Evolution and co-evolution of the suck behaviour, a postcopulatory female resistance trait that manipulates received ejaculate

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Abstract

Sexual conflicts over the post-mating fate of received ejaculate can favour traits in one sex that are costly to the other. Reciprocally mating hermaphrodites face unique challenges as they mate simultaneously in both the male and female role, potentially leading to receipt of unwanted ejaculate. Reciprocal mating can then give rise to postcopulatory female resistance traits that allow manipulation of received ejaculate. A putative example is the suck behaviour, observed in the flatworm genus *Macrostomum*. It involves the sperm recipient placing its pharynx over its own female genital opening and appearing to suck, likely removing received ejaculate after mating. The genus also contains hypodermically-inseminating species that presumably exhibit unilateral mating and have not been observed to suck. Here, we examine the evolution of the suck behaviour in *Macrostomum*, aiming to document the mating behaviour in 64 species. First, we provide videographic evidence that ejaculate is indeed removed during the suck behaviour in a reciprocally mating species, *Macrostomum hamatum*. Next, we show evolutionary positive correlations between the presence, duration and frequency of reciprocal mating behaviour and the suck behaviour, providing clear evidence that the suck behaviour co-evolves with reciprocal mating behaviour. Finally, we show an association between reproductive behaviour and reproductive morphology, suggesting that reproductive morphology can be used for inferring the behavioural mating strategy of a species. Together our study demonstrates sexual antagonistic coevolution leading to the evolution of a postcopulatory behavioural trait that functions as a female counter-adaptation allowing individuals to gain control over received ejaculate in a hermaphroditic sexual system.

45 ***Keywords***

46 Sexual conflict; sexual selection; simultaneous hermaphrodite; co-evolution; reproductive

47 traits; postcopulatory mechanisms; mating behaviour

Introduction

Sexual conflict is defined as the conflict between the two sexes over their evolutionary interests involving reproduction (Charnov, 1979; Parker, 1979; Arnqvist & Rowe, 2005). The primordial cause of sexual conflict is anisogamy, in which the male sex produces more but smaller gametes (called sperm in animals), whereas the female sex produces fewer but larger gametes (called eggs in animals) (Parker, 2011). Because of this asymmetry, eggs are often a limiting resource for reproductive success, resulting in divergent interests between the two sexes (Bateman, 1948; Lehtonen *et al.*, 2016). Furthermore, these conflicting interests can give rise to traits expressed by one sex that are costly to the other sex, resulting in antagonistic co-evolution between the sexes (Holland & Rice, 1998; Arnqvist & Rowe, 2005). Although work on sexual conflict has primarily focussed on separate-sexed organisms, sexual conflict is also pervasive in the lesser-studied hermaphroditic organisms (Charnov, 1979; Leonard, 1991; Michiels, 1998; Abbott, 2011; Schärer *et al.*, 2015).

Of particular interest is the biology of simultaneous hermaphrodites (referred to as hermaphrodites hereafter), which involves unique sexual conflicts. For example, there can be conflicts between the mating partners over the sex role exhibited in a mating, namely mating as a sperm donor, a sperm recipient, or both. Depending on the costs and benefits of mating in each role, this may lead to sex role preferences (Michiels, 1998; Schärer *et al.*, 2015). These are linked to Bateman's principle, a term coined by Charnov (1979), which reflects the notion that there is a "greater dependence of males for their fertility on frequency of insemination" (Bateman, 1948). In his seminal paper, Charnov (1979) explored the proposal that Bateman's principle also applies to simultaneous hermaphrodites. He concluded that, if true, hermaphroditic individuals may often mate more in order to give away sperm than to receive sperm, resulting in a mating conflict between the partners.

72 This conflict over the sex roles can be resolved via different mating strategies. One such
73 strategy is reciprocal mating (also called reciprocal copulation), in which the partners
74 simultaneously mate in both the male and female role. Each sperm donor is thus also a sperm
75 recipient, and while multiple mating offers more opportunities to donate sperm, it may also
76 lead to receipt of unwanted ejaculate from the partners. While this strategy seems like a
77 cooperative conflict resolution, it could shift the conflict from the precopulatory to the
78 postcopulatory arena (Schärer *et al.*, 2015). In the presence of sperm competition, a donor—in
79 order to secure a greater share of paternity—may often donate more sperm than the recipient
80 requires for fertilisation, thereby potentially causing direct costs, such as a risk of polyspermy
81 (Frank, 2000). But even if there are no direct costs posed by the received ejaculate, mating
82 with multiple partners—which is probably the norm for most species (Jennions & Petrie,
83 2007; Kokko & Mappes, 2013; Arbuthnott *et al.*, 2015)—could lead to the evolution of
84 cryptic female choice (Charnov, 1979; Eberhard, 1996; Hemmings & Birkhead, 2017). Thus,
85 receipt of excessive or unwanted ejaculate can favour the evolution of female resistance traits
86 that allow postcopulatory control and rejection of the received ejaculate, e.g. via sperm
87 digestion (Charnov, 1979).

88 Female resistance traits can in turn favour the evolution of male persistence traits, including
89 other mating strategies. Such counter-adaptations may allow the sperm donor to either
90 counteract or bypass the female resistance traits, thereby retain or regain access to the
91 recipient's eggs (Charnov, 1979; Schärer *et al.*, 2015). An example of such an alternative
92 mating strategy involves forced unilateral hypodermic insemination (also called hemocoelic
93 insemination; Charnov 1979). Here one of the partners mates in the male role and donates
94 sperm, while the other mates in the female role, potentially against its interests, and receives
95 sperm hypodermically via a traumatic male copulatory organ (Lange *et al.*, 2013; Reinhardt *et*
96 *al.*, 2015). With both types of mating strategy these sexual conflicts could then lead to the

97 evolution of multiple male persistence and female resistance traits (spanning behaviour,
98 morphology and physiology) that act jointly to either gain access to eggs, or to control and
99 reject the received ejaculate, respectively (Arnqvist & Rowe, 2005). Therefore, we might
100 expect behavioural mating strategies to be involved in sexually antagonistic coevolution, and
101 thus to be correlated with morphological and/or physiological traits.

102 A putative example of a behavioural female resistance trait is the suck behaviour, originally
103 documented in the free-living flatworm, *Macrostomum lignano* (Schärer *et al.*, 2004). Studies
104 in this reciprocally-mating simultaneous hermaphrodite have shown that matings are often
105 followed by the suck behaviour, during which the worm bends down and places its pharynx
106 over its own female genital opening (which is connected to the female antrum, the sperm-
107 receiving organ) and then appears to suck. The suck behaviour is hypothesised to be a
108 postcopulatory behaviour used for removing sperm or other ejaculate components received
109 during mating and thus to function as a female resistance trait (Vizoso *et al.*, 2010). However,
110 while there have been multiple studies on this behaviour (Schärer *et al.*, 2004, 2011, 2020;
111 Marie-Orleach *et al.*, 2013, 2017; Patlar *et al.*, 2020), there has to date been no direct
112 evidence for sperm and/or ejaculate actually being removed during the suck behaviour.
113 Moreover, if the suck functions as a postcopulatory sexual selection process, it could affect
114 the strength of sperm competition and potentially impact the optimal sex allocation (i.e. the
115 amount of resources allocated to the male and female function) (van Velzen *et al.*, 2009;
116 Schärer & Pen, 2013). Indeed, studies have documented both inter- and intra-specific
117 variation in sex allocation in *Macrostomum* (Singh *et al.*, 2020b; Brand *et al.*, 2022a; Singh &
118 Schärer, 2021), with mating behaviour predicting the evolution of a species' sex allocation
119 (Brand *et al.*, 2022a), but not the evolution of its sex allocation plasticity (Singh & Schärer,
120 2021).

121 Interestingly, *Macrostomum* species exhibit different combinations of reproductive
122 morphological traits that are likely associated with the reciprocal mating and hypodermic
123 insemination strategies (Figure 1A,B). Indeed, a previous study demonstrated an association
124 between certain male and female reproductive traits and the mating strategy in 16
125 *Macrostomum* species, naming the two alternative outcomes the reciprocal and hypodermic
126 mating syndrome, respectively (Schärer *et al.*, 2011). A more recent study has used a refined
127 composite measure, called the inferred mating syndrome, derived from the observation of
128 additional components of the reproductive morphology, in an attempt to classify 145
129 *Macrostomum* species as showing either the reciprocal or hypodermic inferred mating
130 syndrome, respectively (Figure 1B) (Brand *et al.*, 2022b). The lateral bristles on the sperm in
131 reciprocally mating species are hypothesized to represent a male persistence trait that allows
132 the sperm to remain anchored in the female antrum and not be pulled out during the suck
133 behaviour (Vizoso *et al.*, 2010), whereas the thick female antrum wall might prevent internal
134 injury resulting from the male genitalia during mating. In contrast, the sharp needle-like stylet
135 tip of hypodermically inseminating species likely allows sperm injection through the partner's
136 epidermis, while the simple sperm design presumably aides its movement through the
137 partner's body (Schärer *et al.*, 2011; Brand *et al.*, 2022b).

138 Although sexual conflict has been studied in many organisms spanning different reproductive
139 systems, studies on female resistance traits in hermaphrodites have been fewer, particularly in
140 a phylogenetic context (Koene & Schulenburg, 2005; Beese *et al.*, 2006, 2009; Anthes *et al.*,
141 2008; Sauer & Hausdorf, 2009; Schärer *et al.*, 2011; Brand *et al.*, 2022b). In our study, we
142 examine the evolution of the suck behaviour, aiming to document reproductive behaviour in a
143 total of 64 *Macrostomum* species. As a result of this, we, for the first time, provide
144 videographic evidence that ejaculate is indeed removed during the suck behaviour, supporting
145 the previously proposed hypothesis for the function of this postcopulatory behaviour (Vizoso

146 *et al.*, 2010). Using this extensive behavioural data set, we examine correlations between
147 different aspects of the mating and suck behaviour, and between reproductive morphology
148 and the behavioural mating strategies, while accounting for the phylogenetic
149 interrelationships. If the suck behaviour has indeed evolved as a postcopulatory strategy, we
150 predict positive correlations between the presence, duration, and frequency of the mating
151 behaviour and the suck behaviour. This could occur, e.g., if longer/frequent matings lead to
152 more ejaculate being transferred, which would need longer/frequent sucks to remove the
153 ejaculate. We might also expect a trade-off between copulation duration and frequency, if
154 species that spend a lot of time in copulation cannot copulate that often, e.g. due to ejaculate
155 limitation or mating taking up a lot of the total time (so that fewer mating could be done over
156 a period, i.e., an autocorrelation). Similarly, if suck functions to remove ejaculate, we may
157 expect a trade-off between suck duration and frequency, if shorter sucks necessitate the need
158 for more frequent sucks to remove the ejaculate. Finally, we also expect the reproductive
159 morphology to be a good proxy for inferring the behavioural mating strategy as a result of
160 coevolution.

161

Materials and Methods

Study organisms

Species in the genus *Macrostomum* are small (~0.3 to 3.0 mm body length) aquatic free-living flatworms that are highly transparent, permitting detailed observations of internal structures (for the general morphology see Figure 1A,B). The sperm and eggs are produced in the paired testes and paired ovaries, respectively, with studies documenting inter- and intra-specific variation in both testis and ovary size across the genus (Singh *et al.*, 2020b; Brand *et al.*, 2022a; Singh & Schärer, 2021). The female antrum is located anterior to the male antrum, connected to the outside, respectively, via a female genital opening (also female genital pore or vagina) and the male genital opening (also male genital pore). The stylet (male intromittent organ) resides within the male antrum and it is proximately connected via the vesicula granulorum (not shown) to the seminal vesicle, which contains sperm to be transferred during mating. In both reciprocally-mating and hypodermically-inseminating species, the female antrum serves as the egg-laying organ, while in reciprocally-mating species it additionally serves to receive the stylet during mating and as the sperm-storage organ (Vizoso *et al.*, 2010; Schärer *et al.*, 2011).

We obtained multiple specimens for a large number of *Macrostomum* species, collected from a range of locations and habitats, using a variety of extraction techniques, which we report on in more detail as part of separate studies on the phylogenetic interrelationships (Brand *et al.*, 2022c) and reproductive character evolution in this genus (Brand *et al.*, 2022b). Briefly, most specimens were sampled directly from natural field sites, while some were sampled from artificial ponds, or from aquaria containing other study organisms, and they were generally observed within a few days of collection. Other specimens were obtained from short- and long-term laboratory cultures maintained either by our group or by colleagues.

186 Following Brand *et al.* (2022b), 38 of a total of 64 species *Macrostomum* included in the
187 current study were classified as exhibiting the reciprocal inferred mating syndrome, because
188 they had a blunt tip of the stylet (the male intromittent organ), and of these all but one had
189 received sperm in the antrum. A further 6 species with a sharp stylet were also classified as
190 exhibiting the reciprocal inferred mating syndrome because they had complex sperm with
191 lateral bristles and we observed sperm in the antrum (Figure 2). In contrast, 15 species were
192 classified as exhibiting the hypodermic inferred mating syndrome because allosperm was
193 exclusively found hypodermically. An additional 4 species without observation of received
194 sperm were classified as exhibiting the hypodermic inferred mating syndrome because they
195 had a simple female antrum (no thickening of the antrum wall and no visible cellular valve), a
196 sperm design with reduced or absent bristles and a sharp stylet tip. Finally, one species
197 (*Macrostomum sp*. 101) was classified as intermediate because received sperm was observed
198 both in the antrum and within the tissue (Figure 2).

199 ***Observation methodology***

200 We aimed at documenting the mating behaviour of all 64 *Macrostomum* species, by placing
201 the worms in mating chambers (Schärer *et al.*, 2004). A mating chamber consisted of the
202 worms being placed between two microscope slides in small drops (i.e. either freshwater or
203 water with different salinity, depending on the collection habitat), with a certain number of
204 spacers (separating the slides), and sealed with pure white Vaseline (note that we generally
205 also placed 4-6 empty drops around, to reduce evaporation). We adjusted the spacer number
206 and drop volume depending on the size and number of worms in a drop, respectively.
207 Usually, for a pair of worms of the size of *M. lignano* (~1.5 mm body length), we used 2
208 spacers (each spacer being ~105 µm) and a drop size of ~3 µl. Movies were recorded when
209 specimens were available and therefore across several sampling campaigns. Consequently, the
210 recording setups differed (macro lenses, cameras or lighting conditions). However from a

211 previous detailed study of two *Macrostomum* species we know that these minor setup
212 differences are unlikely to bias our observations (Singh *et al.*, 2020a). Usually, the movies
213 were recorded in QuickTime Format using BTV Pro (http://www.bensoftware.com/) at 1
214 frame s^{-1}, but for some species we also generated detailed close-up movies, where worms
215 were manually tracked at higher magnifications under a compound microscope and filmed at
216 higher frame rates (see next section). All worms were visually checked for sexual maturity
217 (defined as having visible gonads or eggs), either before or after filming.

218 ***Detailed observation of mating and suck behaviour in Macrostomum hamatum***

219 While earlier work documented sperm sticking out of the female antrum after the suck
220 behaviour in *M. lignano* (Schärer *et al.*, 2004, 2011), direct observations of ejaculate removal
221 have not been reported to date. Here we could document ejaculate removal in detailed close-
222 up movies of *M. hamatum*, possibly since field-collected specimens of this species appeared
223 to be more transparent than other species. This allowed us to clearly visualise the deposition
224 and subsequent removal of ejaculate during the mating and suck behaviour, respectively.
225 Specifically, we examine the mating behaviour of *M. hamatum*, collected on 27. July 2017
226 directly in front of the Tvärminne Zoological Station, Finland (N 59.84452, E 23.24986), in a
227 detailed close-up movie (Supplementary Movie S1). Note that while we describe and
228 illustrate only one such instance (in an extract from a longer movie), we also observed
229 ejaculate removal in other detailed close-up movies of *M. hamatum* that we also deposit
230 (https://doi.org/10.5281/zenodo.6354683), and these observations corroborated our finding as
231 described here.

232 ***Scoring of mating and suck behaviour across species***

233 We scored the mating behaviours from the mating movies by visual frame-by-frame analysis
234 (Supplementary Table S1). A reciprocal mating was scored when the tail plates of two worms

235 were in ventral contact and intertwined, such that the female antrum was accessible to the
236 partner's stylet and vice-versa, which would allow reciprocal transfer of ejaculate. In most
237 species, the copulatory posture is accompanied by the pair being tightly interlinked (like two
238 interlocking Gs, see Figure 3), and thus similar to the mating behaviour originally described
239 for *M. lignano* (Schärer *et al.*, 2004). Note that in some species the mating posture can deviate
240 from that observed in *M. lignano* (see Supplementary Table S2), such as, for example, in
241 *Macrostomum* sp. 57 and *Macrostomum* sp. 61 (Supplementary Movie S2A,B). The mating
242 duration was measured from the frame when the tail plates were in ventral contact (and
243 usually tightly intertwined), to the frame where the tail plates were no longer attached to each
244 other. We defined behaviours as matings only if the pair was in the above-described posture
245 for at least 3 s. The suck duration was measured starting from the frame when an individual
246 placed its pharynx over its female genital opening, up to the frame where the pharynx
247 disengaged. Note that in some cases, individuals do not lie on their side while sucking (as
248 generally seen in *M. lignano*), which can sometimes make it more difficult to observe the
249 suck behaviour. For each replicate drop, we divided the total number of matings and sucks by
250 the number of worms and the movie duration to obtain a standardized value. We then
251 averaged the frequency and duration estimates across all replicate drops for each species to
252 obtain the species estimates of the respective behaviours.

253 While we also invested significant effort into observing hypodermic insemination (see Table
254 S1 and Results), we only saw some rare behavioural instances in a few species that could
255 possibly represent cases of hypodermic insemination, such as, for example, in
256 *Macrostomum* sp. 1 and *M. gabriellae* (Supplementary Movie S3A,B). Possible reasons for
257 not observing hypodermic insemination could be that in many species such matings occur
258 very rapidly or that they mate less frequently, possibly since they try to avoid sperm receipt
259 (Apelt, 1969; Michiels, 1998). Given that we could not confirm the presence of hypodermic

260 insemination, we scored species either as having reciprocal mating being present (when it was
261 observed) or absent (when it was not observed) (Figure 2). Note, however, that the absence of
262 observations of reciprocal mating does not necessarily imply the presence of hypodermic
263 insemination. Instead, it could also result from a reciprocally-mating species not mating under
264 laboratory conditions and/or from an overall low mating frequency of a species. Similarly,
265 while the presence of the suck behaviour can clearly be identified in many species, the
266 absence of observations of the suck behaviour does not necessarily mean that a species never
267 shows this behaviour.

268 ***Evolution of the mating and suck behaviour across the genus Macrostomum***

269 To perform phylogenetic comparative analyses, we used a trimmed version of a recently
270 published ultrametric large-scale phylogeny of the genus *Macrostomum* (i.e. the C-IQ-TREE
271 phylogeny of Brand *et al.*, 2022c). This phylogeny is based on an amino acid alignment of
272 385 genes from 98 species, supplemented with Sanger sequences from a *28S rRNA* fragment,
273 which allowed the addition of a further 47 species, and calculated using a maximum
274 likelihood approach (Brand *et al.*, 2022c), covering all the species we included in the current
275 study. Specifically, we determined 1) whether the presence/absence of reciprocal mating is
276 correlated with the presence/absence of the suck behaviour, 2) whether the presence/absence
277 of reciprocal mating is correlated to the presence/absence of the reciprocal inferred mating
278 syndrome, and 3) whether there are correlations between the frequency and the duration of the
279 reciprocal mating and suck behaviours among the species that show these behaviours.

280 *Presence/absence of reciprocal mating and the suck behaviour*: We used the DISCRETE
281 model in BayesTraits V.3.0.1 to test for correlated evolution between reciprocal mating and
282 the suck behaviour (both scored as present/absent), using the Reversible Jump Markov Chain
283 Monte Carlo (RJ MCMC) approach (Pagel, 1994; Pagel & Meade, 2006; Meade & Pagel,
284 2016). Specifically, we compared the marginal likelihood of a dependent model, in which the

285 presence of suck depends on the presence of reciprocal mating, to an independent model, in
286 which the suck behaviour and reciprocal mating evolve independently. Each RJ MCMC chain
287 was run for twelve million iterations and the first one million iterations were discarded as
288 burn-in, after which the chain was sampled every 1000th iteration. We used a gamma
289 hyperprior (gamma 0 1 0 1), and placed 1000 stepping stones (with each iterating 10000
290 times) to obtain the marginal likelihood values for the models. We ran three separate chains
291 each for the dependent and independent model to check for the stability of the likelihood
292 values and convergence. Using the R package coda (Plummer *et al.*, 2006), we confirmed that
293 the chains had converged (Gelman & Rubin, 1992; Brooks & Gelman, 1998) and that the
294 Effective Sample Size was >200 for all parameters. In addition, we also confirmed that the
295 acceptance rate was between 20-40% (Pagel & Meade, 2006). We compared the alternative
296 models with the Log Bayes Factor (BF), using the convention that BF values > 2 are
297 considered as positive support for the best-fit model, while values between 5-10 and > 10 are
298 considered as strong and very strong support for the model, respectively (Pagel & Meade,
299 2006). To examine the robustness, we repeated the analysis for a reduced dataset, by
300 excluding six species that had in total been observed for < 21 h (~10% quantile). For the
301 dependent models of the full dataset, we estimated the transition rates among the different
302 trait states by calculating Z values. This value can be understood as the percentage of times a
303 transition rate was set to zero, with a high value thus indicating that the transition between
304 two states is unlikely. We expect a correlation between the presence of reciprocal mating and
305 the presence of the suck behaviour, which would corroborate that the suck behaviour indeed
306 is a postcopulatory behaviour that is linked to reciprocal mating, rather than possibly serving
307 a function that is also present in species with hypodermic insemination.

308 *Presence/absence of reciprocal mating and the reciprocal inferred mating syndrome*: We
309 checked for an association between reciprocal mating (scored as present/absent) and the

310 inferred mating syndrome (scored as reciprocal/hypodermic), using the DISCRETE model in
311 BayesTraits V.3.0.1 (as above). One of the species, *Macrostomum* sp. 101, had a morphology
312 that was scored intermediate between reciprocal and hypodermic (Brand *et al.*, 2022b), but
313 since the discrete method in BayesTraits only allows binary trait states, we excluded this
314 species from this analysis. We expect a correlation between the presence of reciprocal mating
315 behaviour and the reciprocal inferred mating syndrome, which could indicate that behaviour
316 and morphology coevolve.

317 *Correlations between the frequency and the duration of mating behaviours*: In preparation for
318 phylogenetic correlation analyses we estimated the phylogenetic signal for the continuous
319 traits (i.e. the duration and frequency of both the reciprocal mating and the suck behaviour;
320 log-transformed for all the analyses) using Pagel's λ (Pagel, 1999; Revell, 2012). A λ value of
321 1 indicates a strong phylogenetic signal, while a value around 0 indicates no/low phylogenetic
322 signal (Pagel, 1999). We found phylogenetic signal that was significantly different from 0 for
323 the suck frequency (λ=0.67, P=0.02), the suck duration (λ=0.76, P=0.005), and the reciprocal
324 mating frequency (λ=0.50, P=0.05), but only marginally so for the mating duration (λ=0.46,
325 P=0.06). For each trait, we then fitted four different models of trait evolution, i.e. Brownian
326 motion, Ornstein–Uhlenbeck, Early-burst, and Lambda models (Harmon *et al.*, 2008). We
327 found that the Lambda model had the highest sample-size corrected Akaike Information
328 Criterion (AICc) weights (ω_i) (Supplementary Table S3), and this model was hence chosen
329 for further PGLS analysis.

330 For the species that exhibited both reciprocal mating and the suck behaviour, we then
331 investigated if there was a correlation between the frequency and duration of the reciprocal
332 mating and suck behaviours, using phylogenetic generalized least-squares (PGLS) regression
333 implemented in the *caper* package version 1.0.1 (Orme *et al.*, 2014). PGLS accounts for the
334 non-independence of the data by incorporating the phylogenetic relationships between species

335 into the error structure of the model. For each analysis using the frequency and duration of the
336 reciprocal mating and suck behaviours, the phylogenetic signal (Pagel's λ) was estimated
337 using the maximum likelihood approach. We examined the residuals of each model for
338 normality and homogeneity (Mundry, 2014). Additionally, we scrutinized for influential cases
339 (species) in each PGLS model, by excluding one species at a time from the data and rerunning
340 the analysis, and comparing the results obtained with the results for the entire dataset
341 (Mundry, 2014). And finally, we evaluated the robustness of our results by repeating the
342 PGLS for a reduced dataset, which excluded five species in which mating or suck had only
343 been observed in one replicate (note that this reduced dataset is different from the reduced
344 dataset used in the above BayesTraits analysis).

345 We performed our analysis in R, version 3.6.1 (R Core Team, 2019).

346

347 ## *Results*

348 ### *Sperm deposition and removal during mating and suck behaviour in Macrostomum*
349 ### *hamatum*

350 The general anatomy of the reproductive organs of *M. hamatum* is similar to that of many
351 other reciprocally-mating *Macrostomum* species (Figure 1A,B). In the detailed movie of
352 *M. hamatum*, the worms are already interlinked in the reciprocal copulatory position at the
353 beginning of the clip (Figure 3, Supplementary Movie S1), and we consider this as t = 0 s
354 (hereafter we refer to the worm on the right as Orange and the worm on the left as Grey,
355 respectively). At this timepoint "the tail plates touch each other ventrally in opposing
356 directions, while the anterior ventral surface of each worm touches the posterior dorsal
357 surface of the partner", as previously described for the copulatory position in *M. lignano*
358 (Schärer *et al.*, 2004). Interestingly, in *M. hamatum* the copulatory position resembles a
359 square with rounded corners, as opposed to *M. lignano,* where it is more circular. This may in
360 part be due to a strikingly different position of the tail plate, which in *M. hamatum* stands at a
361 90° angle from the posterior body axis and appears to poke into the anterior ventral surface of
362 the partner, leading to a dorsal bulge in both Orange and Grey.

363 Moreover, *M. hamatum* has a much more prominent erection (i.e., a translucent finger-like
364 structure on the ventral tail plate, likely formed by the eversion of the muscular male antrum),
365 which pokes into the posterior ventral surface of the partner in the region of the female genital
366 opening (although it is unclear if the erection actually enters the partner). The stylet of Grey—
367 while moving inside of the relatively stationary erection—then performs poking movements
368 that are directed towards Orange's female antrum, initially without any transfer of ejaculate.
369 At t = 3-5 s, the stylet of Grey is seen repeatedly poking against the dorsal side of Orange's
370 female antrum wall, each time leading to a visible bulge on Orange's dorsal side. In some of

371 these frames one can see the sharp hook-shaped distal end of the stylet that is typical for
372 *M. hamatum*. Eventually, Grey begins to deposit ejaculate (seen as a visible darkening of
373 Orange's female antrum lumen starting at about t = 5s). During this process the seminal
374 vesicle of Grey empties (as seen at the base of the erection, see also drawing in Figure 3 at 0 s
375 for location), while the female antrum of Orange fills up with ejaculate over the next ~21 s
376 (see Figure 3 from 3.7 s). Note that we here mainly focus on the sperm transfer from Grey to
377 Orange, but in the meantime, Orange also pokes and eventually enters the female antrum of
378 Grey (t = 16-20 s) and sperm is also transferred from Orange to Grey (between t = 21-27 s),
379 although this is more difficult to follow in the movie.

380 At t = 28 s, Orange pushes out its female antrum region, places its pharynx over its female
381 genital opening, and then sucks. The received ejaculate can be seen leaving Orange's female
382 antrum (i.e. the visible darkening in the female antrum lumen moves towards the pharynx
383 between t = 29-30s). In total, the suck behaviour lasts for 7 s. Interestingly, during the suck
384 the stylet of Grey remains anchored in Orange's female genital opening (probably involving
385 the above-mentioned hook). At t = 52 s, the mating ends after a mating duration of ~64 s
386 (recall that the worms were already in copula at t = 0 s). At t = 56 s, only Grey is in frame and
387 the received ejaculate in its female antrum is clearly visible. It continues to have a small
388 erection despite the mating being over. At t = 78 s, Grey pushes its female antrum region out
389 and some sperm is ejected from the female antrum at t = 80 s, notably before the pharynx
390 makes contact (Figure 4, Supplementary Movie S1). At t = 81 s, Grey puts its pharynx over
391 its female genital opening and then sucks for 10 s. After the suck, some sperm can still be
392 seen sticking out of the female antrum (similar to *M. lignano*, Schärer *et al.*, 2004), especially
393 at 92 s, but most of the ejaculate has been removed from the female antrum. The female
394 antrum remains slightly everted and the erection somewhat visible until at least 108 s.

395 ***Evolution of the mating and suck behaviour across the genus Macrostomum***

396 We observed a total of 2796 worms across 64 *Macrostomum* species, with a mean of 44
397 worms and 76.7 hours of observation time per species, for a total observation time of 4908
398 hours. Of the 64 species, 30 species exhibited reciprocal mating behaviour, 31 species
399 exhibited the suck behaviour, and 25 species exhibited both the reciprocal mating and suck
400 behaviour (Figure 2).

401 *Presence/absence of reciprocal mating and the suck behaviour*: We found strong support for
402 the dependent model over the independent model of evolution for the correlation between the
403 presence of reciprocal mating and the presence of the suck behaviour, with all three runs for
404 each model providing highly consistent values (average marginal likelihood, independent=-
405 89.25, dependent=-83.37, BF: 11.75; see also Supplementary Table S4a). This showed that
406 the presence of reciprocal mating and the presence of the suck behaviour are strongly
407 correlated. And the result was robust to observation time, since excluding the 6 species that
408 were observed for < 21 h gave similar results (Supplementary Table S4a).

409 The transitions from the absence of both the reciprocal mating and suck behaviour to the
410 presence of either of these traits were found to be the most unlikely, as is evident from the
411 low transition rates and the high Z values (Figure 5a). Interestingly, the other transitions,
412 including losing reciprocal mating or the suck behaviour from the state when they are both
413 present, are all similarly likely. This contrast suggests that once both reciprocal mating and
414 suck are lost or absent in a species, it is highly unlikely to regain either.

415 *Presence/absence of reciprocal mating and the reciprocal inferred mating syndrome*: There
416 was a clear correlation between the presence of reciprocal mating behaviour and the
417 reciprocal inferred mating syndrome, as evident from the strong support for the dependent
418 model over the independent model of evolution, with similar values for the three independent
419 runs of each model (average marginal likelihood, independent=-69.09, dependent=-65.60, BF:

420 6.99; see also Supplementary Table S4b), suggesting that the reproductive morphology of a
421 species can serve as a good proxy for its mating behaviour. And as before, our result was
422 robust, as the reduced dataset gave us similar results (Supplementary Table S4b).

423 Transitions from the presence of reciprocal mating and the presence of the reciprocal inferred
424 mating syndrome to the absence of either were moderately likely, while the converse
425 transitions were very likely (Figure 5b). Similarly, transitions from the absence of reciprocal
426 mating and the absence of the reciprocal inferred mating syndrome to the presence of either
427 were either unlikely or relatively unlikely, while the converse transitions were very likely.
428 Together this suggests that there is a strong association between reciprocal mating behaviour
429 and morphological traits characterizing the reciprocal inferred mating syndrome (and between
430 absence of reciprocal mating behaviour and the hypodermic inferred mating syndrome), such
431 that species are attracted to these states and evolve away from states where the morphology
432 and behaviour are mismatched.

433 *Correlations between the frequency and the duration of mating behaviours*: Among the
434 species that exhibited reciprocal mating (n=30), the average mating frequency was 0.84 hr^{-1}
435 (range: 0.02-7.82 hr^{-1}, Figure 6A) and the average mating duration was 283.7 s (range: 5.2-
436 4609 s, Figure 6B), with some sibling species showing fairly divergent values. Moreover,
437 among the species that showed the suck behaviour (n=31), the average suck frequency was
438 0.54 hr^{-1} (range: 0.01-3.7 hr^{-1}, Figure 6A) and the average suck duration was 9.6 s (range: 4.7-
439 16.1 s, Figure 6C).

440 In line with our predictions, we found significant positive relationships between both
441 reciprocal mating frequency and suck frequency (Figure 7A), and reciprocal mating duration
442 and suck duration (Figure 7B); while there was no significant relationship between reciprocal
443 mating frequency and reciprocal mating duration (Figure 7C), and suck frequency and suck

444 duration (Figure 7D). The reduced dataset also gave qualitatively similar results for all

445 analysis (Supplementary Table S5).

446

447 ## *Discussion*

448 Sexual conflict can give rise to antagonistic coevolution in all sexual systems (Charnov, 1979;
449 Bedhomme *et al.*, 2009). Here we documented the widespread occurrence of a putative
450 female resistance trait, the suck behaviour, in >30 species in the hermaphroditic flatworm
451 genus *Macrostomum*. Moreover, the direct observation of ejaculate removal in one species,
452 *M. hamatum*, corroborates the hypothesis that the suck functions as a female resistance trait to
453 remove received ejaculate (Schärer et al. 2004; Vizoso et al. 2010; Schärer et al. 2011), and
454 this interpretation is also supported by significant evolutionary correlations between different
455 aspects of reciprocal mating and suck behaviour. Finally, we could also show that the
456 reproductive morphology is a good proxy for inferring the mating strategy of a species,
457 presumably also as a result of coevolution. In the following we discuss these findings in more
458 detail.

459 ### ***Sperm deposition and removal during mating and suck behaviour in Macrostomum***
460 ***hamatum***

461 While multiple studies in *Macrostomum* have examined aspects of the suck behaviour
462 (Schärer *et al.*, 2004, 2011, 2020; Marie-Orleach *et al.*, 2013, 2017; Patlar *et al.*, 2020; Singh
463 *et al.*, 2020a), its involvement in removing received ejaculate components has so far only
464 been hypothesized. Our detailed observations of mating interactions in *M. hamatum* provide
465 the first direct evidence that ejaculate is indeed removed during this postcopulatory
466 behaviour. Interestingly, compared to *M. lignano* (Schärer *et al.*, 2004), *M. hamatum* has a
467 more rectangular mating posture (possibly due to the angular position of the tail plate), a
468 larger erection around the stylet, and the worms prominently evert the female antrum just
469 before the suck behaviour, likely as a result of muscular contractions. This could result from
470 differences in the female antrum morphology: while *M. hamatum* has a strong musculature

471 and an inner second chamber connecting to the main female antrum (Luther, 1947),
472 *M. lignano* has a somewhat simpler female antrum with a single chamber (Ladurner *et al.*,
473 2005; Vizoso *et al.*, 2010). Similarly, the prominent erection of the male antrum could result
474 from a muscular morphology that is similar to the muscular cirrus seen in species of the sister
475 genus, *Psammomacrostomum* (Ax, 1966; Janssen *et al.*, 2015). The combination of a rather
476 prominent female antrum and the relatively transparent specimens may have helped us
477 visualise the function of the suck behaviour better in *M. hamatum* than in other *Macrostomum*
478 species observed to date.

479 While we see ejaculate being removed during the suck behaviour, we cannot clearly
480 determine whether it is ingested. Although sperm digestion is widespread in hermaphrodites
481 (Charnov, 1979; Baur, 1998; Dillen *et al.*, 2009; Koene *et al.*, 2009), it usually occurs inside
482 an organ connected to the individual's reproductive system, unlike in the case of the suck
483 behaviour. To our knowledge, there have been only two earlier reports of sperm being orally
484 taken up in hermaphrodites, one in the arrow worm *Spadella cephaloptera* (John, 1933) and
485 the other in the leech *Placobdella parasitica* (Myers, 1935). Thus, the suck behaviour seems
486 to be a novel trait, which to date has only been observed in species of the Macrostomidae
487 (including a member of the sister genus *Psammomacrostomum*; P. Singh, pers. obs.). Similar
488 to the suck behaviour, females of the ladybird beetle, *Adalia bipunctata*, consume a
489 spermatophore after mating (Perry & Rowe, 2008). Moreover, there is also sperm dumping in
490 many separate-sexed species, in which the female physically ejects received sperm from her
491 reproductive tract, and this, at least in some cases, is thought to be a mechanism of cryptic
492 female choice (Snook & Hosken, 2004; Peretti & Eberhard, 2010; Firman *et al.*, 2017). If the
493 suck behaviour also functioned in cryptic female choice, we might expect individuals to
494 remove or retain sperm of certain partners more frequently (e.g. Pizzari & Birkhead, 2000).
495 This has also been observed in *M. lignano*, where the propensity of the recipient to suck is

496 affected by the mating status (Marie-Orleach *et al.*, 2013) and the genotype of its partners
497 (Marie-Orleach *et al.*, 2017). However, it is difficult to ascertain whether the suck implies an
498 active choice by the recipient or whether it is sometimes prevented as a result of a
499 manipulation by the donor (Patlar *et al.*, 2020). Moreover, our study documents in detail the
500 reciprocal transfer and deposition of sperm by both mating individuals during a reciprocal
501 mating in *Macrostomum* (but see Ax & Borkott, 1968 which documents mating and unilateral
502 sperm transfer in *M. salinum*, now considered to be *M. romanicum*).

503 ***Evolution of the mating and suck behaviour across the genus Macrostomum***

504 We found a significant evolutionary correlation between the presence of reciprocal mating
505 and the suck behaviour (Figure 5a). In reciprocally-mating species, ejaculate is deposited in
506 the female antrum allowing its removal during the suck behaviour, while in hypodermically-
507 inseminating species, sperm is injected potentially anywhere in the body (Schärer *et al.*, 2011;
508 Brand *et al.*, 2022b). Given that the function of the suck behaviour indeed appears to be the
509 removal of ejaculate, we do not expect to see the suck behaviour in hypodermically-
510 inseminating species. Performing a suck at a site of hypodermic insemination might not
511 permit effective ejaculate removal (particularly also given the above-mentioned active
512 participation of the female antrum musculature), but instead would more likely lead to
513 additional tissue damage. Interestingly, the transition rates showed that while it is unlikely for
514 a species that lacks both the reciprocal mating and suck behaviour to gain either of these
515 traits, the loss of either reciprocal mating or the suck behaviour was estimated as being more
516 likely. These transitions could represent transitional steps towards hypodermic insemination,
517 which might arise as a means to bypass the female control and allow access to the eggs
518 (Charnov, 1979; Brand *et al.*, 2022b). Moreover, this interpretation is also supported by the
519 finding that there are multiple origins of hypodermic insemination in the genus *Macrostomum*
520 (Brand *et al.*, 2022b; Singh & Schärer, 2021). There are at least nine independent shifts from

521 reciprocal mating to hypodermic insemination in *Macrostomum*, while no transition is
522 observed in the converse direction (Brand *et al.*, 2022b).

523 However, it is important to point out that some of these findings could also have resulted from
524 a lack of observations of either the reciprocal mating and/or suck behaviour (despite being
525 present in a species), leading to an overestimation of these transition rates. Specifically, there
526 were six species that showed only the reciprocal mating behaviour and five species that
527 showed only the suck behaviour (Supplementary Table S1). These mismatches usually
528 appeared in species for which we had comparatively few observation hours (for more detail
529 see Supplementary Figure S3), suggesting that additional observations could help to further
530 ascertain the actual presence/absence of reciprocal mating or the suck behaviour, respectively.
531 Moreover, mismatches could result from a species not exhibiting some behaviours under our
532 laboratory conditions, or they might indicate that a species indeed lacks a behaviour. In
533 addition, if a species mates only rarely, individuals might be less inclined to remove the
534 sperm they receive, and in our study the species that showed reciprocal mating but did not
535 suck, had low or intermediate mating frequencies (see *Macrostomum* sp. 43, *Macrostomum*
536 sp. 67, *M. distinguendum, M. gieysztori,* and *M. poznaniense* in Figure 6A). Alternatively,
537 species might actually lack reciprocal mating, but losing a resistance trait like the suck
538 behaviour might take longer, particularly if the suck behaviour does not impose costs on the
539 fecundity. Moreover, the suck behaviour could have additional functions, such as possibly
540 removing egg material that remains in the antrum after egg laying. Species are predicted to
541 lose defensive or resistance traits only after the persistence traits have become substantially
542 less harmful, leading to a time lag (Parker, 1979). A study on the seed beetle, *Callosobruchus*
543 *maculatus*, showed that, while large males evolved relatively reduced length of genital spines
544 under monogamy, there was no detectable evolution in female genitalia within the same time
545 period (Cayetano *et al.*, 2011). And finally, since the worms we observed may often have

546 mated before we placed them into the mating chambers, some of the observed sucks might
547 have occurred in response to unobserved earlier matings, since sucks do not only occur
548 immediately after mating (Schärer et al. 2004).

549 The significant evolutionary correlation between the presence of reciprocal mating and the
550 purely morphologically-derived reciprocal inferred mating syndrome (Figure 5b) confirms
551 previous findings (Schärer *et al.*, 2011). It shows that persistence and resistance are not
552 generally limited to single traits, but are often composite suites of behavioural, morphological
553 and physiological traits acting together (Arnqvist & Rowe, 2005). For example, the thickened
554 female antrum wall and the suck behaviour might be different components of female
555 resistance. While the former might prevent injury resulting from the male genitalia when
556 mating reciprocally, the suck behaviour serves to remove unwanted ejaculate received during
557 mating. Similar adaptations of the female reproductive tract are also seen in the seed beetle
558 *C. maculatus,* where a thicker female tract lining serves as a resistance trait against harm by
559 male genitalia (Dougherty *et al.*, 2017). Moreover, resistance and persistence traits can also
560 occur at the proteomic level. A study in *M. lignano* identified two seminal fluid transcripts,
561 experimental knock-down of which caused mating partners to suck more often (Patlar *et al.*,
562 2020). This suggests that the seminal fluid proteins derived from these transcripts might be
563 counter adaptations by the donor to prevent the suck behaviour by the recipient.

564 In our dataset, there was one species each that exhibited the hypodermic inferred mating
565 syndrome morphology and showed both reciprocal mating and the suck behaviour
566 (*M. rostratum*), only reciprocal mating (*M. distinguendum*), or only the suck behaviour
567 (*M. finlandense*) (Supplementary Table S1). Interestingly, the three species represent at least
568 two, but possibly three, of the above-mentioned multiple independent origins of the
569 hypodermic inferred mating syndrome (Brand *et al.*, 2022b). Conversely, there were 12
570 species that exhibited the reciprocal inferred mating syndrome, but in which neither reciprocal

571 mating, nor the suck behaviour was observed. As above, this mismatch occurred mainly in
572 species for which we had relatively few observation hours (Supplementary Figure S3),
573 suggesting that, if these species have a low mating frequency, then more observation time
574 may be needed to avoid falsely inferring the absence of the mating and suck behaviour. And
575 finally, for many of the species that showed the hypodermic inferred mating syndrome we had
576 considerable amounts of observation hours (Supplementary Figure S3), so that it seems
577 unlikely that the absence of mating and suck observations in these species were due to a lack
578 of effort.

579 *Macrostomum* species showed large interspecific variation in behaviour, with a nearly 900-
580 fold variation in mating duration, a 3-fold variation in the suck duration, and a nearly 400-fold
581 variation in the mating and suck frequency across the genus (Figure 6). Remarkably, despite
582 this extensive interspecific variation in behavioural traits, we see clear correlations between
583 both the mating and suck duration, as well as the mating and suck frequency, suggesting that
584 the mating and suck behaviour have coevolved. If a longer mating duration or more frequent
585 mating implies more sperm transfer, then we expect selection for a longer suck duration
586 and/or a more frequent suck behaviour (particularly if ejaculate receipt is associated with
587 fitness costs). In some species, at least, a longer mating duration does imply more ejaculate
588 transferred (Engqvist & Sauer, 2003), and is often used as a proxy for ejaculate size (Kelly &
589 Jennions, 2011). Alternatively, such a correlation could also emerge as a result of variation in
590 genital complexity, e.g. if it takes longer to insert and remove more complex male genitalia,
591 and to suck out ejaculate from more complex female antra. Interestingly in *Macrostomum*,
592 male and female genital complexity are indeed correlated (Brand *et al.*, 2022b). Moreover, a
593 positive correlation between reciprocal mating and suck could also appear, if some species do
594 not do well under our laboratory conditions, leading to an overall low behaviour frequency.
595 Note, however, that we confirmed that the individuals we used for mating movies were adults

596 with visible testes and ovaries, and we also established the robustness of the observed
597 correlations by excluding species in which mating or suck had only been observed in one
598 replicate (Supplementary Table S5). We did not find any correlations between the frequency
599 and duration for either reciprocal mating or suck. While we might have expected mating
600 duration to trade off with mating frequency, mating duration only made up a relatively small
601 percentage of total tine, potentially posing no trade-off. Similarly, if sucking is not very
602 costly, the suck duration and frequency may not trade-off; and could even be positively
603 correlated, since both help to remove ejaculate.

604 Finally, mating frequency (and possibly mating duration) could be positively correlated with
605 allocation towards the male function (e.g., testes). Indeed, studies in *Macrostomum* have
606 shown interspecific variation in sex allocation towards the male and female functions, such as
607 testes and ovaries (Singh *et al.*, 2020b; Brand *et al.*, 2022a; Singh & Schärer, 2021). This
608 interspecific variation could potentially relate to the mating behaviour, as we can expect
609 species that have a longer mating duration or higher mating frequency to have larger testes, if
610 longer and/or more frequent mating implies that more sperm are transferred (Janicke &
611 Schärer, 2009). Mating duration could also correlate with the complexity of genitalia, such
612 that more complex genitalia might require longer mating duration (King *et al.*, 2009), and
613 future studies should investigate the correlations between different aspects of reproductive
614 behaviour and reproductive morphology in *Macrostomum*.

615 ***Conclusions***

616 Our study provides direct observational evidence for ejaculate removal during the
617 postcopulatory suck behaviour in the species *M. hamatum*, compelling support for the
618 coevolution between the reciprocal mating and suck behaviour, and detailed information in a
619 phylogenetic context on the occurrence and interspecific variation of the suck behaviour.
620 Moreover, we show that reproductive morphology can be a good proxy to infer the

621 behavioural mating strategy. Taken together our study shows the presence of a postcopulatory
622 female behavioural resistance trait that co-evolves with mating strategy and allows
623 manipulation of received ejaculate in a simultaneously hermaphroditic sexual system. Thus,
624 our study adds to the repertoire of information on traits involved in sexual conflict in
625 *Macrostomum* genus and demonstrates the genus as an excellent model system for
626 understanding sexual antagonistic coevolution by allowing us to examine the evolution of
627 diverse female resistance and male persistence traits, spanning behavioural and morphological
628 traits, simultaneously.

629

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638

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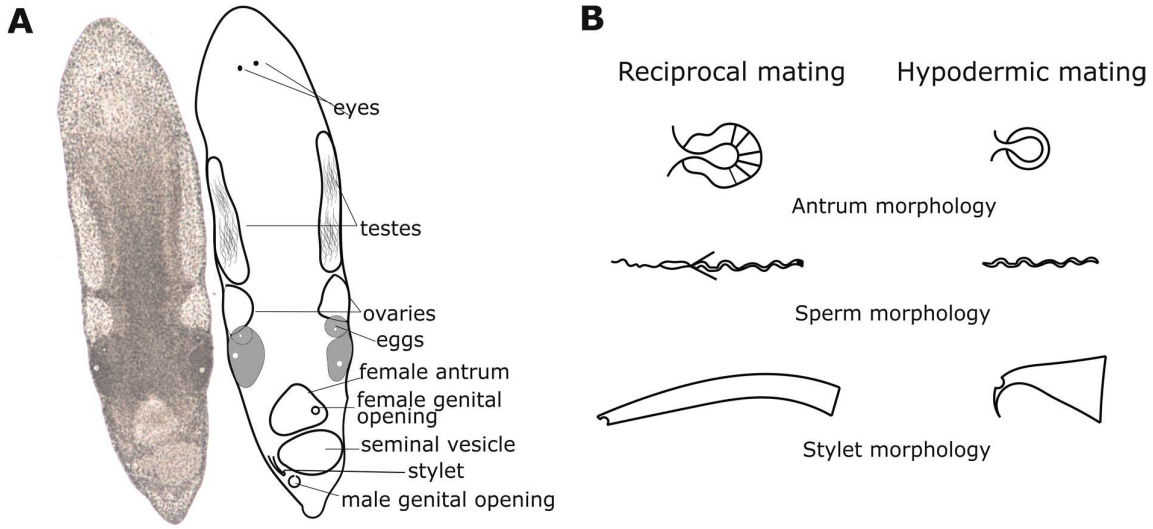
830 ***Figures***

831



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833 Figure 1. (A) Photograph and line drawing of an adult *Macrostomum cliftonense* (previously
834 *M. cliftonensis*, name updated following Zhang *et al.*, 2021), showing some of the
835 components of the reproductive system to help understand the mating behaviour observations
836 (total length ~1.2 mm). (B) Schematic drawings of the typical morphology of the antrum
837 (female reproductive organ), sperm, and stylet (male intromittent organ) of *Macrostomum*
838 species with reciprocal mating (i.e. complex antrum and sperm, and stylet with a blunt distal
839 end) and hypodermic mating (i.e. simple antrum and sperm, and a needle-like stylet) (see also
840 Brand *et al.*, 2022b).

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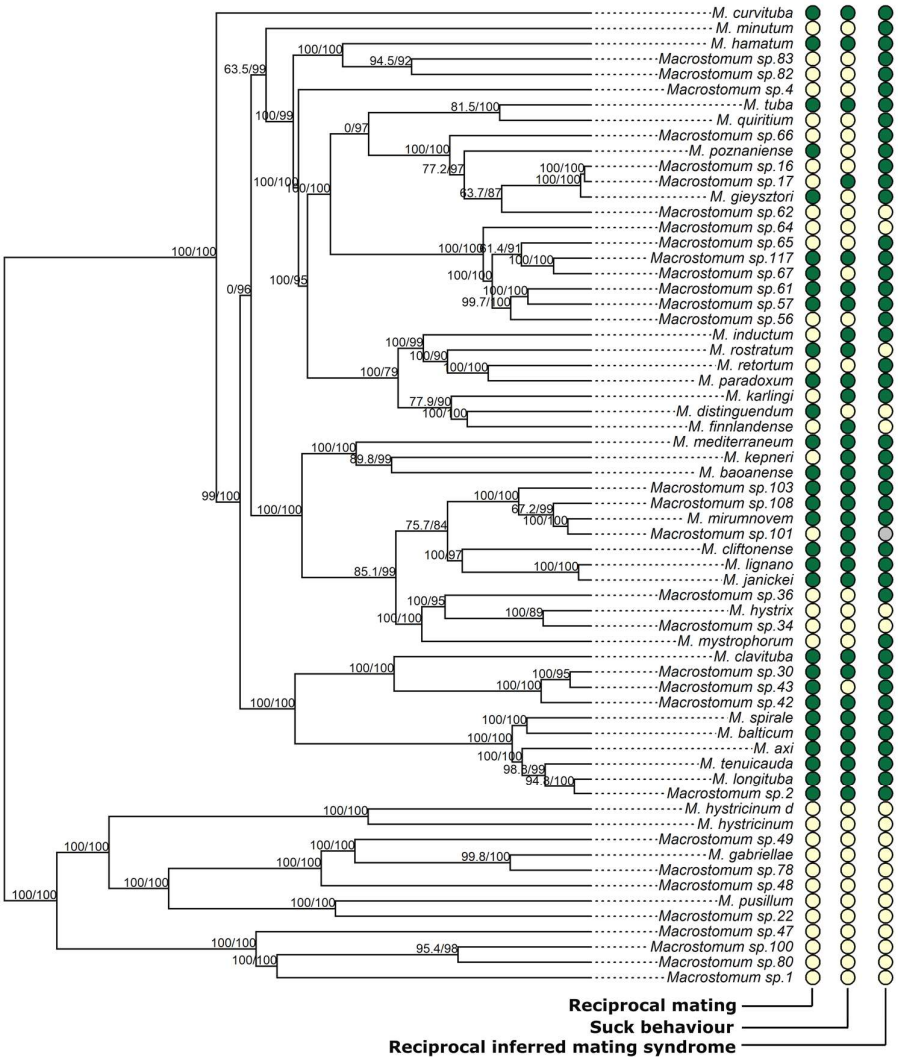

Figure 2. Presence (green) or absence (yellow) of reciprocal mating, the suck behaviour, and the reciprocal inferred mating syndrome across the *Macrostomum* phylogeny (for a total of 64 *Macrostomum* species, see Brand *et al.*, 2022c for full phylogeny). Note that for the behaviourally-inferred traits an absence may be due to a lack of sufficient data for observing the behaviour, and that for the reciprocal inferred mating syndrome the absence represents the hypodermic inferred mating syndrome (except for *Macrostomum* sp. 101, which showed an intermediate inferred mating syndrome, grey). Branch supports are indicated by ultrafast bootstrap (first number) and approximate likelihood ratio tests (second number), respectively (from Brand *et al.*, 2022c).

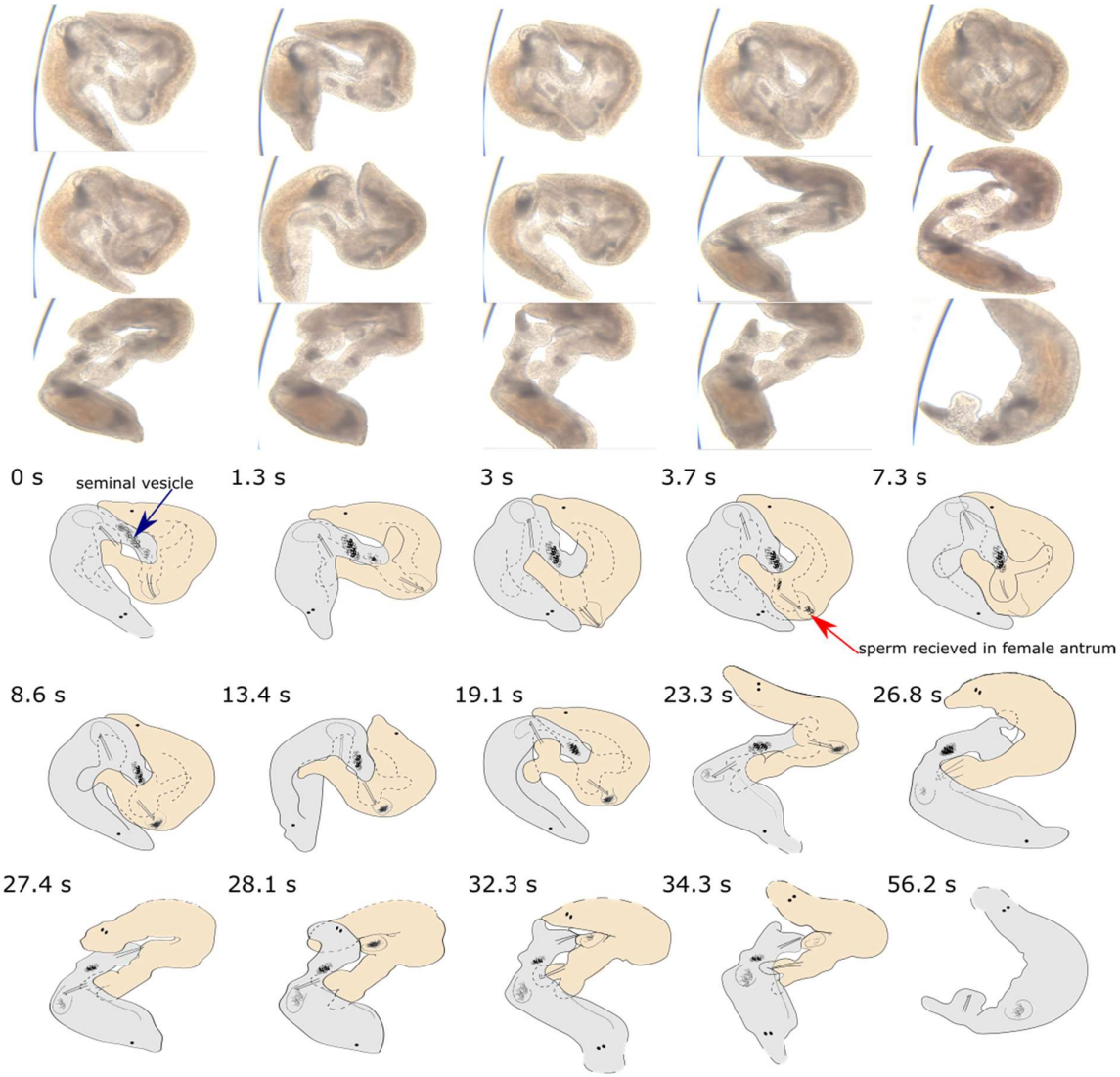

Figure 3. Reciprocal mating followed by a postcopulatory suck in *Macrostomum hamatum*, including ejaculate deposition by Grey (the worm on the left at 0 s) and its subsequent removal during the suck behaviour by Orange (the worm on the right at 0 s). Before transfer, the sperm is stored in the seminal vesicle of Grey (blue arrow in first frame), which is connected to its stylet. Ejaculate (dark mass indicated by red arrow) can be seen being deposited by Grey from the seminal vesicle starting from 3.7 s in the female antrum of Orange, followed by Orange pushing its female antrum region out (at 27.4 s) and sucking (note that Orange is also depositing ejaculate in Grey from 23.3 s). There is a visible reduction in the quantity of received ejaculate in the female antrum of Orange after the suck

ends. Note that we call the frame from where we start describing the movie as t = 0 s, but the mating had already started before that timepoint. In some frames, parts of the worms are not visible on the video, and the presumed outlines are drawn using stippled lines. A high-resolution version is provided in Supplementary Figure S1A.

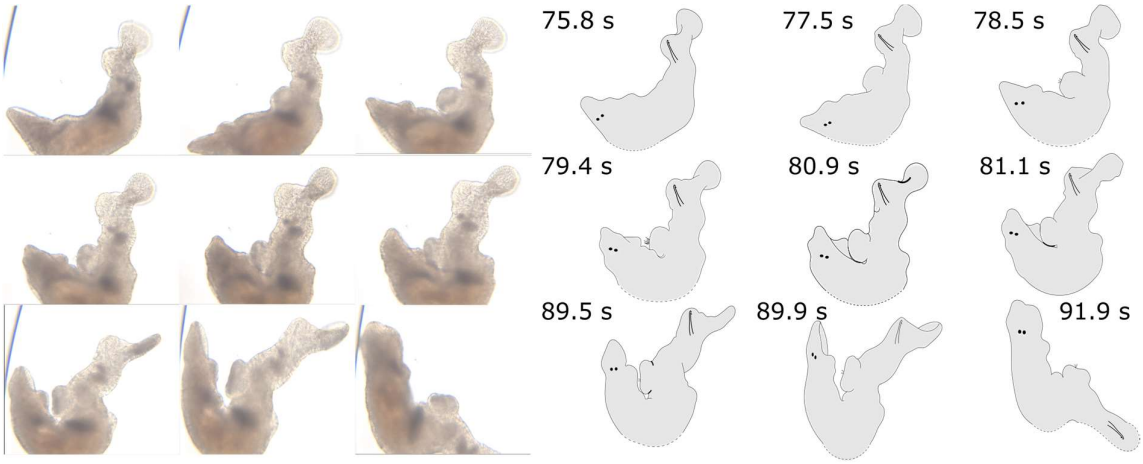

Figure 4. A postcopulatory suck following the reciprocal mating shown in Figure 3 (a continuation of the same movie), performed by Grey (i.e. the individual that had not yet sucked). Grey completely pushes out its female antrum region at t = 78 s (which leads to some sperm appearing near the female genital opening at t = 78.5 s), puts its pharynx over the female genital opening, and then sucks out most of the previously deposited ejaculate over a period of 10 s (from t = 81 s). Moreover, some sperm can be seen sticking out of the female genital opening after the suck ends. In some frames, part of the worm is not visible on the video, and the presumed outline is thus drawn using stippled lines. A high-resolution version is provided in Supplementary Figure S1B.

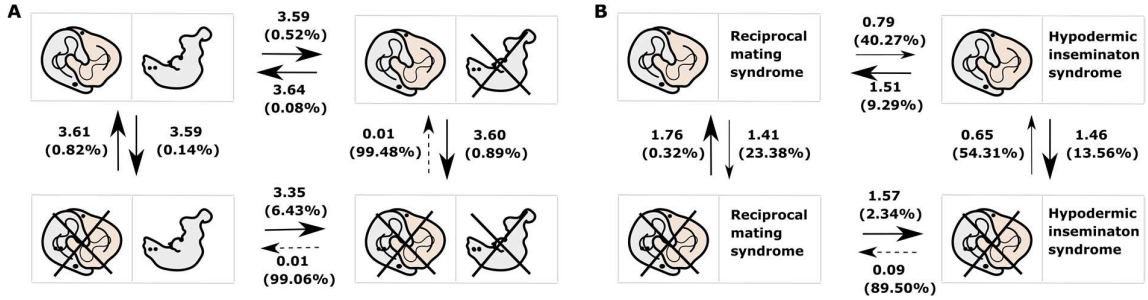

Figure 5. Correlated evolution of behavioural character states. The panels show the transition rates and the Z values (in brackets, expressed as %) for transitions between (A) the presence or absence of reciprocal mating and the suck behaviour (crossed out when absent), and (B) the presence or absence of reciprocal mating (crossed out when absent) and the inferred mating syndrome (from Brand *et al.*, 2022c). For the transition rates, the mean of the posterior distributions across all runs is given. The Z value can be understood as the percentage of times the transition rate was set to zero, amongst all the sampled parameters. The different arrows represent different probabilities of transitions between the states: high probability (strong black arrows, Z value < 15%), moderate probability (thin black arrows, Z value 20-55%), and low probability (dashed black arrows, Z value > 85%). The posterior distributions of the transition rate parameters are given in Supplementary Figure S2.

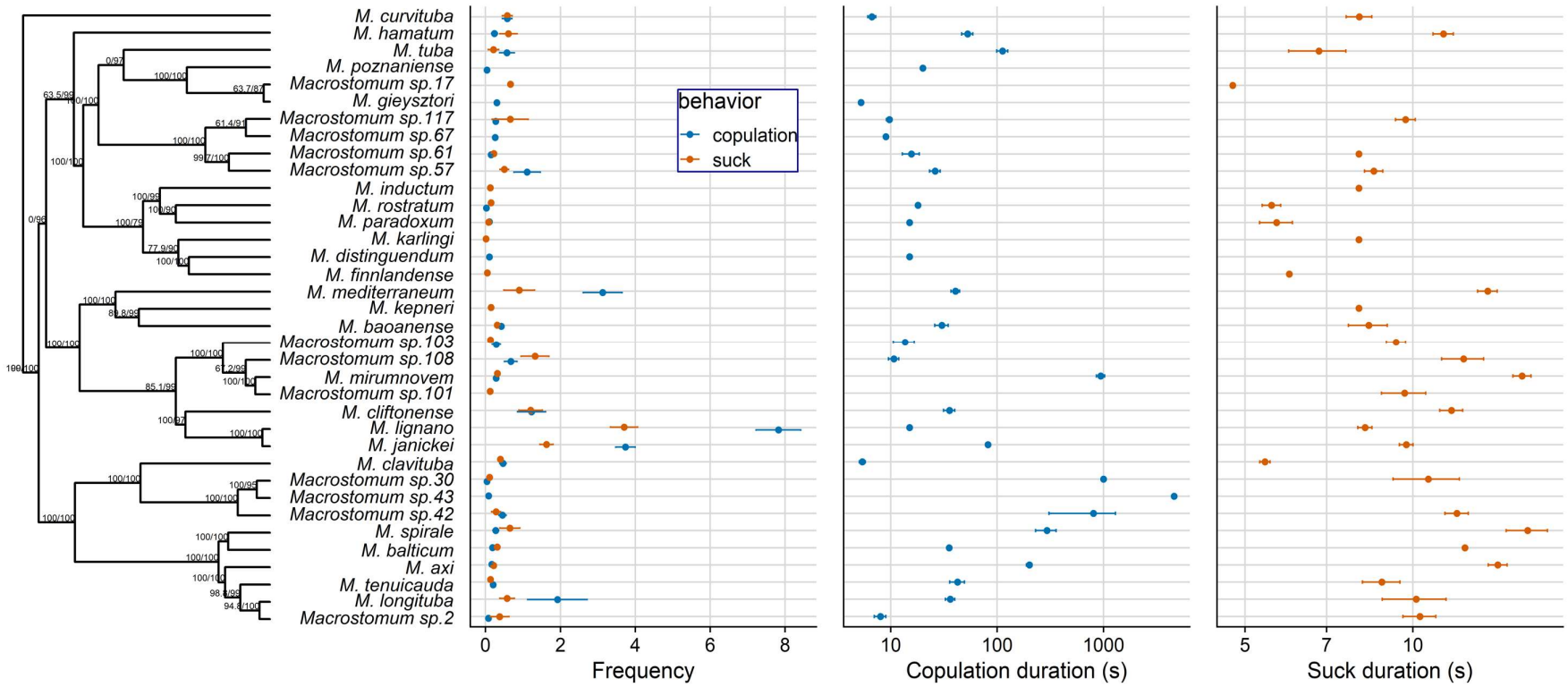

Figure 6. Trimmed phylogeny of the 36 *Macrostomum* species that showed reciprocal mating and/or the suck behaviour alongside data on means and standard errors of (A) reciprocal mating and suck frequency, (B) reciprocal mating duration (log-transformed), and (C) suck duration (log-transformed). Note that some species exhibited either only reciprocal mating or only the suck behaviour. Also note that for the species in which a

896 behaviour had been observed in only 1 replicate, we report only that single value. The branch support values are indicated by ultrafast bootstrap

897 (first number) and approximate likelihood ratio tests (second number), respectively (from Brand *et al.*, 2022c).

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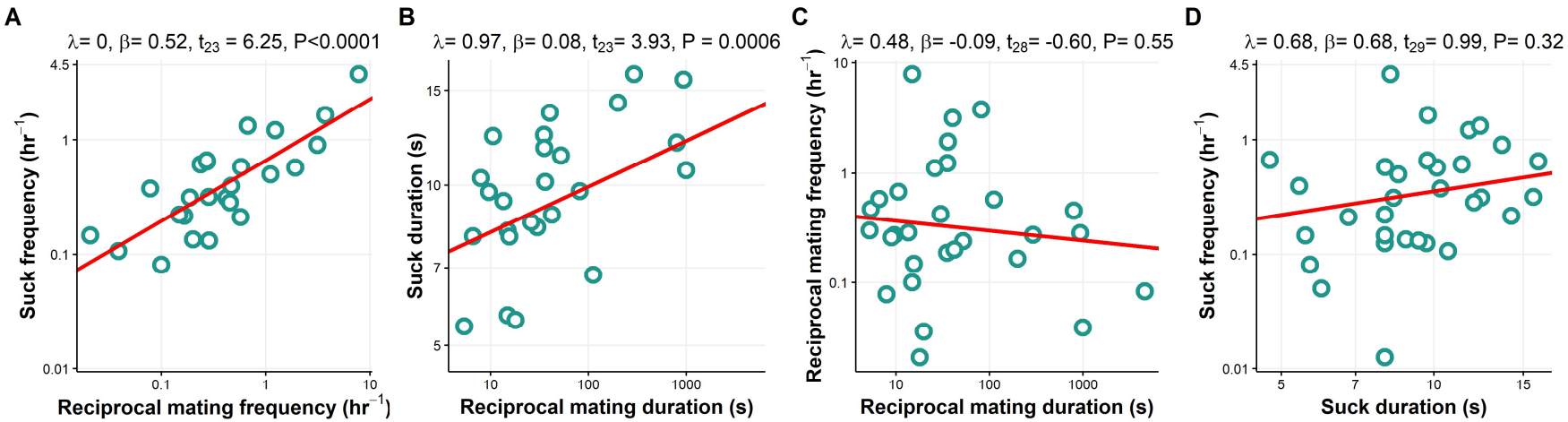

Figure 7. Relationships between (A) reciprocal mating frequency and suck frequency, (B) reciprocal mating duration and suck duration, (C) reciprocal mating duration and frequency, and (D) suck duration and frequency for *Macrostomum* species. Note that (A-D) show values plotted on log-transformed axes with PGLS results.