostyles like these were commonly observed near the entrance of the
1167   spermatheca or the spermathecal duct with individual sperm predominately
1168   occupying the apical regions of the spermatheca. These masses were frequently
1169   difficult to break apart and mirrored the shape of the spermatheca.

1170   Some female harpaline ground beetles use different storage organs for
1171   different parts of a male's sperm conjugate. *Galerita* females appear to use one storage
1172   organ for sperm and another storage organ for the spermatostyles that males make
1173   (Figs. 12A–C). We dissected females of two different *Galerita* species, and discovered
1174   that the large balloon-shaped structure that has been called the spermatheca by
1175   Liebherr and Will, (1998) held large numbers of bare spermatosyles (Fig. 12A) and
1176   the notably smaller spherical structure termed a secondary spermathecal gland by
1177   Liebherr and Will, (1998) contained only individual sperm (Fig. 12C). These two
1178   storage sites are physically separated from one another and are connected via
1179   separate ducts to a larger common duct that joins the bursa copulatrix (Fig. 12 A;
1180   Liebherr and Will, 1998; Hunting, 2008). Nothing is known regarding sperm use by
1181   *Galerita* females, but we speculate that conjugates arrive to the balloon-shaped
1182   structure, dissociate or become dissociated from their sperm, and sperm travel or are
1183   moved to the functional spermatheca. Near relatives of *Galerita* possess similar female
1184   reproductive tract forms (Hunting, 2008), and it seems likely that this pattern of
1185   decoupled sperm and spermatostyle storage applies more broadly.

1186   *Sperm motility observations*. *Agonum piceolum* sperm conjugates swim in a typical
1187   helical fashion, and they appear to swim faster than individual sperm (Supporting
1188   Information MV1–MV2).

1189

1190   **5. Discussion**

1191   *Trends in carabid sperm evolution*

1192   One of the obvious ways in which sperm vary is in their length, a trait which
1193   may covary with conjugation. Long sperm have historically received much attention,
1194   and long sperm can be ornaments evolving under sexual selection (Lüpold et al.,
1195   2016). Various studies have shown that longer sperm are more costly to produce than
1196   shorter sperm (e.g., Pitnick, 1996). Considering only this factor, we would expect that
1197   through time sperm would evolve to become shorter (Parker, 1970; 1998). Sperm,
1198   instead, show a wide range in lengths in response to a variety of post-mating selection
1199   pressures (Lüpold and Pitnick, 2018). We found that carabid sperm vary in length
1200   from 48–3400μm, with variation in either direction towards long or short sperm
1201   occurring in several large groups of ground beetles, suggesting that sperm size is an

1202   evolutionarily labile trait (Figs. 2–4). Many species with singleton sperm tend to make
1203   long sperm of over 1mm in length, and perhaps sperm length is correlated with
1204   conjugation state or loss of the spermatostyle (Figs. 2–3; Higginson et al., 2012a).
1205   Because the spermatostyle competes for space with sperm in male and female
1206   reproductive tracts, it may be the case that loss of conjugation with a spermatostyle
1207   allows for more space for sperm, which might allow for either longer sperm or more
1208   numerous sperm. In diving beetles, sperm length does not correlate with conjugation
1209   (Higginson et al., 2012a), and conjugation may have more to do with occupying a site
1210   favorable for fertilization rather than conferring any motility advantages to sperm
1211   (Higginson et al., 2012b).

1212   Drag and efficient sperm packaging for conjugation may explain why ground
1213   beetle sperm are rarely broad-headed. The vast majority of carabid beetles studied
1214   make filamentous sperm with visually indistinct heads that are usually no broader
1215   than the remainder of the cell (e.g., Fig. 8F). Sperm head length varies from 0.5–
1216   270µm (Fig. 4B), but most species have sperm heads that measure under 20µm (Table
1217   2), suggesting that head length evolves much slower than sperm total length. Because
1218   sperm live in a low Reynolds number environment where viscous forces dominate
1219   over inertial forces (Vogel, 1994), drag is likely an important physical variable in
1220   sperm evolution (e.g., Ishimoto and Gaffney, 2015). If drag is an important variable in
1221   sperm evolution, you would expect sperm to be broad-headed only rarely (Humphries
1222   and Evans, 2008). At the same time, the physical joining of sperm to each other or to a
1223   spermatostyle might covary with head shape through time (Higginson and Pitnick,
1224   2011). Higginson et al., (2012a) found that the gain or loss of broad-headed sperm
1225   was evolutionarily correlated with qualitative changes to sperm conjugate type.
1226   Unlike ground beetles, many diving beetles have broad-headed sperm, but diving
1227   beetles do not make sperm conjugates with a spermatostyle (Higginson et al., 2012a).
1228   Thick- or broad-headed sperm (1.0–6.3µm) are found in only a few ground beetles
1229   such as some Dyschiriiini (Figs. 9I–J, L–N), members of the genus *Omophron* (Figs. 6G,
1230   I), genus *Trachypachus* (Fig. 5G), *Eucamaragnathus oxygonus* (Fig. 8H), and *Metrius*
1231   *contractus* (Fig. 10H). Based on the taxonomic distribution of broad-headed sperm
1232   (Fig. 4C), it seems likely that broad-headed sperm evolved from slender-headed
1233   sperm only a few times in Carabidae. Sperm head width may be more constrained
1234   functionally or developmentally than other aspects of sperm form. The scope of sperm
1235   head width variation is limited, and head width generally varies little between closely
1236   related species with narrow-headed sperm. Perhaps broad sperm heads are difficult
1237   to produce or are evolutionarily unstable. Diversification models of diving beetle
1238   sperm suggest that being broad-headed and single is an evolutionarily unstable state
1239   for sperm (Higginson et al., 2012a). All ground beetle species with thickened or broad
1240   heads make rod conjugates except for the singleton sperm of *Eucamaragnathus*
1241   *oxygonus*. Sperm conjugate type may not covary with sperm head width in ground
1242   beetles, but we surmise that the number of sperm in a conjugate depends on the form
1243   of the sperm head. For instance, the asymmetrical and broad-headed sperm of
1244   *Omophron* must stack and pair up in such a way to allow for the side bearing the

1245   flagellum to be lateral (Figs. 6G, I). Smaller heads might offer less drag, and it may be
1246   possible to group together more sperm by their heads when they are small. Species
1247   that make small-headed sperm (sperm head l x w under 0.5μm x 0.5μm) typically
1248   make conjugates with large numbers of sperm. Whether sperm head width is
1249   correlated with the number of sperm in a conjugate remains to be evaluated more
1250   thoroughly.

1251   *Trends in carabid sperm conjugate evolution*

1252   We identified several different qualitative types of sperm conjugates in ground
1253   beetles (Figs. 2–3; Table 2). Within each conjugate type, we found variation in sperm
1254   form, spermatostyle form, sperm number, and arrangement of sperm in a conjugate.
1255   The different conjugate types therefore only capture a small amount of the continuum
1256   of variation found in ground beetle sperm conjugates.

1257   Sperm conjugation with a spermatostyle appears to have been present early in
1258   the history of Carabidae. Several studied species make only singleton sperm, but most
1259   carabid beetles make sperm conjugates, and the distribution of sperm conjugates
1260   suggests an early origin. Most early diverging lineages of ground beetles such as
1261   Trachypachinae, Elaphrinae, and Carabinae (Maddison et al., 2009, Maddison et al.,
1262   unpublished data) make rod conjugates with a spermatostyle and unbounded flagella
1263   (Figs. 2, 5A–H). The one exception is subfamily Nebriinae, which make singleton
1264   sperm (Figs. 6B–D). The exact position of Nebriinae is an outstanding question in
1265   carabid systematics (Arndt et al., 2005; Maddison et al., 1999; 2009), and it may be
1266   that the ancestor of all carabids made singleton sperm. However, wherever Nebriinae
1267   might be placed, the extent of conjugates throughout carabids outside of Harpalinae
1268   suggests that sperm conjugation was present early in the history of Carabidae, and is
1269   ancestral for a majority of the family.

1270   Sperm conjugation in ground beetles almost always involves a spermatostyle,
1271   and carabids with conjugated sperm tend to make either rod conjugates or sheet
1272   conjugates (Figs. 2–3; Sasakawa, 2007). Sheet conjugates occur in several putatively
1273   unrelated tribes of Harpalinae (e.g., Figs. 11F–G, J–K) and the tribe Clivinini (Figs. 9A–
1274   B). If rod sperm conjugates are ancestral, this would mean that there have been many
1275   independent transitions to sheet conjugates.

1276   The distribution of singleton and conjugated sperm across ground beetles
1277   suggests that conjugation has been lost at least three times independently. Our low-
1278   resolution phylogeny implies a loss of conjugation in Cicindelinae, Paussini, and at the
1279   base of Trechitae (Fig. 2). Additional occurrences of singleton sperm are known from
1280   other phylogenetically scattered lineages such as Apotomini, Hiletini, and Gehringiini
1281   (Fig. 2; Maddison et al., 1999; 2009), but the lack of phylogenetic resolution for these
1282   groups prohibits deeper insights into the gain or loss of conjugation in ground beetles.

1283   The subfamily Trechinae is one clade in which patterns of sperm evolution
1284   appear evident, in part as there is a well-supported phylogenetic tree on which to
1285   examine our results (Maddison et al., 2019). Trechinae males vary in sperm
1286   conjugation presence and type (Figs. 2, 7). Some trechines make sperm conjugates
1287   with small spermatostyles and short sperm (Figs. 7A–B), some make conjugates
1288   without a spermatostyle (Figs. 7D–E), many make singleton sperm (Fig. 7C), and some
1289   make long sperm that grapple onto one another, forming haphazard groupings of
1290   sperm (Figs. 7G–J; mechanical conjugation). Ancestral Trechinae appear to have had
1291   sperm conjugates with short sperm and simple rod-like spermatostyles as seen in the
1292   Patrobini (Figs. 7A–B). We hypothesize that the spermatostyle and conjugation were
1293   lost in several Trechitae before sperm got longer. Once singleton sperm became
1294   longer, some Tachynini sperm gained a different type of conjugation that does not
1295   include a spermatostyle or cementing material: their long sperm form large loops
1296   (Figs. 7I–J) that turn while they swim, grabbing adjacent sperm in the process.
1297   Because mechanical conjugation is thus far known only from muroid rodents and the
1298   data we have are limited, further research is needed to confirm that this represents
1299   another example of this phenomenon. We note that this sperm evolution model
1300   assumes that the loss of sperm conjugation with a spermatostyle is more probable
1301   than its being gained and that the mechanical conjugates of some tachyine carabids
1302   are not homologous (as conjugates) with the rod conjugates of Patrobini or
1303   Bembidiini.

1304   *The spermatostyle as an understudied example of biological novelty*

1305   Conjugation with a spermatostyle is an interesting phenomenon because it
1306   entails a trade-off between sperm and spermatostyles. The more resources in terms of
1307   space, energy, and nutrients a male dedicates to spermatostyle production, the fewer
1308   resources are available for sperm production. Reducing sperm production seemingly
1309   reduces direct opportunities for paternal DNA to be passed to the next generation.
1310   Thus, increasing spermatostyles could reduce potential fertilizations, unless
1311   spermatostyles increase the per-sperm probability of successful fertlization. This
1312   trade-off in resource utilization also extends to sperm storage in the female's
1313   reproductive tract. In spite of this, most carabids make spermatostyles, and the
1314   spermatostyle is frequently large and elaborate among many different species. We
1315   know little about the chemical composition of the spermatostyle, but histological
1316   evidence suggests that it is a matrix of proteins and carbohydrates (e.g., Hodgson et
1317   al., 2013; Schubert et al., 2017). Males sometimes make sperm conjugates with bare
1318   regions that lack sperm (e.g., Figs. 6F, 9A, 12E) suggesting that the spermatostyle is
1319   more than just a device for joining together sperm.

1320   Evolution has explored a vast amount of morphological space in
1321   spermatostyles in ground beetles, and our data suggest that spermatostyles evolve at
1322   a much faster rate than sperm. Spermatostyles vary along several axes including size,
1323   shape, texture, and thickness. These spermatostyle traits frequently vary between
1324   species in a given higher-level taxonomic group (Fig. 2), and we suspect that there is

1325 much divergence and convergence in spermatostyle phenotype across Carabidae.
1326 Spermatostyle production can also occur without sperm conjugation (Fig. 3), as in
1327 Broscinae. Broscinae sperm are singleton, but their sperm are joined to individual
1328 spermatostyles (Figs. 8A–C). If spermatostyles evolve faster than sperm, then closely
1329 related species should vary more in spermatostyle and conjugate form than sperm
1330 form. Our observations of multiple species within 20 genera (*Agonum*, *Agra*, *Amara*,
1331 *Anisodactylus*, *Ardistomis*, *Brachinus*, *Bradycellus*, *Calleida*, *Carabus*, *Chlaenius*,
1332 *Cymindis*, *Galerita*, *Haplotrachelus*, *Lachnophorus*, *Lebia*, *Pterostichus*, *Scarites*,
1333 *Sphaeroderus*, *Stolonis*, *Tetragonoderus*) indicate that closely related species are more
1334 likely to differ in spermatostyle form than sperm form similar to the findings of
1335 Takami and Sota, (2007).

1336   We hypothesize that the spermatostyle can be an ornament evolving under
1337 postmating sexual selection and that because it is non-cellular, it has been freed from
1338 constraints that may be operating on sperm. Spermatostyles that contain large bare
1339 regions are particularly interesting from a post-mating sexual selection perspective. If
1340 sperm are like lottery tickets (Parker, 1970), this is akin to going to the racing downs
1341 and spending lots on beer and little on tickets. It may be the case that large conjugates
1342 with few sperm and large spermatostyles are similar to the exaggerated ornaments of
1343 some *Drosophila* sperm (Lüpold and Pitnick, 2018; Lüpold et al., 2016). Perhaps the
1344 spermatostyles modulate female mating behavior and are essential for successful
1345 fertilization like the anucleate parasperm of some butterflies (Cook and Wedell, 1999;
1346 Sakai et al., 2019).

1347   Other insects make sperm conjugates (e.g., Higginson and Pitnick, 2011;
1348 Higginson et al., 2012a; 2015), but only carabids and some whirligig beetles are
1349 known to make sperm conjugates with spermatostyles (Breland and Simmons, 1970;
1350 Gustafson and Miller, 2017; Higginson et al., 2015). Whirligig beetles are close
1351 relatives of ground beetles (McKenna et al., 2015; Maddison et al., 2009; Zhang et al.,
1352 2018), but the exact phylogenetic position of whirligigs relative to ground beetles is
1353 unclear. Whirligigs are usually placed with other aquatic adephagan beetles in a clade
1354 that is sister to ground beetles (McKenna et al., 2015; Zhang et al., 2018) or are
1355 inferred to be the sister to all remaining Adephaga (Beutel and Roughley, 1988).
1356 Evidence from whirligig systematics suggests that the spermatostyle is a derived trait
1357 within the group (Gustafson, personal comm.; Gustafson and Miller, 2017; Higginson
1358 et al., 2015), which implies that the spermatostyle in carabid beetles and whirligig
1359 beetles is convergent.

1360 *Insight into sperm-female interactions*

1361   The female reproductive tract can be considered a morphological
1362 representation of female sperm preference traits (Birkhead, 1998; Eberhard, 1996),
1363 and we found preliminary evidence that females exert pressure on different
1364 components of a male's sperm conjugate. We recovered sperm from the spermathecae
1365 of several females, and sperm conjugates clearly arrive at the spermatheca before

1366   dissociating (Figs. 12E–J). This observation confirms that sperm compete with
1367   spermatostyles for storage within a female's reproductive tract. This means that a
1368   male that invests more in spermatostyle size sacrifices spermathecal space for sperm.
1369   Spermatostyles recovered from female preparations were typically thinner or
1370   compacted compared to spermatostyles recovered from males' seminal vesicles, and
1371   most conjugated sperm in our female preparations had separated from their cohort
1372   sperm (Figs. 12E–J). Because sperm conjugates arrive to the spermatheca but only
1373   individual sperm can fertilize eggs, sperm must dissociate at some point (Higginson
1374   and Pitnick, 2011). Because sperm dissociation occurs within the female, females may
1375   be able to exert control over how quickly a male's conjugated sperm dissociate
1376   (Pitnick et al., 2009b). It is possible that males vary in how easily their conjugated
1377   sperm dissociate.

1378   A more compelling piece of evidence for cryptic female choice operating on
1379   sperm conjugates in ground beetles is the discovery that some females have separate
1380   storage organs for different parts of the sperm conjugate. Some *Galerita* females have
1381   a large balloon-shaped organ that stores spermatostyles and a small spherical
1382   spermatheca for sperm (Figs. 12A–C). This discovery suggests that female *Galerita*
1383   may have partially decoupled sperm evolution from spermatostyle evolution in males.
1384   Very little is known regarding sperm use by *Galerita* females, but we suspect that
1385   conjugates arrive to the balloon-shaped structure, dissociate or become dissociated
1386   from their sperm, and the sperm travel or are moved to the functional spermatheca.
1387   Near relatives of *Galerita* also possess a similar configuration of female reproductive
1388   tract structures (Hunting, 2008), and it seems likely that this pattern of sperm use
1389   applies more broadly.

1390

1391   *Are sperm conjugates greater than the sum of their parts?*

1392   Closely related ground beetles generally differ most at the level of the
1393   conjugate rather than the sperm themselves. We found variation among ground beetle
1394   sperm at multiple levels; in their sperm, spermatostyles, and how these are joined, i.e.,
1395   the sperm conjugates (Fig. 2; Table 2). Conjugate-level variation likely evolves rapidly,
1396   as indicated by the significant variation we observed at this level in our sampling. The
1397   sperm of closely related species frequently differ only at the level of the conjugate. If
1398   sperm conjugates change rapidly, you would predict to see within-species or within-
1399   male variation in sperm conjugates. We found several instances of intraspecies or
1400   intramale variation in sperm conjugate form throughout Carabidae (e.g., Fig. 5E), with
1401   males making monomorphic sperm but packaging them into sperm conjugates that
1402   overlap in size or with males making two sperm conjugate size classes (Takami and
1403   Sota, 2007). *Brachinus elongatulus* males make monomorphic sperm but package
1404   them into two morphologically distinctive conjugates (Fig. 11A). Conjugate size
1405   polymorphism in ground beetles commonly entails variation in spermatostyle size,
1406   but we also observed variation beyond conjugate size among closely related species.

1407   Ground beetle conjugates vary between species in the number of included
1408   sperm, and conjugates include anywhere from a few to thousands of sperm (Table 2).
1409   Because we often had difficulty estimating the number of included sperm in very large
1410   conjugates, our data surely underreport the variation that is present in Carabidae. The
1411   largest conjugates we observed were all conjugates with large spermatostyles. The
1412   density and arrangement of sperm along the spermatostyle determines the number of
1413   sperm cells in a given conjugate. As spermatostyles get larger, there is more space for
1414   sperm attachment, which may correlate with sperm size and number of sperm
1415   embedded in a conjugate. We generally found that larger spermatostyles included
1416   more sperm and/or longer sperm, but there are many exceptions.

1417   Variation at the conjugate-level is usually the result of sperm number and
1418   distribution patterns rather than orientation of sperm. In some closely related species
1419   the conjugates have similar numbers of included sperm but different arrangements of
1420   sperm. One such example comes from the sperm conjugates of *Dyschirius tridentatus*
1421   and *D. dejeanii*. Both species make long rod-like spermatostyles of similar length with
1422   sperm of similar length. However, in *D. tridentatus* about 35 sperm are distributed one
1423   at a time throughout the length of the spermatostyle (Figs. 9F–G) whereas in *D.*
1424   *dejeanii* sperm are all located on one end of the spermatostyle in a small cluster of
1425   about 7 sperm (Fig. 9H).

1426   A likely functional consequence of variation in number and placement of sperm
1427   in a conjugate is variation in motility, but motility alone likely does not explain the
1428   variation observed in carabid conjugates (Pitnick et al., 2009a). Evidence from muroid
1429   rodents and diving beetles indicates that sperm motility varies with conjugate form
1430   (Fisher et al., 2014; Higginson et al., 2012a). Our preliminary data from *in vitro*
1431   observation of ground beetle sperm suggests that motility differs among conjugate
1432   forms (Supporting Information MV1–MV16). Although we did not systematically
1433   investigate this topic enough to warrant firm conclusions, we suspect that sperm
1434   conjugate motility is dependent on the composition and arrangement of their sperm
1435   in a conjugate (Fisher et al., 2014) as well as interactions with the female reproductive
1436   tract epithelium (Lüpold and Pitnick, 2018). Given the wide range of variation
1437   observed in conjugate form, it seems unlikely that selection for variation in motility is
1438   the only proximate mechanism behind the morphological diversification of ground
1439   beetle sperm conjugates.

1440   Our data indicate that the ingredients used to make sperm conjugates likely
1441   evolve slower than the arrangement of sperm in a conjugate. This pattern has also
1442   been found in other animal groups with a history of sperm conjugation (Higginson
1443   and Pitnick, 2011; Higginson et al., 2012a; Immler et al., 2007). The conclusion that
1444   sperm and spermatostyles evolve slower than the joining of these two in a conjugate
1445   aligns well with research on emergent patterns (Maynard Smith and Szathmary, 1995;
1446   Michord, 2007; Parrish and Edelstein-Keshet, 1999; Turing, 1952). Evolutionary
1447   theory on emergence predicts that as a group becomes more inclusive, it will show
1448   more variation (Novikoff, 1945). The highest levels of organization in emergent

1449   patterns contain the most variation (Novikoff, 1945). Our data suggest that there are
1450   bottom-up trends in the evolution of emergent patterns where the parts (sperm and
1451   spermatostyles) drive change in the group (conjugate) as well as top-down trends
1452   where the group drives changes in the parts. Sperm conjugation evolution may follow
1453   trends common to other emergent patterns in nature like schooling in fish, flocking in
1454   birds, and colony-formation in unicellular organisms (Parrish and Edelstein-Keshet,
1455   1999). If so, the study of sperm conjugation might yield new insights into the
1456   evolution and development of complex novel traits.

1457

1458   *Concluding remarks*

1459   The variation we observed in ground beetle sperm, spermatostyles, and sperm
1460   conjugates hints at rapid evolution at the molecular level. Understanding how genetic
1461   and epigenetic networks shape phenotype is a focus in the study of organic evolution
1462   (Jablonka and Lamb, 2005) and has previously been identified as a high-priority
1463   research goal in the study of post-mating sexual selection (Birkhead and Pizzari, 2002;
1464   Lüpold and Pitnick, 2018). As genetic tools are ever improving and becoming more
1465   accessible to non-model taxa (e.g., Ellegren, 2014; Russell et al., 2017), studies that use
1466   ground beetles and their diverse sperm and genitalia to answer questions on the
1467   genetics and epigenetics of post-mating sexual selection will soon be viable.

1468   There are, of course, gaps in our dataset as we were unable to study the sperm
1469   form of carabid beetles from every major split in the tree of Carabidae. Sperm
1470   morphology of unsampled early diverging carabids such as Southern Hemisphere
1471   carabines (beetles of the genus *Pamborus* and *Ceroglossus*) and members of the tribes
1472   Migadopini and Cicindini could be particularly valuable for inferring transitions in
1473   sperm conjugate evolution. We did not study any male Psydrini, and we were also
1474   unable to study the sperm of any Moriomorphini, which are the sister-group of the
1475   large clade comprised of Brachininae and Harpalinae (Maddison et al., 2009;
1476   Maddison and Ober, 2011). The study of Broscinae sperm may shed light on the
1477   transition from sperm conjugation (with a spermatostyle) to singleton sperm (without
1478   a spermatostyle). We suspect that the study of sperm in Broscinae worldwide could
1479   yield new insights into the intersection of evolution and development of sperm,
1480   spermatostyles, and sperm conjugation. In addition, advances in ground beetles
1481   phylogenetics from genome and transcriptome sequencing stand to greatly improve
1482   the accuracy of our inferences about sperm diversification.

1483   Our survey did not principally focus on female reproductive tract form, and we
1484   acknowledge that we focused on only one side of the story (Ah-King et al., 2014). We
1485   agree with Ah-King et al., (2014) that future studies that regularly incorporate female
1486   reproductive tract data will be essential to a more holistic understanding of
1487   morphological evolution in sperm. We look forward to future research that more fully

1488   incorporates female reproductive trait data in the study of post-mating sexual
1489   selection in ground beetles.

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Table 1. Taxon and specimen sampling for sperm data.

	Tribe	Genus	Species	# male	# female	total (n)
Carabinae	Carabini	*Calosoma*	*C. peregrinator*	2	-	2
Carabinae	Carabini	*Carabus*	*C. nemoralis*	1	-	1
Carabinae	Carabini	*Carabus*	*C. taedatus*	1	-	1
Carabinae	Cychrini	*Cychrus*	*C. tuberculatus*	-	1	1
Carabinae	Cychrini	*Scaphinotus*	*S. marginatus*	1	1	2
Carabinae	Cychrini	*Sphaeroderus*	*S. schaumii*	2	-	2
Carabinae	Cychrini	*Sphaeroderus*	*S. stenostomus*	3	2	5
Elaphrinae	Elaphrini	*Elaphrus*	*E. purpuratus*	1	4	5
Elaphrinae	Elaphrini	*Blethisa*	*B. oregonensis*	2	-	2
Trachypachinae	Trachypachini	*Trachypachus*	*T. inermis*	1	-	1
Trachypachinae	Trachypachini	*Trachypachus*	*T. slevini*	-	4	4
Loricerinae	Loricerini	*Loricera*	*L. decempunctata*	1	1	2
Loricerinae	Loricerini	*Loricera*	*L. foveata*	-	2	2
Nebriinae	Nebriini	*Nebria*	*N. brevicollis*	1	-	1
Nebriinae	Notiophilini	*Notiophilus*	*N. sylvaticus*	3	-	3
Nebriinae	Opisthiini	*Opisthius*	*O. richardsoni*	1	1	2
Omophroninae	Omophronini	*Omophron*	*O. americanum*	1	-	1
Omophroninae	Omophronini	*Omophron*	*O. ovale*	4	-	4
Trechinae	Patrobini	*Patrobus*	*P. longicornis*	3	1	4
Trechinae	Patrobini	*Diplous*	*D. filicornis*	-	1	1
Trechinae	Anillini	*gen. nov.*	Anillini *gen. nov sp. nov.*	3	1	4
Trechinae	Bembidiini	*Bembidion*	*B.* sp. nr. transversale	4	-	4
Trechinae	Bembidiini	*Bembidion*	*B. incrematum*	2	1	3
Trechinae	Bembidiini	*Bembidion*	*B. iridescens*	1	1	2
Trechinae	Bembidiini	*Bembidion*	*B. kuprianovi #2*	1	-	1
Trechinae	Bembidiini	*Bembidion*	*B. obliquulum*	1	1	2
Trechinae	Bembidiini	*Bembidion*	*B. sejunctum*	1	-	1
Trechinae	Bembidiini	*Bembidion*	*B. zephyrum*	3	-	3
Trechinae	Bembidiini	*Lionepha*	*L. sp. nov.*	3	-	3
Trechinae	Pogonini	*Diplochaetus*	*D. planatus*	5	-	5
Trechinae	Tachyini	*Mioptachys*	*M. flavicauda*	3	1	4
Trechinae	Tachyini	*Tachyta*	*T. inornata*	1	1	2

Trechinae	Tachyini	*Tachyura*	*T. rapax*	-	1	1
Trechinae	Tachyini	*Paratachys*	*P.* sp. 1	1	-	1
Trechinae	Tachyini	*Paratachys*	*P.* sp. 2	2	-	2
Trechinae	Trechini	*Pachydesus*	*P.* sp.	3	1	4
Trechinae	Trechini	*Perileptus*	*P.* sp.	1	1	2
Trechinae	Trechini	*Trechodes*	*T.* sp.	-	1	1
Trechinae	Trechini	*Trechosiella*	*T. scotti*	3	-	3
Trechinae	Trechini	*Trechus*	*T. humboldti*	3	1	4
Broscinae	Broscini	*Zacotus*	*Z. matthewsii*	1	3	4
Broscinae	Broscini	*Broscodera*	*B. insignis*	1	1	2
Non-Harpalinae Carabidae *incertae sedis*	Psydrini	*Psydrus*	*P. piceus*	-	1	1
Non-Harpalinae Carabidae *incertae sedis*	Gehringiini	*Gehringia*	*G. olympica*	2	-	2
Non-Harpalinae Carabidae *incertae sedis*	Apotomini	*Apotomus*	*A.* sp.	1	3	4
Non-Harpalinae Carabidae *incertae sedis*	Promecognathini	*Promecognathus*	*P. laevissimus*	1	1	2
Non-Harpalinae Carabidae *incertae sedis*	Hiletini	*Eucamaragnathus*	*E. oxygonus*	1	2	3
Scaritinae s. l.	Clivinini	*Ardistomis*	*A. obliquata*	3	-	3
Scaritinae s. l.	Clivinini	*Ardistomis*	*A. schaumii*	5	-	5
Scaritinae s. l.	Clivinini	*Aspidoglossa*	*A. subangulata*	2	-	2
Scaritinae s. l.	Clivinini	*Clivina*	*C. fossor*	3	-	3
Scaritinae s. l.	Clivinini	*Paraclivina*	*P. bipustulata*	2	-	2
Scaritinae s. l.	Clivinini	*Schizogenius*	*S. litigiosus*	2	1	3
Scaritinae s. l.	Clivinini	*Semiardistomis*	*S. viridis*	6	-	6
Scaritinae s. l.	Dyschiriini	*Akephorus*	*A. obesus*	2	3	5
Scaritinae s. l.	Dyschiriini	*Dyschirius*	*D. dejeanii*	1	-	1
Scaritinae s. l.	Dyschiriini	*Dyschirius*	*D. globosus*	5	-	5
Scaritinae s. l.	Dyschiriini	*Dyschirius*	*D. haemorrhoidalis*	1	-	1
Scaritinae s. l.	Dyschiriini	*Dyschirius*	*D. pacificus*	1	1	2
Scaritinae s. l.	Dyschiriini	*Dyschirius*	*D. thoracicus*	4	-	4
Scaritinae s. l.	Dyschiriini	*Dyschirius*	*D. tridentatus*	1	-	1
Scaritinae s. l.	Dyschiriini	*Striganoviella*	*S. vanhillei*	5	1	6
Scaritinae s. l.	Pasimachini	*Pasimachus*	*P. californicus*	2	1	3

Scaritinae s. l.	Scaritini	*Haplotrachelus*	*H. atropsis*	1	-	1
Scaritinae s. l.	Scaritini	*Haplotrachelus*	*H. cf. latesulcatus*	2	-	2
Scaritinae s. l.	Scaritini	*Haplotrachelus*	*H.* sp.	2	-	2
Scaritinae s. l.	Scaritini	*Scarites*	*S. marinus*	2	1	3
Scaritinae s. l.	Scaritini	*Scarites*	*S. (Distichus)* sp.	1	-	1
Scaritinae s. l.	Scaritini	*Scarites*	*S. (Parallelomorphus)* sp.	2	-	2
Rhysodinae	Clinidiini	*Clinidium*	*C.* sp. nr. *guatemalenum*	1	1	2
Rhysodinae	Omoglymmiini	*Omoglymmius*	*O. hamatus*	3	-	3
Cicindelinae	Amblycheilini	*Omus*	*Omus audouini*	5	2	7
Cicindelinae	Amblycheilini	*Omus*	*Omus dejeanii*	2	1	3
Cicindelinae	Cicindelini	*Brasiella*	*B. wickhami*	1	-	1
Cicindelinae	Cicindelini	*Cicindela*	*C. haemorrhagica*	2	1	3
Cicindelinae	Megacephalini	*Tetracha*	*T. carolina*	2	1	3
Paussinae	Metriini	*Metrius*	*M. contractus*	2	1	3
Paussinae	Ozaenini	*Goniotropis*	*G. parca*	3	-	3
Paussinae	Ozaenini	*Ozaena*	*O.* sp.	-	1	1
Paussinae	Ozaenini	*Pachyteles*	*P.* sp.	-	1	1
Paussinae	Paussini	*Cerapterus*	*C.* sp.	1	-	1
Paussinae	Paussini	*Paussus*	*P. cucullatus*	-	2	2
Paussinae	Paussini	*Paussus*	*P. (Bathypaussus)* sp.	1	-	1
Brachininae	Brachinini	*Brachinus*	*B. elongatulus*	4	3	7
Brachininae	Brachinini	*Brachinus*	*B. ichabodopsis*	1	-	1
Brachininae	Brachinini	*Mastax*	*M.* sp.	3	1	4
Brachininae	Brachinini	*Pheropsophus*	*P.* sp. 1	4	1	5
Brachininae	Brachinini	*Pheropsophus*	*P.* sp. 2	1	-	1
Harpalinae	Abacetini	*Abacetus*	*A.* sp.	3	1	4
Harpalinae	Abacetini	*Stolonis*	*S. intercepta*	1	1	2
Harpalinae	Abacetini	*Stolonis*	*S.* sp.	1	-	1
Harpalinae	Anthiini	*Anthia*	*Anthia (Termophilum)* sp.	1	-	1
Harpalinae	Anthiini	*Cycloloba*	*C.* sp.	1	-	1
Harpalinae	Catapiesini	*Catapiesis*	*C.* sp.	-	1	1
Harpalinae	Chlaeniini	*Chlaenius*	*C. cumatilis*	2	-	2
Harpalinae	Chlaeniini	*Chlaenius*	*C. glaucus*	1	-	1
Harpalinae	Chlaeniini	*Chlaenius*	*C. harpalinus*	1	1	2

Harpalinae	Chlaeniini	*Chlaenius*	*C. leucoscelis*	2	-	2
Harpalinae	Chlaeniini	*Chlaenius*	*C. prasinus*	1	-	1
Harpalinae	Chlaeniini	*Chlaenius*	*C. ruficauda*	2	-	2
Harpalinae	Chlaeniini	*Chlaenius*	*C. sericeus*	1	-	1
Harpalinae	Chlaeniini	*Chlaenius*	*C. tricolor*	1	-	1
Harpalinae	Ctenodactylini	*Leptotrachelus*	*L.* sp.	1	-	1
Harpalinae	Cyclosomini	*Tetragonoderus*	*T. fasciatus*	3	2	5
Harpalinae	Cyclosomini	*Tetragonoderus*	*T.* sp. nr. *latipennis*	1	-	1
Harpalinae	Dryptini	*Drypta*	*D.* sp.	2	1	3
Harpalinae	Galeritini	*Galerita*	*G. atripes*	-	1	1
Harpalinae	Galeritini	*Galerita*	*G. bicolor*	-	1	1
Harpalinae	Galeritini	*Galerita*	*G. forreri*	1	-	1
Harpalinae	Galeritini	*Galerita*	*G. lecontei*	2	-	2
Harpalinae	Graphipterini	*Graphipterus*	*G.* sp.	1	-	1
Harpalinae	Harpalini	*Anisodactylus*	*A. alternans*	1	2	3
Harpalinae	Harpalini	*Anisodactylus*	*A. anthracinus*	2	-	2
Harpalinae	Harpalini	*Anisodactylus*	*A. similis*	1	-	1
Harpalinae	Harpalini	*Bradycellus*	*B.* sp. 1	1	-	1
Harpalinae	Harpalini	*Bradycellus*	*B.* sp. 2	1	-	1
Harpalinae	Harpalini	*Discoderus*	*D.* sp.	1	-	1
Harpalinae	Harpalini	*Euryderus*	*E. grossus*	2	-	2
Harpalinae	Harpalini	*Harpalus*	*H. affinis*	2	2	4
Harpalinae	Harpalini	*Polpochila*	*P. erro*	2	-	2
Harpalinae	Harpalini	*Selenophorus*	*S.* sp.	-	1	1
Harpalinae	Harpalini	*Stenolophus*	*S.* sp.	1	-	1
Harpalinae	Harpalini	*Stenomorphus*	*S. convexior*	3	-	3
Harpalinae	Helluonini	*Helluomorphoides*	*H. papago*	1	1	2
Harpalinae	Helluonini	*Macrocheilus*	*M.* sp.	1	-	1
Harpalinae	Lachnophorini	*Ega*	*E. sallei*	1	-	1
Harpalinae	Lachnophorini	*Lachnophorus*	*L. elegantulus*	2	-	2
Harpalinae	Lachnophorini	*Lachnophorus*	*L.* sp. nr. *elegantulus*	1	-	1
Harpalinae	Lebiini	*Agra*	*A.* sp. 1	1	-	1
Harpalinae	Lebiini	*Agra*	*A.* sp. 2	-	1	1
Harpalinae	Lebiini	*Apenes*	*A. lucidula*	1	-	1
Harpalinae	Lebiini	*Calleida*	*C. bella*	1	-	1
Harpalinae	Lebiini	*Calleida*	*C. decora*	-	1	1
Harpalinae	Lebiini	*Calleida*	*C. jansoni*	2	-	2
Harpalinae	Lebiini	*Cymindis*	*C. punctifera*	1	-	1
Harpalinae	Lebiini	*Cymindis*	*C. punctigera*	1	-	1
Harpalinae	Lebiini	*Cymindis*	*C. basipunctata*-group sp.	1	-	1
Harpalinae	Lebiini	*Lebia*	*L. deceptrix*	1	1	2
Harpalinae	Lebiini	*Lebia*	*L. subgrandis*	1	-	1

Harpalinae	Lebiini	*Lebia*	*L. viridis*	1	1	2
Harpalinae	Lebiini	*Phloeoxena*	*P. nigricollis*	1	-	1
Harpalinae	Lebiini	*Stenognathus*	*S. quadricollis*	-	1	1
Harpalinae	Lebiini	*Syntomus*	*S. americanus*	1	1	2
Harpalinae	Lebiini	*Thyreopterus*	*T. flavosignatus*	1	-	1
Harpalinae	Licinini	*Badister*	*B. ferrugineus*	1	-	1
Harpalinae	Licinini	*Dicaelus*	*D. suffusus*	1	2	3
Harpalinae	Licinini	*Diplocheila*	*D. nupera*	1	-	1
Harpalinae	Morionini	*Morion*	*M.* sp.	1	-	1
Harpalinae	Odacanthini	*Colliuris*	*C. pensylvanica*	3	1	4
Harpalinae	Oodini	*Anatrichis*	*A. minuta*	1	-	1
Harpalinae	Oodini	*Oodes*	*O. fluvialis*	8	-	8
Harpalinae	Oodini	*Stenocrepis*	*S. elegans*	1	-	1
Harpalinae	Panagaeini	*Panagaeus*	*P. sallei*	3	-	3
Harpalinae	Peleciini	*Disphaericus*	*D.* sp.	1	1	2
Harpalinae	Pentagonicini	*Pentagonica*	*P.* sp.	2	-	2
Harpalinae	Perigonini	*Perigona*	*P. nigriceps*	1	-	1
Harpalinae	Platynini	*Agonum*	*A. piceolum*	4	1	5
Harpalinae	Platynini	*Agonum*	*A. muelleri*	2	-	2
Harpalinae	Platynini	*Rhadine*	*R. dissecta*-group sp.	1	-	1
Harpalinae	Platynini	*Sericoda*	*S. bembidioides*	1	-	1
Harpalinae	Pseudomorphini	*Pseudomorpha*	*P.* sp.	-	1	1
Harpalinae	Pterostichini	*Abaris*	*A. splendida*	2	1	3
Harpalinae	Pterostichini	*Cyrtomoscelis*	*C.* cf. *dwesana*	3	-	3
Harpalinae	Pterostichini	*Cyclotrachelus*	*C. dejeanellus*	2	1	3
Harpalinae	Pterostichini	*Hybothecus*	*H. flohri*	-	1	1
Harpalinae	Pterostichini	*Poecilus*	*P. laetulus*	1	1	2
Harpalinae	Pterostichini	*Poecilus*	*P. scitulus*	-	1	1
Harpalinae	Pterostichini	*Pterostichus*	*P. infernalis*	2	3	5
Harpalinae	Pterostichini	*Pterostichus*	*P. lama*	1	-	1
Harpalinae	Pterostichini	*Pterostichus*	*P. melanarius*	-	1	1
Harpalinae	Sphodrini	*Calathus*	*C. peropacus*	2	1	3
Harpalinae	Sphodrini	*Synuchus*	*S. dubius*	3	2	5
Harpalinae	Zabrini	*Amara*	*A. aenea*	6	2	8
Harpalinae	Zabrini	*Amara*	*A. farcta*	-	1	1
Harpalinae	Zuphiini	*Pseudaptinus*	*P. horni*	-	1	1
Harpalinae	Zuphiini	*Pseudaptinus*	*P. simplex*	-	1	1
Harpalinae	Zuphiini	*Pseudaptinus*	*P. tenuicollis*	1	-	1

Table 2. Summarized sperm morphological data for 177 species of ground beetles. In order to limit column width, the term rod is used in place of spermatostyle. All measurements are reported in microns (μm) excluding the last column, which lists the average number of sperm found in a conjugate.

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Abacetus sp.	Sheet	4	590	1.5	0.3	2300	45	490	200
Abaris splendidula	Sheet	3	170	1.5	0.4	190	7.9	12	24
Agonum muelleri	Simple rod	2	240	1.2	0.6	220	10	6.2	51
Agonum piceolum	Simple rod	5	260			160	8.1	16	66
Agra sp. 1		1	74						
Agra sp. 2	Simple rod	1	140			90	5.2	19	
Akephorus obesus	Simple rod	5	860	2.9	6.3	53	6.9	1.0	7.2
Amara aenea	Simple rod	8	940	1.4	0.4	1600	7.8	3.3	190
Amara farcta		1	540	2.4	0.5				
Anatrichis minuta		1	720	4.3	0.5				
Anillini *gen. nov. sp. nov.*	Singleton	4	290						
Anisodactylus alternans	Simple rod	3	410	2.2	0.4	3700	21	60	120
Anisodactylus anthracinus	Simple rod	2	730	2.3	0.4	3900	17	94	880
Anisodactylus similis	Simple rod	1	380			3300	19		120
Anthia (Termophilum) sp.		1	1200			5800	13		
Apenes lucidula	Simple rod	1	200	2.3	0.5	470	4.6	12	31
Apotomus sp.	Singleton	4	2700						
Ardistomis obliquata	Simple rod	3	990			1400	21		220
Ardistomis schaumii	Simple rod	5	660			840	11	72	77
Aspidoglossa subangulata	Simple rod	2	790			6600	29	190	160
Badister ferrugineus	Simple rod	1	660	3.5	0.3	3800	19		

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Bembidion incrematum	Singleton	3	300						
Bembidion iridescens	Aggregate	2	290						
Bembidion kuprianovi #2	Singleton	1	520						
Bembidion obliquulum	Singleton	2	860						
Bembidion sejunctum	Aggregate	1	760						6.8
Bembidion sp.nr. *transversale*	Singleton/ Aggregate	4	710						6.9
Bembidion zephyrum	Singleton	3	420						
Blethisa oregonensis	Simple rod	2	51	11	0.8	260	11	9	220
Brachinus elongatulus	Simple rod	5	130	0.8	0.3	17 / 350	5.2 / 5.1	9.8 / ?	51 / ?
Brachinus ichabodopsis	Simple rod	1	120			640			
Bradycellus sp. 1	Simple rod	1	170	0.9	0.4	4900	11		900
Bradycellus sp. 2	Simple rod	1	130	1.2	0.4	2200	8.9	72	2100
Brasiella wickhami	Singleton	1	110						
Broscodera insignis	Singleton with rod	2	1500	73	0.6	92	1.8	11	
Calathus peropacus	Simple rod	3	210	2.4	0.5	3800	24	130	2900
Calleida bella	Simple rod	1	190			77	4.1	1.9	
Calleida decora	Sheet	1	160	2.1	0.4				
Calleida jansoni	Sheet	2	220			230	8.8		
Calosoma peregrinator	Simple rod	2	78	18	0.8	44	11	2.9	48
Carabus nemoralis	Simple rod	1	72	11	0.6	46	17	26	47
Carabus taedatus	Simple rod	1	81	11	0.7	51	14	4.7	120

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Catapiesis sp.		1	280	1.0	0.3				
Cerapterus sp.	Singleton	1	390	26	0.8				
Chlaenius cumatilis	Simple rod	2	480	1.7	0.4	660	6.3	15	77
Chlaenius glaucus	Simple rod	1	1200	2.9	0.4	910	8.5	82	
Chlaenius harpalinus		2	510	3.7	0.5	490	5.3		
Chlaenius leucoscelis	Simple rod	2	340	3.0	0.5	49	13	4.3	81
Chlaenius prasinus	Simple rod	1	360	3.0	0.4	17	8.6	3.2	56
Chlaenius ruficauda	Sheet	2	1000	6.7	0.4	5000	8.1	79	
Chlaenius sericeus	Simple rod	1	330	1.4	0.5	450	5.1	22	130
Chlaenius tricolor	Simple rod	1	250	3.5	0.7	250	3.9	13	
Cicindela haemorrhagica	Singleton	3	97						
Clinidium sp. nr. *guatemalenum*	Simple rod	2	270	16	0.5				
Clivina fossor	Sheet	3	91			3100	37	2600	370
Colliuris pensylvanica	Simple rod	3	330	2.1	0.4	840	18	8.1	340
Cychrus tuberculatus	Simple rod	1	50	9.1	0.7				
Cycloloba sp.		1	260			4400	15		
Cyclotrachelus dejeanellus	Sheet	3	480	4.3	0.4	6200	10	130	
Cymindis basipunctata-group sp.	Sheet	1	240			800	3.5		
Cymindis punctifera	Simple rod	1	240	1.6	0.3	1100	16	6.1	85
Cymindis punctigera	Simple rod	1	210	1.6	0.3	550	10	59	130
Cyrtomoscelis cf. dwesana	Sheet	3	700				20		
Dicaelus suffusus	Simple rod	3	490						
Diplochaetus planatus	Singleton	5	2200	46	0.4				

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Diplocheila nupera	Simple rod	1	570			13000	62		
Diplous filicornis	Simple rod	1	73	1.8	0.6				
Discoderus sp.	Sheet	1	300	1.8	0.4	860	6.9	3.8	34
Disphaericus sp.	Simple rod	2	790			600	11		54
Drypta sp.		3	180						
Dyschirius dejeanii	Simple rod	1	790	3.4	1.3	1000	4.6	7.9	7.3
Dyschirius globosus	Simple rod	6	320	15	0.5		3.5	21	20
Dyschirius haemorrhoidalis	Simple rod	1	550	2.0	1.9	280	4	13	7.7
Dyschirius pacificus	Simple rod	2	990	3.5	1.1				7.2
Dyschirius thoracicus	Simple rod	4	360	4	4.3	40	7	3	7.5
Dyschirius tridentatus	Simple rod	1	820	4.0	4.5	1000	8.1	34	35
Ega sallei		1	1100						
Elaphrus purpurans	Simple rod	5	73	13	0.9	83	4.9	3.5	54
Eucamaragnathus oxygonus	Singleton	3	62	8.5	1.7				
Euryderus grossus	Simple rod	2	630	2.1	0.4	2900	13	84	1100
Galerita atripes		1	260						
Galerita bicolor		1	300						
Galerita forreri	Sheet	1	340			4800	41	76	
Galerita lecontei	Sheet	2	280			3500	26	56	
Gehringia olympica	Singleton	2	500						
Goniotropis parca	Simple rod	3	48	12	0.6	100	9.2	5.3	94
Graphipterus sp.	Simple rod	1	960	0.5	0.4	2200	20		
Haplotrachelus atropsis	Simple rod	1	470	0.6	0.2	120	13		96

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Haplotrachelus cf. *latesulcatus*	Simple rod	2	420	0.8	0.3	180	18	23	130
Haplotrachelus sp.	Simple rod	2	410	0.9	0.3	140	16	11	75
Harpalus affinis	Simple rod	4	730			1800	12	510	
Helluomorphoides papago		2	450						
Hybothecus flohri		1	360	2.0	0.4				
Lachnophorus elegantulus	Simple rod	2	290			710	22	25	130
Lachnophorus sp.	Sheet	1	290			610	13		
Lebia deceptrix	Sheet	2	340	1.2	0.3	430	5.2	4.5	
Lebia subgrandis	Sheet	1	370	2.0	0.3	520	6.5	3.2	
Lebia viridis	Simple rod	2	340	0.8	0.4	800	14	31	
Leptotrachelus sp.	Sheet	1	240	2.1	0.4	1100	63	120	
Lionepha sp. nov.	Aggregate	3	400						6.7
Loricera decempunctata	Simple rod	3	130	12	0.9	1400	5.5	8.7	1500
Loricera foveata	Simple rod	2	120	13	0.9				
Macrocheilus sp.		1	510			1400	8.0		
Mastax sp.	Simple rod	4	170	0.8	0.3	20	2.7	3.5	21
Metrius contractus	Simple rod	3	150	7.8	1.0	84	7.2	5.0	220
Mioptachys flavicauda	Singleton	4	380	130	0.6				
Morion sp.		1	140						
Nebria brevicollis	Singleton	1	1400	26	0.6				
Notiophilus sylvaticus	Singleton	3	2000	43	0.5				
Omoglymmius hamatus	Simple rod	3	290	44	0.6	69	9.1	6.2	66
Omophron americanum	Simple rod	1	750	4.1	2.3	870	6.2	700	130

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Omophron ovale	Simple rod	4	280	6.5	2.9	140	6.4	74	40
Omus audouini	Singleton	7	120						
Omus dejeanii	Singleton	3	130						
Oodes fluvialis	Simple rod	8	880	1.3	0.4	520	8.3		
Opisthius richardsoni	Singleton	2	500	13	0.9				
Ozaena sp.		1	86						
Pachydesus sp.	Singleton	4	250	1.3	0.4				
Pachyteles sp.		1	130						
Panagaeus sallei	Simple rod	3	1400	6.7	0.5	1100	20	430	610
Paraclivina bipustulata	Sheet	2	540			1900	64	1100	370
Paratachys sp. 1	Singleton	1	290						
Paratachys sp. 2	Singleton	2	450	29	0.5				
Pasimachus californicus	Simple rod	3	390	0.8	0.3	86	16	24	66
Patrobus longicornis	Simple rod	4	76	1.3	0.3	74	4.8	0.6	23
Paussus (Bathypaussus) sp.	Singleton	1	420	22	0.5				
Paussus cucullatus	Singleton	2	370	31	0.7				
Pentagonica sp.	Simple rod	2	260	1.1	0.3	250	5.4	41	26
Perigona nigriceps	Simple rod	1	150			88	3.7	14	33
Perileptus sp.	Singleton	2	200	0.9	0.4				
Pheropsophus sp. 1	Simple rod	5	130	1.0	0.3	41000	7.8	8.5	
Pheropsophus sp. 2	Simple rod	1	120				5.2		
Phloeoxena nigricollis	Simple rod	1	710			21	3.1	12	
Poecilus laetulus	Simple rod	2	420	3.9	0.5	6100	140		

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Poecilus scitulus	Simple rod	1	420	2.2	0.4				
Polpochila erro	Simple rod	2	320			810	34	100	
Promecognathus laevissimus	Simple rod	2	290	21	0.7	2000	35	58	420
Pseudaptinus horni		1	390						
Pseudaptinus simplex		1	450						
Pseudaptinus tenuicollis		1	400			1600	5.7		
Pseudomorpha sp.		1	200						
Psydrus piceus		1	260	13	0.8				
Pterostichus infernalis	Sheet	5	330	2.6	0.4	2100	11	100	87
Pterostichus lama	Sheet	1	340	2.2	0.4	9600	27	110	
Pterostichus melanarius	Sheet	1							
Rhadine dissecta-group sp.	Simple rod	1	350	1.7	0.6	450	12	4.2	280
Scaphinotus marginatus	Simple rod	2	54	11	0.7	620	14	2.3	1500
Scarites (Distichus) sp.	Simple rod	1	260	1.0	0.4	160	3.9		19
Scarites (Parallelomorphus) sp.	Simple rod	2	330			40	7.1		64
Scarites marinus	Simple rod	3	200			18	3.2	10	17
Schizogenius litigiosus	Sheet	3	1000			960	4.3	44	7.2
Selenophorus sp.		1	260	2.3	0.4				
Semiardistomis viridis	Simple rod	6	540			2200	3.1	150	42
Sericoda bembidioides	Simple rod	1	250						
Sphaeroderus schaumii	Simple rod	2	61	15	0.7	1400	6.4	2.7	1100
Sphaeroderus stenostomus	Simple rod	5	57	16	0.6	1700	8.2	3.4	1500
Stenocrepis elegans	Simple rod	1	400			1900	31		1000

Taxon	Conjugate type	Specimens examined (n)	Sperm length (avg)	Head length (avg)	Head width (avg)	Rod length (avg)	Rod width (avg)	Rod bare apex length (avg)	Sperm in conjugate (avg)
Stenognathus quadricollis		1	550						
Stenolophus sp.	Sheet	1	500	1.4	0.3	5300	25	60	
Stenomorphus convexior	Sheet	3	490	3.8	0.4	760	6.5	4.4	
Stolonis intercepta	Simple rod	2	520	12	0.5	2000	21	260	1100
Stolonis sp.	Simple rod	1	370			590	21		
Striganoviella vanhillei	Simple rod	6	410	2.0	1.9	220	4.0	21	8.3
Syntomus americanus	Sheet	2	190	2.1	0.3	340	8.5	35	55
Synuchus dubius	Simple rod	5	340	4.4	0.4	520	5.7	60	94
Tachyta inornata	Mechanical	2	3400	270	0.5				
Tachyura rapax	Mechanical	1	2200	82	0.8				
Tetracha carolina	Singleton	3	137						
Tetragonoderus fasciatus	Sheet	5	250	1.2	0.4	2200	47	450	310
Tetragonoderus sp. nr. *latipennis*	Sheet	1							
Thyreopterus flavosignatus	Sheet	1	250	1.5	0.3	330	25	12	270
Trachypachus inermis	Simple rod	1	530	8.5	1.5	87	9.4	12	12
Trachypachus slevini	Simple rod	4	840	12	1.9				
Trechodes sp.	Singleton	1	100						
Trechosiella scotti	Singleton	3	96	0.7	0.3				
Trechus humboldti	Singleton	4	110	1.3	0.3				
Zacotus matthewsii	Singleton with rod	4	550	18	0.7	17	4.3		

Figure Captions

Figure 1. Many carabid beetles make sperm conjugates by pairing their sperm to a non-cellular structure or spermatostyle. (A) illustration of a *Scaphinotus marginatus* sperm conjugate (B, C). (B) DIC light microscope image of same. (C) fluorescence microscope image of DAPI-stained sperm heads. (D) TEM of a cross section through a *S. marginatus* sperm conjugate. (E) closeup of D showing details of indiviual sperm. ax = axoneme, mt = mitochondrial derivatives, sty = spermatostyle, sz = spermatozoa, szN = sperm nuclei. Scale bars: 1 μm (E), 5 μm (D).

Figure 2. Variation in studied sperm quantitative traits across major taxonomic groupings of ground beetles (on logarithmic scale; lengths in μm). Colored boxes beside taxon names refer to qualitative conjugate types (see text and Fig. 3). (A) sperm length. (B) sperm head length. (C) sperm head width. (D) spermatostyle length. (E) spermatostyle width. (F) number of sperm included in a conjugate. Note that there are fewer rows in plots D–F because some ground beetles do not make a spermatostyle and/or they lack sperm conjugation. In order to avoid negative transformed values for small-headed sperm in plots B and C, we adjusted all values by 1.2μm prior to log transformation.

Figure 3. A genus-level phylogenetic visualization of ground beetle sperm data. Colored boxes refer to different qualitative types of sperm conjugation, which are described in the text (see methods) and illustrated below the tree. The tree is not derived from any one particular phylogenetic analysis but is meant to summarize

current understanding of ground beetle phylogeny (see methods). Sperm length and spermatostyle length are illustrated in μm on a logarithmic scale to the right of the tree. Grey circles in place of gray bars indicate that a spermatostyle was observed but was not measured. When reporting sperm length and spermatostyle length, we chose one species per genus. In cases where we studied more than one species per genus, we chose one species arbitrarily. The asterisk beside *Scarites* refers to the fact that one species in the genus makes two distinct sperm forms, one of which is singleton and another that is involved in conjugation (Sasakawa 2009). Branches colored black in the tree are supported by molecular phylogenetic studies. Branches colored gray refer to low-resolution placements of taxa, which have not been previously sampled or whose placement is contentious

Figure 4. A genus-level phylogenetic visualization of ground beetle sperm data among higher-grade Carabidae (subfamilies Brachininae and Harpalinae). See Fig. 3 caption for more details.

Figure 5. Sperm and sperm conjugate morphological variation in Carabinae (A–E), Elaphrinae (F), and Trachypachinae ground beetles (G–H). (A–C) rod conjugates of *Carabus nemoralis*. (D) rod conjugates of *Sphaeroderus stenostomus*, note conjugate size polymorphism. (E) histograms of sperm conjugate size variation in four Carabinae species. (F) rod conjugate of *Elaphrus purpurans*. (G) composite image of *Trachypachus inermis* sperm heads (lower inset) and sperm conjugates. (H) *Trachypachus slevini* female reproductive tract with stored sperm and several sperm

conjugates with added thin white line to help visualize sperm storage organ and its adjoining duct. (A, F, G) stacked image of DIC and Fluoresecence microscopy images. (B, D, upper inset of G) DIC microscopy. (C, lower inset of G, H) Fluorescence images with only DAPI-stained structures visible. cj = conjugate, sd = spermathecal duct, sty = spermatostyle, sp = spermatheca, sz = spermatozoa, szN = sperm nuclei. Scale bars: 10 μm (G lower inset), 20 μm (B–C, F–H excluding lower inset of G), 100 μm (A, D).

Figure 6. Sperm and sperm conjugate morphological variation in Loricerinae (A, E), Nebriinae (B–D), and Omophroninae (F–J) ground beetles. (A) large rod conjugates of *Loricera decempunctata* include approximately 1500 sperm. (B) slender and elongate sperm heads of *Notiophilus sylvaticus*. (C) sperm heads of *Opisthius richardsoni*. (D) singleton sperm of *Opisthius richardsoni*. (E) close-up of A. (F–I) complex rod conjugates of *Omophron*. (F) *Omophron ovale* rod conjugate, note the posterior placement of sperm in spermatostyle. (G) composite image of *O. ovale* sperm head and sperm conjugate, note the asymmetry of sperm heads and the stacking of heads. (H) *Omophron americanum* rod conjugate, note the prominent bare region of the spermatostyle anteriorly. (I) *O. americanum* sperm and sperm conjugate. (J) *O. americanum* spermatozoon, note the asymmetrical attachment of the flagellum. (A–C, G, I) Fluorescence images with only DAPI-stained structures visible. (D) stacked image of Darkfield and Fluoresecence microscopy images. (E–F, J) stacked image of DIC and Fluoresecence microscopy images. (H) DIC microscopy. sty = spermatostyle, sz = spermatozoa, szN = sperm nuclei. Scale bars: 5 μm (G inset), 20 μm (B–D, F–J excluding inset of G), 50 μm (E), 100 μm (A).

Figure 7. Sperm and sperm conjugate morphological variation in Trechinae ground beetles. (A) rod conjugates of *Patrobus longicornis*. (B) rod conjugate of *Diplous filicornis* recovered from the spermatheca of a female specimen. (C) *Trechus humboldti* sperm showing two regions of fluorescence corresponding to the minute nucleus and large mitochondrial derivatives. (D) aggregate conjugate with 9 sperm in an undescribed species of *Lionepha*. (E) aggregate conjugate of *Bembidion* sp. nr. *transversale*, note the lack of a spermatostyle. (F) elongate sperm heads of *Diplochaetus planatus*. (G–H) elongate sperm heads of *Tachyta inornata*, note the zig-zag shape, the extensive pre-nuclear area, and the interaction between two spermatozoa. (I) *Tachyta inornata* appear to form mechanical conjugates by forming hairpin loops with their flagella and grappling with adjacent sperm. (J) *Tachyta inornata* sperm recovered from a female spermatheca forming characteristic loops as they swim (Supporting Information MV12–MV14 of live *T. inornata* sperm). (A, D–E, H, I) DIC microscopy. (B–C, F–G) Fluorescence images with only DAPI-stained structures visible. (J) Brightfield microscopy. loop = flagellar loops, mt = mitochondrial derivatives, sd = spermathecal duct, sp = spermatheca, sty = spermatostyle, sz = spermatozoa, szh = sperm head, szN = sperm nuclei. Scale bars: 20 µm.

Figure 8. Sperm and sperm conjugate morphological variation in Broscinae (A–C) and ground beetles that we considered non-Harpalinae Carabidae of uncertain position (D–H). (A–B) *Broscodera insignis* sperm are singleton but are individually joined to a spermatostyle. (C) Composite image of *Zacotus matthewsii* sperm. Singleton sperm

appear broad-headed but are filamentous and are paired with a short and broad spermatostyle (see inset). (D–E) Rod sperm conjugate of *Promecognathus laevissimus* with its large corkscrew-shaped spermatostyle. The apex of the spermatostyle is frequently variable, and in *P. laevissimus* the apex is spoon-shaped (D). (F) *Promecognathus laevissimus* spermatozoon. (G) *Psydrus piceus* sperm head. (H) *Eucamaragnathus oxygonus* sperm head. (A) Brightfield microscopy. (B, D) DIC microscopy. (C, F) stacked image of DIC and Fluoresecence microscopy images. (inset of C, E, G, H) Fluorescence images with only DAPI-stained structures visible. apex = apex of spermatostyle, cj = conjugate, sty = spermatostyle, sz = spermatozoa, szN = sperm nuclei. Scale bars: 5 µm (G, H), 20 µm (A–C, E–F), 100 µm (D).

Figure 9. Sperm and sperm conjugate morphological variation in Clivinini (A–D) and Dyschiriini (E–N) (Scaritinae *partim*) ground beetles. (A) sheet conjugate of *Clivina fossor*. The spermatostyle of *C. fossor* contains a central cavity where sperm are housed. (B) large sheet conjugate of *Aspidoglossa subangulata*, note the asymmetrical attachment of sperm. (C) rod conjugate of *Ardistomis obliquata*. (D) sheet conjugate of *Schizogenius litigiosus*, note the wrapping of sperm around the spermatostyle. (E) rod conjugate of *Akephorus marinus*, note the broad and triangular sperm heads. (F–G) rod conjugate of *Dyschirius tridentatus*, note the regular distribution of sperm in the spermatostyle (G). (H) rod conjugate of *Dyschirius dejeanii*. Although the spermatostyle and sperm of *D. tridentatus* and *D. dejeanii* are similar, the arrangement of their sperm is very different. (I–N) *Dyschirius* sperm heads: (I) *D. dejeanii*, (J) *D. pacificus*, (K) *D. globosus*, (L) *D. haemorrhoidalis*, (M) D. *thoracicus*, (N) *D. tridentatus*.

(A, E, F, H) stacked image of DIC and Fluoresecence microscopy images. (B–D) DIC microscopy. (G, I–N) Fluorescence images with only DAPI-stained structures visible. sty = spermatostyle, sz = spermatozoa, szN = sperm nuclei. Scale bars: 5 μm (I–N), 20 μm (B–H), 100 μm (A).

Figure 10. Sperm and sperm conjugate morphological variation in ground beetles of the subfamilies Scaritinae (excluding Clivinini and Dyschiriini) (A–D), Rhysodinae (E), Cicindelinae (F–G), and Paussinae (H–K). (A–C) rod conjugates of *Pasimachus californicus* with small weakly fluorescent sperm heads (B–C) and large intensely fluorescent mitochondrial derivates (B). (D) rod conjugate of *Scarites marinus*, note the conspicuous mitochondrial derivatives. (E) rod conjugates of *Omoglymmius hamatus* include sperm with only one obvious region of fluorescence following DAPI staining. (F–G) singleton sperm of *Brasiella wickhami*, note the small gap in fluorescence between the suspected nucleus and mitochondrial derivatives (G). (H) Composite image of *Metrius contractus* rod conjugate and slightly broad sperm head (inset). (I) conjugate size polymorphism in *Metrius contractus*. (J) closeup of rod conjugate of *Goniotropis parca*, note the linear arrangement of slender-headed sperm. (K) singleton sperm of an unidentified species of *Cerapterus*. (A) DIC microscopy. (D–E, H) stacked image of DIC and Fluoresecence microscopy images. (F, K) stacked image of Darkfield and Fluoresecence microscopy images. (B–C, G, inset of H, I–J) Fluorescence images with only DAPI-stained structures visible. cj = conjugate, mt = mitochondrial derivatives, sty = spermatostyle, sz = spermatozoa, szN = sperm nuclei. Scale bars: 5 μm (inset of H, J), 20 μm (A–G, I, K).

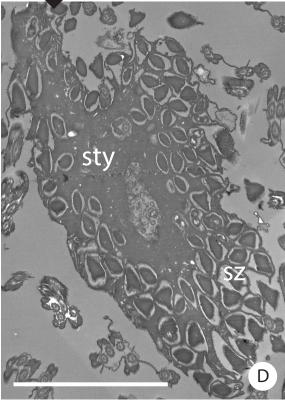**Figure 11**. Sperm and sperm conjugate morphological variation in Brachininae (A–D) and Harpalinae (E–K) ground beetles. (A) *Brachinus elongatulus* males have monomorphic sperm but package them into two distinct rod conjugates. (B) closeup of small rod conjugate of *Brachinus elongatulus* showing the small weakly fluorescent sperm nuclei and the large intensely fluoresecent mitochondrial derivatives. (C–D) giant sperm conjugates of *Pheropsophus*, which reach up to 5.8 cm. (E) rod conjugate of *Agonum piceolum*, (F) sheet conjugate in an unidentified species of *Leptotrachelus*. (G) slinky-like sheet conjugate of *Chlaenius ruficauda*. (H) rod conjugate in an unidentified speices of *Bradycellus*. (I) rod conjugate of *Stenocrepis elegans*, note the thin, ribbon-like spermatostyle. (J) feather-like sheet conjugate of *Calleida jansoni*. (K) sheet conjugate of *Tetragonoderus fasciatus*, note the wavy spermatostyle and the bilateral attachment of sperm. (A, D, E, G, I–J) DIC microscopy. (B, F, H, K) Fluorescence images with only DAPI-stained structures visible. (C) Brightfield microscopy. cj = conjugate, mt = mitochondrial derivatives, sty = spermatostyle, sz = spermatozoa, szN = sperm nuclei. Scale bars: 5 μm (inset of H), 20 μm (A–B, D–G, I–K), 100 μm (C, H).

Figure 12. Sperm + female reproductive tract interactions observed in ground beetles. (A–C) *Galerita* sperm + female interactions. (A) *Galerita bicolor* female reproductive tracts include two sperm storage organs that store different parts of a male's conjugate, redrawn from Liebherr and Will (1998). (B) A large mass of bare spermatostyles recovered from the large balloon-like spermatheca of *Galerita atripes*. (C) A large bolus of sperm recovered from the smaller spherical sperm storage organ

(= secondary spermathecal gland of Liebherr and Will (1998)) of *Galerita bicolor*. (D) closeup of the spermatostyle of *Galerita forreri*, note the presence of a groove where we suspect sperm are attached. (E–J) sperm conjugates before and after storage in female reproductive tracts, note the dissociation of sperm from spermatostyles and morphological changes to spermatostyles. (E, G, I) sperm from male preparations. (F, H, J) sperm from female spermathecae. (E–F) *Harpalus affinis*. (G–H) *Elaphrus purpurans*. (I–J) *Sphaeroderus stenostomus*. bc = bursa copulatrix, cj = conjugate, co = common oviduct, grv = groove, sg = spermathecal gland, sp1 = spermatheca 1, sp2 = spermatheca2, sty = spermatostyle, sz = spermatozoa, szN = sperm nuclei. Scale bars: 20 μm (D, G–J), 100 μm (B–C, E–F).

Fig. 1

Fig. 2

A — sperm length [μm]

B — sperm head length [μm]

C — sperm head width [μm]

D — spermatostyle length [μm]

E — spermatostyle width [μm]

F — sperm per conjugate (n)

Carabinae
Elaphrinae
Trachypachinae
Loricerinae
Nebriinae
Omophroninae
Trechinae: Patrobini
Trechinae: Trechitae
Broscinae
Carabidae *inc. sed.*
Clivinini + Dyschiriini
Scaritinae partim.
Rhysodinae
Cicindelinae
Paussinae
Brachininae
Harpalinae

Fig. 3

Scaphinotus
Sphaeroderus
Carabus
Calosoma — Carabinae
Blethisa
Elaphrus — Elaphrinae
Trachypachus — Trachypachinae
Loricera — Loricerinae
Nebria
Opisthius
Notiophilus — Nebriinae
Omophron — Omophroninae
Patrobus
Diplous — Trechinae: Patrobini
Trechodes
Trechosiella
Pachydesus
Perileptus
Trechus
anilline
Bembidion
Lionepha
Diplochaetus
Mioptachys
Tachyta
Tachyura
Paratachys — Trechinae: Trechitae
Zacotus
Broscodera — Broscinae
Psydrus
Gehringia
Eucamaragnathus
Apotomus
Promecognathus — Non-Harpalinae Carabidae *incertae sedis*
Akephorus
Dyschirius
Ardistomis
Semiardistomis
Aspidoglossa
Schizogenius
Clivina
Paraclivina — Clivinini + Dyschiriini
*Scarites**
Haplotrachelus
Pasimachus — Scaritinae excl. Clivinini + Dyschiriini
Clinidium
Omoglymmius — Rhysodinae
Omus
Tetracha
Brasiella
Cicindela — Cicindelinae
Metrius
Pachyteles
Goniotropis
Ozaena
Paussus
Cerapterus — Paussinae

length [μm]
0 10 10² 10³ 10⁴

sperm length ——
spermatostyle length ▬▬

Brachininae + Harpalinae

singleton | singleton w/ spermatostyle | simple rod-type | sheet-type | aggregate-type | mechanical-type

sperm with spermatostyle   sperm conjugation

Fig. 4

simple rod-type

sheet-type

length [µm]

sperm length
spermatostyle length

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12